Maladaptive fear and L-type voltage gated calcium channel subtypes

by

Stephanie A. J. Temme

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Doctoral Committee:

Associate Professor Geoffrey G. Murphy, chair
Professor Lori L. Isom
Professor Audrey F. Seasholtz
Professor Edward L. Stuenkel
Associate Professor Michael M. A. Sutton

“it is a truth very certain that, when it is not in our power to determine the most true opinions we ought to follow the most probable.”
― René Descartes

Dedication

This thesis is dedicated to the two loves of my life:

To my loving husband Jacob, your love and support made it all possible

and

To my beautiful daughter Catalina, may you never lose your spark of curiosity and your sense of joy at every discovery

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List of Abbreviations

ºC	celsius
129S1	129S1/SvImJ
129S6	129SvEvS6/Tac
129S6/B6	B57Bl/6, 129SvEv F2 hybrid
aCSF	artificial cerebral spinal fluid
AHP	afterhyperpolarization
AL	adjacent left
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP	action potential
AR	adjacent right
BA	basal amygdala
BLA	basolateral amygdala
BrdU	5-Bromodeoxyuridine
C57B6	C57BL/6NTac
CA1	*Cornu Ammonis* 1
CA2	*Cornu Ammonis* 2
CA3	*Cornu Ammonis* 3
CaMKII	Calmodulin Dependent Protein Kinase II

CEA	central amygdala
cKO	conditional knockout
DHP	dihydropyridine
DNA	deoxyribonucleic acid
EPSP	excitatory postsynaptic potential
EPSC	excitatory postsynaptic current
F1	filial 1
F2	filial 2
GAD	generalized anxiety disorder
GAD65	Glutamic Acid decarboxylase 65
GAD67	Glutamic Acid decarboxylase 67
h	hour
HES1	Hairy/Enhancer of split 1
HVA	high voltage activated
Id2	Inhibitor of DNA binding 2
IEI	inter-event interval
ip	intraperotoneil
IPSP	inhibitory postsynaptic potential
IPSC	inhibitory postsynaptic current
ISI	inter-spike interval
KO	knockout
LA	lateral amygdala
LTD	long term depression

LTP	long term potentiation
LVA	low voltage activated
LVGCC	L-type voltage gated calcium channel
MΩ	megaOhm
mA	milli-Ampere
mEPSC	miniature excitatory postsynaptic current
mGluR5	metabotropic glutamate receptor 5
mIPSC	miniature inhibitory postsynaptic current
ms	millisecond
mV	millivolt
NMDA	N-methyl-D-Aspartate
NVGCC	N-type voltage gated calcium channel
Opp	opposite
pA	pico-Amperes
PBS	phosphate buffered saline
PTSD	post-traumatic stress disorder
PVGCC	P-type voltage gated calcium channel
ROI	region of interest
RVGCC	R-type voltage gated calcium channels
Sec	second
sEPSC	spontaneous excitatory postsynaptic current
sIPSC	spontaneous inhibitory postsynaptic current
siRNA	small interfering ribonucleic acid

SSLP	simple sequence length polymorphism
Syn1	Synapin1a
VGCC	voltage gated calcium channel
SEM	standard error of the mean
TBS	tris buffered saline
TVGCC	T-type voltage gated calcium channel

Abstract

Fear learning can be adaptive and maladaptive. Learned fear to a harmful stimulus is adaptive and helps organisms survive in a given environment. However, learned fear can be maladaptive when it is persistent or when it is generalized to a non-threatening stimulus. These types of fears are often diagnosed as trauma or anxiety-related disorders in humans. Despite being the number one most common mental health disorder in the United States, very little is understood about the formation of these disorders and underlying maladaptive fears.

Studies have implicated a class of voltage-gated calcium channels (VGCCs), known as L-type VGCCs (LVGCCs), in both the formation and extinction of conditioned fear. Additionally, LVGCCs have been linked to changes in the neuronal plasticity in structures known to be involved in fear learning including the amygdala and the hippocampus. While it is clear that LVGCCs are involved with these forms of fear related learning, it is not clear to what extent LVGCCs and the two LVGCC neuronal subtypes, $Ca_V1.2$ and $Ca_V1.3$, mediate adaptive and maladaptive fears or the neurophysiology associated with fear. Understanding the contribution of these LVGCCs to maladaptive fear learning could provide insights into the neurobiological mechanism underlying trauma and anxiety-related disorders.

The goal of the research presented in this thesis was to investigate adaptive and maladaptive fear phenotypes, as well as explore the individual role of $Ca_V1.2$ and

$Ca_V1.3$ in fear related learning and physiology. Utilizing mice with a conditional knockout of $Ca_V1.2$ in neurons in the brain, mice with a global knockout of $Ca_V1.3$, and various pure-bred mouse strains and sub-strains, five main points are illustrated: 1) Two forms of maladaptive fear learning, persistent fear and generalized fear exist, 2) $Ca_V1.2$ mediates generalized fear, likely through the dentate gyrus and adult neurogenesis, 3) $Ca_V1.2$ mediates persistent fear, likely through alterations in the inhibitory/excitatory synaptic activity onto the amygdala. 4) Deletion of $Ca_V1.2$ alters neurophysiological correlates of learning in the amygdala, including intrinsic excitability and synaptic plasticity. 5) $Ca_V1.2$ appears to alter behavior and neurophysiology in a LVGCC subtype specific manner.

CHAPTER 1

INTRODUCTION

Learning is an important feature of human life. From birth to old age, we are constantly being bombarded with information about our environment and our experiences. While this information may seem unimportant and transient, our brains encode this information as memories for later recall. These memories in turn influence the way we live our lives and the way we view our world. While some memories may influence small factors in our lives, such as the title of a favorite book or where you parked your car, other memories can have a profound influence on our life. Evolutionarily, these memories are important because they influence our chances of survival and reproduction, such as the memory of an injury or a first love. These types of memories are often tied to emotion. In fact, learning and memories tied to strong emotions are often acquired rapidly and recalled easily, aiding in their adaptive nature (Burke, Heuer et al. 1992, Cahill and McGaugh 1998, Hamann, Ely et al. 1999). However, the acquisition and recall of emotional memory can quickly become problematic in the case of unwanted memories that interfere with daily life.

1.1 Adaptive and maladaptive emotional learning

Emotional learning, such as learned fear, is vital to human adaptation to various environments (Bracha 2006). Learning to fear a noxious stimulus is adaptive and helps individuals avoid injury and harm. However, learned fear can become maladaptive when it is generalized to a non-noxious stimulus or is persistent across time. In humans, maladaptive fear that interferes with an individual's quality of life is often diagnosed as trauma-related and anxiety-related disorders (McKay 2009, American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force. 2013). This group of disorders, which include generalized anxiety disorder, phobias, panic disorder and post-traumatic stress disorder, is the most common mental health illness in the United States (Anxiety and Depression Association of America, 2015) and has a 25% lifetime prevalence rate (Kessler, Berglund et al. 2005). Treatment for these disorders usually involve a combination of behavioral therapies and pharmacology. However, emotional memories are resilient to change and the passage of time making trauma-related and anxiety-related disorders difficult to treat behaviorally and prone to relapse (Bouton 1988, Rasmusson and Charney 1997, Gale, Anagnostaras et al. 2004).

1.1.1. Pavlovian conditioning

In 1927, Ivan Pavlov described a form of associative learning that could be easily explored and investigated in a laboratory setting (Pavlov and Anrep 1927). In this form of learning, now termed Pavlovian conditioning, two stimuli are paired so that one stimulus comes to predict the other. The ability of the one stimulus to predict the second stimulus is usually measured using a conditioned response. In Pavlov's experiments, he conditioned dogs to predict the arrival of food with an auditory cue, such as a bell, by repeated presentations of the bell and the food together. After pairing, the bell was

found to produce a salivary response in the dogs, a response normally elicited by food. Using the salivary response, Pavlov could evaluate the dogs association of the bell and the food. Fear learning is often studied in a laboratory setting using a form of Pavlovian conditioning known as Pavlovian fear conditioning (Maren 2001). In Pavlovian fear conditioning, a neutral stimulus, such as a context or a tone, is paired with a noxious unconditioned stimulus (US), such as a footshock, which naturally produces a fear response. After pairing, presentation of the neutral stimulus, now termed a conditioned stimulus (CS), alone is enough to drive a conditioned response (CR) of fear. While there are many ways to quantify this fear response, one of the most common ways is to evaluate the percent of time the animal spends freezing, or inactive except for respiration (Maren 2008). Though the CS is not inherently threatening, the mice form an associative memory between it and the noxious stimulus, with the CS as the predictor of the US, similar to Pavlov's dogs. Given that, in many cases, the only experience the animal has had with the CS in Pavlovian fear conditioning has been paired with a negative outcome, one can easily see how conditioned fear to this stimulus could be considered adaptive. While this type of associative fear learning is common in humans, and is often the source of fear to threatening stimuli, one of the clearest and well documented cases of conditioned fear in humans is the account of little Albert (Watson and Rayner 1920). In these studies, which occurred before the development of the Institutional Review Board (IRB) to review the ethical nature of human studies, an infant boy named Albert was conditioned to fear a small white rat by pairing the small white rat, the CS, with the sound of a hammer hitting a steel bar, the US. While initial presentation of the white rat alone did not induce a negative response in little Albert, the

loud noise, generated by the banging of a hammer on a steel bar, produced a strong fear response in young Albert. After pairing, presentation of the white rat alone elicited a fearful crying response in little Albert. As with the pairing of a context or tone with an aversive foot shock, Albert's experiences with the white rat demonstrate the adaptive and implicit nature of learned fear.

1.1.2 Fear Extinction

Learned fear to the CS can be reduced in a laboratory setting using fear extinction. In extinction, the CS is presented repeatedly in the absence of a US. After repeated presentations, the CS begins to exhibit a reduction in the fear response, or freezing. Importantly, fear extinction is not an erasure of the original fear memory, but the formation of a new memory in which the CS does not predict the negative outcome (Rescorla and Heth 1975, Rescorla 2001). This new extinction memory competes with the pre-existing fear memory to determine the animal's reaction to the CS. The existence of the original fear memory after extinction has been demonstrated in the laboratory setting through the observations of reinstatement, renewal, and spontaneous recovery in which the original fear memory out competes the extinction memory (Rescorla and Heth 1975, Bouton and King 1983, Rescorla 2004). In the case of reinstatement, presentation of the US, in the absence of the CS, is enough to produce a resurgence of the fear memory and a fear response to the CS (Rescorla and Heth 1975). In renewal, the fear memory to the CS reappears when the CS is presented in a different setting then that used for extinction, illustrating the contextual limitations of extinction (Bouton and King 1983). In spontaneous recovery, fear to the CS returns with the simple passage of time (Rescorla 2004). The ability to extinguish a fear memory is of

particular interest to researchers as a way to model behavioral therapies for the treatment of trauma and anxiety related disorders in humans.

1.1.3 Maladaptive Fear

Learned fear to a harmful stimulus, such as in Pavlovian fear conditioning is considered adaptive. Additionally, the ability to adjust ones representation and behavioral response to a given CS based on additional information, such as in fear extinction, is also considered adaptive (Milad, Rauch et al. 2006, Rauch, Shin et al. 2006).

Similar to adaptive fear, maladaptive fear, such as persistent fear or overgeneralized fear, can be evaluated in a laboratory setting. In the case of persistent fear, fear is considered maladaptive when it cannot be reduced easily through fear extinction (Milad, Rauch et al. 2006, Rauch, Shin et al. 2006). Given that the extinction memory competes with the fearful memory, this could be due to either a weak extinction memory or a stronger fear memory. A weak or strong memory could result from the ease or difficulty in the encoding or recall of the memory. While persistent fear can be described as a failure to extinguish, generalized fear can be seen as fear to a non-conditioned stimulus. For example, fear conditioning to a tone of a given frequency producing fear to both the original tone frequency, but also to another tone frequency. Generalization of fear was also seen in the case of little Albert (Watson and Rayner 1920). After being conditioned to fear a white rat, little Albert began to show fear to a variety of items with a furry appearance ranging from a white rabbit, Santa Claus mask and beard, to a fur coat. While some amount of generalization is probably adaptive,

such as the ability to generalize fear from a burn acquired from touching an oven to a fear of touching other objects that are hot, non-specific generalization is maladaptive, such as the generalization of fear to a hot oven producing fear to the kitchen in which the oven is located (Kheirbek, Klemenhagen et al. 2012). In this way, generalized fear can interfere with everyday life, leading to trauma and anxiety-related disorders. Similar to persistent fear, generalized fear could be produced through a variety of mechanisms. Generalized fear could be produced through an inability to cognitively differentiate between, or pattern separate, between two similar stimuli, or an increased state of fear and anxiety to novel or unfamiliar stimuli induced by fear conditioning.

1.2 Neurobiology of learning

Learning requires a variety of structures within the brain. The exact structures involved in learning are dependent on the type of memory formed and the information encoded.

1.2.1 The Hippocampus

Spatial and contextual information requires a structure in the brain known as the hippocampus. The hippocampus, which is made up of the hippocampus proper, entorhinal cortex, subiculum, and the dentate gyrus, is a highly organized structure that has been found to be conserved across a variety of species (Amaral and Witter 1989). In the hippocampus proper, projection neurons reside in a single cell body layer that can be subdivided into three regions: CA1, CA2, and CA3. In the dentate gyrus, projection neurons reside in the granular cell layer. Information in the hippocampus is thought to travel in a serial manner with information entering from the entorhinal cortex to the cell

bodies of the dentate gyrus via the perforant path (Andersen, Bliss et al. 1971). Information is then relayed from the dentate gyrus to the CA3 subregion of the hippocampus proper via the mossy fiber pathway, and from CA3 to CA1 via a pathway known as the Schaffer collaterals. Information from CA1 is then relayed to the subiculum where it sent on to other brain structures. Damage to, or removal of, the hippocampal structure in humans have been tied to deficits in memory formation (Grunthal 1947, Glees and Griffith 1952, Scoville and Milner 1957, Morris, Garrud et al. 1982, Nadel and Moscovitch 1997). Additionally, lesions of the hippocampus in animal models have been found to produce profound deficits in spatial learning and contextual coding (Zola-Morgan and Squire 1985, Squire and Cave 1991, Jarrard 1993, Alvarez, Zola-Morgan et al. 1995, Kessels, de Haan et al. 2001). Lesions of the hippocampus proper are also associated with a failure to fear condition to a context as well as extinguish fear to a context or tone (Phillips and LeDoux 1992, Maren, Aharonov et al. 1997, Corcoran and Maren 2001, Corcoran, Desmond et al. 2005).

The dentate gyrus has been tied to the ability to differentiate between two similar patterns or stimuli. In particular, the dentate gyrus is involved in the encoding and recall of memories in terms of previously encoded information via pattern completion and pattern separation (Gilbert, Kesner et al. 2001, Saxe, Battaglia et al. 2006, Hunsaker, Rosenberg et al. 2008, Clelland, Choi et al. 2009, Kheirbek, Klemenhagen et al. 2012, Nakashiba, Cushman et al. 2012). In terms of fear-related learning, the dentate gyrus has been tied to the ability to discriminate between two familiar stimuli, thereby reducing fear generalization (Saxe, Battaglia et al. 2006, Sahay, Scobie et al. 2011, Nakashiba, Cushman et al. 2012, Tronel, Belnoue et al.

2012). The dentate gyrus is believed to modulate these forms of learning and memory via the birth of adult-born neurons (Kheirbek, Klemenhagen et al. 2012). In fact, increases and decreases in neurogenesis within the dentate gyrus have been tied to increases and decreases in context discrimination, respectively (Saxe, Battaglia et al. 2006, Sahay, Scobie et al. 2011, Nakashiba, Cushman et al. 2012).

1.2.2 The Amygdala

In terms of emotional learning, including fear learning, memories are thought to rely on a structure of the brain known as the amygdala (Maren and Fanselow 1996, LeDoux 2000, Maren 2001). This heterogeneous structure consists of a variety of substructures including the lateral amygdala (LA) and basal amygdala (BA), often grouped together as the basolateral amygdala complex (BLA), and the central amygdala (CEA). Lesions of the amygdala, either BLA or CEA, have been found to produce significant impairments in fear learning as well as an inability to express previously learned fears (Sananes and Davis 1992, Cousens and Otto 1998, Maren 1999, Goosens and Maren 2001). Similar to the hippocampus, evidence suggests that information in the amygdala travels serially (Jimenez and Maren 2009, Orsini and Maren 2012). Information about our environment is relayed to the amygdala via brain structures including the cortex, hippocampus, and thalamus into the LA. From the LA, information is relayed to the BA and the CEA where it triggers an emotional response. While information in the hippocampus is thought to be relayed through excitatory projection neurons, information and activity in the amygdala is relayed via a variety of excitatory and inhibitory projection neurons vital for fear-related learning (Ehrlich, Humeau et al. 2009, Lee, Kim et al. 2013, Duvarci and Pare 2014). Additionally,

inhibitory interneurons are thought to play an important role in amygdala function by gating excitation within the BLA (Ehrlich, Humeau et al. 2009). In fact, removal of inhibition within the BLA has been found to produce an increase in the excitability of pyramidal cells within the BLA and an enhanced state of fear and anxiety leading to a generalization of fear in behaving animals (Shaban, Humeau et al. 2006, Bergado-Acosta, Sangha et al. 2008, Wiltgen, Godsil et al. 2009). Fear conditioning has also been associated with a decrease in inhibition within the amygdala while extinction produces enhancements (Stork, Ji et al. 2002, Lin, Mao et al. 2009, Wolff, Grundemann et al. 2014).

1.3 Neurophysiological Basis of learning

While human interest in learning and memory has existed since the early days of Descartes' discussion of a corporeal memory (Sutton 1998), it wasn't until the late 1800's and the scientific studies of Santiago Ramon y Cajal that we truly began to understand the biological underpinnings of learning (Ramón y Cajal and Azoulay 1894). Ramon y Cajal proposed that the brain was made up of individual cells that each served as a single unit of a larger picture. Furthermore, Ramon y Cajal believed that these units could communicate with each other through modifiable connection points and that the modification of these connections could provide the bases of memory storage. These theories and hypotheses, put forth by Ramon y Cajal over 100 years ago, still represent the foundation by which current research studies learning and memory.

Neurobiologically, learning is thought to occur through the repeated firing of neurons and neuronal networks within the brain, producing an enhancement in the

electrophysiological properties of the brain (Martin, Grimwood et al. 2000, Zhang and Linden 2003, Rudy 2014). This includes changes in the strength of the connections between neurons, known as synaptic plasticity (Martin, Grimwood et al. 2000), and changes in individual neuronal properties, known as intrinsic plasticity (Zhang and Linden 2003).

1.3.1 Synaptic Plasticity

In general, neurons communicate with each other through chemical signaling at the connection point between two neuron, known as a synapse. Activity of the presynaptic cell, usually in the form of an action potential, causes the opening of calcium channels at the presynaptic terminal and the release of neurotransmitter into the synapse. Neurotransmitter released from the presynaptic cell then binds to and activates ligand gated ion channels on the postsynaptic cell. Once these channels are open, charge carrying ions can enter the neuron and produce a change in the membrane potential of the postsynaptic cell. A depolarization in the membrane potential in response to synaptic activity is termed an excitatory postsynaptic potential (EPSP), while a hyperpolarization in the membrane potential is termed an inhibitory postsynaptic potential (IPSP). The ability of activity in the presynaptic cell to generate an EPSP or IPSP in postsynaptic cells is often studied as a measure of the synaptic strength between the two neurons. It is believed that the array of synapses and the strength of synapses within the brain can mediate the storage of memories (Barnes 1995, Stevens 1998, Neves, Cooke et al. 2008).

The ability to alter the strength of the synapse between two neurons is termed synaptic plasticity. In the late 1940s, a theory was proposed that if two neurons that were connected were active at the same time, the synapse between them would be strengthened (Hebb 1949). In the 1970s, a set of experiments were performed in the mammalian brain that demonstrated the strengthening of synaptic connections in response to stimulation and supported this theory (Bliss and Gardner-Medwin 1973, Bliss and Lomo 1973). Through stimulation of the axonal fibers from the entorhinal cortex and measurement of the resulting EPSP in granule cells in the dentate gyrus, experimenters were able to measure the synaptic strength between these two neuronal populations. After high frequency stimulation of the afferents from the entorhinal cortex, experimenters noted a substantial increase in the synaptic strength, or size of the EPSP, induced from single stimulations of these fibers. This type of increase in synaptic strength, termed long term potentiation (LTP) has since been found in a variety of structures throughout the brain in electrophysiological recordings *in vivo* (in alive animals) and *in vitro* (in tissue preparations from animals) (Lee 1982, Gerren and Weinberger 1983, Racine, Milgram et al. 1983, Patneau and Stripling 1992).

While studies have not been able to directly link LTP and synaptic plasticity with learning, the 1970s studies of the marine mollusk, *Aplysia*, supported the idea of changes in behavior in response to previous experience with external stimuli being associated with synaptic plasticity (Castellucci, Pinsker et al. 1970). This was done by looking at the neurobiology that linked the stimulation of the siphon of *Aplysia* with the defensive withdrawal of the animals gill. Using a dissected preparation of the animal, studies found that stimulation of the siphon produced action potential firing in the

associated sensory neuron and an EPSP in the motor neuron connected with the gill withdrawal reflex. After repeated stimulation of the siphon over a long period of time, a single stimulation of the siphon now produced a decreased gill withdrawal response, as well as a decreased EPSP in the motor neuron associated with the gill response. Since then, studies have found a variety of similarities in synaptic plasticity, in particular the induction of LTP, and the formation of memory, supporting the idea of synaptic plasticity as a cellular analog of learning. In particular, LTP and memory formation appear to rely on similar mechanisms, such as: dependence on calcium influx, gene regulation, and activation of calcium dependent second messenger systems (Goosens and Maren 2001, Baudry, Zhu et al. 2014).

The formation of LTP has now been studied in many brain regions associated with learning, including the hippocampus (Bliss and Collingridge 1993, Malenka 1994, Baudry, Zhu et al. 2014) and the amygdala (Goosens and Maren 2002, Maren 2005). Along with the studies by Bliss and Lomo which, demonstrated LTP in the perforant path within the hippocampus, LTP has been studied within the mossy fiber pathway (Hopkins and Johnston 1988, Jaffe and Johnston 1990, Weisskopf, Castillo et al. 1994) and the Schaffer collaterals (Korte, Carroll et al. 1995, Stella, Schweitzer et al. 1997). Correlations have also been found between the magnitude of the LTP seen in the perforant path and the ability of animals to acquire associative fear learning, supporting LTP as an analog of learning (Laroche, Doyere et al. 1989). Interestingly, avoidance learning in rats has also been found to produce increases in synaptic transmission in Schaffer collaterals and that this increase can occlude LTP, suggesting a similar mechanism between learning and LTP in this pathway (Whitlock, Heynen et al. 2006).

In the amygdala, studies have noted increases in the neuronal activity within the BLA in response to CS presentation after fear conditioning compared to CS presentation prior to conditioning, indicative of an increase in the synaptic connection between neurons carrying information about the CS and neurons in the BLA (Maren, Poremba et al. 1991, Quirk, Repa et al. 1995, Quirk, Armony et al. 1997, Collins and Pare 2000). Additionally, *in vitro* studies have demonstrated LTP induction in the lateral amygdala in response to high frequency stimulation of afferents from the cortex and hippocampus (Chapman, Kairiss et al. 1990, Bauer, LeDoux et al. 2001, McKinney, Sze et al. 2009) and the thalamus (Chapman and Bellavance 1992, Li, Phillips et al. 1995, Weisskopf, Bauer et al. 1999, Bauer, Schafe et al. 2002), structures associated with the processing of the CS and US.

1.3.2 Intrinsic Plasticity

In addition to synaptic forms of plasticity, long term changes in intrinsic excitability have also been tied to learning and memory (Daoudal and Debanne 2003, Zhang and Linden 2003). Intrinsic excitability is important for determining whether a neuron fires an action potential in response to an EPSP and at what rate it fires in response to depolarization (Henze and Buzsaki 2001).

The intrinsic excitability of a neuron is maintained by the regulation of ionic concentrations and gating of ionic currents within a cell, including sodium, potassium, and calcium, which shape how and when a neuron fires an action potential (Cohen 1973, Bean 2007). In particular, sodium is strongly associated with the action potential threshold and the rising phase of an action potential (Gilly and Armstrong 1984, Kole,

Ilschner et al. 2008). Potassium is thought to contribute to the falling phase of the action potential, the width of the action potential, as well as the afterhyperpolarization (AHP) after an action potential or burst of action potentials (Sah and McLachlan 1992, Kim, Wei et al. 2005). While calcium enters cells during the course of the action potential, it is not the influx of the positive charge on the calcium, but the activation of calcium activated potassium channels that is the means by which calcium is thought to shape excitability (Lancaster and Adams 1986). The ability of these ions to regulate intrinsic excitability is determined by the ability of these ions to flow into and out of the cell at different membrane potentials. The conductance, or flow, of these ions during neuronal activity is heavily regulated by voltage-gated, and in the case of potassium, calcium-gated, ion channels. The frequency at which cells can fire action potentials is mediated by components of the action potential shape, membrane repolarization, and the balance of inward and outward currents that bring the membrane potential close to threshold (Bean 2007). Changes in intrinsic excitability, or intrinsic plasticity, is thought to be mediated by changes in the conductance of ions, in particular via changes in ion channels that mediate the shape and rate of action potential firing (Daoudal and Debanne 2003). Decreases in action potential threshold can result in an increased probability of action potential firing in response to EPSPs while decreases in action potential afterhyperpolarization could allow for an increased number of action potentials over a given period of depolarization.

Alterations in intrinsic excitability can influence the probability of synaptic plasticity by mediating a neuron's capability of translating an EPSP into an action potential. Additionally, alterations in intrinsic excitability, such as the shape of an

action potential can influence synaptic plasticity by altering the degree of neurotransmitter released onto postsynaptic cells (Borst and Sakmann 1999, Kress and Mennerick 2009).

Similar to studies of synaptic plasticity, early evidence for intrinsic plasticity in response to learning was found in a marine mollusk, in this case, *Hermissenda* (Crow and Alkon 1978, Alkon, Shoukimas et al. 1982, Alkon 1984, Alkon, Farley et al. 1984, Connor and Alkon 1984). Like other mollusks, *Hermissenda* exhibit positive phototaxis, or movement toward light. When phototaxic responses were paired with manual rotation of the animal, *Hermissenda* display long term changes in their response to light, namely an increase in the latency between light onset and the movement of the animal (Crow and Alkon 1978). This associative behavior was also seen as a change in the neuronal connections associated with phototaxis with a change in the potassium (Alkon, Shoukimas et al. 1982) and calcium (Alkon 1984) currents in response to light onset and phototaxic responses alone than prior to pairing. Intrinsic plasticity has also been noted in mammals (Brons and Woody 1980, Franklin, Fickbohm et al. 1992, Li, Jia et al. 1996, Moyer, Thompson et al. 1996, Oh, Kuo et al. 2003, Ohno, Sametsky et al. 2006, Sehgal, Ehlers et al. 2014). When an auditory click was paired with a glabella tap in cats, the pairing produced a conditioned eyeblink and nose twitch response to the click alone. Measurements of intrinsic excitability of the neurons associated with the conditioned response found a decrease in the current required to produce an action potential after the association (Brons and Woody 1980). Along with changes in action potential threshold, several studies have correlated changes in the action potential AHP, which can mediate the firing rate of neurons, with hippocampal-dependent forms of

learning (Moyer, Thompson et al. 1996, Oh, Kuo et al. 2003, Ohno, Sametsky et al. 2006). Additionally, changes in intrinsic excitability, including decreased AHP and increased neuronal firing have been noted in the LA in rats who underwent fear conditioning compared to rats who did not undergo fear conditioning (Sehgal, Ehlers et al. 2014).

1.4 Calcium as a neuronal modulator

Calcium is vital to the regulation of neuronal activity, including intrinsic excitability and synaptic activity (Berridge 1998, Raymond and Redman 2006, Clapham 2007, Burgoyne and Haynes 2014). While calcium lies in abundance extracellularly, intracellular levels of calcium in the cytoplasm, or free calcium, are minimal and tightly maintained. This tight regulation of free calcium means that changes in intracellular free calcium in the neuron can produce significant alterations in neuronal response in a time and location dependent manner. Small changes in free calcium concentration are more likely to affect neuronal function through localized and short lived methods, such as the effect of calcium on neurotransmitter release. In contrast, larger and longer lasting changes in free calcium concentration can alter neuronal function on a larger time scale and can even alter transcription and translation through activation of calcium dependent second messenger systems (Murphy, Worley et al. 1991, Dolmetsch, Pajvani et al. 2001).

1.4.1 Voltage gated calcium channels

One important regulator of calcium flow within neurons is voltage gated calcium channels (VGCCs) (Zamponi 2005). Voltage gated calcium channels open in

response to membrane depolarization and are selectively permeable to calcium. In this way VGCCs time the action of calcium within neurons with neuronal activity. Initial studies of VGCCs divided these channels into two types: low-voltage activated (LVA) and high-voltage activated (HVA) (termed I and II at the time) based on the membrane potential at which the channels open (Hagiwara, Ozawa et al. 1975). Additionally, LVA channels were found to deactivate at a slower rate and show decreased levels of current run down compared to HVA channels. Further investigation of VGCCs identified several classes of VGCCs based on the size of their currents, measured using barium, and their sensitivity to various agonists and antagonists (Catterall, Perez-Reyes et al. 2005). These VGCC classes include: T-type, N-type, P/Q-type, R-type, and L-type calcium channels. T-type calcium channels (TVGCCs) were named for their tiny and transient Barium currents (Nowycky, Fox et al. 1985). N-type calcium channels (NVGCCs) were named for their specificity to neuronal populations and their intermediate barium currents (Nowycky, Fox et al. 1985). P/Q-type voltage gated calcium channels (PVGCCs) were identified based on their localization in Purkinje neurons in the cerebellum (Llinas, Sugimori et al. 1989). R-type voltage gated calcium channels (RVGCCs) were named because they were resistant to the known agonists and antagonists at the time (Cribbs, Lee et al. 1998). Finally, L-type voltage gated calcium channels (LVGCCs) were named for their large and long lasting barium currents and their sensitivity to a class of drugs known as dihydropyridines (DHPs) (Nowycky, Fox et al. 1985). While TVGCCs were categorized as low voltage activated, all the other classes, including LVGCCs, were identified as high voltage activated. Along with being

high voltage activated, these VGCC classes exhibited calcium and voltage-dependent inactivation (Imredy and Yue 1994, Xu and Lipscombe 2001).

Voltage gated calcium channels within the brain are composed of a variety of subunits including an alpha pore forming subunit and a set of auxiliary subunits including alpha2, gamma, delta, and beta (Catterall, de Jongh et al. 1993). While the auxiliary subunits help mediate various channel characteristics, the alpha pore forming subunit regulates the voltage sensitivity of the channel, the basic channel kinetics, and contains binding sites for various channel modulators, including regulation by agonists and antagonists (Catterall 2000). Identification of VGCCs based on their pore forming subunits has resulted in the identification of a variety of subtypes of calcium channels for each VGCC class. Identification of L-type voltage gated calcium channels based on their alpha pore forming subunits lead to the identification of 4 different L-type voltage gated calcium channels, $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$ (Ertel, Campbell et al. 2000). Of these subtypes, $Ca_V1.1$ and $Ca_V1.4$ exhibit limited expression patterns with $Ca_V1.1$ having only been found in skeletal muscles (Kugler, Weiss et al. 2004) and $Ca_V1.4$ only found in the retina (McRory, Hamid et al. 2004). In contrast, $Ca_V1.2$ and $Ca_V1.3$ have been found in a variety of tissues throughout the body including cardiac, smooth muscle, skeletal, and, in particular, $Ca_V1.2$ and $Ca_V1.3$ have been found to be heavily expressed in the brain (Hell, Westenbroek et al. 1993, Sinnegger-Brauns, Huber et al. 2009).

Recent investigation into the function of the two neuronal LVGCC subtypes, $Ca_V1.2$ and $Ca_V1.3$, demonstrated substantial differences between these two channels including: activation voltage, inactivation, sensitivity to DHPs, and neuronal

distribution. (Hell, Westenbroek et al. 1993, Koschak, Reimer et al. 2001, Xu and Lipscombe 2001, Lipscombe, Helton et al. 2004). Investigation of $Ca_V1.2$ and $Ca_V1.3$ in an *in vitro* cell line revealed activation of $Ca_V1.3$ channels at significantly more hyperpolarized potentials than $Ca_V1.2$, with an activation voltage of around -55mV in $Ca_V1.3$ and around -35mV in $Ca_V1.2$ (Koschak, Reimer et al. 2001, Xu and Lipscombe 2001). These results were supported by *ex vivo* recordings from $Ca_V1.3$ knockout mice in which low-threshold activating calcium currents in hair cells were attenuated in the absence of $Ca_V1.3$ (Zhang, Xu et al. 2002). In addition, differences have been noted in activation and inactivation kinetics between the two subtypes with $Ca_V1.3$ activating more rapidly and inactivating slower than $Ca_V1.2$ (Koschak, Reimer et al. 2001). Examination of the effects of LVGCC agonists and antagonists on $Ca_V1.2$ and $Ca_V1.3$ in *in vitro* cell lines also revealed a stronger sensitivity of $Ca_V1.2$ to DHPs (Koschak, Reimer et al. 2001, Xu and Lipscombe 2001) , with $Ca_V1.3$ requiring up to 5 times the concentration of nifedipine and nitrendipine to produce inhibition at the level of $Ca_V1.2$ (Sinnegger-Brauns, Huber et al. 2009). However, as $Ca_V1.3$ was found to be inhibited at up to 50% with >90% blockade of $Ca_V1.2$ with DHPs, these drugs cannot be considered selective for $Ca_V1.2$ (Xu and Lipscombe 2001).

Using radioreceptor assays with the dihydropyridine, isradipine, it was found that $Ca_V1.2$ makes up roughly 89% of the LVGCC population within the brain with $Ca_V1.3$ making up the remainder (Sinnegger-Brauns, Huber et al. 2009). Though studies suggest that both $Ca_V1.2$ and $Ca_V1.3$ are heavily expressed in the hippocampus, cortex, and the cerebellum, as well as other brain structures, immunohistological investigation found differences in the subcellular distribution of these two LVGCC subtypes within

these structures. While $Ca_V1.2$ was found to be expressed in the cell body and proximal dendrites with a uniform distribution, staining for $Ca_V1.3$ was found to be localized mainly in the cell body with a punctated expression, indicative of high density clusters of $Ca_V1.3$ channels (Westenbroek, Ahlijanian et al. 1990, Hell, Westenbroek et al. 1993). Similar subcellular localization of $Ca_V1.2$ channels were seen in pyramidal cells in the basolateral amygdala as in the hippocampus, with small levels of $Ca_V1.2$ expressed in inhibitory cells within this structure (Pinard, Mascagni et al. 2005).

1.4.2 L-type voltage gated calcium channels in learning and neurobiology

Given the substantial differences in the function and kinetics of $Ca_V1.2$ and $Ca_V1.3$, it is believed that these LVGCC subtypes likely have differential roles in neuronal regulation and learning and memory. L-type voltage gated calcium channels have been implicated in learning and memory and neuronal physiology both as a class, using pharmacological antagonists, and as LVGCC subtypes $Ca_V1.2$ and $Ca_V1.3$, using subtype specific knockout or conditional knockout mice (**Table 1**) (Striessnig, Koschak et al. 2006, Berger and Bartsch 2014). Of particular interest in fear related learning is the role of the LVGCCs in the hippocampus and the amygdala.

1.4.2.1 L-type voltage gated calcium channels in Neurophysiology

Physiologically, L-type voltage gated calcium channels have been linked to LTP within the hippocampus and the amygdala. The addition of nifedipine blocked an NMDA-independent form of LTP in CA1 in response to stimulation of the Schaffer collaterals (Grover and Teyler 1990). In addition, LVGCCs have been found to increase the activity dependent transcription of calcium dependent genes in CA1 neurons which

may mediate this form of LTP (Murphy, Worley et al. 1991, West, Chen et al. 2001) Similar studies using a $Ca_V1.2$ conditional knockout mouse found that deletion of $Ca_V1.2$ in excitatory neurons in the forebrain inhibited NMDA-independent LTP induction at Schaffer collateral/CA1 synapses (Moosmang, Haider et al. 2005). Inhibition of LVGCCs using nimodipine also blocked the induction of NMDA-dependent LTP at the mossy fiber terminals using a brief- high frequency tetanic stimulation (Kapur, Yeckel et al. 1998) However, the same study did not find an effect of nimodipine on LTP induced using a long tetanic stimulation. Additional studies of the mossy fiber pathway found LVGCC mediated phosphorylation of CREB and CRE-dependent gene transcription which have been linked to LTP formation (Impey, Mark et al. 1996, Dolmetsch, Pajvani et al. 2001).

Similar to the hippocampus, LVGCCs in the amygdala have been linked to LTP induction in a variety of pathways. In the thalamo-amygdala pathway, pharmacological inhibition of LVGCCs was found to impair NMDA-independent LTP induced by paired stimulation (Weisskopf, Bauer et al. 1999, Bauer, Schafe et al. 2002, Humeau, Shaban et al. 2003). In the cortico-amygdala pathway, blockade of LVGCCs also inhibited the formation of NMDA-independent LTP (Humeau, Shaban et al. 2003, Schroeder and Shinnick-Gallagher 2004, Schroeder and Packard 2004, Drephal, Schubert et al. 2006, Fourcaudot, Gambino et al. 2009). Further studies found that deletion of $Ca_V1.3$ produced impairments in the cortico-amygdala pathway (McKinney, Sze et al. 2009).

In addition to LTP, LVGCCs have been linked to intrinsic excitability within these structures. Several studies have noted significant decreases in the AHP in the hippocampus, in particular the slow component of the AHP in response to LVGCC

blockade (Rascol, Potier et al. 1991, Marrion and Tavalin 1998, Shah and Haylett 2000, Power, Wu et al. 2002, Lima and Marrion 2007). Further studies have found a role of $Ca_V1.3$, but not $Ca_V1.2$ on the AHP within the hippocampus, as well as neuronal firing rates (Gamelli, McKinney et al. 2011). Studies have also tied the regulation of the AHP and neuronal firing in the amygdala to the function of VGCCs (Washburn and Moises 1992, Faber, Callister et al. 2001, Faber and Sah 2002). These studies in the amygdala did not investigate the role of LVGCCs specifically. However, deletion of $Ca_V1.3$ was found to produce a significant decreases in the slow AHP recorded from pyramidal neurons in the LA (McKinney, Sze et al. 2009). While some studies have not found an effect of conditional deletion of $Ca_V1.2$ on intrinsic excitability in the LA (Langwieser, Christel et al. 2010), detailed investigation of the role of $Ca_V1.2$ in amygdala intrinsic excitability has not been done.

1.4.2.2 L-type voltage gated calcium channels in learning and behavior

Behaviorally, LVGCCs have been tied to both hippocampal dependent and amygdala dependent learning tasks. Intra-amygdala infusions of LVGCC antagonists were found to block fear conditioning to a context or a cue (Bauer, Schafe et al. 2002) and intra-amygdala infusions (Davis and Bauer 2012), or systemic infusions (Cain, Blouin et al. 2002, Suzuki, Josselyn et al. 2004, Busquet, Hetzenauer et al. 2008) were found to block fear extinction. Though it is important to note that systemic injections of LVGCC antagonists have been found to produce non-associative freezing behavior, most likely due to the effects of blockade of LVGCCs in the peripheral nervous system (McKinney, Sze et al. 2008). Exploration of the contribution of $Ca_V1.2$ and $Ca_V1.3$ found a role of $Ca_V1.3$ but not $Ca_V1.2$ in fear consolidation and but did not find a role of

either subtype in fear extinction (McKinney and Murphy 2006, McKinney, Sze et al. 2008). However, previous studies have only investigated the effects of deletion of $Ca_V1.2$ in glutamatergic neurons in the forebrain in fear learning and extinction and therefore may be discounting a role of $Ca_V1.2$ in other neuronal populations.

Studies investigating the role of LVGCCs in terms of hippocampal-dependent learning have reported mixed results. While some studies have implicated $Ca_V1.2$ in spatial learning (Moosmang, Haider et al. 2005), with deficits in the labyrinth maze and a visible platform version of the Morris water maze, additional studies have failed to find deficits in the classic Morris water maze in a $Ca_V1.2$ conditional knockout mouse with deletion in glutamatergic neurons in the forebrain (White, McKinney et al. 2008). However, deficits in remote spatial memory, ~30 days after learning, were noted in these mice. Studies have not supported a role of $Ca_V1.3$ in hippocampal dependent learning with normal spatial learning in the Morris water maze despite deficits in fear learning, suggesting that these deficits in fear learning are likely not due to a loss of $Ca_V1.3$ in the hippocampus (McKinney and Murphy 2006).

1.4.2.3 L-type voltage gated calcium channels in gene regulation and neurogenesis

Gene regulation is believed to be important for both learning and the neuronal plasticity. Similar to studies of LVGCCs in neurophysiology and behavior, LVGCCs as a class and $Ca_V1.2$ and $Ca_V1.3$ as LVGCC subtypes have been linked to gene transcription and translation (Sheng, McFadden et al. 1990, Graef, Mermelstein et al. 1999, Mao and Wiedmann 1999, Weick, Groth et al. 2003, Gomez-Ospina, Tsuruta et al. 2006, Lu, Sirish et al. 2015). Studies of LVGCCs have found that calcium influx

through these channels can regulate gene expression through the activation of transcription factors within the nucleus (Sheng, McFadden et al. 1990, Graef, Mermelstein et al. 1999, Mao and Wiedmann 1999, Weick, Groth et al. 2003). Calcium influx through LVGCCs is thought to regulate these factors via diffusion of calcium to the nucleus (Hardingham, Arnold et al. 2001) or through activation of calcium dependent second messenger proteins located near the channel pore (Deisseroth, Heist et al. 1998, Dolmetsch, Pajvani et al. 2001). Studies have also identified portions of the C-terminal domain of the alpha pore forming subunits, Cav1.2 (Gomez-Ospina, Tsuruta et al. 2006) and Cav1.3 (Lu, Sirish et al. 2015) that, when cleaved, appear to translocate into the nucleus and function as a transcription factors.

Among the various genes found to be regulated by calcium influx through LVGCCs is a set of genes associated with cell fate determination including: HES1, Id2, and NeuroD (Deisseroth, Singla et al. 2004). Perhaps related to the role of LVGCC in the regulation of these genes, LVGCCs have been implicated in cell proliferation and neurogenesis embryonically (D'Ascenzo, Piacentini et al. 2006, Piacentini, Ripoli et al. 2008, Brustein, Cote et al. 2013) and into adulthood (Deisseroth, Singla et al. 2004, Luo, Zhu et al. 2005, Zhu, Zhou et al. 2012).

1.4.3 $Ca_V1.2$ voltage gated calcium channels in disease states

Along with their role in learning and memory, LVGCCs have been linked to a variety of human pathologies and psychiatric conditions (Casamassima, Hay et al. 2010, Bhat, Dao et al. 2012). Recent genome-wide association studies have found links between several types of polymorphisms in the gene *CACNA1C,* that encodes $Ca_V1.2$,

and psychiatric disorders. These psychiatric disorders include bipolar disorder (Ferreira, O'Donovan et al. 2008, Sklar, Smoller et al. 2008, Casamassima, Huang et al. 2010), major depression (Sullivan, de Geus et al. 2009, Green, Grozeva et al. 2010), and schizophrenia (Green, Grozeva et al. 2010). Mutations in *CACNA1C* have also been linked with a condition known as Timothy syndrome in which patients present with cognitive deficits thought to be due to altered neuronal physiology (Splawski, Timothy et al. 2004, Barrett and Tsien 2008, Pasca, Portmann et al. 2011, Krey, Pasca et al. 2013). Influx of calcium through and the regulation of LVGCCs have also been linked with human cognition, with increases in calcium via LVGCCs associated with decreases in age-related cognitive abilities and age-related changes in neuronal function (Thibault and Landfield 1996, Norris, Halpain et al. 1998, Shankar, Teyler et al. 1998, Veng, Mesches et al. 2003).

1.5 Aims

Given this body of literature, it is clear that LVGCCs have a complex role in learning and memory, as well as psychiatric and neuronal pathologies in humans. Despite significant pharmacological studies investigating the role of LVGCCs in the brain, very little is understood about the role of individual LVGCC subtypes, $Ca_V1.2$ and $Ca_V1.3$, in various structures and functions in the brain (**Table 1**). Understanding the role of these two subtypes in neuronal function and behavior could provide important insights into both basal learning and memory, as well as the potential effects mutations in these channels have on the brain, such as those seen in psychiatric conditions.

This thesis explores the role of LVGCCs in neuronal function and learning associated with basal fear learning, as well as maladaptive fear learning more indicative of psychiatric disorders such as trauma and anxiety-related disorders. Particular emphasis will be placed on the role of $Ca_V1.2$ in these forms of learning given its recent ties to various psychiatric conditions. In chapters two through five I will explore the formation of adaptive and maladaptive fear in a laboratory and the neurobiology that may underlie the formation of these fears in mice

- In Chapter two I explore the expression of adaptive and maladaptive fear in a laboratory setting using two sub-strains of the commonly used 129 mouse inbred strain: 129S1 and 129S6. Mice are assessed for changes in fear extinction and the ability to discriminate between similar contexts.
- In Chapter three I assess the role of LVGCC subtypes $Ca_V1.2$ and $Ca_V1.3$ in the overgeneralization of fear, measured as changes in context discrimination, and in simple versus complex hippocampal dependent tasks which could be indicative of deficits in dentate gyrus function. I also assess the effects of neuronal deletion of $Ca_V1.2$ on cell proliferation and neurogenesis in the dentate gyrus, which have been linked to context discrimination.
- In Chapter four I assess the role of LVGCC subtype $Ca_V1.2$ in persistent fear, measured as a deficit in fear extinction. I also assess the balance of inhibitory and excitatory synaptic activity onto the amygdala which could alter amygdala function and fear expression.

- In Chapter five I assess the effects of neuronal deletion of $Ca_V1.2$ on the intrinsic excitability of pyramidal cells in the lateral amygdala, including basal membrane properties, action potential properties, AHP, and repetitive firing. I also assess the effects of deletion of $Ca_V1.2$ and $Ca_V1.3$ on synaptic plasticity in the lateral amygdala, specifically long term potentiation in the thalamo-amygdala pathway.

Through these studies five main points are illustrated: 1) Two forms of maladaptive fear learning, persistent fear and generalized fear exist, 2) $Ca_V1.2$ mediates generalized fear, likely through the dentate gyrus and adult neurogenesis, 3) $Ca_V1.2$ mediates persistent fear, likely through alterations in the inhibitory/excitatory synaptic activity onto the amygdala. 4) Deletion of $Ca_V1.2$ alters neurophysiological correlates of learning in the amygdala, including intrinsic excitability and synaptic plasticity. 5) $Ca_V1.2$ appears to alter behavior and neurophysiology in a LVGCC subtype specific manner.

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CHAPTER 2

COMPARISON OF INBRED MOUSE SUBSTRAINS REVEALS SEGREGATION OF MALADAPTIVE FEAR PHENOTYPES

2.1 Summary

Maladaptive fear, such as fear that is persistent or easily generalized to a nonthreatening stimuli, is associated with anxiety-related disorders in humans. In the laboratory, maladaptive fear can be modeled in rodents using Pavlovian fear conditioning. Recently, an inbred mouse strain known as 129S1/SvImJ, or 129S1 have been reported as exhibiting impairments in fear extinction and enhanced fear generalization. With a long-term goal of identifying segregating genetic markers of maladaptive fear, we used Pavlovian fear conditioning to characterize a closely related substrain designated as 129S6/SvEvTac, or 129S6. Here we report that, like 129S1 animals, 129S6 mice exhibit appropriate levels of fear upon conditioning, but are unable to extinguish fear memories once they are consolidated. Importantly, the maladaptive fear phenotype in this inbred stain can be segregated by sub-strain when probed using conditioning protocols designed to assess generalized fear. We find that unlike the 129S1 substrain, mice from the 129S6 sub-strain do not generalize conditioned fear to previously novel contexts and can learn to discriminate between two similar contexts when trained using a discrimination protocol. These results suggest that at least two

forms of maladaptive fear (deficits in fear extinction and fear generalization) can be can be functionally segregated, further suggesting that the underlying neurobiology is heritable. Given the observation that two closely related sub-strains can exhibit different constellations of maladaptive fear suggests that these findings could be exploited to facilitate the identification of candidate genes for anxiety-related disorders.

2.2 Introduction

Fear can be both adaptive and maladaptive (Bracha 2006). While adaptive fear helps protect against injury or death, maladaptive fear often result in anxiety-related and trauma-related disorders, such as post-traumatic stress disorder (PTSD) (Bracha 2006).

In a laboratory setting, fear is often studied through Pavlovian fear conditioning, in which a neutral conditioned stimulus (CS), such as a context or a tone, is paired with an aversive unconditioned stimulus (US), such as a footshock (Maren 2005, Milad, Rauch et al. 2006, Orsini and Maren 2012). Following paired presentations of the CS and US, the previously neutral CS alone is enough to produce a fear response, often quantified as freezing, or inactivity of the animal except for that required for respiration. Freezing to a conditioned stimulus can be subsequently reduced through Pavlovian fear extinction, in which the CS is presented multiple times in the absence of the US. It is generally accepted that the extinction of conditioned fear represents the learning of a new association and not the erasure of the original fear memory (Rescorla and Heth 1975, Bouton and King 1983, Rescorla 2001) Under normal conditions, fear extinction learning is viewed as adaptive (Milad, Rauch et al. 2006, Rauch, Shin et al. 2006). Conversely, deficits in fear extinction are considered to be maladaptive and may be

related to anxiety-related and trauma-related disorders (Milad, Rauch et al. 2006, Rauch, Shin et al. 2006).

In an attempt to identify genetic components that may modulate maladaptive fear learning and anxiety-related disorders, previous studies have compared various inbred mouse lines for abnormalities in Pavlovian fear conditioning or extinction (Trullas and Skolnick 1993, Crawley, Belknap et al. 1997, Bolivar, Pooler et al. 2001, Holmes, Wrenn et al. 2002, Balogh and Wehner 2003, Bothe, Bolivar et al. 2004, Hefner, Whittle et al. 2008, Camp, Norcross et al. 2009, Wilkinson, Turner et al. 2013). In these studies, the C57B6 strain is considered to exhibit "normal" fear learning and extinction. Studies examining the 129 inbred strain have found that the 129S1 substrain acquires and consolidates fear memories (Bolivar, Pooler et al. 2001, Hefner, Whittle et al. 2008), but is unable to extinguish fear of a previously conditioned stimulus when compared to the C57B6 inbred strain (Hefner, Whittle et al. 2008). Further studies of the 129S1 substrain found that, once conditioned to fear a context, 129S1 mice over-generalized their fear to non-conditioned contexts relative to C57B6 mice (Camp, Macpherson et al. 2012). Based on these studies, genetic comparison of 129S1 and C57B6, using techniques such as DNA microarrays could result in a list of genes that mediate maladaptive fear. However, significant genetic diversity between the 129S1 and C57B6 inbred strains make the identification of these genes difficult. If differences in maladaptive fear could be found between two more genetically similar substrains, such as 129S1 and 129S6 mice (Simpson, Linder et al. 1997), the selection of candidate genes contributing to the 129S1 phenotype could be significantly facilitated. While a previous study has demonstrated that additional 129 substrains exhibit similar deficits in

extinction (Camp, Norcross et al. 2009), little else is known about the behavior and neurobiological differences between 129 substrains. In particular, it remains unknown whether the commonly used 129S6 substrain exhibits similar maladaptive fear. Based on the development of the 129 lineages and substrains, the genetic variation between the 129S1 and 129S6 should be significantly reduced compared to C57B6 and other inbred mouse strains (Simpson, Linder et al. 1997, Threadgill, Yee et al. 1997).

To determine whether 129S6 mice exhibit maladaptive fear, we fear conditioned 129S6 mice to a context or tone and compared their levels of fear consolidation, fear extinction, and fear generalization to C57B6 mice. In addition, we directly compared 129S1 and 129S6 in context discrimination and generalization.

The results from these studies suggest that the 129S6 and 129S1 substrains share some aspects of maladaptive fear and not others. While the 129S6 substrain conditions to fear normally, mice in this substrain are unable to extinguish this conditioned fear, similar to the previous published 129S1 mice. However, the 129S6 did not exhibit aberrant fear generalization or context discrimination while 129S1 mice do, illustrating key differences in maladaptive fear between the two strains. Based on these studies, we conclude that deficits in fear extinction and fear generalization/discrimination likely represent independent forms of maladaptive fear. In this way, further comparison of 129S1 and 129S6 mice may help shed light on the genetic underpinnings of these psychiatric disease states. Additionally, this divergence in forms of maladaptive fear may be important for understanding the development and maintenance of anxiety-related disorders.

2.3 Materials and Methods

2.3.1 Mice

All mice were either obtained from their respective vendors or bred within our colony using naïve mice from the same vendors. The 129SvEvS6/Tac and C57BL/6NTac mice were obtained from Taconic Farms (Hudson, NY) and 129S1/SvImJ mice were obtained from Jackson Laboratories (Bar Harbor ME) and are referred to hereafter as 129S6, C57B6, and 129S1, respectively. To obtain B57Bl/6:129SvEv hybrid mice with an F2 genetic background, 129S6 mice were first breed to C57B6 mice to produce an F1 B57Bl/6:129SvEv cross. Non-sibling mice from the F1 cross were then breed to produce the F2 B57Bl/6:129SvEv hybrid mice used for experimentation. Hybrid B57Bl/6:129SvEv are referred to hereafter as 129S6/B6.

Studies were conducted using mice aged 3-6 months at the time of testing with approximately equal numbers of males and females. All mice were housed by sex in groups of two to five. Mice were maintained in micro-isolation cages with a 14-h/10-h light/dark cycle for a minimum of one week prior to behavioral studies. The average ambient temperature was 22°C and mice were provided with ad libitum food and water. All experiments were conducted according to the National Institute of Health guidelines for animal care and were approved by the University Committee on the Use and Care of Animals of the University of Michigan.

2.3.2 Behavioral Procedures

2.3.2.1 Conditioning Apparatus and Contexts:

All experiments were conducted in fear conditioning chambers with clear acrylic backs and doors, aluminum sides, stainless steel grid floors spaced 1/8 inches, and stainless steel drop pans (Med Associates). Shocks were administered through the grid via solid-state shock scramblers and electronic constant-current shock sources controlled by a desktop PC running Actimetrics Freezeframe software (Wilmette, IL). The same computer and software were used to record behavior which was digitized using individual cameras mounted above each chamber. Individual chamber details and room lighting were altered to create three experimental contexts termed "same, A", "similar, B", and "different, C". Context A was created using the basic conditioning chamber described above and white room lights set at 150 watts. Chambers and floor pans were cleaned with 70% ethanol to provide a distinct background odor. Context B was identical to context A with the addition of rubber speckled floor coverings over smooth acrylic coverings to hide grid floors. Context C included smooth opaque white acrylic coverings over the floor and walls which produced the appearance of a semicircular chamber. The chamber and floor pans in context C were cleaned with 2% acetic acid and red room lights at 60 watts were used. For experimental sessions using contexts A or B, mice were transferred to a holding room prior to the beginning of the session. For context C, mice were transferred directly from their housing room to the experimental set-up at the start of the session. In all contexts, freezing was defined as a lack of motion, except that required for respiration, for one second or more and was calculated using a sensitive global motion-detection algorithm (FreezeFrame and FreezeView software; Actimetrics; Wilmette, IL)

2.3.2.2 Protocols

Context Protocols

During context conditioning, mice were trained for three days using one training session per day. Throughout training, mice were placed in context A in individual conditioning chambers. Each training session was composed of three minutes of baseline activity in context A followed by three unsignaled footshock (2 seconds) with 30 second postshock intervals . Mice remained in the conditioning chamber for 30 seconds following the last footshock. Based on previous literature (Smith, Gallagher et al. 2007, Matynia, Anagnostaras et al. 2008) and our prior work, strain specific shock intensities were used (0.5mA for 129S, 129S6, and 129S6/B6 mice and 0.75mA for C57B6 mice) to elicit similar levels of freezing while preventing over/undertraining. After training, mice were tested in one of three contexts: same, A; similar, B; and different, C. All context tests consisted of five minutes of context exposure.

For experiments that examined context extinction, mice were counterbalanced for their test context and subsequently divided into two groups: extinction and no extinction. Twenty-four hours after testing, mice in the extinction group were extinguished to context A using sixty minutes of context exposure divided evenly across two days. For 129S6/B6 mice, mice in the extinction group received one hundred and twenty minutes of context exposure divided evenly across two days. Mice in the no extinction group remained in their home-cage as a retention control group. Twenty-four hours after extinction, both extinction and no extinction mice were tested for their fear in context A using 5 minutes of context exposure.

During context discrimination, mice were trained to discriminate through exposure to both context A and context B each day for nine days, separated by a minimum of six hours. The order of exposure to contexts A and B was alternated each day. In context A, mice were trained each day using three minutes of context exposure followed by one unsignaled footshock for 2 seconds at a strain specific intensity (129S1 and 129S6 at 0.5mA and C57B6 at 0.75mA). Mice were removed from the conditioning chambers 30 seconds after the footshock. In context B, mice received context exposure for three minutes and thirty-two seconds, comparable to the time spent in context A with no unsignaled footshock. Mice were tested for their fear to contexts A and B on day ten using three minutes and 30 second exposure to each context in the absence of a footshock. Roughly twenty-four hours later, mice were tested in the different context, context C, using five minutes of context exposure to test for increases in basal anxiety.

Tone Protocols

Mice were fear conditioned to a tone in context A using three training sessions, one per day for three days. During training, mice were exposed to context A for three minutes followed by three tone-shock presentations in which a 30 second tone (75dB, 2.8kHz) co-terminated with a 2 second footshock, with 30 seconds between tones. Mice remained in the conditioning chamber for 30 seconds following the last tone-shock pairing. Mouse strains 129S6 and 129S1 received footshocks at 0.5mA and strain C57B6 at 0.75mA. Seventy-two hours later, mice were tested to their fear of the tone in context C using one minute context exposure followed by three 30 second tone-alone presentations spaced 30 seconds apart. Mice were removed from the chambers thirty seconds after the last tone presentation. For tone extinction experiments, mice were

divided into two groups following training: extinction and no extinction. Twenty-four hours after training, mice in the extinction group received two days of extinction training in context B with each day consisting of two minutes of context exposure followed by thirty 30-second tone-alone presentations spaced thirty seconds apart. Mice were removed from the conditioning chambers thirty seconds after the last tone presentation. In lieu of extinction, mice in the no extinction group were placed in context B for thirty-two minutes per day for two days with no tone presentations. Twenty-four hours after extinction training, no extinction and extinction mice were tested for their fear of the tone using the testing protocol described above. To study acquisition of fear to the tone, fear of the tone in the no extinction group during testing was observed.

2.4 Results

2.4.1 Similar acquisition and consolidation of fear memories in the 129S6 and C57B6 strains

To assess whether 129S6 mice showed deficits in fear acquisition and/or consolidation, 129S6 mice were compared to the commonly used C57B6 mice using Pavlovian fear conditioning to a context and tone.

Mice were fear conditioned to a context using three minutes of context exposure followed by three unsignaled footshocks per day for three days (**Figure 2.1A**). Acquisition of fear across training days was analyzed as the average percent freezing over the first three minutes of context exposure for each day (**Figure 2.1B**). Analysis of fear acquisition across training days using a repeated measures ANOVA showed a

significant effect of training ($F_{(2, 26)} = 103.19$, $p < 0.0001$), but no significant difference between 129S6 and C57B6 strains ($F_{(1, 13)} = 0.008$, $p = 0.9321$). Twenty-four hours after fear conditioning, mice were tested for fear to the trained context using five minutes of context exposure (**Figure 2.1C**). Analysis of context testing using an unpaired t-test showed no significant difference in percent time freezing between strains ($p = 0.966$) with 129S6 and C57B6 mice freezing at an average of 80% and 75% respectively. A separate group of mice were fear conditioned to a tone using three minutes of context exposure followed by three tone-shock pairings per day for three days (**Figure 2.1D**). Average freezing to the tone was calculated per day. Analysis of fear acquisition across tone training days using a repeated measures ANOVA showed no significant difference between strains ($F_{(1, 6)}=0.167$, $p = 0.689$) but a significant effect of training ($F_{(2,12)} = 119.35$, $p < 0.001$) (**Figure 2.1E**) . Seventy-two hours later mice were tested for their fear to a tone in a different context using five tone-alone presentations (**Figure 2.1F**). During testing, there was no significant difference between strains ($p = 0.6512$) with 129S6 mice showing an average freezing level of 69% compared with 75% in C57B6 mice. These results demonstrate that the 129S6 mice exhibit normal, and adaptive, fear learning to a context and tone.

2.4.2 Mouse strain 129S6 shows significant deficits in cued and contextual fear extinction compared to C57B6 mice.

Our results demonstrate that the 129S6 strain exhibit normal acquisition and consolidation compared to C57B6, which is similar to results obtained with the 129S1 strain (Hefner, Whittle et al. 2008). To determine whether 129S6 mice have deficits in fear extinction, 129S6 and C57B6 mice were conditioned to a context or a tone then

extinguished by repeated CS exposures. Mice were first fear conditioned to a context using three minutes of context exposure followed by three unsignaled footshocks per day for three days, same as the context fear conditioning described above. Twenty-four hours later, mice were tested for fear generalization (see below and **Figure 2.3A**). The following day mice were counter balanced and divided into extinction and no extinction groups. Mice in the extinction group received 30 minutes of context exposure per day for two days while mice in the no extinction group remained in their home cage (**Figure 2.2A**).

To assess fear extinction learning between strains, freezing responses to the trained context were analyzed in 5 minute time bins with bins 1-6 representing extinction day 1 and bins 7-12 representing extinction day 2 (**Figure 2.2B**). Within session fear extinction was analyzed using a repeated measures ANOVA which revealed a significant effect of extinction training in 129S6 mice on day 1 ($F_{(5, 50)}$ = 4.037, p = 0.0032), but not on extinction day 2 ($F_{(5, 50)}$ = 1.871, p = 0.1129). In comparison, analysis of within session fear extinction in C57B6 mice revealed a significant effect of extinction training on extinction day 1 ($F_{(5, 60)}$ = 24.970, p < 0.0001) and extinction day 2 ($F_{(5, 60)}$ = 7.334, p < 0.0001). Additionally, analysis of within session extinction using a repeated measures ANOVA also revealed a significant effect of strain on both extinction day 1 ($F_{(1, 27)}$ = 7.334, p = 0.0116) and day 2 ($F_{(1, 27)}$ = 25.907, p < 0.0001). Twenty-four hours after extinction, all mice were tested for their fear to the trained context. Analysis of context freezing using a two-way ANOVA followed by post hoc analysis with a planned unpaired t-test found a significant effect of

extinction training ($F_{(1,42)}$ = 18.772, p < 0.0001), which was significant in C57B6 mice (p < 0.0001), but not 129S6 mice (p = 0.0991) (**Figure 2.2C**).

To determine whether 129S6 mice exhibit deficits in tone fear extinction, mice were trained to a tone using three tone shock pairings per day for three days, as described above. Twenty-four hours later, mice were split into extinction and no extinction groups (**Figure 2.2D**). Mice in the extinction group were extinguished in a novel context, Context C, using thirty tone alone presentations per day for two days. Mice in the no extinction group were placed in the novel context, without tone presentations, for an equivalent length of time. Extinction training was plotted similarly to context extinction with bins of five tones and bins 1-6 representing extinction day 1 and 7-12 representing extinction day 2 (**Figure 2.2E**). Analysis of within-session fear extinction using repeated measures ANOVA revealed a no significant effect of extinction training in 129S6 mice on extinction day 1 ($F_{(5,25)}$=0.704, p=0.6239) or extinction day 2 ($F_{(5,25)}$=0.866, p=0.5138). In comparison, analysis of within-session extinction in C57B6 mice found a significant effect of extinction training on both extinction day 1 ($F_{(5,20)}$=11.284, p<0.0001) and extinction day 2 ($F_{(5,20)}$=3.734, p<0.0001). Analysis of within-session fear extinction between strains using a repeated measures ANOVA revealed a significant effect of strain on extinction day 1 ($F_{(1, 30)}$ = 37.894, p < 0.0001) and day 2 ($F_{(1, 30)}$ = 7.761, p = 0.0092). Twenty-four hours later mice were tested for their fear to the trained tone (**Figure 2.2F**). Analysis of tone testing using a two-way ANOVA found a significant effect of extinction training ($F_{(1, 30)}$ = 10.580, p = 0.0030). Further analysis using a planned unpaired t-test revealed that the effect of extinction was limited to the C57B6 mice (p = 0.0064) with no significant

difference between the extinction and no extinction groups in the 129S6 mice ($p = 0.2221$). This data indicates that, like 129S1 mice, 129S6 mice have significant deficits in fear extinction to a context as well as a tone. This is consistent with the previously published data suggesting extinction deficits in various genetically similar 129 strains (Hefner, Whittle et al. 2008, Camp, Norcross et al. 2009).

2.4.3 Mouse Strain 129S6 shows comparable/normal levels of fear generalization as C57B6 mice

Based on the similarities in fear conditioning and extinction phenotypes between 129S1 and 129S6, we hypothesized that 129S6 mice would also show an overgeneralization of fear from a trained context to a novel and similar context, as previously seen in 129S1 mice (Camp, Macpherson et al. 2012). To test for fear generalization, mice that were trained to context "A", using the context training protocol described above were tested for generalization of fear to an untrained context. Twenty-four hours after training, animals were tested for their fear to either the trained context, A, a similar context, B, or a completely different context, C (**Figure 2.3A**). See "methods" for further details pertaining to the similarities and differences between contexts. In mice considered to generalize normally, context B should produce some level of generalized fear due to its similarities to context A, but this fear, represented as percent time freezing, would be expected to be significantly lower than that seen in the trained context. Due to the substantially different nature of context C, mice considered to generalize normally should show significantly lower levels of freezing compared to that seen in the trained context, and in many cases, significantly lower levels of freezing than that seen to the similar context. Fear was analyzed as an average percent freezing

in each context and compared using a two-way ANOVA with strain and context as factors. Analysis of context generalization showed no interaction between strain and context ($F_{(1, 43)} = 0.075$, $p = 0.7855$), but a significant effect of context ($F_{(2, 86)} = 22.444$, $p < 0.0001$). Using an unpaired t-test between contexts it was found that both C57B6 and 129S6 froze significantly more in the same context, at 80% in 129S6 mice and 75% in C57B6 mice, than in either the similar context (129S6:$p = 0.0481$, C57B6:$p = 0.0271$) or different context (129S6:$p = 0.0027$, C57B6:$p < 0.0001$). Furthermore, both C57B6 and 129S6 froze at a significantly higher level in the similar context, at 58% in 129S6 mice and 57% in C57B6 mice, than the different context (129S6:$p = 0.0156$, C57B6:$p = 0.0106$). Both 129S6 and C57B6 mice also showed low levels of freezing in the different context at 28% and 33% respectively (**Figure 2.3B**). This data indicates that 129S6 mice show similar levels of fear generalization as C57B6 mice, suggesting normal levels of fear generalization. While these data clearly suggest normal levels of fear acquisition in 129S6 mice, this does not match previously published data showing a strong overgeneralization phenotype in the genetically similar 129S1 substrain (Camp, Macpherson et al. 2012).

To better understand the potential differences in 129S6 and 129S1 mice in fear generalization and to rule out a difference in generalization parameters between current and previously reported studies in the mouse phenotypes observed, 129S1 mice were compared to 129S6 mice in the fear generalization protocol previously described. Analysis of fear generalization between 129S1 and 129S6 mice using a two-way ANOVA showed a significant interaction of strain and context ($F_{(2, 170)} = 8.404$, $p = 0.0003$) as well as context ($F_{(2, 170)} = 23.580$, $p < 0.0001$) (**Figure 2.3C**). Post hoc

analysis using planned unpaired t-tests showed significantly higher levels of freezing of 129S6 mice in the same context at 79% compared with the similar context at 53% ($p = 0.015$) and different context at 36% ($p < 0.001$). Conversely, there was no significant difference between 129S1 freezing in the same context at 63% compared to the similar ($p = 0.173$) and different ($p = 0.842$) contexts. Taken together, these results support a strong fear overgeneralization phenotype in 129S1 mice which is absent in 129S6 mice, despite the similarities between these two substrains in persistent/extinction-resistant fear.

2.4.4 Unlike 129S1 mice, 129S6 can be trained to discriminate between similar contexts.

Our results demonstrate that unlike the 129S1 substrain, the 129S6 mice exhibit an intact generalization of fear gradient. We hypothesized that the differences in 129S6 and 129S1 substrain fear generalization might be due to an increase in anxiety-like behavior in the 129S1 strain. To further investigate the putative substrain differences and to better understand whether the overgeneralization in 129S1 mice represents an inability to cognitively discriminate between two similar contexts or an overall increase in anxiety-related behavior independent of context, we subjected both substrains to a context discrimination paradigm. During context discrimination training, mice were exposed to two contexts, contexts A and B, each day for 10 days. In context A, mice received three minutes of context exposure followed by one unsignaled footshock and were then removed thirty seconds later. In context B, mice did not receive a footshock and were instead allowed an equivalent period of time (3.5 minutes) of context exposure and removed from the chamber. The order in which mice received contexts A and B

was alternated each day to control for order and time of day effects (**Figure 2.4A**). On day 10 mice were placed back in contexts A and B in the absence of a footshock. Twenty-four hours after training, mice were placed in a novel context, context C. Fear learning to each context was analyzed throughout training by comparing the percent freezing in the first three minutes of exposure to each context per day across days. Analysis using a repeated measures ANOVA revealed a significant effect of training day in context A ($F_{(4, 120)} = 87.133$, $p < 0.0001$) and no significant difference between strains ($F_{(1, 30)} = 0.8795$, $p = 0.6194$) (data not shown). Discrimination ratios were calculated for each day of training as the percent freezing in context A divided by the sum of freezing in contexts A and B. A discrimination ratio of 0.5 represents a lack of context discrimination. To control for the effect of time of day on freezing to each context, discrimination ratios are represented as an average of two days (**Figure 2.4B**). When compared to chance (a discrimination ratio of 0.5) 129S6 mice were able to discriminate between the two similar contexts by the fifth/sixth day of training ($p = 0.0079$), whereas the discrimination ratio for mice in the 129S1 strain never exceeded chance ($p = 0.2344$ on days nine/ten) (**Figure 2.4B**). These data, similar to the generalization data presented above, suggest that 129S1 mice may be prone to overgeneralization of fear in context B. Both 129S6 and 129S1 mice show an average freezing level of over 60% in context A with no significant difference between the strains on day 10 (**Figure 2.4C**). However, while 129S6 mice showed a significantly lower level of freezing in context B, compared to context A ($p = 0.0377$), 129S1 mice showed high levels of freezing in context B that was indistinguishable from that seen in context A ($p = 0.5192$) and significantly higher than 129S6 mice ($p = 0.026$). If the

inability to actively discriminate between context A and context B in the 129S1 strain is due to an enhanced anxiety state, one would predict that the 129S1 mice would exhibit high levels of freezing in a novel context compared to 129S6. However, when mice were exposed to a novel context (context C) both 129S1 and 129S6 mice exhibited similarly low levels of freezing ($p = 0.5864$). This data suggests that 129S1 mice, unlike 129S6 mice, overgeneralize their fear of a trained context to a similar context and that this generalization is likely due to an inability to discriminate between the two contexts versus an increase in anxiety-like behavior due to fear conditioning.

2.4.5 Despite deficits in 129S6 mice, hybrid 129S6/B6 mice exhibit normal fear extinction to a conditioned context

Due to the presence of significant deficits in fear extinction in 129S6 mice, but not C57B6 mice, and recent work utilizing a hybrid 129S6/B6 mice background to investigate the effects of deletion of L-type voltage gated subtypes in fear related learning (McKinney and Murphy 2006, McKinney, Sze et al. 2008, McKinney, Sze et al. 2009), we wanted to test whether an F2 hybrid cross of 129S6 and C57B6 mice could extinguish their fear to a conditioned context. Mice were generated using an F2 cross of naïve 129S6 mice and C57B6. Hybrid 129S6/B6 mice were then conditioned to a context as using the same protocol as was used for pure 129S6 and C57B6 mice. Twenty four hours after conditioning, all mice underwent extinction training. Extinction training consisted of one hundred and twenty minutes of context exposure split evenly across two days (**Figure 2.5A**) Twenty-four hours after the last day of extinction mice underwent a context test consisting of five minutes of context exposure. Analysis of freezing across extinction training and context testing using a one way repeated

measures ANOVA revealed a significant effect of context exposure on freezing levels ($F_{(12,192)} = 4.487$, $p < 0.0001$). Further analysis using a planned unpaired t-test found a significant drop in freezing levels between the first ten minutes of extinction day one and the first ten minutes of extinction day two ($p = 0.003$), but not between the first ten minutes of extinction day two and test ($p = 0.9075$) . Additionally, comparison of freezing levels in 129S6/B6 mice during the five minute test, or post-extinction, to the first five minutes of context exposure on extinction day one, labeled pre-extinction, using an unpaired t-test demonstrated a significant decrease in freezing in 129S6/B6 mice after training with freezing levels of 38% and pre-extinction freezing levels of 65% ($p = 0.0301$) (**Figure 2.5B**). This data suggests that while 129S6 mice exhibit a significant deficit in fear extinction to a context, hybrid 129S6/B6 mice can extinguish their fear to a conditioned context as a significant reduction in fear to the conditioned context throughout extinction training and testing.

2.5 Discussion

Through a series of fear related studies, we were able to segregate different forms of maladaptive fear in two genetically similar substrains of inbred mice. While 129S6 mice show strong deficits in fear extinction, similar to the previous published 129S1 mice; 129S1, but not 129S6 mice, show substantial fear over generalization and a lack of context discrimination.

Our studies found that 129S6 mice exhibit severe deficits in fear extinction in spite of normal learning and consolidation of fear memories. While 129S6 and C57B6 mice froze at comparable levels throughout training and at the start of extinction

training, 129S6 mice continued to freeze at high levels throughout the extinction training sessions. In addition, unlike the C57B6 mice, the 129S6 mice maintained high levels of freezing when tested on subsequent test days, exhibiting similar levels of freezing to mice in the no extinction group. These findings are consistent with previous experiments that have described similar deficits in other 129 substrains (Hefner, Whittle et al. 2008, Camp, Norcross et al. 2009).

Despite 129S6 mice exhibiting clear aberrant fear processing in terms of extinction learning, data from our experiments demonstrates normal levels of fear generalization in the 129S6 substrain. Both 129S6 and C57B6 mice showed high levels of fear to the trained context, illustrating normal fear learning, and significantly lower levels of fear to both a similar and completely novel context, suggesting a normal generalization of fear gradiant. However, when we directly compared the 129S6 and 129S1 substrains, 129S1 mice exhibited overgeneralization of fear with high levels of freezing in both the trained and similar, as well as in the completely novel context. These data suggest an inability of 129S1 mice to distinguish between the contexts or an enhanced state of anxiety induced by fear conditioning. However, our studies comparing context discrimination between the 129S1 and 129S6 mice found a lack of discrimination in the 129S1 mice which was independent from an increase in overall anxiety. While 129S6 mice exhibited significantly higher levels of fear to the trained context versus the similar context, illustrated by a discrimination ratio of greater than 0.5, 129S1 mice showed a similar fear response in both contexts resulting in a discrimination ratio of roughly 0.5. However, 129S1 and 129S6 exhibited similarly low levels of freezing to a completely different context. Taken together with the fear

generalization data, this deficit in context discrimination suggests that the differences in 129S1 mice regarding overgeneralization is likely due to an inability to cognitively separate the two contexts. While unexpected, these discrepancies between two genetically similar substrains suggests clear differences in the mechanisms underlying fear overgeneralization and persistent fears that may be tied to the subtle differences in genotypes.

In recent years a great deal has been uncovered regarding the neurobiology that underlies fear learning and extinction (Myers and Davis 2007, Milad and Quirk 2012, Orsini and Maren 2012). Previous studies have linked deficits in fear extinction to a context or cue to various brain structures including the hippocampus (Corcoran, Desmond et al. 2005), prefrontal cortex (Milad and Quirk 2002), and amygdala (Likhtik, Popa et al. 2008). In addition, a number of cellular changes have been implicated, including presynaptic changes (Stork, Ji et al. 2002, Tsvetkov, Carlezon et al. 2002) and alterations in protein synthesis (Hernandez and Abel 2008) . While the mechanism underlying of the extinction deficits in 129S6 substrain has yet to be determined, previous reports comparing 129S1 mice and C57B6 mice have found altered neuronal activity in the infralimbic cortex of the prefrontal cortex (PFC), basolateral amygdala, and the central amygdala in 129S1 mice associated with fear extinction (Hefner, Whittle et al. 2008). These changes in neuronal activity were measured as alterations in the expression of immediate early genes (IEG) c-Fos and Zif268 and implicate the prefrontal cortex and amygdala function in the extinction deficits seen in 129S1 mice, and perhaps other 129 substrains, such as 129S6. These results were further supported by studies suggesting that a zinc restricted diet improved

fear extinction learning in 129S1 mice and normalized the expression of IEGs in the amygdala and prefrontal cortex compared to C57B6 mice (Whittle, Hauschild et al. 2010). While it is possible that these changes in neuronal activity in the PFC and amygdala in129S1 mice may also be responsible for the generalization and context discrimination deficits described in this substrain, the presence of extinction deficits, but not overgeneralization in the 129S6 substrain suggests a disassociation between these two forms of maladaptive fear.

In this case, deficits in the cognitive ability to learn and distinguish two contexts likely involves structures known to be involved in memory formation. In particular, the ability to encode fine details regarding a pattern or context and distinguish this information from other patterns or contexts has been linked to the dentate gyrus of the hippocampal formation (Gilbert, Kesner et al. 2001, Leutgeb, Leutgeb et al. 2007, McHugh, Jones et al. 2007) and more specifically, the adult-born granule cells in the dentate gyrus of the hippocampal formation (Clelland, Choi et al. 2009, Sahay, Scobie et al. 2011, Nakashiba, Cushman et al. 2012, Tronel, Belnoue et al. 2012) . For example, several studies that examined context discrimination have found that ablation of adult-born neurons prior to learning prevented discrimination of similar contexts (Clelland, Choi et al. 2009, Sahay, Scobie et al. 2011, Nakashiba, Cushman et al. 2012, Tronel, Belnoue et al. 2012). Conversely, increasing adult neurogenesis resulted in enhanced context discrimination (Sahay, Scobie et al. 2011). Interestingly, previous studies of various mouse strains have noted significant differences in basal rates of adult neurogenesis in dentate gyrus, including lower levels of neurogenesis in 129S1 mice compared to C57B6 mice (Kempermann, Kuhn et al. 1997, Kempermann and Gage

2002). At present it remains unknown to what extent these differences in basal levels of neurogenesis might alter behavioral output measures in general, or whether these differences in adult neurogenesis might account for differences in context discrimination, as seen in the 129S1 and 129S6 mouse strains. In addition, it remains unknown whether strain dependent variations in survival and functional integration of adult born neurons may also play a role in learning and memory. Careful examination of strain dependent behavioral differences, similar to the current study, in conjunction with detailed cellular analysis could be used in the future to further explore the putative link between adult neurogenesis and cognitive function.

Given recent and ongoing work utilizing 129S6 mice to produce a F2 hybrid 129S6/B6 genetic background for studies of various genes, particularly L-type voltage gated calcium channel genes (McKinney and Murphy 2006, McKinney, Sze et al. 2008, McKinney, Sze et al. 2009), in fear related learning and significant deficits in 129S6 mice in fear extinction, we investigated the ability of 129S6/B6 mice to extinguish. We found that hybrid 129S6/B6 mice were capable of extinguishing their fear to a conditioned context over two days of extinction training with lower levels of freezing to the context during testing than at measured at the beginning of extinction training. Interestingly though, analysis of fear throughout experimentation revealed a significant decline in freezing in 129S6/B6 mice between the start of days one and two of extinction training, but not between day two of extinction and tests. This may suggest that while 129S6/B6 exhibit significant reductions in fear, they are limited in the extent to which they can extinguish. This will likely be valuable for current individuals

utilizing a 129S6/B6 cross for behavioral experimentation and any individuals considering this cross for the generation and maintenance of transgenic mice.

In the current study we describe significant substrain differences in aberrant generalization of fear and context discrimination but not extinction of conditioned fear. Functionally, similarities between 129S6 and 129S1 in extinction deficits may be linked to the similarities in substrain genetics absent in C57B6 mice, while differences in 129S6 and 129S1 mice in fear generalization and context discrimination may be mediated by differences in genetics between the two substrains. These similarities and differences in 129S6 and 129S1 mice may highlight important differences between two types of maladaptive fear: that associated with an inability to extinguish to a conditioned stimulus and that associated with an overgeneralization of fear to an unconditioned stimulus. This dissociation between maladaptive fears suggests different mechanisms and underlying genetics that give rise to these two forms of maladaptive fear. Identification of the genes and cellular mechanisms involved in the distinct forms of maladaptive fear may lead to a better understanding of their roles in anxiety-related disorders and their treatments. While the use of inbred mouse strains to model anxiety-related and trauma-related disorders is limited due to the genetic homogeny and limited behavioral variability of inbred mouse strains compared to the heterogeneity of the human population, this homogeneity can be useful in the identification of genes and mechanisms mediating specific types of behaviors (Sankoorikal, Kaercher et al. 2006). Though unable to directly model the psychiatric disorder itself, discoveries such as these can inform studies aimed at understanding specific components leading to various pathological behaviors and conditions (Lifsted, Le Voyer et al. 1998, Mahler, Bristol et

al. 1998, Qi, Fujita et al. 2005, Sankoorikal, Kaercher et al. 2006). In this way, 129S1 and 129S6 mice can function as key tools for studying the mechanisms underlying these maladaptive fears individually. Given the presumed genetic similarities of the 129S1 and 129S6 substrains (Simpson, Linder et al. 1997) and the divergent forms of maladaptive fear described here, we suggest that further comparison of the two substrains may reveal valuable insights into psychiatric disease states thought to be related to maladaptive fear learning and or memory.

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Figure 1

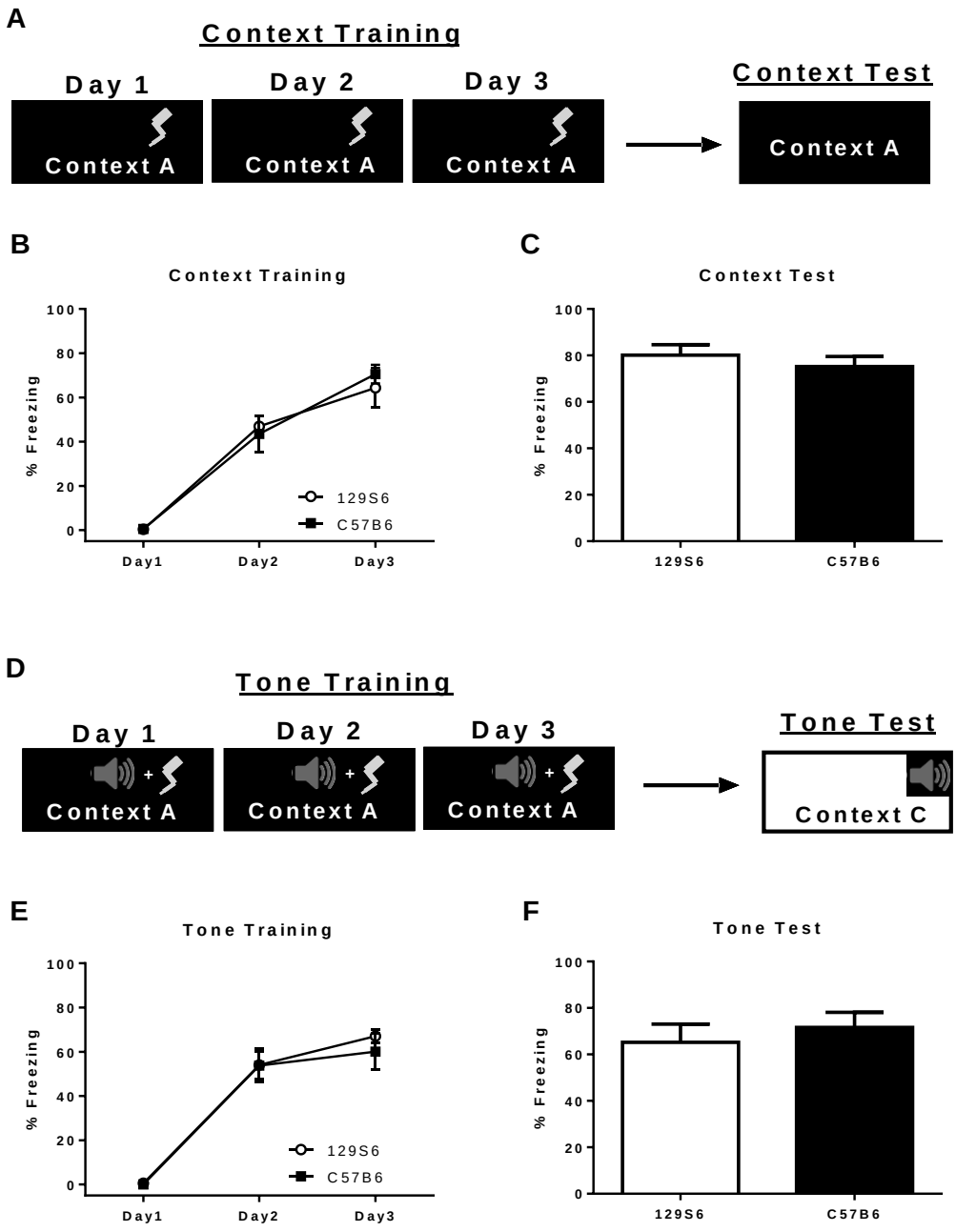


Figure 2.1: Inbred mouse strains, 129S6 and C57B6, have comparable levels of fear acquisition and consolidation.

(**A**) Schematic representation of context fear conditioning. Mice were trained to a context or tone using 3 unsignaled footshocks or 3 tone-shock pairings per day for three days. Acquisition of fear across training is represented as the average percent freezing to the context or tone prior to shock for each day. Mice were tested twenty-four hours

later. (**B**) The 129S6 mice (n = 7) showed a significant increase in freezing across context training days comparable to that seen in C57B6 mice (n = 8). (**C**) During testing, both 129S6 and C57B6 mice show high levels of freezing to the context, not significantly different from each other. (**D**) Schematic represention of tone fear conditioning. (**E & F**) Similar results were obtained when 129S6 and C57B6 mice (n = 4 and 4 respectively) were conditioned to a tone and tested 72 hours later**.** Data are presented as mean ± SEM. *$p<0.05$.

Figure 2

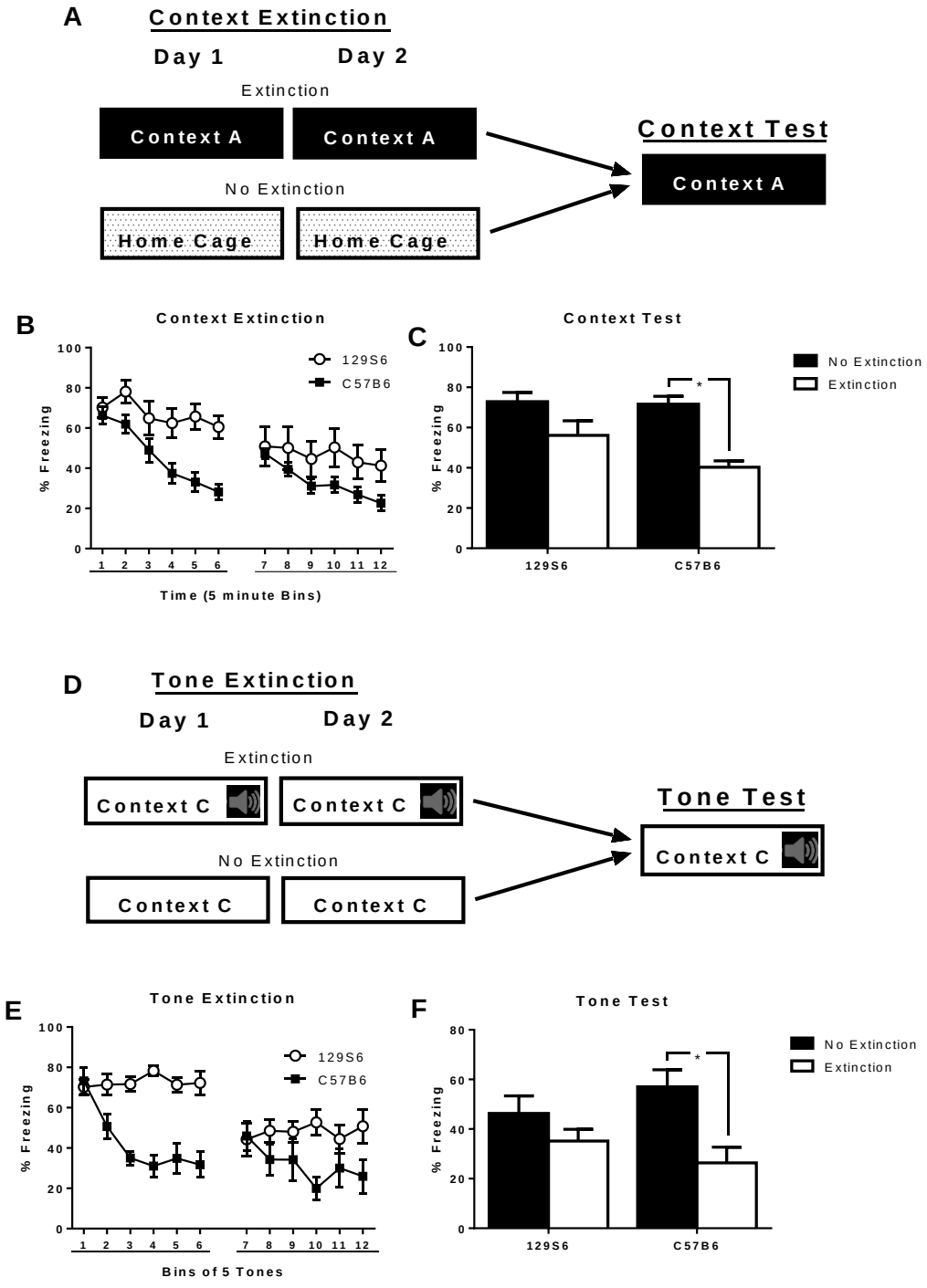


Figure 2.2: Inbred mouse strain 129S6 shows deficits in context and tone extinction.

(**A**) Schematic representation of context extinction. Mice were trained to a context or tone using 3 tone-shock or unsignaled footshock presentations per day for three days. Subsequently, mice were split into extinction and no extinction groups. Mice that were trained to a context were extinguished to the training context using sixty minutes of context exposure split across two days. Mice that were trained to fear a tone were extinguished with sixty tone-alone presentations split across two days. Extinction data is binned in groups of 5 minutes or 5 tones with bins 1-6 representing day 1 of extinction and bins 7-12 representing day 2. Twenty four hours later all mice (both the extinction and no extinction groups) were exposed to the training context or the training tone in a novel context. (**B**) During context extinction, 129S6 showed high levels of freezing within extinction days 1 and 2 compared to C57B6 mice. (**C**) During testing, C57B6 mice in the extinction group (n = 16) showed significantly less freezing than the no extinction controls (n = 8), while 129S6 mice exhibited similar levels of freezing across both groups (n = 13 and 7 for the extinction and no extinction groups, respectively). (**D**) Schematic representation of tone extinction. (**E & F**) Similar results were observed when C57B6 mice (n = 7 and n = 8; extinction and no extinction groups, respectively) and 129S6 mice (n = 8 and n = 9; extinction and no extinction groups, respectively) were extinguished and tested with a tone. Data are represented as mean ± SEM. *$p<0.05$.

Figure 3

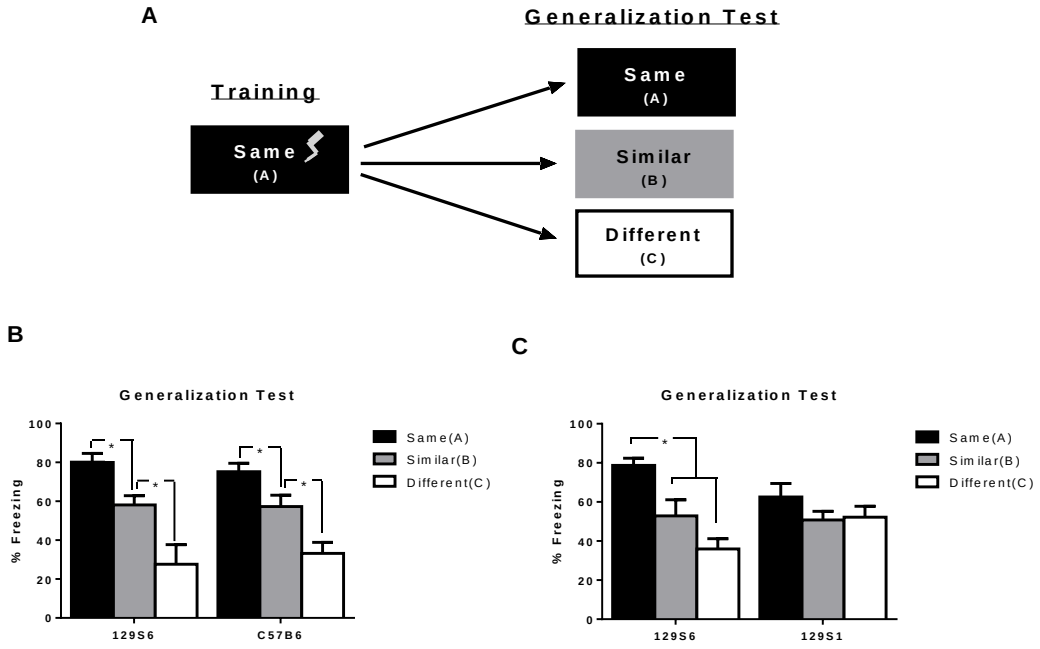


Figure 2.3: Inbred mouse Strain 129S6, but not 129S1, has normal context generalization

(**A**) Schematic representation of experiment. Mice were trained to a context using 3 unsignaled footshock presentations per day for three days. During generalization testing, mice were tested for fear in either the same training context, a similar context, or a different context. Data are represented as the average percent freezing in each context. (**B**) When tested, both 129S6 and C57B6 mice displayed high levels of freezing in the same context (C57B6 n = 8, 129S6 n = 7), intermediate levels of freezing in the similar context (C57B6 n = 8, 129S6 n = 6), and low levels of freezing in the different context (C57B6 n = 8, 129S6 n = 7). (**C**) In a separate experiment, fear generalization in the 129S6 and 129S1 substrains were compared. Consistent with previous reports, the 129S1 mice exhibited similar levels of freezing regardless of the context (same, n = 8; similar, n = 8; different, n = 8). In contrast, and consistent with our previous experiment, the 129S6 exhibited graded levels of freezing in the three contexts (same, n = 8; similar, n = 8; different, n = 8). Data are represented as mean ± SEM. *$p<0.05$.

Figure 4

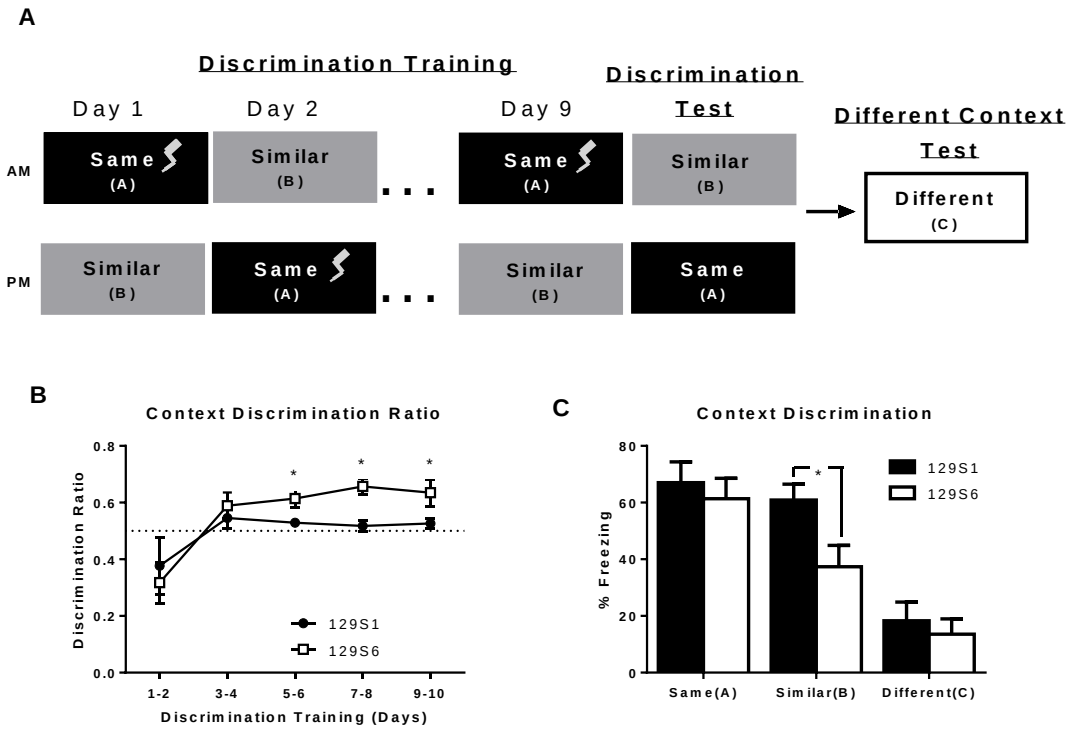
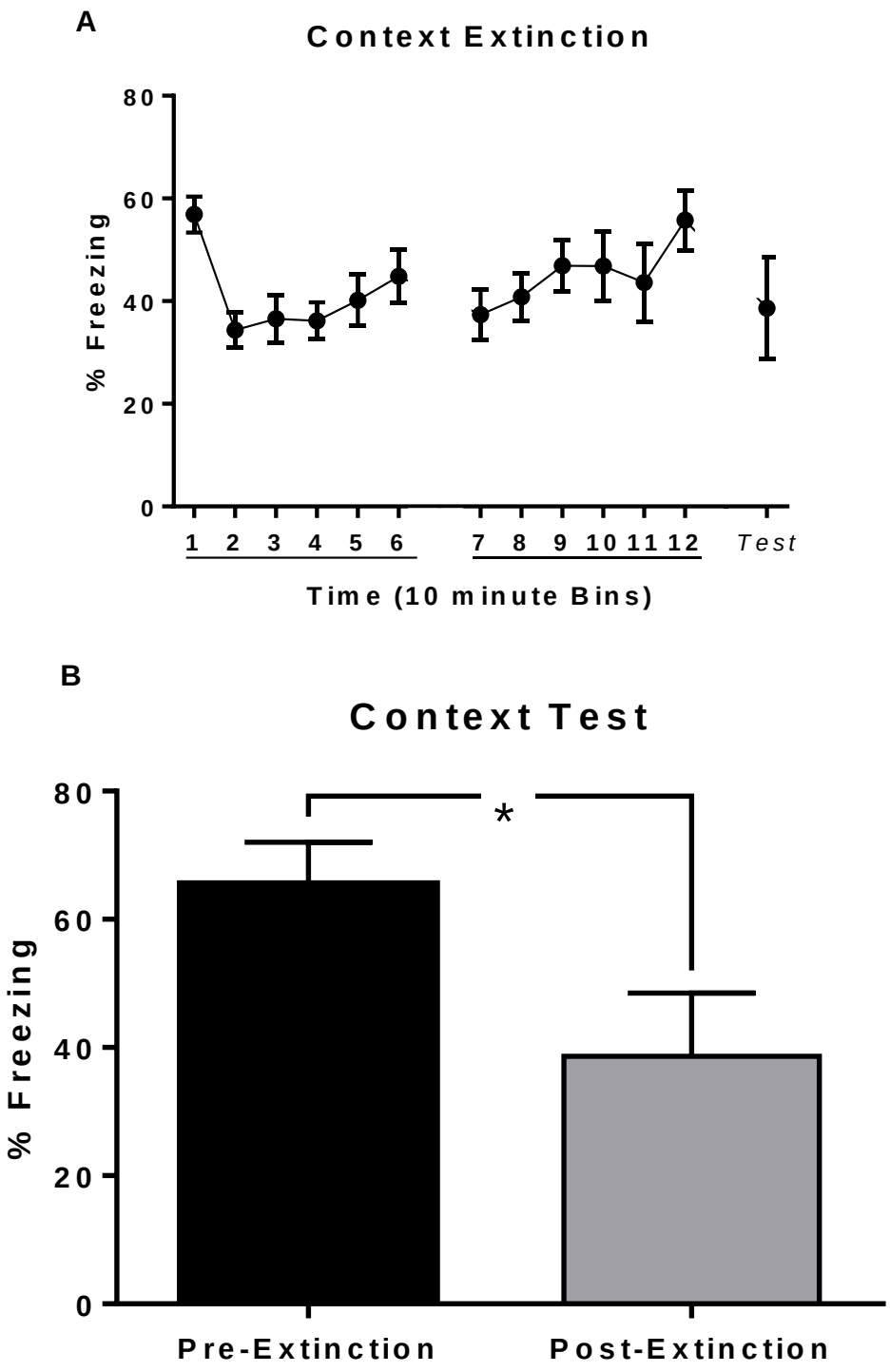


Figure 2.4: Inbred mouse strain 129S1, but not 129S6, fail to discriminate between two similar contexts independent of an increase in basal anxiety.

(**A**) Mice were trained to context discriminate using a daily exposure to context A and context B in which only the exposure to context A was paired with a single unsignaled footshock on days 1 through 9. On day 10 mice were returned to context A and context B and the shock was omitted. Mice were tested for fear to a different context (context C) one day later. (**B**) Context Discrimination across training days is represented as a discrimination ratio of freezing in context A divided by freezing in context B with 0.5 representing no discrimination (dotted line). Ratios are averaged every two days to counterbalance for order of context exposure. During context discrimination training, 129S6 mice show significant context discrimination on day 5-10, while 129S1 mice failed to exhibit significant context discrimination. * $p < 0.05$ compared to chance (0.5 ratio; dotted line). **(C)** On day 10, exposure to context A produced similar levels of freezing in both 129S6 and 129S1 substrains. However, in context B, 129S1 mice exhibited significantly more freezing compared to 129S6 mice. Conversely, both substrains exhibited similarly low levels of freezing when exposed to a novel context (context C). Data are presented as mean ± SEM; * $p < 0.05$ n= 16 for 129S1 and 129S6.

Figure 5


A
Context Extinction
80
60
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1 2 3 4 5 6
7 8 9 10 11 12
Test
Time (10 minute Bins)
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Context Test
80
60
40
20
0
% Freezing
*
Pre-Extinction
Post-Extinction

Figure 2.5: Hybrid 129S6/B6 mice exhibit significant fear extinction to a conditioned context.

Mice were trained to a context using one tone-shock presentation per day for two days (n = 17). Mice were extinguished to the training context using one hundred and twenty minutes of context exposure split across two days. Mice were tested for their fear to the context after extinction using five minutes of context exposure. Extinction data is binned in groups of 10 minutes with bins 1-6 representing day 1 of extinction and bins 7-12 representing day 2. (**A**) During context extinction, hybrid 129S6/B6 mice show a significant decline in fear across extinction training. (**B**) Comparison of freezing after-extinction, during context testing, and before-extinction, at the start of extinction day one, revealed a significant decrease in freezing response to the context in 129S6/B6 mice. Data are represented as mean ± SEM. *$p<0.05$.

CHAPTER 3

NEURONAL DELETION OF $CA_V1.2$, BUT NOT $CA_V1.3$ IMPAIRS DENTATE GYRUS ASSOCIATED MEMORY TASKS AND ADULT NEUROGENESIS

3.1 Summary

L-type voltage gated calcium channels (LVGCCs) have been implicated in various forms of learning, memory, and synaptic plasticity. Within the hippocampus, the LVGCC subtype, $Ca_V1.2$ is prominently expressed throughout the dentate gyrus. However, despite the apparent high levels of $Ca_V1.2$ expression in the dentate gyrus, the role of $Ca_V1.2$ in dentate gyrus-dependent learning remains unknown. Using transgenic mice lacking $Ca_V1.2$, we investigated the role of $Ca_V1.2$ in dentate gyrus-dependent tasks. Mice were examined using Pavlovian fear conditioning and a version of the Morris water maze in which spatial cues were degraded to study pattern separation and spatial encoding. Consistent with our previous results, deletion of $Ca_V1.2$ did not impair acquisition of fear to the conditioned context associated with the footshock. However, mice lacking $Ca_V1.2$ exhibited deficits in the ability to discriminate between a context in which they received footshock and that in which they did not, suggesting deficits in pattern separation. Similarly, when $Ca_V1.2$ knockout mice were trained using a traditional form of the Morris water maze, knockout mice were able to learn and recall the location of the hidden platform as well as their wild-type littermates. When the task was made more difficult by restricting the number of available spatial cues, a form of

the task more likely to be dependent on the dentate gyrus, mice lacking $Ca_V1.2$ were unable to encode the location of the hidden platform. These results suggest that deletion of $Ca_V1.2$ preferentially impacts dentate gyrus function and dentate gyrus dependent learning.

3.2 Introduction

Calcium is a key modulator of neuronal activity within the brain (Berridge 1998, Clapham 2007, Burgoyne and Haynes 2014). Changes in intracellular free calcium concentrations impact a wide range of neuronal functions, including the intrinsic excitability of the cell (Wong and Prince 1978, Hotson and Prince 1980, Shao, Halvorsrud et al. 1999) and the transcription and translation of new proteins thought to induce long term potentiation of synaptic connections (Sheng, McFadden et al. 1990, Crabtree 2001). One major source of intracellular calcium is calcium influx through voltage gated calcium channels. In particular, L-type voltage gated calcium channels (LVGCCs) are known to regulate intrinsic excitability through their involvement in the action potential afterhyperpolarization (AHP) (Tanabe, Gahwiler et al. 1998, Bowden, Fletcher et al. 2001, McKinney, Sze et al. 2009, Gamelli, McKinney et al. 2011) and NMDA-independent and dependent forms of long term potentiation (LTP) (Huber, Mauk et al. 1995, Weisskopf, Bauer et al. 1999, McKinney, Sze et al. 2009). Through these means, LVGCCs are thought to modulate learning and memory. Though LVGCCs can be broken down into four subtypes: $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$, only $Ca_V1.2$ and $Ca_V1.3$ have been found to be expressed in the mammalian central nervous system, (Hell, Westenbroek et al. 1993, Sinnegger-Brauns, Huber et al. 2009). Differences in channel kinetics (Xu and Lipscombe 2001) and neuronal distribution

(Hell, Westenbroek et al. 1993) between $Ca_V1.2$ and $Ca_V1.3$ suggest differential roles of these channels in neuronal function and behavior.

Investigation of the role of LVGCCs in learning and memory have utilized pharmacological agonists and antagonists and subtype specific transgenic mice. Pharmacological blockade of LVGCCs has been found to produce deficits in Pavlovian fear conditioning (Bauer, Schafe et al. 2002, Langwieser, Christel et al. 2010), as well as the extinction of conditioned fear (Cain, Blouin et al. 2002, Davis and Bauer 2012). Additionally, LVGCCs have been implicated in some forms of spatial learning (Moosmang, Haider et al. 2005, McKinney and Murphy 2006). Investigation of the LVGCC subtypes, $Ca_V1.2$ and $Ca_V1.3$, in learning revealed a potential role of $Ca_V1.3$ in fear learning and $Ca_V1.2$ in spatial learning. While global deletion of $Ca_V1.3$ was found to produce deficits in contextual fear learning (McKinney and Murphy 2006), impairments were not noted in other hippocampal dependent learning tasks (Clark, Nagano et al. 2003, McKinney and Murphy 2006). In comparison, conditional deletion of $Ca_V1.2$ in forebrain glutamatergic neurons, using either a CamKIIα Cre-driver line or a Nestin Cre-driver line, did not affect fear learning or acquisition of spatial memories in the Morris water. (McKinney, Sze et al. 2008, White, McKinney et al. 2008, Langwieser, Christel et al. 2010). However, deficits in remote (~30 days) spatial memory for the platform location in the Morris water maze were observed (White, McKinney et al. 2008). In contrast, studies in which $Ca_V1.2$ was conditionally deleted using a Nex promoter to drive Cre-recombinase expression, which is also thought to delete $Ca_V1.2$ in forebrain glutamatergic neurons, found deficits of Cav1.2 conditional knockout mice in the hippocampal dependent version of the visible platform water maze

and the labyrinth maze (Moosmang, Haider et al. 2005). While this apparent discrepancy in the role of $Ca_V1.2$ in hippocampal dependent learning could be due to the deletion pattern and temporal expression of the various Cre drivers, it is also possible that this discrepancy in the role of $Ca_V1.2$ in hippocampal dependent learning is related to the difficulty of the task and the hippocampal subregions utilized. The hippocampal formation is a highly organized structure which can be divided into many substructures including the hippocampus proper and the dentate gyrus (Amaral and Witter 1989). The hippocampus proper consists of a c-shaped cell body layer which can be split into three subregions: CA1, CA2, and CA3. The dentate gyrus, on the other hand, is made up of a v-shaped cell body region. Unlike other regions of the hippocampal formation, the dentate gyrus is the location of continual cell proliferation and neurogenesis which continues into adulthood (Altman and Das 1965, Gage 2002). Adult born neurons within the dentate gyrus have been linked to some forms of hippocampal dependent learning (Gould, Tanapat et al. 1999, Shors, Miesegaes et al. 2001, Winocur, Wojtowicz et al. 2006), but not others (Shors, Townsend et al. 2002, Jaholkowski, Kiryk et al. 2009). Lesion studies have demonstrated an important role of the hippocampus proper in contextual fear conditioning (Logue, Paylor et al. 1997, Maren, Aharonov et al. 1997) and the classic water maze (Logue, Paylor et al. 1997), with lesions of this structure producing deficits in the learning of these tasks. However, disruptions in neurogenesis within the dentate gyrus did not impair the ability of animals to acquire these tasks (Shors, Townsend et al. 2002, Jaholkowski, Kiryk et al. 2009). In contrast, studies have suggested that more difficult learning tasks rely on the dentate gyrus and are impaired when neurogenesis is decreased (Shors, Townsend et al.

2002). In fact, several studies have found that decreases in adult neurogenesis within the dentate gyrus negatively impact the ability to learn a form of contextual fear learning known as context discrimination (Saxe, Battaglia et al. 2006). Similarly, alterations in neurogenesis negatively impacts more challenging versions of the water maze in which the spatial cues around the maze are slowly eliminated (Nakashiba, Cushman et al. 2012).

In order to determine whether $Ca_V1.2$ differentially impairs hippocampal dependent learning tasks based on the difficulty of the task, we utilized a $Ca_V1.2$ conditional knockout mouse in which Cre recombinase expression was driven by the synapsin1 promoter which produces Cre expression broadly throughout neuronal populations (Zhu, Romero et al. 2001, Cui, Costa et al. 2008). $Ca_V1.2$ conditional knockout mice were examined for deficits in simple and complex versions of spatial and contextual learning tasks. Simple spatial and contextual learning tasks included contextual fear conditioning and the classic Morris water maze. In contrast, complex versions of these spatial and contextual learning tasks included context discrimination and a limited cues version of the Morris water maze. While the simple version of these tasks, fear conditioning and the Morris water maze, have been previously shown to be hippocampal dependent and dentate gyrus/neurogenesis independent, the complex version of the tasks, context discrimination and the limited cues water maze, are thought to be more difficult and therefore more likely to involve additional structures such as the dentate gyrus. We found that mice with neuronal deletion of $Ca_V1.2$ learned normally in context fear conditioning and the classic Morris water maze, but exhibited significant deficits in context discrimination and the limited cues water maze. To

determine whether these deficits could be due to a role of $Ca_V1.2$ in the dentate gyrus, $Ca_V1.2$ conditional knockout mice were assessed for alterations in cell division in the dentate gyrus. Interestingly, we found a decrease in cell proliferation, measured using 5-Bromodeoxyuridine (BrdU) labeling, and a decrease in immature neurons, measured by doublecortin staining in $Ca_V1.2$ conditional knockout mice. To determine whether these deficits in hippocampal dependent learning based on the difficulty of the task were specific to $Ca_V1.2$, or a characteristic of LVGCCs, we tested $Ca_V1.3$ global knockout mice for deficits in context discrimination and the limited cues water maze. We found that $Ca_V1.3$ global knockout mice did not show deficits in these two forms of hippocampal learning, supporting the theory that $Ca_V1.2$ regulates dentate gyrus dependent learning, cell proliferation and neurogenesis in a LVGCC subtype specific manner.

3.3 Materials and Methods

3.3.1 Mice

Studies were conducted using mice aged 3-7 months at the time of behavioral experimentation or tissue collection. Approximately equal numbers of males and females were used. Mice in each line were housed by sex and in groups of three to five. Throughout the course of all experiments, the investigator remained blind to the genotype of the mice. Mice were maintained in micro-isolation cages with a 14-h/10-h light/dark cycle with an average ambient temperature 22°C and *ad libitum* food and water. All experiments were conducted according to the National Institute of Health

guidelines for animal care and were approved by the University Committee on the Use and Care of Animals of the University of Michigan.

3.3.1.1 $Ca_V1.2$ conditional knockout mice

$Ca_V1.2$ conditional knockout mice with neuron-specific deletion of $Ca_V1.2$ and their wild-type littermates were used. Mice used in this study were on a B57Bl/6:129SvEv F2 genetic background. Mice with a floxed $Ca_V1.2$ exon two allele ($Ca_V1.2^{f/+}$ or $Ca_V1.2^{f/f}$) (White, McKinney et al. 2008) and maintained on a 129SvEv genetic background were first breed to transgenic mice expressing the Cre recombinase regulated by the synapsin1 promoter (Syn1-$Cre^{Cre/+}$) (Zhu, Romero et al. 2001, Cui, Costa et al. 2008) and maintained and a C57BL/6 background, producing an F1 cross. Using non-littermate offspring from the F1 cross, heterozygous floxed, Cre positive ($Ca_V1.2^{f/+}$ Syn1-$Cre^{Cre/+}$) mice were then crossed with heterozygous floxed, Cre negative ($Ca_V1.2^{f/+}$ Syn1-$Cre^{+/+}$) mice to produce homozygous floxed, Cre positive ($Ca_V1.2^{f/f}$ Syn1-$Cre^{Cre/+}$) conditional knockout mice as well as mice categorized as wild-type or control. Mice were considered wild-type if they were Cre positive, but lacked the floxed alleles ($Ca_V1.2^{+/+}$ Syn1-$Cre^{+/+}$). Mice were considered control if they were homozygous or heterozygous for the floxed allele and Cre negative ($Ca_V1.2^{f/f}$ Syn1-$Cre^{+/+}$; $Ca_V1.2^{f/+}$ Syn1-$Cre^{+/+}$), or if they were Cre positive but lacked the floxed alleles ($Ca_V1.2^{+/+}$ Syn1-$Cre^{Cre/+}$). For all experiments control mice were compared to and collapsed with wild-type mice when no significant difference was detected.

3.3.1.2 $Ca_V1.3$ knockout mice

$Ca_V1.3$ global knockout mice were generated using a targeted mutation of exon two of the $Ca_V1.3$ allele using a neomycin cassette (Platzer et al. 2000). Similar to $Ca_V1.2$ conditional knockout mice, $Ca_V1.3$ knockout mice used in this study were on a B57Bl/6:129SvEv F2 genetic background. Mice with the targeted mutation were maintained on a purebred 129SvEv background. Mice on a 129SvEv background heterozygous for the $Ca_V1.3$ mutant allele ($Ca_V1.3^{+/-}$) were breed to purebred B57Bl/6 mice to produce an F1 cross. Non-sibling heterozygous mice ($Ca_V1.3^{+/-}$) from the F1 cross were then crossed to produce an F2 cross containing homozygous $Ca_V1.3$ knockout mice ($Ca_V1.3^{-/-}$) and wild-type littermates ($Ca_V1.3^{+/+}$) for experimentation.

3.3.2 Behavioral Paradigms

3.3.2.1 Pavlovian Fear Conditioning

Fear conditioning experiments were conducted as previously described (Temme, Bell et al. 2014). Fear conditioning chamber were composed of clear acrylic backs and doors, aluminum sides, stainless steel grid floors spaced 1/8 inches, and stainless steel drop pans (Med Associates). Throughout experimentation, chambers and floor pans were cleaned with 70% ethanol. Chambers were illuminated using room lights set at 150 watts. Shocks were administered through the grid via solid-state shock scramblers and electronic constant-current shock sources. Shocks were controlled by a desktop PC running Actimetrics freezeframe software (Wilmette, IL). Behavior of each mouse was recorded and digitized using individual cameras mounted above each chamber using the Actimetrics freezeframe software. Mice were fear conditioned to a context using one training session per day for two day. Each fear conditioning session consisted of three

minutes of context exposure to the training chamber followed by three unsignalled-footshocks (0.5mA, 2sec) with thirty seconds between footshocks. Mice were then removed from the training chamber thirty seconds after the last footshock. Twenty-four hours after the last training session, mice were tested for fear to the trained context using five minutes of context exposure.

3.3.2.2 Context Discrimination

During context discrimination, mice were trained to discriminate between two contexts through exposure to both contexts each day for ten days, separated by a minimum of six hours. One context was termed context A and consisted of the fear conditioning chamber as described above, room lights at 150 watts, a scent of 70% ethanol and white noise. The second context was termed context B and was composed of red room lights, a scent of 2% acetic acid, no white noise, and the floor is the fear conditioning chamber was covered with a speckled rubber floor covering. In context A, mice were trained each day using three minutes of context exposure followed by one unsignalled footshock (2 sec, 0.5mA). Mice were removed from the conditioning chambers 30 seconds after the footshock. In context B, mice received context exposure for three minutes and thirty-two seconds, comparable to the time spent in context A with no unsignaled footshock. The order of exposure to contexts A and B was alternated each day. Mice were tested for their fear to contexts A and B on day ten using three minutes and 30 second exposure to each context in the absence of a footshock.

3.3.2.3 Classic Morris water maze

The Morris water maze was performed as previous described (McKinney and Murphy 2006, White, McKinney et al. 2008). The water maze was composed of a round white acrylic pool sized 1.2 meters in diameter. Throughout experimentation, the pool was filled with water that was made opaque using nontoxic white tempera paint and heated to 27 degrees Celsius. A round platform made of clear acrylic and measuring 10 cm in diameter was submerged just below the surface of the water, Mice were allowed to find the hidden platform using two training sessions per day for nine days. For each session, mice were released pseudo-randomly into the maze facing the outside of the maze and their time to find the platform was recorded. For all sessions, mice were given sixty seconds to find the platform after which point mice would be guided to the platform. Each day, mice were individually placed on the platform for fifteen seconds before the start of session one and session two. After the completion of both training sessions, mice were allowed to remain on the platform for a period of fifteen seconds before being placed back in their home cage. Mice were tested for their memory of the platform location using a probe test twenty four hours after the last day of training. During the probe test the platform is removed and the amount of time mice spend within a specific vicinity of the platform is measured. The probe test was performed by releasing each mouse individual into the pool opposite of the original platform location. Mice were then allowed to explore the maze for a period of sixty seconds before being removed near the original platform location.

3.3.2.4 Limited Cues water maze

During the limited cues water maze, a blue plastic barrier was used to encircle the entire water maze blocking all visual cues in the room except four discreet visual

cues that were mounted evenly around the maze. Mice were trained to the limited cues water maze using two sessions per day for twelve days. Aside from the increase in number of training days, training was conducted in the same manner as in the classic Morris water maze. Mice were tested for their memory of the platform location using two probe tests: one at the beginning of day nine of experimentation and one twenty-four hours after the last day of training. Probe tests were conducted as described in the classic Morris water maze.

3.3.3 Cell Labeling

3.3.3.1 BrdU

Mice were administered BrdU(100mg/kg) dissolved in sterile phosphate buffered saline (PBS), via intraperitoneal injections, once a day for five days in order to assess cell birth rates in the dentate gyrus. Mice were anesthetized using isoflurane prior to each BrdU injection. Roughly twenty-four hours after the last injection, mice were perfused using 0.9% sodium chloride followed by ice cold 4% paraformaldehyde in PBS. Brains were then removed and post-fixed overnight in 4% paraformaldehyde after which time they were transferred to 30% sucrose for a minimum of 48 hours or until saturation of the brain in sucrose allowed the brains to sink in solution. The brains were then frozen on dry ice, and coronal slices were made at 40μm, and stored, free floating, in cryoprotectant buffer at -20 °C for later use. Brains sections were labeled for BrdU expression using immunofluorescent histochemistry. Selected tissue sections were rinsed of cryoprotectant buffer using an overnight wash in Tris (pH7.4). Sections were then washed using Tris buffered saline (TBS, pH7.4) and incubated in 2M HCl for 30

minutes at 32 °C to denature the DNA. Sections were then neutralized using 0.1M Sodium Borate for 10 minutes (pH 8.5). Following neutralization, sections were again rinsed in TBS followed by incubation in blocking buffer (10% normal horse serum and 0.3% Triton X-100 in TBS) for 1 hour and incubation in sheep anti-BrdU (1:250,abcam #ab1893) in blocking buffer overnight at 4°C. The following day, slices were washed in TBS and incubated for 1.5 hours in donkey anti-sheep (Alexa Fluor 594, 1:200, abcam #ab150184) at room temperature. Once labeled, sections were washed in TBS, mounted, and cover slipped using anti-fade containing dapi (Prolong Gold, Molecular Probes #P36931).

3.3.3.2 Doublecortin

Doublecortin studies were performed using tissue collected from BrdU injected mice. Tissue sections were washed in a 1X Tris buffer (pH 7.6) prior to incubation in 1% H_2O_2 for 30 minutes to quench endogenous peroxidase activity. Sections were then washed in the 1X Tris buffer and cells were permeabilized using 0.1% Triton in Tris for 15 minutes. Sections were then incubated in a solution of 0.05% Bovine Serum albumin and 0.1% Triton in Tris for 15 minutes prior to blocking in a blocking buffer solution of 0.05% Bovine Serum albumin, 10% natural horse serum, and 0.1% Triton in Tris for one hour. After blocking, tissue was rinsed in 0.1% Triton X-100 in Tris and 0.1% Triton X-100 with 0.05% Bovine Serum albumin in Tris for a period of 15 minutes each prior to incubation in rabbit anti-doublecortin (1:1000; Abcam #AB18723) in blocking buffer solution overnight at 4°C. Sections were again rinsed in 0.1% Triton X-100 in Tris and 0.1% Triton X-100 with 0.05% Bovine Serum albumin in Tris for a period of 15 minutes each prior to incubation in a biotinylated anti-rabbit IgG (1:200;Vector

Laboratories #BA-1000) for 2 hours at room temperature. Sections were then briefly rinsed in 0.1% Triton X-100 with 0.05% Bovine Serum albumin in Tris and incubated with avidin-biotin complex (1:1000; ABC Kit, Vector Laboratories #PK-6100, Burlingame, CA) in the same solution for a period of 1 hour. Tissue was then incubated in diaminobenzidene with nickel according to manufacturer instructions (Vector Laboratories #SK-4100, Burlingame, CA) for approximately 2 minutes and washed four times in 1xTris. Sections were then mounted and cover slipped.

3.3.3.3. BrdU and Doublecortin Cell Counting

Brain slices labeled for BrdU and Doublecortin were imaged at 10x magnification using a 1344 x 1024 CCD camera (Orca-ER, Hamamatsu; Hamamatsu City, Japan) on a LeicaDMI6000B microscope (Leica; Wetzlar, Germany). Images were subsequently analyzed offline using ImageJ (ImageJ 1.48v; National Institute of Health, USA). Individual images of the granule cell layer were concatenated and a region of interest (ROI) consisting of labeled cells within 35µm of the subgranular zone was selected and straightened. The number of cells positive for BrdU or Doublecortin were then counted visually by an experimenter blind to genotype and divided by the ROI producing a density value. Density values were then normalized to the density of BrdU positive or doublecortin positive cells in wild-type mice.

3.3.3.4. Dentate Gyrus Granular Cell Layer Measurements

The overall width of the granular cell body layer of the dentate gyrus was assessed using a custom designed Matlab code designed by Jacob Temme (Temme, Wabel et al. 2015) to calculate an average width for a region of interest. For dentate

gyrus images, the code was designed to identify the cell body layer of both the superior and inferior blades of the dentate gyrus. The width of the cell body layer of each blade was calculated along the length of each blade and averaged. The average cell body layer width for each blade was then average by individual dentate gyri prior to averaging by mouse then genotype.

3.3.4 Statistical Analysis

Analysis of behavioral experiments was carried out using repeated measures ANOVAs, two way ANOVA and planned unpaired t-tests comparing $Ca_V1.2$ conditional knockout mice with their wild-type counterparts. Learning across fear conditioning discrimination ratio, the classic Morris water maze, the limited cues water maze, and the visible platform test in the limited cues water maze were analyzed using repeated measures ANOVA, with genotype and training as factors. Discrimination freezing, and platform preference during water maze probe tests were analyzed using a two-way ANOVA. Probe test values were further analyzed using a fisher post-hoc test. The context test and differences in BrdU and doublecortin cell densities were analyzed using an unpaired t-test. In experiments in which values were compared to chance, a one group t-test was utilized.

3.4 Results

3.4.1 Conditional deletion of $Ca_V1.2$ does not affect simple hippocampal dependent learning

Mice in which deletion of Cav1.2 is restricted to glutamatergic neurons in the forebrain do not exhibit deficits in either contextual fear conditioning (McKinney, Sze

et al. 2008) or the Morris water maze twenty-four hours after training (White, McKinney et al. 2008). However, it is possible that deletion of $Ca_V1.2$ in neurons throughout the brain could generate deficits in these forms of learning. Using the synapsin1-cre CaV1.2 conditional knockout mice, we first investigated the role of $Ca_V1.2$ in contextual fear conditioning. Mice were fear conditioned to a context across two days using one session per day. Each session consisted of three minutes of context exposure followed by three unsignalled footshocks (0.5mA, 2sec) with an intershock interval of 30 seconds. Acquisition and consolidation of fear across training days was analyzed by comparing the average percent freezing during the three minutes of context exposure for each day (**Figure 3.1A**). Analysis of context conditioning using a two way ANOVA found a significant effect of training day ($F_{(1,32)} = 66.582$, $p < 0.0001$), but not genotype ($F_{(1,32)} = 0.121$, $p = 0.7302$). Both wild-type and $Ca_V1.2$ conditional knockout mice showed an increase freezing across training with an average percent freezing of 0.2 % on day one and 34 % on day two in wild-type mice and 1 % on day one and 31 % on day two in Cav1.2 conditional knockout mice. Twenty-four hours after the last training session, mice were tested for their fear to the context using five minutes of context exposure. Analysis of freezing behavior during context testing using an unpaired t-test found no significant difference between genotypes ($p = 0.8618$) with an average percent freezing of 63 % in wild-type mice and 61 % in Cav1.2 conditional knockout mice (**Figure 3.1B**). This data suggests normal fear conditioning in mice lacking $Ca_V1.2$ in excitatory neurons in the forebrain (McKinney, Sze et al. 2008).

To further explore the effects of $Ca_V1.2$ on hippocampal dependent learning, we tested $Ca_V1.2$ conditional knockout mice in the classic Morris water maze task (Morris

1984). In the classic Morris water maze, mice learn to use the complex spatial cues located around the water maze to locate a hidden platform placed just below the surface of the pool (**Figure 3.2A**). Mice were trained to find the location of the hidden platform using two sessions per day for nine days. During each session, mice were pseudo-randomly released into the pool and allowed sixty seconds to locate the hidden platform. Roughly twenty-four hours after the last day of training, mice were tested for their spatial memory of the platform location using a probe test. Analysis of the latency to find the hidden platform across training days using a repeated measures ANOVA exhibited a significant reduction in latency to find the platform across training days ($F_{(8,320)} = 25.883$, $p < 0.0001$) with a decrease in average latency from 50.098 seconds to 23.417 seconds in wild-types and 53.577 seconds to 19.500 seconds in $Ca_V1.2$ conditional knockout mice, but no difference between genotypes ($F_{(1,40)} = 0.2102$, $p = 0.1549$) (**Figure 3.2B**).

During the probe test, mice were analyzed for the percent of time spent in the location in which the platform had been located. Exploration in the probe test is analyzed by splitting the maze into four quadrants with the original platform location in the center of one quadrant, titled the target quadrant. Percent time spent in the target quadrant is then compared to percent time spent in the other quadrants and chance, 25%. During the probe test, both $Ca_V1.2$ conditional knockout mice and their wild-type littermates spent a significant percent of time in the target quadrant compared to chance ($p < 0.0001$ and $p < 0.0001$, respectively, one sided t-test) Analysis of the percent of time spent in each quadrant between quadrants, using a repeated measures ANOVA, revealed a significant effect of quadrant ($F_{(3,120)} = 26.245$, $p < 0.0001$), but not genotype

($F_{(1,40)}$ = 0.327, p = 0.5708). Further analysis using a fisher post-hoc test revealed a significant difference between percent time spent in the target quadrant and all other quadrants (p < 0.0001, all quadrants) (**Figure 3.2C**). This is further illustrated using a heat map to illustrate the average percent time spent throughout the probe test (**Figure 3.2D**). Examination of the heat map revealed a large period of time in the target quadrant where the hidden platform was located. A significant amount of time was also noted in the opposite quadrant which corresponded with the location at which mice were released into the pool and likely represents the brief period during which the mice spent orienting themselves from the wall of the pool. Similar to previously published literature, this data suggests that $Ca_V1.2$ is not involved in spatial learning in the classic Morris water maze when assessed twenty-four hours after training (White, McKinney et al. 2008). Taken together with the data demonstrating no effects of deletion of $Ca_V1.2$ on fear conditioning to a context, this suggests that $Ca_V1.2$ does not affect these forms of learning and memory, previously found to be neurogenesis independent forms of hippocampal dependent learning tasks (Shors, Townsend et al. 2002, Jaholkowski, Kiryk et al. 2009).

3.4.2 Neuronal deletion of $Ca_V1.2$ produces deficits in neurogenesis associated learning tasks

To determine whether neuronal deletion of $Ca_V1.2$ produces deficits in more difficult versions of hippocampal dependent learning $Ca_V1.2$ conditional knockout mice were tested in two alternate versions of contextual and spatial learning, context discrimination and a limited cues version of the Morris water maze. During context discrimination, mice were trained to discriminate between two contexts, contexts A and

context B, through exposure to each context once a day for ten days (**Figure 3.3A**). For nine days, context A, was paired with an unsignalled footshock (0.5mA, 2sec), on day ten, mice were placed in context A, but no footshock was given. For all ten days mice were placed in context B for the same period of time as context A without a footshock and the order in which the mice experienced each context was counterbalanced. Discrimination between contexts was calculated as a discrimination ratio, calculated as the average percent of freezing in the trained context divided by the total percent freezing in both contexts per day. Data was analyzed as the average of every two days to control for time of day effects. Due to the order in which the contexts were presented, mice were analyzed for a freezing response to context A on day one prior to the first pairing of the context and footshock, while the first exposure to context B occurred after the pairing of context A with the footshock. Therefore, the context discrimination ratio from days 1-2 do not accurately represent the measurement of discrimination between contexts. Analysis of context discrimination ratios across training days using a two way repeated measures ANOVA revealed a significant effect of genotype ($F_{(1,33)} = 5.183$, $p = 0.0294$) with higher discrimination ratios seen in wild-type (**Figure 3.3B**). While wild-type mice showed a significant discrimination ratio above 0.5 starting on days 3-4 of context discrimination ($p = 0.0182$, one group t-test), $Ca_V1.2$ conditional knockout mice exhibited a discrimination ratios that were no better than chance up until days 9-10 of training ($p = 0.0159$, one group t-test). Additionally, analysis of context discrimination through the comparison of freezing levels between the two contexts during discrimination test using a two way ANOVA revealed a significant effect of genotype ($F_{(1,?)} = 15.659$, $p = 0.0002$) and context ($F = 12.661$, $p = 0.0007$) (**Figure**

3.3C). Further analysis of freezing during the context discrimination test using a planned unpaired t-test revealed significantly higher levels of freezing to the trained context A versus the similar context B in wild-type mice ($p = 0.0029$) , but not in $Ca_V1.2$ conditional knockout mice ($p = 0.0680$). These data suggest a moderate, but significant deficit in context discrimination in mice with a pan-neuronal deletion of $Ca_V1.2$.

To further investigate the effects of neuronal deletion of $Ca_V1.2$ in a more difficult spatial learning task, we assessed the performance of $Ca_V1.2$ conditional knockout mice in a version of the water maze we refer to as the "limited cues" water maze. During the limited cues water maze, complex spatial cues around the room were eliminated using a blue plastic barrier, in their place, four discreet cues were spaced evenly around the maze (**Figure 3.4A**). Mice were trained to the limited cues water maze using two, sixty second training session per day, for eleven days. Training sessions were performed as in the classic Morris water maze. Mice were probed for their preference for the platform location on day nine, similar to the classical water maze, and on day twelve, twenty four hours after the last training session.

Analysis of the latency to find the hidden platform across training using a repeated measures ANOVA and genotype and training day as factors revealed a significant effect of both genotype ($F_{(1.30)} = 12.362$, $p = 0.0014$) and training day ($F_{(13,390)} = 8.343$, $p < 0.0001$) with $Ca_V1.2$ conditional knockout mice exhibiting a significant deficit in training compared to wild-type mice (**Figure 3.4B**). While wild-type mice showed a decrease in latency to find the hidden platform from 52.097 seconds on day one to 21.923 seconds on day eleven, $Ca_V1.2$ conditional knockout mice

only showed a minor improvement in latency to find the hidden platform from 53.348 seconds on day one to 38.207 seconds on day eleven. During the probe test one, on day nine of training, neither wild-type nor $Ca_V1.2$ conditional knockout mice showed a significant preference for the target quadrant, represented as percent time, compared to chance ($p = 0.2297$ and $p = 0.9809$, respectively; one group t-test) (**Figure 3.4C**). Comparison of the percent time spent in the target quadrant and other quadrants between genotypes using a repeated measures ANOVA revealed no significant difference between genotype ($F_{(1,30)} = 0.844$, $p = 0.3657$), but a significant effect of quadrant ($F_{(3,90)} = 9.099$, $p < 0.0001$). Further analysis using a Fisher post-hoc test found significantly greater percent of time spent in the opposite quadrant, the quadrant in which they were released, compared to all other quadrants ($p = 0.0190$, target; $p < 0.0001$, AL; $p = 0.0002$, AR). This data demonstrates that, after eight days of training, neither $Ca_V1.2$ conditional knock mice nor wild-type mice have a spatial memory for the platform location and suggest that this version of the water maze is indeed more difficult to learn. During probe test two, on day twelve of experimentation, wild-type mice exhibited a significant preference for the target quadrant compared to chance ($p = 0.0021$) (**Figure 3.4D)**. However, $Ca_V1.2$ conditional knockout mice did not spend significantly more time searching in the target quadrant compared to chance ($p = 0.8677$). Comparison of the percent time spent in each quadrant for each genotype using a repeated measure ANOVA revealed a significant effect of quadrant in wild-type mice ($F_{(1,30)} = 0.006$, $p = 0.9386$), but not $Ca_V1.2$ conditional knockout mice ($F_{(3,90)} = 3.286$, $p = 0.0243$). Further analysis using a fisher post-hoc test revealed significantly more time spent in the target quadrant compared to all other quadrants ($p < 0.0001$, AL; $p =$

0.0003, AR; p = 0.0112, Opp). These data suggest that wild-type, but not $Ca_V1.2$ conditional knockout mice have formed a spatial memory of the platform location. Following probe test two, $Ca_V1.2$ conditional knockout mice and wild-type mice were tested for their ability to find a visible platform across six trials (**Figure 3.4E**). Poor performance on the visible platform could suggest an inability for mice to properly view the spatial cues around the maze. Comparison of the latency to find the visible platform between genotypes using a repeated measures ANOVA revealed no significant difference in latency to find the platform between genotypes ($F_{(1,30)} = 2.424$, $p = 0.1300$) but a significant effect of training trials ($F_{(5,150)} = 3.010$, $p = 0.0128$) suggesting that deficits in spatial learning in $Ca_V1.2$ conditional knockout mice are not likely due to a deficit in their ability to see the visible cues or their ability to perform the task. Taken together, this data suggests that neuronal deletion of $Ca_V1.2$ produces deficits in difficult hippocampal tasks, such as those associated with dentate gyrus neurogenesis (Shors, Townsend et al. 2002, Saxe, Battaglia et al. 2006).

3.4.3 Neurogenesis and Cell Division in the Dentate Gyrus

LVGCCs has been implicated in cell proliferation and neurogenesis prenatally (D'Ascenzo, Piacentini et al. 2006, Piacentini, Ripoli et al. 2008, Brustein, Cote et al. 2013) and into adulthood (Deisseroth, Singla et al. 2004, Luo, Zhu et al. 2005, Zhu, Zhou et al. 2012) using pharmacological blockade of LVGCCs. Given the ties of context discrimination and difficult hippocampal tasks to adult neurogenesis in the dentate gyrus (Saxe, Battaglia et al. 2006, Sahay, Scobie et al. 2011), we hypothesized that the deficits we observed in the $Ca_V1.2$ conditional knockout mice may reflect changes in cell proliferation and neurogenesis in the dentate gyrus. To determine

whether neuronal deletion of $Ca_V1.2$ alters cell proliferation and adult neurogenesis, we labeled dividing cells in the dentate gyrus in naïve mice using five days of 100 mg/kg Bromodeoxyuridine (BrdU) (del Rio and Soriano 1989) injections administered via intraperitoneal (i.p.) injections. Twenty-four hours after the final BrdU injection, mice were perfused and sections containing the subgranular zone of the dentate gyrus were collected and processed for BrdU using a red fluorescent secondary antibody (**Figure 3.5A$_1$, 3.5A$_2$**). The subgranular zone of the dentate gyrus was isolated for each image (**Figure 3.5B$_1$, 3.5B$_2$**). BrdU positive cells were counted and normalized to the average density of BrdU in three to four month old wild-type mice. Analysis of the density of BrdU positive cells between genotypes revealed a significant decrease in BrdU density in $Ca_V1.2$ conditional knockout mice compared to wild-type mice ($p = 0.0447$, unpaired t-test) with a decrease in BrdU positive cell density of 24.2% in $Ca_V1.2$ conditional knockout mice (**Figure 3.6B**). To determine whether neuronal deletion of Cav1.2 also altered the size of the dentate gyrus, we calculated the average width of the granular cell layer for each image mice (**Figure 3.6C**). Analysis of the width of the cell body layer of the dentate gyrus using an unpaired t-test revealed no significant difference between genotypes ($p = 0.8782$).

To determine whether this decrease in cell proliferation was associated with a decrease in immature neurons, we performed immunohistochemistry on sections from mice injected with BrdU for a marker of immature neuronal populations, doublecortin (Brown, Couillard-Despres et al. 2003) (**Figure 3.6A$_1$, 3.6A$_2$**). Similar to BrdU studies, doublecortin analysis was limited to the subgranular zone of the dentate gyrus. Analysis of the number of immature neurons in the dentate gyrus using doublecortin staining in

the dentate gyrus revealed a significant decrease in the density of doublecortin labeled cells($p = 0.0430$) with $Ca_V1.2$ conditional knockout mice exhibiting a decrease in doublecortin labeled cells of 34.7% compared to wild-type mice (**Figure 3.6B**). From these studies, it appears that neuronal deletion of $Ca_V1.2$ decreases cell proliferation and neurogenesis in the dentate gyrus which could result in deficits in hippocampal dependent learning, such as that seen in $Ca_V1.2$ conditional knockout mice.

3.4.4. Deletion of $Ca_V1.3$ does not alter complex spatial and contextual learning

To determine whether the deficits in difficult hippocampal-dependent learning in $Ca_V1.2$ conditional knockout mice were specific to $Ca_V1.2$, or a property of L-type voltage gated calcium channels as a whole, we tested mice with global deletions of $Ca_V1.3$ in context discrimination and the limited cues version of the Morris water maze. Context discrimination and limited cues water maze were performed in the same manner for $Ca_V1.3$ global knockout mice as for $Ca_V1.2$ conditional knockout mice. During context discrimination, $Ca_V1.3$ global knockout mice and their wild-type counterparts exhibited significant discrimination between context A and context B starting on days 3-4 of discrimination training, measured as a discrimination ratio greater than 0.5 using one group t-tests ($p = 0.0013$ and $p < 0.0001$ respectively) (**Figure 3.7A**). Additionally, comparison of the discrimination ratios between genotypes across discrimination training using a repeated measures ANOVA revealed no significant differences between genotypes ($F_{(1,60)} = 1.345$, $p = 0.2508$). Analysis of the percent freezing on days 9-10 using a two way ANOVA revealed no significant difference between genotypes ($F_{(1,34)} = 0.3928$, $p = 0.3928$), but a significant effect of context ($F_{(1,34)} = 7.824$, $p = 0.0084$) with lower levels of freezing in the similar context

B than the trained context A (**Figure 3.7B**). When trained in the limited cues water maze, $Ca_V1.3$ conditional knockout mice exhibited a similar reduction in latency to find a hidden platform as wild-type mice ($F_{(1,18)} = 2.437$, $p = 0.1359$, repeated measures ANOVA) (**Figure 3.7C**). Additional analysis also revealed a significant effect of training day on the latency to find the platform ($F_{(11,198)} = 9.535$, $p < 0.0001$). When mice were probed for their spatial memory of the platform location on day nine of experimentation, both wild-type mice and $Ca_V1.3$ knockout mice spent more time in the target quadrant than chance ($p = 0.0400$ and $p = 0.0274$ respectively, one group t-test) (**Figure 3.7D**). Additionally, analysis of percent time spent in the quadrants between genotypes using a repeated measures ANOVA revealed a significant effect of quadrant ($F_{(3,54)} = 7.045$, $p = 0.0004$), but not genotype ($F_{(1,18)} = 1.967$, $p = 0.1777$). Taken together, these studies suggest that $Ca_V1.3$ does not play a role in dentate gyrus associated tasks and that the effects of deletion of $Ca_V1.2$ on dentate gyrus associated tasks is specific to $Ca_V1.2$ versus L-type voltage gated calcium channels as a class.

3.5 Discussion

Using transgenic mice with a pan-neuronal deletion of $Ca_V1.2$, we investigated the role of the L-type voltage gated calcium channel subtype $Ca_V1.2$, in complex versus simple forms of hippocampal dependent learning. We found that $Ca_V1.2$ conditional knockout mice exhibited significant deficits in difficult hippocampal tasks, such as those associated with neurogenesis in the dentate gyrus, but normal learning in hippocampal tasks thought to be independent of neurogenesis. Additionally, examination of the dentate gyrus morphology in $Ca_V1.2$ conditional knockout mice

revealed decreased levels of cell proliferation and adult neurogenesis which could serve as a mechanism for the observed behavioral deficits.

We tested $Ca_V1.2$ conditional knockout mice in two types of simple hippocampal dependent learning tasks: Pavlovian fear conditioning and the classic Morris water maze. When fear conditioned, $Ca_V1.2$ conditional knockout mice exhibited normal acquisition, consolidation, and expression of fear to a context. We also found that $Ca_V1.2$ conditional knockout mice acquired a spatial memory of the location of a hidden platform in the classic Morris water maze at comparable rates and levels as that observed in wild-type mice.

Additionally, we tested $Ca_V1.2$ conditional knockout mice in more difficult versions of these hippocampal dependent tasks. During context discrimination mice were trained to discriminate between two contexts, one which was paired with a footshock and one which was not. When tested in the context discrimination test, $Ca_V1.2$ conditional knockout mice exhibited a significant deficit in the discrimination of the two contexts throughout training compared to their wild-type littermates. While wild-type mice were able to discriminate between the two contexts early in training, as seen by a discrimination ratio above 0.5 on days 3-4, $Ca_V1.2$ conditional mice were unable to discriminate between the two contexts until days 9-10. Despite a discrimination ratio significantly above 0.5, analysis of the percent of time freezing in the two contexts across days nine and ten revealed high levels of freezing in the untrained context as well as the trained context in $Ca_V1.2$ conditional knockout mice. Next, $Ca_V1.2$ conditional knockout mice were tested in the limited cues water maze, in which the number and diversity of spatial cues are limited. In the limited cues water

maze, $Ca_V1.2$ conditional knockout mice exhibited a significant deficit in the latency to find the hidden platform across training days compared to wild-type mice. Additionally, $Ca_V1.2$ conditional knockout mice failed to show a preference for the target quadrant when probed for their memory for the platform location after days nine and days twelve of training.

In this set of studies, we have defined simple hippocampal dependent learning tasks as those tasks previously found to be dependent on the hippocampus proper, but independent of neurogenesis in the dentate gyrus. Consistent with this definition, previous studies investigating the role of neurogenesis within the dentate gyrus and learning and memory have suggested that there is no effect of decreased neurogenesis on contextual fear conditioning or the Morris water maze (Shors, Townsend et al. 2002, Jaholkowski, Kiryk et al. 2009). In addition, we defined our difficult hippocampal tasks as similar tasks, which involved similar brain structures, but were more cognitively challenging and therefore required additional training in order to learn. Studies suggest that hippocampal dependent tasks that are more difficult are more likely to require the dentate gyrus and adult born neurons within this structure (Shors, Townsend et al. 2002). This appears to be the case for context discrimination in which decreases in neurogenesis impair an animal's ability to learn to discriminate between two contexts (Saxe, Battaglia et al. 2006), while increases in neurogenesis enhance an animal's performance in this task (Nakashiba, Cushman et al. 2012). While there is no direct literature investigating the effects of adult neurogenesis on the limited cues water maze, we believe that the limited cues water maze is more difficult than the classic water maze. We found that while wild-type mice were able to learn the location of a hidden

platform in the classic Morris water maze after only 8 days of training, wild-type mice trained in the limited cues version of the water maze exhibited chance performance after eight days of training and required an additional three days of training (six additional training sessions) to learn the location of the platform. These data are consistent with several studies suggesting an increased role of adult neurogenesis on complex, more difficult versions of the Morris water maze (Nakashiba, Cushman et al. 2012).

Previous studies investigating the role $Ca_V1.2$ in hippocampal dependent learning have yielded conflicting results. However, given our current findings, supporting a role of $Ca_V1.2$ in difficult, but not simple version of hippocampal learning, both sets of literature would appear to be correct. Normal spatial and contextual learning in $Ca_V1.2$ conditional knockout mice in simple hippocampal learning tasks in the current study supports previous studies which reported normal hippocampal learning in mice lacking $Ca_V1.2$ in excitatory neurons in the forebrain in fear conditioning (McKinney, Sze et al. 2008) and twenty-four hour spatial learning in the Morris water maze (White, McKinney et al. 2008). The presence of impairments of $Ca_V1.2$ conditional knockout mice in complex learning in the current study would also be consistent with a previous report which utilized $Ca_V1.2$ conditional knockout mice in which Cre recombinase expression was driven by the NEX promoter and describes deficits in a visible platform version of the Morris water maze and the labyrinth maze (Moosmang, Haider et al. 2005). In the visible platform version of the water maze task mice must learn to discriminate the spatial location of two visible platforms, one which is fixed and another which sinks (Arns, Sauvage et al. 1999, Steckler, Weis et al. 1999, Kleppisch, Wolfsgruber et al. 2003, Moosmang, Haider et al. 2005). In the labyrinth

maze, mice learned to transverse a brightly lit maze in order to be returned to their shadowed home cages. The maze was made up of nine interactions at which point mice must make a spatial decision in direction (Tang, Shimizu et al. 1999, Moosmang, Haider et al. 2005) Though the exact difficulty of these tasks in comparison to other hippocampal dependent tasks is hard to assess and no studies have been done to examine the role of neurogenesis within the dentate gyrus in this task, impairments of $Ca_V1.2$ in difficult but not simple hippocampal learning tasks may explain the discrepancy in previous literature examining the role of $Ca_V1.2$ in learning.

Due to the link between hippocampal learning of difficult tasks and the birth of new neurons in the dentate gyrus (Shors, Townsend et al. 2002), in particular the strong link drawn between context discrimination and neurogenesis (Saxe, Battaglia et al. 2006, Nakashiba, Cushman et al. 2012), we sought to investigate the potential role of $Ca_V1.2$ in adult born neurogenesis in the dentate gyrus. We labeled dividing cells in the dentate gyrus using BrdU and immature adult born neurons using doublecortin. Our studies found that mice with neuronal deletion of $Ca_V1.2$ had decreased levels of cell proliferation and immature adult-born neurons in the dentate gyrus compared to wild-type mice. While these data do not directly examine rates of neurogenesis in $Ca_V1.2$ conditional knockout mice, a reduction in both cell proliferation and immature neurons strong suggests a decrease in adult neurogenesis associated with deletion of $Ca_V1.2$. We believe that these decreases in cell proliferation and immature neurons in $Ca_V1.2$ conditional knockout mice could be sufficient to produce deficits in hippocampal learning dependent on the dentate gyrus, such as in context discrimination and, we believe, the limited cues water maze. Pharmacological studies have found a potential

link between L-type voltage gated calcium channels and cell proliferation and neurogenesis prenatally (D'Ascenzo, Piacentini et al. 2006, Piacentini, Ripoli et al. 2008, Brustein, Cote et al. 2013) and into adulthood (Deisseroth, Singla et al. 2004, Luo, Zhu et al. 2005, Zhu, Zhou et al. 2012) using pharmacological blockade of LVGCCs. In the case of adult neurogenesis, studies found that blockade of LVGCCs prevented induced cell proliferation (Zhu, Zhou et al. 2012) and neurogenesis (Deisseroth, Singla et al. 2004, Luo, Zhu et al. 2005). Additionally, activation of LVGCCs using channel agonists increased the percent of neural progenitor cells that survived to become adult neurons (Deisseroth, Singla et al. 2004). However, these studies provide little information about the role of LVGCCs in basal levels of cell proliferation and neurogenesis in adulthood in vivo. Additionally, nothing is known about individual LVGCC subtypes in these processes. Therefore, the results from the current study, implicating $Ca_V1.2$ in the maintenance of cell proliferation and with a decreased number of immature neurons, suggestive of decreased neurogenesis, represent to our knowledge, the first study to directly implicate $Ca_V1.2$ specifically in basal neurogenesis and in the adult dentate gyrus.

Given the similarities between L-type voltage gated calcium channel subtypes, $Ca_V1.2$ and $Ca_V1.3$, we wanted to determine whether deficits in difficult hippocampal learning tasks were specific to $Ca_V1.2$ conditional knockout mice or were shared by other L-type voltage gated calcium channel subtypes, specifically $Ca_V1.3$ global knockout mice $Ca_V1.2$ and $Ca_V1.3$ share significant channel homology and are both found within the hippocampus proper and dentate gyrus (Hell, Westenbroek et al. 1993). However, differences in $Ca_V1.2$ and $Ca_V1.3$ properties such as activation

voltages (Xu and Lipscombe 2001), rates of inactivation (Xu and Lipscombe 2001), and even distribution within neurons (Hell, Westenbroek et al. 1993) may represent a differential function of these channels in a variety of neuronal functions. Previous studies of the role of $Ca_V1.3$ in hippocampal dependent, neurogenesis independent learning found mild deficits in contextual fear conditioning, which could be overcome with additional training, but normal performance in the classic Morris water maze (McKinney and Murphy 2006). The presence of deficits in contextual fear learning, but not spatial learning suggests an amygdala associated deficit and normal hippocampal function. Our studies found that $Ca_V1.3$ knockout mice showed normal fear and discrimination learning during the context discrimination task and a similar decrease in latency to find a hidden platform and a preference for the platform quadrant as wild-types in the limited cues water maze. These data, along with previous literature, suggest normal learning in both simple and difficult hippocampal dependent tasks, in terms of hippocampal function. Given the differences in LVGCC subtypes $Ca_V1.2$ and $Ca_V1.3$ this discrepancy in the role of each subtype in various learning tasks is not unexpected. Importantly, these data also indicate that $Ca_V1.2$, and not $Ca_V1.3$, modulates difficult hippocampal learning and that it likely does so in a $Ca_V1.2$ specific manner.

While we note decreases in cell proliferation and immature adult-born neurons in the dentate gyrus and deficits in difficult, seemingly neurogenesis dependent, hippocampal learning tasks, it is possible that these two observations are correlational in nature. The structures involved in context discrimination and the limited cues water maze are likely numerous. Given that $Ca_V1.2$ is expressed throughout the brain it is possible that deletion of this channel may impact multiple structures which in turn could

differentially effect context discrimination and limited cues water maze. However, given that we did not observe deficits in $Ca_V1.2$ conditional knockout mice in simpler versions of these tasks, which likely involve similar structures, this seems unlikely. However, studies directly deleting $Ca_V1.2$ in the dentate gyrus alone or, more specifically, in new born neurons within the dentate gyrus could more directly examine the role of $Ca_V1.2$ in this structure and in neurogenesis associated learning tasks. Finally it should be noted that the synapsin1 promoter to drive Cre expression used in our $Ca_V1.2$ conditionally knockout mice is activated prenatally, at embryonic age E12-E15 (Zhu, Romero et al. 2001). Therefore, effects of loss of $Ca_V1.2$ on complex hippocampal learning could be due to altered neuronal development in the absence of $Ca_V1.2$ in structures throughout the brain, including the dentate gyrus. This early alteration in neuronal development could produce impairments in difficult hippocampal tasks through changes in the function of neurons in a number of structures, including the dentate gyrus and in this way may also alter neurogenesis. Specific deletion of $Ca_V1.2$ in neurogenesis, or an inducible form of Cre, which could be turned on in adulthood, would likely address this question.

The data presented here have provided strong evidence that $Ca_V1.2$ does in fact mediate hippocampal dependent learning, but does so based on the task difficulty and likely the hippocampal structures involved. Additionally, we have demonstrated an important candidate role of $Ca_V1.2$ in modulating adult neurogenesis in the hippocampal structure, the dentate gyrus and this modulation as the mechanism by which $Ca_V1.2$ mediates hippocampal dependent learning. More work needs to be performed to determine the true mechanism by which $Ca_V1.2$ mediates neurogenesis

and whether direct deletion of $Ca_V1.2$ in these cells, or dentate gyrus is sufficient to replicate the results seen here. However, this work provides important insights into a previously uninvestigated and unknown mechanism of $Ca_V1.2$ in modulating hippocampal function and learning.

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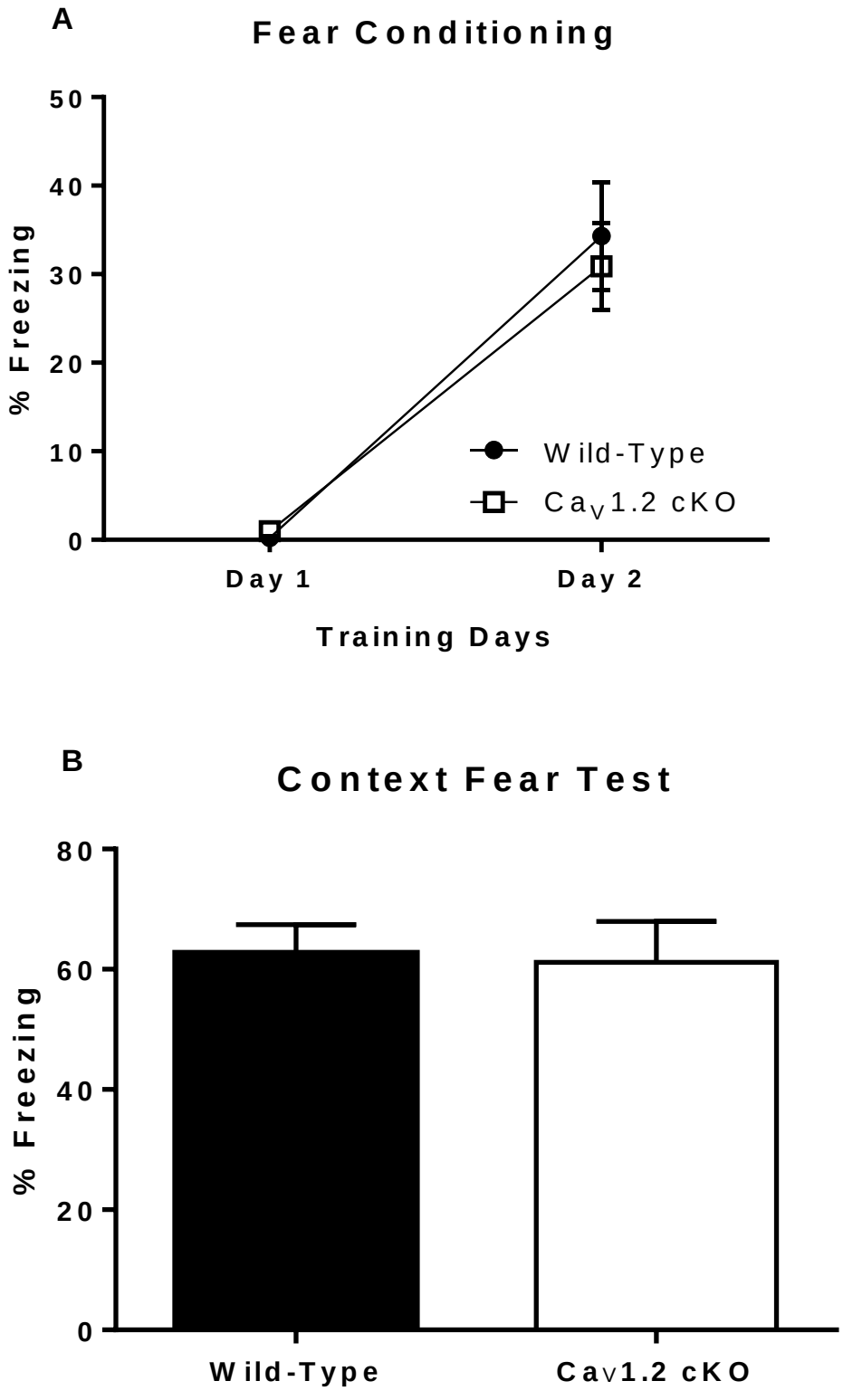
Figure 1


A
Fear Conditioning
50
40
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20
10
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% Freezing
Wild-Type
Ca$_V$1.2 cKO
Day 1
Day 2
Training Days
B
Context Fear Test
80
60
40
20
0
% Freezing
Wild-Type
Cav1.2 cKO

Figure 3.1: Neuronal deletion of $Ca_V1.2$ does not impair fear acquisition or consolidation to a conditioned context

Mice were fear conditioning to a context using three unsignaled footshocks per day for two day. (**A**) Both $Ca_V1.2$ conditional knockout mice (n = 17) and wild-type mice (n = 17) exhibited a significant enhancement in freezing to the conditioned context between days one and two of training. (**B**) When tested for their fear to the context, $Ca_V1.2$ conditional knockout mice exhibited high levels freezing comparable to wild-type mice. Data are represented as mean ± SEM.

Figure 2

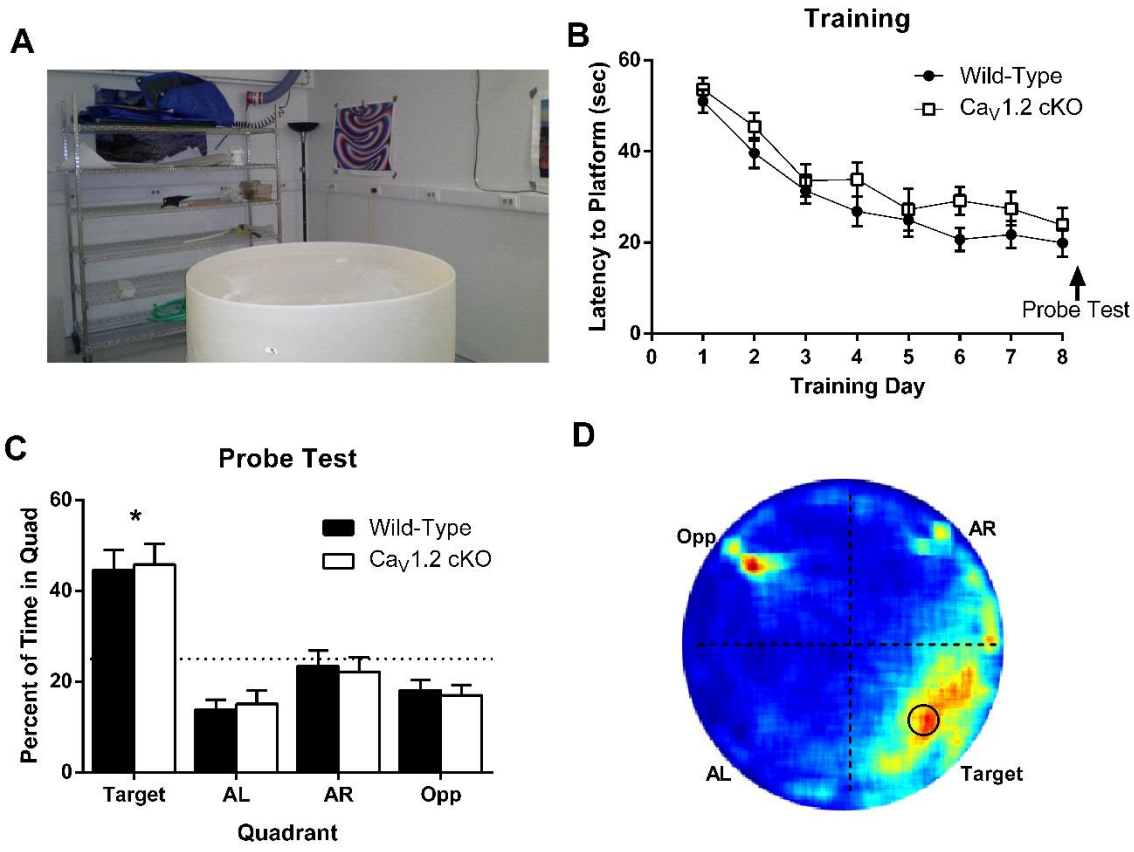

Figure 3.2: Neuronal deletion of Cav1.2 does not impair spatial learning in the Morris water maze

(**A**) Mice were trained to find a hidden platform in a Morris water maze using complex spatial cues around the room. Mice were trained across eight days with a probe test on day nine. (**B**) Both $Ca_V1.2$ conditional knockout mice (n = 17) and wild-type mice (n = 23) exhibited a significant decline in the latency to find the hidden platform across training days. (**C**) When probed for their memory of the platform location, both $Ca_V1.2$ conditional knockout mice and wild-type mice spend significantly more time in the target quadrant than chance. (**D**) Representation of the time spent throughout the water maze during the probe test using a heat map illustrated a strong preference for the platform location and the target quadrant, with an additional strong signal at the location at which mice were released into the pool.

Figure 3

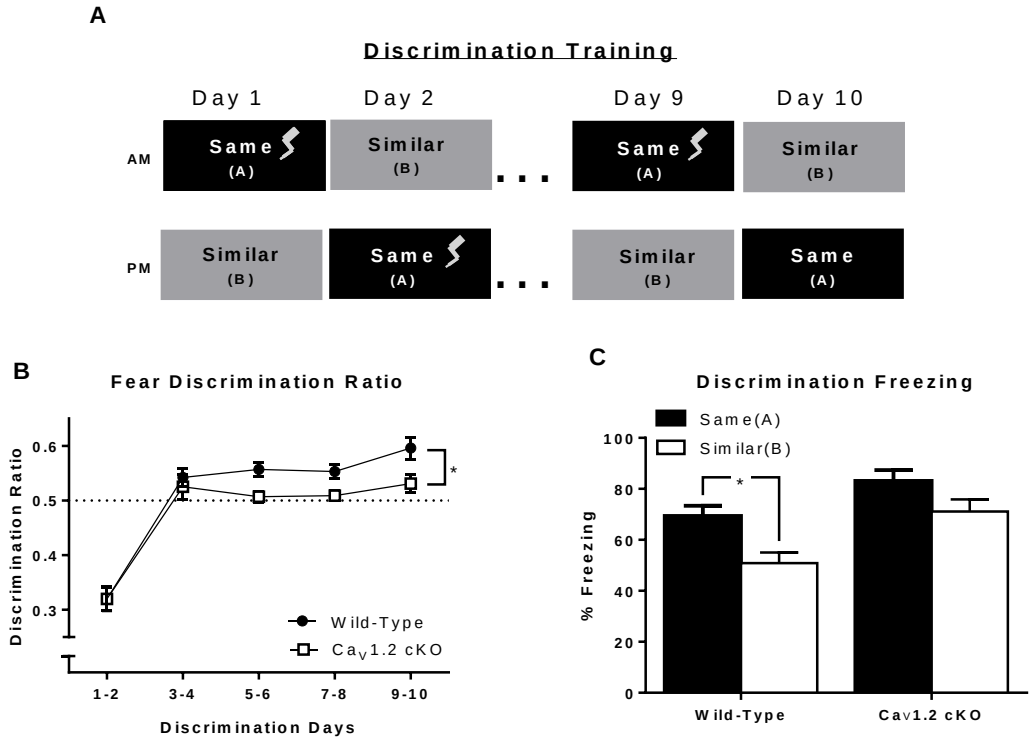

Figure 3.3: Cav1.2 conditional knockout mice exhibit significant deficits in context discrimination

(**A**) Mice were trained to discrimination between two similar contexts through context exposure to each context once a day for ten days with one context, the same context, paired with a footshock. Context discrimination throughout training was assessed using a discrimination ratio, with a ratio of 0.5 representing a lack of discrimination between the two contexts. (**B**) While wild-type mice (n = 18) displayed a significant discrimination ratio above 0.5 by day 3-4 of training, $Ca_V1.2$ conditional knockout mice (n = 17) fail to show a significant discrimination ratio till days 9-10. Additionally, analysis between genotypes revealed a significant deficit in context discrimination in $Ca_V1.2$ conditional knockout mice compared to wild-type mice. (**C**) Comparison of freezing levels between the trained context, A and the similar context, B on days 9-10 of training showed significantly lower level of freezing in context B than A in wild-type mice, but not in $Ca_V1.2$ conditional knockout mice. Data are represented as mean ± SEM. *$p < 0.05$.

Figure 4

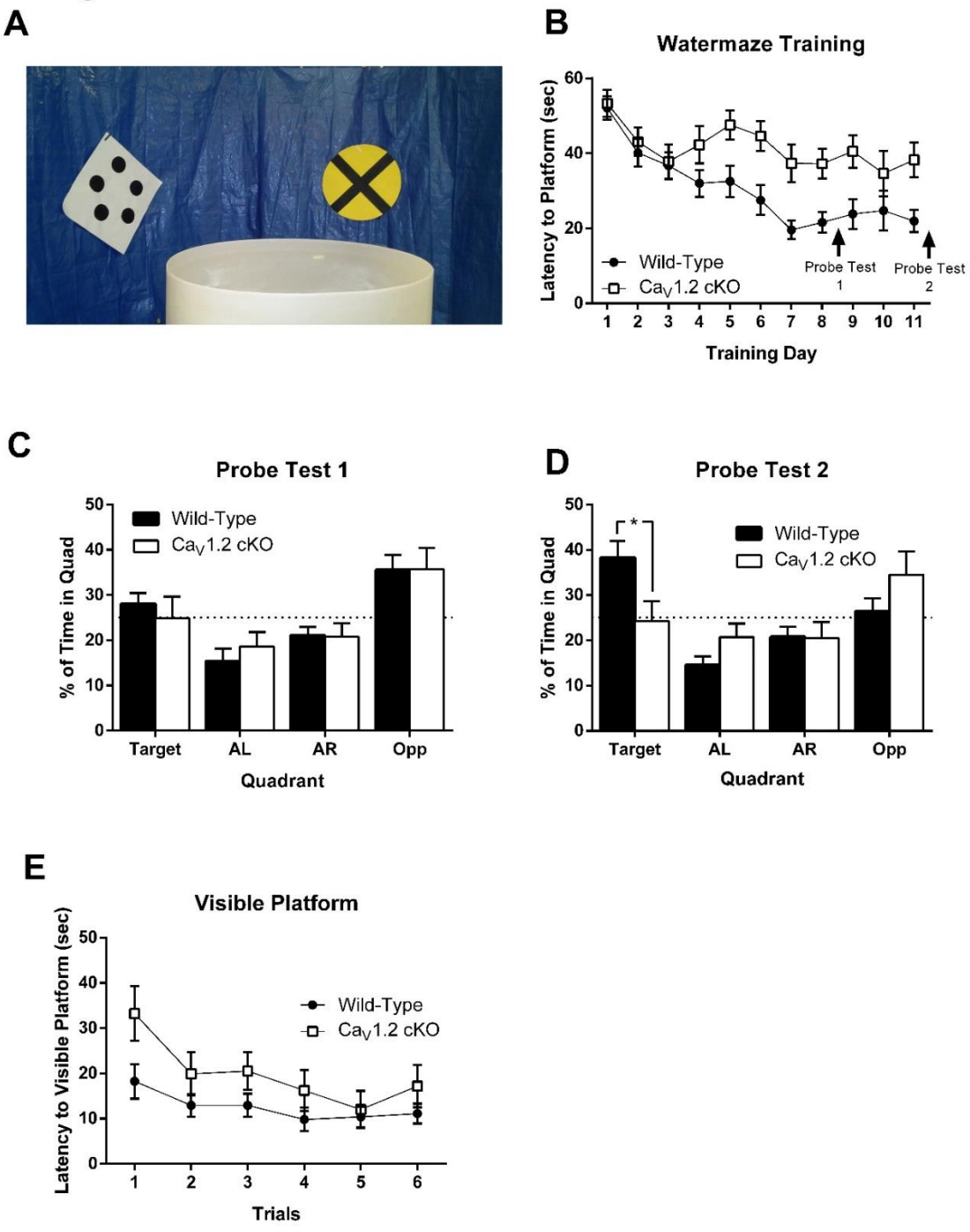

Figure 3.4: Cav1.2 conditional knockout mice exhibit significant impairments in the acquisition of spatial memory in the Limited Cues version of the Morris water maze

(A) To test for deficits in complex hippocampal learning, mice were tested in a version of the Morris water maze in which the visible cues around the room were limited. Mice were trained across 11 days and tested for their spatial memory of the platform location on days 9 and 12 of experimentation. (**B**) Throughout training, $Ca_V1.2$ conditional knockout mice (n = 14) exhibited a significant deficit in the reduction in latency compared to wild-type mice (n = 18). (**C**) During the first probe test, neither $Ca_V1.2$ conditional knockout mice nor wild-type mice exhibited a significant preference for the target quadrant compared to chance or the other quadrants. (**D**) During the second probe test, wild-type, but not $Ca_V1.2$ conditional knockout mice exhibited a significant preference for the target quadrant over the other quadrants and over chance. (**E**) When tested for their ability to find a visible platform, both $Ca_V1.2$ conditional knockout mice and wild-type mice were able to find the platform across six trials. Data are represented as mean ± SEM. *$p < 0.05$.

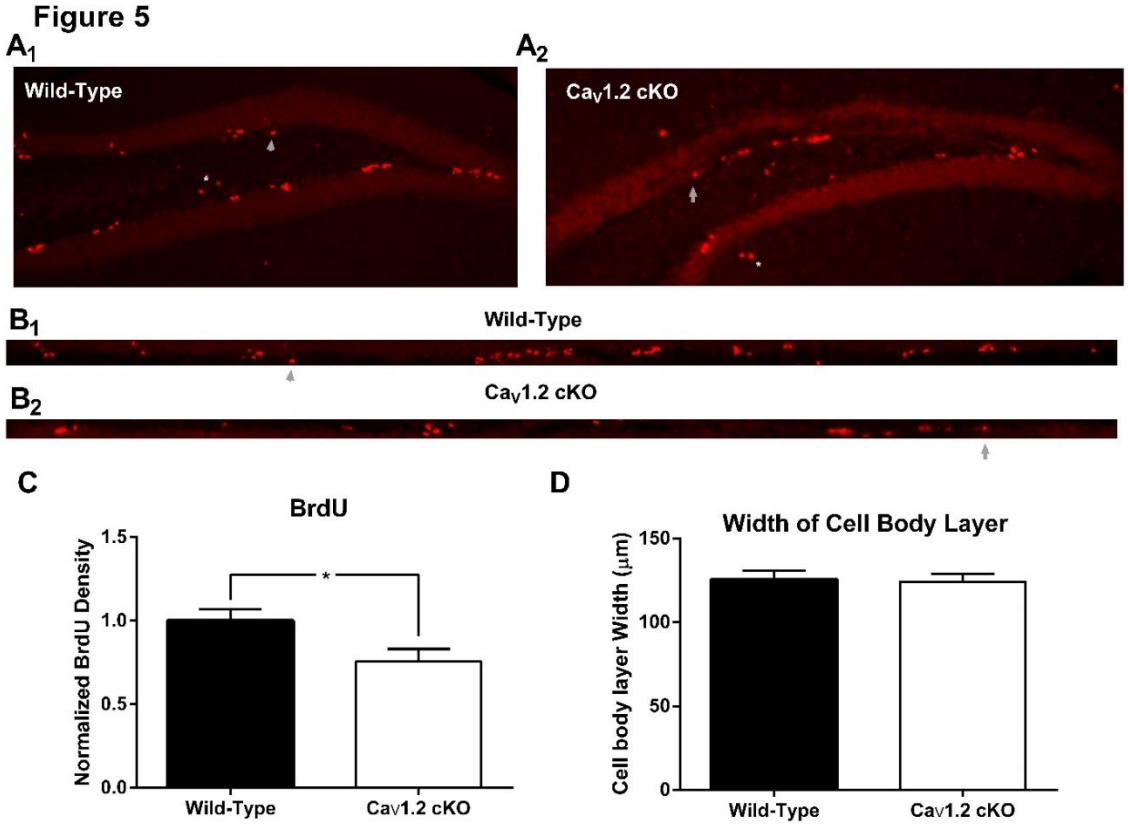

Figure 3.5: $Ca_V1.2$ conditional knockout mice exhibit decreased levels of cell division in the adult dentate gyrus

(**A_1** & **A_2**) Both $Ca_V1.2$ conditional knockout mice (n = 10) and wild-type mice (n = 7) were assessed for the rates of cell division in the adult dentate gyrus using five injections of BrdU across five days. (**B_1** & **B_2**) Analysis of BrdU labeled cells limited the subgranular zone of the dentate gyrus. Arrows indicate examples of BrdU positive cells within the area of interest. Astricks represent examples of cells outside of the area of interest. (**C**) Comparison of the density of BrdU positive cells in the dentate gyrus between genotypes revealed a significant decrease in cell division in $Ca_V1.2$ conditional knockout mice versus wild-type mice. (**D**) However, decreases in cell division in the dentate gyrus was not correlated with alterations in the width of the dentate gyrus. Data are represented as mean ± SEM. *$p < 0.05$.

Figure 6

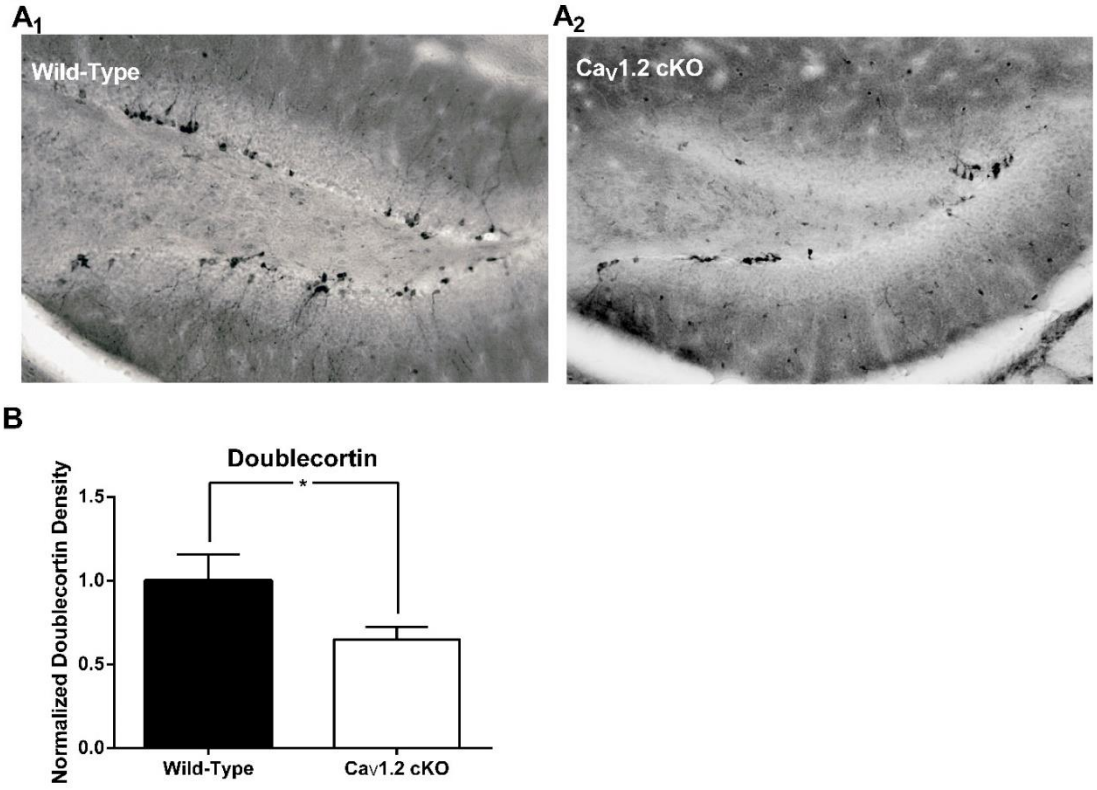

Figure 3.6: Ca$_V$1.2 conditional knockout mice exhibit decreased levels of immature neurons in the adult dentate gyrus

(**A$_1$** & **A$_2$**) Both Ca$_V$1.2 conditional knockout mice (n = 10) and wild-type mice (n = 7) were assessed for adult-born immature neurons in the dentate gyrus through immunohistological labeling of doublecortin positive cells in the subgranular zone of the dentate gyrus. (**B**) Analysis of the density of doublecortin positive cells revealed a significant decrease in adult-born immature neurons in Ca$_V$1.2 conditional knockout mice compared to wild-type mice. Data are represented as mean ± SEM. *$p < 0.05$.

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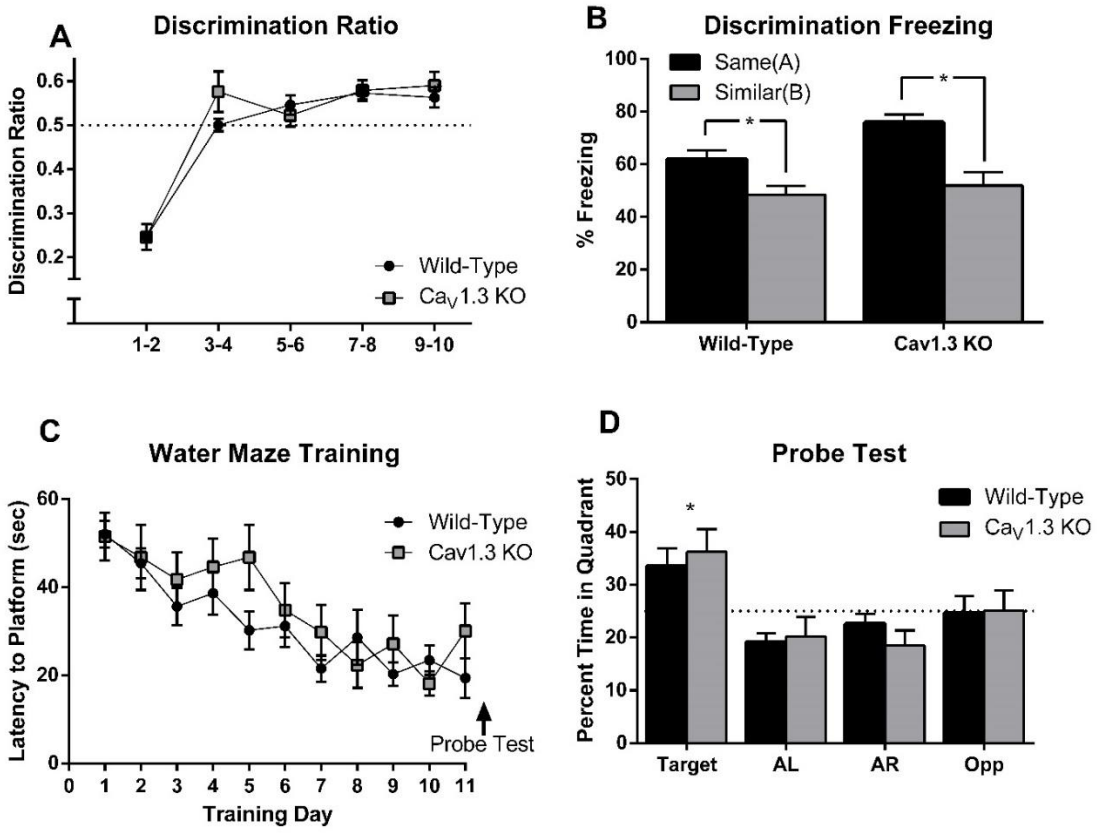

Figure 3.7: Global deletion of $Ca_V1.3$ does not impair context discrimination or spatial learning in the Limited cues version of the Morris water maze

$Ca_V1.3$ global knockout mice were assessed for deficits in complex hippocampal dependent learning tasks using context discrimination and the limited cues version of the Morris water maze. (**A**) $Ca_V1.3$ knockout mice (n = 5) exhibited similar discrimination ratios as wild-type mice (n = 9) throughout discrimination training. (**B**) Both wild-type and $Ca_V1.3$ knockout mice displayed significantly higher levels of freezing in the trained context A versus the similar context B on days 9-10 of discrimination training. (**C**) In the limited cues water maze, both $Ca_V1.3$ knockout mice (n = 7) and wild-type mice (n = 13) exhibited a significant reduction in latency to find the platform across training. (**D**). During the probe test, both $Ca_V1.3$ knockout mice and wild-type mice exhibit a preference for the target quadrant significantly above chance. Data are represented as mean ± SEM. *$p < 0.05$.

CHAPTER 4

THE L-TYPE VOLTAGE GATED CALCIUM CHANNEL, $Ca_V1.2$. MEDIATES FEAR EXTINCTION AND SYNAPTIC REGULATION OF THE LATERAL AMYGDALA

4.1 Summary

L-type voltage gated calcium channels (LVGCCs) have been implicated in both the formation and the reduction of fear through Pavlovian fear conditioning and extinction. Despite the implication of LVGCCs in fear learning and extinction, studies of the individual LVGCC subtypes, $Ca_V1.2$ and $Ca_V1.3$, using transgenic mice have failed to find a role of either subtype in fear extinction. This discontinuity between the pharmacological studies of LVGCCs and the studies investigating individual subtype contributions could be due to the limited neuronal deletion pattern of the $Ca_V1.2$ conditional knockout mice previously studied to excitatory neurons in the forebrain. To investigate the effects of deletion of $Ca_V1.2$ in all neuronal populations, we bred a $Ca_V1.2$ conditional knockout mouse using a synapsin1a cre promoter. Neuronal deletion of $Ca_V1.2$ did not alter basal anxiety or fear learning. However, neuronal deletion of $Ca_V1.2$ resulted in a significant deficit in fear extinction, implicating LVGCCs, specifically $Ca_V1.2$, in extinction learning. Further exploration on the effects of deletion of $Ca_V1.2$ on inhibitory and excitatory input onto the principle neurons of the lateral amygdala found a significant shift in inhibitory/excitatory balance. This shift in the

inhibitory/excitatory balance of synaptic input into the lateral amygdala could disrupt the homeostatic balance of excitability within the amygdala, producing alterations in fear-related learning. Together these data illustrate an important role of $Ca_V1.2$ in fear extinction and the synaptic regulation of activity within the amygdala. illustrates an important role of $Ca_V1.2$ in fear extinction and the synaptic regulation of activity within the amygdala.

4.2 Introduction

Throughout life, humans establish adaptive behaviors through the formation of fear-related memories to aversive stimuli. While associations such as these serve to protect us from harm, pathological fear, such as fear to a non-threatening stimulus, can interfere with an individual's quality of life. Pathological fears such as these are often associated with psychiatric disease, including acute stress disorder and post-traumatic stress disorder (Association 2013). Treatments for such disorders are often only moderately effective, and typically involve attempts to reduce or eliminate the learned fear response through techniques such as exposure therapy (Hofmann and Smits 2008, Gordon, Heimberg et al. 2013, Furini, Myskiw et al. 2014). The development of more effective behavioral and pharmacological treatments of trauma and anxiety-related disorders will likely require a deeper understanding of the neurobiological substrates that underlie the acquisition, consolidation, and modification of fear memories.

In the laboratory, the formation and modification of learned fear is studied using Pavlovian fear conditioning and extinction (Maren 2001, Garakani, Mathew et al. 2006, Furini, Myskiw et al. 2014). During Pavlovian fear conditioning, a neutral

conditioned stimulus (CS), such as a tone or context, is paired with a naturally aversive unconditioned stimulus (US), such as a footshock. After as little as one pairing, the conditioned stimulus alone is sufficient to trigger a fear response (Kalish 1954, Maren and Fanselow 1996). Fear of the CS can then be reduced through fear extinction. During fear extinction the CS is presented repeatedly in the absence of the US (Kalish 1954, Myers and Davis 2002). Studies attempting to uncover the neurobiology underlying Pavlovian fear conditioning and fear extinction have identified the amygdala as the key brain structure involved in the processing of fearful memories (Maren and Fanselow 1996, Barad, Gean et al. 2006, Pape and Pare 2010). Regulation of basal activity in the amygdala and plasticity within amygdala-associated circuits is thought to play a key role in both the establishment and extinction of conditioned fear (Maren 1996, Sah, Westbrook et al. 2008, Pape and Pare 2010).

It is well appreciated that changes in intracellular calcium can influence basal activity as well as neuronal plasticity (Blair, Schafe et al. 2001, Clapham 2007). One major source of intracellular calcium is the influx of calcium through voltage-gated calcium channels. Blockade of L-type voltage gated calcium channels (LVGCCs) using non-subtype specific antagonists has revealed a potential role of LVGCCs in plasticity within the amygdala (Weisskopf, Bauer et al. 1999, Bauer, Schafe et al. 2002), as well as fear learning and extinction (Bauer, Schafe et al. 2002, Cain, Blouin et al. 2002, Davis and Bauer 2012). Specifically, studies investigating the role of LVGCCs in fear learning have found that intra-amygdala infusions of the LVGCC antagonist, verapamil, prior to fear conditioning to a context or tone, prevented the acquisition and consolidation of the learned fear (Bauer, Schafe et al. 2002). Additionally, studies using

systemic or intra-amygdala infusions of the LVGCC antagonists, verapamil, nifedipine, and nimodipine, found that blockade of LVGCCs can produce deficits in fear extinction (Cain, Blouin et al. 2002, Davis and Bauer 2012).

Despite numerous studies demonstrating a clear role of LVGCCs in fear learning and extinction, the specific identity of the LVGCC subtypes involved is unclear. Of the four LVGCC subtypes expressed throughout the body, $Ca_V1.2$ and $Ca_V1.3$ are known to be abundantly expressed within the brain (Hell, Westenbroek et al. 1993, Sinnegger-Brauns, Huber et al. 2009). While the $Ca_V1.2$ and $Ca_V1.3$ subtypes share extensive similarities in structure and binding sites of LVGCC antagonists, substantial differences in activation voltages and inactivation rates (Xu and Lipscombe 2001) and neuronal distribution (Hell, Westenbroek et al. 1993) suggest important differences in $Ca_V1.2$ and $Ca_V1.3$ function. Using a mouse in which *Cacna1d* (the gene that encodes $Ca_V1.3$) was deleted, it was determined that Cav1.3 was involved in the consolidation of conditioned fear, but not fear acquisition or fear extinction (McKinney and Murphy 2006). However, when similar studies were performed using mice in which $Ca_V1.2$ was conditionally deleted in excitatory forebrain neurons using a CaMKIIα cre-driver line, no deficits were observed, suggesting that $Ca_V1.2$ was not necessary for the consolidation or extinction of learned fear (McKinney, Sze et al. 2008). While these studies demonstrate a clear role of Cav1.3 in fear consolidation, due to the limited neuronal deletion pattern of the previously used $Ca_V1.2$ conditional knockout mice, the potential role of $Ca_V1.2$ in fear learning and extinction remains unclear.

In order to better understand the neuronal contribution of $Ca_V1.2$ to fear learning and extinction, we crossed mice in which neuronal expression of cre

recombinase was driven by the synapsin 1 promoter (Zhu, Romero et al. 2001, Cui, Costa et al. 2008) with mice which have been engineered to have loxP sites flanking exon two of the gene that encodes $Ca_V1.2$ (*Cacna1c*) (White, McKinney et al. 2008)

Using these $Ca_V1.2$ conditional knockout mice, we found that neuronal deletion of $Ca_V1.2$ did not alter fear acquisition or consolidation to a tone or context. However, deletion of $Ca_V1.2$ did produce a significant deficit in fear extinction to a conditioned context. To investigate the neurophysiological consequences of deleting $Ca_V1.2$ within the amygdala, we recorded spontaneous inhibitory post-synaptic currents (sIPSCs) and spontaneous excitatory post-synaptic currents (sEPSCs) in principle neurons within the lateral amygdala. We found that neuronal deletion of $Ca_V1.2$ led to a shift in the balance of sIPSC and sEPSC activity by increasing the sIPSC frequency and reducing both the frequency and amplitude of sEPSCs. Taken together, our data suggests that $Ca_V1.2$ is not only necessary for the extinction of a conditioned fear, but that deletion of $Ca_V1.2$ produces an imbalance in the synaptic regulation of lateral amygdala activity. Understanding the contribution of $Ca_V1.2$ in fear extinction and amygdala excitability could lead to a better understanding of the biological underpinnings of treatments of trauma and anxiety-related disorders, such as exposure therapy, as well as a potential target for drug therapies.

4.3 Materials and Methods

4.3.1 Mice

For all experiments, $Ca_V1.2$ conditional knockout mice with neuron specific deletion of $Ca_V1.2$ and their wild-type littermates were used. Mice used in these studies

were on a B57Bl/6:129SvEv F2 genetic background. Mice with a floxed $Ca_V1.2$ exon two allele ($Ca_V1.2^{f/+}$ or $Ca_V1.2^{f/f}$) and maintained on a 129SvEv genetic background (White, McKinney et al. 2008) were first bred to transgenic mice expressing the cre recombinase regulated by the synapsin 1 promoter (Syn1-$Cre^{Cre/+}$) and maintained on a C57BL/6 background (Zhu, Romero et al. 2001, Cui, Costa et al. 2008), producing an F1 cross. Using non-littermate offspring from the F1 cross, heterozygous floxed, cre positive ($Ca_V1.2^{f/+}$ Syn1-$Cre^{Cre/+}$) mice were then crossed with heterozygous floxed, cre negative ($Ca_V1.2^{f/+}$ Syn1-$Cre^{+/+}$) mice to produce homozygous floxed, cre positive ($Ca_V1.2^{f/f}$ Syn1-$Cre^{Cre/+}$) conditional knockout mice as well as mice categorized as wild-type or control. Mice were considered wild-type if they were cre positive, but lacked the floxed alleles ($Ca_V1.2^{+/+}$ Syn1-$Cre^{+/+}$). Mice were considered control if they were homozygous or heterozygous for the floxed allele and cre negative ($Ca_V1.2^{f/f}$ Syn1-$Cre^{+/+}$; $Ca_V1.2^{f/+}$ Syn1-$Cre^{+/+}$), or if they were cre positive but lacked the floxed alleles ($Ca_V1.2^{+/+}$ Syn1-$Cre^{Cre/+}$). For all experiments control mice were compared to and collapsed with wild-type mice due to no significant difference between the two groups. This collapsed group is termed wild-type from here after.

Studies were conducted using mice that were 3-7 months old at the time of testing or slice preparation. Approximately equal numbers of males and females were used. Mice were housed by sex in groups of three to five mice. Mice were kept in micro-isolation cages with a 14-h/10-h light/dark cycle with an average ambient temperature of 22°C and ad libitum food and water. All experiments were conducted according to the National Institute of Health guidelines for animal care and were

approved by the University Committee on the Use and Care of Animals of the University of Michigan.

4.3.2 Behavioral Procedures

4.3.2.1 Open Field

The open field experiment was performed in a large white acrylic chamber (71x71x30cm) with indirect white light and a light level of 200 lux at the center of the chamber. During testing, mice were placed in the center of the chamber and allowed to explore for five minutes. Mouse movement throughout the chamber was recorded and analyzed using Limelight software by Actimetrics. During analysis, the open field chamber was divided into an 8 x 8 grid with a center zone of 53.25 cm x 53.25 cm and an outer zone of 8.875 cm around the border. Mouse performance was analyzed for total distance traveled and percent time in the center.

4.3.2.2 Light-Dark Box

The light-dark box experiment was conducted using an acrylic chamber with the light portion making up two thirds of the length and composed of white acrylic and an enclosed dark portion composed of black acrylic making up one third of the length (McKinney, Chow et al. 2008, McKinney, Schneider et al. 2008). Light levels measured 200 lux at the center of the light compartment. During testing, mice were placed individually in the center of the light compartment and were allowed to explore for a period of five minutes. Mouse movement was collected and analyzed using Limelight by Actimetrics. Mouse behavior was analyzed for time spent in the light compartment and the number of light dark transitions.

4.3.2.3 Fear Conditioning and extinction

Fear conditioning experiments were carried out as previously described (Temme, Bell et al. 2014). Fear conditioning experiments were conducted in fear conditioning chambers with clear acrylic backs and doors, aluminum sides, stainless steel grid floors spaced 1/8 inches, and stainless steel drop pans (Med Associates). Chambers and floor pans were cleaned with 70% ethanol throughout experimentation and illuminated with white room lights set at 150 watts. Shocks were administered through the grid via solid-state shock scramblers and electronic constant-current shock sources controlled by a desktop PC running Actimetrics, FreezeFrame software (Wilmette, IL). The same computer and software were used to record behavior which was digitized using individual cameras mounted above each chamber. Mice were fear conditioned to a tone using two training sessions, one per day for two days. During training, mice were exposed to the training context for three minutes followed by three tone-shock presentations in which a thirty second tone (75dB, 2.8kHz) co-terminated with a two second footshock, with thirty seconds between tones. Mice remained in the conditioning chamber for thirty seconds following the last tone-shock pairing. Twenty-four hours after training mice were tested for their freezing behavior to the conditioned tone in a different context (context B) consisting of smooth opaque white acrylic coverings over the floor and walls which produced the appearance of a semicircular chamber. The chamber and floor pans were cleaned with 2% acetic acid and red room lights at sixty watts were used. During tone testing, mice were placed in context B followed by five, thirty second tone alone presentations with thirty seconds between tones. Mice were removed from the chambers thirty seconds after the last tone

presentation. Seventy-two hours after tone testing, mice were tested for their fear to the training context. Mice were tested to their fear to the context using five minutes of context exposure. Fear was measured as freezing in each session. Freezing was defined as a lack of motion, except that required for respiration, for one second or more and was calculated u sing a sensitive global motion-detection algorithm (FreezeFrame and FreezeView software; Actimetrics; Wilmette, IL).

In experiments involving fear extinction, mice were trained to a context over two days, one session per day, with sessions consisting of three minutes of context exposure followed by one unsignaled footshock (0.5mA, 2 sec) and thirty seconds context exposure after the shock. After conditioning, mice were split into extinction and no extinction groups. Twenty-four hours after training, mice in the extinction group were extinguished to their fear to the context using two one-hour sessions of context exposure, one per day for two days. Twenty-four hours after the last extinction session, extinction and no extinction mice were tested for their fear to the context using five minutes of context exposure.

4.3.3 Electrophysiology

4.3.3.1 Slice Preparation

Mice (3-7 months old) were anesthetized with isoflurane prior to dissection. Brains were rapidly removed and incubated in an oxygenated, ice slurry of sucrose based cutting solution: (in mM): 206.0 sucrose, 2.8 KCl, 1.25 $MgCl_2$ $6H_2O$, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 1.25 $CaCl_2$, 10.00 D-Glucose, 26.00 $NaHCO_3$, 0.4 Ascorbic Acid, for a period of one to two minutes. Brains were then bisected and placed in the slicing

chamber with 1.5% agar blocks as support and submerged in ice cold sucrose cutting solution. The brain tissue was sliced in a coronal plane through the amygdala at 300µm. Slices were then incubated in room temperature oxygenated artificial cerebral spinal fluid (aCSF; in mM): 124.0 NaCl, 2.8 KCl, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 10.0 D-Glucose, 26.0 $MaHCO_3$, 0.4 ascorbic acid, for a minimum of one hour prior to recording.

4.3.3.2 Electrophysiological recordings

Electrophysiology recordings were made using a Dagan 3900A integrating patch clamp amplifier and digitized using an Axon Instruments 1322A Digidata. Recordings were made using glass electrodes made from borosilicate glass with filament (1.5mm x 0.86mm; Sutter Instruments) and a resistance between 4 to 8 mega ohms. Whole-cell voltage clamp recordings of spontaneous inhibitory post-synaptic currents (sIPSCs) and spontaneous excitatory post-synaptic currents (sEPSCs) were made from principle neurons in the LA. Spontaneous IPSC recordings were made using a cesium chloride internal solution (in mM): 130 CsCl, 1.0 KCl, 1.0 NaCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 10.0 HEPES, 7.0 phosphocreatine, 4.0 Na_2ATP, 0.3 TrisGTP, 0.2 EGTA, 0.1% biocytin, in a submersion chamber with continuous aCSF perfusion (~1.5ml/min) containing 4.0 mM kynurenic acid (Sigma) and heated to 31°C. Spontaneous EPSC recordings were made using cesium gluconate internal solution (in mM): 100 cesium gluconate, 0.2 EGTA, 5 $MgCl_2$, 2 ATP, 0.3 GTP, 40 HEPES, 0.1% biocytin, and aCSF perfusion containing 20µm picrotoxin (Sigma). For both sIPSC and sEPSC experiments, the resting membrane potential was clamped at -70mV and synaptic activity was recorded for a

period of five minutes. Recordings of sIPSCs and sEPSCs were analyzed offline using Mini Analysis (Synaptosoft).

4.3.4 Statistical Analysis

Analysis of behavioral experiments was carried out using unpaired t-tests comparing $Ca_V1.2$ conditional knockout mice with their wild-type counterparts. Learning across fear conditioning and extinction training was analyzed using a two way ANOVA and repeated measures ANOVA, respectively, with genotype and training as factors. Electrophysiological recordings of sIPSCs and sEPSCs frequency, as an inter-event interval, were analyzed as a cumulative frequency distribution using the Kolomonov-Smirnov test. Average inter-event intervals and amplitudes between genotypes were also compared using an unpaired t-test.

4.4 Results

4.4.1 Neuronal deletion of Cav1.2 does not alter basal anxiety

Prior to studying the effects of deletion of $Ca_V1.2$ on fear learning and extinction, we first sought to determine whether neuronal deletion of $Ca_V1.2$ alters basal anxiety within the mice. Alterations in basal anxiety have been previously observed with the use of L-type voltage gated calcium channel antagonists (Matsumoto, Kataoka et al. 1994, El Ganouni, Tazi et al. 1998) and could hinder the study of learned fear. To study basal anxiety, we utilized the open field and light/dark box tests. During the open field test, mice were placed in a large arena where the well-lit center of the field represents an anxiety provoking stimulus compared to the sheltered perimeter. During experimentation, mice were placed individually in the center of the arena and monitored

for a period of five minutes to determine time spent in the anxiety-provoking center of the maze as well as the total distance traveled. Analysis of the percent of time spent in the center of the maze revealed no alterations in anxiety-like behavior in $Ca_V1.2$ conditional knockout mice compared to wild-type mice ($p = 0.42$ unpaired t-test) (**Figure 4.1A**). As a measure of overall locomotor performance, total distance traveled with the arena was compared between genotypes (**Figure 4.1B**). Analysis of the total distance traveled between genotypes using an unpaired t-test found no significant difference ($p = 0.96$). In the light/dark box test, mice were placed in a chamber consisting of a well lit open arena attached to an enclosed dark arena for five minutes. During the light-dark box test, the well lit open arena was considered anxiety provoking compared to the enclosed dark arena. Mice were assessed for the percent of time spent in the light arena (**Figure 4.1C**), as well as the number of transitions between the light and dark compartments (**Figure 4.1D**). Analysis of both the percent of time spent in the light arena and the number of transitions between light and dark compartments using a planned unpaired t-test revealed no significant difference in genotype in either category ($p = 0.56$ and $p = 0.46$, respectively). Based on the results from the open field test and the light/dark box test, it appears that neuronal deletion of $Ca_V1.2$ does not alter basal anxiety.

4.4.2 Cav1.2 conditional knockout mice exhibit normal fear acquisition and consolidation, but impaired extinction to a conditioned context

To determine whether neuronal deletion of $Ca_V1.2$ altered fear acquisition or consolidation, we fear conditioned transgenic $Ca_V1.2$ mice to a tone and analyzed freezing levels to the tone, as well as the trained context throughout training and during

tone and context tests. Mice were fear conditioned using one training session per day for two days. During training, mice were given three minutes of context exposure in the conditioning chamber followed by three tone presentations, each which co-terminated with a two second footshock. Tone-shock presentations were separated by thirty seconds with an additional thirty seconds after the final tone presentation. Acquisition of fear to the tone was analyzed across training as an average percent freezing to the tone for each training day (**Figure 4.2A**). Analysis of tone freezing using a repeated measures ANOVA found no significant effect of genotype ($F_{(1,38)} = 1.024$, $p = 0.32$) but a significant effect of training day ($F_{(1,38)} = 164.895$, $p < 0.0001$). Acquisition of fear to the trained context was also assessed across training days as an average of the three minutes of context exposure for each conditioning day (**Figure 4.2B**). Analysis of context freezing across training using a repeated measures ANOVA found no significant difference between genotypes ($F_{(1,38)} = 0.076$, $p = 0.7842$) but a significant effect of training day ($F_{(1,38)} = 140.253$, $p < 0.0001$). Twenty-four hours after the last day of training, mice were tested for freezing to the tone in a novel context. Tone testing consisted of one minute of context exposure followed by five tone alone presentations separated by thirty seconds. Freezing to the tone was assessed as an average percent freezing across all tone presentations (**Figure 4.2C**). Comparison of the average percent freezing to the tone between genotypes was assessed using an unpaired t-test which found no significant differences between $Ca_V1.2$ conditional knockout mice and their wild-type counterparts ($p = 0.82$) with freezing levels at 53% and 56% respectively. Seventy-two hours after tone testing, mice were tested for their fear to the conditioned context using five minutes of context exposure to the trained context. Comparison of the

average percent freezing during context testing using an unpaired t-test also revealed no significant difference between genotypes ($p = 0.67$) with $Ca_V1.2$ conditional knockout mice freezing at 53% and wild-type mice at 55%. (**Figure 4.2D**). These studies suggest that neuronal deletion of $Ca_V1.2$ does not alter consolidation and express of conditioned fear.

To determine whether neuronal deletion of $Ca_V1.2$ alters extinction of a conditioned fear, $Ca_V1.2$ conditional knockout mice were conditioned to a context across two days. Each day mice were given three minutes of context exposure followed by one unsignaled footshock (0.5mA, 2sec). Conditioned freezing to the training context was plotted across training days using the average percent freezing of the three minute context exposure each day (**Figure 4.3A**). Analysis of the percent freezing across training days using a repeated measures ANOVA revealed a significant effect of training ($F_{(1,32)} = 75.354$, $p < 0.0001$), but not of genotype ($F_{(1,32)} = 1.585$, $p = 0.22$).Twenty-four hours after training mice were split into extinction and no extinction groups. Freezing to the trained context was extinguished using two hours of context exposure, one hour of context exposure per day for two days, while mice who did not receive extinction remained in their home cages. Extinction training was plotted as an average percent freezing per ten minute bins, with bins one through six representing extinction day one and bins seven through twelve representing extinction day two (**Figure 4.3B**). Analysis of freezing within extinction days found a significant effect of extinction training on day one ($F_{(5,80)} = 5.343$, $p = 0.0003$) and day two ($F_{(5,80)} = 2.577$, $p = 0.0331$), but no effect of genotype on either day (day one: $F_{(1,16)} = 0.028$, $p = 0.870$ and day two: $F_{(1,16)} = 0.118$, $p = 0.7361$). Twenty-four hours after extinction training,

mice in the extinction and no extinction groups were tested for their fear to the conditioned context using five minutes of context exposure (**Figure 4.3C**). Analysis of context testing using a two-way ANOVA found a significant effect of extinction training ($F_{(1,33)} = 26.251$, $p < 0.0001$) and genotype ($F_{(1,33)} = 7.109$, $p = 0.0084$). Further analysis of genotypes using a planned t-test revealed no significant difference in context freezing between wild-type and $Ca_V1.2$ conditional knockout mice in either the extinction ($p = 0.2415$) or no extinction groups ($p = 0.5684$). However, additional analysis of extinction training using a planned unpaired t-test revealed a significant decrease in freezing in mice that received extinction training versus mice who did not receive extinction training that was present in wild-type ($p = 0.0194$), but not $Ca_V1.2$ conditional knockout mice ($p = 0.2713$). These data suggest that neuronal deletion of $Ca_V1.2$ leads to a deficit in extinction to a previously conditioned context. This supports previous pharmacological work suggesting an important role of L-type voltage gated calcium channels in fear extinction. Taken together with previous studies showing normal fear extinction in mice lacking $Ca_V1.2$ in excitatory neurons in the forebrain, but a significant decline in fear extinction with intra-amygdala infusions of LVGCC antagonists, we hypothesized that the deficit in fear extinction found in mice with a pan-neuronal deletion of $Ca_V1.2$ is likely due to the role of these channels in inhibitory neuronal population within the amygdala.

4.4.3 Neuronal deletion of Cav1.2 alters synaptic inhibitory and excitatory input onto pyramidal cells in the LA

To determine whether $Ca_V1.2$ neuronal deletion alters inhibition within and onto the amygdala, we made whole cell voltage clamp recordings of spontaneous inhibitory post-synaptic currents (sIPSCs) in principle neurons of the lateral amygdala. Given the potential role of $Ca_V1.2$ in inhibitory neurons in mediating extinction to a conditioned context, particularly the importance of intra-amygdala inhibition of principle neurons in the lateral amygdala in mediating fear extinction (Likhtik, Popa et al. 2008, Lee, Kim et al. 2013, Trouche, Sasaki et al. 2013), we hypothesized that deletion of $Ca_V1.2$ alters inhibition within the lateral amygdala. Electrophysiological recordings of sIPSCs were made using naïve $Ca_V1.2$ conditional knockout and wild-type mice, three to seven months in age (**Figure 4.4A**). Analysis of sIPSCs within the principle neurons of the lateral amygdala using a Kolmogorov-Smirnov test revealed a significant change in sIPSC inter-event interval cumulative distribution between $Ca_V1.2$ conditional knockout mice and wild-type mice ($p < 0.0001$) (**Figure 4.4B**). Spontaneous IPSC inter-event interval distribution in $Ca_V1.2$ conditional knockout mice can also be seen as a leftward shift from wild-type mice when assuming a Gaussian distribution (**Figure 4.4C**). Analysis of spontaneous IPSCs using a Gaussian distribution predicted a mean of the log value of 2.795, equal to 623.73 ms, and a variance of 0.5930, equal to 3.95 ms, in wild-type mice and a mean of 2.656, equal to 452.90 ms, and a variance of 0.6499, equal to 4.47 ms, in $Ca_V1.2$ conditional knockout mice. Finally, comparison of the average inter-event interval of sIPSCs between genotypes using an unpaired t-test showed a significant increase in sIPSC frequency, seen as a decrease in inter-event interval, compared to wild-type mice ($p = 0.0135$) (**Figure 4.4D**). However, comparison of the average sIPSC amplitude between genotypes using an unpaired t-test revealed no

significant change ($p = 0.46$) (**Figure 4.4E**). Along with producing significant deficits in the consolidation of fear extinction, neuronal deletion of $Ca_V1.2$ significantly increases the frequency of spontaneous inhibitory activity onto principle neurons in the lateral amygdala, without altering the amplitude of these events. While increases in inhibition within the amygdala are more often linked to deficits in fear learning and expression, a correct balance of inhibitory and excitatory input is believed to be crucial for proper amygdala function.

To fully assess potential alterations in the balance of inhibitory and excitatory input in the amygdala in $Ca_V1.2$ conditional knockout mice, we performed whole cell voltage clamp recordings of spontaneous excitatory post-synaptic potentials onto principle neurons within the amygdala (**Figure 4.5A**). Analysis of sEPSC inter-event intervals using a Kolmogorov-Smirnov test found a significant difference in the sEPSC inter-event interval cumulative distribution between $Ca_V1.2$ conditional knockout mice and their wild-type counterparts ($p < 0.0001$) (**Figure 4.5B**). This change in sEPSC inter-event interval distribution was reflected as a rightward shift in the $Ca_V1.2$ conditional knockout distribution compared to wild-type mice, assuming a Gaussian distribution (**Figure 4.5C**). Analysis of sEPSC inter-event intervals using a Gaussian distribution predicted a mean of 2.968, equal to 928.97 ms, and a variance of 0.5408, equal to 3.47 ms, in wild-type mice and a mean of 3.102, equal to 1264.74 ms, and a variance of 0.6574, equal to 4.54 ms, in $Ca_V1.2$ conditional knockout mice. Comparison of the average inter-event interval between genotypes using an unpaired t-test further supported an increase in inter-event interval in $Ca_V1.2$ conditional knockout mice ($p = 0.003$) (**Figure 4.5D**). Comparison of the average sEPSC amplitude between genotypes

using an unpaired t-test also found a significant decrease in sEPSC size in $Ca_V1.2$ conditional knockout mice compared to their wild-type counterparts (**Figure 4.5E**). These data further support the hypothesis of altered inhibitory and excitatory balance in synaptic input onto pyramidal cells in the LA in the lateral amygdala produced through neuronal deletion of $Ca_V1.2$.

4.5 Discussion

Using a mouse with floxed $Ca_V1.2$ exon 2 alleles crossed with a mouse expressing cre recombinase driven by a synapsin1 promoter, we assessed the neuronal contribution of $Ca_V1.2$ in basal anxiety, fear learning, extinction and network dynamics pyramidal cells in the lateral amygdala. Our studies found that neuronal loss of $Ca_V1.2$ did not alter basal anxiety or fear learning and consolidation. However, neuronal deletion of $Ca_V1.2$ lead to significant deficits in the extinction of a conditioned fear that was not observed in previous studies using other deletion patterns of $Ca_V1.2$. In addition, $Ca_V1.2$ neuronal deletion altered sIPSC and sEPSC activity within the amygdala, producing a shift in the inhibitory/excitatory balance onto principle neurons in the lateral amygdala.

In order to understand the potential role of $Ca_V1.2$ in fear related learning, we tested whether neuronal deletion of $Ca_V1.2$ leads to an increase in basal anxiety-like behavior independent of learned fear. When examined in the open field test and light/dark box test, no changes in basal anxiety were noted in the $Ca_V1.2$ conditional knockout mice. When $Ca_V1.2$ conditional knockout mice were examined in the open field test, they exhibited equal amounts of time in the open, center portion of the arena as their wild-type littermates. Similarly, when $Ca_V1.2$ conditional knockout mice were

observed in the light-dark box test, they spent similar quantities of time in the light side of the arena compared to their wild-type counter parts. These results were further supported by the analysis of locomotion during each test demonstrating roughly equal amount of distance traveled in the open field test and number of light-dark arena transitions in the light/dark box test suggesting normal motor capability and anxiety within $Ca_V1.2$ conditional knockout mice. These data suggest that neuronal deletion of $Ca_V1.2$ does not alter basal anxiety. Interestingly, previous studies have provided conflicting results pertaining to the role of $Ca_V1.2$ and LVGCCs in the regulation of anxiety-like behaviors. While decreases in anxiety-like behavior has been noted in rats with intracerebroventricular injections of the LVGCC antagonist verapamil (Matsumoto, Kataoka et al. 1994, El Ganouni, Tazi et al. 1998), forebrain specific and prefrontal cortex specific deletion of $Ca_V1.2$ in mice resulted in an increase in anxiety-like behaviors (Lee, Ra et al. 2012). Discrepancies between our results and previously published results could be due to the well documented non-specific effects of LVGCC antagonists (Das, Bell-Horner et al. 2004, Edraki, Mehdipour et al. 2009, Thompson, Duke et al. 2011) or the effect of deletion of $Ca_V1.2$ on a different genetic background (Crawley, Belknap et al. 1997, Temme, Bell et al. 2014). Substantial differences in various transgenic mouse lines have been noted using difference mouse strains (Bergren, Chen et al. 2005, Abdolvahab, Brinks et al. 2014, Dora, Collinson et al. 2014). Despite potential discrepancies in anxiety-like behavior related to LVGCCs, the lack of an anxiety-related phenotype in our mice lead us to conclude that any differences in fear related learning in our $Ca_V1.2$ conditional knockout mice are likely

due to the effect of deletion of $Ca_V1.2$ in fear related circuits rather than a change in basal anxiety.

In addition to conflicting literature pertaining to the role of LVGCCs in basal anxiety, studies of LVGCCs have found mixed results regarding the role of these channels in fear related learning. Pharmacological studies have implicated deficits in both fear learning as well as fear extinction in the presence of systemic and intra-amygdala infusions of LVGCC antagonists (Bauer, Schafe et al. 2002, Cain, Blouin et al. 2002, Davis and Bauer 2012). Further studies have identified a specific role of the LVGCC subtype $Ca_V1.3$ in fear learning, using a global knockout mouse, but not $Ca_V1.2$, using an excitatory forebrain specific conditional knockout mouse (McKinney and Murphy 2006, McKinney, Sze et al. 2008). However, deficits in fear extinction have not been replicated with the use of these mice (McKinney and Murphy 2006, McKinney, Sze et al. 2008). While these studies suggest a lack of $Ca_V1.2$ involvement in fear learning and fear extinction, the use of a $Ca_V1.2$ conditional knockout mouse with a limited deletion pattern may occlude the role of $Ca_V1.2$ in inhibitory neurons and other brain structures in mediating fear related learning.

In our studies, $Ca_V1.2$ conditional knockout mice with neuronal deletion of $Ca_V1.2$ throughout the brain displayed normal fear acquisition, consolidation, and expression. Fear conditioned, $Ca_V1.2$ conditional knockout mice and their wild-type counterparts showed similarly high levels of freezing when tested for their fear to either the context or tone. These results suggest normal fear learning in mice lacking neuronal $Ca_V1.2$ and further support previously published data suggesting an important role of $Ca_V1.3$ (McKinney and Murphy 2006), but not $Ca_V1.2$ in fear consolidation

(McKinney, Sze et al. 2008, Langwieser, Christel et al. 2010). However, $Ca_V1.2$ conditional knockout mice failed to show a reduction in fear response to a conditioned context after extinction training. When mice underwent extinction of the conditioned context, $Ca_V1.2$ conditional knockout extinction mice displayed a high level of freezing to the conditioned context, with no notable difference in freezing between mice that received extinction training and mice that did not. Interestingly, this deficit in context extinction was observed during extinction testing and across extinction days, but not during within session extinction training. These data confirm previously published literature indicating a role of LVGCCs in fear extinction (Bauer, Schafe et al. 2002, Cain, Blouin et al. 2002, Davis and Bauer 2012), as well as the theory that LVGCCs effects the consolidation, but not the acquisition or expression of extinction learning (Davis and Bauer 2012). Taken together with previous studies showing normal extinction in mice lacking $Ca_V1.3$, these data indicate a subtype specific role for $Ca_V1.2$ in modulating fear extinction and $Ca_V1.3$ in the modulation of fear learning.

The presence of an extinction phenotype in $Ca_V1.2$ conditional knock mice with a neuronal specific deletion, but not in the previously studied $Ca_V1.2$ conditional knockout mice in which $Ca_V1.2$ was deleted in excitatory neurons in the forebrain (McKinney, Sze et al. 2008) suggests that $Ca_V1.2$ may mediate the consolidation of fear extinction through its role in inhibitory neurons that mediate the activity of the amygdala. While fear conditioning has been associated with an increase in excitation and a stimuli specific reduction in basal inhibition within the amygdala (Wolff, Grundemann et al. 2014), fear extinction is often associated with a resurgence of inhibitory tone originating from within the amygdala itself, as well as other structures

thought to be involved in fear extinction, such as the prefrontal cortex and hippocampus (Maren and Quirk 2004, Barad 2006, Pape and Pare 2010).

To address the effects of deletion of $Ca_V1.2$ on inhibition and excitation within the amygdala, whole cell voltage clamp recordings of sIPSCs and sEPSCS in principle neurons within the lateral nucleus of the amygdala were made. Recordings of sIPSCs and sEPSCs revealed a significant shift in neuronal input onto principle neurons in the amygdala. Specifically, recordings from pyramidal neurons in the $Ca_V1.2$ conditional knockout mice exhibited an increase in sIPSC frequency, measured as a decrease in sIPSC inter-event interval. Along with an increase in sIPSC frequency, $Ca_V1.2$ conditional knockout mice were also found to have a significant decrease in sEPSC frequency and amplitude in pyramidal neurons in the amygdala. When taken together, these moderate changes in sIPSC and sEPSC constitute a much larger shift in the overall inhibitory/excitatory balance in neuronal input onto principle neurons in the lateral amygdala. While one might predict deficits in fear extinction, such as those observed in the $Ca_V1.2$ conditional knockout mice studied here, to be associated with a decrease in inhibition or enhanced excitation within the amygdala, we hypothesize that the shift in the inhibitory/excitatory input onto projection neurons in the lateral amygdala alters the homeostatic balance of activity within the amygdala which in turn disrupts extinction. The lateral amygdala is considered the input structure of the amygdala (Maren 1999, Maren 2000, Jimenez and Maren 2009). Tight regulation of activity within this structure is vital for mediating fear memories (Lang and Pare 1997, Royer, Martina et al. 1999, Pare, Royer et al. 2003). Basal alterations of inhibitory tone within the LA and BLA have been shown to significantly change the activity pattern in

the amygdala producing distinct differences in amygdala associated behaviors. In fact, reductions in local inhibition within the amygdala have been demonstrated to produce non-associative and generalized fear expression (Shaban, Humeau et al. 2006, Bergado-Acosta, Sangha et al. 2008, Wiltgen, Godsil et al. 2009). Similarly, increases in inhibition have been found to alter the firing activity of neurons within the amygdala (Royer, Martina et al. 1999, Pare, Royer et al. 2003). Enhanced activity of inhibitory interneurons within the amygdala has been associated with a reduced probability of long term plasticity induction at the thalamo-amygdala pathway (Bissiere, Humeau et al. 2003). Additionally, firing of inhibitory interneurons within the basolateral amygdala has been found to inhibit spiking of pyramidal neurons (Lang and Pare 1997) and alters synchronized firing of principle neurons in the amygdala (Woodruff and Sah 2007). In fact changes in sEPSCs frequency and amplitude, such as those seen in our studies, could be due to an increase in inhibitory input onto the excitatory afferents that form synapses onto principle neurons in the amygdala. Additionally, while increases in inhibitory/excitatory balance are not normally tied to the extinction of fear, the exact neurons that are altered by the shift in inhibition may matter in mediating fear behavior. For example, while some excitatory cells in the BLA have been observed to decrease their firing to a fearful stimulus after extinction, other excitatory cells, such as extinction cells, do not show an alteration in firing rates in the presence of fear conditioning, but do show an increased rate of activity upon fear extinction (Herry, Ciocchi et al. 2008).

Given the complex interaction between inhibitory/excitatory input onto lateral amygdala neurons and amygdala related behavior and plasticity, more information

would need to be gathered to understand how an increase in inhibitory/excitatory balance could produce deficits in fear extinction. In order to address this question in the $Ca_V1.2$ conditional knockout mice, additional studies would need to be performed to investigate the source of enhanced sIPSC activity in projection neurons within the lateral amygdala, whether it originates from external regions associated with fear extinction, such as the prefrontal cortex and hippocampus, or from within the amygdala itself. Similarly, additional studies will need to be completed to determine the source of the altered sEPSC activity within the lateral amygdala, as well as whether the changes in sEPSC activity are due to the loss of $Ca_V1.2$ within excitatory neurons that synapse onto principle neurons in the amygdala, or due to an increase in inhibitory input onto these excitatory afferents. Finally, more complex studies to identify the types of neurons, such as those that fire in response to fear learning or to extinction learning, that are altered by the change in inhibitory/excitatory balance, could help in the identification of the $Ca_V1.2$ mediated neural circuitry involved in fear extinction.

In conclusion, our work has clearly identified a role of the LVGCC subtype, $Ca_V1.2$ in fear extinction. In addition to altering fear extinction, neuronal deletion of $Ca_V1.2$ was also found to significantly alter the sIPSC/sEPSC balance of activity onto principle projection neurons within the lateral amygdala. We postulate that this imbalance in activity may produce significant alterations in amygdala function and output and may alter fear extinction in a $Ca_V1.2$ dependent manner.

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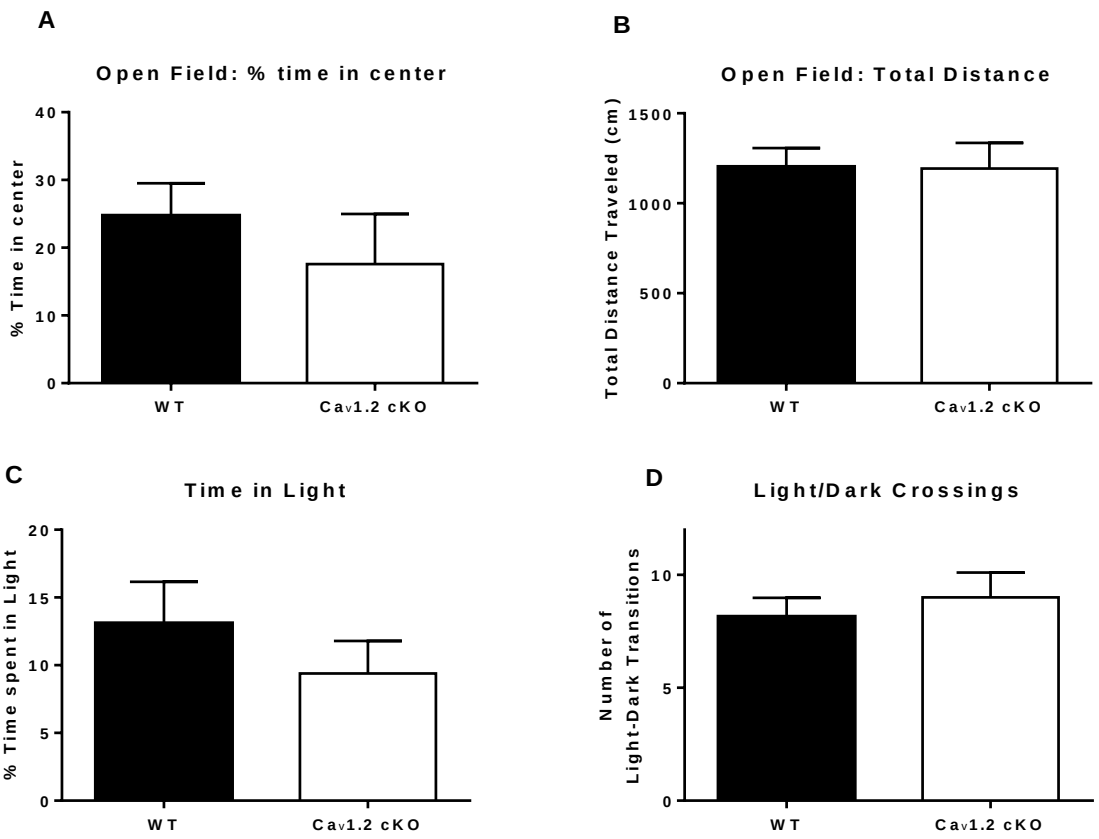

Figure 4.1: $Ca_V1.2$ conditional knockout mice exhibit normal basal anxiety and locomotion

Mice were tested for alterations in basal anxiety using the open field test and light-dark box test. (**A**) In the open field test, $Ca_V1.2$ conditional knockout (n = 12) mice spent a similar amount of time in the center of the open arena as their wild-type (n = 28) littermates (**B**) $Ca_V1.2$ conditional knockout and wild-type mice also travelled a comparable distance around the arena throughout the open field test. (**C**) In the light-dark box test, the same $Ca_V1.2$ conditional knockout mice spent equivalent amounts of time in the light portion of the chamber as wild-type mice. (**D**) Similarly, $Ca_V1.2$ conditional knockout mice and wild-type mice made a comparable number of transitions between the light and dark portions of the light-dark test. Data are represented as mean ± SEM.

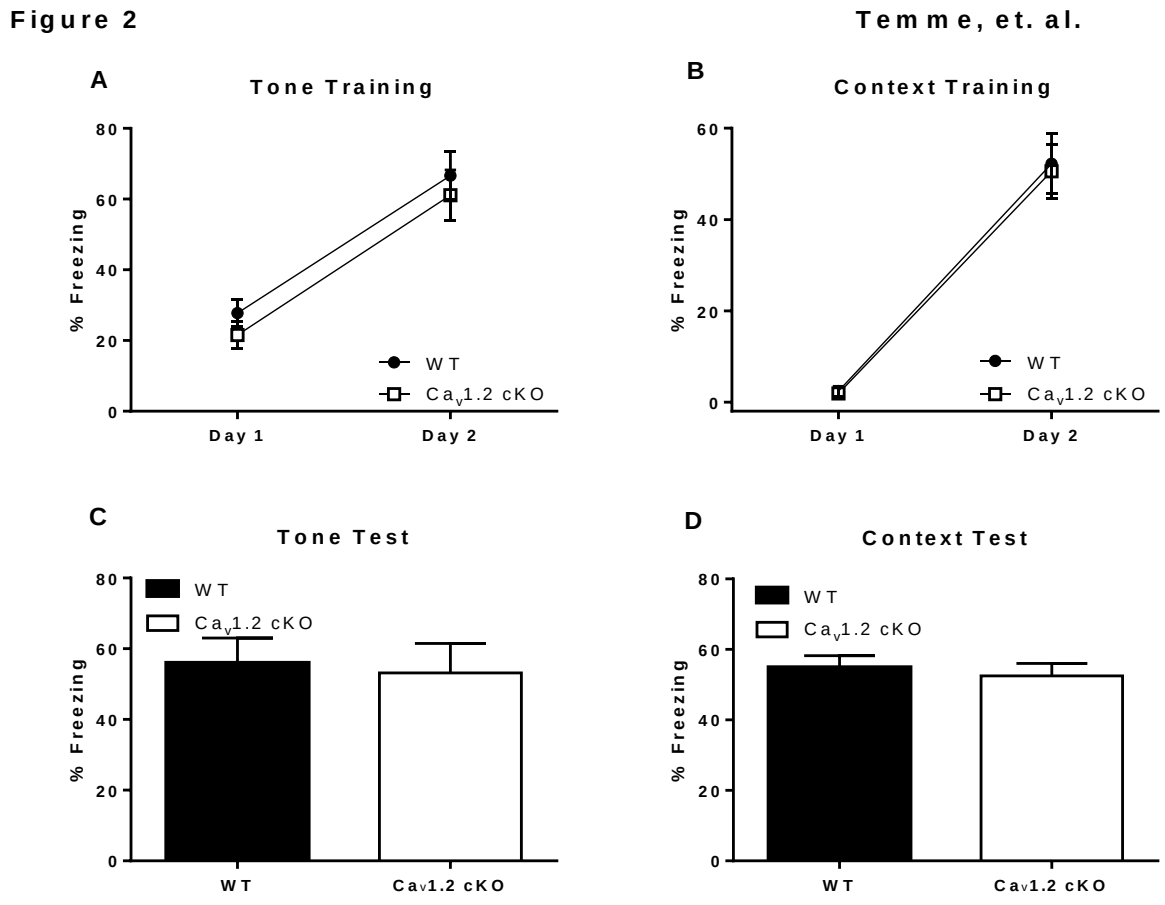

Figure 4.2: Neuronal deletion of Cav1.2 does not alter fear conditioning to a context or tone

Mice were fear conditioned to a context and tone using three minutes of context exposure followed by three tone-footshock presentations per day for two days. Mice were subsequently exposed to the training context alone or the trained tone alone to assess for acquisition and consolidation of fear. (**A & B**) Across training days, both $Ca_V1.2$ conditional knockout (n = 12) and wild-type mice (n = 28) displayed a significant enhancement in freezing to the context and cue. (**C & D**) $Ca_V1.2$ conditional knockout and wild-type mice also showed similarly high levels of freezing to both the context and tone when tested for fear acquisition and consolidation. Data are represented as mean ± SEM.

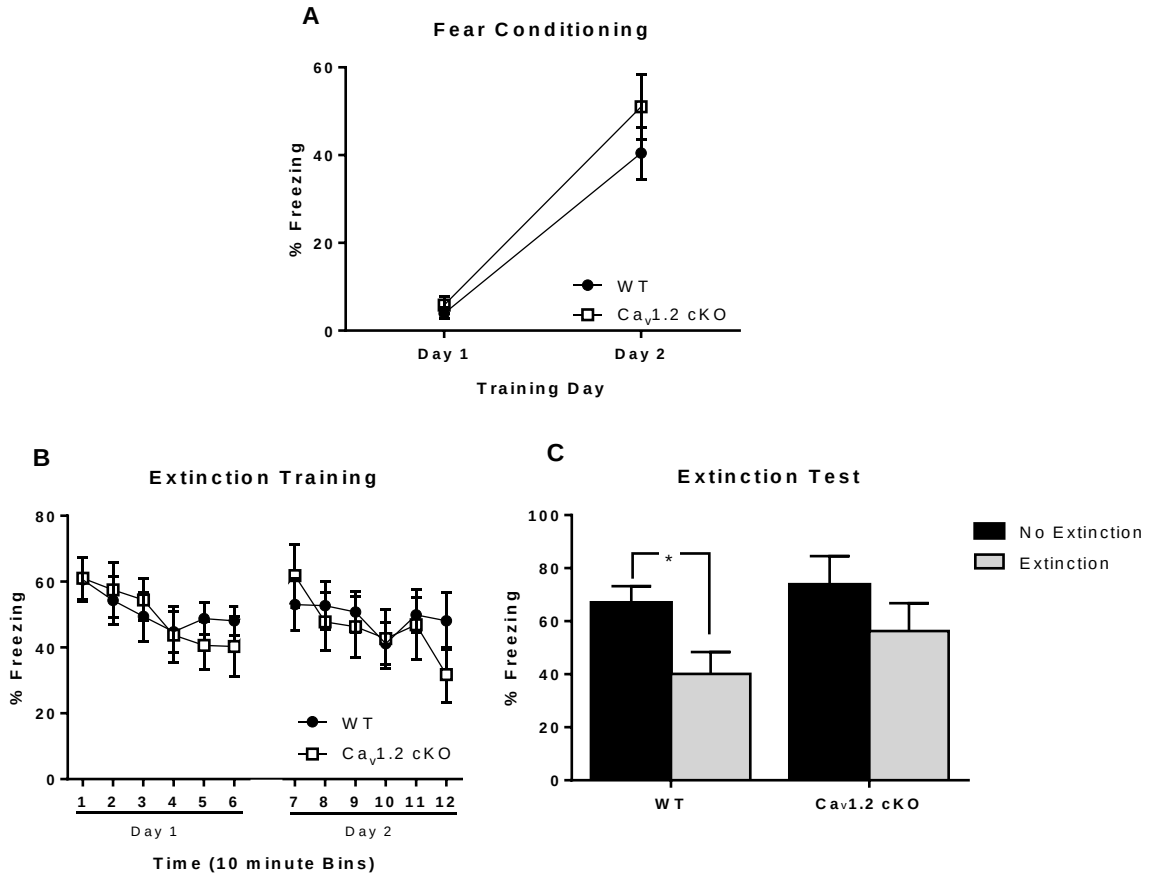

Figure 4.3: Cav1.2 conditional knockout mice exhibit significant deficits in fear extinction to a conditioned context

Mice were fear conditioned to a context using three minutes of context exposure followed by one unsignalled footshock presentation per day for two days. Mice were then randomly assigned to extinction (n = 7 and 10 for $Ca_V1.2$ conditional knockout and wild-type respectively) or no extinction (n = 7 and 10 for $Ca_V1.2$ conditional knockout and wild-type respectively) groups. Mice in the extinction group were extinguished using two hours of context exposure split across two days. Following extinction, both extinction and no extinction mice were assessed for fear to the conditioned context during the extinction test. (**A**) $Ca_V1.2$ and wild-type mice showed similar fear acquisition across context training days. (**B**) During context extinction, $Ca_V1.2$ conditional knockout and wild-type mice exhibit within session extinction in extinction day one, represented as time bins 1-6 and extinction day two, represented as time bins 7-12. (**C**) During extinction test, $Ca_V1.2$ conditional knockout mice who underwent

extinction failed to show a decline in freezing to the trained context compared to their no extinction counterparts while wild-type mice in the extinction group did. Data are represented as mean ± SEM. *$p < 0.05$.

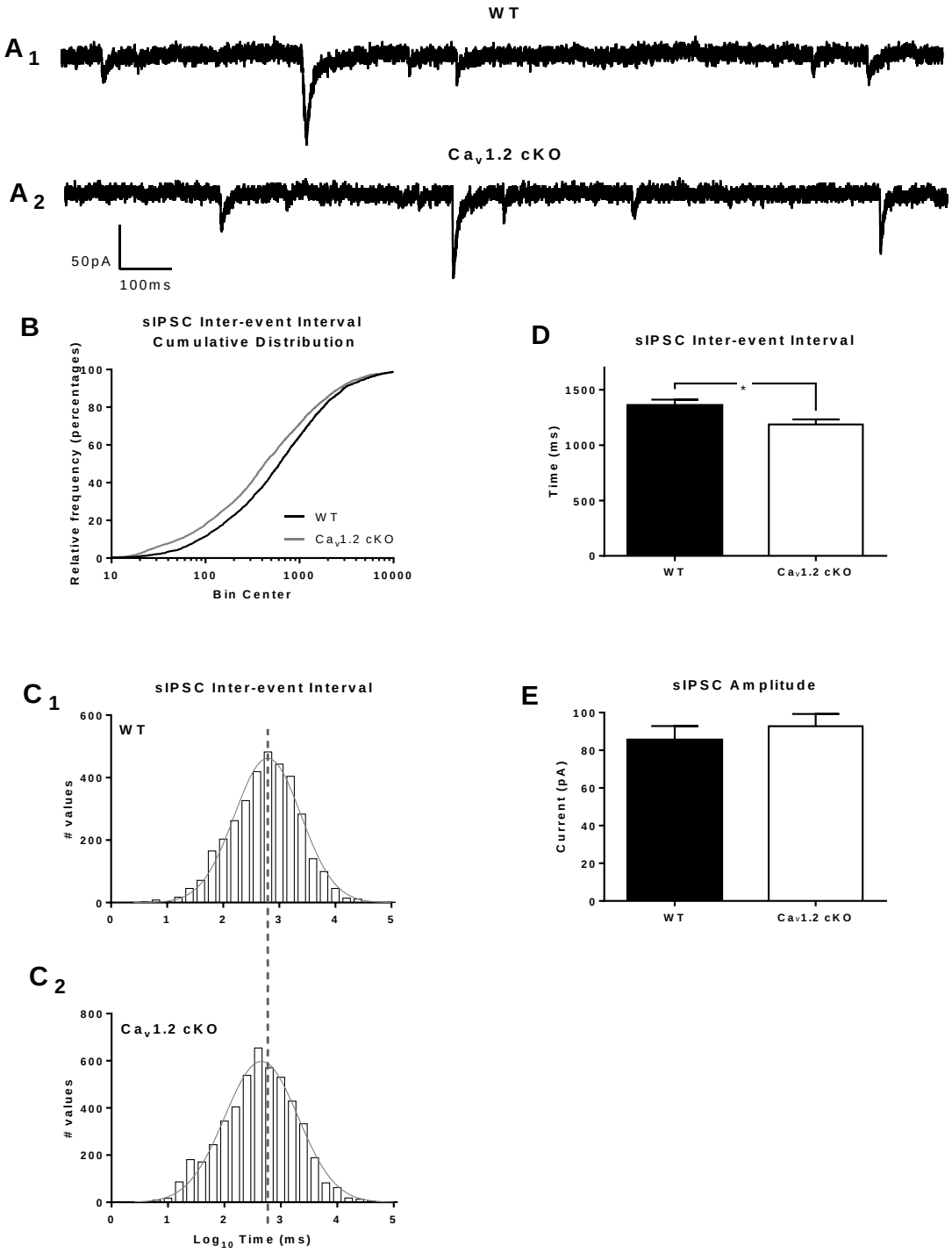

Figure 4.4: Neuronal deletion of Cav1.2 results in an increase in sIPSC activity in principle neurons of the lateral amygdala

Representative recordings from wild-type (**A_1**) and $Ca_V1.2$ conditional knockout mice (**A_2**) of spontaneous IPSCs in inhibition onto principle neurons of the lateral amygdala using whole cell voltage clamp. (**B**) $Ca_V1.2$ conditional knockout mice exhibited a significant change in sIPSC inter-event interval cumulative distribution compared to wild-type mice. (**C_1** & **C_2**) Representation of the sIPSC inter-event intervals using a histogram and a fitted Gaussian distribution, showed a leftward shift in $Ca_V1.2$ conditional knockout inter-event intervals compared to wild-type mice. (**D & E**) $Ca_V1.2$ conditional knockout mice exhibited a significant decrease in the average sIPSC (n = 4884 events in 23 cells) inter-event interval compared to wild-type littermates (n = 3442 events in 19 cells), but no change in sIPSC amplitude. Data are represented as mean ± SEM. *$p < 0.05$.

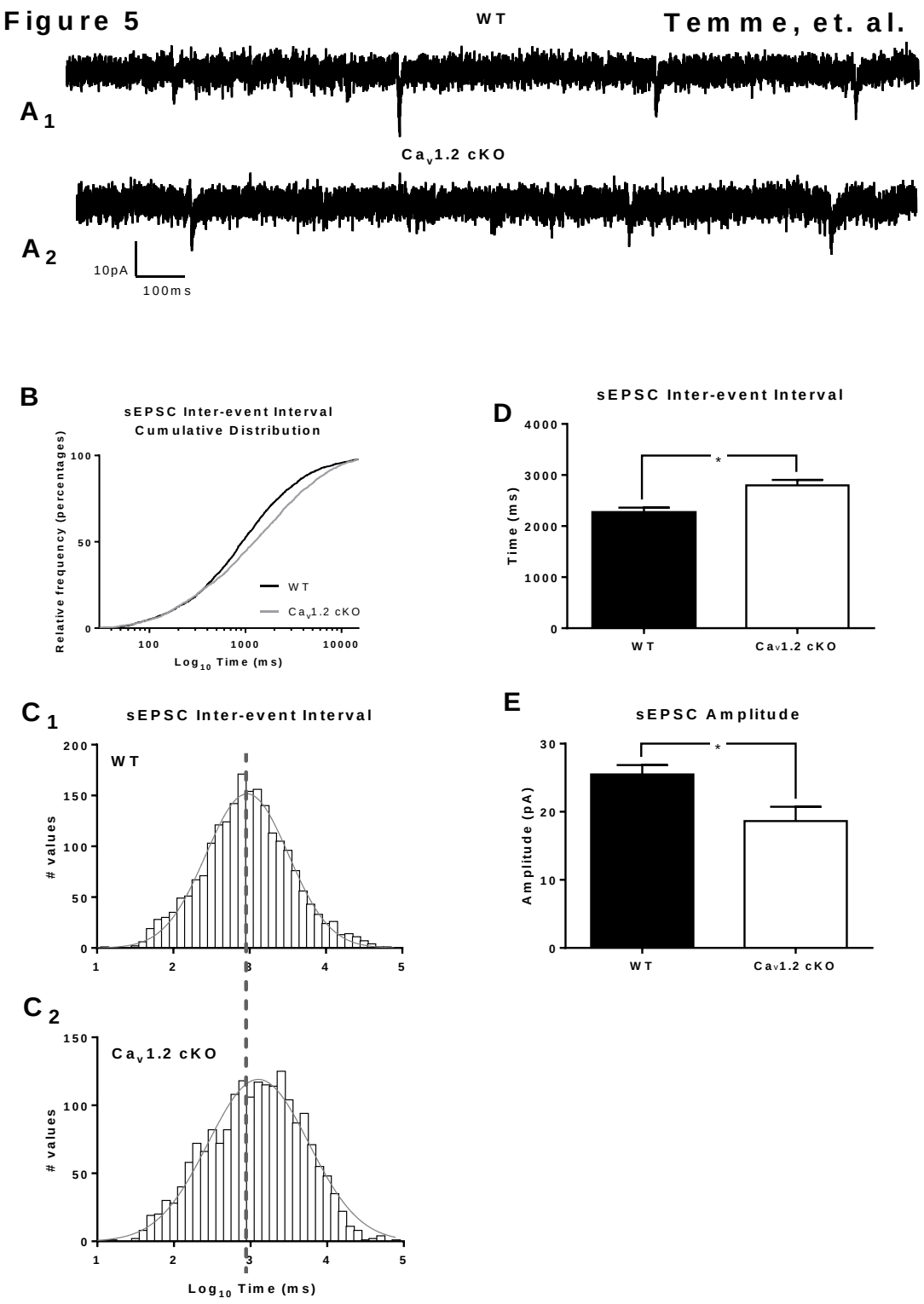
Figure 5
WT
Temme, et. al.
A 1
Ca$_v$1.2 cKO
A 2
10pA
100ms
B
sEPSC Inter-event Interval
Cumulative Distribution
Relative frequency (percentages)
100
50
0
WT
Ca$_v$1.2 cKO
100
1000
10000
Log$_{10}$ Time (ms)
D
sEPSC Inter-event Interval
Time (ms)
4000
3000
2000
1000
0
*
WT
Ca$_v$1.2 cKO
C 1
sEPSC Inter-event Interval
WT
values
200
150
100
50
0
1
2
3
4
5
C 2
Ca$_v$1.2 cKO
values
150
100
50
0
1
2
3
4
5
Log$_{10}$ Time (ms)
E
sEPSC Amplitude
Amplitude (pA)
30
20
10
0
*
WT
Ca$_v$1.2 cKO

Figure 4.5: Neuronal deletion of $Ca_V1.2$ results in an increase in sEPSC inter-event interval and a decrease in sEPSC amplitude in principle neurons in the lateral amygdala

Representative recordings from wild-type (**A_1**) and $Ca_V1.2$ conditional knockout mice (**A_2**) of spontaneous EPSCs in inhibition onto principle neurons of the lateral amygdala using whole cell voltage clamp. (**B**) Representation of the sEPSC inter-event interval as a cumulative distribution with a significant difference in the Kolomonov-Smirnov test between $Ca_V1.2$ conditional knockout and wild-type mice (**C_1** & **C_2**) Representation of the sEPSC inter-event intervals using a histogram, and a fitted Gaussian distribution shows a rightward shift in $Ca_V1.2$ conditional knockout inter-event interval compared to wild-type mice. (**D**) $Ca_V1.2$ conditional knockout mice exhibited a significant increase in the average sEPSC (n = 1927 events in 20 cells) inter-event interval compared to wild-type littermates (n = 2094 events in 16 cells). (**E**) $Ca_V1.2$ conditional knockout mice also showed a significant decrease in the average size of the sEPSCs. Data are represented as mean ± SEM. *$p < 0.05$.

CHAPTER 5

L-TYPE VOLTAGE GATED CALCIUM CHANNEL $CA_V1.2$ DEMONSTRATES SUBTYPE SPECIFIC MODULATION OF NEURONAL EXCITABILITY AND LONG TERM POTENTIATION

5.1 Summary

Calcium is a vital regulator of neuronal function in the brain. L-type voltage gated calcium channels (LVGCCs) are a class of calcium channels which open in response to large depolarizing shifts in membrane potential and modulate a wide array of neuronal functions via the influx of calcium into cells. Pharmacological studies have implicated LVGCCs in the maintenance of intrinsic neuronal excitability, as well as in the induction of long term potentiation in various brain structures, including the amygdala. Despite these studies, little is known about the relative contribution of the two LVGCC neuronal subtypes, $Ca_V1.2$ and $Ca_V1.3$ to these processes. This is due, in large part, to the lack of subtype specific pharmacological antagonists. Previous studies utilizing transgenic mice in which $Ca_V1.3$ was deleted have demonstrated that $Ca_V1.3$ is important for various components of intrinsic excitability in the amygdala, as well as LTP at the cortico-amygdala pathway. While $Ca_V1.2$ has been implicated in LTP formation at the thalamo-amygdala pathway, it remains unknown whether $Ca_V1.2$ plays a similar role in the forms of intrinsic neuronal excitability found to be altered by deletion of $Ca_V1.3$. Additionally, it is unclear to what extent $Ca_V1.3$ contributes to LTP

induction at the thalamo-amygdala pathway. To address these questions, we made *in vitro* whole-cell recordings from lateral amygdala pyramidal neurons in slices prepared from mice in which Cav1.2 or Cav1.3 was deleted. We found that neuronal deletion of $Ca_V1.2$ produced a significant increase in evoked firing rates in the absence of other changes in intrinsic excitability. Additionally, we found that deletion of $Ca_V1.2$, but not $Ca_V1.3$ significantly reduced pairing induced LTP at the thalamo-amygdala pathway. Taken with previous literature, these data suggest a differential role of $Ca_V1.2$ and $Ca_V1.3$ in neurophysiological properties within the lateral amygdala.

5.2 Introduction

Calcium is a key modulator of neuronal function (Berridge 1998, Raymond and Redman 2006, Clapham 2007, Burgoyne and Haynes 2014). Voltage-gated calcium channels allow for tight coupling of membrane potential and intracellular calcium levels which in turn mediate various components of neuronal activity and plasticity (Catterall 1998). Tight regulations of calcium concentrations intrinsically means that small changes in calcium concentration effect neuronal function through localized and short lived methods, such as neurotransmitter release (Perez-Reyes 2003, Kamp, Hanggi et al. 2012). In contrast, larger and longer lasting changes in free calcium concentration can alter neuronal function on a longer time scale and across farther distances, even altering the transcription of new genes through activation of second messenger systems (Murphy, Worley et al. 1991, Dolmetsch, Pajvani et al. 2001). Calcium through L-type voltage gated calcium channels has been tied to a variety of neurophysiological properties. L-type voltage gated calcium channels are known to influence the action potential afterhyperpolarization (AHP) (Rascol, Potier et al. 1991, Moyer, Thompson et

al. 1992, Tanabe, Gahwiler et al. 1998, White, McKinney et al. 2008, McKinney, Sze et al. 2009). L-type voltage gated calcium channels are also thought to contribute to the induction of LTP in various brain structures (Kapur, Yeckel et al. 1998, Weisskopf, Bauer et al. 1999, Bauer, Schafe et al. 2002, Moosmang, Haider et al. 2005, Fourcaudot, Gambino et al. 2009, McKinney, Sze et al. 2009). L-type voltage gated calcium channels have been classified into four subtypes based on the channel's pore forming subunit: $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$. Of the four different LVGCC subtypes, $Ca_V1.2$ and $Ca_V1.3$ have been found to be expressed in the brain (Hell, Westenbroek et al. 1993, Sinnegger-Brauns, Huber et al. 2009). Despite significant similarities in the structure of the two channel subtypes and their ability to be blocked by known LVGCC antagonists, differences in neuronal distribution (Hell, Westenbroek et al. 1993) and channel kinetics (Platzer, Engel et al. 2000, Xu and Lipscombe 2001) suggest that these two subtypes represent two very different forms of LVGCCs which may modulate neuronal function in significantly different manners.

Due to the absence of LVGCC subtype specific antagonists, studies have begun to utilize transgenic mice to investigate the contribution of each subtype to neuronal function and memory. Behaviorally, investigation of LVGCC subtypes in brain function have focused on the amygdala due to studies which found that blockade of LVGCCs produced deficits in fear related phenotypes (Bauer, Schafe et al. 2002, Cain, Blouin et al. 2002, Drephal, Schubert et al. 2006, McKinney and Murphy 2006, McKinney, Sze et al. 2008, Davis and Bauer 2012) as well as amygdala physiology and LTP (Weisskopf, Bauer et al. 1999, Bauer, Schafe et al. 2002, Schroeder and Shinnick-Gallagher 2004, Drephal, Schubert et al. 2006, Fourcaudot, Gambino et al. 2009, Langwieser, Christel et

al. 2010). These studies found that deletion of $Ca_V1.3$ resulted in a significant reduction in the slow AHP and AHP area, and an increase in spike accommodation and frequency in the basolateral amygdala (McKinney, Sze et al. 2009). Additionally, deletion of $Ca_V1.3$ produced deficits in the induction of LTP in the cortical-amygdala pathway. No alterations were noted in cells from $Ca_V1.3$ knockout mice in terms of resting membrane potential, resistance or action potential properties (McKinney, Sze et al. 2009). Investigation of the role of $Ca_V1.2$ on amygdala function, using transgenic mice with deletion of $Ca_V1.2$ in excitatory neurons in the forebrain found deficits in LTP in the thalamo-amygdala pathway (Langwieser, Christel et al. 2010). While these studies suggest a role of both $Ca_V1.2$ and $Ca_V1.3$ in LTP within the amygdala currently only $Ca_V1.3$ has been found to alter intrinsic excitability. However, a comprehensive study examining the impact of $Ca_V1.2$ deletion on intrinsic neuronal excitability in the lateral amygdala has yet to be completed. Additionally, studies of $Ca_V1.2$ and $Ca_V1.3$ knockout mice have investigated the role of these channels in LTP induction in the thalamo-amygdala and cortico-amygdala pathways (respectively) but the relative contribution to LTP induction in a single pathway (the thalamo-amygdala pathway) has yet to be determined.

To address these questions, we set out to determine whether $Ca_V1.2$ modulates intrinsic excitability in the amygdala, in particular those forms of intrinsic excitability altered by deletion of $Ca_V1.3$. Further, we investigated whether $Ca_V1.2$ and $Ca_V1.3$ may have differential contributions to LTP induction at thalamo-amygdala synapses in the lateral amygdala (LA). To do so, we utilized a $Ca_V1.2$ conditional knockout mouse in which the exon 2 allele of the gene for $Ca_V1.2$ (CACNA1C) was flanked by loxP sites

(White, McKinney et al. 2008) and expression of cre recombinase was driven in all neuronal populations by the synapsin 1 promoter (Zhu, Romero et al. 2001, Cui, Costa et al. 2008). $Ca_V1.2$ conditional knockout mice were assessed for alterations in resting membrane properties, action potential properties, properties of repetitive firing, and post-burst AHP in pyramidal neurons in the LA. Long term potentiation in the thalamo-amygdala pathway was assessed in $Ca_V1.2$ conditional knockout mice, as well as $Ca_V1.3$ global knockout mice previously investigated in amygdala neuronal physiology (McKinney, Sze et al. 2009). We found that mice with neuronal deletion of $Ca_V1.2$ exhibited normal input resistance, resting membrane potential and action potential firing in pyramidal cells in the LA. Interestingly, $Ca_V1.2$ conditional knockout mice exhibited no change in spike accommodation or the post-burst AHP properties of pyramidal neurons in the LA, but a significant change in firing frequency. These results suggest significantly different roles for $Ca_V1.2$ and $Ca_V1.3$ in intrinsic excitability in the principle neurons of the LA. To determine whether these channel also play distinct roles in plasticity within the amygdala, we investigated LTP in the thalamo-amygdala pathway using $Ca_V1.2$ conditional knockout mice and $Ca_V1.3$ global knockout mice and a paired induction protocol. We found that deletion of $Ca_V1.2$ produced a significant deficit in LTP induction in the thalamo-amygdala pathway while $Ca_V1.3$ mice exhibited normal LTP induction. These data suggest that $Ca_V1.2$ does play a role in the intrinsic excitability in the LA and LTP induction. Taken along with previous reports demonstrating a role of $Ca_V1.3$ in spike accommodation, AHP, and LTP at the cortical amygdala pathway, these results support a significant functional segregation of the LVGCC subtypes $Ca_V1.2$ and $Ca_V1.3$ in amygdala excitability and plasticity.

5.3 Methods

5.3.1 Mice

Electrophysiological experiments were performed using tissue from mice aged 3-7 months. Approximately equal numbers of males and females were used. The experimenter was kept blind to the genotype of the mice throughout experimentation. In all studies, experimental mice were compared to wild-type littermates. All experiments were conducted according to the National Institute of Health guidelines for animal care and were approved by the University Committee on the Use and Care of Animals of the University of Michigan.

5.3.1.1 $Ca_V1.2$ conditional knockout mice

$Ca_V1.2$ conditional knockout mice with neuron-specific deletion of $Ca_V1.2$ were created by crossing mice with a floxed $Ca_V1.2$ exon two allele with mice expressing cre driven by synapsin1a. Mice with a floxed $Ca_V1.2$ exon two allele ($Ca_V1.2^{f/+}$ or $Ca_V1.2^{f/f}$) (White, McKinney et al. 2008), and maintained on a 129SvEv genetic background, were first breed to transgenic mice expressing Cre recombinase regulated by the synapsin1 promoter (Syn1-$Cre^{Cre/+}$) (Zhu, Romero et al. 2001, Cui, Costa et al. 2008) and maintained on a C57BL/6 background, producing an F1 cross. Using non-littermate offspring from the F1 cross, heterozygous floxed, cre positive ($Ca_V1.2^{f/+}$ Syn1-$Cre^{Cre/+}$) mice were then crossed with heterozygous floxed, cre negative ($Ca_V1.2^{f/+}$ Syn1-$Cre^{+/+}$) mice to produce homozygous floxed, cre positive ($Ca_V1.2^{f/f}$ Syn1-$Cre^{Cre/+}$) conditional knock-out mice as well as mice categorized as wild-type or control. Mice were considered wild-type if they were cre positive, but lacked the floxed alleles ($Ca_V1.2^{+/+}$

Syn1-Cre$^{+/+}$). Mice were considered control if they were homozygous or heterozygous for the floxed allele and cre negative ($Ca_V1.2^{f/f}$ Syn1-Cre$^{+/+}$; $Ca_V1.2^{f/+}$ Syn1-Cre$^{+/+}$), or if they were cre positive but lacked the floxed alleles ($Ca_V1.2^{+/+}$ Syn1-Cre$^{Cre/+}$). For all experiments control mice were compared to and collapsed with wild-type mice when no significant difference was detected.

5.3.1.2 $Ca_V1.3$ global knockout mice

$Ca_V1.3$ global knockout mice were generated using a targeted mutation of exon two of the $Ca_V1.3$ allele using a neomycin cassette (Platzer et al. 2000). Similar to $Ca_V1.2$ conditional knock-out mice, $Ca_V1.3$ knock-out mice used in this study were on a B57Bl/6:129SvEv F2 genetic background. Mice with the targeted mutation were maintained on a purebred B57Bl/6 background. To produce an F2 cross, mice heterozygous for the $Ca_V1.3$ mutant allele ($Ca_V1.3^{+/-}$) and on the B57Bl/6 background were breed to purebred 129SvEv mice to produce an F1 cross. Non-sibling heterozygous mice ($Ca_V1.3^{+/-}$) from the F1 cross were then crossed to produce an F2 cross containing homozygous $Ca_V1.3$ knock-out mice ($Ca_V1.3^{-/-}$) and wild-type littermates ($Ca_V1.3^{+/+}$) for experimentation.

5.3.2 Electrophysiology

5.3.2.1 Slice Preparation

Mice aged 3-7 months were anesthetized with isoflurane prior to dissection. Brains were rapidly removed and incubated in an oxygenated, ice slurry of sucrose based cutting solution: (in mM): 206.0 sucrose, 2.8 KCl, 1.25 $MgCl_2$ $6H_2O$, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 1.25 $CaCl_2$, 10.00 D-Glucose, 26.00 $NaHCO_3$, 0.4 Ascorbic Acid, for a

period of one to two minutes. Brains were then bisected and placed in the slicing chamber with 1.5% agar blocks as support and submerged in ice cold sucrose cutting solution. The brain tissue was sliced in a coronal plane through the amygdala at 300µm. Slices were then incubated in room temperature oxygenated artificial cerebral spinal fluid (aCSF; in mM): 124.0 NaCl, 2.8 KCl, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 10.0 D-Glucose, 26.0 $MaHCO_3$, 0.4 ascorbic acid, for a minimum of one hour prior to recording.

5.3.2.2 Electrophysiological recordings

Electrophysiology recordings were made using a Dagan 3900A integrating patch clamp amplifier and digitized using an Axon Instruments 1322A Digidata. Recordings were made using glass electrodes made from borosilicate glass with filament (1.5mm x 0.86mm; Sutter Instruments) and a resistance between 4 to 8 mega ohms. Experiments were performed using whole-cell current clamp recordings of principle neurons in the LA. Action potential AHP recordings were performed using a potassium methylsulfate based internal solution (in mM): 120 potassium methylsulfate, 20 KCl, 10 HEPES, 4 Na2-ATP, 2 MgCl2, 0.3 GTP, 0.2 EGTA, and 7 phosphocreatine, and 0.1% Biocytin. All other recordings were performed using a potassium gluconate based internal solution (in mM): 120 KGluconate, 20 KCl, 10 HEPES, 10 phosphocreatine, 4 MgATP, 0.3 NaGTP, 0.1% Biocytin. All experiments were carried out, in a submersion chamber with continuous aCSF perfusion (~1.5ml/min) and heated to 31°C. Cells were considered to be a healthy if they met 3 criteria: (1) the initial resting membrane potential was -60mV or less, (2) the resting membrane potential varied no more than 5mV throughout the recordings, (3) they fired action potentials with amplitudes that

exceeded 0mV. Pyramidal neurons were identified based on their morphology and their ability to demonstrate spike accommodation in the presence of prolonged depolarization. After electrophysiological examination, slices were collected and stained for the presence of biocytin in order to confirm the location and morphology of recorded cells (**Figure 5.1A**). Single action potentials were generated using a 10 ms long depolarization step. Action potential properties, except half-width, were calculated from spikes produced with the smallest level of depolarization sufficient to elicit an action potential. Repetitive firing was examined using one second long current steps ranging from 100pA to 150pA in 10pA increments. Input resistance was calculated using the peak currents induced by one second long current steps ranging from -50pA to 50pA as the slope of the input/output curve. Action potential half-widths were measured from 500 ms long depolarization steps at the minimal current required to elicit five action potentials. For AHP experiments, cells were held at a membrane potential 10 mV below action potential threshold and the AHP was measured using a 500 ms long depolarization step at the minimal current required to elicit five action potentials. For LTP experiments, excitatory postsynaptic potentials (EPSPs) were recorded in principle neurons in the LA in response to 100 μs stimulation of the thalamic afferents into the amygdala using a 2-contact cluster electrode (FHC; Bowdoinham, ME). Stimulation intensity was selected based on the current intensity which produced an EPSP between one-quarter to half the maximum inducible EPSP with a clear mono-synaptic component. Excitatory post-synaptic potentials were sampled at a rate of 0.067 Hz for five minutes prior to LTP induction and for forty-five minutes post-LTP induction. Long term potentiation was induced through paired stimulation of thalamo-amygdala

pathway and postsynaptic depolarization of lateral amygdala neurons of 1.2nA for three milliseconds using three bouts of 100 stimulations at 33Hz with an inter-bout interval of 10 seconds. Long term potentiation was calculated by comparing the initial slope of the EPSP. All data was acquire using ClampEx and analyzed using Clampfit (pClamp9.2, Molecular Devices)

5.3.3 Statistical Analysis

Thalamo-amygdala LTP data was analyzed per slice and averaged by mouse prior to analysis as genotype. For all other recordings, data were analyzed by cell then averaged by mouse with genotype representing the mouse average. Comparison of individual values between genotypes was performed using an unpaired t-test. Measurements with multiple values were analyzed using a repeated measures ANOVA with genotype as a factor. Synaptic efficacy was analyzed using comparison of fit of a non-parametric line. Analyses were considered significant if they had a p-value less than 0.05.

5.4 Results

5.4.1 Neuronal deletion of $Ca_V1.2$ does not alter passive neuronal properties or action potential properties

To determine whether neuronal deletion of $Ca_V1.2$ altered basic properties of the principle cells in the LA (**Figure 5.1A**), measurements of input resistance and resting membrane potential were made along with analysis of action potential firing properties. Input resistance in $Ca_V1.2$ conditional knockout mice was not significantly different from wild-type littermates ($p = 0.4671$, unpaired t-test) (**Figure 5.1B**). Additionally

deletion of $Ca_V1.2$ did not alter the resting membrane potential compared to wild-type mice ($p = 0.4496$) (**Figure 5.1C**, unpaired t-test).

Action potential were recorded from wild-type and $Ca_V1.2$ conditional knockout mice using 10 ms long depolarization steps with action potential analysis occurring at the minimal intensity to induce a full action potential (**Figure 5.2A$_1$ and A$_2$**). No significant difference was noted between $Ca_V1.2$ conditional knockout mice and wild-type mice in the minimal current intensity to generate a full action potential ($p= 0.0694$, unpaired t-test) (**Figure 5.2B**). Analysis of the action potential threshold, measured as the change in membrane voltage from rest to action potential induction, in $Ca_V1.2$ conditional knockout mice and wild-type mice using an unpaired t-test revealed no significant different from wild-type mice ($p = 0.1836$) with an average current shift of 38 mV in wild-types and 34 mV in $Ca_V1.2$ conditional knockout mice (**Figure 5.2C**). Similarly, analysis of the average action potential height from the point of action potential induction to the peak of the action potential using an unpaired t-test also found no significant difference between genotypes ($p = 0.4810$) with an average action potential height of 64 mV in wild-type mice and 68 mV in $Ca_V1.2$ conditional knockout mice (**Figure 5.2D**). Action potential half-width was assessed across a train of five action potentials generated from a depolarization of 500 ms and the minimal current intensity required to reliably generate five action potentials (**Figure 5.2E**). Comparison of the average action potential half-width across action potentials using a repeated measures ANOVA found a significant effect of order of action potential occurrence in half-width ($F_{(4,44)} = 105.624$, $p < 0.0001$), but no effect of genotype ($F_{(1,11)} = 0.636$, $p =$

0.4419). From these data, it appears that $Ca_V1.2$ does not affect basal neuronal properties and action potential firing.

5.4.2 Neuronal deletion of $Ca_V1.2$ enhances action potential frequency, but not accommodation

To determine whether deletion of $Ca_V1.2$ alters intrinsic excitability of the cell, repetitive action potential firing was assessed. Firing rates were assessed in $Ca_V1.2$ conditional and wild-type mice by measuring the number and inter-event interval of action potentials generated in response to a one second long depolarization of variable current intensity (**Figure 5.3A$_1$ and A$_2$**). Analysis of the number of action potentials in response to various current injections using a repeated measures ANOVA with current intensity and genotype as factors revealed a significant effect of current intensity ($F_{(5,100)}$ = 165.093, $p < 0.0001$) and a significant effect of genotype ($F_{(1,20)}$ = 4.809, $p = 0.0403$) with an increase in the number of action potentials in response to various current intensities in $Ca_V1.2$ conditional knockout mice (**Figure 5.3B**). Spike accommodation was assessed by analyzing the action potential inter-event intervals generated from the minimal current required to generate seven action potentials in one second. Analysis of spike accommodation between genotypes using a repeated measures ANOVA revealed significant accommodation in both $Ca_V1.2$ conditional knockout mice and wild-type mice ($F_{(1,25)}$ = 2.741, $p = 0.1103$), but no significant alteration in spike accommodation between genotypes ($F_{(5,125)}$ = , $p < 0.0001$) (**Figure 5.3C**). These data suggest that unlike $Ca_V1.3$, $Ca_V1.2$ does not play a role in spike accommodation in the LA, however, $Ca_V1.2$ does affect spike frequency.

5.4.3 Neuronal deletion of $Ca_V1.2$ does not alter neuronal afterhyperpolarization

To assess whether, like $Ca_V1.3$, $Ca_V1.2$ produces alteration in the AHP in pyramidal neurons in the BLA we recorded the AHP generated from a train of five action potentials while the cell was held at 10 mV below action potential threshold. The size of AHP was assessed in wild-type (**Figure 5.4A$_1$**) and $Ca_V1.2$ conditional knockout mice (**Figure 5.4A$_2$**) through measurement of the AHP peak, the size of the AHP 200 ms and 1000ms after the end of the last action potential to assess the medium AHP and s AHP respectively, and the total area of the AHP. When analyzed for changes in the AHP, $Ca_V1.2$ conditional knockout mice exhibited no changes in the AHP peak and the AHP 200 ms after the last action potential compared to wild-type mice using an unpaired t-test ($p = 0.8767$ and $p = 0.4655$, respectively). Interestingly, $Ca_V1.2$ conditional knockout mice also exhibited no change the AHP 1000 ms after the last action potential when compared to wild-type mice ($p = 0.8183$, unpaired t-test). These results were supported by analysis showing no significant difference in the overall area of the AHP between $Ca_V1.2$ conditional knock-out mice and wild-type mice ($p = 0.5868$, unpaired t-test). These data suggest that $Ca_V1.2$ does not affect the AHP in the LA, and given previous literature supporting a role of $Ca_V1.3$ in the AHP in the LA, supports a differential role of these two LVGCC subtypes in amygdala function.

5.4.4 Neuronal deletion of $Ca_V1.2$ reduces long term potentiation in the thalamo-amygdala pathway

Given the significant discrepancy in the role of $Ca_V1.2$ in physiology of pyramidal neurons within the lateral amygdala compared to reports of $Ca_V1.3$ in the

literature, we wanted to determine whether $Ca_V1.2$ and $Ca_V1.3$ may play differential roles in amygdala LTP. Long term potentiation in the thalamo-amygdala pathway was induced using paired presynaptic stimulation of thalamo-amygdala afferents and postsynaptic synaptic depolarization (**Figure 5.5A**). Specifically, LTP was induced using three bouts of 100 stimulations of thalamo-amygdala pathway at 33 Hz and paired postsynaptic depolarization of lateral amygdala neurons at 1.2nA for three milliseconds at the same rate (**Figure 5.5B and C**). The temporal relationship between postsynaptic depolarization and presynaptic stimulation was altered for each cell to produce postsynaptic action potentials timed to occur near the peak of the presynaptically induced EPSPs. Bouts of paired stimulation were separated by an inter-bout interval of 10 seconds. Recordings of excitatory postsynaptic potentials were made from pyramidal neurons in the lateral amygdala before and after LTP induction (**Figure 5.6A$_1$ and A$_2$**). Analysis of EPSP strength was made by calculating the initial slope of the EPSP (**Figure 5.6B$_1$ and B$_2$**). Long term potentiation was assessed over the post-LTP induction period in five minute blocks as an average of the EPSP slopes recorded in each block and normalized to the average of the EPSP slopes recorded prior to LTP induction for each genotype (**Figure 5.6C**). Following LTP induction, wild-type mice and $Ca_V1.2$ conditional knockout mice exhibited an increase in normalized EPSP slope of 40% and 24% compared to pre-LTP induction respectively ($p = 0.0001$, $p = 0.0035$ respectively, one group t-test). Comparison of the normalized EPSP slope values between genotypes across the post-LTP induction period using a repeated measures ANOVA revealed a significant effect of genotype ($F_{(1,16)} = 6.215$, $p = 0.0240$) with a significant decrease in LTP in $Ca_V1.2$ conditional knockouts strength compared to wild-

type mice. These enhancements in EPSP slope appear to be stable for each genotype and maintained for a minimum of 45 minutes, with an ending normalized EPSP slope of 1.475 in wild-type mice and 1.190 in $Ca_V1.2$ conditional knockout mice compared pre-LTP induction. Further analysis of the final five minutes of the post-LTP induction period using an unpaired t-test supported a significant increase in normalized EPSP slope compared to the pre-LTP period in both wild-type and $Ca_V1.2$ conditional knockout mice ($p = 0.0013$ and $p = 0.0049$ respectively) (**Figure 5.6D**). Comparison of the final five minutes of post-LTP induction between genotypes using a unpaired t-test also revealed a significant reduction of LTP in $Ca_V1.2$ conditional knockout mice ($p = 0.0403$). To verify that these deficits in LTP were not due to alterations in synaptic connectivity in $Ca_V1.2$ conditional knockout mice, EPSPs were recorded from a subset of mice using variable stimulation intensities of the thalamo-amygdala pathway (**Figure 5.6E**). Analysis of synaptic efficacy using a comparison fit of a non-parametric line between the two genotypes found no significant difference between genotypes ($F_{(2,490)} = 2.615$, $p = 0.0742$). These data are consistent with previously published data in $Ca_V1.2$ conditional knockout mice lacking $Ca_V1.2$ in excitatory neurons (Langwieser, Christel et al. 2010), and supports a partial role of $Ca_V1.2$ in LTP in the thalamo-amygdala pathway.

5.4.5 Global deletion of $Ca_V1.3$ does not alter long term potentiation in the thalamo-amygdala pathway

To determine the potential involvement of $Ca_V1.3$ in LTP at the thalamo-amygdala pathway, we performed whole cell recordings and analysis similar to that used in $Ca_V1.2$ conditional knockout mice. Excitatory post-synaptic potential were

recorded from pyramidal neurons in the lateral amygdala in response to stimulation of the thalamo-amygdala pathway (**Figure 5.7A$_1$ and A$_2$**). As described above, changes in the strength of synaptic connectivity were analyzed using the initial slope of the EPSP before and after LTP induction (**Figure 5.7B$_1$ and B$_2$**). Comparison of the normalized EPSP slope values across the post-LTP induction recordings between genotypes revealed no significant alteration in EPSP slope in $Ca_V1.3$ knockout mice ($F_{(1,16)}$ = 0.169, p = 0.6867), repeated measure ANOVA) (**Figure 5.7C**). Further analysis of the final five minutes of the post-LTP induction period using an unpaired t-test compared to the normalized pre-LTP levels found a significant increase in normalized EPSP slope in both wild-type and $Ca_V1.3$ knockout mice (p = 0.0004 and p = 0.0005 respectively) (**Figure 5.7D**). Additionally, comparison of the normalized EPSP slope between genotypes during the post-LTP induction period also found no significant difference between genotypes (p = 0.6302, unpaired t-test). Synaptic efficacy was recorded in a subset of mice and also revealed normal strength of EPSPs in response to variable thalamo-amygdala stimulation in $Ca_V1.3$ knockout mice compared to their wild-type counterparts ($F_{(2,401)}$ = 0.1671, p = 0.6217, comparison of fit of a non-parametric line between genotypes (**Figure 5.7E**). Taken together with data illustrating a role of $Ca_V1.2$ in thalamo-amygdala LTP, these data support a differential role of $Ca_V1.2$ and $Ca_V1.3$ in thalamo-amygdala LTP, as well as intrinsic excitability.

5.5 Discussion

L-type voltage gated calcium channels have been linked to fear related behavior (Bauer, Schafe et al. 2002, Cain, Blouin et al. 2002, Moosmang, Haider et al. 2005, McKinney and Murphy 2006, Davis and Bauer 2012) as well as neuronal excitability

and plasticity in the amygdala (Weisskopf, Bauer et al. 1999, Bauer, Schafe et al. 2002, Moosmang, Haider et al. 2005, McKinney, Sze et al. 2009). Using $Ca_V1.2$ conditional knockout mice in which exon 2 of the $Ca_V1.2$ allele is floxed by loxP sites (White, McKinney et al. 2008) and cre recombinase was driven in all neuronal populations by the synapsin 1 promoter (Zhu, Romero et al. 2001, Cui, Costa et al. 2008), we examined the role the of the LVGCC subtype, $Ca_V1.2$ in the neurophysiological function of pyramidal neurons in the LA. Additionally, $Ca_V1.2$ and $Ca_V1.3$ were assessed for their role in LTP induction in the thalamo-amygdala pathway using $Ca_V1.2$ conditional knockout mice and $Ca_V1.3$ global knockout mice. Mice with neuronal deletion of $Ca_V1.2$ demonstrated an increase in action potential frequency, but normal action potential properties, spike accommodation, and AHP in pyramidal neurons in the LA. We also found that neuronal deletion of $Ca_V1.2$, but not $Ca_V1.3$, produced a deficit in LTP in the thalamo-amygdala pathway. Taken together with previous literature showing a role of $Ca_V1.3$ in the AHP and spike accommodation in the LA, these results demonstrate a differential role of $Ca_V1.2$ and $Ca_V1.3$ in amygdala plasticity and excitability.

$Ca_V1.2$ conditional knockout mice were examined for changes in input resistance and resting membrane potential as well as changes in action potential, repetitive firing, and AHP properties in pyramidal neurons in the LA. $Ca_V1.2$ conditional knockout mice exhibit normal input resistance and resting membrane potential similar to their wild-type counterparts. Additionally, $Ca_V1.2$ conditional knockout mice exhibited no alterations in action potential firing with similar current to first action potential, action potential threshold, and action potential height. Analysis of

a train of five action potentials illustrated a normal action potential half-width and increase in half-width with each consecutive spike. When analyzed for repetitive firing, $Ca_V1.2$ conditional knockout mice were found to have an increase in firing frequency, but normal spike accommodation. Lastly, analysis of the AHP in pyramidal neurons from $Ca_V1.2$ conditional knockout mice revealed normal AHP peak, medium AHP (AHP at 200ms), slow AHP (AHP at 1000ms), as well as overall AHP area when compared to wild-type mice. Overall, this data suggests that $Ca_V1.2$ alters the intrinsic excitability of pyramidal neurons in the LA.

Pharmacological studies of the role of LVGCCs in intrinsic excitability have found a significant decrease in the size of the AHP and spike accommodation in the presence of LVGCC blockers (Rascol, Potier et al. 1991, Marrion and Tavalin 1998, Shah and Haylett 2000, Power, Wu et al. 2002, Lima and Marrion 2007, McKinney, Sze et al. 2009). While our current study fails to find a role of $Ca_V1.2$ in the AHP and firing accommodation within the LA, previous studies utilizing the $Ca_V1.3$ global knockout found that deletion $Ca_V1.3$ produced an increase in spike accommodation and a decrease in the slow component of the AHP in the amygdala (McKinney, Sze et al. 2009). This suggests that $Ca_V1.2$ and $Ca_V1.3$ may have differential roles in the regulation of these neurophysiological properties within the brain, including the amygdala. In fact, a study utilizing the same $Ca_V1.3$ knockout mouse and a $Ca_V1.2$ conditional knockout mouse in which $Ca_V1.2$ has been deleted in excitatory neurons in the forebrain, also demonstrated a significant effect of $Ca_V1.3$, but not $Ca_V1.2$ on the size of the AHP in the hippocampus (Gamelli, McKinney et al. 2011). These data suggest that $Ca_V1.3$ and not $Ca_V1.2$ may mediate the effect of LVGCCs on the

formation of the AHP in the amygdala and hippocampus, as well as other structures throughout the brain.

Similar to Cav1.3 global knockout mice, $Ca_V1.2$ conditional knockout mice demonstrated a significant increase in firing frequency in pyramidal cells in the LA. While changes in action potential frequency in the previous published $Ca_V1.3$ global knockout mice (McKinney, Sze et al. 2009) were attributed to alterations in the slow component of the AHP also observed in Cav1.3 global knockout mice. However, $Ca_V1.2$ conditional knockout mice did not show alterations in the AHP. Therefore, changes in firing frequency in $Ca_V1.2$ conditional knockout mice must be generated through a different mechanism, separate from the function of Cav1.3. Calcium is known to regulate a variety of functions within the cell which could influence excitability (Berridge 1998, Raymond and Redman 2006, Clapham 2007, Burgoyne and Haynes 2014). While calcium influx through LVGCCs is known for its role in the modulation of the AHP (Rascol, Potier et al. 1991, Marrion and Tavalin 1998, Shah and Haylett 2000, Power, Wu et al. 2002, Lima and Marrion 2007, McKinney, Sze et al. 2009), calcium through these channels could be effects other calcium regulated cell functions that may produce changes in excitability. For example, increases in calcium influx are known to activate various calcium-dependent ion channels including potassium channels (Bowden, Fletcher et al. 2001, Sah and Faber 2002, Lu, Sirish et al. 2015) and intracellular calcium channels (Leong and MacLennan 1998). While LVGCCs are thought to regulate the AHP by activation of some calcium-activated potassium channels, $Ca_V1.2$ could be mediating other calcium-activated potassium channels, perhaps not involved in the AHP, or not involved in the features of the AHP assessed, to

modulate excitability. It is also possible that deletion of $Ca_V1.2$ produces alterations in in other features of the refractory period between action potentials, such as the reactivation of voltage activated sodium channels responsible for action potential firing. Changes in the reactivation rates of these sodium channels could produce changes in the firing of repetitive action potentials, but not a single action potential or the AHP.

Additionally, neuronal deletion of $Ca_V1.2$ could be mediating an increase in the firing frequency of pyramidal neurons via compensatory mechanisms. Recent studies of mice with neuronal deletion of $Ca_V1.2$ found a significant shift in inhibitory and excitatory inputs onto pyramidal cells in the LA (Chapter 4). Long term alterations in the inhibition of pyramidal neurons could produce a compensatory response in excitability to maintain the homeostatic balance of inhibition and excitation within the amygdala. Therefore $Ca_V1.2$ may not be altering the firing frequency of pyramidal cells directly, but through their action on the inhibitory and excitatory afferents onto these cells. This data may also explain why deletion of $Ca_V1.2$ would alter the firing frequency, but not spike accommodation. To eliminate the possibility that changes in the inhibitory tone of the amygdala are producing a compensatory increase in the firing frequency of pyramidal neurons in the LA, recordings of repetitive firing could be made in $Ca_V1.2$ conditional knockout mice in which $Ca_V1.2$ is deleted in excitatory neurons. Lastly, it is possible that changes in spike frequency in the amygdala of mice with neuronal deletion of $Ca_V1.2$ could be due to the time point at which cre recombinase is expressed prenatally. A loss of $Ca_V1.2$ early in prenatal development could alter the manner in which neuronal circuits within the amygdala form and the function of neurons within these circuits.

Investigation of the effects of deletion of $Ca_V1.2$ and $Ca_V1.3$ in our studies also revealed a differential role of these two channels with a significant deficit in LTP formation in $Ca_V1.2$ conditional knockout mice, but not $Ca_V1.3$. Interestingly, previous literature has demonstrated a role for $Ca_V1.3$ when LTP in the amygdala is induced in the cortico-amygdala pathway, suggesting that $Ca_V1.2$ and $Ca_V1.3$ both mediate plasticity in the amygdala, but may do so in a pathway specific manner. Additionally, because $Ca_V1.2$ and $Ca_V1.3$ are both expressed in pyramidal cells in the amygdala, differential effects of these subtypes on different forms of LTP suggest that these channels may regulate LTP formation through different subcellular mechanisms. LVGCCs are thought to induce LTP formation via second messenger systems and activation of transcription and translation (Sheng, McFadden et al. 1990, Dolmetsch, Pajvani et al. 2001, Moosmang, Haider et al. 2005). Different mechanism of induction of LTP between $Ca_V1.2$ and $Ca_V1.3$ could be due to a difference in the second messenger systems activated. Differences in the composition and structure of $Ca_V1.2$ and $Ca_V1.3$ may result in different binding and regulation sites that can mediate LTP induction. Additionally, while LTP in the thalamo-amygdala pathway is generally believed to be due to post-synaptic changes, LTP in the cortical pathway has been tied to changes pre-synaptically (Fourcaudot, Gambino et al. 2009). In fact, despite the prevailing view that LVGCCs are only expressed on dendrites and somas (Hell, Westenbroek et al. 1993, Pinard, Mascagni et al. 2005), recent studies in the hippocampus have provided evidence that suggest presynaptic localization of LVGCCs (Tippens, Pare et al. 2008). Additionally, $Ca_V1.3$ is known to play a vital role in neurotransmitter release in ribbon synapses (Brandt, Striessnig et al. 2003, Sheets,

Kindt et al. 2012).Therefore, $Ca_V1.2$ and $Ca_V1.3$ could be acting via differential distributions in the cell, whether it be through localization to additional signaling mechanisms, like $Ca_V1.3$ and SK channels, or presynaptic modulation.

In summary, our data support a significant role of $Ca_V1.2$ in the amygdala in the modulation of action potential frequency and long-term potentiation at the thalamo-amygdala pathway. These data also suggest a significant differential effect of $Ca_V1.2$ and $Ca_V1.3$ on amygdala function, and perhaps neuronal function throughout the brain. Confirmation of this hypothesis would require additional studies of the effects of $Ca_V1.2$ and $Ca_V1.3$ deletion in a variety of neuronal classes and brain structures. Importantly, this supports a growing body of literature demonstrating significant difference in $Ca_V1.2$ and $Ca_V1.3$ characteristics, and the re-evaluation of the functions and kinetics assigned to the L-type class of voltage gated calcium channels.

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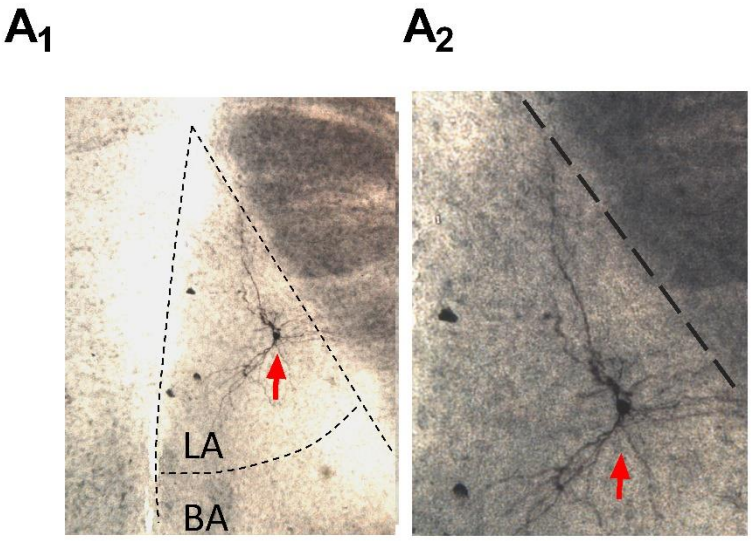
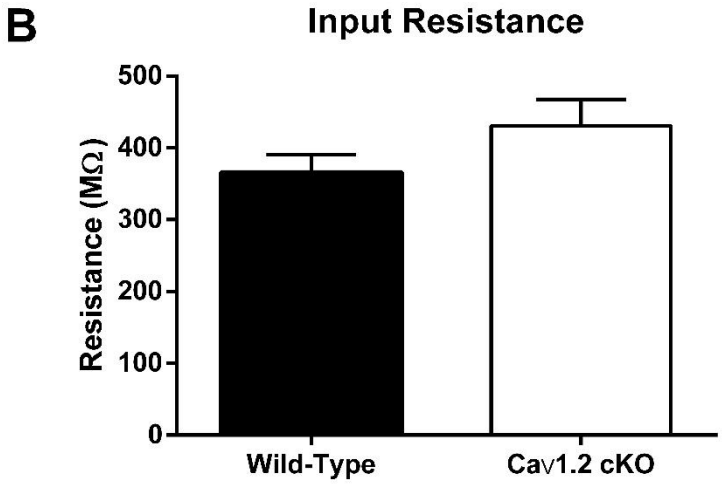

A1
A2
LA
BA


B
Input Resistance
Resistance (MΩ)
500
400
300
200
100
0
Wild-Type
Cav1.2 cKO

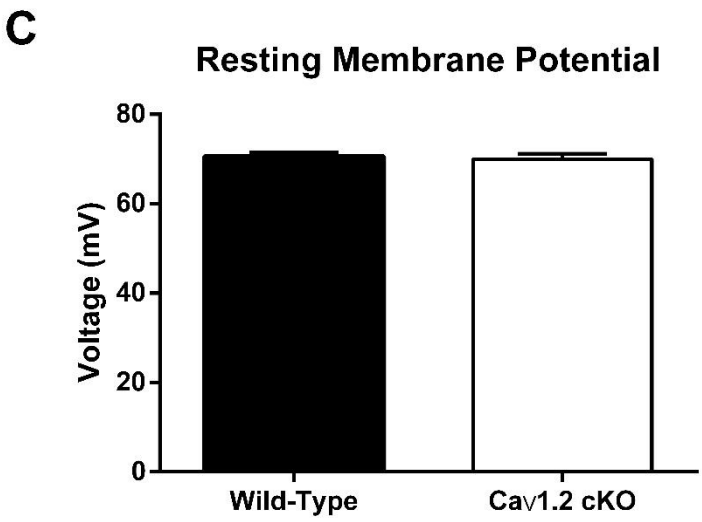

C
Resting Membrane Potential
Voltage (mV)
80
60
40
20
0
Wild-Type
Cav1.2 cKO

Figure 5.1: Neuronal deletion of $Ca_V1.2$ does not alter passive properties of the pyramidal neurons in the lateral amygdala.

Whole cell current clamp recordings were made from pyramidal neurons in the lateral amygdala in $Ca_V1.2$ conditional knockout mice (n = 8) and wild-type mice (n = 11). (**A_1 and A_2**) The red arrow indicates an example of a pyramidal neuron in the LA stained for biocytin after recording. (**B**) Pyramidal cells in $Ca_V1.2$ conditional knockout mice exhibited a similar input resistance and (**C**) resting membrane potential as wild-type mice. Data are represented as mean ± SEM.

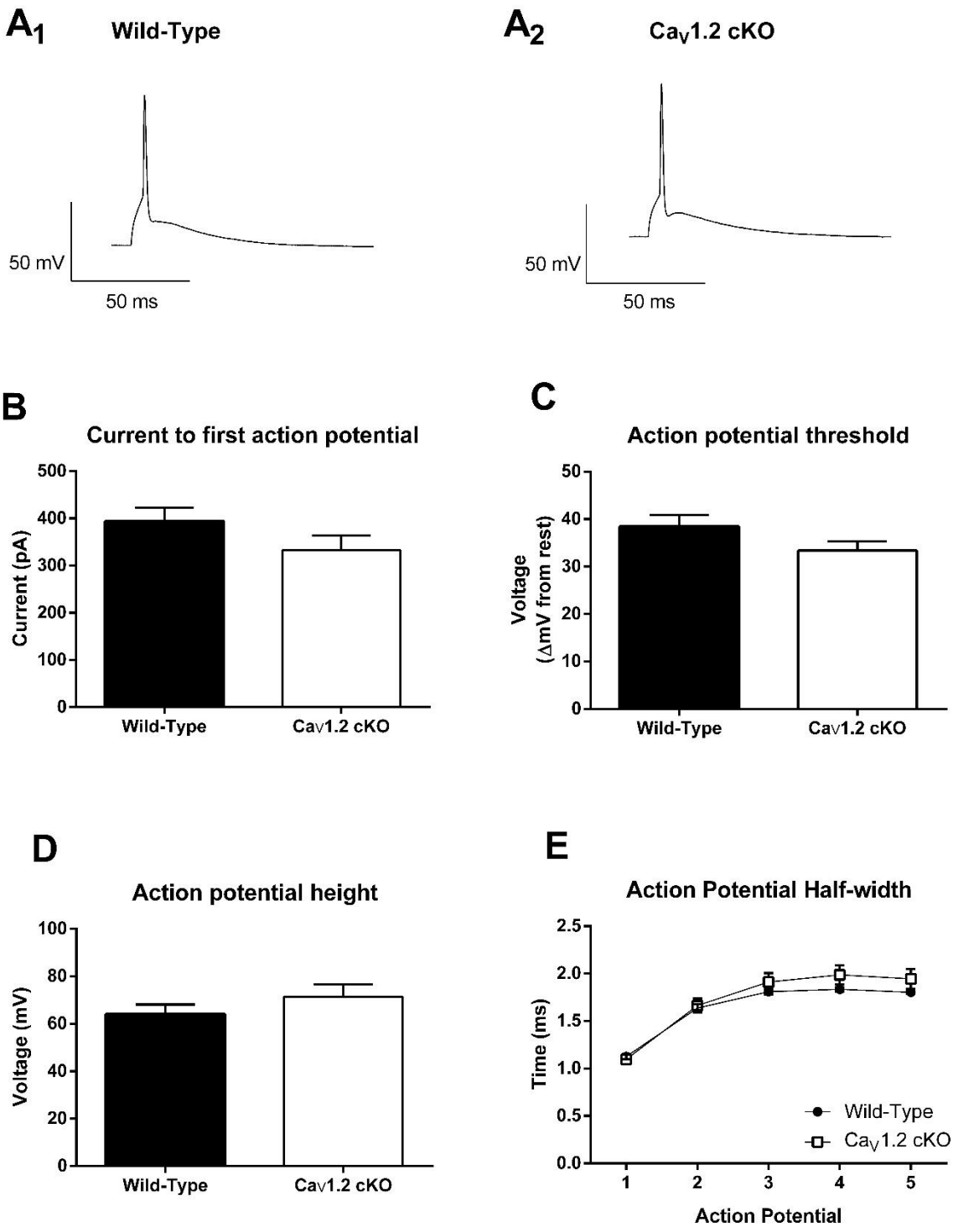

A1
Wild-Type
50 mV
50 ms
A2
CaV1.2 cKO
50 mV
50 ms
B
Current to first action potential
Current (pA)
500
400
300
200
100
0
Wild-Type
CaV1.2 cKO
C
Action potential threshold
Voltage (ΔmV from rest)
50
40
30
20
10
0
Wild-Type
CaV1.2 cKO
D
Action potential height
Voltage (mV)
100
80
60
40
20
0
Wild-Type
CaV1.2 cKO
E
Action Potential Half-width
Time (ms)
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
Action Potential
Wild-Type
CaV1.2 cKO

Figure 5.2: Neuronal deletion of $Ca_V1.2$ does not alter the action potential properties of pyramidal neurons in the lateral amygdala

Representative recordings of a single action potential, elicited by a 10 ms long depolarization step, in wild-type (n = 11) (**A_1**) and $Ca_V1.2$ conditional knockout (n = 8) (**A_2**) mice. (**B**) Pyramidal cells in $Ca_V1.2$ conditional knockout mice required a similar current to elicit a single action potential as wild-type mice. Additionally, action potentials in $Ca_V1.2$ conditional knockout mice did not show a significant change in the action potential threshold (**C**) or action potential height (**D**) when analyzing a single action potential. (**E**) Using a train of 5 action potentials, pyramidal cells in $Ca_V1.2$ conditional knockout mice (n = 8) exhibit comparable action potential half-width as wild-type mice (n = 5) for all action potentials in the train. Data are represented as mean ± SEM.

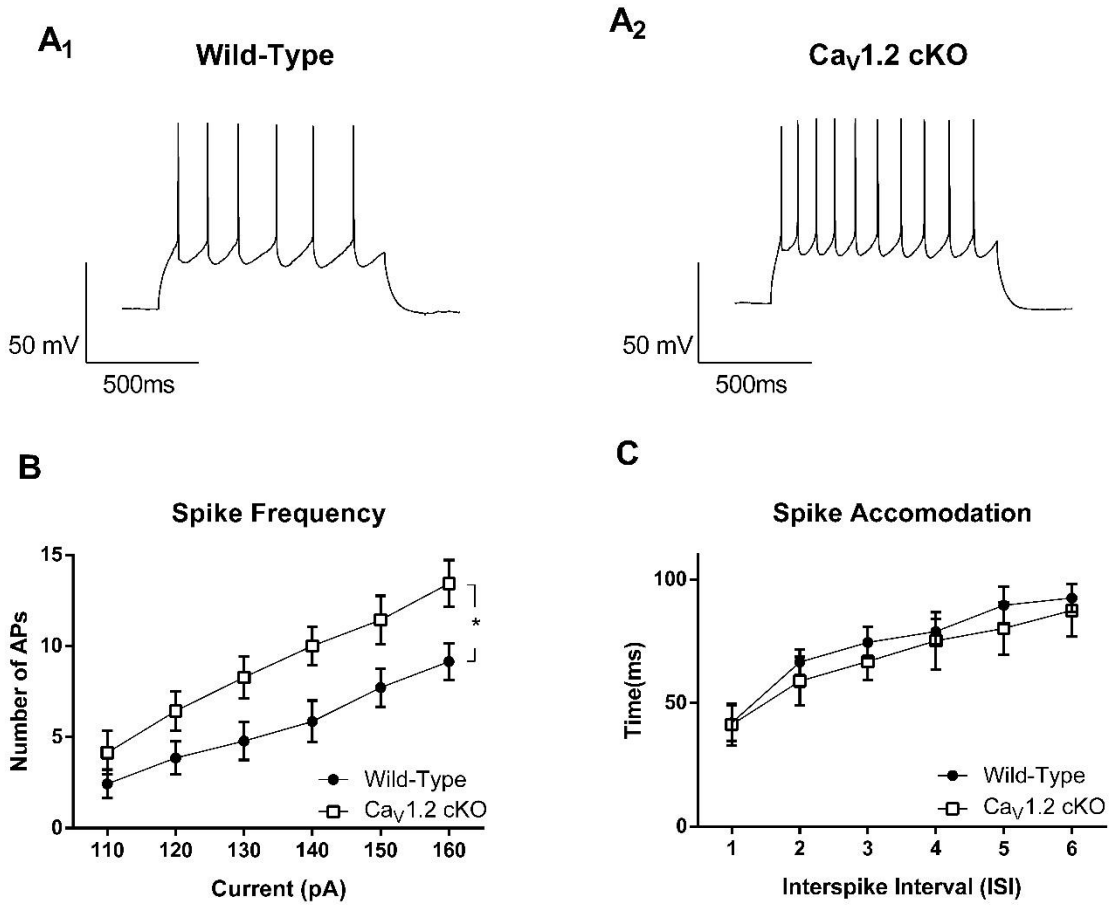


Figure 5.3: Neuronal deletion of $Ca_V1.2$ produces an increase in action potential firing frequency in pyramidal neurons in the LA

Representative recordings of a train of action potentials, elicited using a 1 second long depolarization step in wild-type (n= 11) (**A_1**) and $Ca_V1.2$ conditional knockout (n = 8) (**A_2**) mice. (**B**) Pyramidal cells in $Ca_V1.2$ conditional knockout mice exhibited a significant increase in the number of action potentials elicits per current injection compared to wild-type mice. (**C**) However, analysis of the spike accommodation produced by the first current step to elicit seven action potentials, found no significant alteration between $Ca_V1.2$ conditional knockout mice and wild-type mice. Data are represented as mean ± SEM. *$p < 0.05$.

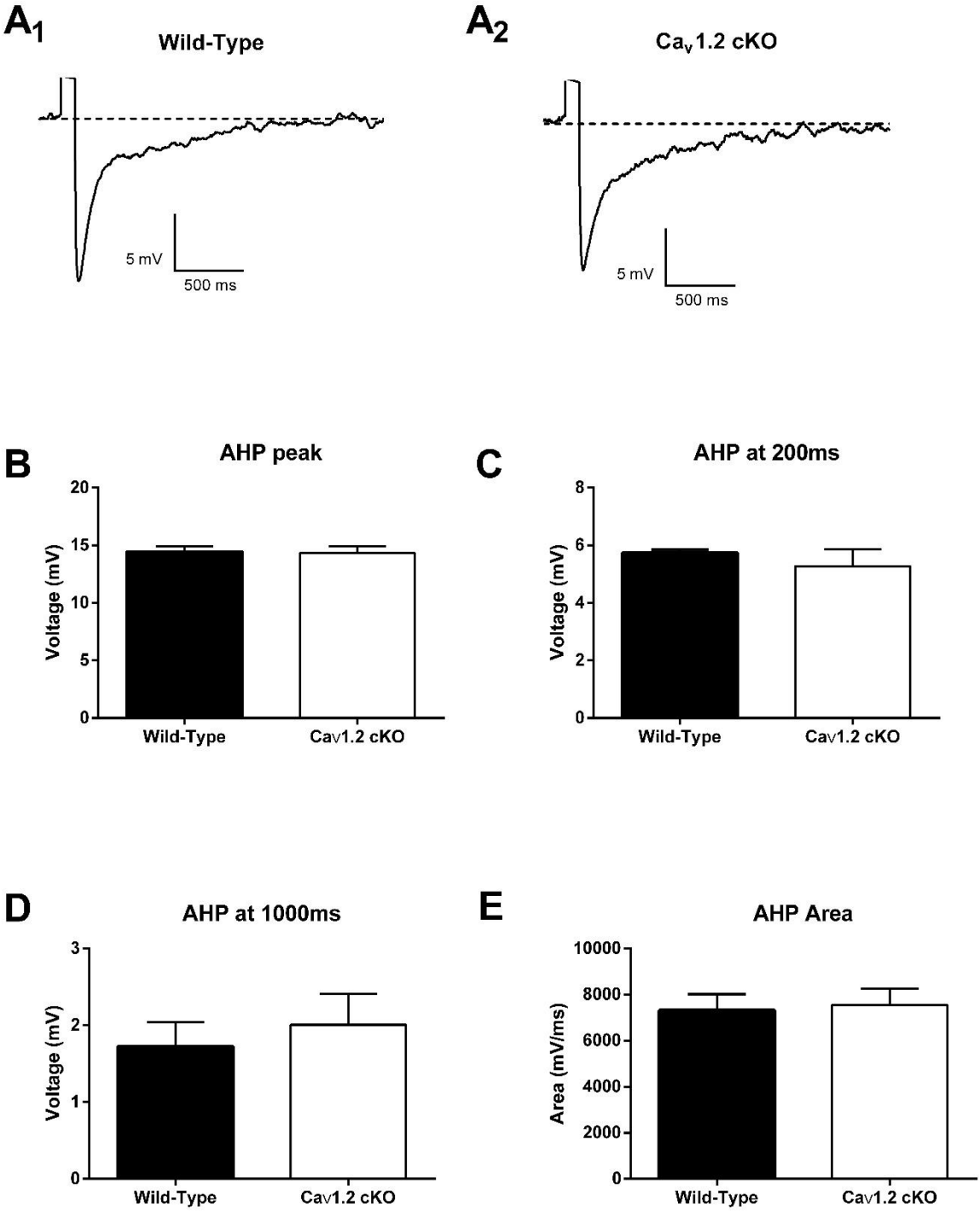

A1
Wild-Type
5 mV
500 ms
A2
Cav1.2 cKO
5 mV
500 ms
B
AHP peak
Voltage (mV)
20
15
10
5
0
Wild-Type
Cav1.2 cKO
C
AHP at 200ms
Voltage (mV)
8
6
4
2
0
Wild-Type
Cav1.2 cKO
D
AHP at 1000ms
Voltage (mV)
3
2
1
0
Wild-Type
Cav1.2 cKO
E
AHP Area
Area (mV/ms)
10000
8000
6000
4000
2000
0
Wild-Type
Cav1.2 cKO

Figure 5.4: Neuronal deletion of $Ca_V1.2$ does not alter the post-burst afterhyperpolarization in pyramidal neurons in the LA

Representative recordings of the afterhyperpolarization (AHP) elicited after a burst of 5 action potentials in wild-type (n = 11) (**A_1**) and $Ca_V1.2$ conditional knockout (n = 8) (**A_2**) mice. Action potentials were elicited using a 500 ms long depolarization step at the minimal current to elicit five action potentials. Pyramidal cells in $Ca_V1.2$ conditional knockout mice exhibited similar membrane voltages at the peak of the AHP (**B**), the AHP at 200 ms (**C**), and the AHP at 1000ms as wild-type mice. (**D**). Pyramidal cells in $Ca_V1.2$ conditional knockout mice also exhibit similar area of the AHP as wild-type mice (**E**). Data are represented as mean ± SEM.

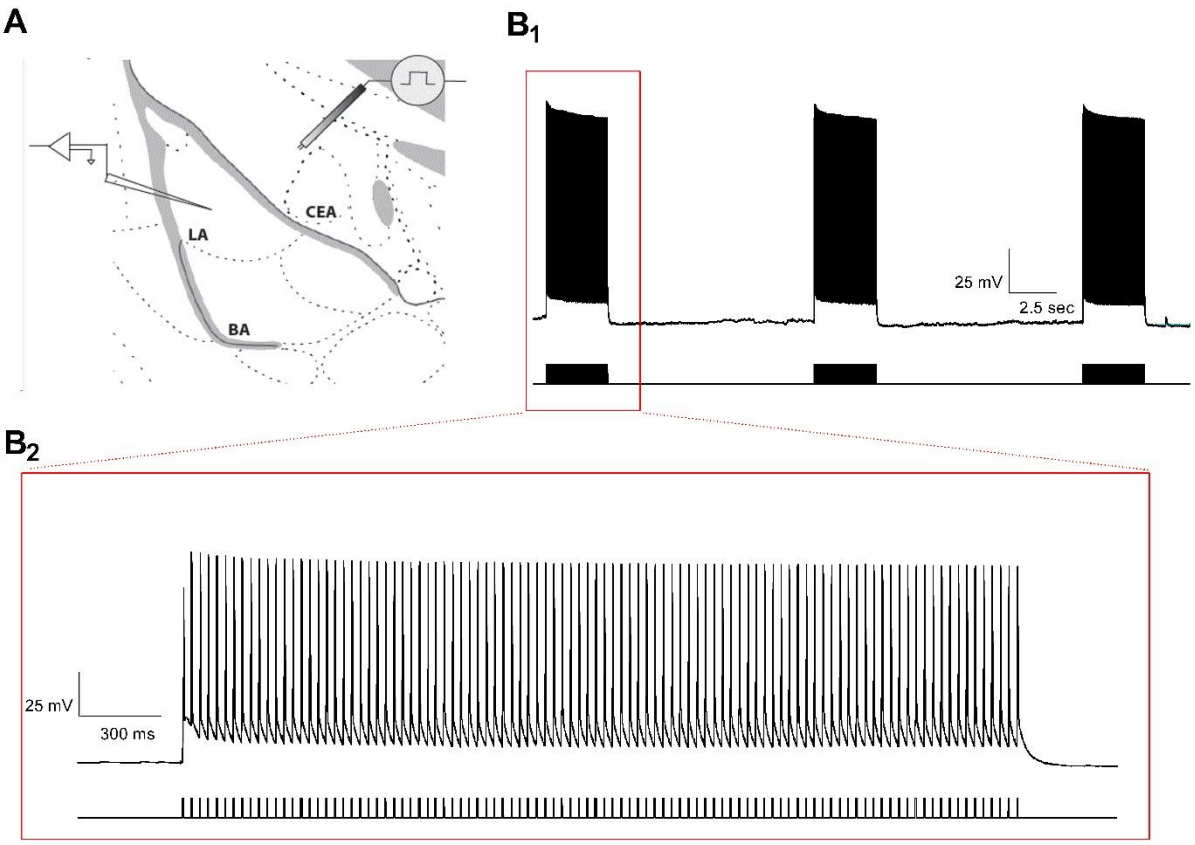


Figure 5.5: Long term potentiation was induced in the thalamo-amygdala pathway using a paired stimulation protocol.

(**A**) The formation of LTP in the thalamo-amygdala pathway was assessed using whole cell current clamp recordings of pyramidal cells in the LA and stimulation of thalamic fibers using a cluster electrode. (**B_1** and **B_2**) LTP was induced using a paired protocol with three bouts of 100 stimulations of thalamo-amygdala pathway at 33 Hz and paired postsynaptic depolarization of lateral amygdala neurons at 1.2nA for three milliseconds at the same rate.

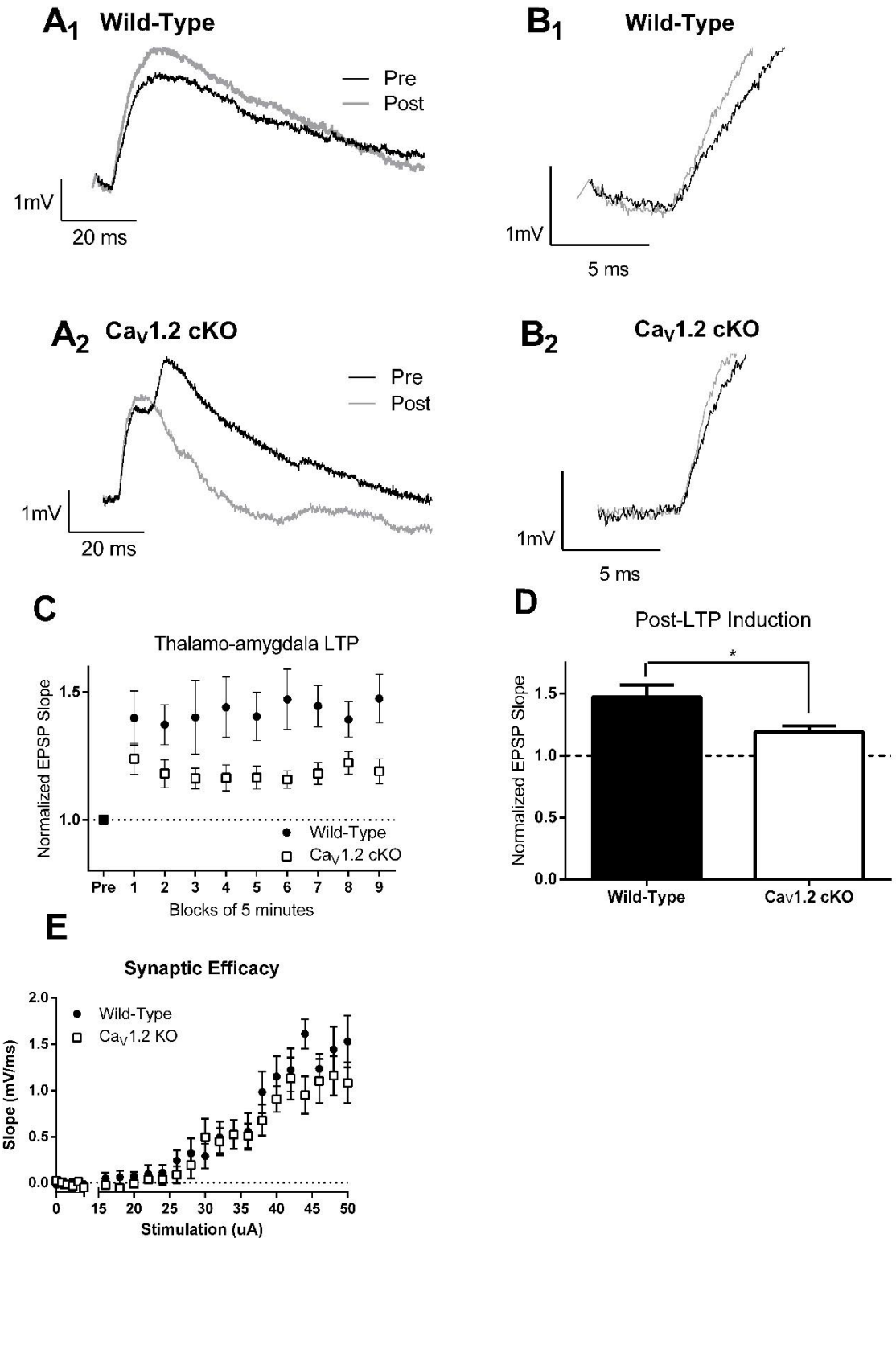

A1 Wild-Type
Pre
Post
1mV
20 ms
B1 Wild-Type
1mV
5 ms
A2 CaV1.2 cKO
Pre
Post
1mV
20 ms
B2 CaV1.2 cKO
1mV
5 ms
C
Thalamo-amygdala LTP
Normalized EPSP Slope
1.5
1.0
Wild-Type
CaV1.2 cKO
Pre 1 2 3 4 5 6 7 8 9
Blocks of 5 minutes
D
Post-LTP Induction
*
Normalized EPSP Slope
1.5
1.0
0.5
0.0
Wild-Type
Cav1.2 cKO
E
Synaptic Efficacy
Wild-Type
CaV1.2 KO
Slope (mV/ms)
2.0
1.5
1.0
0.5
0.0
0 15 20 25 30 35 40 45 50
Stimulation (uA)

Figure 5.6: Neuronal deletion of $Ca_V1.2$ produced a significant deficit in LTP formation in the thalamo-amygdala pathway.

Representative recordings of excitatory postsynaptic potentials before (pre) and after (post) LTP induction in wild-type (n = 7) (**A_1**) and $Ca_V1.2$ conditional knockout (n = 10) (**A_2**) mice in response to stimulation of the thalamo-amygdala pathway. (**B_1** and **B_2**) Recordings were assessed for the initial slope of the EPSP. (**C**) Analysis of the size of the EPSP slope, normalized by pre-LTP baseline, across post-LTP recordings demonstrated a significant deficits in thalamo-amygdala LTP in $Ca_V1.2$ conditional knockout mice compared to wild-type mice. (**D**) Additionally, $Ca_V1.2$ conditional knockout mice exhibits a significantly more moderate increase in the slope of the EPSP during the last five minutes of recording compared to wild-type mice. (**E**) However, no alterations were found in the synaptic efficacy of the thalamo-amygdala pathway in $Ca_V1.2$ conditional knockout mice. Data are represented as mean ± SEM. *$p < 0.05$.

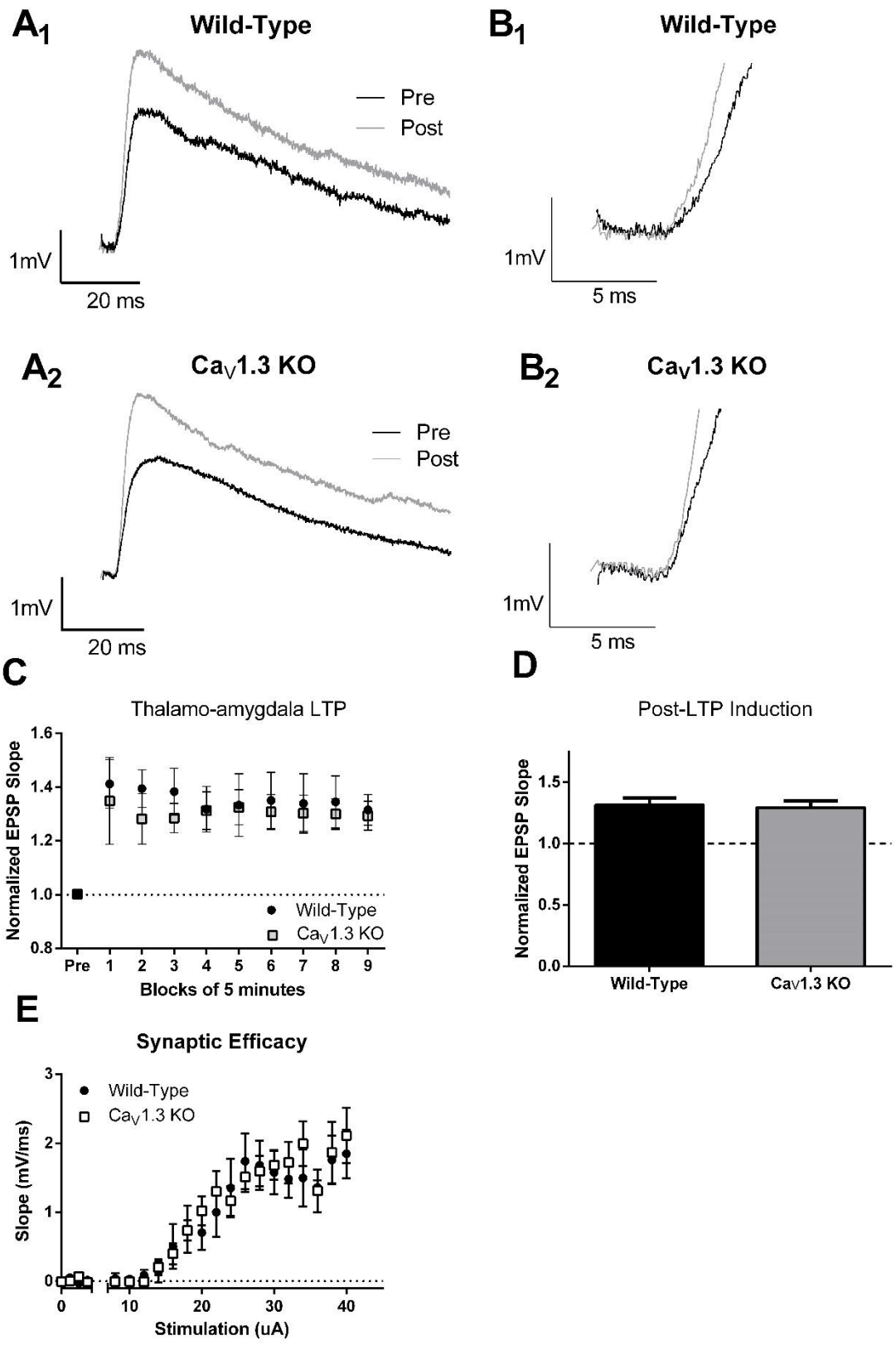

A1
Wild-Type
Pre
Post
1mV
20 ms
B1
Wild-Type
1mV
5 ms
A2
CaV1.3 KO
Pre
Post
1mV
20 ms
B2
CaV1.3 KO
1mV
5 ms
C
Thalamo-amygdala LTP
Normalized EPSP Slope
1.6
1.4
1.2
1.0
0.8
Wild-Type
CaV1.3 KO
Pre 1 2 3 4 5 6 7 8 9
Blocks of 5 minutes
D
Post-LTP Induction
Normalized EPSP Slope
1.5
1.0
0.5
0.0
Wild-Type
Cav1.3 KO
E
Synaptic Efficacy
Wild-Type
CaV1.3 KO
Slope (mV/ms)
3
2
1
0
0 10 20 30 40
Stimulation (uA)

Figure 5.7: Global deletion of $Ca_V1.3$ does not alter LTP formation in the thalamo-amygdala pathway.

Representative recordings of excitatory postsynaptic potentials before (pre) and after (post) LTP induction in wild-type (n = 9) (**A_1**) and $Ca_V1.3$ knockout (n = 8) (**A_2**) mice in response to stimulation of the thalamo-amygdala pathway. (**B_1** and **B_2**) Recordings were assessed for the initial slope of the EPSP. (**C**) Both $Ca_V1.3$ knockout mice and wild-type mice demonstrated a significant increase in the slope of the EPSP after LTP induction. (**D**) Analysis of the size of the slope of the EPSP during the last five minute of post-LTP recording also did not demonstrate a significant difference in LTP formation between $Ca_V1.3$ knockout mice and wild-type mice. (**E**) Additionally, no alterations were found in the synaptic efficacy of the thalamo-amygdala pathway in $Ca_V1.3$ knockout mice. Data are represented as mean ± SEM.

CHAPTER 6

DISCUSSION

6.1 Summary of findings

The regulation of fear and fear learning is vital for human survival and quality of life (Bracha, Bienvenu et al. 2006). While adaptive fears to aversive stimuli reduce the risk of harm and death, maladaptive fears can significantly disrupt an individual's ability to function in everyday life, often leading to trauma and anxiety related disorders and diagnoses. While studies of fear learning over the last several decades have revealed significant information regarding the neurobiology of conditioned fear (Maren and Fanselow 1996, Maren 2001), which could arguably be called adaptive fear, much less is understood about the regulation of maladaptive fears and maladaptive fear learning. Studies of LVGCCs, using LVGCC antagonists, have linked this class of calcium channels with adaptive fear learning and maladaptive fear expression, seen as a deficit in fear conditioning (Bauer, Schafe et al. 2002) and a deficit in fear extinction (Cain, Blouin et al. 2002, Davis and Bauer 2012) respectively. Additionally, pharmacological blockade of LVGCCs has been associated with changes in the neurophysiology associated with learning in both the hippocampus and the amygdala (Grover and Teyler 1990, Moyer, Thompson et al. 1992, Kapur, Yeckel et al. 1998, Weisskopf, Bauer et al. 1999, Bauer, Schafe et al. 2002, Moosmang, Haider et al. 2005,

McKinney, Sze et al. 2009, Langwieser, Christel et al. 2010, Gamelli, McKinney et al. 2011).

L-type VGCCs can be further broken down into two neuronal subtypes, $Ca_V1.2$ and $Ca_V1.3$, with significant differences in channel kinetics and neuronal distribution between these two subtypes (Hell, Westenbroek et al. 1993, Xu and Lipscombe 2001, Lipscombe, Helton et al. 2004). However, due to the lack of subtype specific antagonists, very little was understood about the role of LVGCC subtypes in learning and physiology, including adaptive and maladaptive fear (**Table 1**). However, taking into account the studies in this thesis, it is clear that these two channels have substantially different roles in fear related behavior and neurophysiology (**Table 2**).

The goal of the research presented in this thesis was to investigate adaptive and maladaptive fear phenotypes, as well as explore the individual role of $Ca_V1.2$ and $Ca_V1.3$ in fear related learning and neurophysiology, in particular $Ca_V1.2$. Utilizing mice with a conditional knockout of $Ca_V1.2$ in neurons in the brain, mice with a global knockout of $Ca_V1.3$, and various pure-bred mouse strains and sub-strains, five main points were illustrated in this thesis: 1) Two forms of maladaptive fear learning, persistent fear and generalized fear exist, 2) $Ca_V1.2$ mediates generalized fear, likely through the dentate gyrus and adult neurogenesis, 3) $Ca_V1.2$ mediates persistent fear, likely through alterations in the inhibitory/excitatory synaptic activity onto the amygdala. 4) Deletion of $Ca_V1.2$ alters neurophysiological correlates of learning in the amygdala, including intrinsic excitability and synaptic plasticity. 5) $Ca_V1.2$ appears to alter behavior and neurophysiology in a LVGCC subtype specific manner

The existence of a role of $Ca_V1.2$ in mediating maladaptive fear phenotypes and the potential neurobiology that underlies them may provide insight into the neurobiology mediating the development and expression of trauma-related and anxiety-related disorders in human.

6.1.1 Two forms of maladaptive fear learning

To begin my investigation into adaptive and maladaptive fear learning, in Chapter two I explored the expression of adaptive and maladaptive fear phenotypes in two genetically similar substrains of the 129 inbred mouse strain, 129S1 and 129S6 mice. Examination of fear-related learning in 129S6 and 129S1 mice revealed a segregation of two maladaptive fear phenotypes: persistent fear, in the form of deficits in fear extinction, and overgeneralization of fear, which was seen as a lack of context discrimination. While 129S6 mice displayed a significant deficit in fear extinction, similar to previous published literature in 129S1 mice (Hefner, Whittle et al. 2008), 129S1 , but not 129S6 mice, displayed high levels of freezing to a non-conditioned context in both a fear generalization and a context discrimination protocol. Given the significant similarity and lineage of the two lines (Simpson, Linder et al. 1997, Threadgill, Yee et al. 1997), but the separation of these two maladaptive fear phenotypes, I proposed that persistent fear and fear overgeneralization may represent two separate forms of maladaptive fear learning which may be mediated by different genes and segregate neurobiological mechanisms.

As was mentioned in Chapter two, several differences in the morphology and functionality of the amygdala have been noted between 129S1 mice and C57B6 mice, with C57B6 mice representing normal fear learning (Hefner, Whittle et al. 2008, Camp,

Macpherson et al. 2012). Additionally, differences in the expression of certain proteins in the hippocampus between these two mouse strains have also been published (Camp, Macpherson et al. 2012). Currently, it is not known whether these differences in the amygdala and hippocampus, if any, are present in 129S6 mice. The existence of some of these differences, but not all, could give clues as to which of these differences are linked to each maladaptive fear phenotype. Of course, the interest in studying 129S1 and 129S6 mice is not just in the investigation of different forms of maladaptive fear, but the use of these substrains as a tool for identifying candidate genes involved in the formation and expression of maladaptive fear learning. Currently, only one published study appears to investigate the genetic differences between 129S6 and 129S1 mice (Simpson, Linder et al. 1997). This study found eight simple sequence length polymorphisms (SSLPs) in the DNA between 129S6 and 129S1 mice, though these results are from a specific examination of eighty-six pre-selected SSLP markers from a set of identified SSLPs between inbred mouse strains (Dietrich, Miller et al. 1996). Therefore, these eight SSLPs are only a small glimpse into what is likely thousands of variations in SSLPs between 129S6 and 129S1 mice. Additionally, these SSLPs were not selected or identified based on their behavioral relevance, or even their location to known genes, and are not likely pertinent to differences between these two substrains in fear-related learning. While dense genetic linkage maps have been made of 12 inbred mouse strains (Dietrich, Miller et al. 1996), these maps do not include substrains. The formation of a dense genetic linkage map between 129S1 and 129S6 mice could be valuable in the identification of genes underlying these substrain differences in maladaptive fear learning, this task would be labor intensive and time consuming.

Better approaches to identifying genes of interest could be a candidate gene genetic association study using genes associated with learning and hippocampal and amygdala function, or a combination of comparative genomics and chromosomal heritability, such as was previously used to identify genes linked to obesity traits in pigs and humans (Kim, Lee et al. 2012). Without additional information regarding genetic differences in 129S1 and 129S6 mice, it is difficult to postulate which of the many genes involved in neuronal function, behavior, and learning could be mediating the discrepancies in maladaptive fear learning between these substrains.

Despite the segregation of persistent fear and generalized fear in the 129S1 and 129S6 substrains, suggesting two separate mechanisms by which they occur, we cannot rule out the possibility that these two maladaptive fear phenotypes could be generated by a single cause. The most likely neurobiological culprits, if a single cause is responsible for these two forms of fear learning, would likely lie in the function of the hippocampus or the amygdala. Previous literature has linked the expression and consolidation of extinction memories with the function of the hippocampus, amygdala, and prefrontal cortex and the reciprocal connections between these three structures (Herry, Ciocchi et al. 2008, Orsini, Kim et al. 2011, Orsini and Maren 2012, Maren 2014). While substantial work has been done to investigate the neurobiology behind extinction (Orsini and Maren 2012, Maren 2014), the neurobiology underlying generalized and context discrimination is less understood (Kheirbek, Klemenhagen et al. 2012). From what we do understand, generalization of fear also appears to involves the hippocampus, in particular the dentate gyrus and adult neurogenesis, and the amygdala (Kheirbek, Klemenhagen et al. 2012). A recent study has also implicated the

prefrontal cortex (Likhtik and Paz 2015). However, this is not to say that other brain structures couldn't mediate context discrimination. In fact, one could argue that within the context discrimination protocol mice first express fear to the non-trained context and then are required to extinguish this fear across training days. In this way context discrimination may be relying on the same structures and mechanisms that have been found to be involved in fear extinction.

While these substrains were chosen due to previous literature demonstrating a potent maladaptive fear phenotype in 129S1 mice compared to C57B6 mice (Hefner, Whittle et al. 2008, Camp, Macpherson et al. 2012), a maladaptive fear phenotype in 129S6 mice could directly impact the investigation of these fears in our $Ca_V1.2$ conditional knockout mice and $Ca_V1.3$ global knockout mice as they are maintained on a hybrid 129S6/B6 genetic background. When a F2 genetic cross of 129S6 and C57B6 mice were examined for fear extinction, hybrid 129S6/B6 mice did display normal fear extinction, with a significant reduction in fear after extinction training compared with pre-extinction levels, though they required more extinction training then may be expected for pure C57B6 mice.

Additionally, while I reported robust deficits in 129S6 mice in fear extinction to a context or tone, I noted significant levels of fear extinction learning in 129S6/B6 hybrid mice. It should be noted that 129S6/B6 mice, as well as $Ca_V1.2$ conditional knockout mice, underwent a substantially greater amount of extinction training than was utilized in the studies of 129S6 mice. Therefore, it is possible that with additional extinction training, 129S6 mice could demonstrate a significant reduction in conditioned fear to a context or tone. Moreover, while substantial deficits in fear

extinction were also noted in 129S1 in previous literature (Bolivar, Pooler et al. 2001, Hefner, Whittle et al. 2008) , 129S1 mice were not examined for their ability to extinguish to a conditioned stimuli in our protocol or in comparison with 129S6 mice. Though 129S1 and 129S6 mice both exhibit deficits in extinction learning, there may be differences in the degree of their deficits. Therefore, studies of maladaptive fear in 129S1 and 129S6 mice could be strengthened by the addition of studies to directly compare 129S6 and 129S1 mice using a prolonged extinction training protocol to determine at what point, if any, 129S1 or 129S6 demonstrate a significant reduction in fear to a conditioned stimulus. In fact, differences in the ability of 129S6 mice to extinguish fear to a conditioned stimulus compared to 129S1 mice, along with differences in context generalization/context discrimination could suggest a shared or overlapping neurobiological mechanism of these two forms of maladaptive fear that leads to maladaptive fear phenotypes in 129S1 mice but milder phenotypes in 129S6 mice.

6.1.2 $Ca_V1.2$ mediates generalized fear

To investigate the role of $Ca_V1.2$ in generalized fear, I utilized a $Ca_V1.2$ conditional knockout mouse in which $Ca_V1.2$ was deleted in all neuronal populations, as described in Chapter three. Mice were examined in a classic Pavlovian fear conditioning and context discrimination paradigm. Examination of fear learning, using these two paradigms revealed a significant overgeneralization of fear phenotype, measured as a deficit in context discrimination, in mice with neuronal deletion of $Ca_V1.2$ with no alteration in basic fear learning. As discussed in Chapter one, overgeneralization of fear could occur due to an increase in fear expression independent

of the stimuli present or an inability to cognitively separate the two contexts. Based on the current literature, the inability to cognitively distinguish between similar stimuli would likely involve deficits in the dentate gyrus, and in particular, adult born neurons within this structure (Sahay, Scobie et al. 2011, Kheirbek, Klemenhagen et al. 2012). This has been found to be true in various discrimination paradigms including forms of the context discrimination task utilized here (Gilbert, Kesner et al. 2001, Hunsaker, Rosenberg et al. 2008, Kim and Lee 2011, Sahay, Scobie et al. 2011, Kheirbek, Klemenhagen et al. 2012). Additionally, adult born neurons in the dentate gyrus have been associated with contextual and spatial learning in complex, such as context discrimination, but not simple tasks, such as fear learning (simple) (Shors, Townsend et al. 2002).

Utilizing the concept of dentate gyrus and adult neurogenesis involvement in complex, but not simple spatial learning, subsequent studies presented in Chapter three examined $Ca_V1.2$ conditional knockout mice in spatial learning in two forms of the Morris water maze. These studies found deficits in mice with neuronal deletion of $Ca_V1.2$ in the ability to find a hidden platform in the limited-cues water maze, which could be considered a difficult, and therefore more likely dentate gyrus associated task, but no deficit in spatial learning in the simpler version of the task. Additionally, decreases in the rate of cell birth and the density of immature neurons within the dentate gyrus were noted in mice with neuronal deletion of $Ca_V1.2$. This decrease in cell division and the density of immature neurons in the dentate gyrus, along with the deficits in seemingly dentate gyrus associated tasks, implicate this structure, and decreases in neurogenesis, in the deficits in context discrimination/generalization of fear

maladaptive phenotype in these mice, as was discussed in Chapter three. The studies and the conclusions made in Chapter three could be strengthened with the addition of two studies: First, the investigation of neuronal deletion of $Ca_V1.2$ on rates of neurogenesis could be strengthened by a direct analysis of neurogenesis. Second, evidence for deficits in dentate gyrus function utilizing the Morris water maze could be strengthened by direct studies illustrating a dependence of spatial learning in the limited cues Morris water maze, and not the classic water maze in our lab, on adult neurogenesis in the dentate gyrus.

Neither rates of cell birth, nor density of immature neurons can directly tell us the rates of neurogenesis within $Ca_V1.2$ conditional knockout mice. To determine the rate of neurogenesis directly I would need to label newly born cells over a period of time and track the number of these same cells which differentiate into neurons, therefore determining the number of neurons that were born in that period of time. However, in the absence of this direct measure, measurements of rates of cell division and number of immature neurons are sufficient to suggest a decrease in neurogenesis.

In regards to the studies of the limited cues water maze and the classic water maze performed in Chapter three, no direct studies have been carried out to determine the dependence of this specific task on the dentate gyrus and adult neurogenesis within the dentate gyrus. In fact previous literature investigating the role of the dentate gyrus in spatial learning in the Morris water maze has been somewhat controversial (Gould, Tanapat et al. 1999, Lassalle, Bataille et al. 2000, Shors, Townsend et al. 2002, Jaholkowski, Kiryk et al. 2009). While I present literature which demonstrated the traditional Morris water maze as a form of spatial learning independent of the dentate

gyrus and adult neurogenesis (Shors, Townsend et al. 2002, Jaholkowski, Kiryk et al. 2009), some studies suggest that decreases in adult neurogenesis within the dentate gyrus, or lesions of the dentate gyrus, do produce deficits in this form of the Morris water maze (Lassalle, Bataille et al. 2000). However, it is difficult to say what makes one form of the water maze more difficult than another. Even small differences in the manner by which the Morris water maze is performed between laboratories could make the difference between a simpler form of the water maze, which could appear independent of the dentate gyrus, and one that is more challenging and appears dentate gyrus dependent. To fully evaluate the role of the dentate gyrus in the Morris water maze exhaustive studies would need to be performed utilizing lesions of the dentate gyrus and countless variations of spatial cues. Until that time, there may never be a complete consensus in the scientific community as to the role of the dentate gyrus in the Morris water maze. In lieu of this information, what I can say in regards to the classic Morris water maze and limited cues water maze used in my studies is that the limited cues water maze took longer for wild-type mice to acquire, suggesting a more difficult task. To actually confirm the dependence of spatial learning in the limited cues water maze on the dentate gyrus or adult neurogenesis in the dentate gyrus, I would need to compare mice with lesions of the dentate gyri versus mice with intact dentate gyri, as well as mice with significant knockdown of adult neurogenesis to mice with normal rates of adult neurogenesis in the dentate gyrus in this task. In the absence of this information, I could also perform additional discrimination tasks that have been utilized to differentiate between dentate-gyrus dependent and dentate-gyrus independent tasks, such as tasks requiring spatial discrimination (Kim and Lee 2011)

As previously mentioned, a lack of context discrimination could also be due to an increase in overall fear expression. An increase in overall fear expression would likely be linked to dysfunction within the amygdala, such as an increase in the excitability of this structure. In fact, previous studies have found that increases in the excitability of the amygdala, usually via reduction in inhibitory input into this structure, produces substantial fear generalization and expression independent of fear conditioning (Shaban, Humeau et al. 2006, Bergado-Acosta, Sangha et al. 2008, Wiltgen, Godsil et al. 2009). Interestingly, increases in the excitability of pyramidal neurons in the lateral amygdala were noted in $Ca_V1.2$ conditional knockout mice in Chapter five. Therefore, it is possible that the context discrimination phenotype noted could be due to this increase in amygdala excitability and not the decrease in cell division and immature neurons in $Ca_V1.2$ conditional knockout mice discussed in Chapter three. While this increase in amygdala excitability could possibly explain the deficits in context discrimination, it seems unlikely that that this change in excitability would alter context discrimination, but not basic fear conditioning and anxiety in $Ca_V1.2$ conditional knockout mice. Additionally, it is unlikely that an increase in amygdala excitability would also produce the deficits in the limited cues water maze, but not the classic water maze, as observed in $Ca_V1.2$ conditional knockout mice. Though the amygdala and anxiety have been associated with spatial learning in the Morris water maze (Packard, Cahill et al. 1994, Hatfield and McGaugh 1999), there is no reason to believe that increases in fear and anxiety would affect one form of the water maze and not another. In this case, a different mechanism would likely be mediating the water maze phenotype than the context discrimination phenotype

observed in mice with neuronal deletion of $Ca_V1.2$. Of course, it is also possible that an increase in amygdala excitability and a decrease in immature neurons in the $Ca_V1.2$ conditional knockout mice have been noted, it is possible that these two neurobiological phenotypes could be acting together to produce the observed deficits in context discrimination.

6.1.3 $Ca_V1.2$ mediates persistent fear

To explore the effects of neuronal deletion of $Ca_V1.2$ on persistent fear, I assessed $Ca_V1.2$ conditional knockout mice for deficits in fear extinction to a conditioned context, as described in Chapter four. The investigation of extinction of fear to the conditioned stimulus in $Ca_V1.2$ conditional knockout mice revealed a significant deficit in fear extinction compared to wild-type mice, representing a persistent fear phenotype. Interestingly, previous literature investigating the effects of deletion of $Ca_V1.2$ in excitatory neurons in the forebrain, using cre recombinase driven by a CaMKII promoter, did not find a deficit in fear extinction with this deletion pattern (McKinney, Sze et al. 2008). Given the presence of a fear extinction deficit in mice with neuronal deletion of $Ca_V1.2$ and not forebrain specific deletion of $Ca_V1.2$ in excitatory neurons, these behavior phenotypes are likely due to a difference in the deletion pattern of these two $Ca_V1.2$ transgenic mouse lines. Deletion of $Ca_V1.2$ using Cre recombinase driven by a synapsin1 promoter has three main differences in the deletion pattern of $Ca_V1.2$ compared to $Ca_V1.2$ conditional knockout mice with Cre recombinase driven by CaMKII transgenic mouse lines: 1) Deletion of $Ca_V1.2$ in inhibitory neurons, 2) Deletion of $Ca_V1.2$ in non-forebrain structures, and 3) Deletion of $Ca_V1.2$ early in prenatal development.

The most likely candidate for the difference in extinction deficits noted between mice with these two deletion patterns is deletion of $Ca_V1.2$ in inhibitory neurons. Extinction learning and expression have been found to rely heavily on a variety of inhibitory networks both into and within the amygdala, including inhibition from the hippocampus to the amygdala, prefrontal cortex to the amygdala and hippocampus to prefrontal cortex, as well as local inhibitory neurons in the BLA, and the main projection neurons in the CEA (Ehrlich, Humeau et al. 2009, Lee, Kim et al. 2013). Given previous literature demonstrating similar extinction deficits in rats with intra-BLA infusions of LVGCC antagonists (Davis and Bauer 2012), the most likely candidate would be involvement of $Ca_V1.2$ in inhibitory neurons within the BLA itself. In fact, inhibitory neurons within the BLA are involved in regulating the activity and excitability of the amygdala. As mentioned above, decreases in inhibition within the amygdala can produce increased generalized fear while increased inhibition within the amygdala can reduce fear expression (Shaban, Humeau et al. 2006, Bergado-Acosta, Sangha et al. 2008, Wiltgen, Godsil et al. 2009). Additionally, fear learning and fear extinction have been linked to changes in inhibitory tone within the amygdala (Duvarci and Pare 2014) with fear learning producing a decrease in inhibition onto excitatory neurons in the BLA (Stork, Ji et al. 2002, Wolff, Grundemann et al. 2014) while fear extinction produces an increase in mIPSCs recorded in pyramidal cells of the BLA (Lin, Mao et al. 2009). Therefore in Chapter four, I investigated the effects of deletion of $Ca_V1.2$ on the spontaneous inhibitory synaptic activity onto pyramidal neurons in the lateral amygdala. Recordings of sIPSCs were chosen over mIPSCs due to the links of LVGCCs to forms of intrinsic excitability and action potential firing, but not

neurotransmitter release and synaptic function. In these experiments I found that neuronal deletion of $Ca_V1.2$ produced a significant increase in the frequency of sIPSCs in pyramidal neurons in the lateral amygdala. In Chapter four I postulated that this change in sIPSC frequency, taken together with a change in sEPSC frequency and amplitude observed, could produce a significant shift in the homeostatic balance of the amygdala and therefore could negatively impact extinction learning. However, this increase in sIPSC frequency was both mild, and in the opposite direction of what one may expect in the presence of extinction deficits, given an increase in inhibition of the amygdala has been tied with fear extinction (Lin, Mao et al. 2009, Duvarci and Pare 2014). Additionally, no recordings have been made of sIPSCs or sEPSCs in pyramidal neuron in the LA in mice with deletion of $Ca_V1.2$ in excitatory neurons in the forebrain, therefore, it is possible that this decrease in sEPSC frequency and amplitude was present in these mice as well and did not produce deficits in fear extinction.

However, an increase in the firing frequency of inhibitory neurons that synapse onto pyramidal neurons in the LA would be consistent with the increase in firing frequency of the pyramidal neurons in the LA, noted in Chapter five. It is also possible that this increase in inhibitory frequency could result from an increase in the number of inhibitory synaptic connections onto these pyramidal neurons themselves. An increase in the number of inhibitory synaptic terminals would likely produce an increase in the frequency of mIPSCs as well as sIPSCs. However, additional recordings of mIPSCs in pyramidal neurons in the LA did not reveal a significant difference in frequency, or amplitude, suggesting no significant change in the number of inhibitory synaptic connections (**Appendix A**).

A decrease in the sEPSC frequency would seem counter-intuitive to the previously published role of LVGCCs in excitability and the data presented in Chapter 5 illustrating an increase in firing frequency in pyramidal cells in the LA. However, the increases in excitability reported in Chapter 5 were measured as an increase in evoked, not spontaneous events. The recordings discussed in Chapter 5 were also made from pyramidal neurons in the lateral amygdala which may not represent the role of $Ca_V1.2$ in the excitatory neuronal populations that synapse onto these neurons. Additionally, the sEPSC measurements are not limited to EPSCs generated from spontaneous action potentials, but also contain EPSCs generated from miniature synaptic events (generating mEPSCs). Therefore a change in sEPSC frequency could actually reflect a change in mEPSCs frequency. A change in mEPSC frequency could be a result of changes in the presynaptic machinery associated with synaptic vesicle release, though it is unclear how this would result from alterations in $Ca_V1.2$. A change in presynaptic machinery could also lead to an alteration in the amplitude of the sEPSCs recorded. However changes in sEPSC amplitude could also result from alterations in the postsynaptic structure or number of ligand gated receptors including AMPA and NMDA receptors. To determine whether changes in sEPSC amplitude and frequency are actually due to changes in synaptic function, presynaptically or post-synaptically, or changes in intrinsic excitability of glutamatergic neurons synapsing onto pyramidal neurons in the LA, recordings of mEPSCs could be made.

It is also possible that this change in the frequency and amplitude of sEPSCs is due to deletion of $Ca_V1.2$ in inhibitory neurons, effecting inhibition onto excitatory neurons and not due to deletion of $Ca_V1.2$ in excitatory neurons synapsing onto LA

neurons themselves. To determine whether deletion of $Ca_V1.2$ produced changes in sEPSCs via deletion of $Ca_V1.2$ in inhibitory neurons, or excitatory neurons, recordings of sIPSCs and sEPSCs in pyramidal neurons in the lateral amygdala could be made in mice with deletion of $Ca_V1.2$ in excitatory neurons only or in mice with deletion of $Ca_V1.2$ in inhibitory neurons only.

While I attribute the deficits in fear extinction in the $Ca_V1.2$ conditional knockout mice to changes in inhibitory neuronal populations in the forebrain, specifically the amygdala and it is mainly forebrain structures, including the hippocampus, prefrontal cortex, and amygdala that are associated with fear extinction (Orsini and Maren 2012), it is possible that deletion of $Ca_V1.2$ in a non-forebrain structures could be responsible for the fear extinction results. The amygdala does receive input from structures within the thalamus, the basal ganglia, and areas of the brain stem, including dopaminergic input, (Katona, Rancz et al. 2001, Chhatwal and Ressler 2007, Orsini and Maren 2012) which could regulate the activity of the amygdala and therefore the expression of extinction.

Finally, utilization of transgenic mice in which any gene and protein has been altered can produce compensatory responses in the brain. In particular, deletion of $Ca_V1.2$ using synapsin1a, which is thought to be expressed at embryonic day 14-15 (Zhu, Romero et al. 2001), deletes this channel during a critical point of neuronal development and could produce changes in the way that circuits and structures form. One study in particular, investigating the role of $Ca_V1.2$ within the amygdala, found what is believed to be a significant up regulation of mGlur5 receptor subunits in mice lacking $Ca_V1.2$ and that this up regulation of mGluR5 may compensate for the loss of

$Ca_V1.2$ in LTP and fear learning in the amygdala (Langwieser, Christel et al. 2010). Compensation for the loss of $Ca_V1.2$ could also appear in the form of alterations in other calcium permeable channels, such as $Ca_V1.3$ and NMDA. Within the mice investigated in my studies, potential compensatory mechanisms, such as alterations in the expression of other calcium channels, could be explored by looking at overall protein expression within the brain and within select brain areas using western blots. However, the best way to avoid potential compensatory mechanisms, as well as changes to the circuitry and neurobiology produced from deletion of these channels early in development, would be to utilize more temporally regulated deletion of $Ca_V1.2$ expression. Temporal regulation of the deletion of $Ca_V1.2$ could be done simply by selecting a promoter which is expressed later in life or by using an inducible form of Cre recombinase, in which expression of Cre, and therefore the deletion of $Ca_V1.2$ does not occur until the addition of an exogenous compound such as tamoxifen (Danielian, Muccino et al. 1998, Hayashi and McMahon 2002). Additionally, temporal and region specific deletion could be produced through viral delivery of Cre recombinase (Rohlmann, Gotthardt et al. 1996), or injection of cell permeable Cre recombinase (Jo, Nashabi et al. 2001) directly into specified regions of the brain at the desired time point.

The studies conducted in Chapter four, in particular the exploration of inhibition within the amygdala as the source of extinction deficits, could be strengthened by the addition of whole cell recordings of inhibitory neurons themselves within the LA. Changes in the excitability of inhibitory neurons within the LA would support the theory of a role of $Ca_V1.2$ in inhibitory neurons in the amygdala as a source of deficits in extinction. Additionally, an increase in the excitability and firing frequency of

neurons within the lateral amygdala would support the idea of these neurons representing a source of the increase in sIPSCs in pyramidal neurons within this structure. These recordings could be made by selecting inhibitory neurons in the LA for electrophysiological recording based on an inhibitory/non-pyramidal morphology and the presence of neurophysiology associated with inhibitory neurons. However, given that only a small percentage of neurons within the LA are expected to be inhibitory (Ehrlich, Humeau et al. 2009) and the neurophysiological features associated with inhibitory neurons could be difficult to interpret in the absence of $Ca_V1.2$, it could be challenging to reliably acquire recordings from inhibitory neurons. In this case, the use of a fluorescent marker, such as GFP, expressed in inhibitory neurons may be necessary to differentiate these cells from excitatory cells.

Finally, it is possible that deficits in extinction of fear to a conditioned context could be produced through the decreases in cell division and the density of immature neurons seen in $Ca_V1.2$ conditional knockout mice, seen in Chapter three. In fact, some studies have found that decreases in adult born neurons produces deficits in fear extinction (Pan, Chan et al. 2012, Pan, Storm et al. 2013). Other studies, however, have not supported these results, finding no effect of a loss of adult neurogenesis on extinction learning (Ko, Jang et al. 2009). Similar to the Morris water maze, these differences in experimental findings may be a result of important differences in task design, which result in a simpler or more difficult learning task. The evidence linking adult neurogenesis to extinction is limited at this time, making it difficult to predict whether the observed deficits in cell division in the dentate gyrus of $Ca_V1.2$ conditional knockout mice could mediate the extinction phenotype observed.

6.1.4 $Ca_V1.2$ alters neurophysiological correlates of learning

As normal fear learning and fear expression relies heavily on the functionality of the amygdala, in Chapter five I assessed $Ca_V1.2$ conditional knockout mice for changes in the neurophysiological properties of the main input structure of the amygdala, the LA. Examination of $Ca_V1.2$ conditional knockout mice revealed that deletion of $Ca_V1.2$ increased the excitability of pyramidal neurons within the LA with an increase in the action potential firing frequency. Often, change in firing frequency can be attributed to a change in the AHP (Lorenzon and Foehring 1992, Cloues and Sather 2003), in the action potential threshold (Calvin 1974), or the input resistance of the neuron (Connor, Walter et al. 1977). Previous studies of LVGCCs have observed a decrease in the size of the AHP in the presence of pharmacological blockade of LVGCCs (Tanabe, Gahwiler et al. 1998). Additionally, deletion of $Ca_V1.3$ produced a decrease in the size of the AHP, in particular the slow AHP, and an increase in firing frequency in the amygdala (McKinney, Sze et al. 2009). However, in $Ca_V1.2$ conditional knockout mice, no significant differences were noted in the size of the AHP, measured as the AHP area, or the membrane potential at various points along the AHP. Additionally, deletion of $Ca_V1.2$ did produce alterations in the action potential threshold or the current required to elicit an action potential. Finally, analysis of the input resistance of pyramidal neurons in $Ca_V1.2$ conditional knockout mice using an input output curve did not result in a change in neuronal input resistance compared to wild-type mice. The absence of changes in action potential properties, the AHP, and input resistance in $Ca_V1.2$ conditional knockout mice in the presence of an increased firing frequency is unexpected. However, several explanations for this unexpected increase in firing

frequency could exist, including: 1) Deletion of $Ca_V1.2$ could produce alterations in intrinsic excitability, such as action potential and AHP properties, and that these changes are not being detected using the electrophysiological protocols utilized in this thesis, or 2) The increase in firing frequency is not directly produced through a deletion of $Ca_V1.2$, but a compensatory response to deletion of $Ca_V1.2$ which are independent of the AHP.

Recording conditions and stimulation parameters to determine basic intrinsic excitability properties were based upon previous literature investigating mice with global deletion of $Ca_V1.3$ for alterations in amygdala neurophysiology (McKinney, Sze et al. 2009), however countless variations in the electrophysiological protocols exist to obtain similar measures have been utilized by other studies assessing neurophysiology throughout the brain. Different protocols, based on the length of the protocol, the stimulation in the protocol, as well as whether the neurons were held at a given potential, could produce alterations in which channels are opened or closed and the passive properties of the cells which could produce different results. Initially, analysis of action potential properties were assessed using a short 10 ms long depolarization step of the minimal current required to elicit an action potential. However, additional recordings performed by myself in the lab found pyramidal neurons in the LA required a minimal period of time between the onset of the depolarization and the occurrence of an action potential that appeared to be due to the charging of the membrane (**Appendix B**). While short durations, such as 5 ms could generate an action potential, these action potentials usually took substantially more current and the resulting action potential was obstructed by the passive charging of the membrane. Therefore, it is possible that the

analysis of action potential properties using a step depolarization of only 10 ms in length may not be providing the best and most accurate action potential measurements. However, analysis of the action potentials generated using a 1 second long depolarization step at various current intensities did not reveal any significant shifts in action potential properties in $Ca_V1.2$ conditional knockout mice compared to wild-type mice either (data not shown). Additionally there is no evidence to suggest that blockade of LVGCCs would alter the shape of an action potential. Afterhyperpolarization measurements were generated while holding cells at 10 mV below action potential threshold. This was done in order to increase the driving force of potassium into the cell, providing a larger, more easily assessed AHP. However, it is possible that holding the cell at such a depolarized value could be activating some voltage gated channels, while inactivating others in such a way to mask potential differences in the AHP. Finally, input resistance was calculated using an input/output curve of the change in voltage in response to sub-threshold depolarization and hyperpolarization. At sub-threshold depolarization levels, $Ca_V1.2$ channels would not be expected to be open and therefore deletion of $Ca_V1.2$ would not be expected to alter input resistance. However, it is possible that there is a change in the input resistance of the cells between $Ca_V1.2$ conditional knockout mice and wild-type mice at the voltages at which $Ca_V1.2$ channels are activated. A change in input resistance at these thresholds could alter the firing frequency of cells.

It is also possible that the increase in excitability seen in pyramidal cells within the LA is a result of a compensatory mechanism. Deletion of $Ca_V1.2$ in our $Ca_V1.2$ conditional knockout mice occurs prenatally, prior to a variety of key neuronal

development. Loss of this channel early in development could produce a number of compensatory changes, as described in previous sections. In addition, in Chapter four, $Ca_V1.2$ conditional knockout mice were observed as having a significant alteration in the inhibitory and excitatory balance of synaptic inputs onto pyramidal neurons in the LA. In the presence of prolonged alterations in synaptic input, with an increase in inhibition, alterations in the excitability of pyramidal neurons in the LA as a mechanism in order to maintain the homeostatic balance of excitability and proper gating of amygdala activity. One way the cell could alter excitability without altering the action potential properties, AHP, or input resistance would be to modify the rate at which the cell recovers after an action potential, or refractory period, in particular, the recovery of voltage gated sodium channels. During depolarization near action potential threshold voltage gated sodium channels open, producing the upswing of the action potential, but then inactivate quickly. The ability for the cell to fire again requires the reactivation of these channels in order for them to be opened in response to further depolarization. A significant decrease in the time between inactivation and reactivation of voltage gated sodium channels could increase the firing frequency of the cell without altering the properties of individual action potentials or the AHP. The possibility that the increase in firing frequency is a compensatory result to the increase in the ratio of inhibitory synaptic input could be eliminated by assessing the excitability of pyramidal neurons in the LA in mice with deletion of $Ca_V1.2$ in excitatory neurons in the forebrain used in previous literature. In these mice, deletion of $Ca_V1.2$ would occur post-natally and the presence of an increase in the excitability of these neurons in the absence of changes in

inhibition would suggest that this increase in firing frequency was due to deletion of $Ca_V1.2$ in the excitatory neurons themselves.

Finally it is possible that loss of $Ca_V1.2$ may be acting via an unknown role of LVGCCs in order to modulate firing frequency. While it is difficult to postulate what this unknown mechanism may be, the fact that we have only very recently been able to investigate the individual contribution of $Ca_V1.2$ and $Ca_V1.3$ to neuronal function means there is still a lot to be understood about the ways in which these two channels regulate neuronal function.

In Chapter five I describe how, in addition to changes in firing frequency, neuronal deletion of $Ca_V1.2$ was also found to produce deficits in LTP within the thalamo-amygdala pathway. Such a deficit in LTP, as well as altered intrinsic excitability, suggests an important role of $Ca_V1.2$ in cellular analogs of learning which could be producing alterations in fear related learning and perhaps the persistence and generalization of fear phenotypes observed in $Ca_V1.2$ conditional knockout mice. Interestingly, this deficit in LTP in the thalamo-amygdala pathway could be linked to the increase in inhibitory synaptic activity described in Chapter four. In fact, a previous study found that increases in inhibition within the amygdala produced a significant decrease in LTP formation in this pathway (Bissiere, Humeau et al. 2003). However, other studies in which $Ca_V1.2$ was deleted in excitatory neurons in the forebrain found a similar deficit in LTP formation (Langwieser, Christel et al. 2010). Given that deletion of $Ca_V1.2$ in excitatory neurons in the forebrain would likely not produce the same increase in sIPSCs in pyramidal cells in the LA as our $Ca_V1.2$ conditional knockout

mice, increases in sIPSC frequency are likely not responsible for the LTP deficit observed.

Of course, this decrease in LTP formation suggests only a partial role of $Ca_V1.2$ in this pathway while pharmacological blockade of LVGCCs in this pathway in previous literature results in a complete lack of LTP in this pathway (Bauer, Schafe et al. 2002). While additional studies in Chapter five found no significant role of $Ca_V1.3$ in this form of LTP within this pathway, it is possible that neuronal deletion of $Ca_V1.2$ produced a compensatory change in neuronal function that could be mediating the remainder of the LTP formation, such as an increase in mGluR5 subunit and the calcium permeability of glutamate receptors previously implicated in other $Ca_V1.2$ conditional knockout mice (Langwieser, Christel et al. 2010), or an increase in or recruitment of NMDA receptors involved in other forms of LTP.

6.1.5 $Ca_V1.2$ alter fear related behavior and neurophysiology in a subtype specific manner

Throughout this thesis, studies of $Ca_V1.2$ conditional knockout mice were compared to previous studies of $Ca_V1.3$ global knockout mice, or to acquired data from $Ca_V1.3$ global knockout mice to assess the role of LVGCCs in fear related behavioral and neurophysiological phenotypes. In Chapter three, studies found deficits in context discrimination in $Ca_V1.2$ conditional knockout mice were noted, implicating $Ca_V1.2$ in mediating fear generalization and the associated maladaptive fear phenotype. Additionally, $Ca_V1.2$ conditional knockout mice displayed deficits in a complex version of the Morris water maze, but not a simple form of this task. Furthermore, these deficits in stimulus discrimination and complex spatial learning were correlated with a decrease

in cell division within the dentate gyrus. In contrast, exploration of context discrimination and spatial learning in the limited cues water maze in $Ca_V1.3$ global knockout mice did not find an effect of deletion of $Ca_V1.3$ in these two forms of learning. While not presented in Chapter three, pilot studies into the effects of deletion of $Ca_V1.3$ on cell division and neurogenesis within the brain has found no alteration in the density of BrdU positive cells in the dentate gyrus of $Ca_V1.3$ global knockout mice compared to their wild-type counterparts (**Appendix C**). This data suggests that $Ca_V1.3$ is not involved in the generalization of fear or in dentate gyrus function.

In Chapter four, $Ca_V1.2$ conditional knockout mice were found to exhibit significant deficits in fear extinction to a conditioned context, considered a persistent fear and a separate maladaptive fear phenotype. While these deficits in fear extinction were noted in $Ca_V1.2$ conditional knockout mice, deficits in fear extinction were not noted in previous studies of $Ca_V1.3$ global knockout mice (McKinney and Murphy 2006). This data suggests that $Ca_V1.3$ has no involvement in persistent fear.

Finally, in Chapter five, deletion of $Ca_V1.2$ was found to produce an enhancement in firing frequency in pyramidal neurons in the LA, but no changes in other properties of intrinsic excitability, including action potential properties, AHP, spike accommodation, or input resistance and resting membrane potential. In contrast to these results, previous studies of $Ca_V1.3$ global knockout mice found that deletion of $Ca_V1.3$ produced an increase in various intrinsic excitability factors of pyramidal cells in the LA including the AHP, firing frequency, and spike accommodation. In fact, reductions in the AHP in these cells is believed to be the source of the increase in both firing frequency and spike accommodation. While an increase in firing frequency was

noted in mice with neuronal deletion of $Ca_V1.2$, no change in AHP or spike accommodation was observed. The absence of alteration in AHP and spike accommodation suggest that deletion of $Ca_V1.2$ is likely mediating firing frequency by a different mechanism than deletion of $Ca_V1.3$. Additionally, exploration of LTP within the amygdala, in the thalamo-amygdala pathway discussed in Chapter five found significant deficits in LTP formation in this pathway in $Ca_V1.2$ conditional knockout mice and not $Ca_V1.3$ global knockout mice, in contrast to previous studies have found a role of $Ca_V1.3$ in LTP formation in the cortico-amygdala pathway into the amygdala (McKinney, Sze et al. 2009). This data suggests that both $Ca_V1.2$ and $Ca_V1.3$ are involved in neuronal function of pyramidal neurons within the LA, but that these two LVGCC subtypes are mediating the neuronal function of these neurons in very different manners. This could explain the difference in fear related phenotypes seen in $Ca_V1.2$ conditional knockout mice and $Ca_V1.3$ global knockout mice.

Taken together this data clearly segregates the role of $Ca_V1.2$ and $Ca_V1.3$ in fear related learning and physiology. This also supports a growing body of literature suggesting substantial differences between the LVGCC subtypes, $Ca_V1.2$ and $Ca_V1.3$ in overall brain function.

6.2 Limitations of the current mouse model

While the use of mice with conditional or global knockout of different LVGCC subtypes provides the potential to explore the roles of $Ca_V1.2$ and $Ca_V1.3$ individually, it also involves a variety of caveats which need to be considered.

Firstly, the brain is a heavily adaptive organ. Several studies have found that blockade or deletion of different proteins or molecular pathways can result in compensatory changes in the expression of other proteins or neuronal function (van der Lugt, Domen et al. 1995, Zhang, Goorha et al. 2002, Langwieser, Christel et al. 2010, Kreiner 2015). In the absence of $Ca_V1.2$ or $Ca_V1.3$, it is possible that there is a compensatory change within the brain which may mask the role of $Ca_V1.2$ and $Ca_V1.3$ in various forms of learning and physiology, such as masking a role of $Ca_V1.2$ in the AHP or $Ca_V1.3$ in fear extinction. Likely candidates for compensation would be other calcium permeable channels including other voltage gated calcium channels, calcium permeable AMPA receptors, or NMDA receptors, as well as the remaining LVGCC subtype. Additionally, it has been suggested that deletion of $Ca_V1.2$ may produce alterations in mGluR5 receptors and that this alteration is a compensatory reaction to the deletion of $Ca_V1.2$ (Langwieser, Christel et al. 2010).

Additionally, deletion of $Ca_V1.2$ or $Ca_V1.3$, or any protein, early in embryonically development could alter the structure or function of the brain in adulthood. In particular, LVGCCs has been linked to the induction of calcium transients in neural progenitor cells and neurogenesis (Deisseroth, Singla et al. 2004, D'Ascenzo, Piacentini et al. 2006, Piacentini, Ripoli et al. 2008). The absence or alteration in these calcium transients has been found to negatively impact neurogenesis and the maturation of neurons. This alteration in neuronal development could change the number of neurons or integration neurons within different areas of the brain. Development changes such as these in transgenic mice could produce changes in behavior and physiology independent of the role of the targeted protein in adulthood.

Finally, the creation of transgenic mice requires the alteration of the pre-existing DNA within the mouse. Alterations such as these require precise control of the placement and expression of exogenous DNA. Improper placement or expression of exogenous DNA could result in a variety of negative outcomes. Improper placement of exogenous DNA could result in the disruption of additional genes which could produce confounding results. Alterations in the desired expression of Cre recombinase could occur if the promoter driving Cre expression is weak. Cre could also be expressed in cells outside of the area of interest from non-promoter driven expression of Cre or from expression of Cre in cells that were not previously known to utilize the promoter. Finally, while recombination is thought to occur anywhere Cre and the LoxP sites are located, errors or incomplete recombination can occur in some or all cells if Cre is expressed weakly or the region flanked by the LoxP sites is overly large. Even in the case of a strongly expressed Cre recombinase and a small region for recombination, there will always be some amount of inefficiency of the Cre/recombinase system.

When using transgenic mice, such as the conditional and global knockout mice utilized in my studies, it is important to keep these limitation in mind.

6.3 Future Directions

The studies in this thesis support the existence of two separate forms of maladaptive fear learning: persistent fear and generalized fear, and the existence of two separate mechanism to mediate these fear phenotypes. Additionally, these studies suggest that $Ca_V1.2$ mediates the expression of both of these forms of maladaptive fear learning with generalization of fear being mediated by a role of $Ca_V1.2$ in the dentate gyrus and adult neurogenesis and persistent fear being mediated by a role of $Ca_V1.2$ in

inhibitory tone within the amygdala. An additional role of $Ca_V1.2$ in amygdala neurophysiology and plasticity was also observed. Future studies of persistent fear and generalized fear, as well as the role of $Ca_V1.2$ in the neurobiology and expression of these fears should further explore the segregation of these fears both in pure bred strains and substrains, as discussed in Chapter two, as well as in various $Ca_V1.2$ conditional knockout lines. Additionally future studies should further investigate the effects of deletion of $Ca_V1.2$ on amygdala neurophysiology to determine the mechanism by which the neurophysiological phenotypes observed in mice with neuronal deletion of $Ca_V1.2$ occurred.

6.2.1 Investigation of two forms of maladaptive fears using inbred mouse strains and substrains.

In terms of the studies of maladaptive fear in the two 129 substrains: 129S1 and 129S6, future studies could utilize these mice to explore the structures and genes underlying persistent and generalized fear.

6.2.1.1 Determining the genes involved in maladaptive fear

In terms of the investigation of the different genes involved in persistent versus generalized fear phenotypes, one could compare the genetic differences between 129S1 and 129S6 substrains and C57B6 mice. With respect to persistent fear, as measured as deficits in fear extinction, studies would need to compare genomic differences between C57B6 mice and 129S6 and 129S1 mice. In contrast, studies of generalized fear, measured as either overgeneralization or a lack of context discrimination, would need to compare the genomic differences between 129S1 and 129S6 mice. These studies would

require examination of these mice for differences in pre-selected genes or genome wide association studies to determine a list of genetic differences and genes that could be linked to fear learning. Differences in genes in 129S6 and 129S1 mice, but not C57B6 mice would suggest genes that may be involved in persistent fear. Differences in genes 129S6 mice compared to129S1 mice would suggest genes that may be involved in generalized fear. Further investigation could then be done using knockdown of candidate genes, either transgenically in mice or, perhaps more cost effectively, via siRNA to determine their role in these forms of learning.

6.2.1.2 Determining structures involved in maladaptive fear

In order to identify structures involved in these two forms of maladaptive fear, future studies could assess the different brain structures that are activated in response to the expression of these two forms of maladaptive fear. Activation of different brain structures can be evaluated by assessing for the expression of immediate early genes, such as c-fos, zif268, or Arc, which are considered to be activated by neuronal activity, throughout the brain shortly after exposure to the desired stimuli (Sagar, Sharp et al. 1988, Sheng and Greenberg 1990). In the case of persistent fear, levels of activation of various brain structures in 129S6 and 129S1 mice, but not C57B6 mice in the fear conditioned context after extinction could suggest brain regions that are over or under active in these mice in the presence of deficits in fear extinction. In the case of generalized fear, activation of various brain structures could be assessed between 129S1 and 129S6 mice in response to exposure to the generalized context at the end of context discrimination training. Over or under activation of various brain structures would then suggest structures involved in overgeneralization of fear. During the investigation of

brain structures involved persistent fear and generalized fear, care would need to be taken in the selection of control groups as the presence of any stimuli could induce expression of immediate early genes in structures that may not be directly associated with the maladaptive phenotypes observed. While this technique would not differentiate between inhibitory or excitatory neuronal populations, or the maturation state of the neurons active in the dentate gyrus, it would provide candidate brain structures involved in these forms of maladaptive fear learning which could be further investigated. Additionally, cells could be co-labeled for expression of immediate early genes as well as various neuronal markers, such as GAD65 or GAD67 as an inhibitory cell marker, or CaMKII as an excitatory cell marker, or doublecortin as a marker of immature cells in the dentate gyrus, or BrdU if prior injections of BrdU are performed to birthdate cells, to determine the activation of different cell populations within structures that are activated.

6.2.2 Investigation of $Ca_V1.2$ in two forms of maladaptive fear

6.2.2.1 Determining the role of the amygdala and the dentate gyrus in $Ca_V1.2$ mediated persistent and generalized fears.

Given the observation of both persistent and generalized fears, future studies exploring the role of $Ca_V1.2$ in these maladaptive fear phenotypes should investigate whether these two maladaptive fear phenotypes in mice with neuronal deletion of $Ca_V1.2$ are in fact mediated by separate structures, as is suggested by the work in this thesis and previous literature, or could be linked to deletion of this channel in one structure. To do this, studies could utilize structure specific deletion of $Ca_V1.2$ through delivery of Cre recombinase virally (Rohlmann, Gotthardt et al. 1996), or with a cell

permeable form of Cre recombinase (Jo, Nashabi et al. 2001) infused directly into the amygdala or dentate gyrus in mice with homozygous floxed $Ca_V1.2$ alleles. Additionally, deletion of $Ca_V1.2$ using this method would also eliminate issues related to the deletion of $Ca_V1.2$ during prenatal development. Mice with viral injections to each of these structures could then be assessed for both persistent fear, in the form of a deficit in fear extinction, as well as generalized fear, measured as a lack of context discrimination. Based on the studies in this thesis, one would predict that mice with deletion of $Ca_V1.2$ in the dentate gyrus would display deficits in context discrimination, but not extinction of conditioned fear and that deletion of $Ca_V1.2$ in the amygdala would produce deficits in the extinction of conditioned fear to a context, but not context discrimination. However, the presence of both context discrimination deficits and extinction deficits in mice with deletion of $Ca_V1.2$ in either amygdala, or dentate gyrus, and not the other, would suggest that these two forms of maladaptive fear are regulated by the role of $Ca_V1.2$ via the same structure. In the case of the dentate gyrus, this mechanism could be the role of $Ca_V1.2$ in the modulation of newborn neurons. In the amygdala this mechanism could reflect the role of $Ca_V1.2$ in the excitability or inhibition of pyramidal neurons within the LA. If deletion of $Ca_V1.2$ selectively in the amygdala or dentate gyrus does not alter context discrimination or fear extinction, then the effect of neuronal deletion of $Ca_V1.2$ in our mice on these two forms of maladaptive fear learning could be due to compensatory mechanisms produced from the loss of this channel embryonically, or a role of this channel in a different structure, such as the prefrontal cortex or the hippocampus proper.

6.2.2.2 Determining the role of $Ca_V1.2$ in inhibitory neurons in mediating persistent and generalized fears.

Given the contrast between the deficits in fear extinction in mice with neuronal deletion of $Ca_V1.2$ presented in this thesis and previously published literature using mice with conditional deletion of $Ca_V1.2$ in excitatory neurons in the forebrain (McKinney, Sze et al. 2008), I hypothesized that $Ca_V1.2$ mediates fear extinction through its role in inhibitory neurons. Future studies could investigate the role of $Ca_V1.2$ in inhibitory neuronal populations by crossing mice homozygous for the floxed $Ca_V1.2$ allele with mice expressing Cre recombinase driven by a promoter specific to inhibitory neurons, such as Dlx5/6 (Ruest, Hammer et al. 2003, Taniguchi, He et al. 2011). These mice could then be tested for deficits in fear extinction to a conditioned context. If, as expected, $Ca_V1.2$ mediates fear extinction via its expression in inhibitory neurons, deletion of $Ca_V1.2$ in inhibitory neurons should produce significant deficits in fear extinction. If, however, deletion of $Ca_V1.2$ in inhibitory neurons does not recapitulate the deficits seen in the $Ca_V1.2$ conditional knockout mice utilized in my studies, then $Ca_V1.2$ may be mediating extinction via excitatory neurons in a non-forebrain structure. Additionally, mice with deletion of $Ca_V1.2$ in inhibitory neurons could be assessed in context discrimination. While there is currently no direct evidence for the involvement of inhibitory neurons in the context discrimination phenotype, the effects of deletion of $Ca_V1.2$ in inhibitory neurons in the $Ca_V1.2$ conditional knockout mice utilized in this thesis can not be separated from the effects of deletion of $Ca_V1.2$ in excitatory neurons. Additionally, inhibitory neurons are important for proper amygdala function (Ehrlich,

Humeau et al. 2009) and dentate gyrus function, including modulation of adult neurogenesis (Ge, Pradhan et al. 2007, Song, Sun et al. 2013).

6.2.3 Investigation of $Ca_V1.2$ in the neurobiology associated with maladaptive fear

6.2.3.1 Exploring the role of $Ca_V1.2$ be mediating adult neurogenesis

The presence of a decrease in cell division and the density of immature neurons in the dentate gyrus of mice with neuronal deletion of $Ca_V1.2$ suggests an important role of $Ca_V1.2$ in mediating adult neurogenesis and cell proliferation in the dentate gyrus. However, what remains unclear is how $Ca_V1.2$ could be mediating adult neurogenesis within the dentate gyrus. Previous studies have linked LVGCCs with excitation-neurogenesis coupling, or the increase in neurogenesis in response to excitation of neural stem cells, with activation of LVGCCs inducing an increased rate of neurogenesis (Deisseroth, Singla et al. 2004, D'Ascenzo, Piacentini et al. 2006, Piacentini, Ripoli et al. 2008, Teh, Ishizuka et al. 2014). Additionally, studies have noted a link between LVGCCs and the expression of genes associated with the regulation of neuronal differentiation, including HES1 and Id2, in neural progenitor cells (Deisseroth, Singla et al. 2004). However, these studies note alterations in the percent of new born cells that differentiated into neurons, not an increase in cell proliferation. Additionally, the majority of these studies have utilized cultured neural stem cells or tissue early in prenatal development and may not represent the mechanism by which LVGCCs act in the adult dentate gyrus (Urban and Guillemot 2014). Conversely, other studies have implicated LVGCCs in the induced proliferation of adult born cells within the dentate gyrus (Luo, Zhu et al. 2005, Zhu, Zhou et al. 2012). Though it may be important to note that both of these previous studies investigated

increased adult neurogenesis induced by altered health states, such as ischemic stroke (Luo, Zhu et al. 2005) or Vitamin D(3) deficiency (Zhu, Zhou et al. 2012), which could produce a variety of changes in neuronal function. However, these studies, taken along with our current study, demonstrating decreased cell proliferation in $Ca_V1.2$ conditional knockout mice, do suggest that LVGCCs can influence proliferation of neural progenitor cells in the adult brain.

L-type VGCCs could be altering proliferation of cells within the dentate gyrus via a variety of mechanisms including: action of LVGCCs in neural progenitor cells or action of LVGCCs in external pathways that can regulate the activity of neural progenitor cells. While it is currently unknown whether $Ca_V1.2$ is expressed in neural progenitor cells in the adult dentate gyrus, studies of neural stem cells cultured from mice aged P0 did not find expression of $Ca_V1.2$ in undifferentiated stem cells (D'Ascenzo, Piacentini et al. 2006, Piacentini, Ripoli et al. 2008). These studies suggest that the effects of $Ca_V1.2$ on cell proliferation in the dentate gyrus is likely due to the action of $Ca_V1.2$ in external pathways that regulate the activation and division of neural progenitor cells. One such modulator of cell proliferation in the dentate gyrus is neuronal activity, with increased excitation or LTP increasing cell proliferation (Deisseroth, Singla et al. 2004, Bruel-Jungerman, Davis et al. 2006). Therefore, it seems reasonable to hypothesize that neuronal deletion of $Ca_V1.2$ produces decreases in cell proliferation in the dentate gyrus via a decrease in excitation or an increase in inhibition in the dentate gyrus. Given that neuronal deletion of $Ca_V1.2$ was observed to produce an increase in sIPSCs in pyramidal cells in the LA, it is possible that $Ca_V1.2$ could be increasing inhibitory function in the dentate gyrus. To explore this possibility, future

studies could investigate whether deletion of $Ca_V1.2$ alters inhibition within the dentate gyrus by assessing the intrinsic properties of inhibitory neurons within the dentate gyrus in mice with neuronal deletion of $Ca_V1.2$. An increase in the excitability of inhibitory neurons in the dentate gyrus would support the hypothesis that $Ca_V1.2$ is mediating neurogenesis via alterations in excitation in the dentate gyrus. In contrast, if no change in the intrinsic properties of the inhibitory neurons in the dentate gyrus, or a decrease in excitability is observed, it could contradict this hypothesis. In fact, pilot studies in the lab suggest that neuronal deletion of $Ca_V1.2$ actually produces a substantial enhancement in the induction of LTP within the perforant path (**Appendix D**). However, this result does not eliminate the possibility that $Ca_V1.2$ may be altering inhibition on a smaller scale, in select circuits involved in cell proliferation, to alter cell proliferation. This complicated by the fact that, the exact circuits and signaling mechanisms that modulates cell proliferation in the adult dentate gyrus are still being elucidated. Therefore, it remains unclear as to the exact mechanism by which $Ca_V1.2$ is mediating cell proliferation within the dentate gyrus.

6.2.3.2. Determining the role of $Ca_V1.2$ in mediating thalamo-amygdala LTP and pyramidal neuron excitability

One of the main questions remaining, after neurophysiological studies of the lateral amygdala in mice with neuronal deletion of $Ca_V1.2$, is whether the changes in spontaneous synaptic input, LTP, and intrinsic excitability within the pyramidal neurons are all produced through separate mechanisms of $Ca_V1.2$, or whether these phenotypes could be linked back to one mechanism. Changes in inhibition can result in changes in the likelihood of LTP formation in the thalamo-amygdala pathway (Bissiere, Humeau et

al. 2003). Taken together with the potential for changes in pyamidal neuron excitability to be a compensatory mechanism of deletion of $Ca_V1.2$, one hypothesis is that deletion of $Ca_V1.2$ in inhibitory neurons produces an increase in inhibitory input onto pyramidal neurons in the LA and through this increase in inhibition produces deficits in thalamo-amygdala LTP and a compensatory increase in pyramidal amygdala intrinsic excitability. To investigate this hypothesis, future studies should utilize mice with selective deletion of $Ca_V1.2$ in inhibitory neurons throughout the brain, as mentioned in the previous section. If deletion of $Ca_V1.2$ is mediating these neurophysiological changes via inhibitory neurons, examination of the neurophysiology of pyramidal neurons in the LA in these mice would find an increase in sIPSC frequency, as observed in the $Ca_V1.2$ conditional knockout mice used throughout this thesis, as well as an increase in LA excitability and a decrease in thalamo-amygdala LTP. However, it is also possible that deletion of $Ca_V1.2$ in inhibitory neurons mediates either a decrease in thalamo-amygdala LTP or a compensatory increase in LA excitability, but not the other, which would also be observed with neurophysiological records. If this hypothesis is incorrect and deletion of $Ca_V1.2$ in inhibitory neurons does not effect LTP or the excitability of pyramidal neurons in the LA, examination of the neurophysiological properties of the LA in these mice would reveal an increase in the frequency of sIPSCs, but no changes in the excitability of pyramidal neurons within the LA or LTP formation in the thalamo-amygdala pathway. Alternatively, it is also possible that deletion of $Ca_V1.2$ in inhibitory neurons does not produce a change in sIPSCs at all. This would suggest that the increase in sIPSCs seen in $Ca_V1.2$ conditonal knockout mice reported in this thesis was likely due to compensatory mechanisms due to deletion of $Ca_V1.2$

embyronically, or a compensaory increase in inhibition due to deletion of $Ca_V1.2$ in excitatory neurons.

6.4 Closing

In summary, this thesis provides significant evidence for the existence of two forms of maladaptive fear, persistent fear and generalized fear, and a role of $Ca_V1.2$ in mediating both forms of fear. Experiments within this thesis also suggest that deletion of $Ca_V1.2$ mediates the generalization of fear via a decrease in adult neurogenesis and persistent fear via alterations in amygdala neurophysiology. Additionally, research within this thesis, taken along with previously published research on $Ca_V1.3$ global knockout mice, suggests that $Ca_V1.3$ does not play a role in maladaptive fear learning or expression and that $Ca_V1.2$ mediates the expression of maladaptive fear in a LVGCC subtype specific manner. Future exploration of the role of $Ca_V1.2$ in the modulation of persistent and generalized fear could better isolate the mechanisms mediating these two forms of fear to changes in either the amygdala or the dentate gyrus, as well as to a role of $Ca_V1.2$ in inhibition.

Given the ties of these maladaptive fear phenotypes to the development and diagnosis of trauma-related and anxiety-related disorder in humans, as well as the ties of mutations in $Ca_V1.2$ in the formation of various psychiatric conditions in humans, these results implicate $Ca_V1.2$ in the development and expression of trauma and anxiety-related disorders such as post-traumatic stress disorder, generalized anxiety-disorder, panic disorder and phobias. Future studies investigating the mechanisms by which $Ca_V1.2$ mediates persistent and maladative fear learning may provide important insights into the development and expression of trauma and anxiety related disorders in humans.

Additionally, this thesis demonstrates a significant difference in the function of $Ca_V1.2$ and $Ca_V1.3$ within the amygdala and other areas of the brain.

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Table 1: Literature on L-type voltage gated calcium channels subtypes in learning and physiology

	Subtype	Deletion Pattern	Conclusions
(McKinney and Murphy 2006)	$Ca_V1.3$	Global knockout	Deletion of $Ca_V1.3$ impairs fear consolidation, but not fear extinction
(McKinney, Sze et al. 2008)	$Ca_V1.2$	Conditional knockout using CaMKIIα-cre	Conditional deletion of $Ca_V1.2$ does not impair fear learning or fear extinction
(McKinney, Sze et al. 2009)	$Ca_V1.3$	Global knockout	Deletion of $Ca_V1.3$ decreases the AHP and increases repetitive firing in the BLA, and decreases cortico-amygdala LTP
(White, McKinney et al. 2008)	$Ca_V1.2$	Conditional knockout using CaMKIIα-cre	Conditional deletion of $Ca_V1.2$ impairs remote spatial memories, but not recent spatial memories in the classic Morris water maze
(Gamelli, McKinney et al. 2011)	$Ca_V1.2$ $Ca_V1.3$	$Ca_V1.3$ Global knockout;$Ca_V1.2$ Conditional knockout using CaMKIIα-cre	Deletion of $Ca_V1.3$, but not conditional deletion of $Ca_V1.2$, decreases the AHP in the hippocampus
(Moosmang, Haider et al. 2005)	$Ca_V1.2$	Conditional knockout using Nex-cre	Conditional deletion of $Ca_V1.2$ impairs spatial learning in the labyrinth maze and a visible platform version of the Morris water maze
(Langwieser, Christel et al. 2010)	$Ca_V1.2$	Conditional knockout using Nestin-cre	Conditional deletion of $Ca_V1.2$ impairs fear learning and thalamo-amygdala LTP
(Busquet, Hetzenauer et al. 2008)	$Ca_V1.3$	Global knockout	Deletion of $Ca_V1.3$ did not produce deficits in fear extinction
(Lacinova, Moosmang et al. 2008)	$Ca_V1.2$	Conditional knockout using Nex-cre	Conditional deletion of $Ca_V1.2$ decreases firing frequency in the hippocampus

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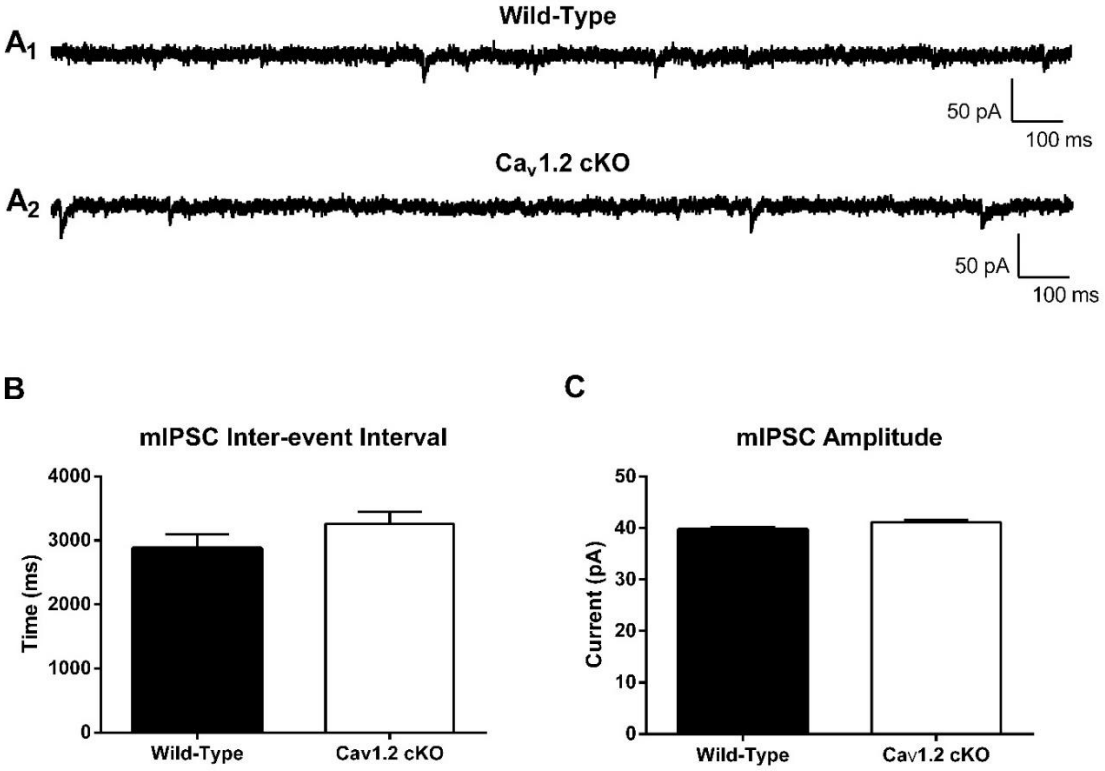
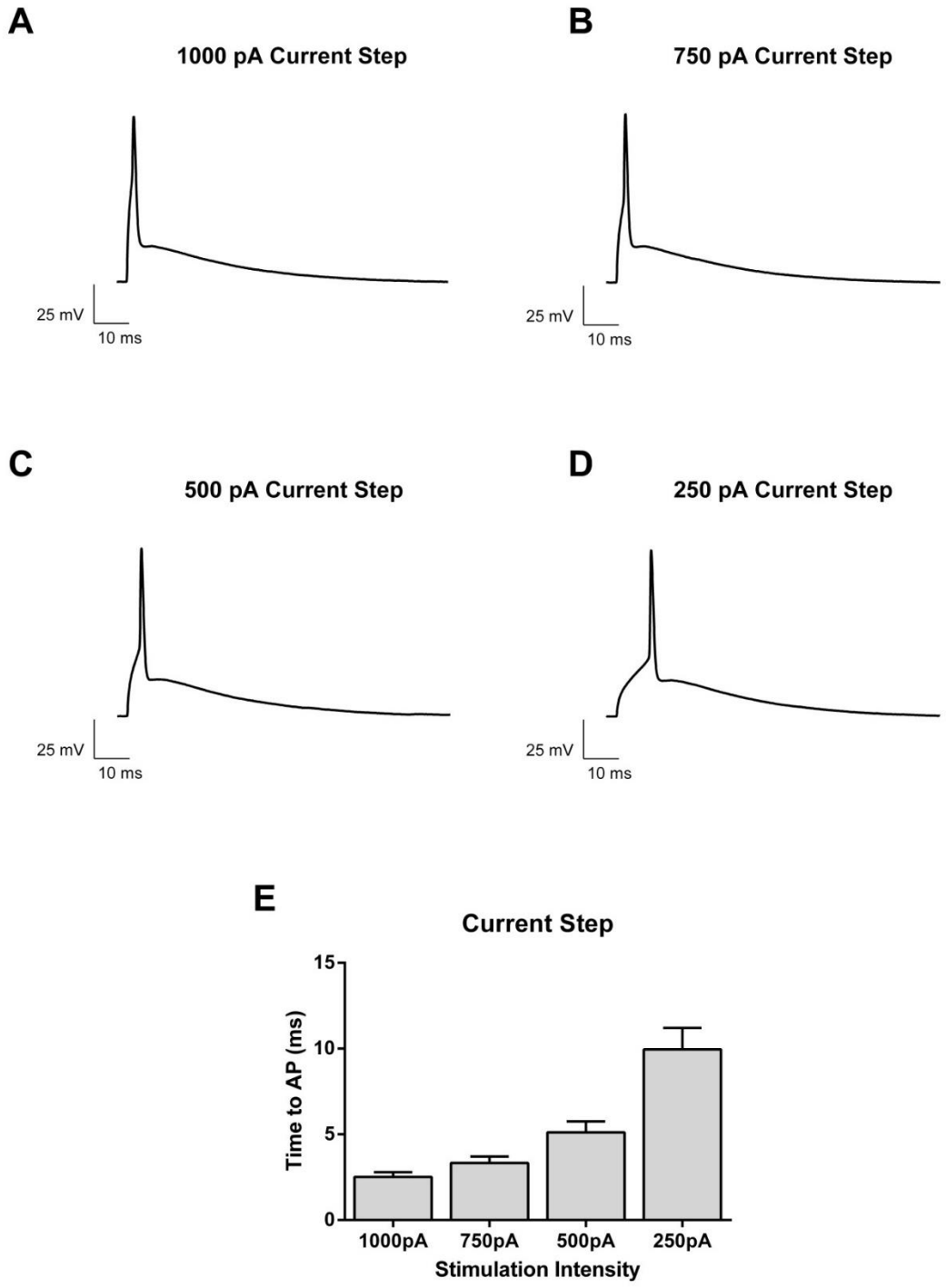
Table 2: Summary of current research of L-type voltage gated calcium channels subtypes in learning and physiology

	Subtype	Deletion Pattern	Conclusions
Chapter 3	$Ca_V1.2$ $Ca_V1.3$	$Ca_V1.2$ conditional knockout using Synapsin1a-cre; $Ca_V1.3$ global knockout	Conditional deletion of $Ca_V1.2$ impairs context discrimination and complex spatial learning in the limited cues water maze Deletion of $Ca_V1.3$ does not impair context discrimination or complex spatial learning
Chapter 4	$Ca_V1.2$	$Ca_V1.2$ conditional knockout using Synapsin1a-cre	Conditional deletion of $Ca_V1.2$ impairs fear extinction and alters spontaneous synaptic activity in the LA
Chapter 5	$Ca_V1.2$ $Ca_V1.3$	$Ca_V1.2$ conditional knockout using Synapsin1a-cre; $Ca_V1.3$ global knockout	Conditional deletion of $Ca_V1.2$ increases firing frequency in pyramidal cells in the lateral amygdala and impairs thalamo-amygdala LTP Deletion of $Ca_V1.3$ does not impair thalamo-amygdala LTP



APPENDIX A: Neuronal deletion of $Ca_V1.2$ does not alter the frequency or amplitude of mIPSCs in pyramidal cells in the lateral amygdala

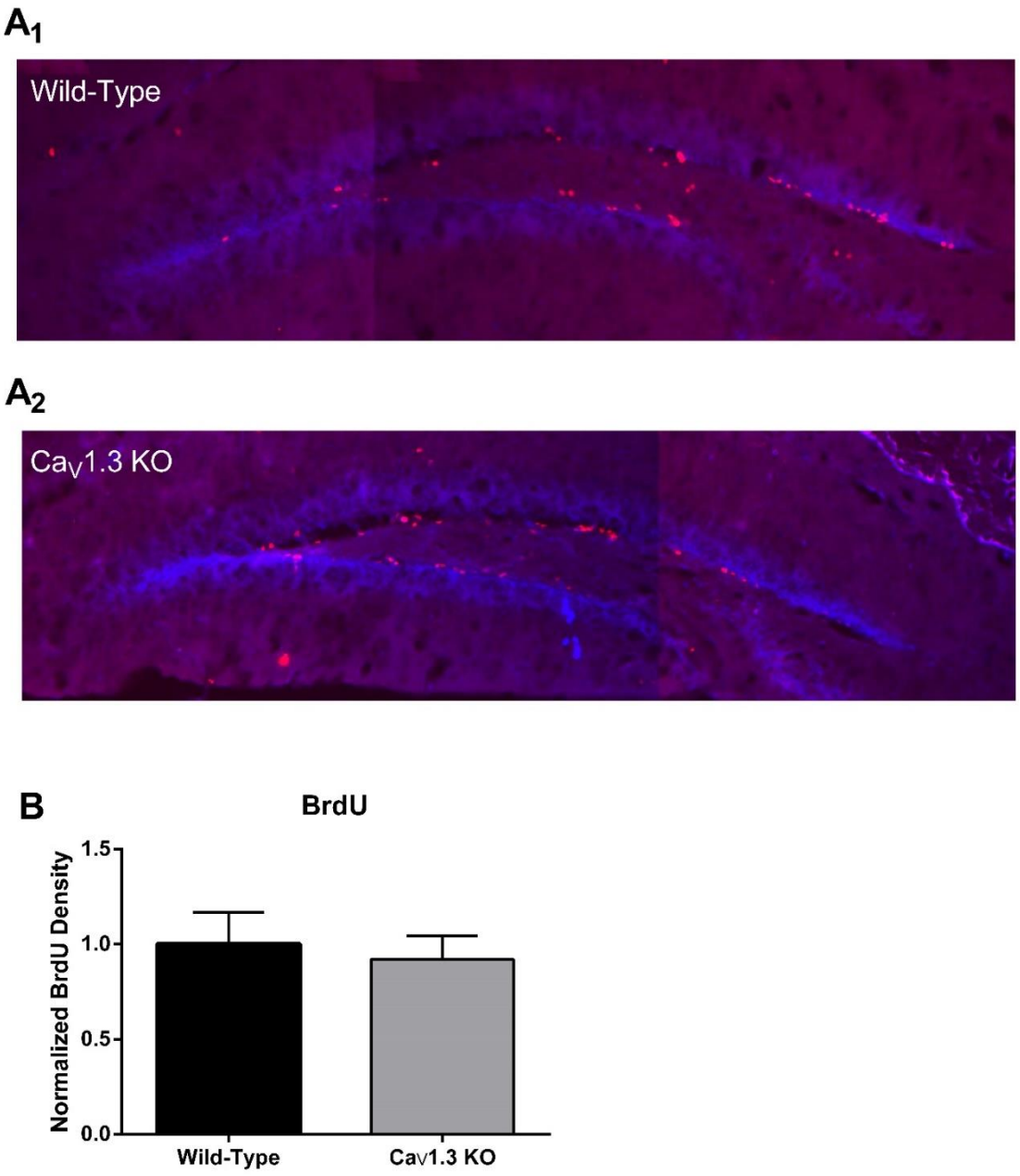
To investigate the effects of neuronal deletion of CaV1.2 for changes in inhibitory synaptic input onto pyramidal cells in the lateral amygdala, whole cell voltage-clamp recordings of miniature inhibitory postsynaptic currents (mIPSCs) were made. Recording electrodes were filled with a cesium chloride internal solution (in mM): 130 CsCl, 1.0 KCl, 1.0 NaCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 10.0 HEPES, 7.0 phosphocreatine, 4.0 Na_2ATP, 0.3 TrisGTP, 0.2 EGTA, 0.1% biocytin in a submersion chamber with continuous aCSF perfusion containing 4.0 mM kynuernic acid (Sigma) and 1 µm tetrodotoxin (Sigma). Events with a decay time greater than 2.0 ms were excluded from analysis. (**A1** & **A2**) Representative traces of mIPSCs in wild-type and $Ca_V1.2$ conditional knockout mice. (**B**) Analysis of the average inter-event interval between mIPSCs using an unpaired t-test revealed no significant difference between $Ca_V1.2$ conditional knockout mice (n = 1049 events, 12 cells, 5 mice) and their wild-type counterparts (n = events, 11 cells, 6 mice) (p = 0.2098). (**C**) Additionally, analysis of the average amplitude of mIPSCs also using an unpaired t-test revealed no significant difference between $Ca_V1.2$ conditional knockout mice and their wild-type counterparts. This suggest no alteration in presynaptic or postsynaptic function with neuronal deletion of $Ca_V1.2$ (p = 0.3579). Data are represented as mean ± SEM.



APPENDIX B: Minimal current intensity required to elicit a single action potential in pyramidal ells in the LA depends on the duration of the current step

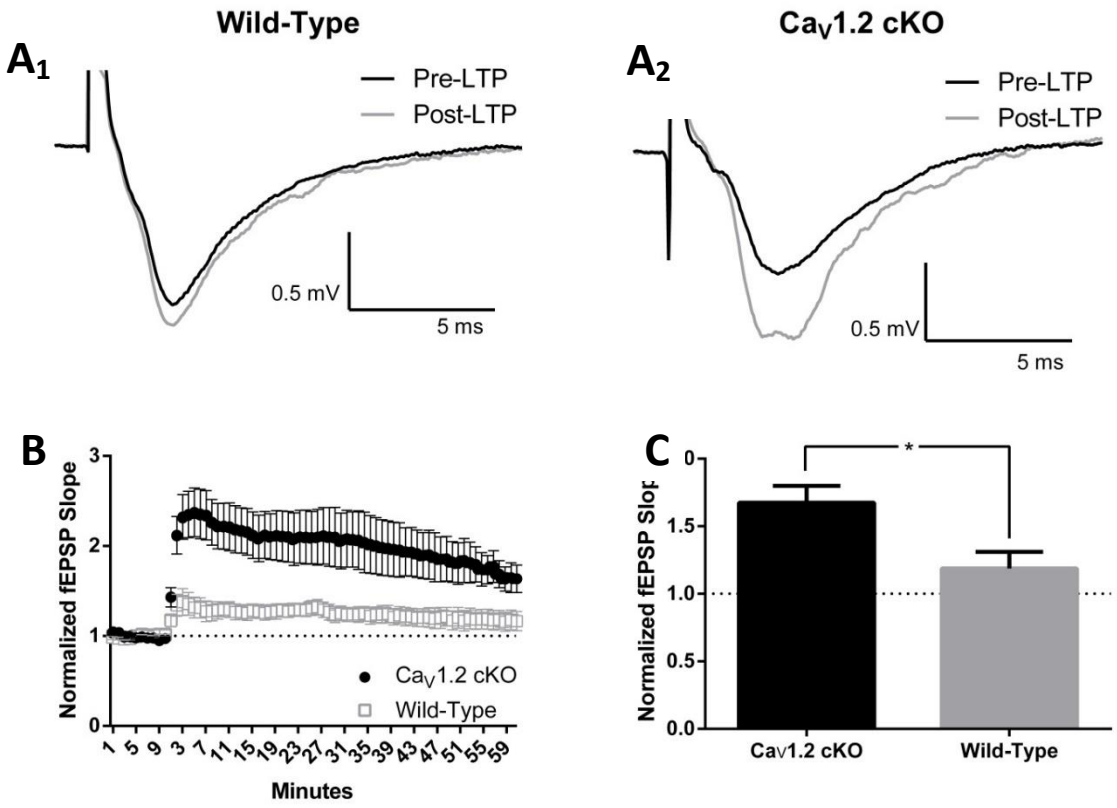
To assess the relationship between the current intensity and depolarization step duration required to elicit a single action potential in pyramidal cells in the LA in 129S6/B6 hybrid mice (5 mice), cells were stimulated with a set current intensities

using depolarization step ranging from 1 millisecond to 15 milliseconds with 0.5 millisecond steps in duration until the first action potential was generated. Time to action potential was measured as the time from the onset of the current to the threshold of the action potential. Current intensities included 1000 pA (**A**), 750 pA (**B**), 500 pA (**C**), and 250 pA **(D**). When the current intensity was set at 1000 pA, action potentials could be produced using only 3 milliseconds (**E**). However, action potential traces exhibited a significant charging component prior to action potential firing which seemed to interfere with the shape of the action potential. As the current intensity decreased, the depolarization step duration required to fire a single action potential increased (Repeated measures ANOVA, $F_{(3,14)} = 20.82$, $p < 0.0001$), but action potential traces did not exhibit as much interference from the charging membrane. Data are represented as mean ± SEM.



APPENDIX C: Global deletion of Cav1.3 does not alter cell proliferation within the dentate gyrus

(**A**$_1$ & **A**$_2$) $Ca_V1.3$ global knockout mice (n = 5 mice, 8 dentate gyri per mouse) and wild-type mice (n = 5 mice, 8 dentate gyri per mouse) were assessed for the rate of cell division in the adult dentate gyrus using five injections of BrdU across five days. Injections were performed by scruffing each mouse individually prior to each injection (**B**$_1$ & **B**$_2$) Analysis of BrdU labeled cells limited the subgranular zone of the dentate gyrus (**C**) Comparison of the density of BrdU positive cells in the dentate gyrus between genotypes revealed a no significant decrease in cell division in $Ca_V1.3$ knockout mice versus wild-type mice (unpaired t-test, $p = 0.7161$). Data are represented as mean ± SEM.



APPENDIX D: Neuronal deletion of Cav1.2 produces an increase in LTP induction in the perforant path

To investigate the effects of neuronal deletion of Cav1.2 on synaptic plasticity within the dentate gyrus, LTP was examined in the perforant path using field potential recordings. LTP was generated using four bouts of 500 ms long, 100 Hz high frequency stimulation. (**A_1 and A_2**) Representative fEPSPs from wild-type (n = 8 slices from 4 mice) and Cav1.2 conditional knockout mice (n = 12 slices from 6 mice) before (pre-LTP) and after (post-LTP) LTP induction. fEPSPs were assessed for the initial slope throughout recording with fEPSP values normalized to the average of the fEPSP slope prior to LTP induction. (**B**) While wild-type mice showed a moderate, but significant increase in the size of the fEPSP slope after LTP induction, Cav1.2 conditional knockout mice demonstrated a significantly greater increase in the fEPSP slope after LTP induction (Repeated measures ANOVA, $F_{(69,1242)} = 15.6646$, $p < 0.0001$). (**C**) Sixty minutes after LTP induction, wild-type mice continued to exhibit a moderate LTP induction, while Cav1.2 conditional knockout mice demonstrated a substantially greater increase in LTP induction (unpaired t-test, $p = 0.016$). Data are represented as mean ± SEM. *$p < 0.05$.