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Hippocampal Neurogenesis and its Contribution to Auditory Fear Generalization

A dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy

in Psychology

by

Vanessa Rodriguez Barrera

2017

ABSTRACT OF THE DISSERTATION

Hippocampal Neurogenesis and its Contribution to Auditory Fear Generalization

by

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Doctor of Philosophy in Psychology

University of California, Los Angeles, 2017

Professor Michael S. Fanselow, Chair

Fear and anxiety disorders affect many individuals and hinders their ability to accurately assess threat (Bar-Haim et al., 2007; Mogg and Bradley, 1998). This type of fear learning has been successfully modeled in the laboratory using Pavlovian fear conditioning and has provided much understanding of the underlying neural mechanisms. Classic theories of animal learning define fear generalization as responding to novel stimuli in the absence of explicit pairing with reinforcement, but as a result of previous training with a similar stimulus (Mackintosh, 1974; Hull, 1947).

Auditory fear conditioning studies show there are significant fear responses to novel auditory stimuli and although associations between tones and shock can be made without and intact hippocampus, lesions of the dorsal hippocampus are able to abolish tone generalization (Duvarci, Bauer, and Pare, 2009; Quinn, Wied, and Fanselow, 2009; Anagnostaras, Maren, and

Fanselow, 2009). In addition, the hippocampus is one of few structures in the brain that undergo neurogenesis, where new granule cells (GCs) are continually added to the dentate gyrus throughout a lifetime (Altman and Das, 1965; Gage, 2000). Therefore, at any given point, the dentate is composed of a heterogeneous population of immature and mature GCs. Adult-generated immature GCs have been shown to exhibit distinct neuronal properties and are preferentially activated during learning and memory tasks (Kee et al., 2007; Synder et al., 2012; Deng, Aimone, and Gage, 2010). More importantly, mice that lack post-natal neurogenesis show enhanced fear generalization to novel auditory stimuli (Cushman et al., 2012). Together, these findings indicate that the tendency to generalize may be regulated by processing within the hippocampus and that adult-born GCs are involved.

My dissertation examines the effect of manipulating hippocampal neurogenesis on auditory fear generalization. This was achieved through use of genetic alterations in mice along with x-irradiation techniques to regulate hippocampal neurogenesis. The effects of these manipulations on conditioned fear behavior were assessed with auditory fear conditioning protocols. In chapter 2, experiments described how we independently tested whether mature, immature, both or neither granule cell population would result in generalization to a novel cue after training with either a pure tone or white noise. Results showed that learning about an auditory cue that predicts shock does not require an intact dentate, however changing the population of GCs that are present produced an effect in amount of generalized freezing to a novel cue at test. In addition, mice with mature GCs silenced showed a normal generalization gradient compared to a control group in which the gradient was biased towards high frequency tones.

Chapter 3 covers an experiment that used only irradiation to investigate how depleting neurogenesis affects generalization to two pure tones after undergoing discrimination training.

Mice learned to discriminate between a high and low frequency tone, but groups that had the high frequency as the CS+ performed better than those with the low frequency as the CS+. During the generalization test, irradiated mice showed little generalization but only if trained with the high frequency tone. Lastly, chapter 4 describes the last study in which a genetic manipulation of neurogenesis is used to measure effects on tone generalization using a high and low frequency auditory stimulus. Mice again showed a generalization bias at test that was dependent on the cue that was used for conditioning.

The overarching pattern of results showed that different populations of dentate GCs may lead to an increased ability to differentiate between stimuli that signal shock and those that do not. This effect, however, is substantially affected by the characteristics of the auditory stimulus that are used during conditioning.

The dissertation of Vanessa Rodriguez Barrera is approved.

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2017

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# CHAPTER ONE

## Introduction

Being able to distinguish between dangerous and safe stimuli is important for survival. Fear serves to help an organism respond in the face of danger and its goal is to continue past the threat at hand. When this defense mechanism is engaged without suitable reason, however, it can become problematic. Inappropriate generalization of fear is a hallmark symptom of fear and anxiety disorders (Pole et al., 2009; Suendermann et al., 2010). For example, according to the Diagnostic and Statistical Manual of Mental Disorders (5th ed.; American Psychiatric Association, 2013) in order to be diagnosed with a specific phobia, one must meet criteria for 'overestimation' of danger; essentially, this means that the fear response is out of proportion to the real life situation/object. It is not difficult to think of a person that shows an exaggerated reaction to stimuli following a traumatic/painful event because these cases are present in substantial numbers among us. The lifetime prevalence of generalized anxiety disorder (6.2%), social phobia (13.0%) or specific phobia (13.8%) to name a few, is enough to warrant concern (Kessler et al., 2012). This suggests that a large portion of the population will encounter a debilitating condition that interferes with every day life. Animal models such as Pavlovian fear conditioning have been very useful in outlining the neural circuitry necessary for fear learning and providing insight into treatment for these conditions (Fanselow and Ponnusamy, 2008). Much research has been devoted to delineating the etiology of such disorders, however the neural underpinnings of fear generalization are not fully understood.

How does the brain do this? How does the fear circuitry that is meant for adaptive responding contribute to an individual behaving in a seemingly irrational manner when confronted with a feared stimulus or worse, when confronted with a stimulus that has never itself posed a threat but is enough to cause the same reaction? This is the question that guided the present dissertation and my thesis work has been focused on the role of hippocampal

neurogenesis in tone fear generalization. Using different transgenic mouse lines and irradiation, the aim was to delineate the role of dentate granule cells in aberrant tone fear. Understanding how granule cells contribute to fear generalization may ultimately serve to inform lines of research on possible treatment for individuals that suffer from these conditions.

**Auditory Fear Conditioning**

Tone fear conditioning is a robust form of learning in which an association is made between an auditory cue (typically a pure tone) and aversive stimulation, such as foot shock. Following the pairing of these two stimuli, the animal will acquire and exhibit a conditional response (CR) when reintroduced to the context in which training occurred but also to the tone that was paired with shock (Romanski and LeDoux, 1992; Fanselow, 1990). The unconditional (US) and conditional stimulus (CS) in this case represent the shock and tone, respectively. Multiple experiments have been conducted to define how information about the CS and the US enter the key structures in the brain to make this type of learning possible. The basolateral amygdala serves as the center of CS-US convergence; information from the painful stimulus reaches both basolateral (BLA) and lateral nuclei (LA) of the amygdala while CS information, such as a discrete tone, enters the lateral amygdala via auditory thalamus and cortex (Davis, 1992; Fanselow and LeDoux, 1999; Fanselow and Gale, 2003). Once these inputs have been associated in the BLA, the behavioral output and the conditional response observed in an animal is a result of central amygdala driving subsequent structures such as periacquductal gray and lateral hypothalamus that produce freezing, heart rate changes, etc.. (Fanselow, 2010; Perusini and Fanselow, 2015). The conditioned response we see after pairing of the CS and US reflects that behavior chosen for the particular level of perceived threat as predicted by predatory imminence theory. Predatory imminence theory postulates that for any given animal, there are a

repertoire of behaviors that are inherent for the species, when an animal enters a state of threat then that repertoire is limited to species-specific defense reactions. The particular defense reaction that is selected will be in line with the perceived level of threat by the animal (Bolles, 1970; Bolles & Fanselow, 1980).

**Role of the Hippocampus**

A large body of literature supports the role of the hippocampus in fear conditioning and in responding appropriately to different stimuli. The hippocampus plays a key role in contextual fear acquisition, navigational learning, and discriminating between similar items and contexts (Conejo et al., 2013; Kim and Fanselow, 1992; Nakashiba et al., 2012). The hippocampus also forms part of a crucial limbic circuit that is required for emotional learning (Gray and McNaughton, 2003). Input from various sensory centers reaches the hippocampus via layer II of entorhinal cortex (EC). This information is then transformed through processing in hippocampal subfields and the output is relayed to the amygdala where the association between a conditional stimulus and an unconditional stimulus is made (Fanselow and Poulos, 2005). Functional MRI studies in humans have shown that the hippocampus is involved in the retrieval of recent and remote episodic memories, and in deciding whether a scene is familiar or novel (Eldrige et al., 2000; Bonnici et al., 2012; Berense, Henson, and Graham, 2011). The hippocampus thus plays a significant role in fear learning and although tone fear can be acquired independently, recent findings have brought attention to its role in fear generalization.

**Hippocampus and Stimulus Generalization in Fear Conditioning**

Freezing to a trained tone happens almost instantaneously and if a previously untrained auditory stimulus is presented at test, there is considerable generalization of fear to this new sound (Duvarci, Bauer, and Pare, 2009; Quinn, Wied, and Fanselow, 2009; Cushman et al.,

2012). A prevalent hypothesis driven by classic learning theories postulates that the amount generalization an animal exhibits is a function of the similarity between the trained and novel cues (Resorla and Furrow, 1977; Guttman and Kalish, 1956; Honig and Urcuioli, 1981). Early studies mostly focused on examining stimulus generalization gradients after instrumental training for rewards but few have investigated generalization of fear after aversive conditioning (Hearst, 1960; Miasnikov and Weinberger, 2012; Bang et al., 2008).

Initial experiments using animals with lesions to the hippocampus showed that the result is flat generalization gradients after eye-blink conditioning in rabbits and brightness discrimination in rats (Wild and Blampied, 1972; Solomon and Moore, 1975). Another example comes from context conditioning experiments in which pre-training lesions of dorsal hippocampus results in more freezing to a context not associated with shock vs shock context compared to sham animals (Frankland et al., 1998). More recently, Quinn and colleagues (2009) paired a tone or a white noise with foot-shock and then made post-training NMDA lesions to the dorsal hippocampus. Rats were tested with both the trained stimulus and a novel auditory CS that had not been presented during training. Those with the hippocampal lesions showed a normal freezing response to the trained cue but much less freezing to the untrained cue compared to sham animals; dorsal hippocampus lesions impaired generalization without affecting acquisition (Quinn, Wied, and Fanselow, 2009). These data suggest that manipulations to the hippocampus may affect processing that dictates whether generalization will ensue.

**The Dentate Gyrus Within the Hippocampal Network**

The dentate gyrus is one of the major subfields that make up the hippocampal formation and a generally accepted version of the way information flows through the hippocampus is based around the trisynaptic circuit. The basic pathway consists of the perforant path projections

synapsing onto GCs; these in turn send mossy fibers to make contact onto CA3 pyramidal cells, which synapse onto CA1 (EC -> DG -> CA3 -> CA1); there are also direct projections from EC onto CA3 pyramidals (Amaral, Scharfman, and Lavenex, 2007). The dentate gyrus is the main input region for CA3 and has characteristics that would make it effective at storing non-overlapping patterns. That is, it has an extremely large number of granule cells in comparison to both its inputs (entorhinal cortex layer II) and outputs (CA3), as well as generally low firing rates and low number of granule cells that fire in any given environment (Jung and McNaughton, 1993; Chawla et al., 2005; Leutgeb et al., 2007), leading to the potential for an improved ability to store a large number of dissociable activity patterns within the synapses of the available cells. This computational principle of similar inputs being stored as dissimilar and dissociable outputs is referred to as pattern separation, and is also attributed to the dentate gyrus by computational models (O'Reilly and Rudy, 2000; Treves and Rolls, 1992; Treves et al., 2008). The structural characteristics of the CA3 region facilitates its ability for memory recall based on cues that are presently available; namely, it contains a strong auto-associative network with a large number of recurrent collaterals among its principle pyramidal cells (Kesner, Lee, and Gilbert, 2004). The full generation of an initial representation can be accomplished with just a small portion of a pattern becoming active, such as with a partial cue during recall. This recall should occur even if the input is "noisy", with some active units that were not in the original pattern. This computational principle is called pattern completion (O'Reilly and Rudy, 2000). The CA1 region and subiculum serve as the main output of the hippocampus (O'Mara, 2005). Behavioral studies support a role for the DG in storing similar inputs, as DG lesions in rats cause impairments in the ability to discriminate between nearby food wells (Gilbert, Kesner, and Lee, 2001) and to remember which nearby arms have already been visited in the radial arm maze, with no effect on

more distant arms (Lee and Solivan, 2010).

**Neurogenesis**

The birth of new cells in the brain occurs in the dentate gyrus throughout the lifespan and this observation has been confirmed in various mammalian species that have been studied to date, including humans (Altman and Das, 1965; Gould et al., 1999; Eriksson et al., 1998). Rather than containing a static population of cells, the DG has a continual birth and maturation of new neurons at any given time point. During the time that newborn cells are undergoing maturation they differ from the neighboring mature granule cells in several ways. Initially, post-mitotic dentate GCs have limited dendrite arborization and not fully developed projections have been formed onto CA3 (Ge et al., 2007; Aimone, Deng, and Gage, 2010). Throughout the first weeks after mitosis, the main synaptic input is GABAergic and these cells show evidence for long term potentiation (LTP) when significant GABA activity takes place (Snyder, Kee, and Wojtowicz, 2001). As they develop into the 4-6 week range, maturing granule cells show significant spine formation, now begin to receive glutamatergic inputs and have a reduced threshold for LTP with a larger LTP magnitude (Schmidt-Heiber, Jonas, and Bischofberger, 2004; Ge et al., 2007). By 7-8 weeks of age adult generated GCs show similar afferent inputs and spiking potential similar to that of mature GCs (Laplangne et al., 2006). The role that these new neurons play within the hippocampal circuit is difficult to predict. The addition of new processing units would seem to improve the differentiating function of the DG, as more unused units are available to decorrelate inputs. However, the increased excitability of these new neurons during development has been suggested to decrease their selectivity, as a lowered threshold may increase responses to a variety of stimuli across contexts, leading to more similar outputs rather than dissimilar outputs.  It is unclear exactly how increasing number of processing

units may interact with the properties of these cells or how GCs influence generalization and discrimination functions supported by the hippocampus. Many attempts have been made to understand their function (Aimone, Wiles, and Gage, 2009; Akers et al., 2014; Sahay, Wilson, and Hen, 2011), yet many questions remain. For example, what are their specific contributions to the circuit when an animal has to make a decision to generalize or not?

**Dentate GCs and Conditioned Fear Generalization**

As adult-generated neurons develop from immature to fully mature and integrated cells in the hippocampal network, they may serve a modulatory function that dictates or at least influences the generalization response. Previous work in our lab shows that selective deletion of all post-natal neurogenesis using a conditional knockout mouse line lacking DNMT1 in GFAP+ positive cells causes greater generalization to novel auditory stimuli (Cushman et al., 2013). In a related line of work, we have also found that silencing the output of mature cells onto CA3 in DG-TeTx transgenic mice results in enhanced discrimination between two very similar contexts but has no effect on contexts that are easily distinguishable (Nakashiba et al., 2012). The fact that both of these manipulations are able to specifically change the way that animals are responding to discrete novel cues and contexts shows support for the idea that adult-born GCs can modulate this behavior and we can further explore the nature of their contribution by controlling their participation in such tasks.

**Dissertation Objective**:

The aim for my thesis work was to focus on the way that the hippocampus, and specifically neurogenesis that occurs in the dentate plays a role in fear responses to auditory cues that have not been explicitly paired with fearful auditory stimuli. This was achieved through use of genetic

alterations in mice along with x-irradiation techniques to regulate hippocampal neurogenesis. The effects of these manipulations on conditioned fear behavior were assessed with auditory fear conditioning protocols. In chapter 2, we were able to independently test whether mature, immature, both or neither granule cell population would result in generalization to a novel cue after being trained with either a pure tone or white noise. Chapter 3 describes an experiment that used only irradiation in a C57/BL6 mouse line to investigate how depleting neurogenesis affects generalization of fear across two pure tones after undergoing discrimination training. Lastly, chapter 4 describes the last study in which a genetic deletion of neurogenesis is used to measure effects on tone generalization using a high and low frequency auditory stimulus. Chapter 5 will conclude with a summary of relevant findings across experiments and discuss implications for how these can help elucidate a view of hippocampal neurogenesis in the context of auditory fear generalization and innate learning biases.

# CHAPTER TWO

**Dentate gyrus granule cells modulate fear generalization to novel auditory cues in DG-**

**TeTx Mice**

**Introduction**

In auditory fear conditioning, a sound stimulus is paired with an aversive unconditional stimulus such as mild foot shock. Following conditioning there is a fear response to the specific tone that was used during training, but previous research shows there can also be considerable generalization to an untrained auditory cue (Quinn, Wied, and Fanselow, 2009). We know that the conditioning of a discrete CS such as a tone does not depend on the hippocampus, however, the fear an animal shows to a novel cue that has never been experienced following training with a separate auditory cue can be dampened by excitotoxic lesions of the dorsal hippocampus (Quinn, Wied, and Fanselow, 2009; Duvarci, Bauer, and Paré, 2009). Fear that does not signal actual danger can be maladaptive.

Within the hippocampus, the dentate gyrus undergoes neurogenesis throughout life and therefore contains immature and mature populations of granule cells. Selective elimination of all postnatal neurogenesis using a conditional knockout mouse line (DNMT1cKO) increases generalization of fear to novel cues not presented during training (Cushman et al., 2012). In a related finding, when discriminating between two similar contexts, mice that have undergone irradiation have a reduced ability to learn a discrimination compared to mice in which mature granule cells have been silenced (Nakashiba et al., 2012). These findings suggest the tendency to generalize may be due to interactions within the dentate gyrus.

We investigated the role that different populations of GCs in the dentate play in auditory generalization using a transgenic mouse line in which the output of mature GCs can be selectively silenced by the conditional expression of tetanus toxin (TeTx), while leaving immature GCs (up to 3 to 4 weeks old) intact (Nakashiba et al., 2012). Furthermore, by using focal x-irradiation on these transgenic mice to ablate immature GCs, we were able to compare

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the effects across groups of mice in which the contribution of either, both, or neither population of dentate granule cells were present (experiment 1). In addition, we were interested in how a generalization gradient is affected by having only mature cells inhibited during training of a tone and testing with a range of tones around the CS+.

**Experiment 1: Materials and Methods**

All procedures were conducted in accordance with the standards set forth by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

*Subjects*

Mice were group housed (2-4 per cage) and kept on a 12h light/12h dark cycle with free access to food and water. A total of 56 DG-TeTX mice were utilized for experiment 1 (n= 14 per group, age of mice was 3-5months). All mice were attained through collaboration with the Tonegawa Lab at MIT and shipped in from their institution. In the DG-TeTx mouse line, a tetanus toxin (TeTx) is expressed under the control of the tetracycline operator and is restricted to dentate granule cells older than 4 weeks of age while sparing other granule cells (Nakashiba et al., 2012). This manipulation allows for the inducible blockage of neurotransmission between mossy fibers of the dentate gyrus that project to CA3. A diet enriched with doxycycline (Dox) prevents the expression of the tetanus toxin, resulting in the normal functioning of all dentate granule cells. When animals are switched off the Dox diet, the tetanus toxin is expressed, leading to inhibition of neurotransmitter release (i.e., synaptic transmission) only in mature granule cells.

*Apparatus*

Two conditioning contexts within a sound attenuating chamber were used that differed in location, odor, floors, and lighting. Context A consisted of offset metal grid floors (18 steel rods,

4mm diameter, 1.5mm apart from center), was cleaned and scented with 10% isopropyl alcohol before each animal, and had an overhead light source in the chamber. In a different room, context B contained opaque plastic floors, was cleaned and scented with 1% acetic acid before each animal, and had no light source in the chamber but the room used a red light bulb. In both contexts, conditioning chambers had aluminum walls and with Plexiglas front, back and top (30 x 25 x 25cm; Med-Associates, St. Albans, VT, USA). Shocks were delivered in context A via a shock scrambler connected to the grid floor (Med-Associates, St. Albans, VT, USA).

*Irradiation and Doxycycline Treatment*

Mice that were part of the irradiation groups underwent focal x-irradiation 6 weeks prior to any behavioral experiments while sham animals received injection of anesthetic but no irradiation. Briefly, each individual mouse received an I.P. injection of ketamine (100mg/kg) and xylazine (7mg/kg) and then placed on a stereotaxic instrument. A lead shield covering the entire body with the exception a window over the hippocampus was secured over the stereotaxic plate and the entire apparatus was placed inside an irradiation chamber. Each mouse in the irradiation group received 2 minutes (5 Gy) of X-ray per day for three days with a day in between each session. All mice were initially maintained on a diet enriched with Dox (10mg/kg, Bioserve). Following the irradiation procedure half of the mice in the sham and half in the irradiation group were switched to a Dox-free diet, the other half remained on the Dox diet; this was maintained throughout the experimental procedures.

*Procedure*

The four experimental groups were composed of the following: mice kept on Dox without irradiation (all dentate GCs intact), mice kept off Dox without irradiation (only immature GCs intact), mice on Dox and given irradiation (only mature GCs intact), and mice off Dox and

given irradiation (neither population of GCs intact). Six weeks after irradiation and Dox treatment all mice underwent auditory fear conditioning. On day 1 mice were placed into context A and after three minutes received either a tone (2800hz 85dB) or a white noise (85dB) immediately followed by a 2 s foot shock (.65mA). This was repeated four times for a total of 5 tone (or white noise)-shock pairings with one minute between each pairing. Mice were taken out of the context 90 s after the last shock and returned to their homecage. During days 2-4, to reduce the amount of baseline freezing at test, each animal underwent extinction of context A. Each mouse was placed into the training context for 15 minutes, no auditory stimuli or shock were administered during this time. On day 5, mice were introduced into a novel chamber, context B, which had different lighting, floors, odors and walls for 15 minutes with no auditory stimuli or shocks. This allowed us to more accurately measure fear to the auditory stimuli and not to the context. On days 6-7 mice were placed into context B and were presented with one auditory stimulus per day (trained or novel, counterbalanced across days). Automated percent time spent freezing was measured during each of the tone or white noise presentations during training and test trials using Video Freeze computer software (Med-Associates, Inc.).

## Experiment 1: Results

*Tone Fear Acquisition*

During tone fear conditioning, freezing was measured during the CS across trials and group means were compared using a repeated measures ANOVA with trained cue (tone or white noise) and neurogenesis manipulation (controls, only mature, only immature, neither) as between-subjects factors. This analysis revealed that trained cue (trial x trained cue interaction: $F(4,192) = 9.981, p < 0.001$) and neurogenesis manipulation (trial x neurogenesis manipulation interaction: $F(12,192) = 1.809, p = 0.049$) significantly influenced freezing scores across trials in

training. Collapsing over groups with different dentate manipulations, the cue used in training affected freezing across trials and vice versa. A significant interaction between these two variables was seen across trials (trained cue x trained cue x neurogenesis manipulation: $F(12,192) = 2.856$, $p = 0.001$). Further inspection of the data shows that all mice learned to acquire fear to the auditory cue used in conditioning, however one of the groups performed at a different rate than the rest and reached a lower level of freezing at the end of training (Figure 2.1A-B). Mice off the Dox diet and irradiated (neither population of GCs intact) showed a much shallower acquisition curve than the other groups, but only if this group was trained with a tone, the case is not true if we look at the same group trained with a white noise.

*Tone Test*

The mean percent freezing to their respective trained auditory cue and a novel cue was measured at test for each animal and group means were compared using a repeated measures ANOVA with trained cue (tone or white noise) and neurogenesis manipulation (controls, only mature, only immature, neither) as between-subjects factors. Results show that there was no statistically significant effect of neurogenesis manipulation (group x trial interaction: $F(3,48) = 0.935$, $p = 0.431$) or trained cue (trained cue x trial interaction: $F(1,48) = 1.851$, $p = 0.180$) on freezing across test trials as well as no significant interaction between these across trials (trial x neurogenesis manipulation x trained cue interaction: $F(3,48) = 1.764$, $p = 0.167$). Although there was no statistically significant interaction between the independent variables, it was inferred beforehand that there might be a significant difference between freezing to the trained vs. novel cue in any individual group of mice. To investigate this, t-tests using a corrected alpha level for number of comparisons were conducted. Figure 2.2 shows that in groups trained with the tone, there was a statistically significant difference between freezing to the trained tone vs. white noise

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for mice that had only the mature GCs intact ($t(6) = 4.616$, $p = 0.004$) and surprisingly for the mice that had neither population of GCs intact ($t(6) = 5.640$, $p = 0.001$). For mice trained with a white noise, there were no statistically significant differences seen in freezing to the white noise vs. the tone at test for any of the four groups ($p > 0.006$).

## Experiment 2: Introduction

In order to test how mice would generalize across a gradient using pure tones, not white noise, we ran an additional experiment training mice with one tone and then tested across a range of five tones that included the trained cue. We were interested in whether using pure tones for training and testing would result in more generalization to the novel cues as seen in experiment 1 for the tone trained group. Due to limited availability of the transgenic line, experiment 2 only consisted of two groups of mice, DG-TeTx controls on a Dox diet (all dentate GCs intact) and DG-TeTx mice off the Dox diet (mature population of dentate GCs silenced).

## Experiment 2: Materials and Methods

*Subjects*

Mice were group housed (2-4 per cage) and kept on a 12h light/12h dark cycle with free access to food and water. A total of 25 DG-TeTX mice were utilized for experiment 2. All mice were attained through collaboration with the Tonegawa Lab at MIT and shipped in from their institution.

*Apparatus*

The same two conditioning contexts from experiment 1 (context A and B) were used; these differed in location, odor, floors, and lighting. Shocks were delivered in context A via a shock scrambler connected to the grid floor (Med-Associates, St. Albans, VT, USA).

*Doxycycline Treatment*

Two groups of mice were use in the present experiment, one of them received regular chow diet (n= 10) and the other had chow supplemented with Dox (n= 15). The group on the Dox diet was fed food containing 10mg Dox per kg (Bioserve) ad libitum and the group on the Dox-free diet was fed regular rodent chow ad libitum. The diet treatment began 3 weeks prior to and maintained during behavioral experiments.

*Procedure*

Two experimental groups were composed of the following: mice with all dentate granule cells intact (mice kept on Dox diet) and mice with mature granule cells silenced (mice kept off Dox diet). Three weeks after diet treatment all mice underwent auditory fear conditioning. On day 1, mice were placed into context A and after three minutes received 5 tone-shock pairings (6KHz, 80dB; 0.65mA) with a 3-minute interstimulus interval. On days 2-3, each mouse was placed into context A for 15 minutes, no auditory stimuli or shock were administered during this procedure to extinguish context fear. On day 4, mice were given pre-exposure to context B with no tones. On Day 5 a generalization test was completed, each mouse in the Dox and Dox-free diet groups were randomly assigned to one of five test stimuli (1,600Hz, 3,800Hz, 6,000Hz, 8,200Hz, or 10,400Hz). Mice were placed into context B, following a 3 min baseline were presented with only one of the tones (20 sec) and removed from the context 90 sec later. Automated percent time spent freezing was measured during the tone presentation at training and test trials using Video Freeze computer software (Med-Associates, Inc.).

**Experiment 2: Results**

*Tone Fear Acquisition*

Freezing during each conditioning trial was measured and subjected to a repeated measures ANOVA with Dox treatment (on vs off) a between subjects factor. Results show

(Figure 2.3A) that both groups of mice effectively learned to fear the conditional stimulus during training (main effect of trial $F(4,92) = 112.928$, $p < 0.001$). In addition, there was a small but significant interaction between trial and Dox treatment ($F(4,92) = 2.568$, $p < 0.043$). An additional t-test revealed that during trial 3 of conditioning the group of mice on Dox froze at a different level than those off Dox ($t(23) = 2.781$, $p = 0.013$). This suggests the rate of learning between for the control group was initially faster than the group on Dox, but this difference between the groups was not present by end of the training trials.

*Generalization Gradient*

During the generalization test, each mouse in the Dox and non-Dox group was tested with only one of five tones and freezing measures of both groups were analyzed using a between-subjects ANOVA with test stimulus (1,600Hz, 3,800Hz, 6,000Hz, 8,200Hz, or 10,400Hz) and Dox treatment (off Dox or on Dox) as the between-subjects factors. Results revealed a no main effects of Dox treatment ($F(1,15) = 1.297$, $p = 0.273$) and test stimulus ($F(4,15) = 1.300$, $p = 0.314$) as well as a non significant interaction between Dox treatment and test stimulus ($F(4,15) = 0.621$, $p = 0.655$). Although no statistical significance was reached, there was a noticeable difference in the gradients between control mice and those with mature GCs silenced (Figure 2.3B).

**Discussion**

In experiment 1, it was shown that DG-TeTx mice show no deficits in learning about a dangerous cue, such as a tone paired with shock. Although the group of mice that was trained with a tone and showed a shallow learning curve compared to the rest of the groups during training, this group showed normal freezing during the generalization test. The deficit in fear expression of this group during training was unexpected and difficult to interpret because

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previous studies (Nakashiba et al., 2012) have shown that DG-TeTx mice are just as capable of acquiring a fear response and show no deficit of fear expression during training. Explanation of these results would point to a possible effect on the circuitry that is working under conditions of irradiation and lack of Dox treatment, however various protocols to characterize this transgenic line have ruled out major changes in the circuitry other than the silencing of mossy fibers. Slice experiments from DG-TeTx mice have also shown that synaptic transmission at perforant path and recurrent collaterals are left intact and are not distinguishable from control mice (Nakashiba et al., 2012). One possibility is that inputs from entorhinal cortex which directly project to CA3, bypassing the dentate gyrus, are able to compensate for the lack of input coming in from DG during conditioning and allow for fear expression during the generalization test.

During the generalization test, we observed what seems to be a pronounced asymmetry in the way mice behaved at test depending on whether they were trained with a pure tone vs. a white noise. The control group showed much more freezing to the untrained cue at test, but only if trained with the tone. In addition, the effects of the manipulations (irradiation and Dox) were the opposite for the groups, again depending on the trained stimulus. Mice that were trained with a tone showed better discrimination when either mature GCs are silenced, immature GCs are irradiated, or when both of these are in effect. Mice that were trained with a white noise however, showed more generalization to the tone when mature GCs are silenced, when immature GCs are irradiated, or when both GC populations are affected. These results cannot be completely explained with any one learning theory. According to configural theories of learning (Pearce, 1987) during conditioning an animal learns about the set of stimuli as a whole when trained with a compound. At test, presenting an element of the compound should produce the same amount of generalization compared to when an element itself is trained and the compound

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stimulus is presented during testing. Elemental models (Rescorla and Wagner, 1972) predict individual elements of a compound stimulus each acquire associative strength during conditioning. These models would hypothesize that amount of generalization essentially depends on the elements present at test that were also present during conditioning and the associative strength of these. For example, when training occurs with a compound stimulus, if one element from a trained compound stimulus is then presented at test, then responding will be reduced compared to having presented the compound itself and assuming associative strength was spread among the individual elements. On the other hand, if you train using only an individual element all the associative strength will be given to the one element. During the test phase, if a compound stimulus is presented (which included the trained element) then there should be no difference in responding between this and presenting the element on its own.
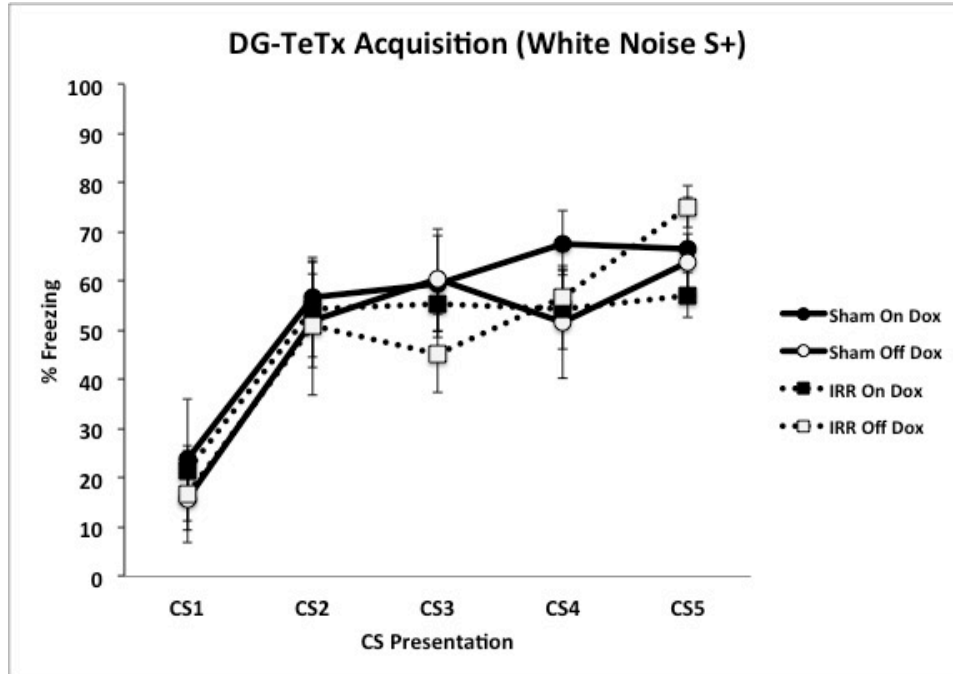
In the present experiment, the white noise may be viewed as a compound stimulus made up of elements, or frequencies of pure tones in this case, and the tone as one element itself.  In trying to parse out the differences observed in the control groups, results showed that training with a white noise and testing with a tone reduced freezing at test; this is not consistent with the configural view but follows elemental models. However, the elemental theories would also state that tone trained animals should show no difference in freezing when tested with a white noise but this was not the case. Other experimenters (Bouton, Doyle-Burr, and Vurbic, 2012) investigating generalization between compound and elements in appetitive conditioning have found similar difficulties accounting for results with only elemental or configural theories when asymmetry is present. Bouton, Doyle-Burr, and Vurbic (2012) trained rats with either a compound stimulus (light + tone) or a single element stimulus (tone) to signal delivery of food pellets; following training, all rats were tested with the compound and the element stimuli. They
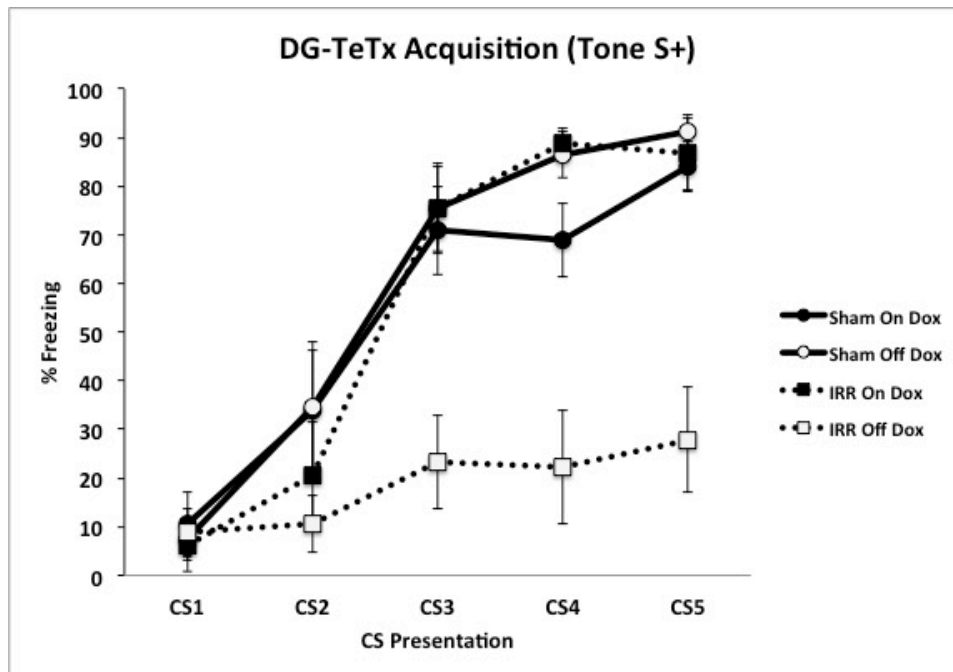
found that generalization of responding was greater when rats were trained with an element and then tested with a compound compared to the responding seen at test with an element after training with a compound. In a separate experiment, they also observed an asymmetry in the generalization of extinction but the effect was in the opposite direction. Generalization of extinction was greater for rats extinguished with a compound and then tested with an element. The pattern of results found in the present experiment align with their findings regarding generalization of extinction, such that training with a white noise stimulus results in more generalization to the pure tone at test.

In experiment 2, we saw a steeper generalization gradient for DG-TeTx mice with only immature GCs intact. Control animals in experiment 2 actually showed increased freezing for higher frequency tones. Like animals in the Nakashiba et al. study (2012) having only the immature population of GCs in the dentate helped differentiate safe and dangerous stimuli. It is unclear whether having only the mature population or neither of these would produce similar results but this would be a next step in follow up studies. Together, these experiments speak to the influence that different populations of GCs have on fear generalization to novel auditory cues and to the ability of trained stimuli to affect responding at test.

A)

**DG-TeTx Acquisition (White Noise S+)**

Legend:
- Sham On Dox
- Sham Off Dox
- IRR On Dox
- IRR Off Dox

B)

**DG-TeTx Acquisition (Tone S+)**

Legend:
- Sham On Dox
- Sham Off Dox
- IRR On Dox
- IRR Off Dox

**Figure 2.1**
Mean (±SEM) percent freezing during each auditory stimulus for groups trained with a white noise (A) or a pure tone (B) (n= 14 for each of the 8 groups). All mice trained with a white noise showed a significant increase in freezing across training trials regardless of which dentate manipulation group they were in. Converseley, if mice were trained with a pure tone, the group with neither population of GC's intact showed a different rate of acquisition than the rest of the groups.

**A)** Generalization Test (White Noise Trained)

**B)** Generalization Test (Tone Trained)
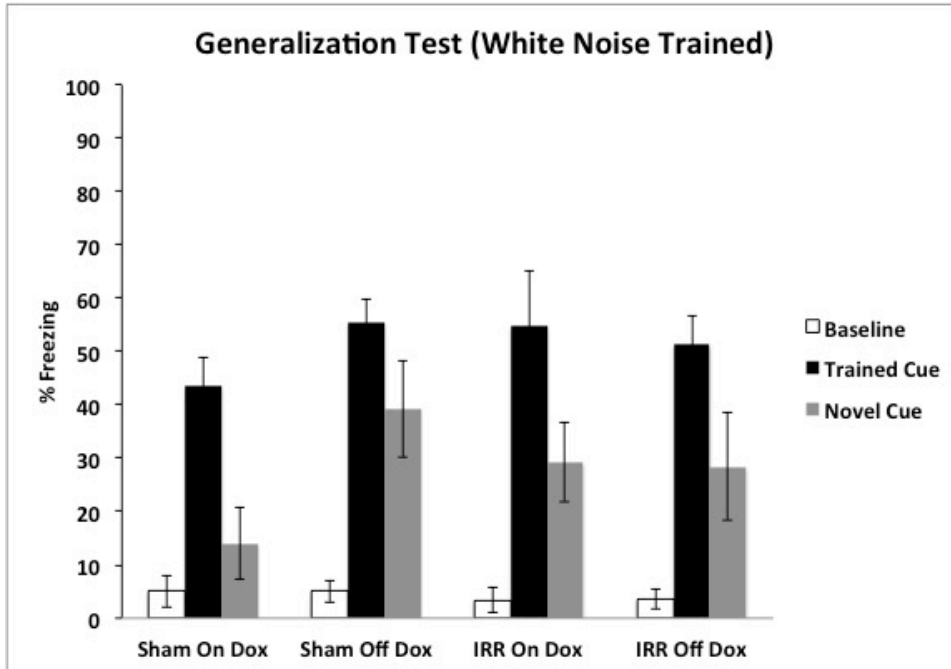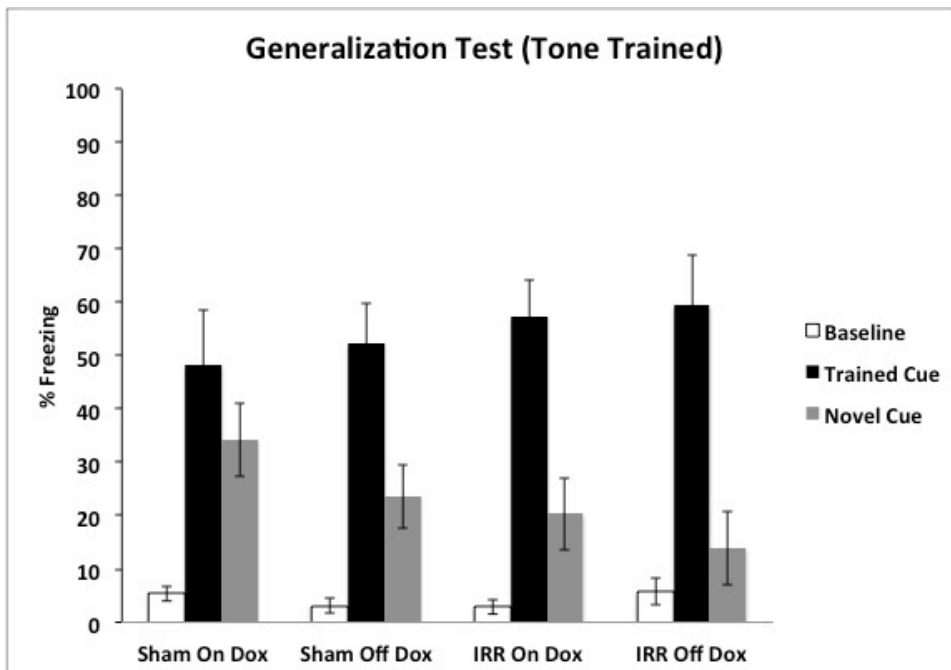
**Figure 2.2**
Mean (±SEM) percent freezing during each cue at test for groups (n= 14 for each of 8 groups) trained with a white noise (A) or a pure tone (B). All mice demonstrated fear to the trained stimulus. Control mice trained with a white noise could differentiate well, but removing either mature or immature GCs increased freezing to the untrained tone. Mice trained with the tone show more freezing to the novel white noise but removing either mature or immature GCs

actually reduces the generalized fear to the tone. Interestingly, the mice that lack both mature and immature GCs peform just as well or better than groups with removal of just one population. Statistical differences were only reached in two groups marked with an asterisk using a corrected alpha level for multiple comparisons.

A)
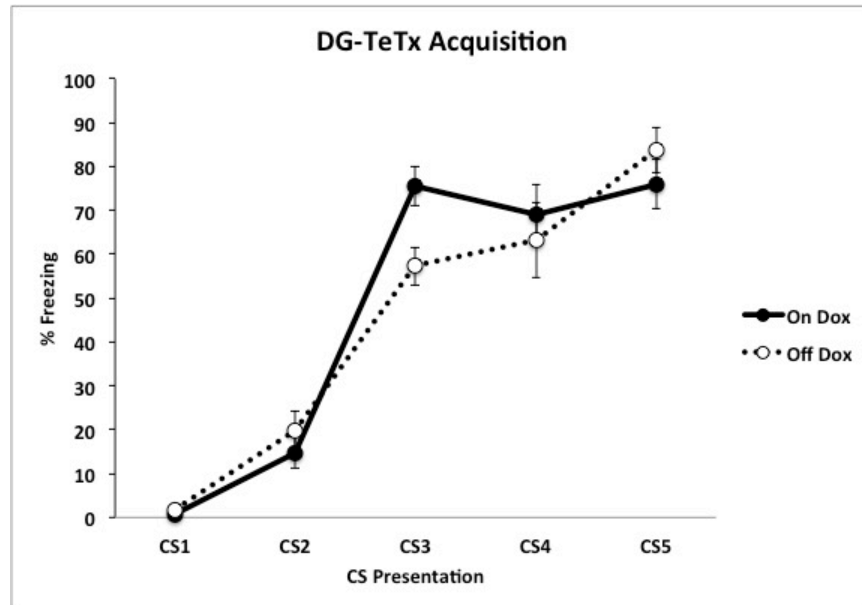


B)
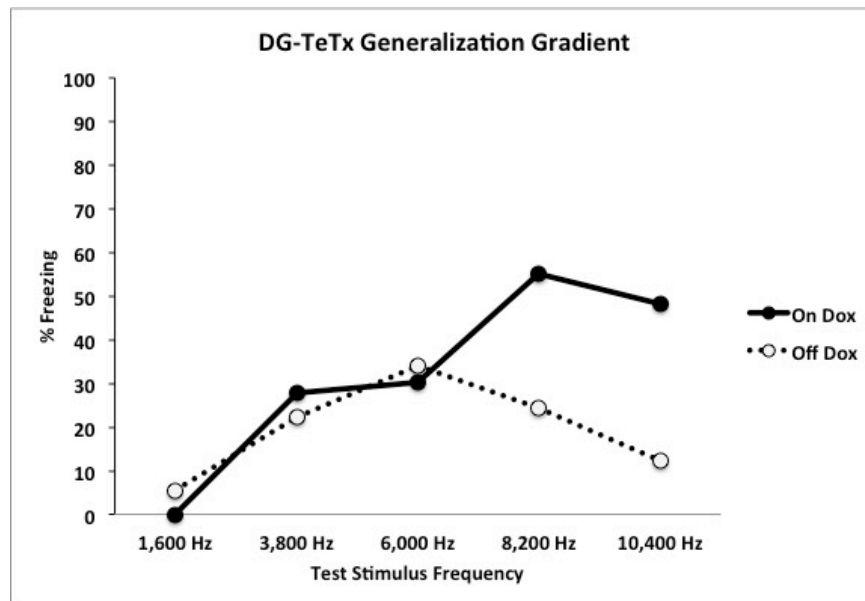


**Figure 2.3**

Mice that are either on or off doxycyline diet show no overall difference in fear acquisition after five tone-shock pairings using a 6,000Hz CS+ (**A**). There was no statistically significant effect of diet at test, however when mice were presented with one of five frequencies, the group with mature dentate GCs silenced (off Dox, n= 15) showed a more appropriate generalization curve than controls (on Dox, n= 10) around the trained CS+ (**B**).

**CHAPTER THREE**

**Loss of neurogenesis via irradiation promotes discrimination following conditioning of high frequencies**

**Introduction**

Multiple studies have looked at the effects of manipulating hippocampal neurogenesis on discrimination between a safe and dangerous contexts (Saxe et al., 2006; Drew et al., 2010; Hernandez-Rabaza et al., 2009 ) but none have looked at discrimination between discrete auditory cues. Results are mixed and a consensus is yet to be reached as to what the exact role, if there is one, adult generated GCs play. There is also a rich literature that examines the nature of stimulus generalization (Guttman and Kalish, 1956; Hanson, 1959), but for the most part these investigations have dealt with appetitive types of reinforcement.

The purpose of the present experiment was to investigate the effect of irradiating immature GCs in the dentate gyrus on discrimination training between two pure tones and generalization of tone fear. A secondary goal was to explore whether asymmetries in generalization of fear could be explained by non-associative aspects of conditioning that had nothing to do with the quality of the auditory stimuli themselves. In order to do this, we used focal x-irradiation to eradicate neurogenesis in C57/BL6 mice and measured fear responses in a tone fear conditioning paradigm.

**Experiment 1: Materials and Methods**

All procedures were conducted in accordance with the standards set forth by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

*Subjects*

A total of 20 C57BL/6 male mice were group housed (2-4 per cage) and kept on a 12h light/12h dark cycle with free access to food and water. Half of the mice received irradiation treatment (n= 10). All mice were handled for 3 days before any experimental manipulations.

*Apparatus*

Two conditioning contexts within a sound attenuating chamber were used that differed in location, odor, floors, and lighting. Context A consisted of offset metal grid floors (18 steel rods, 4mm diameter, 1.5mm apart from center), was cleaned and scented with 10% isopropyl alcohol before each animal, and had an overhead light source in the chamber. In a different room, context B contained opaque plastic floors, was cleaned and scented with 1% acetic acid before each animal, and had no light source in the chamber but the room used a red light bulb. In both contexts, conditioning chambers had aluminum walls and with Plexiglas front, back and top (30 x 25 x 25cm; Med-Associates, St. Albans, VT, USA). Shocks were delivered in context A via a shock scrambler connected to the grid floor (Med-Associates, St. Albans, VT, USA).

*Irradiation*

Mice that were part of the irradiation groups underwent focal x-irradiation 6 weeks prior to any behavioral experiments while sham animals received injection of anesthetic but no irradiation. Briefly, each individual mouse received an I.P. injection of ketamine (100mg/kg) and xylazine (7mg/kg) and then placed on a stereotaxic instrument. A lead shield covering the entire body with the exception a window over the location of the hippocampus was secured on the stereotaxic plate and the entire apparatus was placed inside an irradiation chamber. Each mouse in the irradiation group received 2 minutes (5 Gy) of X-ray per day for three days with a day in between each session.

*Procedure*

Six weeks after irradiated and sham groups underwent the respective treatment, all mice completed tone discrimination training followed by a generalization test. One day of tone habituation in which four presentations of each tone stimulus to be used for training (1,200Hz

and 10,200Hz, 20s, 70dB) were presented during a 35 minute session in context A.

Discrimination training always began 24 h after the tone habituation session also in context A

and spanned 6 consecutive days. During each daily session of discrimination training, all mice

received 4 presentations of one of the tones (1,200Hz or 10,200Hz) followed by shock and four

presentations of the other tone not followed by shock with an interstimulus interval of 4 minutes.

Both tones were played in the same training session but order of the CS+ and CS- were

counterbalanced across days. Following discrimination training, the next day consisted of a 15

minute context pre-exposure to context B where tone fear would be tested to both CS+ and CS-.

Tone generalization testing began 24 h later in context B where mice received 3 presentations of

only CS+ or only CS- without any shock. The stimulus that was not tested on the first day of

tone testing was presented the following day and the stimulus an individual mouse was tested

with first was counterbalanced. Percent time spent freezing was measured during each of the

tone presentations during training and test trials using Video Freeze computer software (Med-

Associates, Inc.).

<center>**Experiment 1: Results**</center>

*Discrimination Training*

Across the six days of discrimination training, freezing to the S+ compared to the S- was

investigated across groups using a repeated measures ANOVA with irradiation (irradiated vs

sham) and trained cue (1,200Hz vs 10,200Hz) as between subject factors. Collapsing all groups,

there was a significant difference in freezing to the S+ and S- across training day (day x CS

interaction: $F(5,175) = 10.526, p < 0.001$) indicating that mice were learning to show differential

freezing to the two conditional stimuli (Figure 3.1A-B). This learning was affected by which cue

the mice were trained with ($F(5,175) = 2.450, p = 0.036$), but not irradiation treatment ($F(5,175)$

<center>29</center>

= 0.970, $p$ = 0.437) and no interaction was found when examining the two variables together across days between freezing to S+ and S- (irradiation x trained cue x day x CS interaction: $F(5,175)$ = 0.409, $p$ = 0.842). All mice showed discrimination between the two tones across training, and the cue these mice received as the S+ influenced their behavior that could not be attributed to irradiation treatment.

*Tone Test*

Measures for freezing during the tone test trials were analyzed using a repeated measures ANOVA with irradiation (irradiated vs sham) and trained cue (1,200Hz vs 10,200Hz) as between subject factors. Combining all groups, mice showed a significant difference in freezing to the S+ vs. S- between test trials (CS x trial interaction: $F(2,70)$ = 3.429, $p$ = 0.038). In addition, the overall freezing across trials was affected by irradiation treatment and the cue mice were trained with (CS x trained cue x irradiation interaction: $F(1,35)$ = 25.578, $p$ < 0.001). This interaction was further analyzed with t-tests corrected for number of comparisons to where differences lie. For mice that were trained with a low frequency tone (Figure 3.2A), sham mice that did not receive irradiation distinguished between S+ and S- on all three test trials (CS trial 1, 2, 3 respectively: $t(9)$ = 4.452, $p$ = 0.002, $t(9)$ = 9.087, $p$ < 0.001, $t(9)$ = 5.965, $p$ < 0.001) while irradiated mice did not show any significant difference in freezing to the two cues at any test trial ($p$ > 0.004 for all tests). For mice that were trained with a high frequency tone (Figure 3.2B), irradiation actually produced a greater discrimination between S+ and S- on all three test trials (CS trial 1, 2, 3 respectively: $t(8)$ =5.200, $p$ = 0.001, $t(8)$ =6.523, $p$ < 0.001, $t(8)$ = 6.688, $p$ < 0.001) while sham mice did not show any significant difference in freezing to the two cues at any of the test trials ($p$ > 0.004 for all tests).

**Experiment 2: Introduction**

As a follow up to the findings for DG-TeTx mice as well as the asymmetry that was observed with C57 mice during the tone test we wanted to know whether simply receiving shocks somehow produces an increase in responding to auditory stimuli that is non associative and if so, whether it happens equally for a high and low frequency tones. In order to rule out whether some of the freezing that we saw in experiment 1 could be due to a sensitization effect, we conducted a control experiment for which all mice were trained with only shocks and then tested with a high frequency tone as well as a low frequency tone and fear responses to these cues were measured.

**Experiment 2: Materials and Methods**

*Subjects*

A total of 8 C57BL/6 male mice were group housed (2-4 per cage) and kept on a 12h light/12h dark cycle with free access to food and water. Half of the mice received irradiation treatment (n= 10). All mice were handled for 3 days before any experimental manipulations.

*Apparatus*

Two conditioning contexts within a sound attenuating chamber were used that differed in location, odor, floors, and lighting. Context A consisted of offset metal grid floors (18 steel rods, 4mm diameter, 1.5mm apart from center), was cleaned and scented with 10% isopropyl alcohol before each animal, and had an overhead light source in the chamber. In a different room, context B contained opaque plastic floors, was cleaned and scented with 1% acetic acid before each animal, and had no light source in the chamber but the room used a red light bulb. In both contexts, conditioning chambers had aluminum walls and with Plexiglas front, back and top (30 x 25 x 25cm; Med-Associates, St. Albans, VT, USA). Shocks were delivered in context A via a shock scrambler connected to the grid floor (Med-Associates, St. Albans, VT, USA).

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*Procedure*

All mice received two days of context conditioning in context A. Mice were placed into the conditioning chamber and received 3 foot shocks (0.65mA, 2s) separated by 4 minutes after a 3 minute baseline. Following one minute after the last shock, all mice were returned to their home cage. Days 3-4 mice were given pre-exposure to context B with no discrete stimuli present to make sure the tone test would accurately measure freezing to the auditory stimulus and not to the context. On day 5-6 mice completed a generalization test where they were presented with one of two tones each day in a counterbalanced order. On day 5, half of the mice were placed into context B and after a 3 minute baseline period received a 10,000Hz tone (75dB, 20 sec) three times separated by 4 minutes; the other half of the animals underwent the same procedure with the exception of the frequency of tone presented which was 1,600Hz (75dB, 20 sec). On day 6, all mice were tested in the same manner as day 5 but received the tone that had not been presented the day before. Percent time spent freezing was measured for 20s following the shock in the training phase and during each of the tone presentations during the tone test using Video Freeze computer software (Med-Associates, Inc.).

**Experiment 2: Results**

*Fear Acquisition*

As shown in Figure 3.3A, a repeated measures ANOVA revealed a significant main effect of trial ($F(5,35) = 13.499$, $p < 0.001$). All mice showed a significant increase in freezing across trials of training over the two days.

*Tone Generalization Test*

At the time of testing, freezing measures from each animal to a 10,000Hz tone vs 1,600Hz tone were compared (Figure 3.3B). An ANOVA revealed no significant main effect of

the tone tested ($F(1,21) = 0.808$, $p = 0.399$) indicating there was no difference in reaction to the two frequencies of the auditory cue, even when looking between trials (tone tested x trial interaction: $F(3,21) = 0.075$, $p = 0.973$). There was a main effect of trial ($F(3,21) = 9.979$, $p = 0..002$), meaning freezing during the tones did increase a bit from the baseline measure taken during the first three minutes of the test. Both auditory cues elicited some freezing behavior but this was not biased towards either frequency. In addition, freezing during the tone test also did not reach levels of freezing during conditioning.

## Discussion

In experiment 1 groups of mice with and without irradiation were trained on a tone discrimination task where two tones served as a CS+ and a CS-, results showed that irradiation of immature GCs did not affect whether any given animal could learn to fear a reinforced tone. There was, however, an effect of the trained cue such that the groups of mice that were trained with the high frequency tone (10,200Hz) as the CS+ showed more differential responding to the two tones compared to the groups of mice that were trained with the low frequency tone (1,200Hz) as the CS+. During the generalization test, we observed opposite effects similar to previous findings (chapter 2) that depend on what cue is used at training. For groups trained with the high frequency tone as the CS+, irradiation actually caused profound discrimination between CS+ and CS-. For groups trained with the low frequency tone as the CS+, irradiation seemed to ruin the ability to discriminate but this may be due to the sub par performance of this particular groups during training. Training with a high frequency somehow helps an animal to determine which cue to freeze to, but only if the immature GCs are out of the picture.

In experiment 2, mice were trained with a shock only protocol to see whether a sensitization effect would be present at test when a low and high frequency tone are delivered. If

the asymmetry that is surfacing between experiments can be explained by one of the auditory cues being sensitized by the shock then there should have been a bias in freezing during the tone test. This was not the case, mice showed no difference in freezing between a high and low frequency cue during testing. This suggests that the differential fear generalization that was observed to the CS- cannot be due to a trigger from merely getting shocked. Importantly, although there was a slight increase in freezing to the unpaired tones at test from baseline, neither tone caused freezing that reached conditioning levels.

A)
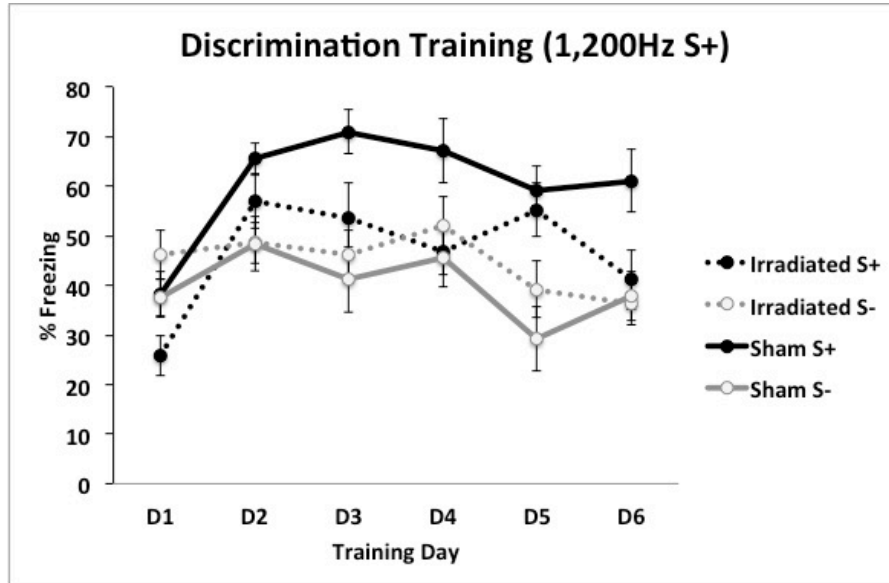


B)
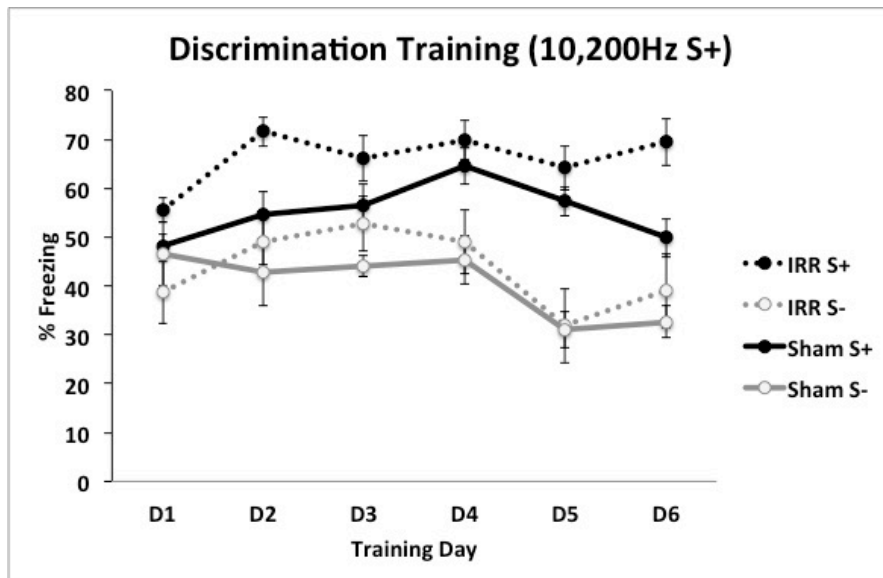


**Figure 3.1**

Mean (±SEM) percent freezing during discrimination training between two tones across six days of training for mice that were trained with either a low frequency tone (A) or a high frequency tone (B). A total of 9-10 mice were used for each of the four groups. All mice showed disrimination between S+ and S- regardless of irradiation treatment, however the cue that served as the S+ had an effect on the groups during training such that mice trained with the high frequency tone, when collapsing across irradiation treatement, showed better discrminiation throughout training.

A)

Tone Test (Trained 1,200Hz)

B)

Tone Test (Trained 10,200Hz)
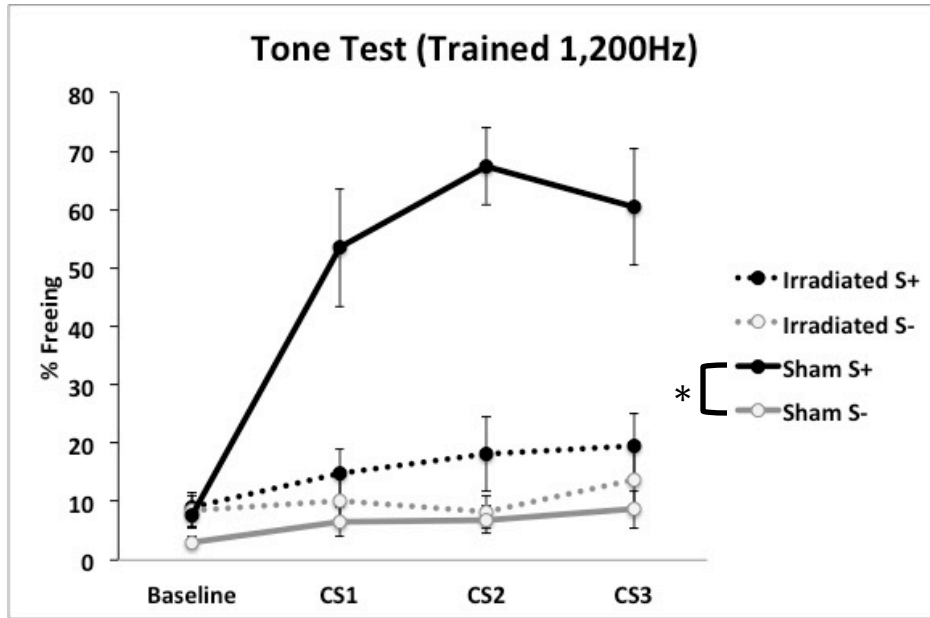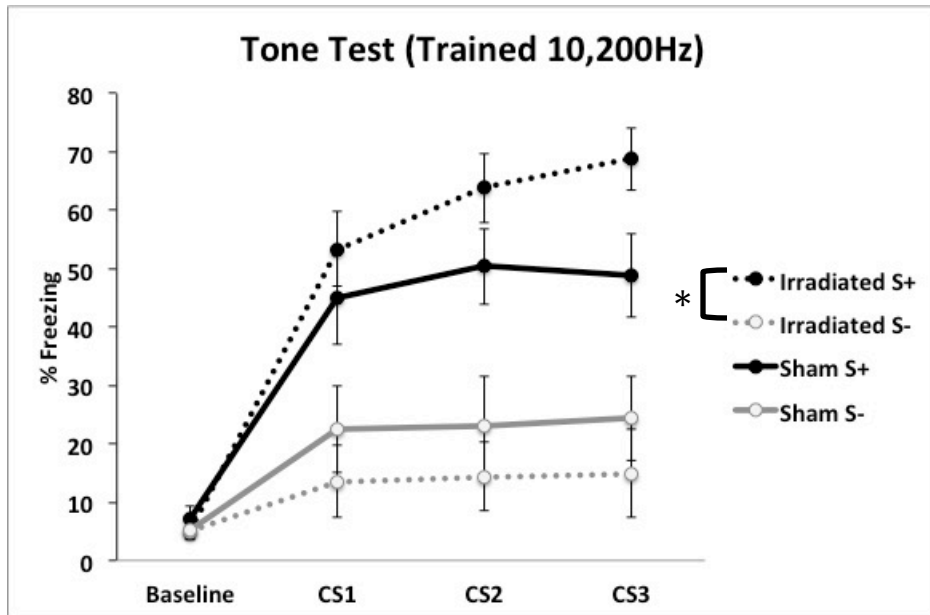
**Figure 3.2**
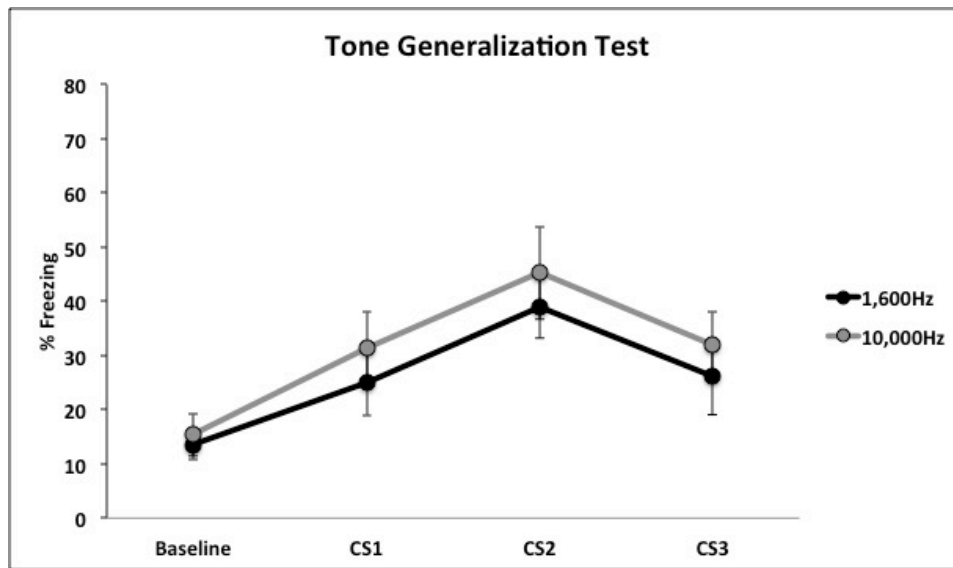Mean (±SEM) percent freezing during the tone test for groups trained with a low frequency tone (A) and groups trained with a high frequency tone (B). A total of 9-10 mice were used for each of the four groups. There was a significant interaction between irradiation treatment and trained cue at test, such that sham animals trained with a low frequency performed better than irradiated mice but the irradiated mice showed better discrimination of tones if they were trained with a high frequency tone.

A)
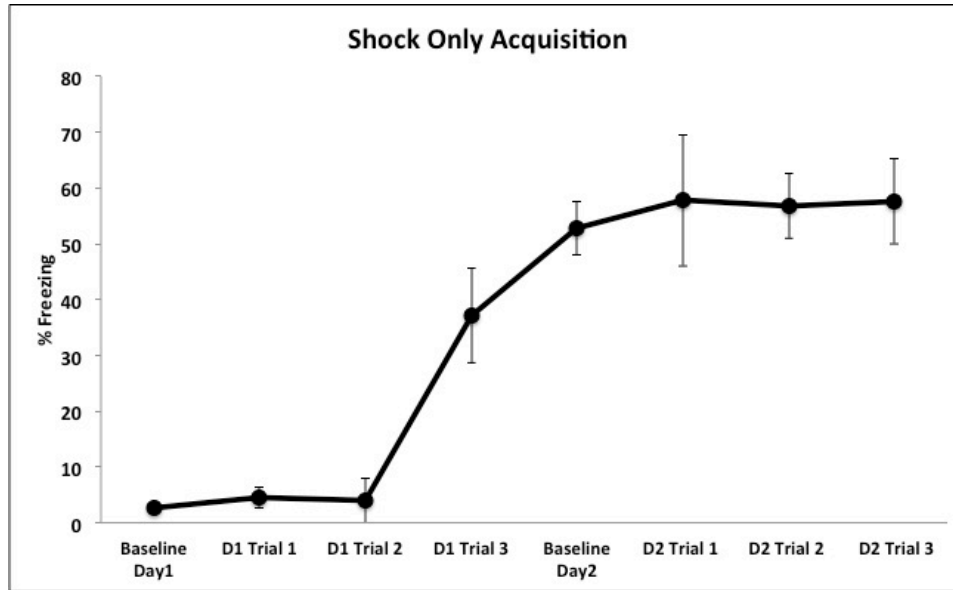


B)

**Figure 3.3**

Mean (±SEM) percent freezing during conditioning with shock only trials across two days (A; n=10). At test (B), mice showed no difference in freezing between a high (10,000Hz) or low frequency (1,600Hz) tone although there was some overal increase in freezing from baseline.

# CHAPTER FOUR

**Reduced generalization in DNMT1 conditional knockout mice when trained with a high**

**frequency tone**

**Introduction**

The hippocampus is one of two places in the brain where birth of new cells occurs over a lifetime (Altman and Das, 1965; Gould et al., 1999). Previous research has shown that lesions of the dorsal hippocampus as well as irradiation of neurogenesis changes the way an animal generalizes to auditory and contextual cues following training (Quinn et al., 2009; Nakashiba et al., 2012). The exact mechanisms by which the central nervous system can lead an animal to generalize or not are not fully understood as well as what the role of the dentate GCs may be.

The focus of the present experiment was to investigate the effect of fear generalization between two tones using mice that have a genetic ablation of neurogenesis and are only trained with one of the CSs. DNMT1-cKO mice were utilized to selectively target postnatal neurogenesis and do so in a manner that required no drug administration or time lapse necessary before behavior could be conducted. The DNMT1-cKO mice were created using two parental lines that when crossed produces permanent ablation of postnatal neurogenesis in the knock out mice while leaving the dentate intact in wild type controls. Deletion of DNA methyltransferase-1 was restricted to cells that were post-mitotic by using one parental line (mGFAP-Cre) in which cre recombinase is expressed only in GFAP+ cells and a second parental line in which the DNMT1 gene had flanked loxP sites (DNMT1-loxP). As a result of crossing these two lines, half of the offspring were DNMT1-loxp/mGFAP Cre-positive and half were DNMT1-loxp/mGFAP Cre-negative. Previous findings (Cushman et al., 2012) have already established that mice lacking neurogenesis show greater levels of generalization when discriminating between a tone and a white noise but given the results (chapter 2-3) showing biased effects of training a particular auditory cue, the goal was to see if results would be replicated when using two pure tones as the conditional stimuli.

## Materials and Methods

All procedures were conducted in accordance with the standards set forth by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

*Subjects*

Mice were housed in groups of 2-4 and kept on a 12h light/12h dark cycle with free access to food and water. A transgenic mouse line with a targeted disruption of DNA methyltransferase (DNMT1) restricted to GFAP+ progenitor cells allowed postnatal neurogenesis to be genetically ablated in the conditional knockout mice (DNMT1cKO) while sparing this in wild type controls (WT). Ten male DNMT1-lox/mGFAP-Cre-positive (DNMT1cKO) and eight DNMT1-lox/mGFAP-Cre-negative (WT) were used for this experiment. Handling of mice and habituation to transport was conducted for 3 days prior to any experimental procedure.

*Apparatus*

Two conditioning contexts within a sound attenuating chamber were used that differed in location, odor, floors, and lighting. Context A consisted of offset metal grid floors (18 steel rods, 4mm diameter, 1.5mm apart from center), was cleaned and scented with 10% isopropyl alcohol before each animal, and had an overhead light source in the chamber. In a different room, context B contained opaque plastic floors, was cleaned and scented with 1% acetic acid before each animal, and had no light source in the chamber but room used a red light bulb. In both contexts, conditioning chambers had aluminum walls and with Plexiglas front, back and top (30 x 25 x 25cm; Med-Associates, St. Albans, VT, USA). Shocks were delivered in context A via a shock scrambler (Med-Associates, St. Albans, VT, USA).

*Procedure*

Mice were placed into context A on day 1 and after a three minute baseline period received three 20s tones of one frequency (10,200Hz or 1,200Hz) followed by a 2s (0.65mA) foot shock with one minute between pairings. In order to reduce contextual freezing and attain a more accurate measure of tone freezing behavior mice received three days of context exposure to context B with no tones or shocks for 15min each day. On day 5 mice were given a tone test in context B with either the trained tone that was given in context A or a novel tone they had no prior experience with. Day 6 was used to test the tone not presented on day 5 and followed the same parameters. The tone test consisted of the same protocol used for training with the exception of the delivery of shocks. Freezing behavior was recorded using VideoFreeze software (Med-Associates) at 30 frames per second.

**Results**

*Tone Fear Acquisition*

Freezing was measured during a three minute baseline period and during the presentation of the three conditional stimuli. A repeated measures ANOVA was performed with trained cue (1,200Hz or 10,200Hz tone) and genotype (Wild Type control or DNMT1cKO) as between subject factors. As shown in Figure 4.1, each group of mice showed acquisition of fear during training ($F_{(3,42)} = 49.168$, $p < 0.001$) that did not differ between the tone used for training ($F_{(3,42)} = 2.505$, $p = 0.072$) or between genotypes ($F_{(3,42)} = 2.065$, $p = 0.119$). All mice showed increase freezing to the tone across the three training trials.

*Tone Generalization Test*

Freezing for each of the three tone presentations during the generalization test was averaged for each mouse before computing group means. A repeated measures ANOVA was

used to compare each groups' freezing to the trained vs. novel tone with trained cue (1,200Hz or 10,200Hz tone) and genotype (Wild Type control or DNMT1cKO) as between subject factors. This analysis showed no main effect of genotype ($F_{(2,28)} = 2.159$, $p = 0.134$) and a nonsignificant interaction between trained cue and genotype ($F_{(2,28)} = 0.010$, $p = 0.990$). However a significant main effect of trained cue was present ($F_{(2,28)} = 39.194$, $p > 0.001$) indicating that freezing between the trained cue and the novel cue at test was affected by which tone mice were trained with as shown in Figure 4.2. This was followed by post hoc t-tests, which revealed mice trained with the 10,200Hz frequency tone were better able to differentiate between that and a novel tone compared to mice trained with a 1,200Hz tone ($t(9) = 7.035$, $p > 0.001$). Even though the interaction between genotype and trained cue was not significant we wanted to further examine the effect of trained cue on generalizing to a novel tone across genotype. Using t-tests corrected for number of comparisons showed that DNMT1cKO mice trained with the high frequency tone were able to differentiate between the two test tones compared to the other groups ($t(5) = 7.330$, $p = 0.001$, see Figure 4.3).

## Discussion

In the present experiment DNMT1cKO mice and wild type controls were trained with one of two auditory stimuli and then given a generalization test to measure freezing across novel and trained cues. The findings demonstrate once again an asymmetry in the fear response to untrained stimuli that depends on the tone used for training.

All mice learned to show fear to their respective CS+ during training. There was a large effect of trained cue on freezing independent of genotype, such that mice trained with a high frequency tone exhibited low responding to the untrained cue and mice trained with the low frequency actually showed more freezing to the untrained cue. This was further examined to see

what was driving the effect seen in the high frequency trained group. Subsequent analysis showed that DNMT1cKO mice trained on the high frequency tone were responsible for the phenomenon. This may be viewed as inconsistent with previous experiments (Cushman et al., 2012) using this transgenic mouse that showed more generalization to a novel cue. However, the present experiment did not use the same auditory cues of white noise and tone. Instead two pure tones were used in training and the same two at test.
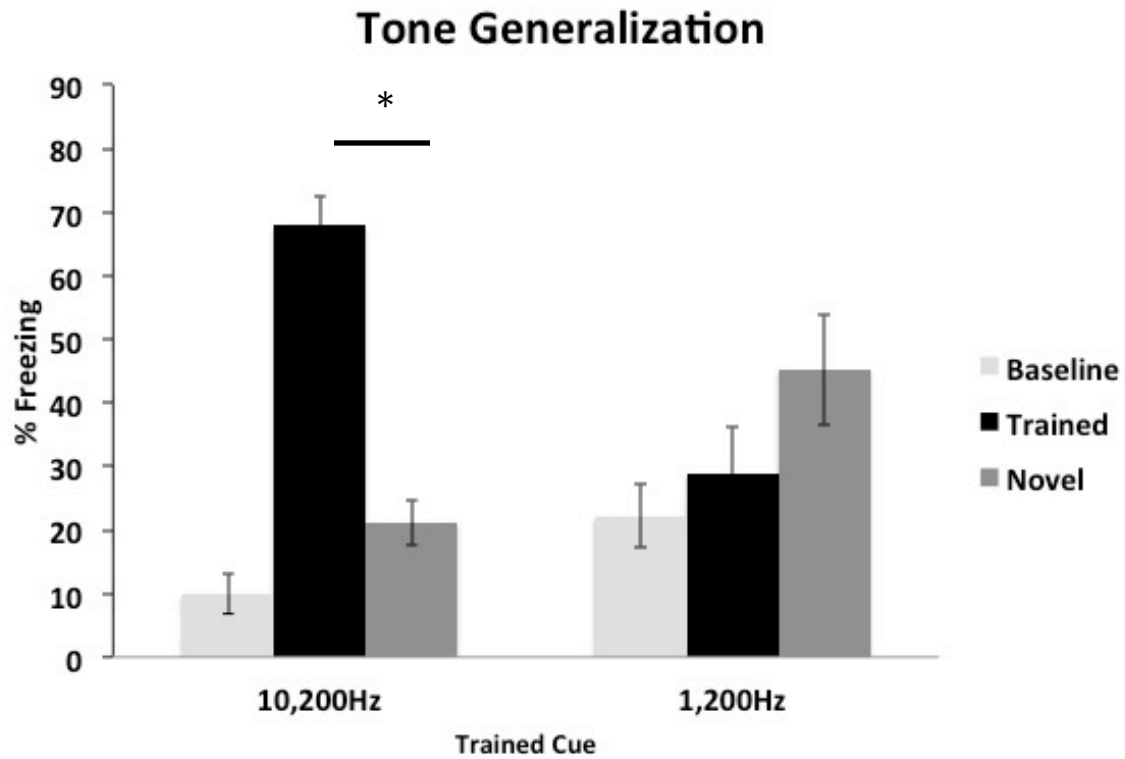
**Acquisition of Tone Fear**
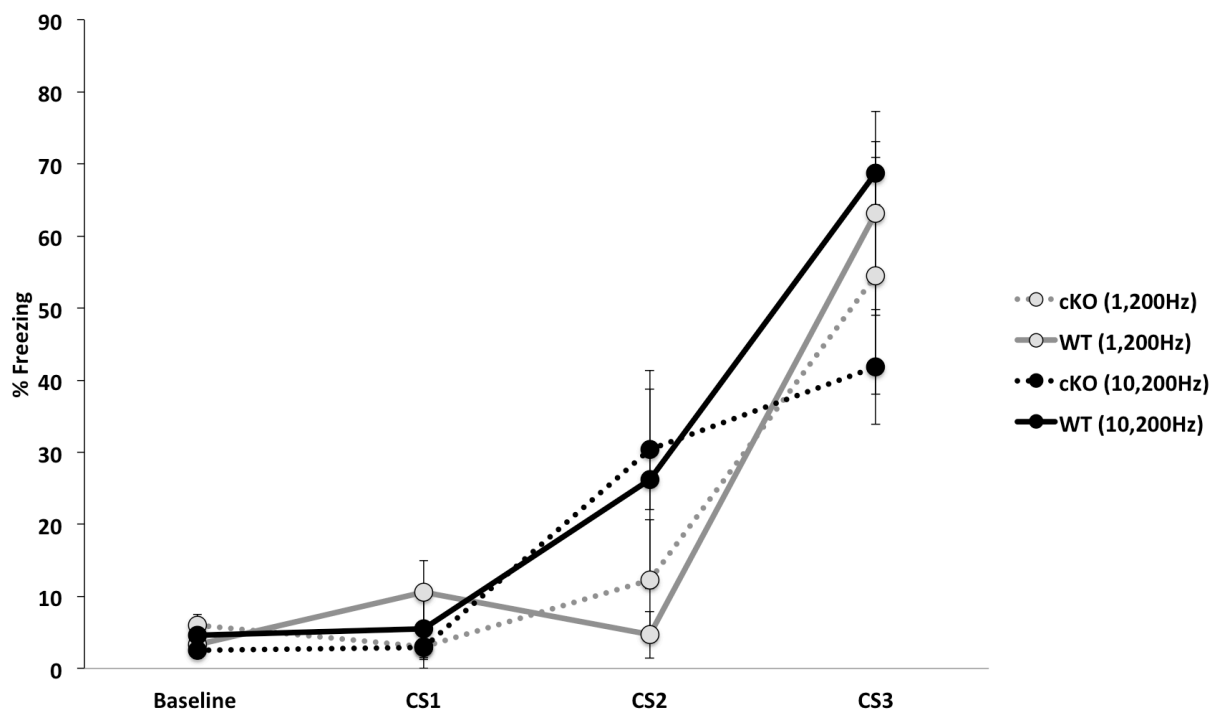
**Figure 4.1**
Mean (±SEM) percent freezing during training for DNMT1cKO (n=10) and wild type (WT; n=8) controls that were either trained with a 1,200Hz tone or a 10,200Hz tone. Regardless of genotype or trained tone frequency (in parentheses), all groups showed fear to the conditional stimulus over the three tone-shock pairings.

## Tone Generalization



**Figure 4.2**
Mean (±SEM) freezing response during the genralization test for mice that were trained with a high frequency tone (10,200Hz; n= 10) and those that were trained with a low frequency tone (1,200Hz; n= 8). Means are collapsed across genotypes. Mice trained with a high frequency tone froze significantly more to this cue compared to a novel tone but this was not the case when mice were trained with a low frequency tone as the S+ during training.

A)
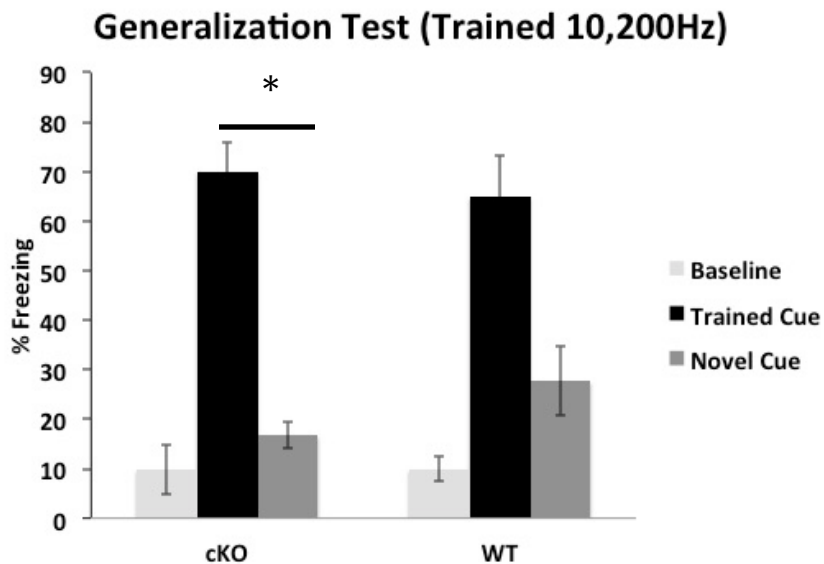


B)
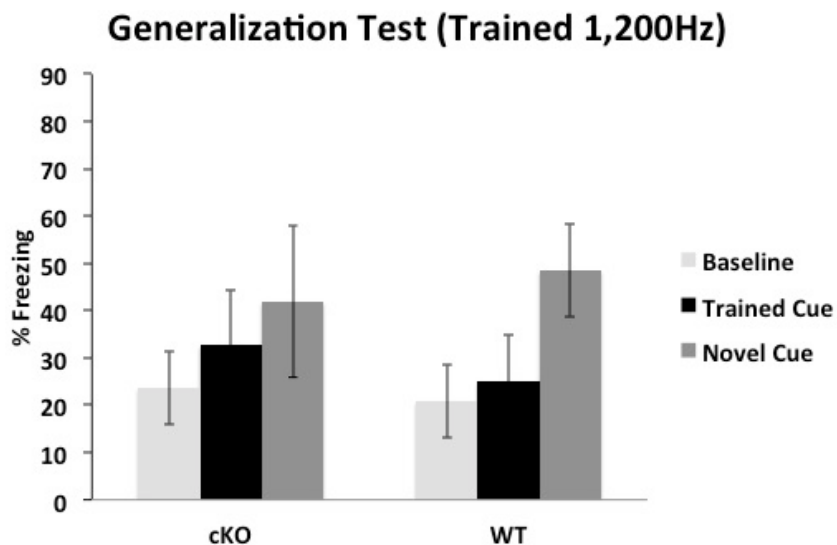


**Figure 4.3**
Mean (±SEM) freezing response during the genralization test for DNMT1cKO (n= 10) and wild type control (WT; n= 8) mice that were trained with a high frequency tone (A) and those that were trained with a low frequency tone (B). Comparisons between freezing to the trained and novel cue across groups showed that DNMT1cKO mice are better able to discriminate between a trained fear cue and a novel one, however this is affected by the the frequency of tone used during conditioning.

**CHAPTER FIVE**

**Summary and Discussion**

The ability to distinguish between stimuli in our environment relies on the capacity to encode and subsequently retrieve information that was previously stored. In order to accomplish a proper form of action, the brain must have a mechanism to distinguish between all of the inputs that can possibly be compared and then respond appropriately. The hippocampus is thought to be one of the key structures that serve this function, many theories about its computational pattern separation and completion abilities abound (Marr, 1970; Aimone, Wiles, and Gage, 2009; Leutgeb et al., 2007). These terms have been introduced and used interchangeably with discrimination and generalization that arise from classic learning theories. The gist of the question most investigators using these terms are after may still be the same though, that is how does the hippocampus with its heterogeneous population of GCs possibly contribute?

The main objective of this dissertation was to delineate the role of hippocampal neurogenesis in tone fear generalization and to dissociate between contributions of mature and immature GCs. Using a factorial design in which irradiation eliminated the population of immature GCs along with a transgenic mouse with inducible silencing of its mature GCs, four complementary experimental groups were studied: mice with only mature, mice with only immature, mice with both or neither populations of GCs. We first observed that overall, these dentate manipulations had no effect on conditioning of a tone or white noise paired with shock; this was in accordance with what hippocampal manipulations have shown (Cushman et al., 2012; Quinn, Wied, and Fanselow, 2009). We also found that by manipulating the dentate population of GCs, we could drive different levels of fear generalization to a novel auditory cue. Further, in the control groups this generalization was greater when mice were trained with a tone and then tested with a white noise than vice versa. No other observations have previously been made that systematically manipulate the populations of GCs in the dentate using auditory conditioning,

although Cushman et al. (2012) used a genetic ablation method to measure auditory fear in mice with and without adult neurogenesis.

In a follow up experiment, we used two of the experimental groups mentioned above that either had all GCs intact or only immature GCs intact to measure generalization gradients around a pure tone CS+. We found that mice with intact immature GCs, showed a much steeper generalization curve around the CS+ compared to control mice, which froze the most to frequencies higher than the CS+. It was unexpected to find that control mice did not show a curve at all but more of a linear increased tendency to freeze at higher frequencies. Having only the mature cells silenced supported appropriate generalization. Mice with only immature GCs showed a normal level of freezing to the trained cue and less freezing as the test frequency moved away from the CS+. One possibility for this result may be the pattern separation function immature GCs have been hypothesized to support. For example, in previously published data with this particular transgenic line, experimenters show that when mature GCs are silenced, there is improved performance in a context discrimination training procedure (Nakashiba et al., 2012). Again, however, no previous findings have been reported on generalization gradients using tone fear and dentate GC manipulations making future experiments investigating this all the more important.

In chapter 3, using irradiation or a sham procedure in C57/BL6 mice we set out to investigate whether neurogenesis could influence discrimination training amongst two pure tones. This was, in part, an attempt to try and avoid the bias observed with the tone/white noise stimuli but also an attempt to see if this manipulation affects generalization after explicit discrimination training. Results showed that irradiation did not affect training at a statistical level, however, the group with the least difference in freezing between S+ and S- was the group

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of irradiated mice trained with a low frequency tone. Irradiated mice trained with a high frequency tone showed similar levels of performance as controls during training. At test, an effect was observed of irradiation treatment in combination with the tone used for training. Irradiated mice showed clear discrimination, but only when the trained cue was a high frequency tone. A follow up experiment using shocks only for training and high and low frequencies at test determined the asymmetry found in the latter experiment was not due to mere sensitization. It was important to rule this out in order to make any possible conclusions about the associative learning that was presumed to be occurring. Supporting this conclusion, it has also been reported that merely presenting high frequency tones to experimentally naive animals in the range of 4kHz-22kHz does not elicit any freezing (Bang et al., 2009). Finally, as described in chapter 4, we used genetic ablation of neurogenesis to show that although fear can be acquired to high and low frequencies of tones equally, high frequency conditioning resulted in better discrimination at test. Genetic ablation of post-natal neurogenesis reduced generalization to a novel tone, but this was only the case if the trained cue was a high frequency. These results contrast with a previous finding using the same manipulation of neurogenesis (Cushman et al., 2012), but training in that particular experiment was conducted with a white noise and a low frequency tone (2,800Hz). The group in the present experiment trained with low frequency displayed much more freezing to the novel cue at test, showing enhanced generalization that is in line with the previous finding mentioned (Cushman et al., 2012). Together, these results make it clear that neurogenesis in the dentate gyrus is not needed for fear acquisition but dysregulation of GC populations can have profound effects on fear expressed to novel auditory cues.

One of the unexpected and robust findings that arose and ultimately guided these experiments was the fact that separate auditory stimuli were able influence behavior at test in

such a way that training with one frequency vs another completely changed the fear response to untrained or unreinforced auditory stimuli. This effect could not be explained by sensitization and was sometimes present above any dentate/neurogenesis manipulation. This asymmetry is consistent with other researchers that have found similar differences in conditioning of different frequencies in rats. In a series of experiments, Bang et al. (2009) first trained rats with either a 19kHz or 4kHz discontinuous tone (pips) as a CS+. Following training, rats were tested with their respective CS+ as well as the other frequency. They found that when the 19kHz pip was used as the CS+ rats generalized much less to the 4kHz pip, but not the other way around. These findings are in support and similar to the high frequency effect we observed. In a separate experiment, the same investigators used either pips or continuous tones for conditioning and found more generalization to the unreinforced stimuli at test when a continuous tone was trained showing another type of bias in auditory fear conditioning. These findings suggest that the quality of auditory stimuli may point to an innate bias in the way these cues are treated such that some tones (generally, the high frequency tones) result in better discrimination performance when they are used for conditioning.

There are previous reports of other types of generalization that might be occurring that is not constrained to quality or characteristics of the stimulus itself but based on categories. In a set of experiments, investigators (Robinson, Whitt, and Jones, 2017) manipulated familiarity of a cue that was paired with shock and subsequently measured its effects on conditioned suppression. Two groups of rats were pre-exposed to either a clicker and a tone (group CT) or a tone only (group T). After the pre-exposure phase, both groups of rats received the same conditioning procedure in which the clicker was paired with foot shock. During the test phase, both groups were tested for suppression of responses upon presentation of the tone stimulus.

Results showed that generalization to the tone was greater for the group that had pre-exposure to both the conditioning and test stimulus as opposed to only the test stimulus. In addition, a follow up control experiment was able to rule out sensory preconditioning. Authors concluded that the generalization observed throughout their experiments was due to the how familiar or novel the test stimuli were. In other words, the rats were generalizing across a dimension not of stimulus frequency or loudness but familiarity. This type of generalized behavior observed towards categories of stimuli may have played a part in the current findings; however, it would only apply under conditions in which there is an association between categories of tone frequencies and shock because merely providing a shock and testing with two very different tones did not elicit a bias in responding.

It is possible that sound stimuli may have some preferred range that result in less generalization once they have been conditioned. Just as some have argued that the characteristics of stimuli should be pertinent to 'biological preparedness' or how some stimuli just by their nature have a higher probability of creating 'selective associations', (Domjan et al., 2004; Garcia and Koelling, 1966), it may be the case that high frequencies are easier to discriminate compared to low frequencies once conditioned. High and low frequencies used in the present experiments were equally likely to drive a fear response, this shows that high frequencies are not easier to condition, but once they have been conditioned show less generalization. Humans show preferential conditioning to stimuli that are more closely associated with naturally fearful stimuli such as pictures snakes as opposed to pictures of a colored square (Schell et al, 1991). Though this does not prove that asymmetries are present in humans for generalizing of stimuli, this may suggest that for humans, there could well be an innate bias in different types of stimuli that result in greater probability for generalization. The possible biases could help explain why there is such

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heterogeneity of symptoms among individuals with fear and anxiety disorders and why not all patients show the same levels of overgeneralization, hypervigilence, and other prominent hallmarks of these conditions.

In conclusion, the present set of experiments adds to the knowledge about how the hippocampus, and specifically the adult generated GCs contribute to aberrant fear after conditioning. Immature and mature granule cells changed the amount of generalization toward novel cues. Importantly, this effect was highly modulated by the nature of the conditional stimulus. Further experiments are needed to decipher how exactly these asymmetries arise and what are the exact neural mechanisms responsible.

Future Directions

One of the follow-up questions that may arise from the findings presented here is how the dentate is able to take what seems to be either already biased inputs or itself highlight a bias in stimuli to provide a code for hippocampus to process and guide behavior? What are the necessary and/or sufficient criteria that determine whether an animal will generalize appropriately or not? Given that the dentate is only a subfield within the hippocampus and has a variety of cell types, a first step in deciphering these types of questions would be to do an in depth analysis of how exactly the different-aged granule cells form part of the circuitry within the dentate and outside of the dentate. Previous studies have focused on inputs that first arrive at these newborn cells, but what are the first targets that projections are sent to and are these different when compared to embryonic GCs? A more complete picture of what all the developmental aspects of these cells are could help in the understanding of their functions. Another line of research that could illuminate the aforementioned questions could be purely behavioral. It is not clear from a handful of present studies how stimulus generalization observed

here in auditory fear conditioning can be explained in the context of classic learning theory when such biases are observed? Further experiments that utilize generalization gradients and discrimination training using auditory stimuli could help in defining what procedural specifications need to be present to detect a bias or maybe even aspects of procedures that can themselves provide an environment for biases to be displayed. Any knowledge that can be added to this area of research would be beneficial because it would ultimately lead to better and more informed investigations in the human populations.

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