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Neural Structure of Perception and Memory

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in

Neurosciences

by

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The Dissertation of Aleena Reneé Garner is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE DISSERTATION

Neural Structure of Perception and Memory

by

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The ability to specifically manipulate functionally defined circuits is crucial for understanding the cellular basis of perception because percepts do not arise from a single brain region or anatomically defined population of neurons but from a distributed, sparse population of neurons whose demographic characteristics are unknown. Furthermore, manipulation of circuits generated by natural sensory experience is necessary to understand how percepts allow comprehension of a coherent world from independent basic sensory input signals. Additionally, controlled regulation of internally generated activity will provide an understanding of the causal role of translational computations on perception generation. Chapter one provides a history of evidence for neural correlates of perception and possible computational roles of internally generated

activity on percept generation. Chapter two discusses a novel approach for studying perception by manipulating sparse, distributed circuits functionally defined by their activity during natural sensory experience. Specifically, the effect of activating a competing, artificially generated neural representation on encoding of contextual fear memory is described. The work was published in *Science* in March 2012. In summary, a cfos based transgenic approach was used to introduce the hM_3D_q receptor into neurons based on their natural activity patterns. Neural activity could then be specifically and inducibly increased in the hM_3D_q expressing neurons by an exogenous ligand. When an ensemble of neurons for one context was artificially activated during conditioning in a distinct context, animals formed a hybrid memory representation. Reactivation of the artificially stimulated network within the conditioning context was required for retrieval of the memory. The memory was specific for the spatial pattern of neurons artificially activated during learning while similar stimulation impaired recall when not part of the initial conditioning. Chapter three considers several research questions that arose as a result of the findings presented in chapter two as well as other previous studies. The chapter describes a new transgenic system using light activation, and proposes experiments to address whether a circuit that represents a new associative memory is constructed entirely *de novo* or if an existing neural network is modified to code for a new experience.

Chapter 1: Investigations of the Neural Correlates of Cognition and Perception

Introduction

The investigation of cognition is metarepresentational. As was stated by the information theorist and psychologist Fred Attneave at the meeting of the Western Psychological Association in 1973 “How can we represent the representational system?” [1]. If we consider the internal brain to be one system and the external world to be another system, how do the two systems interact, and by what translational code does the former represent the latter?

Perception

One facet of cognition is perception. Perception can be thought of as internal, translated code that allows an animal to comprehend the world by giving basic sensory representations functional meaning. Neurons and neural networks can represent integrated concepts and complex percepts. For example, Quiroga et. al. (2005)[2] performed single and multiunit recordings in the anterior hippocampus and amygdala of human patients, and found individual neurons that selectively responded to a picture, a written name, and a spoken name of a specific person. Not only were cells activated by well known people that may have been stored as long-term memories in the patients, but also by multimodal presentations of the researchers performing the recordings who the patients had only known for a few days. These findings reveal a correlation between neural activity and not just simple feature detectors but complex percepts. Futhermore, multi-representational percepts may be created within a few days of novel presentation of the represented object or person.

While these human studies are purely correlational, behavioral studies of animals have shown that they too are capable of integrated and multi-representational perceptions, and therefore can be used as model systems for determining the causal role of molecules and neurons in generating the cognitive processes of representation. For example, Gardner and Gardner, who taught language to chimpanzees, found that the primates would say the name of an animal if they saw the actual animal, saw a picture of the animal, or heard the sound the animal typically made[3]. In fact, pigeons have also been shown to be capable of multi-level representations. Herrnstein and Loveland (1964)[4] showed that the birds will give a conditioned response to entire human figures or to parts of humans, such as hands, that can represent the entire being.

Technology development has allowed a more meticulous, mechanistic understanding of the cellular basis of perception in animals. For example, Boyden et. al. (2005)[5] adapted a light gated cation channel, Channelrhodoposin2 (ChR2) derived from the green alga *Chlamydomonas reinhardtii*[6] for expression in mammalian neurons endowing them with the capability to be optically activated with single spike precision. Expression of ChR2 can be genetically controlled, and because only the neurons expressing the channel will be sensitive to light, it allows activation of sparsely embedded neurons without affecting neighboring cells. Huber et. al. (2008)[7] used this technology to investigate a minimum number of neurons and amount of neural activity that can be detected by an animal and used to drive learned behavior. The authors expressed ChR2 in pyramidal neurons of layer 2/3 in mouse barrel cortex, and

varied the intensity of blue light and number of stimuli pulses to control neural activity with single action potential resolution in mice. The mice were taught to nose-poke in one of two ports for a water reward depending on whether they did or did not perceive the firing of neurons in somatosensory cortex. When 5 action potentials were induced, as few as approximately 60 neurons could be detected and used in the decision making task. When only a single action potential per cell was produced, mice could detect and respond to approximately 300 neurons. While the number of neurons and amount of neural activity that is required to code for or generate a percept or memory of a natural experience may be greater, the number of neurons and level of neural activity that can be detected by animals and used to drive behavior appears to be quite small.

Associations and Associative Memory

One example of a natural perceptual task is making an association between two stimuli or events. For example, if you are looking at a hot oven and the last time you saw a hot oven you ate a delicious cake, then you may become hungry and happy because you are thinking about the cake. However, if the last time you saw a hot oven, you burned your hand, then you may cringe at the thought of the pain you suffered. These associations of hunger and happiness or fear and pain with the visual cue of the oven are internal representations reflecting the external world. The particular representation, that is hunger and happiness or fear and pain, depends on the stimuli that happened to be paired with the sight of the oven, cake or burn.

Associative learning in animals is critical for understanding cognitive tasks such as memory and perception because it allows a functional read-out of neural circuit computations through behavior. The formation of associations in rodents can be measured using classical conditioning[8]. For example, a rodent will learn to associate a cue, such as a tone, with an electric foot-shock if the two stimuli are paired together. In response to the shock, the rodent will acquire a learned freezing fear response, and after learning the association between tone and shock, the rodent will freeze to the tone presented alone. The amount of freezing to the tone can then be used as an assessment of memory strength. The more the rodent freezes, the stronger the memory. Furthermore, one knows that the rodent is perceiving fear if it freezes upon tone presentation[9, 10]. The neural circuits that code for such associative memories likely arise as a result of external stimuli activating cells with the correct temporal pattern for synaptic strengthening as well as internal dynamics and closed loop processing, that is, signal transformation without input from the external world.

Neural Circuits of Associations and Associative Memories

Understanding the formation of neural circuits that code for a learned association between two paired stimuli will allow an understanding of the circuits that lead to percepts because such circuits cannot contain specific feature detectors but must compute subjective relational knowledge about the world. Repa et. al. (2001)[11] recorded from cells in the Lateral Amygdala (LA) of freely behaving rats during tone-shock pairings, and found that approximately 70% of cells had a greater response to the conditioned tone after it was paired with the

shock. Importantly, the increase in neural responsiveness immediately proceeded or occurred simultaneously with changes in behavior (learned freezing response to the tone). Thus, environmental contingencies resulted in neural activity changes underlying an acquired percept and learned behavior in rats. Additionally Rumpel et. al. (2005)[12] showed that GluR1 subunit containing AMPA receptors are incorporated into synapses with afferent fibers from the auditory thalamus and efferent fibers to the LA after tone-shock pairings in rats. When GluR1-receptor synaptic incorporation was molecularly blocked in approximately one-quarter of neurons, rats showed reduced freezing to the tone stimulus. This reveals molecular changes in a neural circuit resulting from an external stimulus that are necessary to drive learned fear behavior.

Perturbation of circuits involved in coding associations and generating the corresponding learned behaviors has revealed that they can be flexible and are likely distributed across different brain regions. Goshen et. al. (2011)[13] expressed the inhibitory photo-activatable receptor eNpHR3.1[14] in excitatory neurons of hippocampal CA1 to determined the effects of inhibition of this sub-region on contextual fear conditioning and recall. When CA1 was selectively silenced during conditioning, mice did not form a contextual fear memory, however, when the same mice were re-trained in the absence of light-induced inhibition, they showed strong memory performance 24 hours later. When CA1 was inhibited during a 24 hour retrieval test, mice failed to recall the fear memory, which is in accord with a long history of lesion studies. Additionally, inhibition of CA1 left intact auditory-cued memory while expression and activation

of the inhibitory opsin in basolateral amygdala (BLA) prevented both contextual and cued fear conditioning demonstrating the functional specificity of the technique. The authors also tested eNpHR3.1 mice four weeks after normal contextual conditioning to address the role of CA1 in remote retrieval because while many hippocampal lesion studies have shown hippocampal independence of remote memories[15-24], several studies which relied heavily on spatial memory[24, 25] and in which more severe medial temporal lobe damage was produced[26-28], have shown complete, non-graded, retrograde amnesia. The authors found that when they silenced CA1 for approximately 30 minutes before retrieval testing, to recapitulate the extended suppression of CA1 in lesion studies, mice showed no contextual memory deficit. Interestingly, when the authors immediately and precisely inhibited CA1 either just prior to retrieval or intermittently during the test, mice showed a severe memory deficit and inhibition of freezing behavior that meticulously followed light administration.

Previous studies[18] have shown that expression of the neural activity induced immediate early genes cfos and zif268 are relatively elevated in the hippocampus, but not anterior cingulate (ACC), after a 24 hour memory retrieval test, but become elevated in the ACC and return to basal hippocampal levels after a 36 day (remote) retrieval test. Goshen et. al. (2011)[13] showed that inhibition of CA1 immediately before 28 day retrieval testing, which results in memory impairment, lead to a slight decrease of cfos expression in CA1 and a large decrease in ACC relative to controls. Prolonged 30 minute inhibition of CA1 before remote memory testing, which does not affect behavioral memory

performance, again lead to a slight reduction of cfos expression in CA1, but in contrast to immediate inhibition, resulted in highly elevated expression of cfos in the ACC compared to control mice. These results show that circuit computations underlying specific perceptions and behaviors such as a memory retrieval can be flexible.

The probabilistic occurrence of an environmental event can also affect the neural computations that underlie an animal's perception of event-related stimuli and responsive behavior. For example, Jaramillo & Zador 2011[29] performed a series of experiments in which rats had to identify a frequency-modulated target in a procession of pure tone distractors. Presentation of the target was made probabilistic by presenting it in 'expect-early' or 'expect-late' blocks. In 'expect-early' blocks, the target sound occurred 300 or 450 ms after pure-tone onset 85% of the time and 1,350 or 1,500 ms after pure-tone onset 15% of the time. In 'expect-late' blocks, this was reversed. The authors also varied the difficulty of the task by making the frequency-modulated target more or less similar to a pure-tone. After learning the task, single unit responses and averaged local field potentials (LFP) were increased following presentation of pure tone cues initiating a trial in expect-early trials. Likewise, spiking of neurons that responded to the target sound also increased, but only when the target matched the neuron's preferred frequency. These results suggest a refinement during learning of the neural circuit required for processing of the auditory task. Neural activity was also correlated with the behavior of the rats. When rats correctly anticipated the timing of a presentation of the target sound, they reacted

significantly faster to receive a water reward even for easy trials in which their accuracy was about 90% correct. Moreover, accurate temporal expectation improved behavioral accuracy especially when the target sound was of intermediate difficulty. This study eloquently demonstrated effects of the temporal structure of external stimuli on modification of neural circuits activated during processing of the stimuli, and furthermore that these circuit modifications were correlated with a change in behavioral performance.

However, specific activity in a particular neural circuit during processing of external stimuli can lead to different circuit functions, as determined by behavioral output, depending on the types of external stimuli present. Lin et. al. (2011)[30] investigated how neural networks underlying aggressive behavior are related to or interleaved with networks responsible for mating behaviors. After an extensive series of experiments defining the ventrolateral sub-region of the ventromedial hypothalamic nucleus (VMHvl) as a locus for offensive attack in male mice, the authors used Cre-dependent expression to target ChR2 specifically into VMHvl neurons. Activation of VMHvl neurons yielded different results depending on the environmental setting. Stimulation did not alter behavior when administered to an isolated mouse, but produced immediate, coordinated, and directed attack behavior when a male, female, or castrated male intruder was present. Interestingly, upon cessation of the light, male mice stopped attacking female mice with a much shorter latency than castrated male mice. Furthermore, low intensity light levels triggered attack more promptly in the presence of a castrated male than a female. When an anaesthetized mouse was

present, illumination resulted in attack by 60% of mice unless the anaesthetized mouse was artificially moved, which resulted in attack by 100% of mice. In fact, illumination resulted in attack of a motionless glove by 25% of mice, but if the glove was artificially moved, 75% of mice attacked. When a female was present, illumination yielded attack 80% of the time before initiation of mating, however during intromission the same light intensity was ineffective. Only after a 4-fold increase in light intensity, did the stimulus induce attack (in approximately 70%) of mice during mating. However, after ejaculation, illumination resulted in attack with the same probability as it did before mating. Finally, the authors selectively silenced VMHvl neurons using genetically directed expression of the *Caenorhabditis elegans* ivermectin (IVM)-gated chloride channel (GluClab), which has been modified to be insensitive to glutamate, and induces hyperpolarization upon IVM binding[31, 32]. This resulted in suppression of offensive attack behavior but has no impact on mating behavior. Thus, the same type of internal activity can generate different types of behaviors depending on the external environmental input present at the time of neural circuit activation. The mechanism by which different environmental settings change the function, that is percept and behavior generated, of neural circuit activity may rely on what is referred to as a schema, a knowledge structure or cognitive framework that allows an internal representation of the world.

Schemas and Attractor States

The ability to organize new or fragmented percepts making them quickly understandable is also thought to depend on schemas. Recently, Tse et. al.

(2007)[33] provided a behavioral demonstration that rats can learn more readily if they have an experientially determined framework to which new information can easily bind. Rats were trained to learn six flavor-place associations, where each place was a sand well in a familiar arena. The rat began each trial in a start-chamber with the presentation of one type of food and then had to find the correct sand well and dig to receive more food reward. Initial training for this task required thirteen sessions, separated by forty-eight hours, of six trials, separated by one hour. The authors then interchanged two of the flavors with novel flavors and new corresponding sand well positions. Now the rats learned the association task with one session of six trials. Moreover, when rats were trained in a completely new environmental context with six new food flavor place associations, they again required thirteen sessions of extensive training to learn the task. These results reveal that the rats did not simply become better learners of associations in general, but that they developed a specific contextual cognitive framework to be able to more quickly interpret and understand novel stimuli in a familiar and predictable world.

A theoretical explanation of a schema may be a stable attractor state that binds new information. Although, the mechanism for how pieces of information signaled by different neurons can be bound together into a coherent picture is a difficult problem and highly debatable[34], attractor state models can be used to explain many of the enigmatic phenomena found empirically. Samsonovich & McNaughton (1997)[35] presented an attractor network model of hippocampal place coding that can account for the persistence and precision of place-cells

regardless of locomotor or motion variance or differences in sensory input that may arise, for example, in different lighting conditions. The model also explains spontaneous re-mapping of place-cell activity without alteration of the basic framework of the spatial map, and also expeditious place-cell formation upon exposure to a novel environment that does not change as exploration time increases. Finally, the occurrence of distinct place-maps for the same environment under different behavioral conditions can also be explained by the model.

Certainly biological examples of attractor states exist in the nervous system. Cossart et. al. (2003)[36] revealed organized ensembles of neurons in visual cortex slices that alternated spontaneously between resting (DOWN) states and hyperexcitable (UP) states with a stereotyped spatial and temporal pattern suggesting the presence circuit attractors. Further support for the idea of attractor states in the nervous system was provided by MacLean et. al. (2005)[37] by comparing spontaneous and evoked activity in thalamocortical slices. Small groups of cells were identified that were spontaneously and coherently active during cortical UP states in layer IV of somatosensory cortex. Intriguingly, when cortex was driven into an UP state by stimulation of thalamic efferent fibers, the same groups of cortical neurons were coherently active.

Kenet et. al. (2003)[38] used voltage sensitive dyes to measure the dynamics of attractor states in the cat visual area 18 *in vivo*. This region shows stereotypic functional maps tuned to orientation when the animal is presented with drifting gratings. Correlation coefficients between two maps generated from

an identical stimulus presented in different recording sessions ranged from 0.7 to 0.8. A comparison of spontaneous activity patterns with orientation maps constructed from evoked activity revealed correlation coefficients in the range of 0.6. Spontaneously generated activity that corresponded to evoked orientation patterns occurred about 20% of the time, and contained most of the information found in stimulus evoked orientation preference maps. These findings suggest the presence of stable nodes of activity that can be activated spontaneously in the absence of a visual stimulus and that may result in representations of visual attributes.

To investigate attractor states with cellular resolution, Ch'ng and Reid (2010)[39] used calcium imaging to measure functional organization in visual cortex *in vivo* during spontaneous and evoked activity. While the activity of hundreds of cells can be simultaneously measured with precise temporal resolution using extra-cellular electrophysiological recordings[40], the specific cell types giving rise to electrical signals and the spatial relationships between them cannot be well determined using this technique. Ca^{++}-imaging however, reveals spatial activity patterns of neurons and because of the broad spatial resolution, it can be used to delineate structure-function relationships of neural circuits even when neurons within the circuits are distributed and sparse[41].

Ch'ng and Reid (2010)[39] measured activity in both the cat, which has a spatially and tonotopically organized map of orientation preference[42-45], and the rat, for which no evidence of spatial organization exists[46, 47]. Interestingly, the authors found that similar to evoked patterns of activity, spontaneously co-active

neurons are spatially clustered in the cat and dispersed in the rat. Moreover, cells with similar orientation tuning in each species were more likely to be co-activated during spontaneous activity. Thus, the clusters of cells in cat visual cortex with similar orientation tuning were synchronously active, and the random pattern of distributed cells in rat visual cortex with similar orientation tuning were synchronously active. Accordingly, the relationship between coordinated activity and distance between cells differed in the cat and rat. In cat visual cortex, coordinated activity decreased sharply with increasing distance between neurons. However in the rat, no relationship was found between coordinated activity and the distance between cells. These findings suggest that functional attractor state dynamics do exist in the brain. Although it is unclear whether attractor states are generated over time as a result of commonalities and probabilities in sensory features and input signals, or whether they are a feature of the intrinsic architecture of neural networks into which information becomes coded, or perhaps a combination of both.

Modulation of Specific Circuits for Associations and Associative Memories

Dispersed neural networks and sparse coding of visual stimuli in rodents are not unique to the visual system or the rodent. For example, a dispersed coding of space in the hippocampus by place cells was beautifully demonstrated by Dombeck et. al. (2010)[48]. Using their previously described virtual navigation system in which head fixed mice can locomote on an air-supported rotational sphere[49, 50], the authors built a two-photon microscope (TFM) that could both accommodate the virtual reality surround screen and block the light source

emitted from the screen. The authors trained mice to traverse a virtual linear track while recording neural Ca^{++}signals to determine how spatial navigation maps are represented. They first showed that hippocampal place cells could be measured using Ca^{++}-imaging similarly to electrophysiological recordings. Then, using the spatial resolution afforded by Ca^{++}-imaging, the authors found that no two- or three-dimensional relationship existed between the location of a place field in the environment and the position of the corresponding place cell in CA1. In addition, no relationship was found between the correlation in activity and distance between cells. More complex percepts and memories, such as those described by Penfield and Perot[51], are also distributed across brain regions and sparsely coded[52, 53]. In fact, associative memory formation can result in modification and sparsification of circuits involved in processing sensory information and relationships between stimuli. For example, Komiyama et. al. (2010)[54] used Ca^{++}-imaging to monitor dynamic neural activity patterns in motor cortex during sensory-motor learning. The authors trained head-fixed mice to lick in response to one odor and suppress licking in response to a second odor. They found correlations in neural activity in the anterior-lateral motor (ALM) and posterior-medial motor (PMM) areas that progressively increased with behavioral learning improvements. Additionally, the proportion of neurons specifically correlated to the task became more sparse as behavior improved.

To investigate the formation and function of a distributed associative memory circuit, Han et. al. (2007)[55] used a molecular genetic manipulation to bias newly encoded information into a particular sub-population of dispersed

neurons. The authors found a similar number of cells expressing an activated form of CREB as were found to undergo synaptic plasticity after tone-shock pairing (about 20%). To test the role of CREB in memory-circuit formation, the authors injected replication defective herpes simplex viral vectors expressing CREB into the LA of mice, which resulted in infection of approximately 18% of cells distributed across the nucleus. The mice were then presented with tone-shock pairings. To identify neurons activated by the paired stimuli, expression of the immediate early gene *Arc* was analyzed. *Arc* is rapidly and transiently induced by neural activity allowing a molecular time-stamp of recently activated neurons [56]. *Arc* expression was about three times as likely to occur in CREB overexpressing neurons than in neighboring uninfected neurons suggesting that neurons expressing the highest levels of CREB are selectively recruited into the associative memory trace. Furthermore, when a weak tone-shock training protocol was used, mice overexpressing CREB showed enhanced memory performance compared to control mice indicating a functional role for CREB in memory formation.

To examine the necessity of CREB-expressing neurons in the output function of the memory circuit (that is, recall), Han et. al. (2009)[57] overexpressed CREB in LA neurons to bias their incorporation into a tone-shock memory trace, and then selectively ablated only those cells after conditioning. The authors used transgenic mice expressing the apoptotic inducer diphtheria toxin receptor (DTR) in a lox-P STOP cre-recombinase-inducible system. Only cells infected with a vector containing cre-recombinase will apoptose after diphtheria toxin

administration. A vector encoding CREB for overexpression in neurons as well cre-recombinase was created and injected in the LA of DTR mice. As shown previously, neurons overexpressing CREB were selectively recruited into the memory trace, however injection of DT before retrieval (tone presentation alone) to specifically kill these neurons resulted in a significant reduction in memory performance. Thus, the authors showed that in the amygdala, associative learning arises from a sparse ensemble code and this engram can be formed through a molecular competition between neurons.

Conclusion

How does cognition arise from the interplay of external factors exerting force on the nervous system and internal dynamics autonomously generated? This chapter provides a review of studies investigating the influence of external input and its temporal structure as well as internally generated activity on the formation and flexibility of neural circuits whose function is the generation of perception. However, these studies were mostly correlational and involved manipulating sensory input and monitoring either changes in neural activity patterns or changes in behavior.

Some studies, such as Huber et. al. (2008)[7] have used recently developed effector molecules, in this case ChR2, to modulate neural activity specifically and directly, however, the circuits being manipulated are not functionally defined and thus may not have any intrinsic meaning to the animal. Goshen et. al. (2011)[13] used the inhibitory effector molecule, eNpHR3.1, to manipulate regions of brain tissue containing functionally defined memory circuits. However, their inhibition

of brain regions did not respect the sparse nature of coding or specific circuits required for memory retrieval. Finally, Lin et. al. (2010)[30] expressed ChR2 in a population of genetically similar cells and found that activation of these neurons, without prior conditioning, resulted in aggressive behavior implying that activity in these cells did have functional meaning to the animal. However, the modulation of these genetically defined cells by natural sensory experience and their causal role in processing input remains unknown. Furthermore, neural stimulation in VMHvl has been historically shown to drive innate behaviors[58], but has not been shown to be involved in complex percepts. Han et. al. (2007)[55] and Han et. al. (2009)[57] provided the first demonstration of identification and manipulation of a sparse circuit required for the perceptual task of memory retrieval. However, manipulation of neural circuits formed through natural sensory input and experience and that give rise to percepts or memories has not yet been accomplished. The ability to specifically orchestrate functionally defined circuits is crucial for understanding the cellular basis of perception because percepts do not arise from a single brain region or anatomically defined population of neurons but from a distributed, sparse population of neurons whose defining, demographic characteristics are not necessarily known.

Additionally, behavioral evidence for the influence of previously learned contextual information on learning of new information has been demonstrated. In parallel, internally driven spontaneous neural activity has been correlated with sensory evoked activity and shown to be capable of containing information about the environment. Although the manipulation of internally generated activity that

is involved with translating basic sensory input signals into meaningful coherent representations of the world has not been shown. Controlled regulation of internally generated activity will provide an understanding of the causal role of translational computations on perception generation.

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Chapter 2: Generation of a Synthetic Memory Trace

Introduction

Chapter one concluded with several main considerations. First, the ability to specifically manipulate functionally defined circuits is crucial for understanding the cellular basis of perception because percepts do not arise from a single brain region or anatomically defined population of neurons but from a distributed, sparse population of neurons whose demographic characteristics are unknown. Second, manipulation of circuits generated by natural sensory experience is necessary to understand how percepts allow comprehension of a coherent world from independent basic sensory input signals[1], and also how perception can result in modification of behavior for successful fitness[2] in an animal's environment[3]. Finally, controlled regulation of internally generated activity will provide an understanding of the causal role of translational computations on perception generation.

Multiple recent clever ingenuities have allowed the establishment of a system that can be used to identify and control functionally defined, naturally derived, sparsely coded circuits as well as affect internal activity independent from external input signals. A circuit whose computations result in a specific operation but whose individual units may be genetically diverse, can be defined by its function instead of its biological profile. However, labeling and manipulation of specific neurons can currently only be achieved through genetically controlled expression of markers and effector molecules. Thus, Reijmers et. al. (2007)[4] cunningly developed a transgenic mouse in which the

activity-regulated cfos promoter is used to drive expression of downstream transgenes. cfos is one of a few hundred genes, termed immediate early genes (IEGs), that are rapidly and transiently activated upon cell stimulation and whose expression cannot be blocked by protein-synthesis inhibitors[5]. They are often transcription factors that affect series of downstream genes ultimately leading to phenotypic changes in cells thereby permitting external signals to influence biological function. cfos is one of the most well studied IEGs in the brain[6], and is thought to interact with other proto-oncogenes to mediate long-term responses to external signals that regulate cellular growth and development[7]. Basal levels of cfos expression are very low and its expression is most dramatically induced after novel exposure to stimuli or during exposure to stimuli after sensory deprivation[6]. In CA1 pyramidal cells, a train of thirty action potentials will robustly result in cfos expression from both the endogenous promoter and a cfos-green fluorescent protein (GFP) transgene[8]. Additionally, expression of cfos has been used extensively as a time stamped measure of neural activity across the brain[6, 9].

Reijmers et. al (2007)[4] specifically used the cfos promoter to drive expression of a tetracycline transactivator (tTA). tTA is a transcription factor that binds the tetO promoter and thereby drives transcription of downstream genes linked to the tetO promoter[10]. However, binding of tTA to tetO is blocked by doxycycline (Dox) and thus only active neurons in the absence of Dox will express tetO linked genes. In summary, this double transgenic system permits activity dependent expression of transgenes only within a specific Dox-regulated

time window. Reijmers et. al (2007)[4] expressed a β-galactosidase enzymatic marker under the tetO promoter, and allowed activity induced expression during tone-shock associative learning by removing Dox only during conditioning. The authors then presented the tone later during a retrieval test and then sacrificed the animals to stain for resulting endogenous cfos expression. Thus, β-galactosidase served as a time stamp of neural activity during learning, or memory formation, and endogenous cfos served as a time stamp of neural activity during recall, or memory retrieval. The authors found that memory retrieval in mice who had learned a tone-shock association, compared to home-caged controls and tone-no shock controls, resulted in significantly more reactivation of cells that had also been active during learning. Moreover, reactivation of cells during retrieval positively correlated with freezing scores suggesting the greater the reactivation of the amygdala circuit that was activated during learning, the better the memory performance. These results are interesting because the temporal relationships of sensory stimuli during encoding of associations is often different than during retrieval and therefore the neuronal representations of the two events could be different. However, the associative percept (as measured by a behavioral, learned fear response) seamed to be best expressed when the neural circuit activated during learning spatially overlapped with the circuit activated during retrieval.

A second ingenuity imparts specific, non-invasive, synthetic control of neural activity. While, direct electrical stimulation provides exquisite temporal control, has been used to define functional domains in the brain, can elicit

stereotyped behavioral responses, drive self-stimulation behavior, and serve as CS or US in conditioning paradigms [11-14], this type of stimulation is relatively focal compared to the entire brain, using either microelectrodes which lack cellular specificity, or more recently, genetically encoded mediators of neural excitability such as ChR2[15-17]. Because electrical and light mediated activation are focal, *a priori* knowledge about the location of the circuit under investigation is required when using these systems. However, synthetic neural activation using a chemical compound that crosses the blood-brain barrier when administered via intraperitoneal injection enables activation of a dispersed circuit throughout the brain and does not necessitate *a priori* knowledge of circuit location. Armbruster et. al (2007)[18] created the ability to chemically control neural activity remotely by engineering a rat muscarinic 3 receptor to have negligible affinity for its endogenous ligand, acetylcholine, but possess potent affinity for the biologically inert and commercially available compound, clozapine-n-oxide (CNO).

Alexander et. al. (2009)[19] linked this humanized G_q-protein coupled receptor (hM_3D_qR) to the tetO promoter and generated transgenic mice with the CamKIIα-tTA[20] and tetO-hM_3D_q transgenes to test the effects of the receptor when expressed in forebrain neurons of transgenic mice. Whole cell recordings of CA1 pyramidal cells in acute hippocampal slices of mice expressing hM_3D_qR in the presence the sodium channel blocker tetrodotoxin (TTX), to measure membrane potential responses, revealed an approximately 8 mV depolarization upon bath application of CNO. In the absence of TTX, to measure synaptic responses, application of CNO resulted in an increase in pyramidal cell firing

rate. When injected IP in transgenic mice, CNO caused a dose-dependent increase in gamma power that initiated about ten minutes post injection and persisted for approximately nine to twelve hours. In parallel, the mice showed hyperactivity and heightened locomotion that followed the time-scale of increased power in the local field potential.

Garner et. al. (2012)[21] combined the activity regulated tTA system[4] with the chemically inducible hM_3D_qR system[19] to be able to synthetically excite a sparse, distributed neural circuit formed during associative learning and functionally defined by its activity during learning. Because spontaneous activity is likely not 'noise' but carries information about the world[22, 23], and because new learning and comprehension are affected by previous experience as well as existing neural network activity[24, 25], the authors used this double transgenic mouse, termed the $hM_3D_q^{fos}$ mouse to investigate how activation of the internal percept or cognitive framework of one environmental context would affect learning and percept formation of a new environmental context.

Perception Results from Both Externally and Internally Generated Signals

In the mammalian cortex there is significant, non-random, spontaneous neural activity that is internally generated rather than arising from sensory inputs, and this activity influences the processing of natural sensory stimuli [26-29]. How does this internally generated activity influence the formation of a new memory representation? To investigate this question transgenic mice in which the hM_3D_q receptor is expressed in an activity dependent manner by a cfos promoter driven tTA transgene ($hM_3D_q^{fos}$ mouse) [4, 19] were used (Fig 2.1A). hM_3D_q is a Gq

coupled receptor that responds specifically to clozapine-N-oxide (CNO) and produces strong depolarization and spiking in pyramidal neurons [19]. Transgenic animals exposed to a particular environmental stimulus will express hM_3D_q in those neurons that are sufficiently active to induce the cfos promoter, and this naturally occurring neural ensemble can be subsequently reactivated artificially in the transgenic mice by delivery of CNO. Artificial activity induced in this manner will retain the spatial character of the neural ensemble, but will not preserve the temporal dynamics achieved by natural-stimuli.

The expression of hM_3D_q is widely distributed in the brain of $hM_3D_q^{fos}$ double transgenic mice in the absence of Doxycycline (Dox), to allow tTA driven transcription (Fig. 2.1 B&C). Within a given brain area expression is limited to a fraction of excitatory neurons based on neural activity driving the cfos promoter. Dox can be used to control the specific time window in which active neurons are genetically tagged with hM_3D_q by modulating tTA driven transcription [4, 30]. To test the kinetics of CNO based neural activation in these animals *in vivo* recordings were performed in the hippocampus of anesthetized animals. Neuronal activity increased reaching a maximum intensity between 30 and 40 minutes post CNO injection (Fig 2.1D). In order to examine more broadly the increase in neural activity after CNO injection, endogenous cfos expression was quantified (Fig 2.1E&F). We found significant increases in cfos labeling across multiple brain regions (ranging from 2-20 fold) in CNO injected $hM_3D_q^{fos}$ transgenic vs. control animals (Table 2.S1). Labeling for cfos was found in both hM_3D_q positive and negative neurons with 91$\pm$ 2% of hM_3D_q positive neurons in CA1 co-labeled with

cfos (Fig. 2.S2).

In standard contextual fear-conditioning animals develop a memory for the conditioning chamber in which they receive a foot-shock. The ability to form the context association is dependent on the hippocampus, which participates in encoding a representation of the environment [31, 32]. To test the effects of competing circuit activation on formation of a memory trace, the fear-conditioning protocol outlined in Fig. 2.2 was designed. On day 1 hM3D^{fos} mice were exposed to a novel context (ctxA) in order to drive expression of the hM_3D_q transgene into neurons activated in that context. On day 2 animals were injected with Dox to inhibit further hM_3D_q receptor expression and with CNO to stimulate activity in the pattern of neurons that expressed the receptor. The mice were then fear conditioned in a distinct context (ctxB), and 24 hours later, memory performance was tested in the absence and presence of CNO. Thus, the neurons that were active in ctxA were being excited while the animals were fear conditioned in ctxB.

Three potential outcomes were anticipated. The strong synthetic activation of ctxA neurons could be dominant and serve as a CS to produce an associative fear memory. This would lead to a fear response to CNO or possibly even a fear response to ctxA itself if the artificial and natural activation of the neurons were sufficiently similar. This was not observed as the level of freezing in ctxA was not significant in transgenic animals either with or without CNO injection (Fig 2.2A). A protocol in which ctxA neurons were activated by CNO and animals were shocked immediately in ctxB (to prevent formation of a ctxB

representation [30]) also failed to produce a CNO dependent memory (Fig. 2.S3). Thus the synthetic activity alone could not serve as a CS in fear conditioning. A second possibility was that the natural sensory experience in ctxB would dominate and transgenic animals would show normal conditioning to ctxB. The hM3D^{fos} animals displayed a severe deficit in freezing to ctxB suggesting that the CNO induced activity was interfering with normal encoding of memory for ctxB (Fig. 2.2B). A third possibility was that animals would form a hybrid representation, incorporating elements of both the CNO induced artificial stimulation and the natural sensory cues from ctxB. This appears to be the case as the transgenic animals showed a significant increase in freezing in response to CNO delivered in the ctxB setting during the 24-hour memory test (Fig 2.2B). Similar results were observed in two separate experiments when a different contextual set-up for ctxA neural labeling was used (Fig. 2.S1, 2.S4). hM_3D_q expression was qualitatively similar in mice exposed to different contextual set-ups for ctxA (Fig. 2.A2). The requirement for reactivation of the transgene expressing neurons during memory retrieval suggests that their activity was incorporated into the memory trace. Consistent with this idea, freezing during memory retrieval positively correlated with the degree of neural activation, assessed by cfos expression in the hippocampus (Fig. 2.2 C&D).

Retrieval of a memory representation likely involves the reactivation of some neurons that were active during the initial learning [4, 33-35]. To test the susceptibility of this spatial code to competing neural network activation, $hM_3D_q^{fos}$ mice were exposed to ctxA to allow expression of the hM_3D_q transgene but then

conditioned in ctxB without CNO stimulation of the ctxA neural ensemble (Fig. 2.3). As expected, these animals developed wild-type levels of freezing to ctxB 24-hours after conditioning. Now, however, activation of the hM_3D_q expressing neurons impaired memory performance during retrieval in ctxB. This suggests that CNO induced activation of a competing neural network interferes with the learned spatial code and degrades recognition if this activity was not present during the initial training. This is not surprising given that even limited focal hippocampal stimulation has been shown to disrupt spatial memory [36].

Does the hybrid fear memory formed by $hM_3D_q^{fos}$ mice incorporate the specific pattern of ctxA neurons activated by CNO during learning or are the animals responding to a less specific alteration in brain state? To distinguish between these possibilities animals were conditioned in the presence of CNO induced firing of ctxA labeled neurons but then placed on Dox to allow turnover of the hM_3D_q receptor. Two days later Dox was removed from the animals' diet, and they were placed in a new home cage to allow de novo expression of the hM_3D_q receptor in a distinct group of neurons (ctxC). Fourteen days after initial conditioning, memory performance was assessed by freezing scores in ctxB in the absence and presence of CNO induced synthetic activation. No increase in freezing in $hM3D_q^{fos}$ mice in response to CNO (Fig 2.4A) was observed, demonstrating a requirement for reactivation specifically of the learned, ctxA, neural ensemble rather than a generalized change in brain state caused by CNO induced activity.

To further address the issue of ensemble specificity, animals were

preexposed to the fear conditioning context (ctxB) on day 1 to express the hM_3D_q receptor in neurons that are activated in that context. The hypothesis behind this manipulation was that the synthetic activation of a ctxB pattern of neurons would more likely overlap with the natural activity during learning in ctxB and should therefore not interfere with the production of a normal ctxB representation. When animals were fear conditioned following injection of CNO to artificially activate the ctxB ensemble during learning they developed wild-type levels of 24-hour context fear memory that was independent of CNO stimulation (Fig 2.4B). This is in contrast to the deficit produced in animals pre-exposed to the novel ctxA and further supports the contention there must be a match in the spatial pattern of neural activity at learning and retrieval. To ensure that these hM_3D^{fos} mice were expressing the hM_3D_q transgene and were expressing cfos at higher levels than control mice, we performed western blots using lysates from cortex and hippocampus (Fig. 2.A1)

Several recent studies have suggested flexibility in the specific neurons incorporated into a fear memory trace in the amygdala through a selection mechanism in which more excitable neurons are preferentially incorporated into the trace [33-35]. The current results do not appear to be due to this type of selection as the reactivation of the neurons with CNO is required for retrieval while in the previous studies the stimulated neurons were part of a representation that could be naturally retrieved. This difference may be due to different requirements for forming simple associations in the amygdala vs more complex representations in the hippocampus and cortex.

In the current study, the artificially stimulated neural ensembles become incorporated into the memory and there must be a match between the pattern of activity at the time of learning and the time of retrieval. In one recent study, ChR2 stimulation of a random population of neurons in the piriform cortex combined with odorant during conditioning found that either the artificial stimulation or the odorant alone could produce recall, suggesting independent and non interfering representations [37]. In contrast, this study found that the CNO activation alone could not act as an independent cue. These studies differed in a variety of parameters including anatomy and size of the artificially stimulated ensembles; one critical difference may be that the activity induced by hM_3D_q is not temporally coordinated in response to the inducing stimulus (CNO), as is the case with ChR2 driven stimulation by light. However, the sensory input during conditioning and retrieval in ctxB may coordinate the activity of CNO depolarized cells to provide some degree of temporal coordination to the CNO driven neurons and account for the requirement for the compound stimulus.

Current views of sensory processing recognize the role of internally generated (spontaneous) neural activity in generating a representation from a given sensory input [27]. This activity is not random but has spatial and temporal structure that is thought to represent defined ensembles formed through previous learning related plasticity. Moreover, in psychology the idea of a schema as a preexisting framework of relationships which modulates learning suggests that new memories are not produced *de novo* from experience but interact with existing circuit activity[24, 25]. While the CNO based stimulation does not replicate

the *temporal* dynamics of this naturally occurring internal activity, the approach allows the activation of a distributed *spatial* pattern of neurons recruited during a specific experience (ctxA exposure). The results demonstrate that this spatial pattern of activity at the time of learning and retrieval must match for appropriate recall. The results imply a strong spatial component to coding in this form of learning and support the idea that the internal dynamics of the brain at the time of learning contribute to memory encoding.

Primary Figures

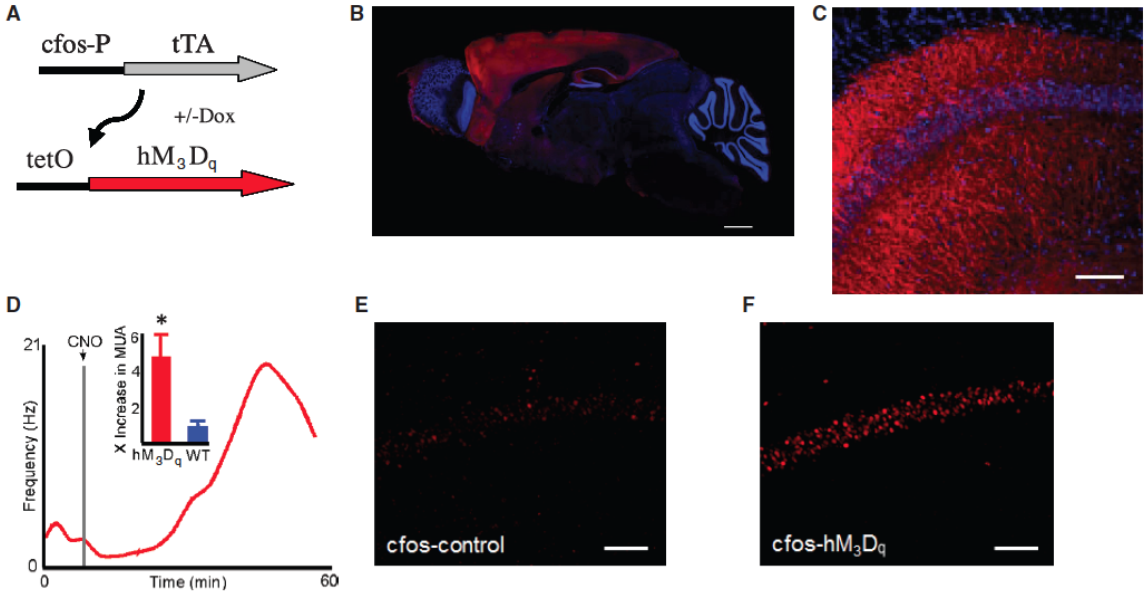

Figure 2.1. Expression and activation of the hM_3D_q transgene.
A) Transgenic mice used in this study carry the 2 transgenes shown allowing Dox regulated and neural activity dependent expression of the hM_3D_q receptor. **B)** Overall spatial expression profile of the hM_3D_q transgene in mice off dox maintained in the homecage. Immunofluorescence was strong in hippocampus, basalateral amygdala, and throughout the cortex. Fluorescence was also observed to a small extent in the pontine nucleus and in brainstem. **C)** Expression in the CA1 region of the hippocampus showing sparse and distributed expression of the hM_3D_q transgene. **D)** CNO injection causes increased neural activity in $hM_3D_q^{fos}$ mice. Red curve shows multi unit activity (MUA) recorded from dorsal CA1 of an anesthetized $hM_3D_q^{fos}$ mouse over time. Inset gives fold increase in MUA (4.76 for $hM_3D_q^{fos}$ vs. .9 for WT, mean 30-40 minutes post-injection/mean pre-injection baseline. n=6 and 6, *=Wilcoxon signed-rank: $P<0.01$). **E & F)** cfos induction 1.5 hours after CNO administration in a control (left) and $hM_3D_q^{fos}$ (right) mouse. $hM_3D_q^{fos}$ mice showed on average a 2.5-fold increase in cfos expression in the hippocampal CA1 region compared to control mice (see supplementary table 2.1 $hM_3D_q^{fos}$ n = 10, control, n = 10, T-test $p < .02$).

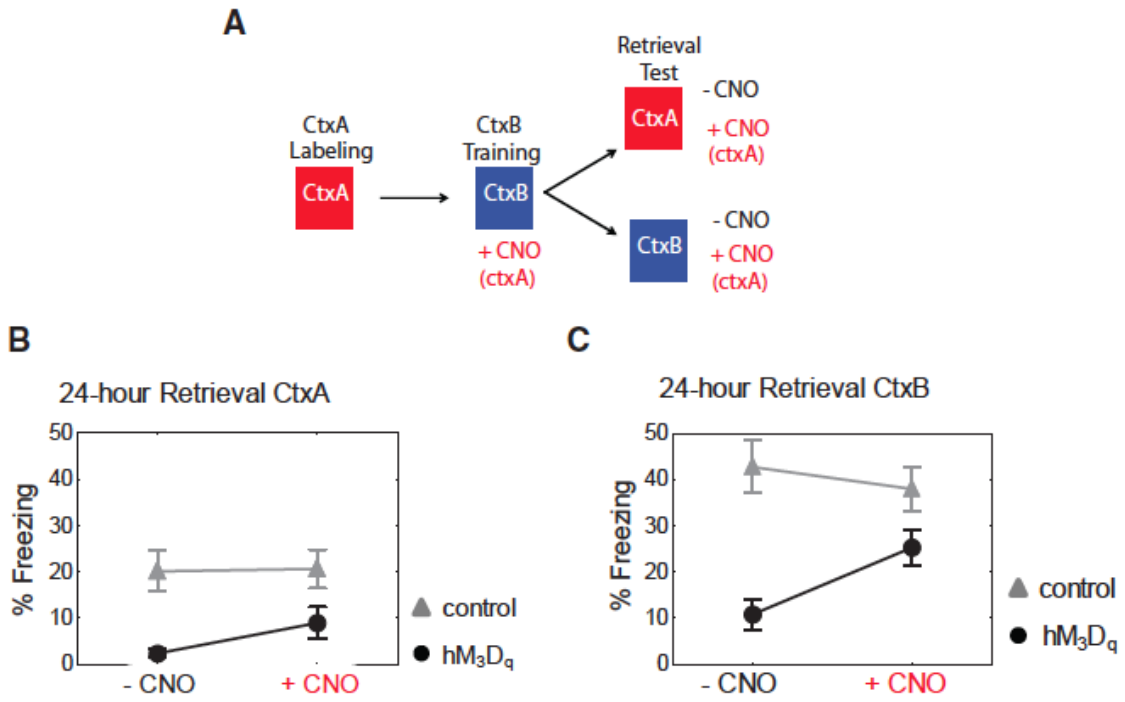

Figure 2.2 Incorporation of Synthetic Neural Activity into a 24-hr Memory Representation.
A) Schematic of experiment **B)** Freezing in ctxA 24-hours after conditioning in ctxB. $hM_3D_q^{fos}$ n = 14, control n = 13. $hM_3D_q^{fos}$ mice freeze significantly less than control mice in ctxA in the absence and presence of CNO. Repeated measures ANOVA main effect of genotype $F(1,26) = 10.96$, $p < .005$. CNO has no significant effect on freezing in either group. Post hoc Bonferroni $hM3D^{fos}$ p = 0.192, control p = 1.00. **C)** Transgenic $hM_3D_q^{fos}$ mice show impaired 24-hour memory for ctxB that is rescued by injection of CNO. Repeated measures ANOVA genotype x CNO interaction $F(1.25) = 10.15$, $p < .005$. Post hoc Fisher's LSD found that $hM_3D_q^{fos}$ mice were freezing significantly less than control mice in ctxB in the absence of CNO, $p < 0.001$, but were statistically similar in ctxB in the presence of CNO, p = 0.117, and showed a significant increase in freezing in ctxB with CNO compared to ctxB alone, $p < 0.001$. **D and E)** Correlation between the difference in freezing scores in the presence and absence of CNO and endogenous cfos expression 1 hour after memory testing in hippocampal area CA1, D, $r = 0.8276$, $p < .005$ and CA3, E, $r = 0.6742$, $p < .05$.

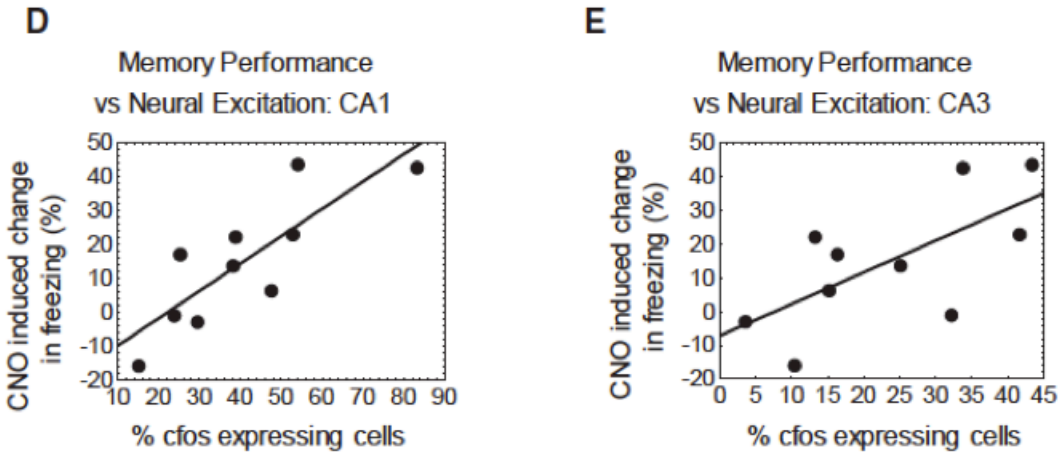

Figure 2.2 Continued. Incorporation of Synthetic Neural Activity into a 24-hr Memory Representation.

D and E) Correlation between the difference in freezing scores in the presence and absence of CNO and endogenous cfos expression 1 hour after memory testing in hippocampal area CA1, D, $r = 0.8276$, $p < .005$ and CA3, E, $r = 0.6742$, $p < .05$.

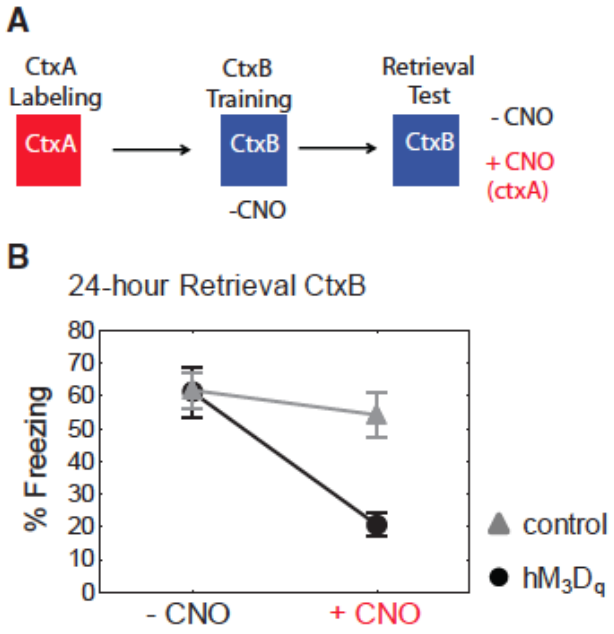

Figure 2.3. Disruption of Memory Retrieval by Synthetic Neural Activation
Transgenic hM3D^{fos} mice develop a normal 24-hr context memory when conditioned in the absence of CNO. This memory is disrupted by CNO injection to activate the competing ctxA representation. hM$_3$D$_q^{fos}$ n = 12, control n = 12. Repeated measures ANOVA main effect of genotype $F(1,22) = 5.3$, $p < .05$, CNO $F(1,22) = 28.6$, $p < 0.001$, and genotype x CNO interaction $F(1,22) = 13.5$, $p = 0.001$. Post-hoc Fisher LSD revealed that hM$_3$D^{fos} mice were freezing significantly less in the presence of CNO compared to before CNO administration $p < 0.001$, and were freezing significantly less that control mice in the presence of CNO $p < 0.001$.

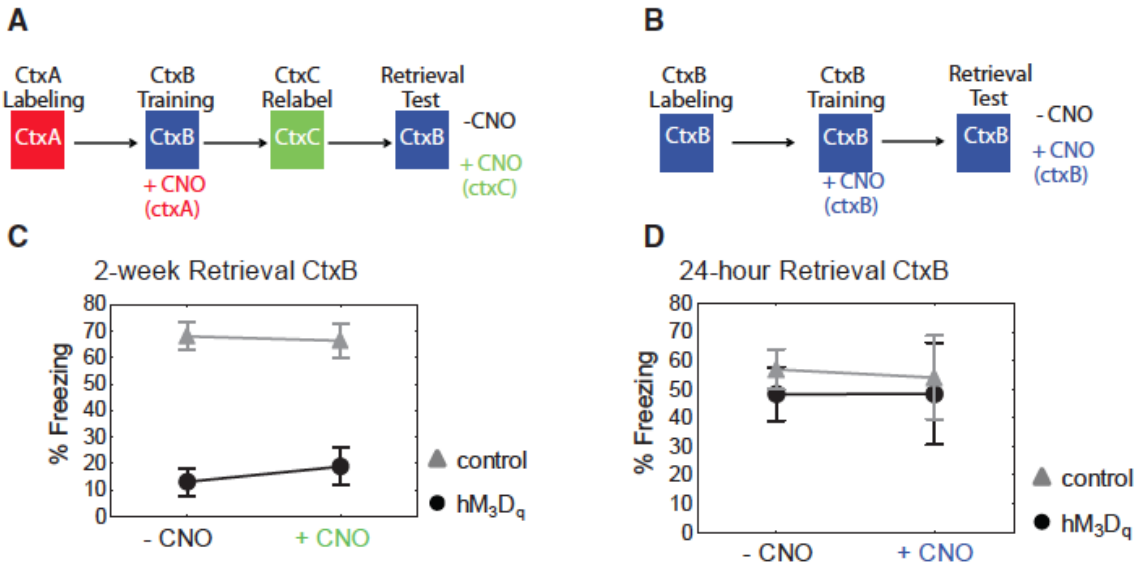

Figure 2.4. Memory performance during synthetic reactivation is network specific.
A) When CNO induced synthetic activation does not occur in identical neural populations during memory formation and memory retrieval, a memory deficit is observed. $hM3D^{fos}$ mice show significantly less freezing than control mice in ctxB both in the absence and presence of CNO. $hM3D^{fos}$ n = 14, control = 17. Repeated measures ANOVA main effect of genotype $F(1,23) = 51.15$, $p < 0.001$.
B) When $hM_3D_q^{fos}$ mice are exposed to ctxB off of dox to induce hM_3D_q expression and then fear conditioned on dox after CNO injection in ctxB, synthetic activation by CNO is not necessary for memory recall in ctxB. ctxB: $hM_3D_q^{fos}$ n = 9, control n = 10, ctxBcno: $hM_3D_q^{fos}$ n = 5, control n = 6 . Repeated measures ANOVA $F(2,18) = 0.0474$, $p = 0.954$.

Materials and Methods

Subjects

cfos-htTA mice [4] were bred with tetO-hM_3D_q mice [19] to produce double transgenic experimental animals. Control animals were single transgenic siblings that underwent all of the same experimental conditions. Mice were housed socially (2-4 animals per cage), allowed free access to food and water, and maintained on a 12 hour light-dark cycle (unless tested between 6:30 and 7:00 AM). To avoid introducing additional stress from handling to read ear tag

numbers, all animals were uniquely labeled with a permanent marker on the tail 2 days before the beginning of an experiment.

Context Conditioning

For context B (ctxB), mice were placed in chambers (30 cm length x 24 cm width x 25 cm height) with a black and white checked-pattern back wall, clear polycarbonate top, stainless steel side walls, and a stainless steel grid floor. One light in each chamber was on to allow motion detection by a digital camera, and the room lights were off. A round wintergreen scent dish was hung from the door of the chamber. When placed into and removed from the chamber, mice were handled with rubber tipped forceps.

For context A (ctxA), mice were first placed into a square plastic box (18 cm length x 18 cm width x 18 cm height) with opaque white walls and floor, and then the box was slid into the context B chamber. The back wall of the chamber was changed from black white checked-pattern to solid white. In addition to a light in the chamber, six room lights mounted at the corners of the room walls and ceiling were on as well. No scent dish was used for context A. When placed into and removed from the box, mice were hand handled.

Pre-exposure to a novel context off of doxycycline was performed between 7 AM and 11 AM and again between 4 PM and 7 PM. Fear conditioning was performed between 7 AM and 12 PM. Testing began between 6:30 AM and 7:30 AM and lasted 2 to 6 hours depending on the number of mice in the experiment and whether or not they were perfused after testing.

During fear conditioning, mice were given 3 minutes 18 seconds to explore the chamber, and were then administered four 0.8 mAmp shocks 2 seconds in duration with an intershock interval of 78 seconds. After the final shock, mice remained in the conditioning chamber for an additional minute before being placed back in their home cage.

Behavior in each context was recorded using a digital camera and motion was quantified and analyzed. The bout length was 1 second, and the threshold for freezing behavior was 10 (determined by eye by 2 students blind to experimental conditions and animal genotype). Freezing scores for context retrieval were calculated by dividing the test session into 1 minute bouts and averaging together all three minutes for each animal.

Injections

All injections were intraperitoneal (IP). Clozapine-N-oxide (CNO) was dissolved in dimethylsulfoxide (DMSO) and then diluted in 0.9% saline solution to yield a final DMSO concentration of 10%. Saline solution for injections also consisted of 10% DMSO. Not more than 1 uL of DMSO per 1 gram mouse was injected into animals [38]. 0.5 mg/kg CNO was injected into mice 28 minutes before behavioral assays. This dose of CNO resulted in behavioral signs of seizure activity [39] in approximately 20% of mice during fear conditioning, which were excluded from behavioral and histological data analysis.

Immunohistochemistry

Sixty minutes after behavioral testing, mice were transcardially perfused with 0.9% saline for approximately 1 minute followed by 4% paraformaldehyde

(PFA) for 6 to 9 minutes. Brains were post-fixed in 4% PFA overnight, sectioned with a thickness of 100 µm on a vibratome, and then stained while free-floating. All sections were blocked for 1 hour at room temperature in 0.8% TX-100 and 10% NGS in PBS followed by 1 hour at room temperature in 0.3% TX-100 and 10% NGS in PBS. Polyclonal rabbit anti-HA primary (Rockland),1:450 dilution, was used to label the HA tag on the hM_3D_q receptor [19]. Polyclonal rabbit anti-cfos (Calbiochem), 1:700 dilution, was used to label the cfos protein. Primary antibodies were diluted in 0.3% TX-100 and 10% NGS in PBS, incubated at 4^{o}C for 48 hours, and rinsed 3 times for 15 minutes in PBS. Cy3- conjugated goat anti-rabbit (Jackson Immuno Research), 1:500 dilution in 0.2% TX-100 and 10% NGS in PBS was then applied to the sections for approximately 2 hours at room temperature followed by 3 rinses for 15 minutes in PBS. Finally, topro3 iodide (Molecular Probes, Eugene, OR), 1:1000 dilution in PBS, was applied to the sections for 15 minutes at room temperature to label cell nuclei. The sections were then rinsed for 10 minutes in PBS, mounted with coverslips on glass slides, sealed with clear nailpolish, and stored at 4^{o}C.

Confocal Microscopy

Confocal microscope was used to collect cfos and hM_3D_q receptor images. PMT, laser power, gain and offset were kept constant between experimental groups. cfos image stacks consisted of 8 slices separated by 10 µm steps.

cfos Quantification

Image J was used to count total number of cells (topro3 positive cells) and cfos positive cells in CA1, CA3, dentate gyrus, lateral amygdala, basalateral

amygdala, and central amygdala. Percent cfos positive cells are reported with respect to total topro3 positive cells to account for differences in selected region sizes. Every other image slice in the z-stack was cell counted yielding cell numbers for 3 different positions along the z-axis. The z-slice with the highest percentage of cfos positive cells was used for further analysis and statistics. All quantification was performed blind to experimental group.

hM_3D_q Receptor- cFos Co-expression

For co-expression experiments (2.S3), a c-fos transgene with a GFP linked reporter was used to quantify cfos expression by measuring endogenous fluorescence [4].

Mice were injected with CNO and one hour 28 minutes later were perfused and the brains were fixed and stained for HA as decribed under *immunohystochemistry*.

***In vivo* Electrophysiology**

For electrophysiological recordings, a total of 12 mice (6 single positive controls and 6 double positive experimental) were used. To mirror the behavioral study, mice were first fear conditioned and returned to their home cage overnight. 24 hours after fear conditioning, the mice were anesthetized with ketamine-medetomidine-atropine and prepped for electrophysiological recording. The head was placed in a stereotaxic frame, the skull was exposed, and a small hole (1 mm in diameter) in the skull was drilled above the left dorsal hippocampus (A, −2 mm from the bregma; L, 1.5 mm from the midline). A bundle of 4 tetrodes comprised of spun 17micron platinum-iridium wire or a 16 channel silicon probe

were slowly advanced into the hippocampus and allowed to settle for a period of 10 minutes before recording. Tetrodes were targeted to the CA1 pyramidal layer, while the sites of the multi-channel silicon probe were positioned to span the hippocampal layers. After reaching the desired depth and rest, a 5 or 10 minute baseline period was recorded followed by injection of CNO and another 50+ minutes of recording. During the recording sessions, neurophysiological signals were amplified, bandpass filtered (0.1 Hz to 6 kHz), and acquired continuously at 32 kHz on a 32-channel system. In the case of the silicon probes, the site closest to the CA1 pyramidal layer was analyzed. Data were analyzed using a combination of custom-written MATLAB software and the MATLAB-based toolbox Chronux. Multi-unit spiking activity (MUA) was quantified by first filtering the fully sampled data (200Hz to 6kHz), then setting a threshold of 5 s.d. above the mean and counting all events over the threshold. The mean MUA (in spikes/second) was calculated for 30 to 40 minutes after CNO injection and then compared to 4 minutes of pre-injection baseline using a Wilcoxin signed-rank test.

Supplementary Data & Figures

Table 2.S1. Percent cfos expression in hippocampus and amygdala. Mice from experiment in Fig. 2.2 were examined 1-hour following the retrieval trial in ctxB and immunostained for cfos expression. Data is presented as a percentage of total cells counted in the corresponding area. hM_3D_q n = 10, control n = 10. Group means were compared by T-test and p-value is indicated.

Brain Region	$hM3D^{fos}$ (mean %)	control (mean %)	x fold increase	P value
CA1	40.8	16.2	2.5	0.0129
CA3	23.5	10.5	2.2	0.0188
DG	46.5	2.24	20.8	0.0067
LA	18.5	8.18	2.3	0.0491
BLA	21.9	8.3	2.6	0.0026
CA	24.8	5.8	4.3	0.0074

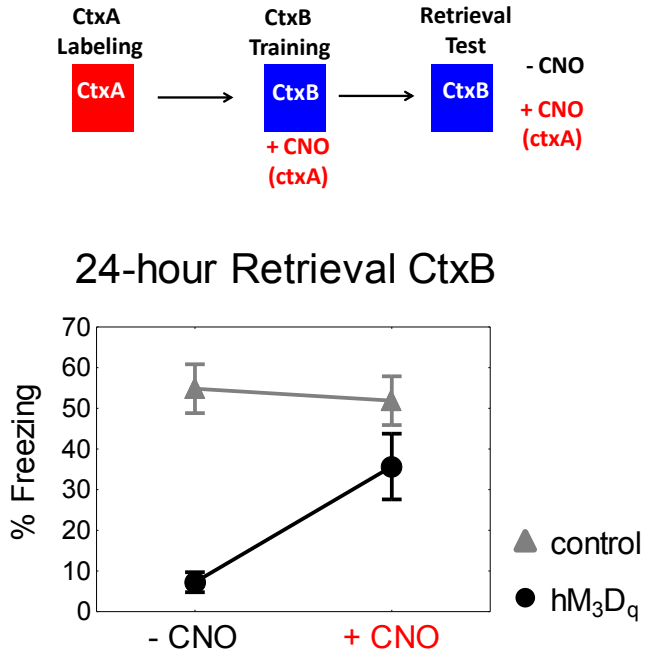

Figure. 2.S1. Using a different context for novel ctxA results in the same effects as shown in experiment 1 (see Fig 2.3C). hM3D^{fos} mice show impaired 24 hour memory for ctxB that is rescued by injection of CNO. hM3D^{fos} n = 15, control n = 17. Repeated measures ANOVA genotype x CNO interaction $F(1,28) = 5.012$, $p < .05$. Post hoc Fisher's LSD test revealed that hM3D^{fos} mice were freezing significantly less than control mice in the absence of CNO, $p < 0.001$, were statistically similar to controls in the presence of CNO, $p = 0.114$, and showed a significant increase in freezing in ctxB in the presence of CNO compared to ctxB alone, $p < .005$.

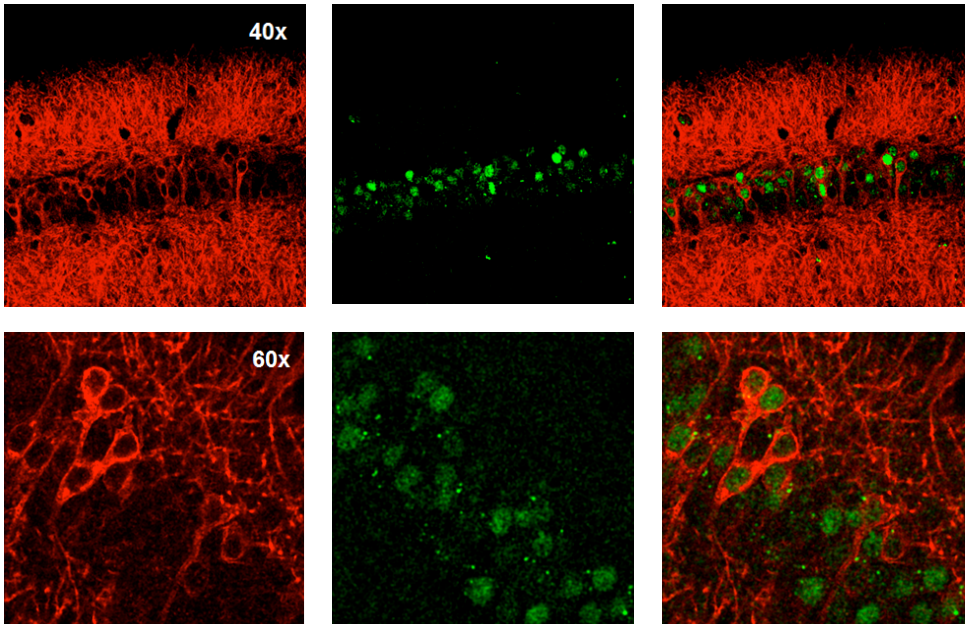

Figure. 2.S2. CNO-induced activity results in cfos expression in both hM_3D_q-expressing and hM_3D_q-non-expressing cells. In CA1, where hM_3D_qR expression can be observed somatically, 91.4 $\pm$ 2% of hM_3D_qR expressing cells also show cfos expression (n = 4) indicating the effectiveness of CNO to induce activity in hM_3D_qR cells. 43.5 $\pm$ 5% (n = 4) of GFP expressing cells do not also express hM_3D_qR indicating activation of additonal cells that do not express the receptor.

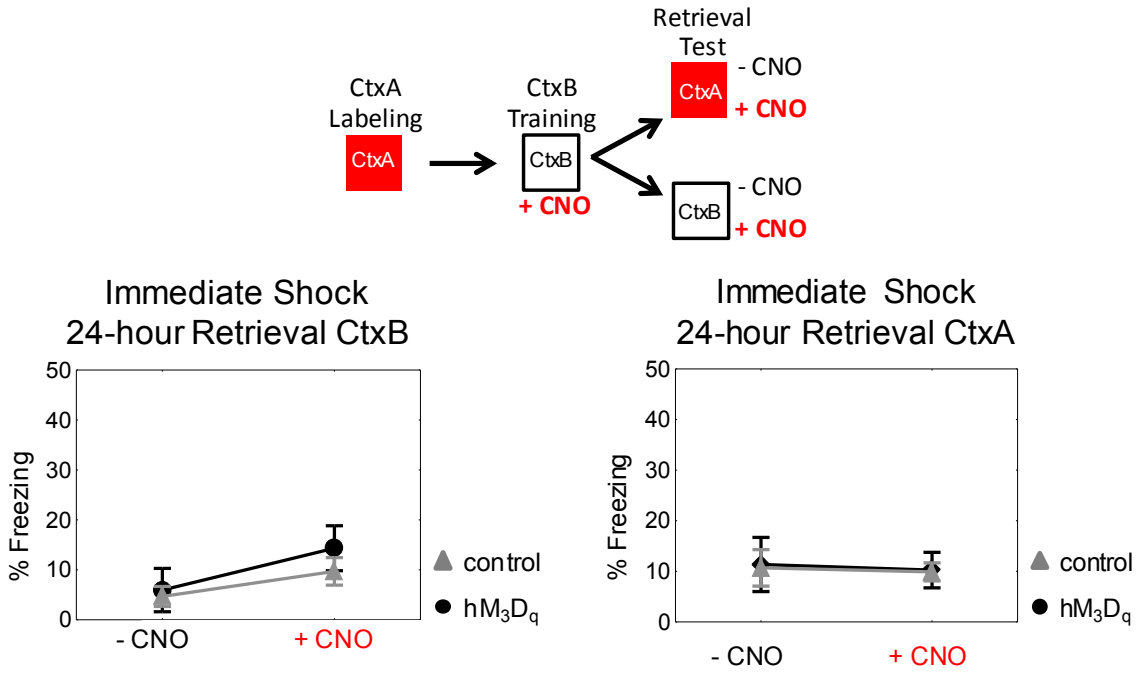

Figure. 2.S3: CNO-induced neural activity cannot be used alone as a conditioned stimulus. $hM_3D_q^{fos}$ n = 4, control n = 6 Repeated measures ANOVA context A $F(1,6) = 0.028$, $p = 0.873$ context B $F(1,6) = 0.336$, $p = 0.583$.

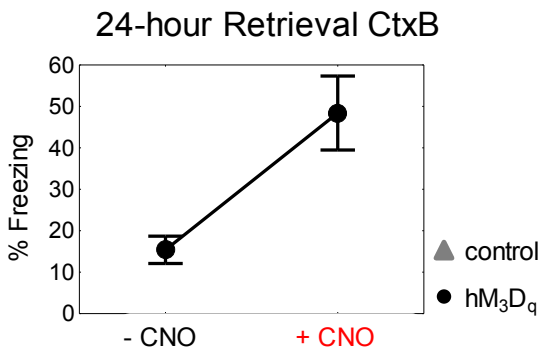

Figure. 2.S4: To ensure that CNO-induced artificial activity during learning is incorporated into the memory representation and does not result in a low level ceiling effect for memory retrieval we repeated the experiment presented in Fig 2.2B and 2.S1. $hM3D^{fos}$ mice that failed to show remote memory recall as described in Fig 2.4A were re-exposed to a novel context A and fear conditioned the following day in the presence of CNO. When tested 24-hours later mice still show impaired memory for ctxB, but this impairment is now rescued by injection of CNO. $hM3D^{fos}$ n = 8. Student's t-test $t = -3.47$, $p = 0.00375$.

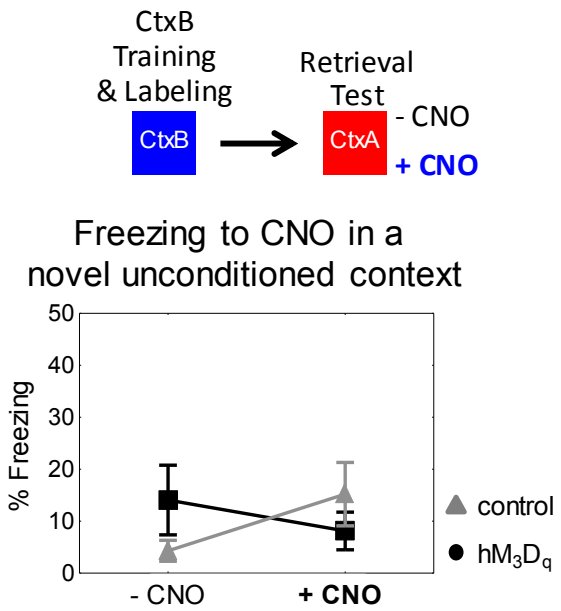

Figure. 2.S5: To test the ability of CNO to activate a contextual representation in a novel unconditioned context we fear conditioned mice off Dox to label shock-associated CtxB neurons and then tested in CtxA in the presence of CNO. Neither hM3D^{fos} or control mice showed significant freezing to the novel unconditioned context in the absence or presence of CNO. hM3D^{fos} n = 6, control n = 6. Repeated measures ANOVA genotype x CNO interaction $F(1,10) = 2.47$, $p = 0.147$

Additional Data & Figures

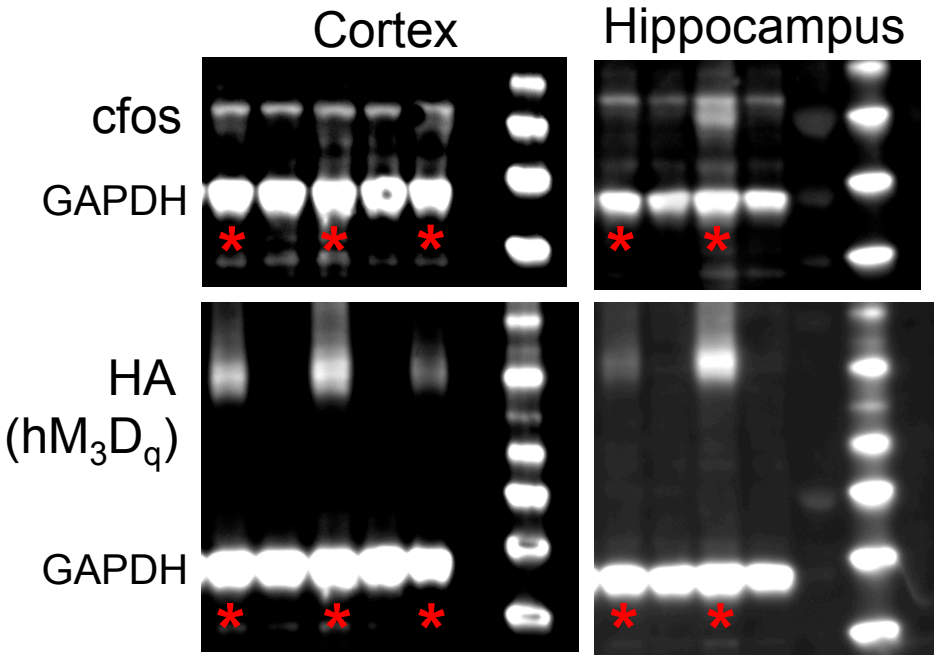

Figure. 2.A1 Western blot analysis of brains from mice in experiment shown in Fig. 2.4B. Red asterisk = hM_3D^{fos} mouse brain lysate. hM_3D^{fos} mice expressed the hM_3D_q transgene and showed a qualitative increase in cfos expression compared to control mice.

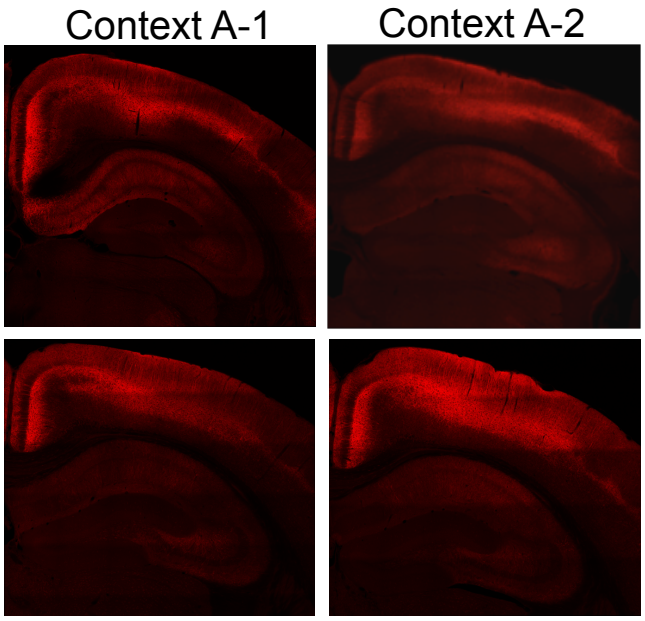

Figure. 2.A2 hM_3D_q expression in hM_3D^{fos} mice exposed to two different set ups for ctxA exposure.

Conclusion

Garner et. al. (2012)[21] provided the first example of manipulating internally generated activity *in vivo* during learning of new information providing a biological example of schemas as well as the ability of neural activity to represent different percepts depending on the cognitive framework to which the activity is bound. To manipulate a dispersed population of neurons naturally activated by experience, the authors took advantage of two recently created transgenic mouse systems. The first system expresses the tetracycline transactivator (tTA) under control of the activity regulated promoter cfos[4] which will induce expression of genes under control of the tetO promoter in the absence of the transcription blocker, doxycycline. The second mouse system expresses a G_q-protein coupled muscarinic receptor, hM_3D_qR, engineered to be unresponsive to acetylcholine but activated by the biologically inert compound clozapine-N-oxide (CNO)[18, 40] under control of the tetO promoter. Thus, the hM_3D_qR will be allocated into neurons naturally activated during experiential learning within a specific doxycycline-dependent time-window. Those neurons can then be subsequently re-activated chemically with the intraperitoneal injectable CNO ligand allowing limited temporal, but broad spatial control.

The authors found that when an ensemble of neurons for one context (context A) was artificially activated during conditioning in a distinct context (context B), animals formed a fear memory for context B that was only expressed when exposure to context B coincided with neural activity from context A.

Neither exposure to context B or synthetic activation of context A neurons alone was sufficient to allow expression of the fear memory.

Furthermore, the authors showed that synthetic re-activation of internally generated activity was specific to the memory trace in two ways. First, the authors conditioned in context B while synthetically activating context A neurons, but then allowed the decay of the hM_3D_qR by putting mice on back on dox. They then induced expression of the hM_3D_qR by exposing mice to a third context (context C), in the absence of dox, before testing two weeks later to express the receptor in a similar but distinct population of neurons from those active during context A. A fear memory for context B could not be achieved when exposure to the context coincided with activation of context C neurons. This evidence supports the idea that information from context B was bound to the internal representation of context A and thus only when neurons active in context B occurred in the attractor state of context A, but not context C, did they signal an associative fear memory. Second, the authors pre-exposed mice to context B itself to induce hM_3D_qR expression in context B neurons. The logic of this condition was that if synthetic activation were activating a specific internal representation of the external world, expression of the hM_3D_qR in context B neurons would result in synthetic activation that closely overlapped with the natural activation of neurons resulting from conditioning in context B. In fact, when context B neurons were synthetically activated during conditioning in context B, a fear memory for context B alone resulted without the need for synthetic re-activation during recall. Thus, information for context B was bound

to an internal representation of context B during learning and re-exposure to context B alone was sufficient to activate context B neurons in the attractor-state of context B.

This study provides the basis for several important questions. First, is the circuit that represents a new associative memory constructed entirely de novo or is an existing neural network modified to code for the new experience of the animal? Second, What percentage of a functional network needs to be activated in order to reactivate the entire network? To address these questions, both spatial and temporal coding dynamics will need to be considered, which will be described in chapter three.

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Aleena R. Garner, David C. Rowland, Sang Youl Hwang, Karsten Baumgaertel, Bryan L. Roth, Cliff Kentros, and Mark Mayford (2012). Generation of a synthetic memory trace. *Science*. 335 (6075): 1513-1516.

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Chapter 3: How do You Know?

Introduction

Cognition allows us to understand the external world by representing it. Neural impulses are nothing like the concrete structure of the world, and therefore must use a code for representation. Thus, some transformation must exist between the external system of the world and the internal system of the brain to enable representation and ultimately comprehension. Studies such as Tse et. al. (2007)[1], Tse et. al. (2011)[2] and Garner et. al. (2012)[3] suggest that learning of new information and the creation of percepts to understand the world do not occur from *Tabula rasa*, but involve and depend on previous experience and pre-existing neural activity. In fact, critical periods for cellular architecture development in sensory systems such as vision[4] and for language production[5] suggest that new learning and processing of new information, especially in the adult brain, must occur through computations in pre-established neural circuitry. However, associative learning can occur between any stimuli or experiences even when the stimuli do not themselves have intrinsic meaning[6, 7]. Is the circuit that represents a new associative memory constructed entirely *de novo* or is an existing neural network modified to code for the new experience of the animal? If a new circuit is created *de novo* during associative learning, how is the functional and structural plasticity achieved and how does this plasticity evolve with the life of the memory? If a pre-existing network is modified to become a new memory circuit, how does the activity of the neural network lead to a different perception

after learning compared to before learning? Additionally, how does the formation of new memories and percepts not overwrite old memories and percepts?

Circuit Formation for Perception

To address these questions, the layout of pre-existing neural circuitry and activity must be established. Measurements of spontaneous activity can produce functional[8, 9] and structural[10] network maps of neural circuits, which can be compared before and after learning. Additionally, ongoing network changes can be monitored during the process of acquiring new associative percepts.

A specific example of an associative learning task using a mouse model system in which the animal is water restricted, is the presentation a visual cue, say a horizontal grating, followed by a water reward. Initially, presentation of the horizontal grating will not produce the unconditioned lick response, and thus should not affect neural activity in lick-related regions of motor cortex. However, after the mouse learns the association, it will lick in response to presentation of the horizontal grading and thus the visual cue will elicit activity in motor cortex. To ensure that this activity is in efferent fibers delivering motor commands to the tongue and not feedback activity from afferent fibers after the tongue moves, the tongue of the mouse can be anesthetized during probe trials of visual cue presentation.

Ca^{++} imaging can be conducted over lick-associated areas anterior-lateral motor (ALM) and posterior-medial motor (PMM) cortex[11] to measure spontaneous activity before learning to asses pre-existing network activity. After learning, spontaneous activity can be compared to patterns of spontaneous

activity before learning to determine if the pattern of internally generated activity was affected by learning. Furthermore, spontaneous activity after learning can be compared to visually evoked activity to determine if both types of activity use the same or similar neural circuits or are providing the same types of neural computation to the animal.

If a new circuit is created *de novo,* several network activity changes may be observed. First, if spontaneous activity is not merely noise in the system but is either representing a percept intrinsically or providing computations for information processing[12], then spontaneous activity patterns before and after learning may be different. Moreover, evoked activity by presentation of horizontal gratings after learning will result in activation of a different group of neurons than was spontaneously active before learning. If evoked activity after learning reveals the same pattern as spontaneous activity after learning, then replay[13-16] of learned activity is likely involved in information consolidation and formation of the neural representation of the learned associative memory. However, If evoked activity after learning results in a pattern of activity that is different from spontaneously generated activity after learning, then spontaneous activity after learning may be involved in transforming neural signals underlying perception[12] but is not a direct neural substrate for memory storage. Another possible outcome is that evoked activity may lead to the same pattern of activated neurons as spontaneous activity but also to an additional population of cells. This would suggest that the population of activated neurons observed during spontaneous activity is the circuit responsible for driving motor behavior.

The additional synchronous neural population active in response to the visual cue may provide contextual specificity and allow the motor output circuit to generate the appropriate motor response given the situation (in this case, a learned response to lick).

Several possible results would support the hypothesis that a pre-existing network is modified to become a new memory circuit. First, evoked activity after learning may result in the same neural activity pattern as spontaneous activity did before learning. If evoked activity after learning results in a different neural activity pattern than spontaneous activity after learning, then spontaneous activity may be a mechanism for encoding new associations by allocating information into the most hyper-excitable neurons[17]. Furthermore, this result would suggest that new learning results in a dampening of spontaneous activity in the population of neurons underlying the representation; perhaps so that new memories do not over-write old ones. However, if evoked activity after learning reveals the same pattern of neural activity as during spontaneous activity, then a temporal, non-spatial, code for representing associations is likely used in cortical networks, and the temporal dynamics of the neural-network's activity would need to be characterized using Ca^{++} imaging[18] or electrophysiological recordings before and after learning.

Generation of an Activity Inducible Light Effector in a Transgenic System

The investigations discussed thus far will yield purely correlational results, but will not determine a causal role for internally generated, or spontaneous, activity in perception generation. Chapter two provides evidence for a system

than can be used to specifically manipulate functionally defined circuits independent of influences from external input signals. However, the $hM_3D_q^{fos}$ mouse is not ideally suited to address questions of primary sensory learning because the neural activating agent is diffusible, which makes the technique better for broadly distributed networks but not for local sensory networks. Additionally, the $hM_3D_q^{fos}$ system cannot provide the temporal resolution that will likely be necessary in regions of cortex involved with primary sensory representations[19].

Therefore, we have developed a transgenic mouse line that expresses a variant of the channelrhodopsin 2 receptor, ChEF[20] under the tetO promoter. By combining this system with the cfos regulated tTA system[21], we have the ability to allocate ChEF into neurons activated in response to sensory or afferent input signals within a specific doxycycline-dependent time-window. Those neurons can then be subsequently activated photonically allowing fine-tuned temporal and local manipulations. We created a tetO-ChEF mouse instead of a tetO-ChR2 mouse because ChEF is an engineered chimera of ChR1 (channelrhodopsin 1) and ChR2, which provides the reduced inactivation character of ChR1 in the presence of continuous light, while maintaining permeability to sodium and potassium ions characteristic of ChR2[20]. The double transgenic mouse will be referred to as $ChEF^{fos}$.

To begin characterization of ChEF receptor expression profiles, individually housed male $ChEF^{fos}$ mice were exposed to a male intruder in the homecage in a separate room from the colony for 10 minutes. The mice did not

fight but did investigate each other for the entire exposure duration. The homecaged mice tended to pursue the intruder mice, while the intruders tended to explore the context (which was novel to them). Twenty-four hours after exposure to an intruder, ChEF^{fos} mice were perfused with 4% formaldehyde and their brains were sectioned, immunostained, and imaged on a confocal microscope. Expression of the receptor was broadly distributed as shown in Fig 3.1. Expression in cortical sensory areas reveals that the proposed experiments could be performed using one of several different sensory cues such as a whisker deflection cue (Fig 3.1D), a visual cue (Fig 3.1 E), or an auditory cue (Fig 3.1 F).

Circuit Function for Perception

To investigate the circuitry leading to the generation of a percept, ChEF^{fos} mice will first learn an associative task such as licking in response to a horizontal grating visual cue. After the animals reach some threshold for accurate performance (licking in response to the visual cue), Dox will be removed from the system in order to label active neurons in visual cortex during the task with the ChEF receptor. Following labeling of these neurons, Dox will be re-administered to the animals to prevent further unrelated labeling. After conditioning, a blue light stimulus, to activate the ChEF receptor, will be applied onto visual cortex and behavioral licking responses will be used to measure the functional output of the circuits being activated. Concurrently, Ca^{++} transients in ALM and PMM will be measured to determine the circuit computations in motor cortex.

Several possible outcomes may occur. Blue light onto visual cortex will result in licking behavior and the same evoked Ca^{++} transients in ALM and PMM as generated by exposure to the natural visual cue. This reveals that activation of visual cortex neurons that were activated in response to the visual cue is sufficient to generate both the perception of the visual cue and the appropriate learned behavioral output. Alternatively, blue light onto visual cortex will result in licking behavior but different evoked Ca^{++} transients in ALM and PMM as generated by exposure to the natural visual cue. This result would support a multiple-trace theory of memory circuits[22]. Activation of the sensory region of the memory-trace is sufficient to produce the appropriate behavioral output, but does not use the precise network activated by the natural visual cue. Perhaps the natural visual cue also activates, for example, emotional, reward, etc. pathways that modulate activity in behavioral output circuitry but not sensory input circuitry. Another possibility is that blue light onto visual cortex will not result in licking behavior but will produce the same evoked Ca^{++} transients in ALM and PMM as generated by exposure to the natural visual cue. This suggests that activation of motor cortex by sensory cortex activation alone is not sufficient to elicit the appropriate behavioral output. Finally, blue light onto visual cortex will not result in licking behavior or the same evoked Ca^{++} transients in ALM and PMM as generated by exposure to the natural visual cue. This suggests that pure activation of the circuit naturally activated by the visual cue is not sufficient to activate the entire sensory-motor network involved in the associative memory and is not sufficient to drive appropriate behavior.

Circuit Activation Requirements for Perception

Focal synthetic stimulation in one region involved in an associative memory to induce activity in the entire circuit required for perception and behavioral output raises a second important question. What percentage of a functional network needs to be activated in order to reactivate the entire network? Using sensory and motor modalities to address formation and function of circuits underlying perception are not as useful for this question because the networks connecting the two modalities after associative learning are likely expansive and diverse. However, the hippocampal region is well suited for addressing this question, when associative memories are hippocampal dependent, because it provides local networks that are known to bind information together[22]. If given a sufficient amount of time to become familiarized with a novel contextual setting, mice will develop a unified complex representation or perceptual map[23-26] that is hippocampus dependent[27, 28]. The advantage of a unified complex representation is that it allows pattern completion[29-31]. For example, an input pattern of 1, 2, 3, 4, 5 will be combined into a single representation so that subsequent presentation of part of the representation, such as 1 and 2 alone, will be sufficient to reactivate the entire representation. Therefore, it is proposed that activation of some fraction of a hippocampal neural network will be sufficient to activate the entire network.

To create a hippocampal-dependent unified contextual fear-memory representation, ChEF^{fos} mice will be allowed to explore a novel context and will then receive four shocks in the context. The ChEF receptor will be expressed in

neurons sufficiently active to drive cfos during the conditioning. Then, hippocampal slices of contextually conditioned mice will then be used for *in vitro* Ca^{++} imaging while stimulating increasing numbers of neurons with various levels of blue light intensity. This will allow the measurement of the fraction of network activation necessary to generate complete network activation. Specifically, cells within CA3 will be stimulated while Ca^{++} imaging cells in CA1[32]. To determine if sufficient levels of ChEF receptor expression would be present in hippocampal regions for subsequent neural activation in slice, $ChEF^{fos}$ mice were conditioned in a contextually rich environment (Fig 3.2 A). The mice were perfused with 4% paraformaldehyde, the brains were sectioned and immunostained with topro3 to label cell bodies, and then sections were mounted onto slides and imaged on a confocal microscope. Expression in CA1, CA3, and dentate gyrus (DG) is shown in Fig 3.2 B.

Conclusion

Manipulation of circuits functionally defined by their activity during perceptual tasks will be required to understand how a percept is generated and also what the role of the underlying circuitry is in behavior. This chapter describes planned experiments for determining the role of intrinsically generated activity in neural computations that give rise to percepts such as learned associations. The chapter also describes a novel system, the $ChEF^{fos}$ mouse, that can be used to determine the type of neural activity that leads to a perception in an animal model. The local regions involved, the temporal coding dynamics, and the amount (percentage) of neural circuit activation required to

generate a percept can be studied using the ChEF^{fos} mouse. In conclusion, the proposal discussed herein attempts to address an ambitious question proposed by Attneave in 1974, "How does the nervous system understand its own language?"[33]

Figures

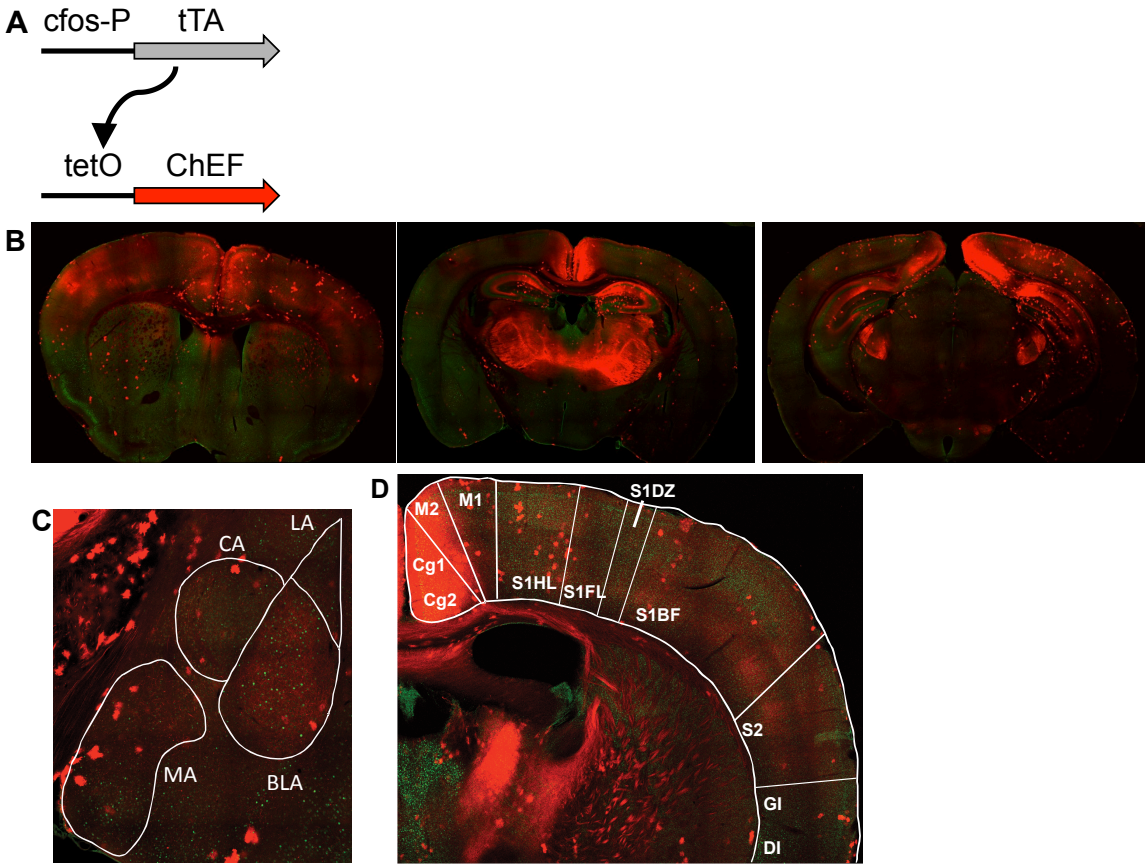

Figure 3.1 Expression of the ChEF transgene
ChEF expression is shown in red, cell bodies are labeled in green.
A) ChEF mice carry the 2 transgenes shown allowing Dox regulated and neural activity dependent expression of the ChEF receptor.
B) 10x sagittal sections showing expression in higher regions of cortex, hippocampus, and thalamus. Left: 0.7 mm, Middle: -1.4 mm, and Right: -3.6 mm to bregma. **C)** 20x amygdaloid nuclei -1.44 mm to Bregma **D)** 20x cortex -0.7 mm to Bregma. Cg1, Cg2: cingulate primary and secondary; M2, M1: secondary and primary motor; S1: primary somatosensory, HL: hindlimb region, FL: forelimb region, DZ: dysgranular zone, BF: barrel field; S2: secondary somatosensory; GI: granular insular; DI: dysgranular insular cortex. **E)** 20x Visual cortex. **F)** 20x Auditory cortex. **G)** Ect: Ectorhinal, Peri: Perirhnal, Ent: Entorhinal cortex

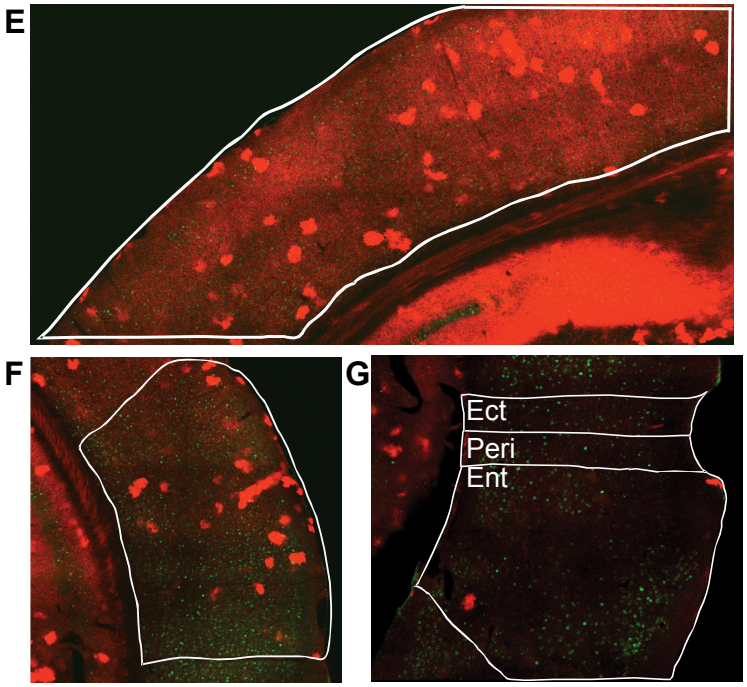

Figure 3.1 Continued. Expression of the ChEF transgene
E) 20x Visual cortex. **F)** 20x Auditory cortex. **G)** Ect: Ectorhinal, Peri: Perirhnal, Ent: Entorhinal cortex

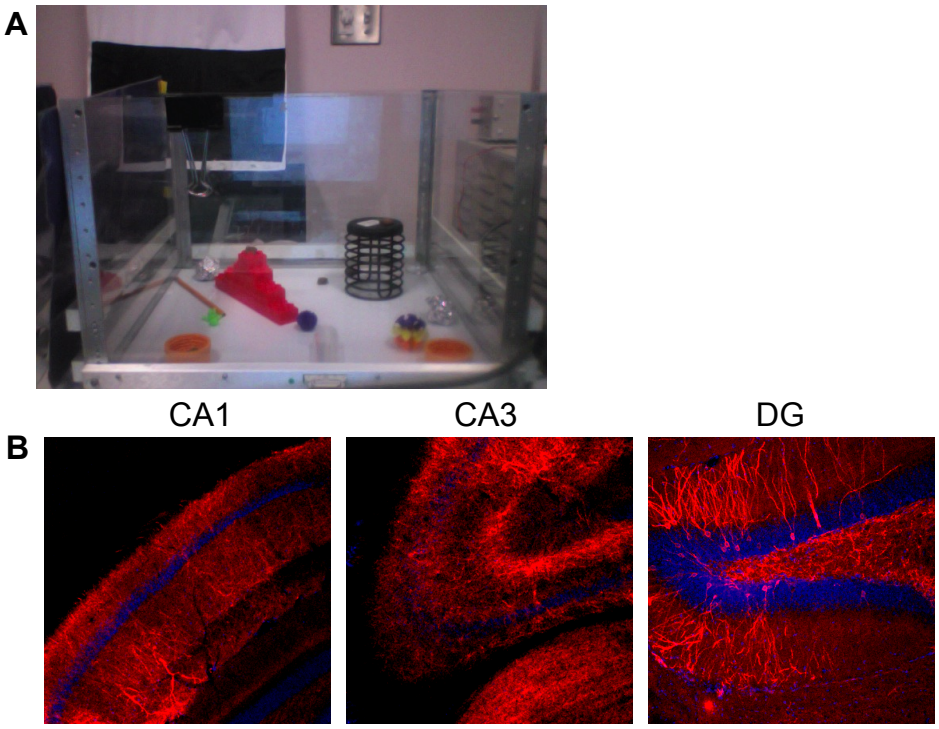

Figure 3.2 Using ChEF mice to investigate a unified trace representation theory in hippocampus
ChEF expression is shown in red, and cell bodies are labeled in blue.
A) Contextually rich conditioning environment to induce a large amount of ChEF receptor expression in hippocampus. **B)** ChEF receptor expression in CA1, CA3, and dentate gyrus (DG) five hours after contextual conditioning.

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