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Insights into the Mechanism of Memory Allocation Through the Trapping and Activating of Emotional Memories

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Insights into the Mechanism of Memory Allocation Through the Trapping and Activating of Emotional Memories

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Neurobiology

by

Thomas William Arundel Rogerson

2013

ABSTRACT OF THE DISSERTATION

Insights into the Mechanism of Memory Allocation

Through the Trapping and Activating of Emotional Memories

by

Thomas William Arundel Rogerson

Doctor of Philosophy in Neurobiology

University of California, Los Angeles, 2013

Professor Alcino Silva, Chair

While the molecular and cellular mechanisms underlying the acquisition, consolidation, reconsolidation and retrieval of memory have attracted a considerable amount of attention in neuroscience, very little is known about memory allocation, the process that determines which neurons in a network store a given memory. Computationally, it is highly advantageous to be able to correctly “file” and “cross-reference” memories in the brain, and memory allocation provides for this. Previous results from our laboratory have suggested that the transcription factor CREB (cAMP-response element binding protein) has a role in memory allocation; increasing the level of CREB in a subset of neurons

increased the probability that these neurons stored a memory. Initially, I explored the functionality of these CREB-biased memory neurons by specifically activating them, utilizing a novel technique called optogenetics. I demonstrated that activation of only the CREB-biased memory neurons is sufficient to elicit recall. This confirms a widely held, yet hitherto largely unproven, belief in the neuroscience community that activating the specific neurons that store a memory will lead to the retrieval of that memory. Although mechanisms for CREB's role in memory allocation have been proposed, to date, none has been validated. Here, I have established that increasing neuronal excitability (the likelihood that a neuron responds to a given input) in a subset of neurons can allocate memories in a way analogous to increasing levels of CREB. These results are highly suggestive that increased neuronal excitability is the mechanism by which CREB allocates memory. This research will advance not only our understanding of CREB's role in memory allocation, it will also elucidate memory mechanisms that could ultimately aid in the development of treatments for disorders thought to be caused by abnormal memory allocation, including post-traumatic stress disorder (PTSD).

The dissertation of Thomas William Arundel Rogerson is approved.

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Dean Buonomano,

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University of California, Los Angeles

2013

DEDICATION

To my parents, for giving me every advantage.

To my wife, for her love and motivation.

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LIST OF ABBREVIATIONS

5HT	serotonin
Ach	acetylcholine
AD	Alzheimer's disease
AFC	auditory-fear conditioning
AHP	afterhyperpolarization
AlstR	allatostatin receptor
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
ARC	activity-regulated cytoskeleton-associated protein
BA	basal amygdala
BLA	basolateral amygdala
BDNF	brain-derived neurotrophic factor
CA1	Cornu Ammonis area 1
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CBP	CREB-binding protein
CeA	central amygdala
ChR2	channelrhodopsin-2
CNO	clozapine-N-oxide
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CR	conditioned response
CRE	cAMP response element

CREB	cAMP-response element binding protein
CS	conditioned stimulus
DA	dopamine
DREADD	designer receptor exclusively activated by designer drug
E-LTP	early long-term potentiation
FISH	fluorescence *in situ* hybridization
GABA	*gamma*-aminobutyric acid
GIRK	G-protein coupled inwardly rectifying K^+
GluR1	glutamate receptor 1
HSV	herpes simplex virus
ITC	intercalated cells
LA	lateral amygdala
LacZ	β-galactosidase
LFS	low frequency stimulation
LTF	long-term facilitation
LTP	long-term potentiation
L-LTP	late long-term potentiation
MAP	mitogen-activated protein
NE	norepinephrine
NMDA	*N*-Methyl-D-aspartic acid
NMDAR	NMDA receptor
PKA	protein kinase A
PKC	protein kinase C

PKM zeta	protein kinase M zeta
PRP	plasticity related proteins
PTSD	post-traumatic stress disorder
RSC	retrosplenial cortex
SFO	step function opsin
STF	short-term facilitation
UR	unconditioned response
US	unconditioned stimulus
VChR1	*Volvox* channelrhodopsin
VEH	vehicle

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With the following exceptions, the work that follows is mine, performed in collaboration with Balaji Jayaprakash and Alcino Silva:

– Chapter 2 was written by me with input from fellow authors Denise Cai, Adam Frank, Manuel Lopez Aranda, Yoshitake Sano, Justin Shobe and Alcino Silva.

– Section 3.2 and 3.3 present data and figures taken from Y. Zhou, J. Won, M.G. Karlsson, M. Zhou, T. Rogerson, J. Balaji, R. Neve, P. Poirazi, A.J. Silva. (2009). "CREB regulates excitability and the allocation of memory to subsets of neurons in the amygdala." *Nature Neuroscience* 12(11): 1438-43.
– Section 3.4 and 3.5 present data and figures from R. Czajkowski, J. Balaji**,** B. Wiltgen, T. Rogerson, M.G. Karlsson, A. Barth, J. Trachtenberg, A.J. Silva. “Spatial memory formation in the retrosplenial cortex.” in preparation.
– Pallavi Bekal assisted in the creation, validation and production of viral constructs used in chapters 4 and 5. Yoshitake Sano performed the electrophysiological recordings presented in chapters 4 and 5.

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PUBLICATIONS

1. A. J. Silva, Y. Zhou, T. Rogerson, J. Shobe, J. Balaji. (2009). "Molecular and cellular approaches to memory allocation in neural circuits." *Science* 326(5951): 391-395.

2. Y. Zhou, J. Won, M.G. Karlsson, M. Zhou, T. Rogerson, J. Balaji, R. Neve, P. Poirazi, A.J. Silva. (2009). "CREB regulates excitability and the allocation of memory to subsets of neurons in the amygdala." *Nature Neuroscience* 12(11): 1438-43.

3. T. Rogerson*, J. Balaji*, S. Yoshitake, P. Bekal, K. Deisseroth, A.J. Silva. “Utilizing memory allocation mechanisms to trap and activate emotional memories.” in preparation.

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PRESENTATIONS

1. T. Rogerson, J. Balaji, Sano Y., Bekal P., Deisseroth K., & Silva A.J. (2012). “Utilizing memory allocation mechanisms to trap and activate emotional memories.” Society for Neuroscience & MCCS, New Orleans, LA.

2. T. Rogerson, J. Balaji, Sano Y., Bekal P., Deisseroth K., & Silva A.J. (2012). “Utilizing memory allocation mechanisms to trap and activate emotional memories.” FENS & MCCS Europe, Barcelona.

3. T. Rogerson*, J. Balaji*, A.J. Silva. (2011). “Utilizing memory allocation mechanisms to trap and activate emotional memories.” Society for Neuroscience & MCCS, Washington D.C.

4. T. Rogerson*, J. Balaji*, Y Zhou, K. Deisseroth, R. Neve, A.J. Silva. (2010). “The role of CREB and excitability in the allocation of an auditory fear memory trace.” Society for Neuroscience & MCCS, San Diego, CA.

5. W. Wu, T. Rogerson, D. Baker, S.L. Zipursky. (2010). “Selective disruption of dscam1 binding specificity reveals an essential role for dscam homophilic recognition *in vivo*.” Society for Neuroscience, San Diego, CA.

CHAPTER I

Introduction

1.1 Rationale for studying memory allocation

A great deal is known about which structures and systems in the mammalian brain subserve particular types of memory. For example, the neural networks within the medial temporal lobe support declarative memory (Scoville and Milner 1957; Squire and Zola-Morgan 1988), and the neural networks within the prefrontal cortex support working memory (Fuster 1980). The neural networks within the amygdala, as this dissertation will make evident, support emotive memory (Bucy and Kluver 1955). A considerable amount of attention has also been paid to the molecular and cellular mechanisms underlying the acquisition, consolidation, reconsolidation and retrieval of memory (Squire and Kandel 1999; Sweatt 2003). However, very little is known about memory allocation, the process that determines which neurons in a neural network store a given memory. The work presented in this dissertation takes advantage of what is known about memory allocation in order to trap and activate an emotional memory. As a result of this work, a potential mechanism for memory allocation, increased neuronal excitability, is validated.

The existence of a mechanism that allocates memories to particular neurons within a neural network is theoretically critical for the efficient storage and recall of information in the brain. Those neurons engaged in the storage of a memory constitute a memory trace. If a memory trace takes up an over-abundance of

neurons, the capacity of the neural network to store more information could be diminished. However, if the memory trace is stored in an overly small subset of neurons, the memory will be susceptible to loss. It appears critical that a balance between too large and too small a memory trace be struck for efficient storage of memory (Marr 1971; Staggs and Perry 1992).

Having a mechanism to allocate memories to particular neurons may also be important for linking contextually similar memories. This could be achieved by storing those memories in overlapping populations of neurons. It is easy to imagine examples where linking of two contextually similar memories is advantageous. For instance, a mouse lives in a hedgerow separating a farmer's garden from his fields. It has seen a dog in the farmer's garden and a fox in the farmer's field but does not know what to make of either creature. If, as might be assumed, the dog and fox appear contextually similar to the mouse, their memory traces may overlap. After entering the garden for the first time, the mouse is set upon by the farmer's dog and narrowly escapes with its life. If the memory of the dog and the fox are stored in overlapping memory traces, the fearful memory associated with the dog will now be linked to that of the fox. Without ever having been attacked by the fox, the mouse has learnt important information about it.

Memory allocation provides a mechanism by which similar memories can be efficiently recalled together, thereby reducing the amount of information needed

to be stored by diminishing redundancy. It may also be evolutionarily advantageous that memories of events that occur close in time be linked. For example, prior to the attack, imagine that the aforementioned mouse was startled by the bang of the farmer's door crashing open as the eager dog bounded through it. If the memory trace for this bang overlapped with the fearful memory of the dog attack, the mouse would have acquired pertinent knowledge of how to avoid future attacks (*i.e.* the bang of the opening door becomes linked to the fear-filled dog attack and, therefore, becomes something to be avoided). Thus, memory allocation can maximize the capacity and efficiency of recall within the brain by linking both contextually similar and temporally related memories.

The idea of related memories being linked also fits well with the phenomenon of priming. Priming is defined as the effect that exposure to an initial stimulus has on the response of an organism to subsequent different stimuli. In the initial experimental study of priming, Meyer and Schvaneveldt demonstrate that related words (*i.e.* bread and butter) are more rapidly identified as words in a words versus non-words identification task than non-related words (*i.e.* bread and tree) (Meyer and Schvaneveldt 1971). Their work suggests that related memories are linked, thereby facilitating each other's recall. Two separate, but not mutually exclusive, models have been postulated to underlie this phenomenon (Brown 1998; Brunel and Lavigne 2009; Buonomano 2011). The association-based model predicts that memories are stored in discrete memory traces (nodes) and that activation of one spreads to associated memory traces (nodes), thereby

priming them (Collins and Loftus 1975; Anderson 1983). This model provides a possible way to link memories without their being stored in overlapping subsets of cells and synapses. Alternatively, the feature-based model, predicts that related memories share features that are stored in a distributed network; the greater the relationship between memories, the more features they will have in common (Masson 1987; Sharkey and Sharkey 1992; Cree and McRae 2003). The supposition is that similar memories will share a common pool of cells and synapses, thereby linking them. Memory allocation potentially provides a cellular and molecular mechanism for the feature-based model and, by extrapolation, priming. Indeed, it has been postulated that memories are stored in component elements and that common features across related memories are stored in the same component elements, thereby linking related memories (Berggren, Bard *et al.* 1995; McClelland, McNaughton *et al.* 1995; Eichenbaum 2004). However, the mechanism by which this allocation may occur remained an open question until groundbreaking work on the transcription factor cAMP response element binding protein (CREB) (Han, Kushner *et al.* 2007).

1.2 cAMP response element binding protein (CREB)

cAMP response element binding protein (CREB) was initially discovered as a 43 kilodalton protein that binds the cAMP response element (CRE) in the promoter region of the somatostatin gene (Montminy and Bilezikjian 1987). CREB is typically activated by an increase in cAMP or Ca+. An elevation of cAMP or Ca+ activates protein kinases that translocate to the nucleus and phosphorylate serine 133 of CREB. Phosphorylation of serine 133 leads to CREB's binding CRE elements in the promoter regions of target genes. CREB then interacts with the CREB-binding protein (CBP) and together they facilitate CREB-dependent transcription. Targets include the genes for c-fos, neurotrophin, BDNF, tyrosine hydroxylase and numerous neuropeptides (Purves 2008; Kandel 2012). CREB and its target genes have been shown to have a ubiquitous and multifaceted role in the consolidation of memory (Silva, Kogan *et al.* 1998). This was initially observed in drosophila genetically engineered to express a dominant negative CREB and in mice genetically engineered to lack two isoforms of CREB. These genetically engineered drosophila and mice were able to form short-term memories but were incapable of forming long-term ones, demonstrating CREB's role in memory consolidation (Bourtchuladze, Frenguelli *et al.* 1994; Yin, Wallach *et al.* 1994). CREB's additional role in memory allocation has only recently been recognized.

The study of how memories are allocated to subsets of neurons has now begun (Han, Kushner *et al.* 2007; Han, Kushner *et al.* 2009; Silva, Zhou *et al.* 2009; Zhou, Won *et al.* 2009). Work in the Silva laboratory has demonstrated that the transcription factor CREB plays a major role in determining which neurons encode a given memory (Han, Kushner *et al.* 2007).

For memory allocation to be able to function as a means to link both contextually similar and temporally related memories, there must be a mechanism that determines where memory is stored in a neural network, as opposed to the memory trace's being stored in every neuron that receives the necessary information to encode the memory. Previous work attests to the fact that memory is not stored in every neuron that receives the necessary information to encode it. In fact, it has been shown that only a portion of those neurons that receive the necessary input to form a memory are incorporated into the memory trace (Quirk, Repa *et al.* 1995; Johansen, Hamanaka *et al.* 2010). In auditory-fear conditioning (AFC), a type of Pavlovian learning (Pavlov and Anrep 1927), in which a neutral conditioned stimulus (CS), a tone, is paired with an unconditioned stimulus (US), a shock, the association between the CS and the US is thought to be made in the lateral amygdala (LA) (Maren and Fanselow 1996). During AFC up to 73% of LA neurons receive information regarding the CS (Repa, Muller *et al.* 2001) and up to 77% of LA neurons receive information regarding the US (Johansen, Tarpley *et al.* 2010). However, only about 33% of neurons go on to encode the memory

(Quirk, Repa *et al.* 1995; Rumpel, LeDoux *et al.* 2005; Johansen, Tarpley *et al.* 2010).

To ascertain whether CREB plays a role in determining which neurons encode memory, Han *et al.* transfected cells of mouse basolateral amygdala (BLA) in three ways: 1) with a virus over-expressing CREB, 2) with a dominant negative CREB with serine 133 mutated to alanine, or 3) with b-galactosidase (LacZ), serving as a control. Han *et al.* then performed AFC and examined whether those neurons containing viral CREB or the dominant negative isoform were disproportionately represented in the memory trace. This was achieved by using viral vectors that delivered the various CREB genes to specific neurons in the BLA, and fluorescence *in situ* hybridization (FISH), which identified those BLA neurons engaged by auditory-fear conditioning (Guzowski, McNaughton *et al.* 1999). The neuronal activity-dependent gene, activity-regulated cytoskeleton-associated protein (Arc), served as a suitable marker of the memory trace, since the transcription of this gene is activated during learning, and the Arc protein is required for memory (Bramham, Worley *et al.* 2008). Han *et al.* found that neurons over-expressing CREB in the BLA were 3 times more likely than other neurons to express Arc. Importantly, neurons expressing the dominant negative form of CREB ($CREB^{S133A}$) were less likely to be incorporated into the auditory-fear conditioned memory trace, as there was a lower colocalization of $CREB^{S133A}$-GFP and Arc than in non-transfected neurons. In the LacZ control, no bias towards or away from the memory trace was detected. Interestingly, the

proportion of Arc positive cells (~25% of eligible neurons) remained constant in all experiments, suggesting that a competitive process determines which neurons will be brought into the memory trace (Han, Kushner *et al.* 2007; Silva, Zhou *et al.* 2009).

Consistent with earlier work in the basal amygdala (Viosca, Lopez de Armentia *et al.* 2009), studies in the Silva laboratory have shown that cells over-expressing CREB were more excitable than neighboring cells or cells over-expressing LacZ (Zhou, Won *et al.* 2009). Furthermore, we found that increases in synaptic strength thought to be critical for memory are greater in CREB over-expressing neurons, suggesting that these neurons hold a disproportionately large component of the auditory-fear memory trace (Zhou, Won *et al.* 2009). Again, this indicates that CREB is playing a role in memory allocation and suggests a possible mechanism by which it may bias memory allocation: cells that are more excitable are more likely to fire in response to sensory input (the tone and shock in AFC) and are therefore more likely to form stronger synaptic connections and thus be over-represented in the memory trace (Hebb 1949).

1.3 Auditory-fear conditioning

A fear memory is a form of learning involving the association of a threatening event and the stimuli associated with it. One of the classic examples of a human fear memory is that of Little Albert. As an infant, Albert was exposed to a white rat and expressed an initial interest in it. However, as Albert's hand reached out to the rat, the experimenter made a loud noise behind Albert's head, causing him to startle and cry. When later exposed to the sight of a white rat, Albert started to cry, demonstrating that he had made an association between the rat and the fear-inducing sound (Watson and Rayner 2000).

This type of associative fear learning is often referred to as Pavlovian fear-conditioning and, as in Pavlov's original experiments, can be thought of in terms of unconditioned and conditioned stimuli and responses. Pavlov's seminal finding was that when an unconditioned stimulus (US), one that has an innate and reflexive response [the unconditioned response (UR)], is paired with a conditioned stimulus (CS), which has no overt behavioral response, the two become associated. A CS presentation in the absence of a US then leads to a conditioned response (CR), which may or may not be the same as the UR. In Pavlov's original experiments involving salivary conditioning, dogs were presented with meat powder (the US), a substance that induces salivation (the UR). If the presentation of the meat powder is paired with a CS, in Pavlov's

experiments a metronome, the two become associated. At a later time, when the CS was presented in the absence of a US, a CR, in this case salivation, was elicited (Pavlov and Anrep 1927). Pavlovian fear-conditioning in the laboratory setting is typically performed on rodents. The US is often a mild foot shock that elicits paroxysms (the UR). When paired with a CS, which is typically a tone or context, an association is made between the two. Later, when the CS is presented in the absence of a US, conditioned responses associated with fear, including freezing, elevated blood pressure and release of cortisol, are observed (Maren and Fanselow 1996). Sensory information relating to the nature of the US and CS of the fear memory converge in the amygdala, where the association is made (LeDoux 2007).

Based upon histological criteria, the amygdala was first identified as a distinct brain region in the medial temporal lobe in the 19th century. Since then a great deal of effort has been exerted to understand the anatomy and circuitry of the structure. The name amygdala (derived from the Greek word for almond) is based upon the shape of what are now referred to as the lateral and basal nuclei of the structure. The amygdala has a cortico-medial region, encompassing the cortical, medial and central nuclei, with the majority of neurons being GABAergic (Cassell, Gray *et al.* 1986; Ehrlich, Humeau *et al.* 2009). The basolateral region, encompassing the lateral, basal and accessory basal nuclei, consists primarily of glutamatergic principle neurons with, to a lesser extent, GABAergic interneurons (McDonald 1982; Ehrlich, Humeau *et al.* 2009). These nuclei can be further

subdivided based upon anatomical and functional differences. In simple terms, the amygdala can be thought of as having an input-output function, in which information enters by way of the basolateral region, and processed information exits via the cortico-medial region (**Fig. I-1A**) (Fanselow and Poulos 2005).

The lateral amygdala (predominantly the dorsal subregion) rapidly receives information regarding the auditory CS from the auditory thalamus. These thalamic inputs originate in the inferior colliculus and terminate in the lateral amygdala by way of the medial geniculate nucleus and posterior intralaminar nucleus (LeDoux, Ruggiero *et al.* 1985; Linke and Schwegler 2000). Cortical projections to the amygdala carry more precise information regarding the CS. However, as this information travels through multiple synaptic connections, it takes longer to arrive in the amygdala (LeDoux 2007). Information about the US travels from the spinothalamic track to the amygdala via the medial geniculate nucleus and posterior intralaminar nucleus (Ledoux, Ruggiero *et al.* 1987; Fanselow and Poulos 2005). Most of the input to the amygdala is excitatory and involves, glutamatergic synaptic connections. Typically, the inputs synapse onto the dendrites of the principle neurons of the lateral amygdala (sometimes referred to as projection neurons). However, some also synapse onto local inhibitory neurons that project to principle neurons, thereby creating inhibitory feedforward circuits. Further feedback inhibition comes from principle neurons that project to inhibitory interneurons, which then project back to the principle neurons (LeDoux 2007). The lateral amygdala also receives CS related

information from other regions of the brain, including the hippocampus, which is important in contextual fear conditioning and in extinction learning.

The initial CS-US association is made on the dendrites of the principle neurons of the lateral amygdala. It is commonly held that the convergence of CS and US input on a local region of the neuron leads to the synaptic strengthening of the CS input, thereby allowing a future presentation of the CS to facilitate an appropriate behavioral response that could not have been elicited prior to synaptic strengthening (**Fig. I-1B**). However, synaptic strengthening at the thalamic-lateral amygdala synapse is in itself insufficient to facilitate a behavioral response. Subsequent plasticity at downstream sites in the amygdala is also necessary (Wilensky, Schafe *et al.* 2006). This makes sense in light of anatomical data demonstrating no direct connection between the lateral amygdala and the central medial amygdala, the amygdala's major output structure. The three major amygdala nuclei that connect the lateral amygdala and central medial amygdala are the basal amygdala, intercalated cells and the central lateral amygdala, all of which may be potential sites of downstream plasticity (Pape and Pare 2010). It is probable that synaptic strengthening of the CS input onto dendrites of the lateral amygdala and the other sites of downstream plasticity occur by way of long-term potentiation (LTP) (Bliss and Lomo 1973).

LTP is an associative form of plasticity in which a strong stimulation paired with a weak stimulation in both space and time lead to the potentiation of the weak synaptic connection, when the weak stimulation alone would not have led to potentiation. In the case of fear conditioning, the CS serves as the weak stimulation and leads to the release of glutamate from presynaptic terminals. Glutamate binds *N*-methyl-D-aspartate (NMDA) and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA), excitatory postsynaptic receptors, but glutamate from the CS alone is not able to depolarize the synapse sufficiently to trigger the calcium influx/release necessary for synaptic plasticity. However, when the CS is paired with a strong stimulation, such as that from a fear-inducing foot shock at a neighboring synapse, the local region of the neuron becomes depolarized, releasing the magnesium block on NMDA receptors (NMDAR). The weak CS stimulation is then sufficient to drive calcium entry into the cell by way of NMDARs and voltage-sensitive calcium channels (**Fig. I-1B**). Calcium entry leads to the phosphorylation of mitogen-activated protein (MAP) kinase and activation of other kinases, including PKC, PKA and CaMKII (Sweatt 2003).

Short term synaptic strengthening occurs when additional AMPA receptors are incorporated into the synapse (Benke, Luthi *et al.* 1998) or when their conductance is increased (Malinow and Malenka 2002) in a calcium-dependent way after phosphorylation by CaMKII or PKC. Long term potentiation also requires elevated calcium levels at the synapse; calcium activates adenyl cyclase, which ultimately activates MAP kinase by way of PKA, B-raf and MEK,

with modulatory influence from ras-coupled receptors and PKC. MAP kinase then translocates to the nucleus and, by way of rsk2, phosphorylates and activates cAMP response element binding protein (CREB) (Sweatt 2003). As mentioned previously, CREB is a stimulus-induced transcription factor that binds to the c-AMP response element (CRE) and has been shown to have a ubiquitous and multifaceted role in consolidation of memory (Shaywitz and Greenberg 1999) as well as memory allocation. CREB is activated when serine 133 is phosphorylated. Serine 133 phosphorylation allows CREB to interact with the CREB-binding protein (CBP) that is then capable of inducing CREB-dependent transcription (Impey, McCorkle *et al.* 2004). Studies involving the positive and negative manipulation of LTP have resulted in the observation of a link between LTP and fear learning (Lee and Silva 2009). This suggests the mechanism by which stable maintenance of the CS-US association is achieved.

Because of LTP, an association between the CS and US may be created in the lateral amygdala. *In vivo* single unit studies in the lateral amygdala have shown that after the formation of the CS-US association, CS presentations elicit greater neuronal activity (more action potentials) (Maren and Quirk 2004). This neuronal activity then flows through the lateral amygdala to the central nuclei of the amygdala by way of the basal nuclei or intercalated cells (**Fig. I-1A and B**).

LTP also occurs between these sub-nuclei and the central medial nuclei of the amygdala. This secondary LTP is necessary for expression of auditory-fear

conditioning (Wilensky, Schafe *et al.* 2006). Even though a CS-US association may have been made in the lateral amygdala, without the strengthening of these downstream amygdala synapses, it is not possible to transmit information to effector regions responsible for the manifestation of fear (Pape and Pare 2010).

The central nucleus is the major output region of the amygdala and has projections to numerous regions of the brain including various modulatory systems, the periaqueducal gray, and the hypothalamus. The modulatory systems to which the central nucleus projects include norepinephrine (NE), dopamine (DA), acetylcholine (Ach) and serotonin (5HT). These neurotransmitters and others then feed back to the amygdala and modulate the interactions between excitatory and inhibitory neurons, possibly facilitating LTP (Ehrlich, Humeau *et al.* 2009). The periaqueducal gray has been shown to be necessary for freezing and the hypothalamus has been shown to modulate physiological responses associated with fear in the rodent (LeDoux 2007). Information (the CS) linked with a harmful US becomes associated with it in the amygdala. Subsequently, when a CS is presented, information flows through the amygdala that would otherwise not, eliciting a behavioral response (**Fig. I-1B**). In the case of rodent fear learning, it is typical to monitor freezing as a marker of this association, although, physiological responses associated with fear could also be used.

Fig. I-1

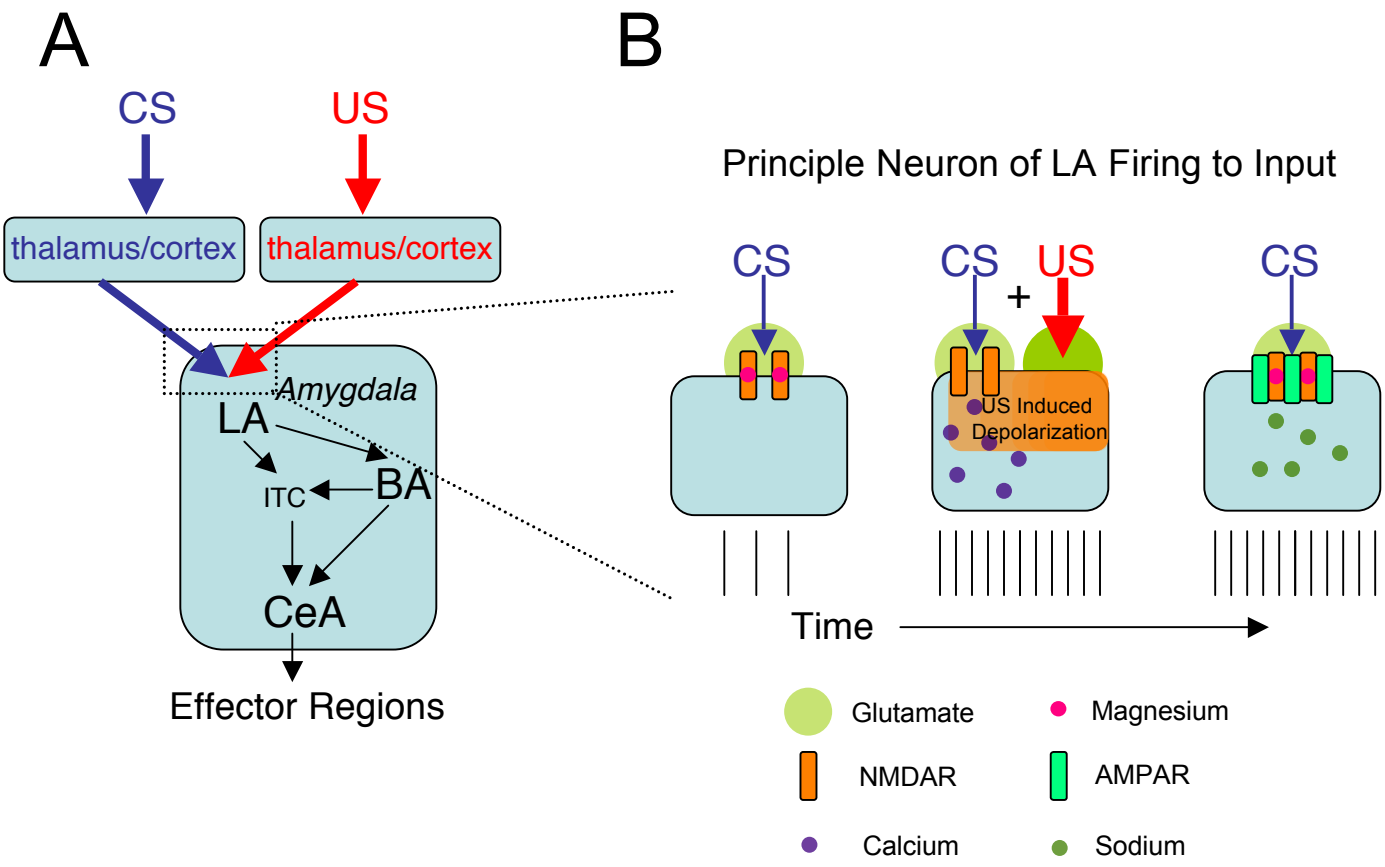


Figure I-1 - Circuitry underlying auditory-fear conditioning

A) Information about the conditioned stimulus (CS) and the unconditioned stimulus (US) arrives at the dendrites of the lateral amygdala (LA), the primary input to the amygdala. Information then flows from the LA to the central amygdala (CeA) by way of the basal amygdala (BA) and intercalated cells (ITC). The CeA, the primary output of the amygdala, modulates the activity of effectors.

B) Synaptic level view of the mechanism governing associative learning. When the CS only is presented, no potentiation occurs and little firing is induced. However, when the CS is paired with a strong stimulation (the US), potentiation occurs and further presentations of the CS elicit strong firing.

1.4 Optogenetics

Optogenetics is a new field pioneered by Edward Boyden and Karl Deisseroth that combines optics, genetics, molecular biology and transgenic or virally mediated gene delivery to control the activity of a genetically and/or stereotaxically defined population of neurons (Boyden, Zhang *et al.* 2005). In 2003, Nagel *et al.* published a paper that demonstrates that an opsin that facilitates phototaxis in the green alga *Chlamydomonas reinhardtii* can be used to depolarize oocytes and human embryonic kidney cells upon blue light illumination (Nagel, Szellas *et al.* 2003). Negal *et al.* named this opsin channelrhodopsin-2 (ChR2). Unlike mammalian rhodopsins, ChR2 does not activate a chemical cascade. Rather, it is a self-contained cation channel comprising seven transmembrane alpha helixes that requires all-*trans*-retinal, a vitamin-A-based chromophore cofactor, to be activated by light. Upon exposure to blue light (~470nm), photoisomerisation of the chromophore elicits a conformational change in ChR2, resulting in the opening of the channel (Nagel, Szellas *et al.* 2003). Edward Boyden and Karl Deisseroth demonstrated the functionality of ChR2 in the mammalian nervous system and developed ways to maximize its utility by pioneering cell type specific transgenic and viral expression systems (Boyden, Zhang *et al.* 2005; Deisseroth, Feng *et al.* 2006; Boyden 2011). Under physiological conditions in the mammalian nervous system, neurons expressing ChR2 that are illuminated with blue light will generate inward depolarizing currents due to the passage of positively charged ions (such as H+

and Na+). This can result in the depolarization of the neurons to such an extent that action potentials are triggered (Boyden, Zhang *et al.* 2005; Zhang, Wang *et al.* 2006; Zhang, Wang *et al.* 2007).

Soon after the utilization of ChR2, numerous other optogenetic tools were developed from variants of ChR2 and other microbial opsins (Zhang, Vierock *et al.* 2011). Some of these new optogenetic tools are advancements on ChR2 as they can pass greater currents, have decreased t_{off} times and are more photo-sensitive. This has resulted in there being channels that can generate greater inwardly depolarizing currents at higher frequencies and with less light stimulation necessary than with the original ChR2. Other variants have red-shifted excitation spectra, which allows for their use in conjunction with the original ChR2 (Mattis, Tye *et al.* 2012). One such example is *Volvox* channelrhodopsin (VChR1), a cation conducting channelrhodopsin from the spheroidal alga *Volvox cateri*. VChR1 has maximum excitation at 589nm, roughly a 70nm shift compared to ChR2 (Zhang, Prigge *et al.* 2008). Separate advances in optogenetic tools have dramatically extended t_{off} times. An extended t_{off} time allows for prolonged depolarization following the offset of light. Channels with these characteristics have been termed Step Function Opsins (SFO) due to their ability to create prolonged steps in excitability. In the case of ChR2-C128S, a SFO variant of ChR2, light activation induces a sustained subthreshold depolarization (Berndt, Yizhar *et al.* 2009). The advent of optogenetics has given

neuroscientists the ability to manipulate the activity of specific populations of neurons in a temporally precise manner that was not previously possible.

A wealth of knowledge relating to the circuitry mediating fear learning, especially in the amygdala, has been acquired from anatomical, pharmacological, lesion and electrophysiological studies (Maren and Fanselow 1996; LeDoux 2007; Ehrlich, Humeau *et al.* 2009). However, until the advent of optogenetics, it was impossible to manipulate the activity of a specific subset of neurons (Boyden, Zhang *et al.* 2005; Zhang, Wang *et al.* 2006). Now that this is feasible, there are exciting opportunities to discover which neurons, when activated, are sufficient to elicit a behavior and to manipulate the excitability of neurons in order to observe whether this affects memory allocation.

1.5 Conclusions

The allocation work reviewed above suggests that CREB is involved in memory allocation. However, many aspects of memory allocation remain obscure. For example, Han *et al.* demonstrated that BLA neurons with the viral CREB are more likely to be part of an auditory-fear memory. Nevertheless, it is unclear whether these CREB biased memory neurons are necessary for memory. Research that I performed in collaboration with Yu Zhou and Rafal Czajkowski in the early years of my Ph.D. addressed this issue. However, it was unclear whether activation of these CREB-biased neurons would be sufficient to trigger memory. These questions were addressed by activating this same subset of neurons and observing whether or not freezing was induced. These neurons were activated with the ChR2 system. The results of this research are presented in Chapter 4. Even though it is hypothesized that increased levels of CREB in a neuron bias a memory to that neuron by increasing excitability, there is as yet a lack of direct evidence for this. Here, we address this problem by enhancing the excitability of a subset of neurons with ChR2-C128S, a strategy independent of CREB. Finding that increases in excitability driven by the ChR2-C128S system also bias memory allocation would provide a strong support for the hypothesis. The results of this research are presented in Chapter 5.

This study of memory allocation and others will form the foundation for a new memory phenomenon. Studies such as those presented in this dissertation will be essential in ultimately revealing the processes of memory allocation that are necessary to correctly “file” and “cross-reference” memories so that the appropriate memories are stored efficiently and retrieved at the appropriate times and in the appropriate context.

CHAPTER II

Synaptic Tagging During Memory Allocation

2.1 Introduction

There is now compelling evidence that memory allocation to specific neurons (neuronal allocation) and synapses (synaptic allocation) in a neurocircuit is not random, and that instead specific mechanisms, such as increases in neuronal excitability, synaptic tagging and capture, determine the exact sites where memories are stored. We propose an integrated view of these processes such that memory allocation, synaptic tagging and capture, and spine clustering, reflect related aspects of memory allocation mechanisms. Importantly, the properties of these mechanisms suggest a set of rules that profoundly affect how memories are stored and recalled.

The molecular, cellular and systems mechanisms underlying the acquisition, consolidation, reconsolidation, retrieval and extinction of memory have attracted a great deal of attention. However, little is known about memory allocation (Silva, Zhou *et al.* 2009), the process that determines which specific neurons and synapses in a neural network will store a given memory. Memory allocation is an active area of research in computer science and artificial intelligence where the representation of data, its structure and site of storage can dramatically affect how the stored information is used (Winston 1977; Aho, Hopcroft *et al.* 1983; Decker 1989; Russell and Norvig 1995).

The significance of having mechanisms that determine the allocation of information to particular neurons and synapses within a neural network is theoretically critical for the efficient storage and recall of that information. Inefficient allocation of information leads to suboptimal use of storage space, whether hard disks or synaptic sites are involved. For example, theoretical studies suggest that there is a balance between the size of a memory representation and the maximum amount of information that can be stored: larger representations can be more stable, but storage space could be wasted; small representations save storage space, but memories are more easily disrupted (Marr 1971; Simoncelli and Olshausen 2001; Olshausen and Field 2004; Quiroga, Kreiman *et al.* 2008).

By directing related information to overlapping populations of neurons, memory allocation mechanisms could link these memories, place them within a common context, save storage space and perhaps alter the memory strength (Silva, Zhou *et al.* 2009). Memory allocation mechanisms may organize the storage of information into component elements that encode features shared across related experiences, thereby linking the storage of these experiences (McClelland, McNaughton *et al.* 1995; Eichenbaum 2004). Memory allocation can be thought of as how the brain “files” and “cross-references” information in brain circuits.

This chapter will present work detailing the mechanisms of memory allocation at both the synaptic (synaptic allocation) and neuronal (neuronal allocation) scale. More importantly, it will attempt to integrate these previously separate areas of memory research into a synergistic theory of how brain circuits regulate which neurons and synapses are committed to store a given memory.

2.2 Neuronal allocation

Whereas mechanisms of synaptic allocation (*i.e.* synaptic tagging and capture, spine clustering, etc.) explain how information is parceled to specific synapses, mechanisms of neuronal allocation account for how specific neurons in a network are committed to a specific memory. For example, previous studies have shown that changes in neuronal excitability triggered by the transcription factor CREB (cAMP-response element binding protein) modulate the probability that a given neuron will be involved in storing a specific memory. Although neuronal and synaptic allocation mechanisms work seamlessly during memory storage, their distinction is useful in designing, interpreting and describing allocation studies.

Molecular, cellular and systems studies of neuronal allocation

The amygdala has a key role in the modulation and storage of fear memories (Maren and Fanselow 1996). Previous studies suggested that only a subset of the amygdala neurons activated during training go on to participate in memory (Quirk, Repa *et al.* 1995; Johansen, Hamanaka *et al.* 2010). Circuits in the lateral amygdala (LA) are thought to store the association between the conditioned stimulus (CS; *e.g.*, a tone) and the unconditioned stimulus (US; foot-shock) in auditory-fear conditioning (Maren and Fanselow 1996). More than 70% of all LA neurons receive information regarding the auditory CS (Repa, Muller *et al.* 2001) or the US (Johansen, Tarpley *et al.* 2010). However, only a small subset of these neurons goes on to encode the memory (Quirk, Repa *et al.* 1995; Rumpel,

LeDoux *et al.* 2005; Johansen, Tarpley *et al.* 2010). Accordingly, only a subset of LA neurons undergoes plasticity following auditory-fear conditioning. Studies with modified AMPA receptors that can electrophysiologically tag synapses involved in learning indicated that only a third of recorded amygdala neurons showed synaptic changes following fear conditioning (Rumpel, LeDoux *et al.* 2005). Other studies described below confirm that only a fraction of LA neurons actually encode memory for auditory-fear conditioning (Reijmers, Perkins *et al.* 2007). This suggests that specific mechanisms govern the allocation of fear memories to specific neurons in the amygdala.

Studies in the cortex have also suggested that the population of neurons encoding a given memory is a subset of the population initially activated during learning (Frostig 2006). Recent studies with two-photon *in vivo* calcium imaging showed that pairing whisker stimulation with footshock led to a decrease in the number of cortical neurons responding to the whisker stimulation (Gdalyahu, Tring *et al.* 2012), suggesting refinement of the memory trace. Importantly, similar findings were also obtained with a whisker signaled trace eye-blink conditioning approach (Ward, Flores *et al.* 2012), suggesting that memory allocation mechanisms shape the neuronal ensembles that determine which neurons are involved in conditioning responses in the somatosensory cortex. It is likely that memory allocation mechanisms not only determine which cells receive the initial inputs but also the pairing down of the cellular ensemble to a more sparse and possibly more efficient storage of the memory trace.

Initial studies using viral vectors demonstrated that changing the levels of CREB within a specific subpopulation of LA neurons could affect the probability with which these neurons are recruited into an auditory-fear memory: increasing the levels of the transcription factor CREB within a subset of lateral amygdala neurons increases the probability that these neurons are involved in fear conditioning, while decreasing the levels of this transcription factor has the opposite effect (Han, Kushner *et al.* 2007; Han, Kushner *et al.* 2009; Zhou, Won *et al.* 2009); .

Three main strategies were used to demonstrate the role of CREB in memory allocation. First, studies with immediate early genes showed that lateral amygdala neurons with higher levels of viral CREB were 3 times more likely to be recruited to the auditory-fear memory trace than their neighboring neurons (using immediate early genes as a marker of the memory trace) than their neighbors. A number of control experiments showed that if learning was blocked, the memory trace was no longer biased to the neurons with higher CREB levels (Han, Kushner *et al.* 2007). Additionally, a form of CREB that interferes with the function of this transcription factor decreased the probability that these neurons were recruited to the memory trace (Han, Kushner *et al.* 2007).

Strategies that either inactivated (Zhou, Won *et al.* 2009) or deleted (Han, Kushner *et al.* 2009) the neurons expressing viral CREB also suggested that the

memory was disproportionally represented in those neurons. For example, inactivation of lateral amygdala neurons expressing viral CREB with the allatostatin system triggered an amnesia for auditory-fear conditioning and conditioned taste aversion, while inactivating a similar number of neurons with normal levels of CREB did not (Zhou, Won *et al.* 2009; Koichi Hashikawa 2013). Similar results were also obtained with conditioned taste aversion, another form of memory that involves amygdala circuits.

Finally, electrophysiological studies showed that following training in auditory-fear conditioning, the LA neurons with the viral-encoded CREB showed higher synaptic strengths than neurons with normal levels of CREB, a result consistent with the idea that memory is encoded in these neurons as increases in synaptic strength (Zhou, Won *et al.* 2009). Importantly, LA neurons expressing viral CREB are more excitable than their neighbors, a finding that could account for their preferential incorporation into LA ensembles encoding auditory-fear conditioning (Zhou, Won *et al.* 2009). This result is consistent with the hypothesis that CREB plays a role in neuronal allocation: neurons with higher CREB levels are more excitable and therefore more likely to fire in response to sensory input, more likely to be involved in synaptic changes underlying memory, and thus are more likely to be over-represented in the memory trace (**Fig. II-1**).

While the aforementioned data strongly support CREB's role in memory allocation in the amygdala, it is unclear if these principles generalize to other

brain regions. However, there is indirect evidence that CREB is involved in regulating memory allocation in hippocampus. First, transduction of CA1 neurons with viral CREB prior to training on the Morris water maze (Sekeres, Mercaldo *et al.* 2012) or in contextual conditioning (Restivo, Tafi *et al.* 2009) enhances these hippocampus-dependent memories. In these experiments, the virus only transduced approximately 25% of neurons in CA1 (Restivo, Tafi *et al.* 2009), resulting in a subset of neurons expressing increased levels of CREB; and these neurons likely recruited a higher proportion of the memory trace in order for the representation to be strengthened by CREB. Importantly, viral transduction had to occur *prior* to training for the behavioral enhancement, as post-training injection of CREB virus did not alter memory performance (Sekeres, Neve *et al.* 2010). This suggests that the enhancement observed was cell-specific and due to recruitment of the memory trace by high levels of CREB in a subset of neurons.

CREB appears to regulate excitability in other structures required for memory, where it may also affect allocation (Dong, Green *et al.* 2006). For example, transgenic mice expressing a constitutively active form of CREB showed reduced afterhyperpolarization currents in hippocampal CA1 pyramidal neurons that led to increased excitability and reduced thresholds for LTP (Lopez de Armentia, Jancic *et al.* 2007). As in the amygdala, there is evidence that this increase in excitability may also affect memory allocation in other brain regions, such as the hippocampus. Whole cell recordings in behaving rats showed that hippocampal

CA1 neurons recruited into encoding a given place (place cells) showed lower spike thresholds and peaked versus flat subthreshold membrane potentials that were sensitive to an animal's location (Epsztein, Brecht *et al.* 2011). Interestingly, this increase in excitability appeared to precede place cell formation during spatial exploration (Epsztein, Brecht *et al.* 2011), as if prior events set the stage for the allocation of place information to a subset of place cells in the hippocampus. Furthermore, increasing excitability by depolarizing the somatic membrane potential of a silent cell (*i.e.* a cell that previously did not fire to a spatially tuned location) during spatial exploration led to the emergence of a spatially tuned place cell (Lee, Lin *et al.* 2012).

More evidence for the idea that prior events determine which place cells will encode a given environment came from studies of a phenomenon called "preplay" (Dragoi and Tonegawa 2011). Preplay is temporally opposite to the better known phenomenon of replay, the recapitulation of place cell sequences experienced during previous spatial explorations (usually while the animal is in quiet rest or sleep). Preplay takes place before (not after) exploration of novel environments. Remarkably, the preplay studies suggested that not only are there mechanisms that allocate which specific hippocampal place cells to encode a given place, these neuronal allocation mechanisms may also determine the sequence in which these future place cells are activated during spatial exploration. It is conceivable that spontaneous firing events prior to actual spatial exploration engage neurons with the highest excitability more often then neurons

with comparatively lower excitability, thus leading to the statistical regularities reflected in preplay. Consequently, during preplay and then spatial exploration, neurons with the lowest spike thresholds would on average be recruited first, followed by others with the next higher spike thresholds and so on. From the perspective of memory allocation, these studies provide yet another piece of evidence that memory allocation is not random and that instead there are mechanisms that determine ahead of time which neurons may be involved in a given memory.

Studies in the piriform cortex also support the idea that increases in excitability have a critical role in determining the neuronal ensemble encoding a given memory (Choi, Stettler *et al.* 2011). The activation of a random sub-population of piriform cortex neurons with Channelrhodopsin-2, paired with either an aversive or an appetitive stimulus, is sufficient to allocate these activated neurons to storing that information. This suggests that in the piriform cortex, as in the amygdala and perhaps the hippocampus, increases in neuronal firing (in this case driven by activation of Channelrhodopsin-2) are sufficient to bias the allocation of both appetitive and aversive memories of specific subpopulations of neurons.

Linking memories with memory allocation mechanisms

One of the proposed roles of memory allocation is to link memories that occur within a defined temporal window (Silva, Zhou *et al.* 2009). The idea is that the

first memory-creating event activates CREB in a subpopulation of neurons; this activation leads to an increase in excitability in these neurons that then biases the storage of memory for a second event to many of the same neurons that stored the first event. Because of the overlap between the memory traces for the two events, recall of one event may also lead to the recall of the other. The result would be the coordinated storage and retrieval of related memories. Although this hypothesis has not been directly tested, the evidence reviewed here is consistent with its predictions.

How could the brain link two separate memories? Recent studies (Garner, Rowland *et al.* 2012) addressed this important question with transgenic mice that express DREADD (designer receptor exclusively activated by designer drug) receptors. The artificial ligand CNO binds to the transgenic DREADD receptors and triggers strong depolarization and spiking. In this study, DREADD was expressed under the control of the activity dependent c-fos promoter and the tetracycline inducible system, so that DREADD could be expressed in an inducible and activity dependent manner. The results showed that in a new environment (*i.e.* context A), these transgenic mice expressed DREADD receptors in active neurons that were turned on by the c-fos promoter. Later this ensemble of neurons expressing DREADD receptors was reactivated by CNO, while the mice were conditioned in a different context (context B). In order to recall the memory both populations of neurons need to be simultaneously activated at a later time. The results suggested that the transgenic mice formed a

memory representation that integrated or linked contexts A and B. It is likely that CNO driven activation of the representation of context A biased the allocation of context B to many of the same neurons that stored context A, thus closely integrating the memories for both contexts. These findings suggest that memory allocation mechanisms could be one of the reasons why recalling one memory while encoding another can result in the linking or integration of the two memories (Howard and Kahana 2002).

Beyond increases in neuronal excitability, what other physiological mechanisms could underlie the integration of separate memories? The findings from a study with calcium imaging and electrophysiological stimulation in rat hippocampal slices suggested that synaptic plasticity could contribute to possible post-training shifts in neuronal allocation (Yuan, Isaacson *et al.* 2011). The results indicated that initially distinct neuronal ensembles (*i.e.* distinct memories?) can become more similar after co-activation of these two neuronal ensembles with stimulation protocols designed to trigger synaptic plasticity. The authors stimulated a set of hippocampal Schaffer collateral inputs and visualized the activated CA1 pyramidal neurons with calcium imaging. This was then repeated for a distinct set of Schaffer collateral inputs. Following paired stimulation of the two inputs expected to trigger synaptic plasticity, the authors showed that the overlap between the ensembles of activated CA1 pyramidal neurons increased significantly. This suggests a mechanism for neuronal allocation in which the neuronal ensembles encoding two distinct memory traces can change and

become linked (*i.e.* greater overlap in the neurons engaged by each memory) due to their coordinated activation (*i.e.* recall?) in the hippocampus.

These examples of neuronal allocation in the amygdala, hippocampus and cortex demonstrate that there are mechanisms that determine which neurons store a given memory in a neurocircuit. As we previously proposed, these neuronal allocation mechanisms may function to link memories and modulate their storage and retrieval (Silva, Zhou *et al.* 2009). Next, we will describe evidence for synaptic allocation mechanisms. Although neuronal and synaptic allocation mechanisms have a different history and have been studied separately, we propose that they are seamlessly integrated.

Fig. II-1

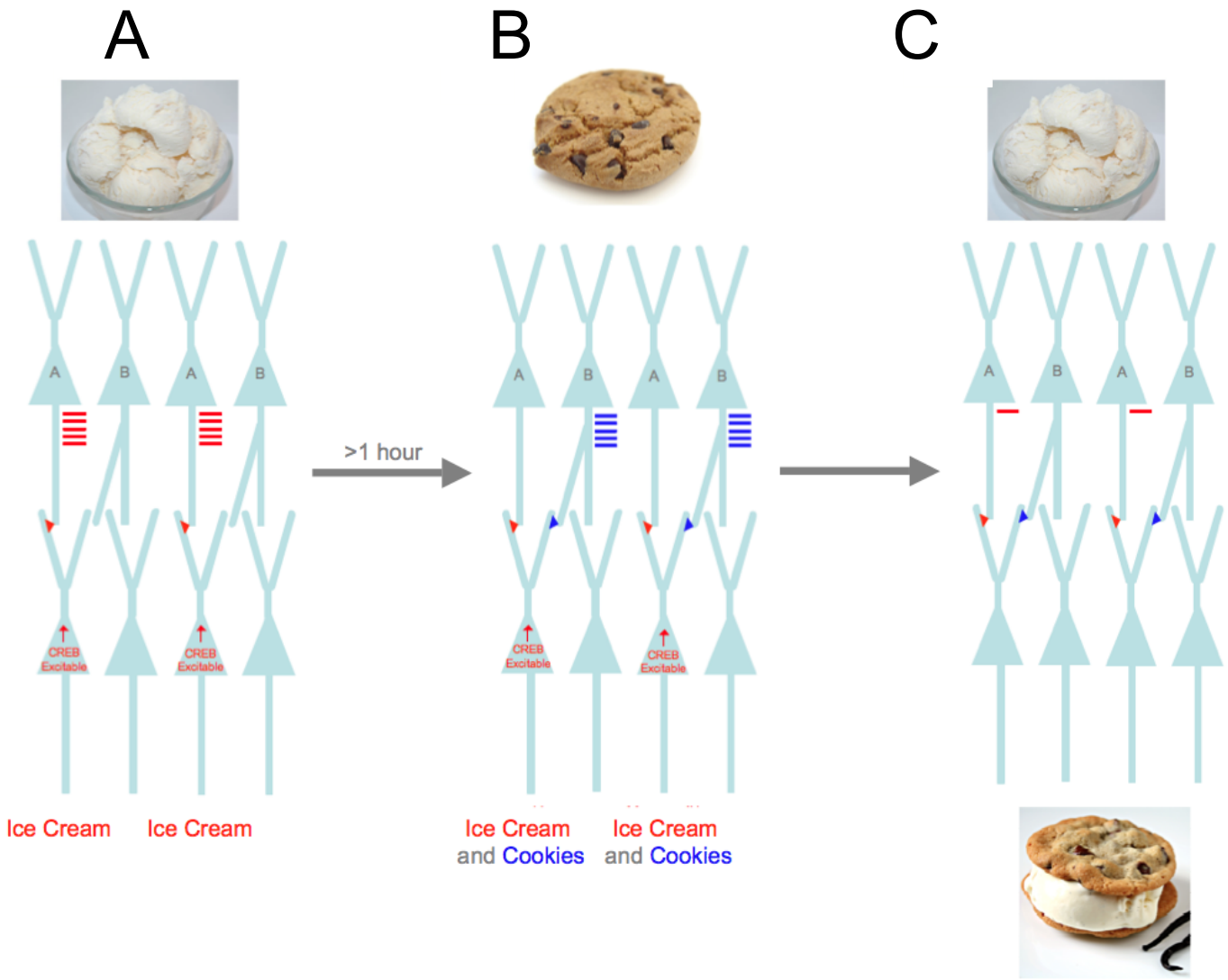


Figure II-1 - Neuronal allocation determines which neuronal ensemble will encode a given memory.

A) Ice cream activates neuronal ensemble A but not neuronal ensemble B. This induces CREB expression in the post-synaptic targets of neuronal ensemble A resulting in their increased excitability.

B) Cookies activate neuronal ensemble B and the memory trace for cookies will be biased towards the post-synaptic neurons it shares with ice cream due the their increased excitability. In this way, ice cream and cookies will be stored in an overlapping population of neurons, thereby linking the two memories.

C) At a later time, recall of ice cream may prompt the recall of ice cream and cookies.

2.3 Synaptic allocation

Synaptic allocation encompasses any mechanism that governs how specific synapses come to store a given memory. Inherent in the idea of synaptic allocation is the concept that multiple synapses could be activated by a given set of inputs, but specific mechanisms determine which synapses actually go on to encode the memory. There is evidence, for example, that synapses do not always respond identically to a given stimulation pattern (Varga, Jia *et al.* 2011) and that a synapse's history of activation can affect its responses, a phenomenon referred to as metaplasticity (Abraham and Bear 1996; Mockett and Hulme 2008). Additionally there is also extensive evidence that the stable potentiation of a given synapse(s) can under certain circumstances affect how other synapses in the same neuron respond to plasticity inducing stimuli, a phenomenon referred to as synaptic tagging and capture (Frey and Morris 1998; Redondo and Morris 2011). All of these mechanisms may shape how synapses are recruited to encode a given memory, how memories become linked in neurocircuits and whether or not they will be remembered.

Molecular and cellular studies of synaptic allocation

Synaptic tagging and capture mechanisms provide a compelling example of memory allocation at the synaptic scale. The idea of synaptic tagging was developed to explain how input specificity is achieved during long-term potentiation (LTP) (Frey and Morris 1998; Redondo and Morris 2011). A

mechanism was needed to account for the fact that many plasticity related proteins (PRP), essential for the maintenance of LTP (Krug, Lossner *et al.* 1984; Frey, Krug *et al.* 1988) and long-term memory (Agranoff and Klinger 1964), are generated in the cell body but only specific synapses (Andersen, Sundberg *et al.* 1977; Lynch, Dunwiddie *et al.* 1977) are potentiated. The synaptic tagging and capture hypothesis proposes that the synapses activated during LTP induction become tagged in a protein synthesis independent manner. These tagged synapses then capture plasticity-related proteins (PRPs) needed for the maintenance of LTP and by extrapolation long-term memory (**Fig. II-2**) (Frey and Morris 1998; Redondo and Morris 2011).

The initial experimental evidence (Frey and Morris 1997) in support of the synaptic tagging and capture hypothesis came from a series of elegant two-pathway in vitro electrophysiological studies in which two stimulating electrodes (S1 and S2) were placed in independent inputs to the same population of rodent hippocampal CA1 neurons. In agreement with previous experiments demonstrating the specificity of LTP (Andersen, Sundberg *et al.* 1977; Lynch, Dunwiddie *et al.* 1977), repeated strong tetanization of S1 could elicit a lasting protein synthesis dependent LTP (late-LTP or L-LTP) in S1 but not in S2 inputs. Surprisingly, following induction of L-LTP in the S1 pathway repeated tetanization of the S2 pathway was able to induce L-LTP even in the presence of protein synthesis inhibitors! Perhaps, the proteins needed for the maintenance of L-LTP, generated during the repeated tetanization of the S1 pathway, could be shared

by synapses tagged during S2 tetanization, and therefore support late-LTP in this second pathway. Accordingly, a weak tetanization of the S2 pathway, that could only elicit a transient potentiation (early-LTP or E-LTP), if preceded one hour earlier by L-LTP induction in the S1 pathway, is capable of inducing L-LTP in the S2 pathway (Frey and Morris 1997). Perhaps, E-LTP was sufficient to tag the S2 set of synapses, that were then capable of capturing the proteins needed for the maintenance of L-LTP generated by repeated tetanization of the S1 pathway.

Later studies (Frey and Morris 1998) using similar techniques confirmed that the sub-threshold E-LTP inducing tetanization could precede the repeated L-LTP tetanization by up to an hour and still be converted to L-LTP! This suggested that the tag set during E-LTP can be maintained for up to an hour. Remarkably, long-term depression (LTD) also appears to be capable of taking advantage of this synaptic tagging and capture mechanism (Sajikumar and Frey 2004): short lived LTD in one set of synapses can be converted into long-lasting or late-LTD by L-LTP at another set of synapses in the same neurons.

Converging evidence for the synaptic tagging and capture hypothesis came from studies conducted on cultured *Aplysia* neurons (Martin, Casadio *et al.* 1997). These compelling studies used a neuronal culture system where a single Aplysia sensory neuron makes synaptic connections with two physically separate motor neurons. In this elegant culture system, five pulses of serotonin to the synapses between the sensory and the motor neurons trigger a long-term facilitation of

synaptic transmission (LTF - *Aplysia* equivalent of L-LTP), whereas a single pulse of serotonin only generates a short-term facilitation (STF - *Aplysia* equivalent of E-LTP). In agreement with the synaptic tagging findings in rodents, STF induced in a set of synapses of the sensory neuron can be converted into LTF, by inducing LTF in another set of synapses of the same sensory neuron. Presumably, STF generates tags that can then be used to capture plasticity proteins generated by LTF in other synapses of the same sensory neuron. Subsequent studies established that the temporal properties of the tag were similar to those in the rodent hippocampus, and that synaptic tagging and capture required PKA activity and CREB function (Casadio, Martin *et al.* 1999). Interestingly, CREB is also needed for neuronal allocation (Silva, Zhou *et al.* 2009).

Recently, pharmacologic studies explored the molecular underpinnings of synaptic tagging (Ramachandran and Frey 2009; Redondo, Okuno *et al.* 2010). These studies suggested that CaMKII and actin remodeling are important for setting the tag, and that the CaMKK-CaMKIV-CREB pathway is important for the synthesis of plasticity proteins presumably shared between tagged synapses. These proteins could include ARC, GluR1, Homer1a and PKM zeta. Interestingly low frequency stimulation (LFS) in conjunction with the application of D1/D5 agonists, brain-derived neurotrophic factor (BDNF), and carbachol can induce L-LTP, suggesting that these molecules may mediate the effects of repeated tetanization, and trigger the production of proteins needed for the maintenance of

L-LTP (Auerbach and Segal 1994; Messaoudi, Ying *et al.* 2002; Navakkode, Sajikumar *et al.* 2007).

Two-photon glutamate uncaging and fluorescence lifetime imaging were used to show that induction of LTP at one spine (*i.e.* reflected in spine enlargement) can affect the probability of LTP induction at a nearby spine given sub-threshold stimulation (Harvey, Yasuda *et al.* 2008). The probability of this synaptic "crosstalk" is inversely related to both distance between spines and time between inducing stimuli. Other studies have also uncovered some of the molecular mechanisms that regulate this local facilitatory process. For example, following LTP induction, calcium-dependent Ras activity increases for ~5min within the activated spine, diffuses ~10um into the adjacent dendrite and invades neighboring spines. This spread of Ras signaling affects the threshold for LTP induction locally, perhaps via its ability to briefly (~1 min) increase AMPAR exocytosis, leading to synaptic strengthening, within and around the stimulated spine (Harvey, Yasuda *et al.* 2008; Patterson, Szatmari *et al.* 2010). Furthermore, activated RhoA is able to briefly (~5 min) diffuse up to 5um from stimulated spines, and could also serve as another mechanism for local synaptic crosstalk (**Fig. II-2**) (Murakoshi, Wang *et al.* 2011).

Recent work has used two-photon uncaging of glutamate paired with two-photon imaging and electrophysiological recordings to study synaptic tagging and capture like processes at the level of individual spines (Govindarajan, Israely *et*

al. 2011). The results indicated that E-LTP at one spine can be converted to L-LTP when L-LTP is previously induced at a nearby spine. LTP was measured as an increase in spine volume with two-photon microscopy and validated with perforated patch electrophysiological recordings. The E-LTP to L-LTP conversion process is protein synthesis dependent, and interestingly, temporally asymmetric. Specifically, prior E-LTP can be converted to L-LTP by L-LTP induction at a nearby spine ~2hrs following E-LTP induction. However, after L-LTP, E-LTP can be converted to L-LTP at a nearby spines within ~1.5hrs after L-LTP induction (Govindarajan, Israely *et al.* 2011).

Beyond time, distance between activated spines also seems to be critical for synaptic allocation mechanisms. Imaging studies suggested that there is an inverse relationship between spine participation in synaptic tagging and capture and inter-spine distance with little to no synaptic tagging and capture induction if spines are more than 70um apart on the same dendritic branch. Further, less synaptic tagging and capture is observed if spines are located on different dendritic branches. Finally, simultaneous induction of L-LTP at two nearby spines causes a reduction in spine growth rate (thought to be correlated with synaptic potentiation), suggesting competition for a limited pool of plasticity related molecules by nearby synapses (Govindarajan, Israely *et al.* 2011). These imaging results extend the original electrophysiological findings concerning synaptic tagging and capture mechanisms. They showed that there are not only temporal constrains, but also structural constrains that limit synaptic tagging and

capture mechanisms. Activity induced protein synthesis, localized to spine neighborhoods (Steward and Schuman 2001; Martin and Kosik 2002), may account for the inverse relationship between distance and the probability that a spine participates in synaptic tagging mechanisms (Govindarajan, Kelleher *et al.* 2006).

Metaplasticity and synaptic allocation

One of the key components of the allocation model introduced at the beginning of this review is that the acquisition and storage of one memory changes a neurocircuit in such a way as to affect the storage and properties of another subsequent memory. Similarly, the synaptic tagging studies described above suggest that one memory can affect the synaptic mechanisms that set the thresholds for storage for a subsequent memory. Analogously, there is extensive evidence that an episode of synaptic plasticity can affect the properties of subsequent synaptic plasticity (*e.g.*, whether a synapse gets potentiated or depressed). Metaplasticity has been coined the plasticity of plasticity and is known to regulate both LTP and LTD (Abraham and Bear 1996; Mockett and Hulme 2008).

Metaplasticity can integrate bouts of synaptic plasticity that are separated by minutes to days (Abraham and Bear 1996; Mockett and Hulme 2008). Homosynaptic metaplasticity and heterosynaptic metaplasticity refer to whether the modulation of the subsequent plasticity is at the same or different synapses.

Cellular excitability has been proposed as one of the mechanisms responsible for heterosynaptic metaplasticity (Frick and Johnston 2005). As we described above, CREB activation during learning induces changes in cellular excitability implicated in neuronal allocation. It is possible that these changes in excitability also help to mediate other potential memory phenomena, such as heterosynaptic metaplasticity.

Metaplasticity, synaptic tagging and capture mechanisms have important implications for memory allocation. They suggest a set of rules that modulate the interaction between memories allocated to an overlapping neuronal population. The studies that were described above suggest that the synaptic mechanisms engaged by one memory can change the synaptic rules for storing a subsequent memory, a finding with profound implications for memory storage. For example, the synaptic tagging studies reviewed above suggest that under certain circumstances, a weak memory, that would otherwise be forgotten, could be strengthened and stabilized by a strong memory provided they were encoded within certain time constraints by synapses in the same neurons. Next, we will review a number of studies that pertain to the impact on memory of allocation mechanisms.

Behavioral implications of synaptic allocation

As discussed earlier, the synaptic tagging and capture hypothesis has three critical components with possible behavioral implications: 1) A weak synaptic

input creates a temporary synaptic-tag; 2) A strong synaptic input (to the same neuron) triggers the induction of plasticity related proteins (PRPs), which can be shared with tagged synapses of weak inputs; 3) Due to the shared PRPs, the synapses of the weak input can undergo long-lasting changes and these changes are dopamine- and protein synthesis-dependent. These properties of synaptic tagging make the following behavioral predictions: 1) A strong, long-lasting memory can convert a short-term weak memory into a stronger long-term memory; 2) This conversion from an unstable to a stable memory by another strong memory should be dopamine- and protein synthesis-dependent.

Behavioral experiments have uncovered interactions between memories that fit the defining features of the synaptic tagging and capture hypothesis (Moncada and Viola 2007). These experiments with rats showed that a weak inhibitory avoidance memory (that only lasted for a few hours) could be converted into a stronger inhibitory avoidance memory (that lasted for days), if the rats were first exposed to a novel environment (but not to a familiar environment) one-hour before training. Moreover, this conversion from a weak to a strong memory was both dopamine and protein synthesis dependent. The implications are that weak inhibitory avoidance training tagged a set of synapses encoding this training, and that the exposure to the novel environment created PRPs that were shared with the tagged synapses allocated to the weak memory. These shared PRPs then strengthened and stabilized the weak memory for the mild inhibitory avoidance training.

A follow-up study showed that weak memories for spatial object recognition, contextual fear conditioning and conditioned taste aversion (all lasting on the order of a few hours) could also be converted into long-lasting memories (lasting for days) if preceded by exposure to a novel experience (Ballarini, Moncada *et al.* 2009). In all cases, protein synthesis was required during exposure to the novel environment. Interesting, human learning and memory studies have shown that changing the context where training takes place can strengthen memory (Dewar, Cuddy *et al.* 1977), a result consistent with these findings and the predictions of the synaptic tagging and capture hypothesis. The idea is that the novel context in these human studies strengthens otherwise weak memories, just as the novel open field strengthens weak memories in rats.

Parallel behavioral and electrophysiological studies of synaptic tagging uncovered compelling evidence for this hypothesis (Wang, Redondo *et al.* 2010). Rats were given one trial per day to find food in different spatial locations, and later had to recall that day's spatial location. A weak food reward led to weak encoding of the food's spatial location that was quickly forgotten. However, when followed by novelty exploration 30 minutes later, a weak encoding episode triggered a long-lasting memory for the food's location. Similarly, strong tetanization, analogous to exposure to a novel environment, both induced late-LTP and converted early- into late-LTP on an independent but convergent

pathway. Again, these processes required hippocampal dopamine D1/D5 receptor function and protein synthesis (Wang, Redondo *et al.* 2010).

Interestingly, behavioral studies, with some of the same tasks used in the experiments above, do not always lead to the predicted memory enhancements derived from these task interactions. For example, a previous study suggested that exposure to a novel open field an hour after inhibitory avoidance training actually impairs the original inhibitory avoidance memory (Izquierdo, Schroder *et al.* 1999). The impairment was observed for both strong and weak avoidance training. There was no impairment, however, with a shorter delay (*i.e.* 5 minutes), a long delay (*i.e.* 6 hours), or when the open field was familiar. These results suggest that the exact parameters (*e.g.*, strength of training, length of intervals, novelty and other characteristics of the open field), of these experiments may matter, and that we have only started to tap the complexity that regulates the interactions between memory-encoding events.

Learning and spine clustering

There is growing evidence for the hypothesis (Poirazi and Mel 2001) that inputs with functional similarities are organized in clusters within the dendrites of pyramidal neurons (Govindarajan, Kelleher *et al.* 2006). Spine clustering is thought to result in the amplification of synaptic inputs due to the non-linear properties of the induction and propagation of dendritic spikes (Losonczy and Magee 2006). Recent findings reviewed above suggest that there are synaptic

allocation mechanisms that account for the clustering of spine changes (Govindarajan, Kelleher *et al.* 2006). A recent study found spine elimination in the hippocampus after fear conditioning (Sanders, Cowansage *et al.* 2012). Remarkably, another study found that fear conditioning and extinction have opposite effects on spine dynamics on layer-V pyramidal neurons in the dorsal medial region of the frontal association cortex, a region implicated in fear condition: auditory-fear conditioning caused a stable *loss* of dendritic spines that correlated strongly with memory on recall (Lai, Franke *et al.* 2012). More striking is the finding that extinction of this specific fear memory induced the formation of spines within 2um on either side of the spines that had been lost following conditioning. Additionally, the spines added following extinction had similar orientation as those lost during conditioning, as if they shared afferents. The implication is that the frontal cortex is involved in fear conditioning and extinction, and that in this region the allocation of memory for extinction is clustered around sites allocated to storing memory for conditioning. This is an interesting and surprising result since there is a large amount of data demonstrating that extinction is not simply the reversal of conditioning (Quirk and Mueller 2008), but instead involves new learning.

A recent elegant demonstration of synaptic clustering of functionally related inputs, paired two-photon *in vivo* imaging (Fu, Yu *et al.* 2012) of synaptic dynamics in motor cortex with a forelimb motor skills task. This study reported clustered addition of spines onto dendrites of Layer 5 pyramidal cells in motor

cortex, while mice learned to reach for a seed. The authors found that following motor learning, spines are added in small groups near existing stable spines. Additionally, this clustered addition of spines occurs in a task dependent manner; that is, spines that appear after training to handle and eat capellini do not cluster with spines that appear after training to reach for a seed, confirming the idea that spine clustering reflects the functional congruence of clustered inputs (Fu, Yu *et al.* 2012). While neuronal allocation mechanisms are thought to primarily link related but distinct memories, multiple exposures to the same information may result in spine clustering and thus strengthening of a given memory.

Molecular mechanisms for synaptic clustering

A potential mechanism for the clustered addition of spines is the aforementioned diffusible molecular crosstalk that occurs near activated spines. Previous studies suggested that signaling molecules synthesized at one spine, such as activated Ras (Harvey, Yasuda *et al.* 2008; Murakoshi, Wang *et al.* 2011), diffuse out and may support spine changes in other sites nearby, thus contributing to spine clustering. Additional two-photon imaging studies with hippocampal slices also implicated another Rho GTPase, Cdc42, in long-term spine volume increases triggered by spine specific stimulation with two-photon glutamate uncaging. These structural changes, as well as the activation of RhoA and Cdc42, were shown to be dependent on a calcium-activated kinase with a critical role in LTP and learning: the calcium calmodulin kinase II (CaMKII) (Harvey, Yasuda *et al.* 2008; Murakoshi, Wang *et al.* 2011). Beyond RhoA, Cdc42 and CaMKII, several

other molecules have been implicated in spine formation and dynamics, such as Tiam1 and beta-PIX. These molecules may also be required for spine clustering (Tolias, Duman *et al.* 2011).

Additionally, it has been shown that induction of LTP leads to addition of new spines which contact the same presynaptic component (Toni, Buchs *et al.* 1999). This hints at a role for transynaptic signaling in synaptic allocation. Later work suggests that this process may in part be regulated my NMDAR activation and nitric oxide signaling (Nikonenko, Jourdain *et al.* 2003).

Fig. II-2

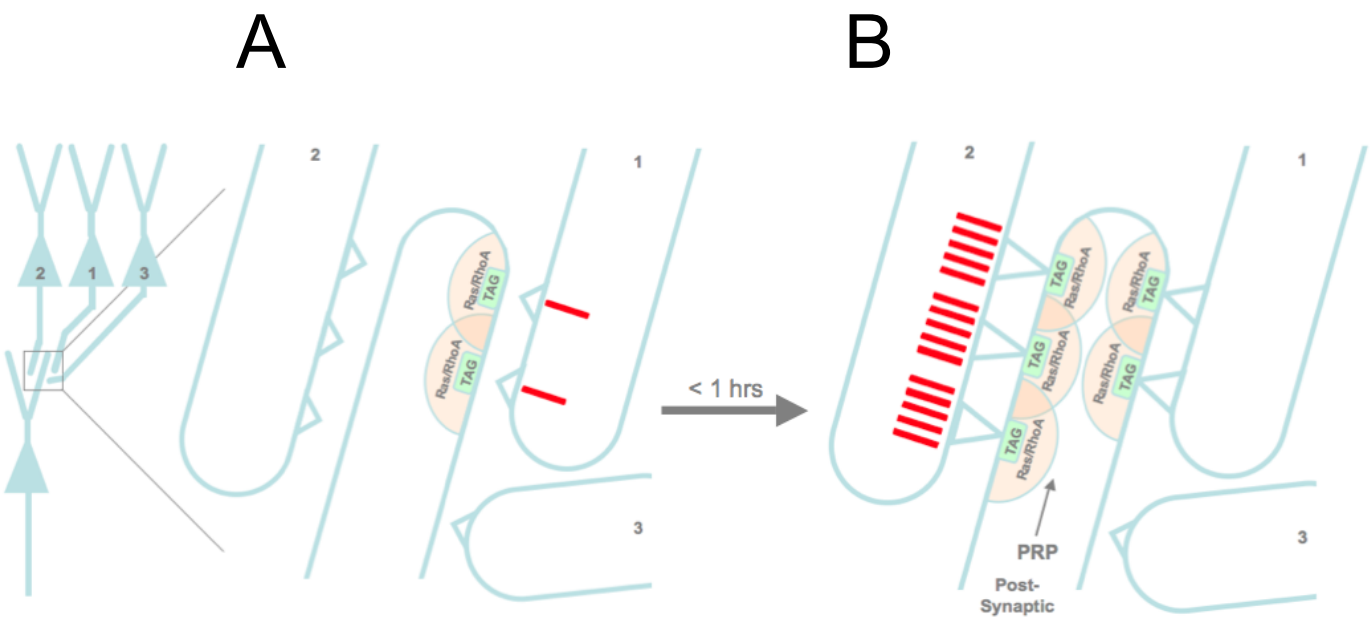


Figure II-2 - Synaptic allocation determines which neuronal ensemble will encode a given memory.

A) Neuron 1 fires weakly, resulting in the formation of synaptic tags at the synapses adjacent to it. This weak firing is not sufficient to result in plasticity-related protein production in the post-synaptic neuron and long term memory consolidation.

B) Within an hour, neuron 2 fires strongly, leading to the formation of synaptic tags at the synapses adjacent to it and to the production of plasticity-related proteins in the post-synaptic neuron needed for synaptic strengthening. These plasticity-related proteins also lead to the strengthening of the synapses tagged due to the firing of neuron 1. The diffusion of Ras and RhoA from activated synapses promotes synaptic clustering. Neuron 3 is inactive and its synapses are not strengthened.

2.3 Synergism of Synaptic and Neuronal Allocation

Although the study of synaptic and neuronal allocation mechanisms have separate histories, these two processes are likely to be closely interconnected. For example, without mechanisms that recruit a given neuron to encode the strong and weak memory events, synaptic tagging and capture could not take place. Similarly, neuronal allocation mechanisms involving CREB-dependent changes in excitability are dependent on mechanisms that recruit specific synapses to store the information in question. Therefore, we propose that synaptic and neuronal allocation mechanisms work hand-in-hand to determine where memories are stored in neurocircuits.

For example, suppose that the formation of a strong memory induces L-LTP in a subset of synapses in neuronal ensemble 1, but not in neuronal ensemble 2. For at least one hour after strong training, neuronal ensemble 1 would be able to share plasticity related proteins that could convert a weak memory into a strong one: The training for the weak memory would be able to elicit L-LTP in neuronal ensemble 1 (due to the presence of plasticity related proteins) but not in neuronal ensemble 2. This is an example of how synaptic tagging and capture could determine which neurons (not just synapses) would encode a given memory (**Fig. II-3)**. Conversely mechanisms typically associated with neuronal allocation (*e.g.* CREB dependent changes in excitability) may also play a role in synaptic allocation. For example, changes in neuronal excitability may affect some

dendrites more than others, thus biasing memory storage to those synapses in dendrites with higher excitability (Larkum and Nevian 2008; Losonczy, Makara *et al.* 2008).

Memory allocation and cognitive deficits

Since memory allocation mechanisms are crucial for determining where memories are stored in neuronal networks, it is possible that deficits of memory allocation could lead to cognitive pathologies. Accordingly, aberrant levels of CREB expression and neuronal excitability have been reported in animal models of human neurological and psychiatric disorders. For example, aberrant CREB activation is associated with Alzheimer's disease (AD) (Yamamoto-Sasaki, Ozawa *et al.* 1999; Satoh, Tabunoki *et al.* 2009). Interestingly overexpression of CBP or CREB in the hippocampal CA1 region seems to rescue the spatial memory deficits of a transgenic mouse model of AD (Caccamo, Maldonado *et al.* 2010; Yiu, Rashid *et al.* 2011). In animal models of AD, elevated amyloid beta causes a deregulation of Ca2+ and K+ channels resulting in abnormal intrinsic neuronal excitability (Santos, Pierrot *et al.* 2010). These findings suggest that elevated amyloid beta results in altered CREB function, which then alters neuronal excitability and consequently could affect memory allocation mechanisms. Alterations of these mechanisms could contribute to the cognitive deficits associated with AD.

Age-related change in neuronal excitability may also lead to aberrations in memory allocation. It is well documented that aging leads to cognitive deficits,

especially hippocampus-dependent memory. It is also known that some of these learning deficits are related to decreases in intrinsic excitability as characterized by the enlarged afterhyperpolarization (AHP) and an increased spike-frequency adaptation (accommodation) of hippocampal neurons in older mice (Disterhoft and Oh 2007). These alterations in excitability may also lead to deficits in memory allocation, specifically; memories that would otherwise be linked and stored together or memories that might strengthen one another are unable to do so in the case where excitability is low.

Future studies of synaptic allocation

While there is strong and growing evidence for synaptic and neuronal allocation, there is much to be done in this exciting young field. For example, it will be critical to identify the detailed molecular mechanisms underlying both synaptic and neuronal allocation mechanisms. What are the molecular mechanisms involved in synaptic clustering, tagging and capture? What are the plasticity related proteins that are captured by tagged synapses? Additionally, there is a pressing need for tools to image and manipulate both synaptic clustering and synaptic tagging, so that it is possible to study the functional significance of these synaptic allocation mechanisms. For example, it would be important to carry out functional behavioral studies with approaches capable of promoting, preventing and imaging *in vivo* synaptic tagging and synaptic clustering. While elegant studies reviewed here uncovered behavioral evidence that is *consistent* with the synaptic tagging and capture hypothesis, there is still no direct evidence that the

interesting behavioral interactions ascribed to synaptic tagging and capture actually involve these mechanisms.

For example, it is possible that the behavioral interactions ascribed to synaptic tagging and capture were caused by protein-synthesis dependent increases in neuromodulators, such as dopamine, that are unrelated to synaptic tagging mechanisms. To obtain direct evidence of the tagging hypothesis, it will be essential to pair behavioral analysis with techniques that allow the labeling, tracking and manipulation *in vivo* of synapses involved in memory. These experiments should be able not only to visualize the synaptic events underlying the behavioral interactions consistent with the synaptic tagging hypothesis, they ought to also manipulate directly and specifically these synaptic changes, and study the impact on the behavioral interactions. Convergence between these tracking and manipulation studies would make a compelling argument for the function of synaptic tagging and capture mechanisms.

Most studies of neuronal allocation have focused on the effects of CREB. Nothing is known about the molecular cascades upstream and downstream of this transcription factor that are specifically involved in memory allocation. What are the receptor systems and signaling cascades that activate CREB and affect allocation? What are the channels that mediate the increase in excitability thought to underlie neuronal allocation? The answers to these questions, not only in the amygdala, where most of previous studies of neuronal allocation have

been carried out, but also in other brain regions, would be critical to advance the understanding of these important phenomena.

Although most studies of neuronal allocation have focused on molecular and cellular mechanisms, future studies could address circuit properties of memory allocation. For example, it would be important to determine how neuronal allocation in one brain region affects allocation in other interacting regions. In this respect, it would be especially interesting to study neuronal allocation during systems consolidation, where interactions between hippocampal and cortical ensembles may shape the semantic structure of information (Squire and Alvarez 1995; Frankland and Bontempi 2005). Similarly, it would be of great interest to determine how phenomena such as extinction and reconsolidation affect synaptic and neuronal allocation of information, in the amygdala, prefrontal cortex and hippocampus (Quirk and Mueller 2008; Nader and Hardt 2009).

Fig. II-3

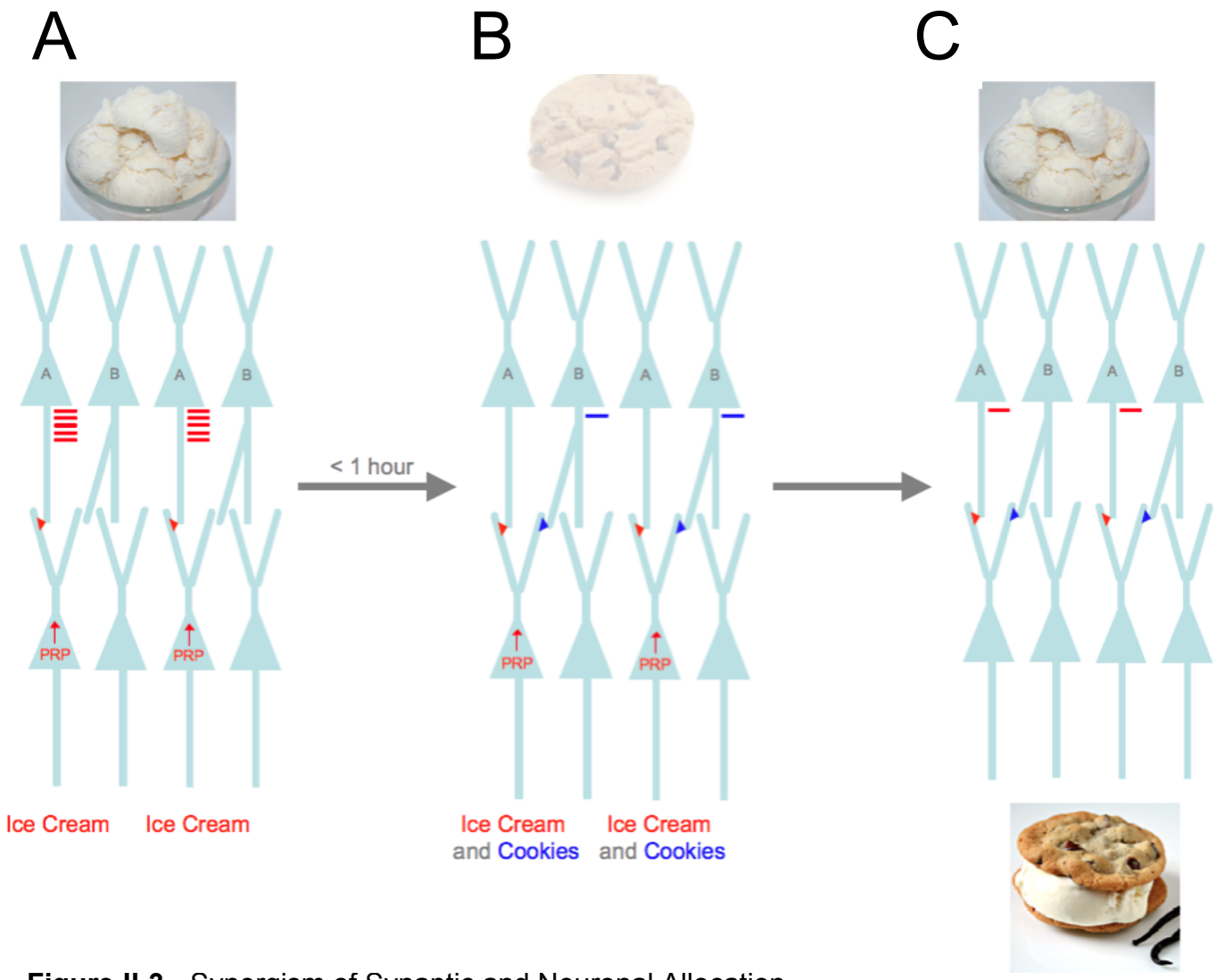


Figure II-3 - Synergism of Synaptic and Neuronal Allocation

A) Suppose that the formation of a strong memory for ice cream (neuronal ensemble A) induces L-LTP in a subset of synapses with post-synaptic target neurons. For at least one hour after strong training, the post-synaptic targets of neuronal ensemble A are able to share plasticity-related proteins that can convert a weak, short-lasting memory into a strong, long-lasting one.

B) A weak memory for cookies (neuronal ensemble B) would be able to elicit L-LTP only onto post-synaptic targets of neuronal ensemble A (due to the presence of plasticity related proteins) but not onto other post-synaptic targets. This is an example of how synaptic allocation can determine which neurons encode a given memory.

C) At a later time, recall of ice cream may prompt the recall of ice cream and cookies.

2.4 Conclusions

The studies reviewed here demonstrate conclusively that there are mechanisms that can determine which synapses and neurons in a neurocircuit will encode a given memory. This process is not random and, instead, is likely to involve mechanisms such as synaptic tagging, spine clustering, metaplasticity, and CREB-dependent changes in excitability. The results also suggest that synaptic and neuronal allocation mechanisms work closely together in shaping the acquisition, stabilization and recall of information in the brain.

CHAPTER III

Silencing of a CREB Allocated Memory Trace

3.1 Introduction

The initial groundbreaking results of Han *et al.* in 2007 demonstrated that CREB plays a role in memory allocation. Han *et al.* infected cells of mouse BLA with either a virus over-expressing CREB or with a control protein β-galactosidase (LacZ). Han *et al.* then performed auditory-fear conditioning and examined whether neurons containing CREB or LacZ were disproportionately represented in the memory trace. Their results showed that neurons over-expressing CREB in the BLA were three times more likely to be incorporated into the memory trace. In the β-galactosidase control, no bias towards or away from the memory trace was detected. Interestingly, the proportion of memory positive cells (~25% of eligible neurons) remained constant in all experiments, suggesting that a competitive process determines which neurons will be brought into the memory trace (Han, Kushner *et al.* 2007; Silva, Zhou *et al.* 2009). For a more detailed explanation of these results, refer to Chapter 1.2. These results prompted new questions, chief among them being whether or not the CREB biased memory neurons are necessary for memory. Research championed by Yu Zhou and Rafal Czajkowski in the nascent years of my Ph.D. directly addressed this issue, refer to Chapter 3.2-3.5. The other pressing question was whether activation of these CREB biased memory neurons would be sufficient to trigger memory. Experiments tackling this question are presented in Chapter 4.

3.2 Inhibition of a CREB allocated emotional memory trace

Yu Zhou, a post-doctoral fellow in Professor Alcino Silva's laboratory when I joined it, spearheaded the project to determine whether or not CREB biased memory neurons are necessary for emotional memory. Yu Zhou mentored me in my early years in the Silva laboratory, and I was fortunate to work with her on these key experiments and to be an author on her paper (Zhou, Won *et al.* 2009). The work presented here is a summary of the parts of that work to which I contributed.

3.3 Methods, results and conclusions

In order to address whether or not CREB biased memory neurons are necessary for emotional memory, we took advantage of the newly discovered allatostatin receptor (AlstR) to inactivate the basolateral amygdala (BLA) neurons infected with CREB (Lechner, Lein *et al.* 2002; Tan, Yamaguchi *et al.* 2006). AlstR is an insect receptor that, when activated, can turn on endogenous mammalian G-protein coupled inwardly rectifying K^+ (GIRK) channels, resulting in a hyperpolarization of the neurons expressing it. The peptide ligand for the AlstR is allatostatin, which is not found in mammals. Jaejoon Won, also an author of the paper, developed a replication defective herpes simplex virus that expressed both CREB and AlstR (HSV-CREB-AlstR) and, as a control, a separate virus that expressed LacZ and AlstR (HSV-LacZ-AlstR) (**Fig. III-1A**). Employing these viruses, it was possible to specifically inhibit the firing of the viral CREB or the viral LacZ expressing neurons by applying allatostatin and to observe the effect that this had on behavior.

Employing methods analogous to those described in Han et al.'s 2007 paper, we infected the BLA of one cohort of mice with HSV-CREB-AlstR and another cohort of mice with HSV-LacZ-AlstR. In both cohorts, only a fraction (~20%) of the total number of BLA neurons was infected. Utilizing *ex vivo* electrophysiological methods, Yu Zhou demonstrated that AlstR could indeed inhibit the neuronal

activity of infected neurons. We hypothesized that inhibiting the activity of HSV-CREB-AlstR infected neurons following auditory-fear conditioning should have a greater effect on memory than inhibition of the control HSV-LacZ-AlstR infected neurons. This hypothesis had its basis in the 2007 work of Han *et al.*, which demonstrated that those neurons with higher levels of CREB contained more of the memory trace.

The HSV-CREB-AlstR and the HSV-LacZ-AlstR cohorts were auditory-fear conditioned and were then divided in half. One half of each cohort then received allatostatin and the other half vehicle. Thirty minutes later all four groups HSV-CREB-AlstR – allatostatin (HSV-CREB-AL), HSV-CREB-AlstR – vehicle (HSV-CREB-VEH), HSV-LacZ-AlstR – allatostatin (HSV-LacZ-AL) and HSV-LacZ-AlstR – vehicle (HSV-LacZ-VEH) were tested for auditory-fear memory (**Fig. III-1B**). The thirty minute time point was chosen in order to observe CREB's role in memory allocation without the confounds of its known role in memory consolidation (Bourtchuladze, Frenguelli *et al.* 1994). Both the HSV-CREB-VEH and the HSV-LacZ-VEH cohorts froze to a similar extent (~50%), demonstrating that CREB was not enhancing memory as would be expected at later times due to its role in consolidation. Critically, the HSV-CREB-AL group did exhibit diminished freezing (~30%), demonstrating that the CREB biased memory neurons are necessary. The HSV-LacZ-AL cohort froze to a similar extent (~50%) to the HSV-CREB-VEH and the HSV-LacZ-VEH cohorts (**Fig. III-1C**). This demonstrates that after auditory-fear conditioning, inactivation of the CREB-

expressing neurons (HSV-CREB-AL), which could have contained ~60-70% of the memory trace (based on data from Han, Kushner *et al.* 2007), led to a pronounced reduction in freezing. Importantly, following auditory-fear conditioning, silencing of a random population of neurons (HSV-LacZ-AL), which by chance could have contained ~10-20% of the memory trace (based on data from Han, Kushner *et al.* 2007), did not lead to a reduction in freezing.

In a separate experiment, two cohorts of mice were infected with HSV-CREB-AlstR and were auditory-fear conditioned and then divided in half. Twenty-four hours later, one half received allatostatin and the other half received vehicle. Thirty minutes after allatostatin or vehicle infusion, both cohorts were tested for auditory-fear memory (**Fig. III-2A**). The HSV-CREB-AlstR group that had the activity of its virally CREB expressing neurons inhibited during testing with allatostatin (AL) showed reduced freezing (~50%) compared to the vehicle (VEH) control (~70%) (**Fig. III-2B**). This again suggests that the memory trace is being biased to the virally CREB expressing neurons and that taking them offline during testing impairs memory. A day later, both groups were tested for auditory-fear memory again, except that the group that had first received allatostatin thirty minutes prior to testing now received vehicle and vice versa. As predicted, the group that received allatostatin but now received vehicle showed the expected level of memory (70%) and the group that had received vehicle but now received allatostatin showed impaired memory (50%) (**Fig. III-2B**).

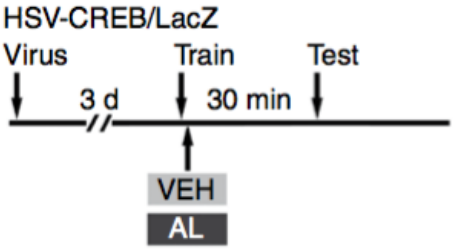
In a third experiment, two cohorts of mice were infected with HSV-CREB-AlstR. Prior to training allatostatin was applied to one cohort and vehicle to the other. One day later, the cohorts were each divided into two groups and received either vehicle or allatostatin thirty minutes prior to auditory-fear testing (**Fig. III-3A**). The cohort that received vehicle prior to training and vehicle prior to testing demonstrated normal learning (~70%). The cohort that received vehicle prior to training and allatostatin prior to testing showed impaired memory (~50%), as would be expected from the previously described experiment. Critically, the cohorts that received allatostatin prior to training and either allatostatin or vehicle prior to auditory-fear testing froze to the same extent (50%) (**Fig. III-3B**). This suggests that taking the virally CREB expressing neurons offline during training resulted in there being no effect on the memory, whether or not those cells were on or offline during testing.

These results are consistent with the hypothesis that CREB plays a role in memory allocation, and also demonstrate that a certain proportion of neurons in the memory trace is necessary for a behavioral manifestation of the memory (freezing). Similar results were obtained with a diphtheria-toxin strategy that ablates neurons expressing viral CREB (Han, Kushner *et al.* 2009).

Fig. III-1

A

B



C

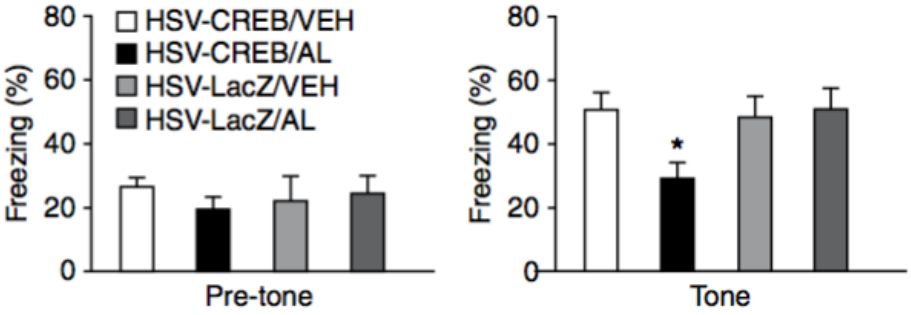


Figure III-1 - Silencing of a CREB biased short-term fear memory

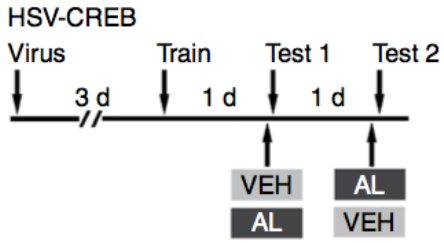
A) HSV-CREB-AlstR and HSV-LacZ-AlstR viral vectors

B) Behavioral design for the four experimental groups: HSV-CREB-VEH, HSV-CREB-AL, HSV-LacZ-VEH and HSV-LacZ-AL. Training (Train) was composed of the presentation of a tone that coterminated with a shock. Animals were then infused with vehicle (VEH) or allatostatin (AL). Thirty minutes later, the animals were presented with the tone in a novel context (Test).

C) Results of the auditory-fear test (Test). For each group, the graph on the left represents the level of freezing before tone presentation (Pre-tone), and the graph on the right represents the level of freezing during tone presentation (Tone). Allatostatin selectively blocked short-term auditory-fear memory in HSV-CREB-AL.

Fig. III-2

A



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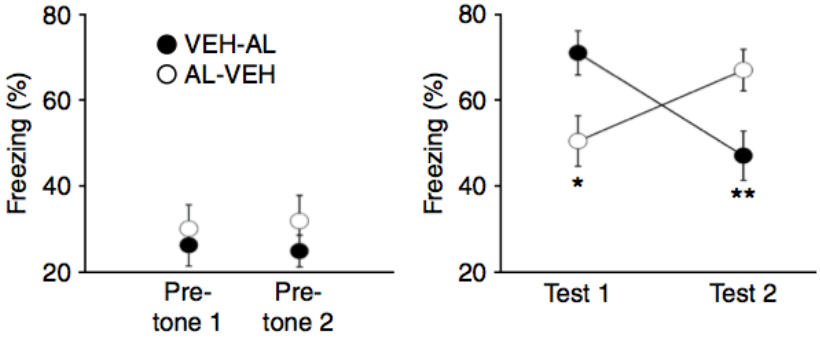


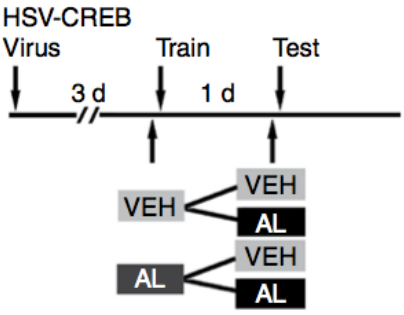
Figure III-2 - Silencing of a CREB biased long-term fear memory

A) Behavioral design for the two experimental groups: VEH-AL and AL-VEH. Training (Train) was composed of the presentation of a tone that coterminated with a shock. One day later, animals were infused with vehicle (VEH) or allatostatin (AL) and presented with the tone in a novel context (Test 1). The next day the animals that were infused with VEH for Test 1 were instead infused with AL (and vice versa) prior to being presented with a tone (Test 2).

B) Results of the auditory-fear test (Test 1 and 2). For each group, the graph on the left represents the level of freezing before tone presentation (Pre-tone 1 and 2), and the graph on the right represents the level of freezing during tone presentation (Test 1 and 2). Allatostatin reversibly blocked long-term auditory-fear memory.

Fig. III-3

A



B

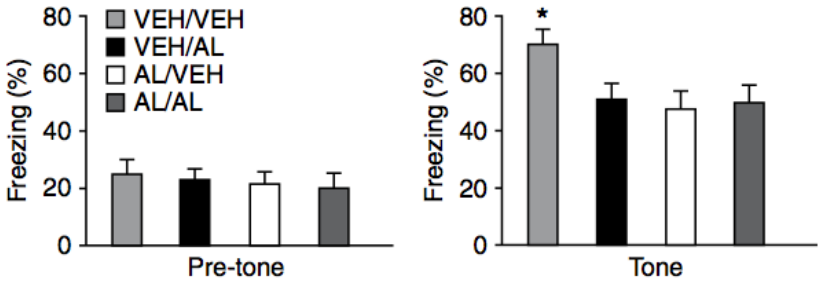


Figure III-3 - Necessity of CREB for silencing of a long-term fear memory

A) Behavioral design for the four experimental groups: VEH/VEH, VEH/AL, AL/VEH and AL/AL. One group of animals was infused with allatostatin (AL) and the other vehicle (VEH) prior to training (Train). Training was composed of the presentation of a tone that coterminated with a shock. One day later, half the animals from each group were infused with VEH the other half received AL. The animals were then presented with the tone in a novel context (Test).

B) Results of the auditory-fear test (Test). For each group, the graph on the left represents the level of freezing before tone presentation (Pre-tone), and the graph on the right represents the level of freezing during tone presentation (Test). Virally CREB expressing neurons need to be online at the time of training to see the affect of AL.

3.4 Inhibition of a CREB allocated spatial memory trace

Rafal Czajkowski, a post-doctoral fellow in Professor Alcino Silva's laboratory at the time I joined the group spearheaded a project to determine the role of the retrosplenial cortex (RSC) in the encoding and recall of spatial memory. Rafal Czajkowski mentored me in my early years in the laboratory, and I was fortunate to be able to work with him on these key experiments and to be an author on his paper (Czajkowski, *et al.* in preparation). The work presented here is a summary of the parts of that work to which I contributed.

3.5 Methods, results and conclusions

The work of Han *et al.* and Zhou *et al.* clearly demonstrates a role for CREB in allocation of emotional memories in the amygdala. Rafal Czajkowski initiated a study of the role of the RSC in spatial memory formation and recall. Part of his study addressed whether CREB has a role in spatial memory formation in the retrosplenial cortex analogous to the one it has for emotional memory in the amygdala. Recently, evidence has emerged that suggests that CREB may play a role in the allocation of spatial memory in the hippocampus. Sekeres *et al.* demonstrated that viral CREB expression in the hippocampus prior to Morris water maze training leads to enhanced spatial memory (Sekeres, Neve *et al.* 2010). An analogous enhancement in memory is seen when viral CREB is expressed in the amygdala prior to auditory-fear conditioning (Josselyn, Shi *et al.* 2001) Professor Josselyn determined that the reason for the memory enhancement in viral CREB overexpressing animals was the fact that the auditory-fear memory was biased to the virally CREB overexpressing neurons (Han, Kushner *et al.* 2007). Yu Zhou *et al.* went on to demonstrate that if memory allocation to viral CREB overexpressing neurons is blocked, there is no memory enhancement (Zhou, Won *et al.* 2009). This provides evidence that an enhancement in memory due to viral CREB overexpression in a subpopulation of neurons is, by necessity, accompanied by memory allocation to that subpopulation of CREB overexpressing neurons (*i.e.* the mere presence of

CREB in a subpopulation of neurons isn't sufficient to enhance memory; for memory enhancement to occur, there must be memory allocation). These results strongly suggest that the CREB enhanced spatial memory that Sekeres *et al.* report is due to memory allocation. Rafal Czajkowski and I built upon what was known about CREB's role in the allocation of memories in the amygdala and hippocampus and applied that to spatial memory in the RSC.

The RSC is connected to the parahippocampal region and anterior thalamus (Jones and Witter 2007; Kononenko and Witter 2012) and is known to play a role in spatial navigation (Aggleton 2008; Vann, Aggleton *et al.* 2009). Utilizing the Morris water maze, a behavioral paradigm designed to test spatial memory in rodents (Morris, Garrud *et al.* 1982), we demonstrated that the RSC is partially necessary for spatial memory. In our experiment, a cohort of mice was trained on the Morris water maze, which is a circular water-filled tank with a small platform just submerged. The mice cannot escape the tank but can seek refuge from swimming by climbing onto the platform, which is not visible to them. The mice are placed in the tank at various positions around the perimeter and come to learn where the platform is by using spatial cues in the room. We trained a group of animals on the Morris water maze for seven days. On the eighth day, the mice were placed in the tank as before, but the platform had been removed (probe trial). By recording the amount of time spent in the vicinity of the now missing platform (target quadrant), it was possible to determine whether the mice had successfully learnt its spatial location (**Fig. III-4A**). The mice spent approximately

60% of their time in the target quadrant, demonstrating that they had learnt the spatial location of the platform (**Fig. III-4B**). The animals were divided into two cohorts of equal spatial learning. They were then subjected to a second probe trial, in which, 10 minutes before placement in the tank, one cohort received an infusion of CNQX to the RSC. CNQX is a transient AMPA receptor antagonist that blocks neurotransmission, resulting in the inactivation of the RSC. The other cohort received saline as a control. The CNQX cohort did not show a deficit in target quadrant occupancy compared to saline control (**Fig. III-4B**) but did show deficits on other more subtle measurements of spatial learning. One measurement that showed such an impairment was the significantly decreased number of target crossings in the CNQX cohort compared to control (**Fig. III-4C**). Another indication of impairment in the CNQX cohort compared to control was the time spent in a circular zone of r=10 cm (but not r=15 cm or r=20 cm) around the target (**Fig. III-4D**). The average proximity to the target during the first 15 seconds (but not at 30s and 60s) was also significantly greater for the CNQX cohort (**Fig. III-4E**). These results demonstrate that inactivating the RSC does subtly impair spatial memory. Since the effect of CNQX is transient, it was possible for both cohorts to be subjected to a third probe trial on the following day. In this trial, no deficits were observed. This data further substantiates the role of the RSC in spatial memory.

In order to determine whether CREB plays a role in spatial memory allocation in the retrosplenial cortex, we utilized the same viral constructs, HSV-CREB-AlstR

and HSV-LacZ-AlstR, that were used in Zhou et al (**Fig. III-1A**). We infected the RSC of one cohort of mice with HSV-CREB-AlstR, resulting in the overexpression of viral CREB in a subpopulation of neurons in this structure. As a control, we infected the RSC of a second cohort with HSV-LacZ-AlstR. We trained both groups on the Morris water maze in an accelerated training protocol that was composed of seven sessions of three trials each in a single day (**Fig. III-5A**). Spatial learning was tested in a probe trial given 24 hours later. We hypothesized that if CREB were playing a role in spatial memory allocation in the RSC, then the HSV-CREB-AlstR cohort would show enhanced learning in a fashion analogous to that seen in previous literature (Josselyn, Shi *et al.* 2001; Zhou, Won *et al.* 2009; Sekeres, Neve *et al.* 2010). As predicted, measurement of target quadrant occupancy, platform crossings and target proximity all showed that the HSV-CREB-AlstR group had learnt to a greater extent than the HSV-LacZ-AlstR infected control (**Fig. III-5B**). To determine whether or not the cells infected with HSV-CREB-AlstR had the spatial memory preferentially allocated to them as predicted, we inactivated these cells using the AlstR receptor system as described in Chapter 3.3. If specific inactivation of the virally CREB expressing neurons resulted in a decrease in the spatial memory enhancemnt, that would provide further proof that the memory was indeed allocated to the virally CREB expressing neurons. Thirty minutes prior to the subsequent probe trial, both cohorts had allatostatin infused into their RSC. In the HSV-CREB-AlstR cohort, the virally CREB overexpressing neurons were hyperpolarized and inactivated. In the case of the HSV-LacZ-AlstR cohort, a random subpopulation of neurons of a

similar size would be inactivated in the RSC. As hypothesized, allatostatin infusion reduced the spatial learning enhancements seen in the HSV-CREB-AlstR cohort (**Fig. III-5B**). This data confirms that CREB plays a role in the allocation of spatial memory in the retrosplenial cortex analogous to the one it plays in emotional memory in the amygdala. Importantly, these results also demonstrate that CREB biased memory neurons are necessary for memory.

The research I performed with Yu Zhou and Rafal Czajkowski directly addressed whether or not CREB biased memory neurons are necessary for emotive and spatial memory. This was accomplished by demonstrating a deficit in memory as a result of specific silencing of CREB biased memory neurons during testing. Clearly, CREB biased memory neurons are indeed necessary for memory.

Fig. III-4

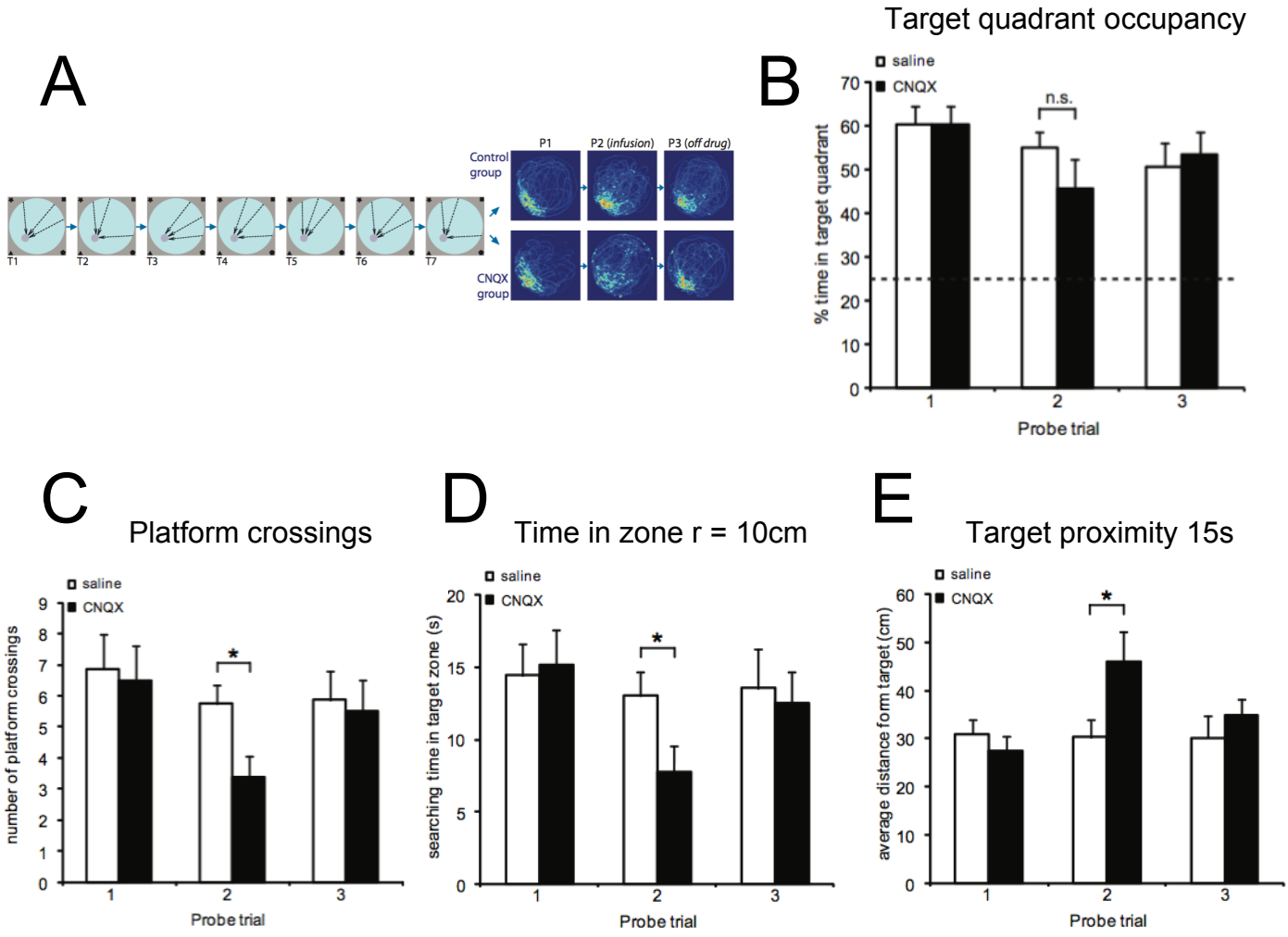


Figure III-4 - The effect of RSC inactivation on retrieval of spatial memory

A) Experimental design and density maps of performance during probe trials (P1,2,3). Mice were trained for 7 days. 24 hours after the last training session, animals were subjected to a probe trial off drug (P1). For the second probe trial (P2) half the animals were infused with CNQX, while the other half were infused with saline. 24 hours later, another probe trial (P3) was performed off drug. Heat maps depict time spent by the animals in each pixel during probe trials.

B) Target quadrant occupancy (percentage of time spent in the quadrant centered on former platform location). CNQX-infused animals are not significantly impaired (P2).

C) Average number of platform crossings, **D)** percent of time spent in the target zone (defined as circular region of radius 10cm) and **E)** average distance from the target during first 15s are all impaired in the CNQX group (P2)

Fig. III-5

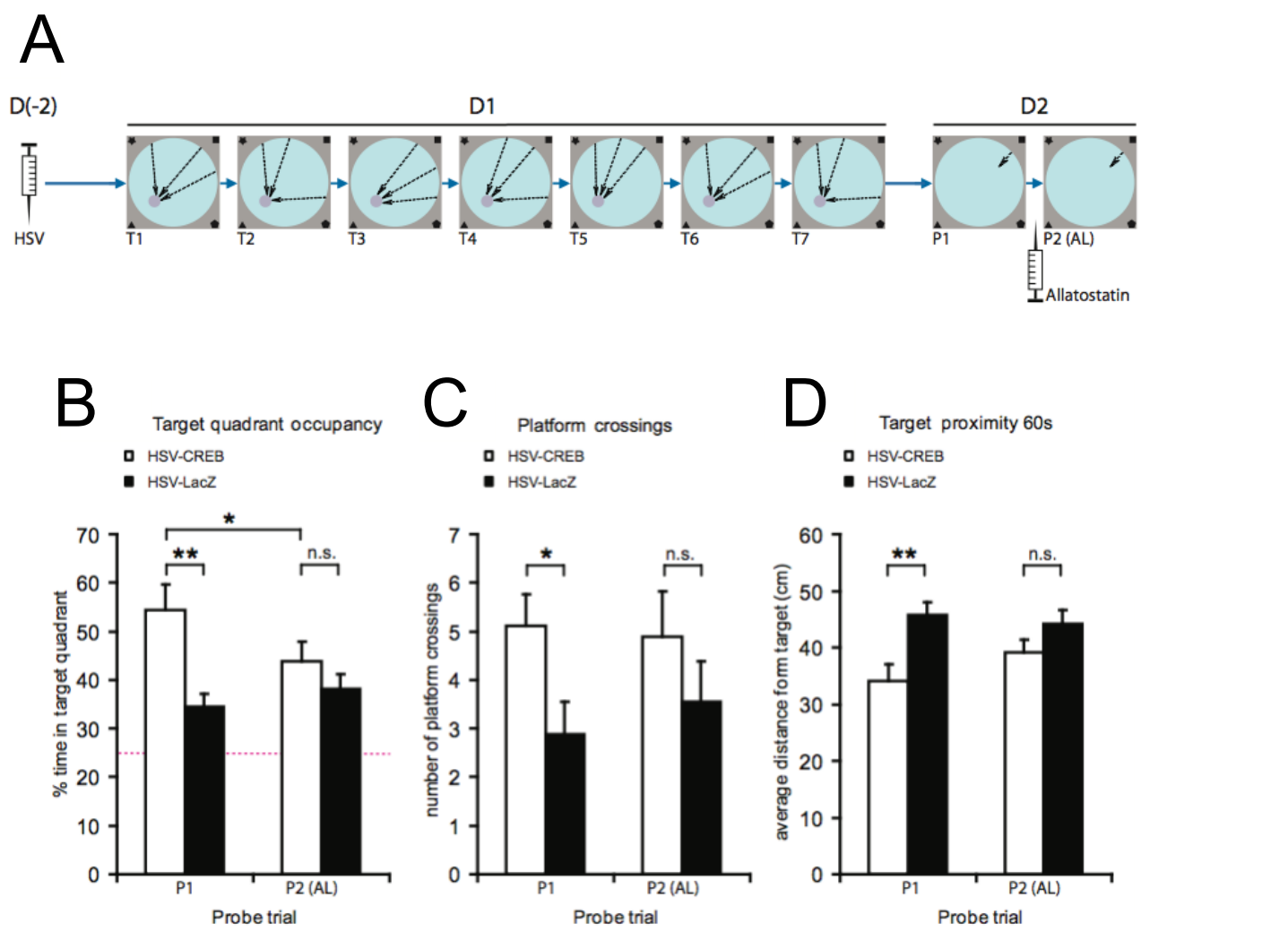


Figure III-5 - Silencing of a CREB biased spatial memory

A) Experimental design. Mice were infused with either HSV-CREB-AlstR (HSV-CREB) or HSV-LacZ-AlstR (HSV-LacZ). On the training day, mice were subjected to 7 training sessions (T1-T7). 24 hours later, the two groups were probed for memory retention (P1), then infused with allatostatin and probed again (P2(AL)).

B) Target quadrant occupancy (percentage of time spent in the quadrant centered on former platform location), **C)** Average number of platform crossing and **D)** the average distance from the target during first 60 seconds are all significantly greater in the HSV-CREB group compared to HSV-LacZ (P1). This effect is abolished by allatostatin infusion (P2).

CHAPTER IV

Activation of a CREB Allocated Memory Trace

4.1 Introduction

There is growing evidence for molecular and cellular mechanisms that regulate the allocation of memories to specific synapses and neurons in a neurocircuit. For example, the transcription factor cAMP-response element binding protein (CREB) has been proposed to regulate memory allocation by modulating neuronal excitability. Here, we used lentiviral vectors to increase the level of CREB in a subset of basolateral amygdala neurons (BLA). Following training in auditory-fear conditioning (AFC), Channelrhodopsin-2 was used to activate these neurons with virally encoded CREB. We show that this activation triggers recall of the memory for auditory-fear conditioning, a result supporting a role for CREB in memory allocation. Similarly, we used a step function opsin to transiently increase excitability in a subset of BLA neurons during AFC, and show that post-training activation of these neurons with Volvox Channelrhodopsin-1 can trigger recall of that memory. Our findings support the hypothesis that CREB regulates memory allocation by modulating neuronal excitability.

Previous results suggest that the transcription factor cAMP-response element binding protein (CREB) has a role in memory allocation in the basolateral amygdala (BLA) (Han, Kushner *et al.* 2007; Won and Silva 2008; Han, Kushner *et al.* 2009; Silva, Zhou *et al.* 2009; Zhou, Won *et al.* 2009), a site required for learning the association between the tone (conditioned stimulus or CS) and foot-

shock (unconditioned stimulus or US) in auditory-fear conditioning (AFC) (Maren and Fanselow 1996; LeDoux 2007; Ehrlich, Humeau *et al.* 2009). Increasing the levels of CREB in a subset of BLA neurons increased the probability that these neurons were engaged in AFC (Han, Kushner *et al.* 2007). Silencing or ablating these neurons triggered deficits in recall (Han, Kushner *et al.* 2009; Zhou, Won *et al.* 2009). Importantly, electrophysiological studies indicated that BLA neurons with higher CREB levels have higher excitability (Zhou, Won *et al.* 2009). Here, we used viral vectors and optogenetic tools to test the hypothesis that CREB modulates memory allocation in the BLA by regulating neuronal excitability.

4.2 Results

We increased CREB levels in a subset of BLA neurons with a lentivirus expressing a CREB gene. The CREB/memory allocation hypothesis (Won and Silva 2008; Silva, Zhou *et al.* 2009) predicts that these neurons would be preferentially chosen to encode a memory for AFC. This lentivirus (pLenti-CREB/ChR2; **Fig. IV-1A**) also included a Channelrhodopsin-2 gene (ChR2) (Boyden, Zhang *et al.* 2005) that allows for the specific activation of the BLA neurons expressing the viral CREB gene. As a control, we used a virus that only expresses ChR2 (pLenti-ChR2; **Fig. IV-1B**). ChR2 is a light-gated ion channel that is activated by blue (488nm) light. To confirm that ChR2 was functioning as expected in our lentiviral constructs, HEK293T cells were infected with pLenti-CREB/ChR2 (**Fig. IV-2A**). Then, whole-cell recordings were carried out with and without 488nm illumination (**Fig. IV-2B**). As expected inward depolarizing currents were observed that lasted for the duration of the 488nm light pulse. To confirm that ChR2 was working as expected *in vivo,* the BLA was infected with either pLenti-CREB/ChR2 or pLenti-ChR2. Subsequently, half of the mice from the pLenti-CREB/ChR2 and pLenti-ChR2 groups had their BLA illuminated with 488nm light, while the other half received sham illumination. Ninety minutes following this manipulation, the mice were sacrificed and the brains were stained for cfos, a common marker of neuronal activity (Morgan and Curran 1991). The pLenti-CREB/ChR2 and pLenti-ChR2 488nm light activated groups (7.82 ±

0.62%, n=5) had a significantly larger population of cfos positive neurons than the sham light activated group (3.15 ± 1.58%, n=6, P<0.05). Thereby indicating that ChR2 in both constructs is capable of activating BLA neurons (**Fig. IV-C**). Therefore, if the BLA neurons with the lentiviral-encoded CREB are more likely to encode a memory for AFC, then activation of these neurons with blue light should trigger stronger recall than activation of BLA neurons transfected with the control lentivirus.

Immunocytochemistry studies with an antibody against mCherry, a marker present in the two lentiviruses described, detected a region 250mm from the tip of the injector transfected by pLenti-CREB/ChR2 and pLenti-ChR2 (**Fig. IV-1D**). To illuminate the transfected neurons and manipulate their activity in behaving mice, an optogenetics setup was created to bilaterally channel light to the BLA with fiber optic cables (**Fig. IV-1C**). Transfected mice were trained with AFC, and 30 minutes later memory was tested by playing the conditioning tone in a novel chamber. Fifteen minutes following the tone test, mice were placed in a third context, and 488nm light was delivered to the BLA for 1 min (Optogenetic Activation) (**Fig. IV-3A**). Both the tone test and optogenetic activation were completed within 60 minutes of training, so as to avoid confounding CREB's role on memory allocation with its well-known role in memory consolidation (Bourtchuladze, Frenguelli *et al.* 1994). Our results show that the levels of freezing during the tone test are similar for mice with the pLenti-CREB/ChR2 virus (Trained–CREB/ChR2; 27.12 ± 6.90%, n=11) and the mice with the pLenti-

ChR2 virus (Trained–ChR2; 29.10 ± 4.93%, n=17) (**Fig. IV-3B**). Freezing, the sessation of all but respiratory movement, is a behavioral response to fear and it is used as a measure of memory strength. Therefore, this result demonstrates that viral-encoded CREB did not affect the acquisition of AFC.

Importantly, optogenetic activation with the 488nm light triggered significantly higher freezing in the Trained–CREB/ChR2 group (48.79 ± 9.21%, n=11) than in the Trained–ChR2 group (20.20 ± 5.87%, n=17, $P<0.05$) (**Fig. IV-3C**). To test the possibility that the higher freezing of the Trained–CREB/ChR2 group reflected a non-specific response to amygdala activation unrelated to learning per se, we included additional controls. A third group of mice infused with plenti-CREB/ChR2 (No Training–CREB/ChR2) was trained and tested as described above, except no foot-shock was delivered during AFC (**Fig. IV-3A**). As expected no AFC was observed in the tone test (3.41 ± 2.11%, n=12) (**Fig. IV-3B**). Optogentic activation of ChR2 in the No Trained–CREB/ChR2 group elicited significantly less freezing (5.47 ± 3.82%, n=12), compared to the Trained–CREB/ChR2 (48.79 ± 9.21%, n=11, $P<0.05$) and the Trained–ChR2 groups (20.20 ± 5.87%, n=17, $P<0.05$) (**Fig. IV-3C**).

Since increasing CREB levels is thought to increase neuronal excitability in the BLA, it is conceivable that the higher freezing levels of the Trained–CREB/ChR2 group is unrelated to learning, and it is instead due to an interaction between training (which should increase BLA excitability), the expected increase in

excitability due to CREB (Viosca, Lopez de Armentia *et al.* 2009; Zhou, Won *et al.* 2009) and ChR2 activation. To control for this possibility, we included a third control group in which the foot-shock was delivered 1 minute before the tone to plenti-CREB/ChR2 transfected mice (Unpaired–CREB/ChR2) (**Fig. IV-3A**). We confirmed that the Unpaired–CREB/ChR2 (11.62 ± 4.89%, n=11) group showed significantly less AFC than the Trained–CREB/ChR2 (27.12 ± 6.90%, n=11, $P<0.05$) and the Trained–ChR2 (29.10 ± 4.93%, n=17, $P<0.05$) cohort, since the CS and the US were unpaired (**Fig. IV-3B**). Accordingly, 488nm optogentic activation also did not trigger significant freezing in this same group of mice (0.06 ± 0.51%, n=11) (**Fig. IV-3C**). These results demonstrate that optogenetic activation of BLA neurons with high CREB in trained mice trigger freezing responses that reflect recall of AFC, a result consistent with the idea that increasing the levels of CREB dramatically enhances the probability that specific BLA neurons encode a memory for AFC. Although optogenetic activation of Trained–ChR2 mice resulted in some freezing, the levels were much lower than those observed for Training–CREB/ChR2. This result suggests that unlike pLenti-CREB/ChR2-transfected neurons, those transfected with pLenti-ChR2 are not preferentially chosen to participate in memory for AFC. In this respect, it is important to note that AFC levels in Trained–ChR2 and Trained–CREB/ChR2 were indistinguishable, a result that demonstrates that the differences in freezing between these two groups during optogenetic activation are not due to differences in AFC learning, but reflect instead the proposed bias of memory allocation towards neurons that express high levels of CREB.

Fig. IV-1

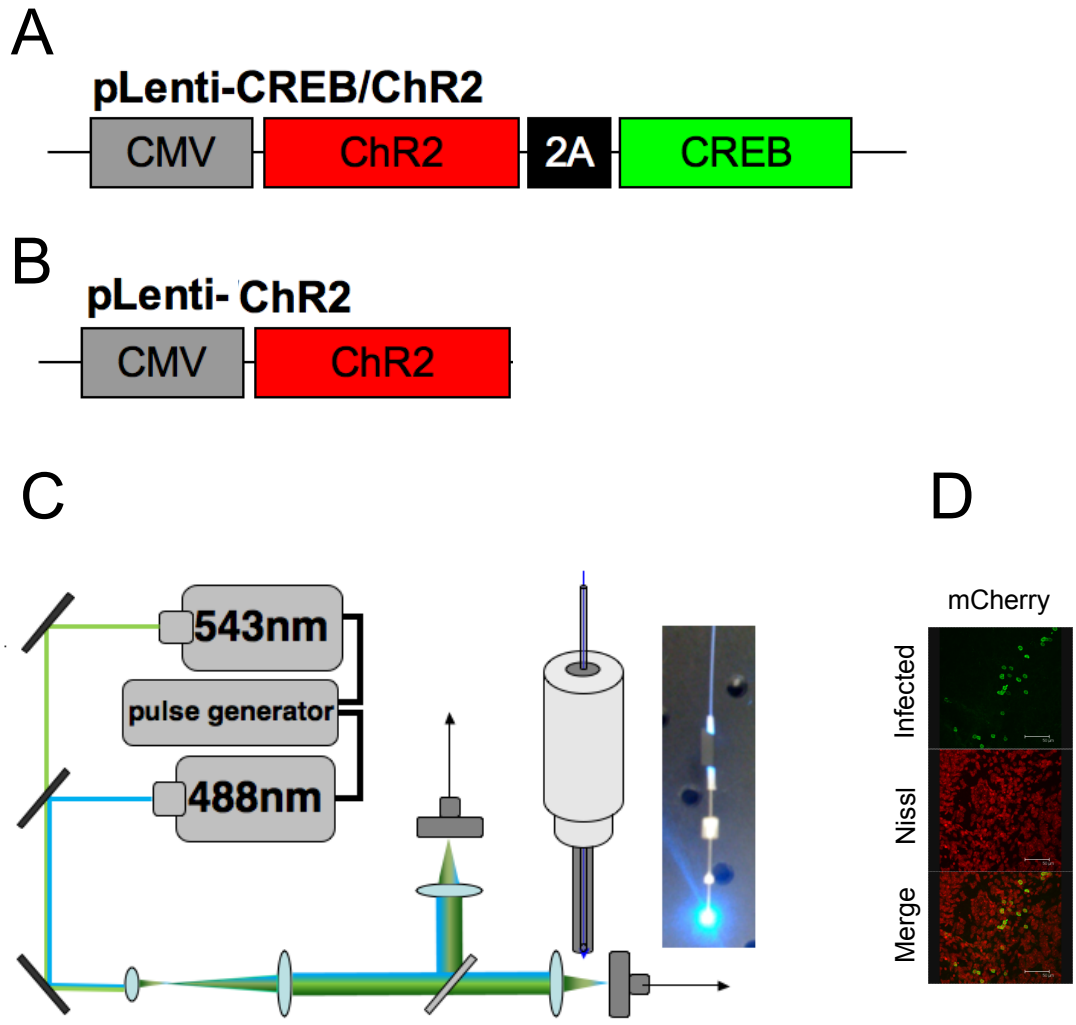


Figure IV-1 - Viral design, optogenetic setup and expression (CREB)

A) pLenti-CREB/ChR2 and **B)** pLenti-ChR2 viral design

C) Optogenetics setup

D) Expression of pLenti-CREB/ChR2 stained for mCherry, neurons are counterstained with Nissl

Fig. IV-2

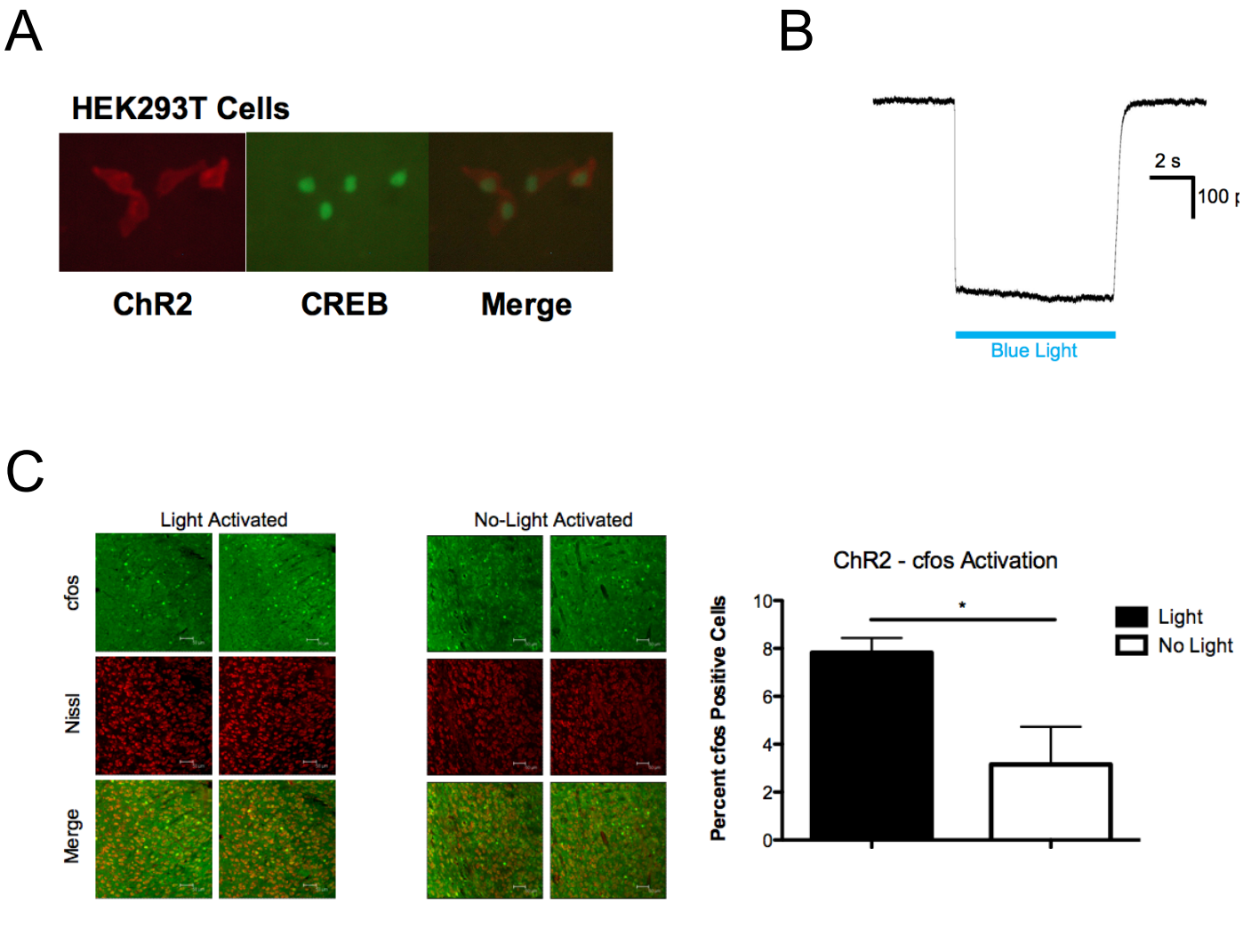


Figure IV-2 -Electrophysiological and molecular characterization

A) HEK293T cells infected with pLenti-CREB/ChR2. ChR2 and CREB were visualized by mCherry and EGFP fluorescence respectively.

B) Whole-cell recordings from pLenti-CREB/ChR2 expressing HEK293T cells. Inward depolarizing currents were observed during 488nm light illumination.

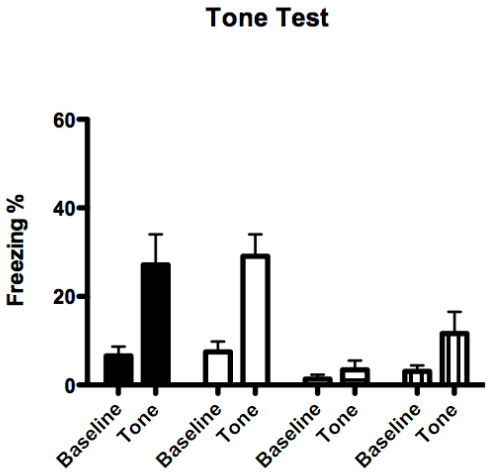
C) The BLA of one cohort of ChR2 expressing animals was illuminated with 488nm light (Light) while the other cohort of ChR2 expressing animals received a sham illumination (No Light). The animals were sacrificed and their brains were stained for cfos. The Light group had a significantly larger population of cfos positive neurons than the No Light group.

Fig. IV-3

A

	Tone Training CtxA	30 min.	Tone Test CtxB	15 min.	Optogenetic Activation CtxC
Trained - CREB/ChR2	CS/US	→	CS	→	Opto. Act.
Trained - ChR2	CS/US		CS		Opto. Act.
No Training - CREB/ChR2	CS		CS		Opto. Act.
Unpaired - CREB/ChR2	US/CS		CS		Opto. Act.

B



C

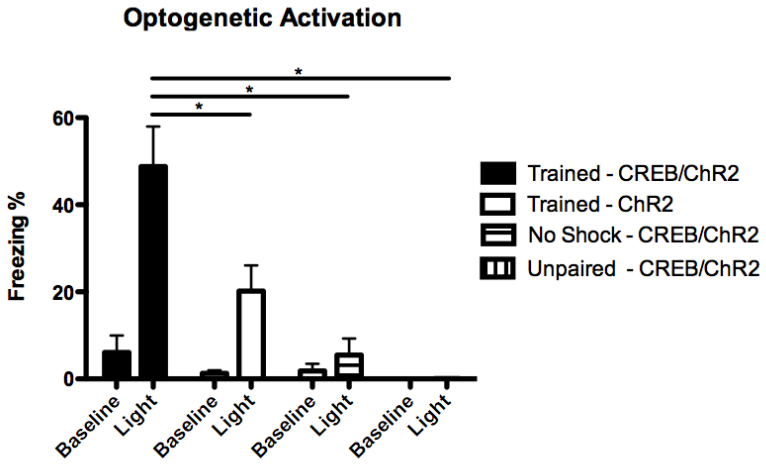


Figure IV-3 - Optogenetic activation of a CREB allocated memory trace

A) Behavioral design for the four experimental groups: Trained - CREB/ChR2, Trained - ChR2, No Training - CREB/ChR2 and Unpaired - CREB/ChR2. The conditioned stimulus (CS) is a 90db, 30 second (Tone Training) or 60 second (Tone Test) tone and the unconditioned stimulus (US) is a 0.7mA, 2 second shock. Optogenetic activation (Opto. Act.) is a 60 second, 488nm, 5Hz, 5mW light.

B) Results of tone test. For each group, the graph includes two bars: the one on the left representing the baseline freezing scores (Baseline) measured before tone presentation, the other showing the level of freezing during tone presentation (Tone).

C) Results of the optogenetic activation. For each group, the graph includes two bars: one on the left representing the baseline freezing scores (Baseline) measured before optogenetic activation, the other showing the level of freezing during optogenetic activation (Light).

4.3 Conclusions

The results presented here provide strong support for the hypothesis that CREB regulates memory allocation. They also demonstrate that it is possible not only to trap a memory for AFC in BLA neurons expressing viral encoded CREB, it is also possible to trigger recall of that memory by optogenetic activation of these neurons.

4.4 Materials and methods

Mice.

Adult F1 hybrid (C57Bl/6NTac × 129S6/SvEvTac) mice were group housed (3–4 per cage) on a 12-h light/dark cycle. These mice were used because they show robust and reliable AFC conditioning. Food and water were available ad libitum throughout the experiment. All procedures were approved by the Chancellor's Animal Research Committee at the University of California at Los Angeles, in accordance with US National Institutes of Health guidelines.

Lenti vectors.

Two vectors were used in this study pLenti-CREB/ChR2 and pLenti-ChR2. The vector pLenti-CREB/ChR2 uses a CMV promoter to drive the expression of a ChR2-mCherry and EGFP-CREB chimera separated by a 2A self-processing viral peptide bridge (Tang, Ehrlich *et al.* 2009). A control virus (pLenti-ChR2) was developed that expresses ChR2 alone. ChR2 is a light gated ion channel derived from Chlamydomonas reinhardtii. When ChR2 absorbs blue light a conformational change occurs that results in the opening of the channel, and consequently in an inward depolarizing current that can trigger action potentials.

Surgery.

All surgeries were done in the Franz Hall animal facility, in accordance with US

National Institutes of Health guidelines. Mice were treated with carprofen (5 mg per kg of body weight, subcutaneous), anesthetized with sodium pentobarbital (30-70 mg per kg, intraperitoneal) and placed in a stereotaxic frame. Core body temperature was maintained at 37.5 C with a heating pad. Eyes were coated with a thin layer of ophthalmic ointment to prevent desiccation. The skin above the skull was retracted and two bore holes were drilled in the skull bilaterally above the lateral amygdala (anterior-posterior = –1.4, medial-lateral = ±3.3, ventral = –4.8 mm from bregma) according to Paxinos and Franklin mouse brain atlas. Cannula (Med Associates) were slowly lowered through the bore holes to the correct depth. At this point, dental cement was used to fix the cannula in place and seal the wound. Caps were inserted in the cannula in order to prevent debris entering the brain. Mice were then removed from the stereotaxic apparatus and allowed to recover on a water-circulating heating pad. When fully alert, mice were returned to their home cage and placed on a two-week course of antibiotics. The body weight and general condition of each mouse was assessed daily.

Viral infusion.

Viral infusion was performed 7 days after surgery to ensure physical recovery. Mice were treated with carprofen (5 mg per kg of body weight, subcutaneous), and anesthetized with sodium pentobarbital (15-35 mg per kg, intraperitoneal). Core body temperature was maintained at 37.5 C with aheating pad. Eyes were coated with a thin layer of ophthalmic ointment to prevent desiccation. Cannula caps were removed and a virus solution (1.0-1.3 µl, bilateral) was delivered to the

basolateral amygdala at a flow rate of 0.065-0.130 µl per minute through an inner injection cannula (Plastic One, 22 gauge) attached by polyethylene tubing to Hamilton microsyringes mounted in an infusion pump (Harvard Instruments). The infusion cannula were left in place an additional 10 minutes to ensure diffusion of the vector. Cannula caps were replaced. Mice were allowed to recover on a water-circulating heating pad. When fully alert, they were returned to their home cage. The body weight and general condition of each mouse was assessed daily. Behavioral experiments were performed three weeks following virus infusions.

Electrophysiology.

HEK293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 4mM L-Glutamine and 1% penicillin/streptomycin. Cells were plated onto coverslips and lentivirus was infected 3 weeks before recording. Photocurrents in HEK cells infected with ChR2, SFO and VChR1 were recorded by conventional whole-cell patch-clamp. The external solution contained [mM]: 135 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 30 d-Glucose (pH 7.4). The internal solution contained [mM]: 8 NaCl, 2 KH_2PO_4, 2 d-Glucose, 10 HEPES, 130 $KMeSO_4$, 4 Mg-ATP, 7 Phosphocreatine Na, 0.3 GTP, 0.5 ADP. Patch pipettes were pulled with micropipette puller model P-97 (Sutter Instrument Co.) from borosilicate glass capillary (World Precision Instruments, Inc.) with around 3 M resistance. Cells were visualized with an upright microscope using infrared or epifluorescent illumination, and whole-cell voltage-clamp recordings were made from cells with Multiclamp 700B. Responses were filtered at 2 kHz and digitized

at 10 kHz. All data were acquired, stored and analyzed using Clampex 10.0 (Axon Instruments).

Histology and immunohistochemistry.

After the behavioral experiments, mice were sacrificed with sodium pentobarbital and fixed with a transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, wt/volume). Brains were sliced coronally (40 μm). The cannula tip locations were confirmed at the end of each experiment. Only those mice with validated bilateral cannula placements were included in the analysis. For cfos immunohistochemistry mice were sacrificed 90 minutes following behavior with sodium pentobarbital and fixed with a transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, wt/volume). Brains were sliced coronally (40 μm). Brains were prepared for immunocytochemistry using either anti cFos (1:200) rabbit polyclonal antibodies (Abcam 7963) and anti mCherry (1:1000) mouse monoclonal antibody (Clonetech 632543) and a Alexa-568 conjugated anti-mouse secondary (1:500) (Invitrogen A-11004). For visualization of neurons a Alexa-455 conjugated Nissl NeuroTrace counter stain was used. Confocal microscopy was used to identify immunoreactive neurons. The basolateral amygdala was anatomically defined according to the Paxinos and Franklin atlas (Paxinos and Franklin 2004). Immunoreactive neurons were counted with a fixed sample window across at least three sections by an experimenter blind to the treatment condition. Scores were expressed as a percentage of immunoreactive neurons in a defined region (250mm from the tip

of the cannula).

Optogenetic Setup.

An optogenetic setup was designed to bilaterally channel 488nm light through fiber optic cables designed to be inserted into the cannula implanted during surgery. Optical components including mirrors (silvered, half-silvered and dichroic), lenses, manipulators and fiber couplers (Thorlabs Inc.) were used to channel 488nm lasers light (Dragon Lasers) into 200mm multimode fiberoptics (Thorlabs Inc.). The frequency and duration of laser stimulation was controlled by a signal generator (B&K Precision). The end of the fiber to be inserted into the cannula was reinforced with a steel tube (Small Parts Inc.). A Plastics One injector and dummy were modified to allow the fiber to be positioned securely at and appropriate depth during behavior (Zhang, Gradinaru *et al.* 2010).

Behavior.

AFC for Trained – CREB/ChR2 and Trained – ChR2 cohorts entailed the placement of mice in a conditioning chamber, and 1 minute later, the presentation of a tone (2800 Hz, 90 dB, 30 seconds) that coterminated with a shock (2 sec, 0.7 mA). Testing for AFC occurred 30 minutes after training (Tone Test). Mice were placed in a novel chamber, and 1 minute later, the tone was presented for 1 minute. The index of memory, freezing, was assessed via automated procedures (Med Associates Inc.). Animals that did not learn were excluded from the remainder of the experiment. 15 minutes later ChR2 activation

was achieved by illuminating the BLA of Trained – CREB/ChR2 and Trained – ChR2 cohorts with a 5mW 488nm laser (5Hz, 5ms pulse width) 1 minute after placement in a novel chamber. Activation continued for 1 minute (Optogenetic Activation). The index of memory, freezing, was assessed via manual scoring procedures by researchers blind to the experimental conditions. The No Training – CREB/ChR2 cohort was treated identically to the Trained – CREB/ChR2 and Trained – ChR2 cohorts expect no shock was administered during AFC. The Unpaired – CREB/ChR2 cohort was treated identically to the Trained – CREB/ChR2 and Trained – ChR2 cohorts except the shock preceded the tone by 1 minute.

CHAPTER V

A Mechanism for Memory Allocation

5.1 Introduction

Previous studies have shown that increasing the CREB levels of BLA neurons results in an increase in their excitability (Viosca, Lopez de Armentia *et al.* 2009; Zhou, Won *et al.* 2009). This finding suggested the hypothesis that the increase in neuronal excitability in BLA neurons with higher CREB levels is responsible for their preferential inclusion into memory traces (Silva, Zhou *et al.* 2009; Zhou, Won *et al.* 2009).

5.2 Results

To test the possible involvement of increases in neuronal excitability in memory allocation, we engineered a new lentiviral vector with a step-function opsin (Berndt, Yizhar *et al.* 2009) (SFO; pLenti-SFO/VChR1; **Fig. V-1A**). The pLenti-SFO/VChR1 vector also included a Volvox Channelrhodopsin-1 (VChR1) (**Fig. V-1A**), which allowed the use of 543nm light to optogenetically activate BLA neurons transfected with this lentivirus (Zhang, Prigge *et al.* 2008). We did not use ChR2 for optogenetic activation in this vector, because the 488nm light required to activate ChR2 would have also activated the SFO present in the vector (See **Fig. V-2**). To confirm that SFO and VChR1 were functioning as expected in our lentiviral constructs, HEK293T cells were infected with pLenti-SFO/VChR1 (**Fig. V-2A**). Then, whole-cell recordings were carried out with and without illumination with 488nm light and (separately) 543nm (green) light (**Fig. V-2B**). SFO activation resulted in a stable depolarizing step in membrane potential that was maintained following the offset of the 488nm light pulse. We also observed VChR1 generated inward depolarizing currents that lasted for the duration of the 543nm light pulses. To confirm that VChR1 was working as expected *in vivo,* analogous cfos experiments to those performed with pLenti-CREB/ChR2 and pLenti-ChR2 were carried out. As expected the 543nm light activated group (10.80 ± 1.66%, n=4) had a significantly larger population of cfos positive neurons than the group that received sham light activation (3.15 ±

1.58%, n=6, P<0.05). This result indicates that VChR1 was capable of activating BLA neurons (**Fig. V-2C**). Immunocytochemistry studies with a EYFP antibody detected a region 250mm from the tip of the injector transfected by pLenti-SFO/VChR1 (**Fig. V-1C**).

Two groups of pLenti-SFO/VChR1 transfected mice were trained with two trials of AFC separated by 24 hours: one of the groups (Step–SFO/VChR1) received a 5 sec long pulse of 488nm light prior to each AFC trial, and the other received sham illumination (no 488nm illumination; Ctrl–SFO/VChR1). The mice received two training trials because the involved manipulation required for SFO activation prior to AFC interfered with the levels of AFC (data not shown). Fifteen minutes after the last AFC training trial, the Step–SFO/VChR1 and Ctrl–SFO/VChR1 groups were tested for tone conditioning in a novel context (**Fig. V-3A**). The results show that the freezing levels measured in both groups were indistinguishable (Step–SFO/VChR1 69.13 ± 5.75%, n=21 vs. Ctr Step–SFO/VChR1 73.67 ± 5.12%, n=13) (**Fig. V-3B**). The following day the same two groups of mice were placed in a third context and underwent optogenetic activation for 1 minute as described above, except that this time we used 543nm light to activate VChR1 (**Fig. V-3A**). The results revealed that the Step–SFO/VChR1 group showed significantly higher levels of freezing (22.14 ± 4.86%, n=21) than the Ctrl–SFO/VChR1 group (8.21 ± 2.75%, n=13, P<0.05) (**Fig. V-3C**).

The results just presented support the hypothesis that increases in excitability underlie memory allocation. This hypothesis makes a crucial prediction that we tested in a second control group of mice also transfected with pLenti-SFO/VChR1: increases in excitability immediately following AFC should not bias memory allocation, since to be effective changes in excitability should be present prior to and during training. To test this prediction, a third group of mice (Reversed–SFO/VChR1) was infused with pLenti-SFO/VChR1, and went through the exact same behavioral procedure as the pLenti-SFO/VChR1 mice described above, except that the 5 seconds pulse of 488nm light was given following the tone/shock presentation (**Fig. V-3A**). As expected, a tone test confirmed that the tone freezing levels of the Reversed–SFO/VChR1 and Step–SFO/VChR1 groups were indistinguishable (Step–SFO/VChR1 69.13 ± 5.75%, n=21 vs. Reversed–SFO/VChR1 65.40 ± 7.75%, n=17) (**Fig. V-3B**). However, optogenetic activation with 543nm light for 1 min, 15 min after the tone test, triggered lower freezing (i.e., weaker recall) in the Reversed–SFO/VChR1 group compared with the Step–SFO/VChR1 (Reversed–SFO/VChR1 8.33 ± 2.49%, n=17 vs. Step–SFO/VChR1 22.14 ± 4.86%, n=21, $P<0.05$; **Fig. V-3C**). These results indicate that increases in excitability following training do not bias memory allocation, a result that supports the conclusion that increases in excitability before and during training bias memory allocation and that this bias accounts for the higher freezing of the Step–SFO/VChR1 group. The freezing levels triggered by optogenetic activation with VChR1 were lower than those triggered with ChR2. This may be

due to the known lower efficiency of VChR1 (Zhang, Prigge *et al.* 2008; Yizhar, Fenno *et al.* 2011).

It is possible that increasing excitability by SFO activation, results in increased anxiety, and that this accounts for the higher freezing of the Step–SFO/VChR1 group following optogenetic activation. Our results indicate that this possibility is unlikely (Tye, Prakash *et al.* 2011). First, both Step–SFO/VChR1 and Ctrl–SFO/VChR1 showed similar freezing scores during tone testing. Second, a group of animals were transfected with pLenti-SFO/VChR1. Anxiety levels were then tested on the elevated plus maze before and after optogenetic stimulation with a 488nm light for 5 seconds (**Fig. V-4A**). We measured the time in the open arms, center and closed arms, common measures of anxiety (Carola, D'Olimpio *et al.* 2002). No increase in anxiety after optogenetic stimulation was detected (**Fig. V-4B**), a result that demonstrates that increased anxiety could not account for the higher levels of freezing detected in the Step–SFO/VChR1 group.

SFO activation could act as an additional US, thus augmenting the effects of foot-shock in our AFC experiments (Johansen, Hamanaka *et al.* 2010). Our findings indicate that this possibility is unlikely. First, both Step–SFO/VChR1 and Ctrl–SFO/VChR1 showed similar freezing levels during tone testing, demonstrating that SFO activation does not strengthen the US in the group with SFO activation prior to training. Second, SFO activation was paired with a tone. The next day the mice were tone tested in a different context (**Fig. V-5A**). No

significant freezing was observed, demonstrating that activation of SFO does not act as a US (**Fig. V-5B**). However, it was conceivable that the activation of SFO may be capable of augmenting the effects of foot shock in AFC. To test this, mice were transfected with pLenti-SFO/VChR1 as described above, and trained in AFC 3 weeks later. The animals were then tone tested 24 hours after training in a different context. The following day, activation of SFO was paired with a tone. The animals were then tone tested in a different context the next day to determine if the SFO/tone pairing strengthened previous learning of AFC. (**Fig. V-6A**). In line with our evidence that SFO activation does not act as a US, we observed no enhancement in freezing due to SFO/Tone pairing (**Fig. V-6B**).

Fig. V-1

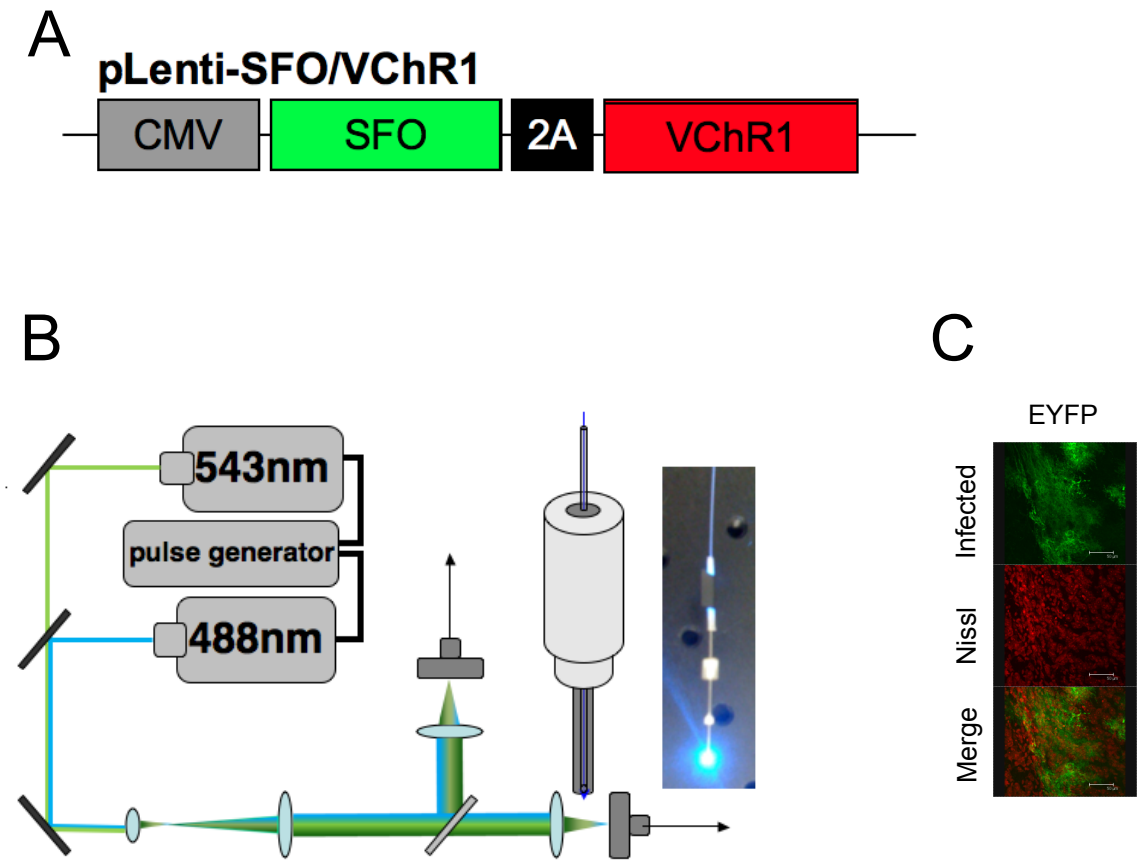


Figure V-1 - Viral design, optogenetic setup and expression (excitability)

A) pLenti-SFO/VChR1 viral design

B) Optogenetic setup

C) Expression of pLenti-SFO/VChR1 stained for EYFP, neurons are counterstained with Nissl

Fig. V-2

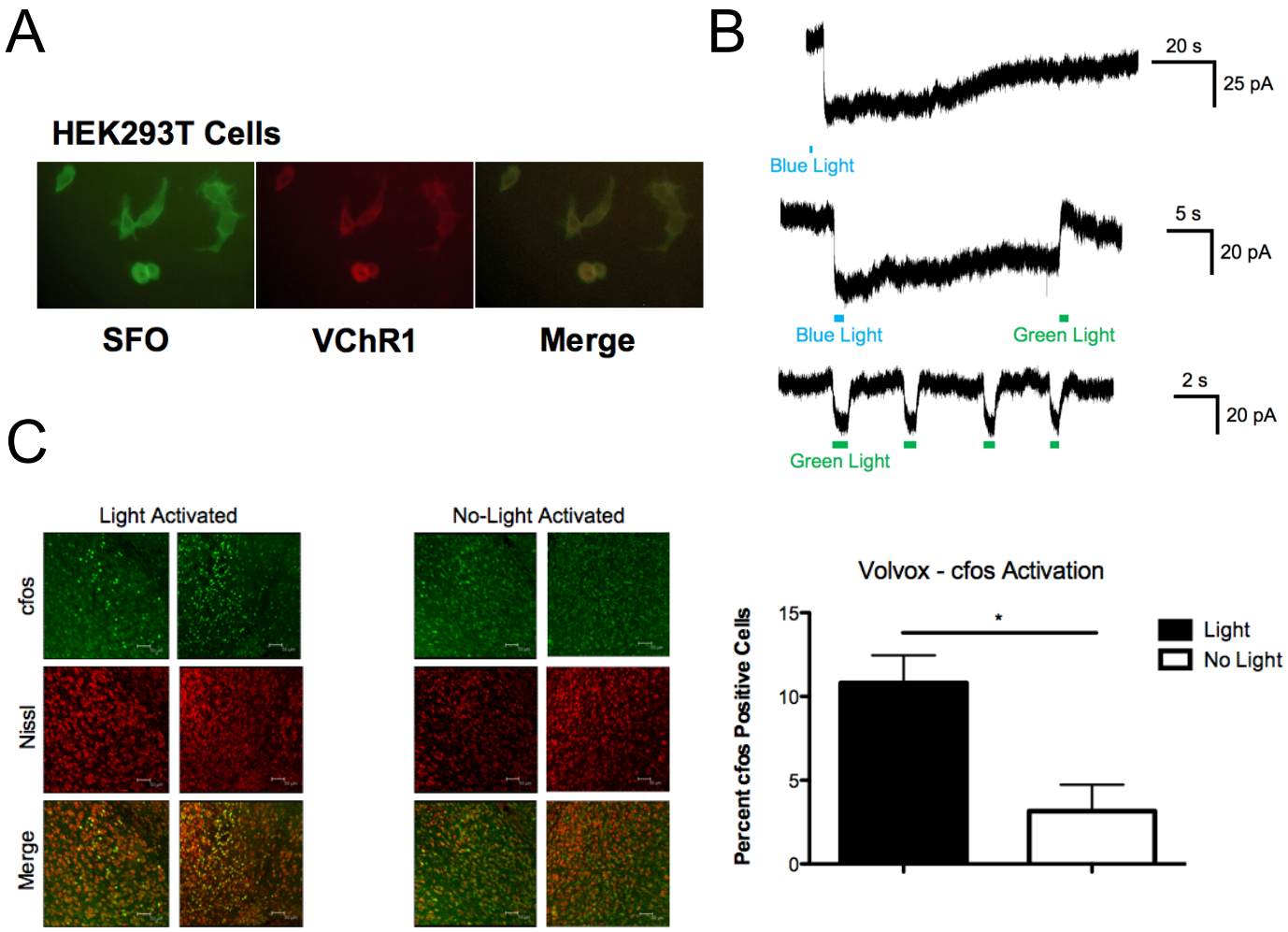


Figure V-2 - Electrophysiological and molecular characterization (excitability)

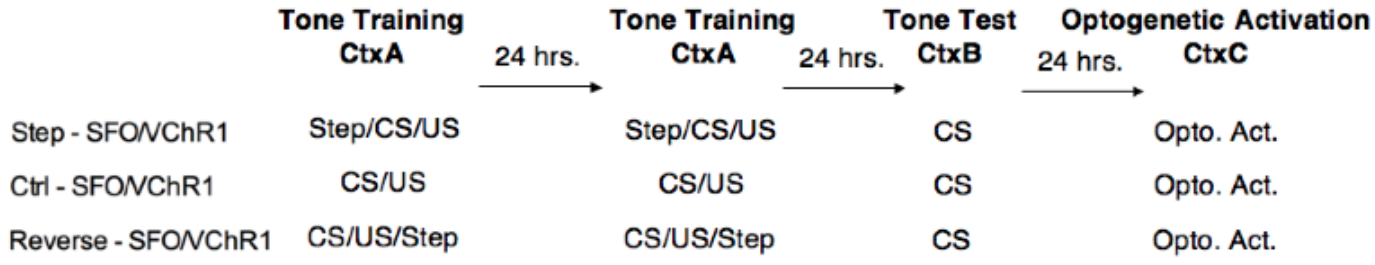
A) HEK293T cells infected with pLenti-SFO/VChR1. SFO and VChR1 were visualized by EYFP and mCherry fluorescence respectively.

B) Whole-cell recordings from pLenti-SFO/VChR1 expressing HEK293T cells. A 488nm light pulse, designed to activate SFO, triggered a stable depolarizing step in membrane potential that was maintained following the offset of the pulse and reversed by a 543nm green light pulse. Additionally, inward depolarizing currents were generated that lasted for the duration of 543nm green light pulses designed to activate VChR1.

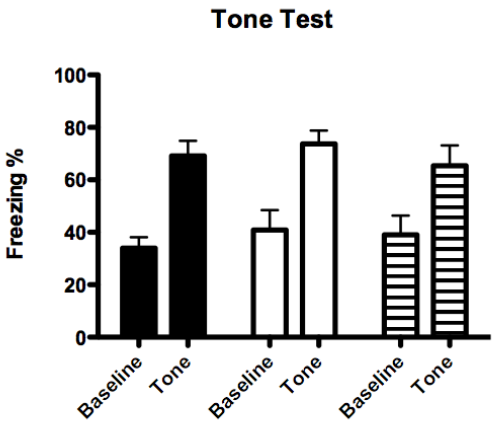
C) The BLA of one cohort of VChR1 expressing animals was illuminated with 543nm green light (Light) while the other cohort of VChR1 expressing animals received a sham illumination (No Light). The animals were sacrificed and the brains were stained for cfos. The Light group had a significantly larger population of cfos positive neurons than the No Light group.

Fig. V-3

A



B



C

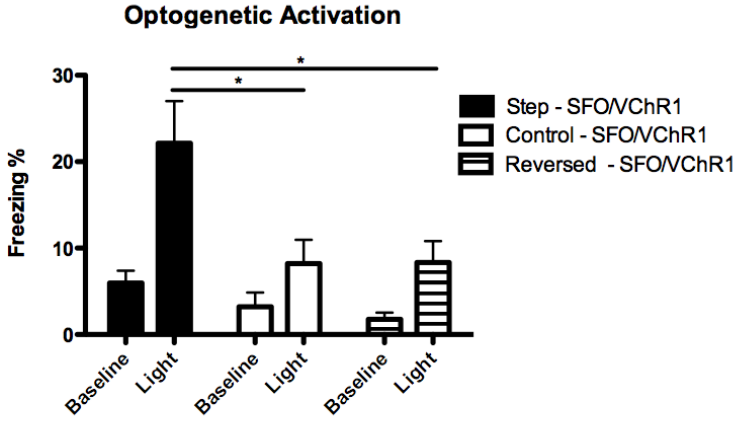


Figure V-3 - Optogenetic activation of a memory trace allocated by excitability

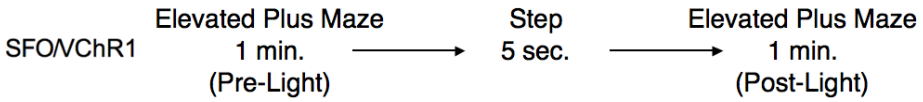
(A) Behavioral design for the three experimental groups: Step - SFO/VChR1, Ctrl - SFO/VChR1 and Reversed - SFO/VChR1. The conditioned stimulus (CS) is a 90db, 30 second (Tone Training) or 60 second (Tone Test) tone, the unconditioned stimulus (US) is a 0.7mA, 3 second shock and the step in excitability (Step) is a 5 second, 488nm, 5mW light. Optogenetic activation (Opto. Act.) is a 60 second, 543nm, 10Hz, 10mW light.

(B) Results of tone test. For each group, the graph includes two bars: the one on the left representing the baseline freezing scores (Baseline) measured before tone presentation, the other showing the level of freezing during tone presentation (Tone).

(C) Results of the optogenetic activation. For each group, the graph includes two bars: the one on the left representing the baseline freezing scores (Baseline) measured before optogenetic activation, the other showing the level of freezing during optogenetic activation (Light).

Fig. V-4

A



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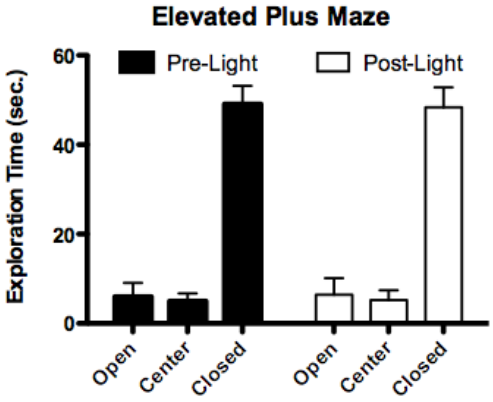


Figure V-4 - Behavioral controls for excitability induced anxiety

(A) Behavioral design for the elevate plus maze (EPM). Animals were placed on the EPM for 1 minute before (Pre-Light) and after (Post-Light) a step in excitability (Step). Step is a 5 second, 488nm, 5mW light.

(B) Results of EPM. The exploration time in the open, center and closed portions of the EPM was monitored before (Pre-Light) and after (Post-Light) a step in excitability.

Fig. V-5

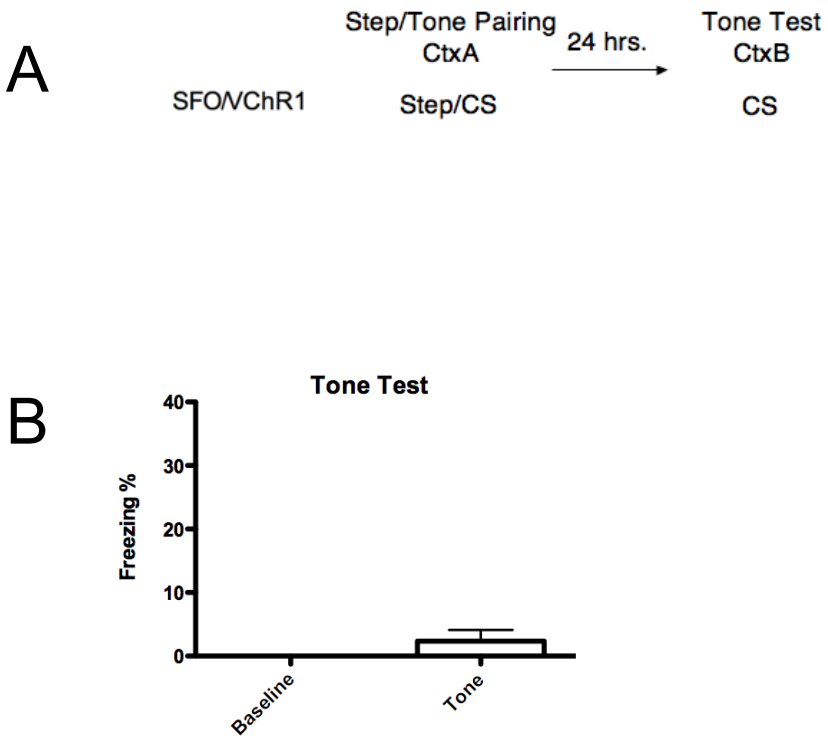


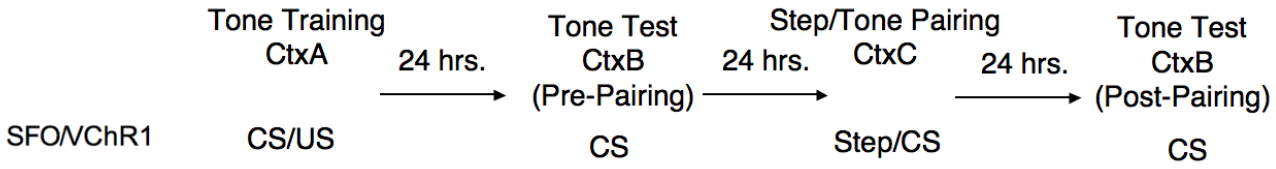
Figure V-5 - Behavioral controls for excitability induced learning (Part 1)

(A) Behavioral design for the Step/Tone pairing. The conditioned stimulus (CS) is a 90db, 30 second tone and Step is a 5 second, 488nm, 5mW light.

(B) Results of tone test. The graph includes two bars: the one on the left representing the baseline freezing scores (Baseline) measured before tone presentation, the other showing the level of freezing during tone presentation (Tone).

Fig. V-6

A



B

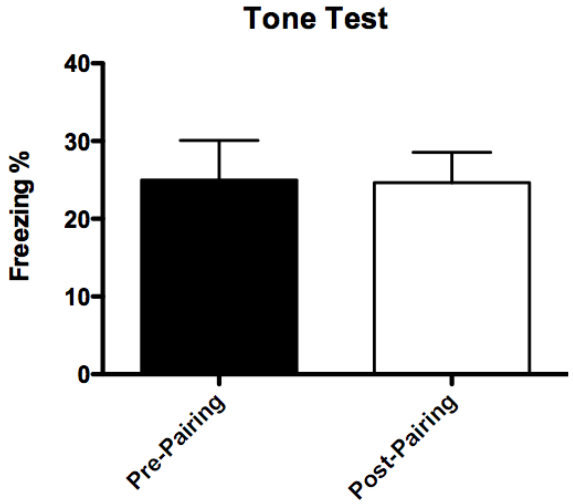


Figure V-6 - Behavioral controls for excitability induced learning (Part 2)

(A) Behavioral design for the trained Step/Tone pairing. The conditioned stimulus (CS) is a 90db, 30 second tone, the unconditioned stimulus (US) is a 0.7mA, 2 second shock and the step in excitability (Step) is a 5 second, 488nm, 5mW light.

(B) Results of Pre-Pairing and Post-Pairing tone test. The graph shows the level of freezing during tone presentation of the Pre-Pairing and Post-Pairing tone test.

5.3 Conclusions

The results presented here provide strong support for the hypothesis that excitability regulates memory allocation. They also demonstrate that it is possible to trap not only a memory for AFC in BLA neurons whose excitability has been increased, but that it is also possible to trigger recall of that memory by optogenetic activation of these neurons.

5.4 Materials and methods

Mice.

Adult F1 hybrid (C57Bl/6NTac × 129S6/SvEvTac) mice were group housed (3–4 per cage) on a 12-h light/dark cycle. These mice were used because they show robust and reliable AFC conditioning. Food and water were available ad libitum throughout the experiment. All procedures were approved by the Chancellor's Animal Research Committee at the University of California at Los Angeles, in accordance with US National Institutes of Health guidelines.

Lenti vector.

One vector was used in this study pLenti-SFO/VChR1. The vector pLenti-SFO/VChR1 uses a CMV promoter to drive the expression of a SFO-EYFP and VChR1-mCherry chimera separated by a 2A self-processing viral peptide bridge (Tang, Ehrlich *et al.* 2009). SFO is a ChR2 with a cysteine 128 mutated to alanine (Berndt, Yizhar *et al.* 2009). Unlike ChR2 a pulse of blue light will result in a stable depolarizing step in membrane potential that is maintained following the offset of blue light. VChR1 is a cation-conducting channelrhodopsin from *Volvox carteri that* functions in an analogous way to ChR2 (Zhang, Prigge *et al.* 2008). However unlike ChR2, VChR1 responds maximally to green light allowing it to be used in conjunction with SFO.

Surgery.

All surgeries were done in the Franz Hall animal facility, in accordance with US National Institutes of Health guidelines. Mice were treated with carprofen (5 mg per kg of body weight, subcutaneous), anesthetized with sodium pentobarbital (30-70 mg per kg, intraperitoneal) and placed in a stereotaxic frame. Core body temperature was maintained at 37.5 C with a heating pad. Eyes were coated with a thin layer of ophthalmic ointment to prevent desiccation. The skin above the skull was retracted and two bore holes were drilled in the skull bilaterally above the lateral amygdala (anterior-posterior = –1.4, medial-lateral = ±3.3, ventral = –4.8 mm from bregma) according to Paxinos and Franklin mouse brain atlas. Cannula (Med Associates) were slowly lowered through the bore holes to the correct depth. At this point, dental cement was used to fix the cannula in place and seal the wound. Caps were inserted in the cannula in order to prevent debris entering the brain. Mice were then removed from the stereotaxic apparatus and allowed to recover on a water-circulating heating pad. When fully alert, mice were returned to their home cage and placed on a two-week course of antibiotics. The body weight and general condition of each mouse was assessed daily.

Viral infusion.

Viral infusion was performed 7 days after surgery to ensure physical recovery. Mice were treated with carprofen (5 mg per kg of body weight, subcutaneous), and anesthetized with sodium pentobarbital (15-35 mg per kg, intraperitoneal). Core body temperature was maintained at 37.5 C with aheating pad. Eyes were

coated with a thin layer of ophthalmic ointment to prevent desiccation. Cannula caps were removed and a virus solution (1.0-1.3 µl, bilateral) was delivered to the basolateral amygdala at a flow rate of 0.065-0.130 µl per minute through an inner injection cannula (Plastic One, 22 gauge) attached by polyethylene tubing to Hamilton microsyringes mounted in an infusion pump (Harvard Instruments). The infusion cannula were left in place an additional 10 minutes to ensure diffusion of the vector. Cannula caps were replaced. Mice were allowed to recover on a water-circulating heating pad. When fully alert, they were returned to their home cage. The body weight and general condition of each mouse was assessed daily. Behavioral experiments were performed three weeks following virus infusions.

Electrophysiology.

HEK293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 4mM L-Glutamine and 1% penicillin/streptomycin. Cells were plated onto coverslips and lentivirus was infected 3 weeks before recording. Photocurrents in HEK cells infected with ChR2, SFO and VChR1 were recorded by conventional whole-cell patch-clamp. The external solution contained [mM]: 135 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 30 d-Glucose (pH 7.4). The internal solution contained [mM]: 8 NaCl, 2 KH_2PO_4, 2 d-Glucose, 10 HEPES, 130 $KMeSO_4$, 4 Mg-ATP, 7 Phosphocreatine Na, 0.3 GTP, 0.5 ADP. Patch pipettes were pulled with micropipette puller model P-97 (Sutter Instrument Co.) from borosilicate glass capillary (World Precision Instruments, Inc.) with around 3 M resistance. Cells were visualized with an upright microscope using infrared or

epifluorescent illumination, and whole-cell voltage-clamp recordings were made from cells with Multiclamp 700B. Responses were filtered at 2 kHz and digitized at 10 kHz. All data were acquired, stored and analyzed using Clampex 10.0 (Axon Instruments).

Histology and immunohistochemistry.

After the behavioral experiments, mice were sacrificed with sodium pentobarbital and fixed with a transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, wt/volume). Brains were sliced coronally (40 μm). The cannula tip locations were confirmed at the end of each experiment. Only those mice with validated bilateral cannula placements were included in the analysis. For cfos immunohistochemistry mice were sacrificed 90 minutes following behavior with sodium pentobarbital and fixed with a transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, wt/volume). Brains were sliced coronally (40 μm). Brains were prepared for immunocytochemistry using either anti cFos (1:200) rabbit polyclonal antibodies (Abcam 7963) and an Alexa-568 conjugated anti-rabbit secondary antibody (1:250) (Invitrogen A-11011), or anti GFP (1:1000) chicken polyclonal antibodies (Aves 1020) and an Alexa-568 conjucated anti-chicked secondary (1:500) (Invitrogen A11041). For visualization of neurons a Alexa-455 conjugated Nissl NeuroTrace counter stain was used. Confocal microscopy was used to identify immunoreactive neurons. The basolateral amygdala was anatomically defined according to the Paxinos and Franklin atlas (Paxinos and Franklin 2004). Immunoreactive neurons were

counted with a fixed sample window across at least three sections by an experimenter blind to the treatment condition. Scores were expressed as a percentage of immunoreactive neurons in a defined region (250mm from the tip of the cannula).

Optogenetic Setup.

An optogenetic setup was designed to bilaterally channel 488 or 543nm light through fiber optic cables designed to be inserted into the cannula implanted during surgery. Optical components including mirrors (silvered, half-silvered and dichroic), lenses, manipulators and fiber couplers (Thorlabs Inc.) were used to channel 488 and 543nm lasers light (Dragon Lasers) into 200mm multimode fiberoptics (Thorlabs Inc.). The frequency and duration of laser stimulation was controlled by a signal generator (B&K Precision). The end of the fiber to be inserted into the cannula was reinforced with a steel tube (Small Parts Inc.). A Plastics One injector and dummy were modified to allow the fiber to be positioned securely at and appropriate depth during behavior (Zhang, Gradinaru *et al.* 2010).

Behavior.

AFC for Step – SFO/VChR1, Ctrl – SFO/VChR1 and Reversed – SFO/VChR1 cohorts entailed the placement of mice in a conditioning chamber, and 1 minute later, the presentation of a tone (2800 Hz, 90 dB, 30 seconds) that coterminated with a shock (3 sec, 0.7 mA). Only the Step – SFO/VChR1 cohort received a 5

second pulse of 5mW 488nm blue light immediately prior to the tone/shock presentation and, thereby, the depolarization of a subset of their neurons during the tone/shock association. The Reversed – SFO/VChR1 cohort received a 5 second pulse of 5mW 488nm blue light 55 seconds after the tone/shock presentation. All three groups were trained a second time 24 hours later. Testing for AFC occurred 24 hours after the second training (Tone Test). Mice were placed in a novel chamber, and 1 minute later, the tone was presented for 1 minute. The index of memory, freezing, was assessed via automated procedures (Med Associates Inc.). 24 hours later VChR1 activation was achieved by illuminating the BLA of all three cohorts with a 10mW 543nm laser (10Hz, 10ms pulse width) 1 minute after placement in a novel chamber. Activation continued for 1 minute (Optogenetic Activation). The index of memory, freezing, was assessed via manual scoring procedures by researchers blind to the conditions of the experiment.

For the elevated plus maze a cohort of pLenti-SFO/VChR1 infused animals were placed on an elevated plus maze and allowed to explore for 3 minutes. Each animal then received a 5 second pulse of 5mW 488nm blue light and allowed to explore the elevated plus maze for another 3 minutes. The time spent in the open, center or closed portions of the maze was assessed with automated procedures.

Pairing of step function opsin and tone entailed the placement of pLenti-SFO/VChR1 infused animals in a conditioning chamber, and 55 seconds later SFO was activated with a 5 second pulse of 5mW 488nm blue light. The presentation of a tone (2800 Hz, 90 dB, 30 seconds) immediately followed the SFO activation. Testing for AFC occurred 24 hours later. Mice were placed in a novel chamber, and 1 minute later, the tone was presented for 1 minute. Freezing was assessed with automated procedures. Pairing of step function opsin and tone on previosly conditioned animals entailed the placedment of pLenti-SFO/VChR1 infused animals in a conditioning chamber, and 1 minute later, a tone (2800 Hz, 90 dB, 30 seconds) was presented that coterminated with a foot-shock (2 sec, 0.7 mA). Testing for AFC occurred 24 hours after training. Mice were placed in a novel chamber, and 1 minute later, the tone was presented for 1 minute (Pre-Pairing Tone Test). 24 hours later mice were placed in the conditioning chamber, and 55 seconds later SFO was activated with a 5 second pulse of 5mW 488nm blue light. SFO activation was immediatley followed by the presentation of a tone (2800 Hz, 90 dB, 30 seconds). Testing for AFC occurred 24 hours later (Post-Pairing Tone Test). Mice were placed in a chamber, and 1 minute later, the tone was presented for 1 minute.

CHAPTER VI

Discussion

6.1 Summary of findings

The research presented in this dissertation is built upon results of work performed previously in the Silva laboratory, which demonstrated that the transcription factor CREB (cAMP-response element binding protein) plays a role in memory allocation; increasing the level of CREB in a subset of basolateral amygdala neurons increased the probability that these neurons would store an auditory-fear memory. Initially, I explored the functionality of these CREB-biased memory neurons by specifically activating them, utilizing a novel technique called optogenetics. I demonstrated that activation of CREB-biased memory neurons is sufficient to elicit recall of an auditory-fear memory. This recall was significantly greater than that seen when activating a similarly sized population of neurons that did not express CREB. Importantly, this non-CREB expressing control group preformed comparably to the CREB expressing group in tone tests. This demonstrated that the recall observed in the CREB expressing group is due to the allocation of the memory trace to the CREB expressing neurons and is not a function of differential learning. It also confirmed a widely held, yet hitherto largely unproven belief in the neuroscience community, that activating the specific neurons that store a memory will lead to the retrieval of that memory.

Two additional controls were performed to validate that these results were indeed due to memory allocation and not to some other mechanism. It was possible that the increased freezing of the CREB group was due to the enhanced excitability of

CREB coupled with the optogenetic activation. To control for this, a group of animals that expressed CREB, but was not trained, received optogenetic activation. As expected, optogenetic activation of this control group did not lead to recall. This result ruled out the possibility that a CREB induced increase in excitability, in conjunction with the effects of optogenetic activation, would be sufficient to trigger non-specific freezing. However, there was still the possibility that a CREB induced increase in excitability and the neuromodulators released following foot shock, in conjunction with optogenetic activation, might be sufficient to trigger recall. To control for this, a group of animals that expressed CREB was trained in a way that exposed the animals to shock but did not lead to auditory-fear learning. As expected, optogenetic activation of this control group did not lead to recall.

A non-memory allocation explanation for these results is that a CREB induced increase in excitability and the encoding of an auditory-fear memory trace in a subset of neurons other than those with increased CREB may have led to recall. A control for this is almost impossible to carry out since expressing CREB in a population of neurons will bias the memory trace to those very CREB expressing neurons, as has been shown previously. Theoretically, it may be possible to design an experiment to test this by inhibiting the activity of a population of CREB expressing neurons during auditory-fear conditioning. This would drive the memory to be stored in a non-CREB expressing subset of neurons. If our hypothesis is correct, activating these CREB expressing neurons at a later time

should not elicit recall. Currently, it is not possible to conduct this experiment due to the technical difficulties of combining a mechanism to activate and inhibit virally CREB expressing neurons. However, subsequent experiments have demonstrated that viral CREB is not needed for the activation of a memory trace, thereby indirectly demonstrating that the recall observed is not due to a combination of a CREB induced increase in excitability and the encoding of an auditory-fear memory trace in a subset of neurons other than those with increased CREB. Collectively, these results demonstrate that activation of CREB biased memory neurons is sufficient to elicit recall of an auditory-fear memory, and that this recall is due to memory allocation.

Another series of experiments established that increasing neuronal excitability in a subset of basolateral amygdala neurons could allocate auditory-fear memories in a way analogous to increasing levels of CREB. This was achieved by specifically enhancing the excitability of a subset of BLA neurons during auditory-fear conditioning with novel optogenetic techniques. Optogenetic activation of these enhanced excitability neurons was sufficient to elicit recall of an auditory-fear memory. Importantly, recall was significantly greater than that seen when activating a similarly sized population of neurons that did not possess enhanced excitability during auditory-fear conditioning. Both groups learnt to the same extent, demonstrating that the recall observed in the enhanced excitability group was due to the allocation of the memory trace to neurons with enhanced excitability, and that it was not a function of differential AFC learning.

Accordingly, the presence of CREB was not necessary for the optogenetic activation of an allocated auditory-fear memory trace. It has been assumed in order for a memory trace to be biased to a population of neurons with enhanced excitability, those neurons must be excitable at the time of auditory-fear conditioning and not after. This was demonstrated by performing an experiment identical to those presented above, with the exception that the excitability of a subset of basolateral amygdala neurons was enhanced following auditory-fear conditioning. Optogenetic activation of these enhanced excitability neurons was not sufficient to elicit recall. These results indicate that increased neuronal excitability may be the mechanism by which CREB allocates memory.

6.2 Amygdala neural circuitry and plasticity

The lateral amygdala (predominantly the dorsal subregion) rapidly receives information regarding the auditory CS from the auditory thalamus. It is on the dendrites of the neurons of the lateral amygdala that the initial CS-US association is made. The convergence of CS and US input on a neuron leads to the synaptic strengthening of the CS input, thereby allowing a future presentation of the CS to facilitate an appropriate behavioral response that could not have been elicited prior to synaptic strengthening (**Fig. VI-1A)**. However, results of our experiments demonstrate that this synaptic strengthening cannot be the only site of plasticity in the amygdala circuit. If this were to be the case, activation of any neurons downstream of the thalamic-lateral amygdala synapses (precisely the neurons manipulated in the experiments presented in this dissertation) would elicit freezing. The results presented here clearly demonstrate that there must be a second site of plasticity, since freezing is only observed when a memory trace is biased to the optogenetically activated neurons (**Fig VI-1B)**. As synaptic strengthening at the thalamic-lateral amygdala synapse is in itself insufficient to facilitate a behavioral response: subsequent plasticity at downstream sites in the amygdala must also be necessary. This makes sense in light of anatomical data demonstrating no direct connection between the lateral amygdala and central medial amygdala, the amygdala's major output structure. Three nuclei of the amygdala that connect the lateral amygdala and central medial amygdala (basal

amygdala, intercalated cells and the central lateral amygdala) are potential sites of downstream plasticity.

Fig. VI-1

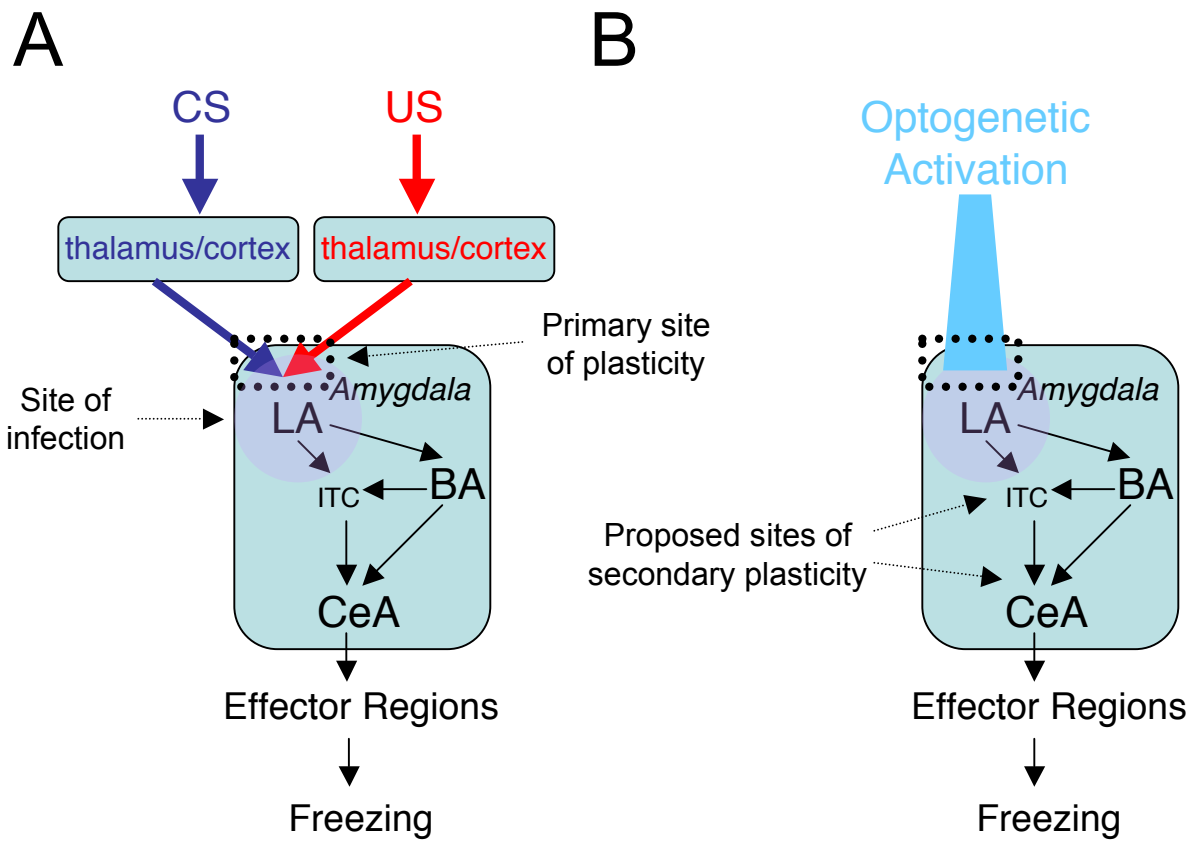


Figure VI-1 - Implications for the circuitry underlying auditory-fear conditioning

A) The primary site of plasticity in auditory-fear conditioning (AFC) is the dendrites of the lateral amygdala (LA), where information about the conditioned stimulus (CS) and the unconditioned stimulus (US) initially meet. The neurons on the LA are the primary site of infection and optogenetic activation. Following AFC presentation of the CS leads to freezing. Information then flows from the LA to the central amygdala (CeA) by way of the basal amygdala (BA) and intercalated cells (ITC). The CeA, the primary output of the amygdala, modulates the activity of effectors.

B) Optogenetic activation of LA neurons leads to freezing only if the AFC memory trace is stored in the neurons being manipulated. Thus, there must be secondary sites of plasticity between the LA and the central amygdala (CeA).

6.3 Global conclusions

The work presented in this dissertation utilized the ChR2 system to demonstrate that post-training activation of neurons with high levels of CREB triggers recall of an AFC allocated memory. Previous results indicated that CREB increases the excitability of BLA neurons, thereby providing a possible mechanism for its role in memory allocation. This was tested by transiently increasing excitability in a subset of BLA neurons during AFC. Data obtained by optogenetically activating this population of neurons demonstrate that excitability plays a key role in memory allocation, a result consistent with the hypothesis that CREB modulates memory allocation by transiently increasing neuronal excitability.

To date, memory allocation has been shown to occur at both the synaptic (synaptic allocation) and neuronal (neuronal allocation) scale. Mechanisms such as synaptic tagging and capture, and the local diffusion of plasticity related proteins have been implicated in synaptic allocation, while CREB induced changes in neuronal excitability have been demonstrated in neuronal allocation. However, distinctions between synaptic and neuronal allocation are somewhat arbitrary, as the two are necessarily intertwined. It is hoped that the study of memory allocation will elucidate the principles that govern which synapses on which neuronal ensembles will encode a given memory in relation to prior and subsequent memory. There are undoubtedly numerous other mechanisms of

memory allocation to be discovered, but some preliminary rules can be postulated. Firstly, and critically, there are mechanisms on both the synaptic and neuronal scale that determine which sub-population of synapses and neurons (from a larger population that receives a similar input) are involved in the encoding of a given memory. This suggests that memory allocation is an active phenomenon and not one solely determined by neuronal wiring. Secondly, synaptic allocation functions to allocate memories on a short timescale, while neuronal allocation functions to allocate memories on longer timescales. Potentially, this temporal separation of functions allows the brain to link memories across greater timescales than would be possible with either synaptic or neuronal allocation alone. Thirdly, on both the synaptic and neuronal scale, the number of synapses and neurons allocated to the encoding of a given memory is regulated. This is critical for the efficient storage and recall of contextually and temporally linked memories.

It will be crucial for future research to further elucidate the mechanisms of memory allocation in structures such as the hippocampus, cortex and striatum, which support various other forms of memory (semantic, episodic, procedural . . .). Another important area of investigation will be how memory allocation functions in the formation of remote memories. A better understanding of the timescales in which the various mechanisms of memory allocation function will lead to clearer insight into how each mechanism fits into the larger picture. For example, the discovery that, for a given window of time, no presently understood

mechanism of memory allocation is applicable, could direct future research. So far, all known mechanisms of memory allocation are synaptic or neuronal. Novel means of memory allocation maybe neither synaptic or neuronal in nature. Since it is recognized that the number of neurons that encode a given memory is regulated, there must be circuit level mechanisms of memory allocation. Investigation of these circuit level mechanisms will surely lead to a quantum leap in understanding.

In order to elucidate the rules governing memory allocation, it will be necessary to observe how memories are allocated in relation to prior and subsequent memories of varying contextual similarity. For example, memories encoded close in time to each other may prove to be encoded in overlapping populations of synapses and neurons. More speculatively, the reconsolidation of a recalled memory may allow it to be linked to a novel memory being encoded at the time of recall. Our present limited level of understanding of memory allocation serves to illuminate the scope and complexity of the issue to be resolved, prompting design of an almost infinite number of exciting and challenging experiments. It will be necessary to observe the activity of entire populations of neurons during the encoding/recall of multiple memories over the space of minutes, days, months and years. It has long been possible to record the activity of neuronal ensembles using single unit recording techniques. Unfortunately, these methods are able to record only a relatively limited population of neurons at any one time, and the type of neuron recorded and its spatial relationship to others cannot be well

defined. Advancements in microelectrodes, microelectrode arrays and spick-sorting software are allowing for data from ever greater numbers of neurons to be recorded (Du, Riedel-Kruse *et al.* 2009; Royer, Zemelman *et al.* 2010). To my knowledge, no studies have yet used single unit recording techniques to directly study memory allocation, but there is every reason to consider it possible. Numerous molecular and genetic techniques currently being developed will address the need to observe the activity of whole populations of neurons. Many have already utilized immediate early genes (IEGs) such as c-fos, arc, homer and zif that are known to be expressed at set time points after neuronal activation (Kubik, Miyashita *et al.* 2007). Guzowski and Worley developed cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (Catfish) to identify the activity of a population of neurons at two time points separated by ~30 minutes. Catfish achieves this by ingeniously taking advantage of the varying cellular locations of arc expression following neuronal activation (Guzowski and Worley 2001). Although Catfish has provided a wealth of knowledge about the relationship between two memories in the brain, it is somewhat constrained by the fact that the two events under investigation must occur within a relatively short time period (Kubik, Miyashita *et al.* 2007). Reijmers *et al.* developed a transgenic mouse that could tag (utilizing the IEG cFos promoter) a population of neurons that were active during a defined window of time. The advantage of this technique over Catfish is that the tag can remain stable for days to weeks. Therefore at a later time, a second behavior can be performed and neural activation (as assessed by the expression of an IEG) can

be compared with that of the transgenic long-lasting tag (Reijmers, Perkins *et al.* 2007). This technique and variants of it have been highly informative to the field, but research is still limited to observing the activity of a population of neurons during two windows in time.

The development of transgenic mouse lines that express fluorescent proteins (*e.g.* GFP) under the control of IEG promoters (Barth, Gerkin *et al.* 2004; Wang, Majewska *et al.* 2006) in combination with two-photon *in vivo* imaging allowed for the activity of populations of neurons to be observed during multiple windows in time (Denk, Strickler *et al.* 1990; Denk, Delaney *et al.* 1994). Two-photon *in vivo* imaging allows for the imaging of cortical neurons expressing GFP down to a depth of a few 100um. Employing this technique, the activity of a population of neurons (as measured by the expression of GFP) has been repeatedly observed following behavior (Yassin, Benedetti *et al.* 2010). However, the researcher is still limited to looking at neurons on the surface of the brain that were active throughout a window of time and not in real time. The development of fluorescent Ca^{+} indicators that can either be bulk-loaded or genetically encoded, in combination with two-photon *in vivo* imaging, has paved the way for real time measurements of neuronal population activity (Stosiek, Garaschuk *et al.* 2003). When neurons fire action potentials, Ca^{+} enters the cell via voltage gated Ca^{+} channels and is also released from intracellular stores, resulting in the Ca^{+} indicator fluorescing. Utilizing two-photon microscopy, the researcher can now observe the activity of entire populations of neurons. Initially, these populations of

neurons had to be situated less than a few 100um from the top of the brain, and the animal was required to be head fixed (Svoboda, Denk *et al.* 1997). However, recent advancements in microscopy have allowed for the miniaturization of the microscope (Helmchen, Fee *et al.* 2001; Flusberg, Nimmerjahn *et al.* 2008; Wilt, Burns *et al.* 2009). This means that it is now feasible to place the microscope on top of the animal's head while it moves freely. A further advancement came with the development of lenses that allow these head-mounted microscopes to image neural populations millimeters from the surface of the brain (Reed, Yan *et al.* 2002). Therefore, observation of the activity of entire populations of neurons anywhere in the freely behaving rodent brain is now theoretically possible. With techniques such as these, novel insights into memory allocation are surely on the horizon.

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