# L-type voltage-gated calcium channels in fear conditioning, fear extinction, and amygdala neurophysiology

by

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# Dedication

I dedicate this work to my parents, Grandma Blanche, and to the loving memory of my grandfathers and Grandma Pearl.

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Abstract

Fear conditioning is a form of learning in which an initially neutral stimulus (e.g., tone or context) comes to be feared after being paired with an aversive stimulus (e.g., shock). After pairing, the neutral stimulus alone can elicit fear responses (e.g., freezing behavior, autonomic arousal, stress hormone release). The initially neutral stimulus is referred to as the "conditioned stimulus" (CS) once it is able to bring about fear responses on its own, the aversive stimulus is the "unconditioned stimulus" (US), and the fear responses elicited by the CS are the "conditioned responses" (CRs). Fear extinction is a process by which the previously conditioned fear responses can be reduced by repeated presentation of the CS alone, in the absence of the aversive US. Fear conditioning and fear extinction are both critically dependent on the amygdala, a medial temporal lobe brain structure.

Interestingly, it has been previously demonstrated that L-type voltage-gated calcium channels (L-VGCCs) have a role in fear conditioning, fear extinction, and amygdala neurophysiology. All of the studies implicating L-VGCCs in these phenomena used L-VGCC antagonists to demonstrate the role for L-VGCCs. There are two brain-expressed L-VGCCs, $Ca_v1.2$ and $Ca_v1.3$, both of which are the targets of currently-available L-VGCC antagonists. In this dissertation, I

address the contribution of each of these L-VGCCs to fear conditioning, fear extinction, and amygdala neurophysiology using mouse models in which the genes for either $Ca_v1.2$ or $Ca_v1.3$ is deleted.

First, I demonstrate that $Ca_v1.3$, but not $Ca_v1.2$, mediates consolidation of fear conditioning. Next, I show that neither $Ca_v1.2$ nor $Ca_v1.3$ alone is necessary for fear extinction. Instead, I find that the L-VGCC antagonist nifedipine used in the previous experiments implicating L-VGCCs in fear extinction impairs locomotion and induces an aversive state. Further, I demonstrate that this aversive state can enter into associations with stimuli present at the time that it is experienced, suggesting that previous studies using nifedipine were likely confounded by drug toxicity. Finally, I show that $Ca_v1.3$ mediates long-term potentiation of afferents to the basolateral amygdala (BLA) as well as the afterhyperpolarization in principal neurons of the BLA.

# Chapter I

# Introduction

## A short history of Pavlovian fear conditioning:

Early in the 20th century, Russian physiologist Ivan Petrovich Pavlov undertook the study of a type of learning now commonly called Pavlovian, or classical, conditioning (Pavlov 1927). His motivation for embarking on this field of study was an observation he made while conducting experiments on the digestive system in dogs, experiments for which he won the Nobel Prize in Physiology or Medicine in 1904 (Pavlov 1967). During these experiments, he noticed that the dogs salivated not only when presented with food, but also in response to objects associated with feeding. From this observation he predicted that, if a particular stimulus in the dog's environment was present when the dog was given food, then this stimulus would become associated with food and cause salivation on its own. This observation so intrigued Pavlov that he devised a systematic experimental approach for studying the phenomenon.

In Pavlov's experimental approach, he would ring a bell or deliver one of a wide variety of neutral stimuli, including whistles, metronomes, tuning forks, and a range of visual stimuli (Johnson 2008) just prior to delivering food to the

mouth of the dog. At first, the dog did not salivate until it got the food. After a few repetitions, however, the dog started to salivate in response to the bell. Finally, Pavlov found that the dog would continue responding to the bell even if it was not followed by food—at least for a while. If Pavlov continued to ring the bell in the absence of food, then the dog would eventually stop salivating to the bell alone. He termed this phenomenon “extinction” and referred to the behavior as being “extinguished”. Pavlov called the food the unconditioned stimulus (US), that is, it was a stimulus to which the dog had a natural response. The response to the US, in this case salivation, was called the unconditioned response (UR). Once the bell came to elicit the UR, it was referred to as the conditioned stimulus (CS) and when it was the CS, rather than the US, that elicited the response, it was referred to as the conditioned response (CR; even if it might be the same behavior as the UR) (Pavlov 1927). Pavlov referred to this learned relationship as a “conditional reflex”, a term that was mistranslated from Russian as "conditioned reflex." Other scientists reading his work reasoned that since such reflexes were conditioned, they must be produced by a process called conditioning, thus this automatic form of learning has come to be known as Pavlovian, or classical, conditioning (Johnson 2008).

Though Pavlov focused mostly on appetitive associative learning, his work has provided a framework for the study of aversive associative learning. Pavlov’s contemporary and fellow countryman, Vladimir Bekhterev, was the first to apply this framework to the study of aversive associative learning when he performed

the first fear conditioning studies nearly 100 years ago. In his initial experiments, volunteer human male subjects (Bekhterev also did similar experiments with dogs) were asked to sit in a chair, in an experimental room, and to rest one foot on a pedal connected to a electric shock generator. Then, sounds of a violin were delivered into the experimental room. The musical sounds alone would not cause any harm or fear. However, the experiment was designed so that every time the music was in the air, the patient received a brief, mild electric shock to his bare foot. Bekhterev found that relatively few such pairings were enough for the subject to learn to withdraw his foot whenever he heard the sounds of the violin. Applying Pavlov's terminology to the above experiment, the music was the CS, the foot shock was the US, and foot withdrawal was the CR (Bekhterev 1923, Bourtchouladze 2004). Though I can find no record that Behhterev attempted to extinguish the foot withdrawal response in these men, one can imagine that if he had continued to play the music in the absence of delivering a footshock, the men might have discontinued withdrawing their feet in response to the music and Behhterev could have been said to have performed the first fear extinction experiment as well.

**The modern fear conditioning and fear extinction paradigms:**

Interestingly, the fear conditioning paradigm is not very different today from when Bekhterev performed the first fear conditioning experiments. Today, subjects more amenable to laboratory study studies than dogs, like mice and rats, are more commonly used. Humans are still used, but less frequently than mice and

rats. The electric shock, however, because of its resistance to habituation (the progressive decrease in behavioral response to repeated stimulation), continues to be the aversive stimulus of choice after all these years. In a typical modern-day fear conditioning experiment, rodents are presented with an emotionally neutral stimulus, such as a tone or light, that is paired in time with an aversive US, usually a mild foot shock. Subsequent presentations of the previously-neutral stimulus elicit a range of fearful responses in rodents, including freezing behavior, the complete absence of non-respiratory movements (Fanselow 1980). In addition to freezing, autonomic arousal occurs, corticosteroid plasma levels increase, sensitivity to pain decreases, startle to unexpected, high intensity stimuli increases, and ongoing instrumental behavior ceases. Other phenomena associated with fear may also occur, including piloerection, defaecation and urination, and vocalization (Fanselow & Poulos 2005). The emergence of fear responses to the previously-neutral stimulus is taken as evidence for associative excitatory learning, associative because it depends on the co-occurrence of the CS and US, and excitatory because the CS now triggers a fear response that it did not trigger before the pairings.

Fear extinction is the reduction of conditioned fear responses through repeated presentation of the CS in the absence of the aversive US. Fear extinction can eliminate all fearful responses with enough unpaired CS presentations, but does not reflect erasure of the original fear memory. The retention of the original association can be uncovered by a variety of maneuvers including changing the

test context (renewal) (Bouton & Bolles 1979), presenting unsignaled USs (reinstatement) (Rescorla & Heth 1975), or simply allowing time to pass (spontaneous recovery) (Baum 1988). Since no further CS–US pairing occurs to retrain the fear association, these phenomena argue that the original association must remain even after extinction and thus, rather than erasure, extinction appears to be new learning that acts to inhibit or compete with the original association. The extinction paradigm serves as an important model for inhibitory learning. Additionally, fear extinction may have implications for the treatment of fear disorders.

**Fear conditioning and fear extinction in human health:**

Everyone experiences fear. Fear serves an important survival function in animals, including humans. Humans are born with some innate fears including heights, sudden noises, potentially predatory animals (Adolph 2000, Anderssen *et al.* 1993), but they also have the ability to adapt based on experience (i.e., learn). Evolution has crafted this form of learning and it is generally advantageous. It allows humans to learn through life experiences about those aspects of the environment to which they do not have innate fear, but are dangerous and potentially threatening to survival. However, if errors in this process occur, it can be disadvantageous. Fear that is inappropriate, excessive, or prolonged can be problematic for both physiology (Sapolsky 1996) and emotional well being (McEwen & Sapolsky 1995)—such problems constitute the body of human health problems collectively referred to as ‘anxiety disorders.’

Anxiety disorders include panic disorder, post-traumatic stress disorder, obsessive-compulsive disorder, generalized anxiety disorder, and phobias (social phobia, agoraphobia, and specific phobia). These disorders are not uncommon. Approximately 40 million American adults ages 18 and older, or about 18.1 percent of people in this age group in a given year, have an anxiety disorder and thus represent a major public health problem (Kessler *et al.* 2005). Unfortunately, the causes of anxiety disorders are poorly understood and treatments for individuals with anxiety disorders are less effective than desirable. Current models of anxiety disorders suggest a role for fear conditioning and extinction in the etiology, maintenance, and treatment of anxiety disorders (Mineka & Oehlberg 2008). Thus, greater knowledge of the neurobiological basis of fear conditioning and extinction may allow for better understanding about how anxiety disorders develop and how they might be treated. The potential health benefits of expanded knowledge of fear conditioning and extinction are not limited to anxiety disorders. For example, anticipatory nausea and vomiting (ANV) in cancer chemotherapy patients results from conditioning to an aversive event. Anticipatory nausea and vomiting develops when a patient forms an association with nausea and vomiting (US) induced by cytotoxic chemotherapeutic agents and environmental stimuli associated with their administration (Stockhorst *et al.* 2007).

Clinically, principles of fear conditioning and extinction already guide some therapies. For example, systemic desensitization and flooding are used to treat some anxiety disorders. Systematic desensitization involves systematically exposing individuals to a feared object or situation in a gradually-increasing hierarchy, until the fear has been extinguished. Generally the therapy will involve the construction of a fear hierarchy of events that gradually escalate to exposure to the object of fear, moving upward through the steps in the hierarchy as the anxiety felt is lessened or eliminated (Frueh *et al.* 1997). Flooding similarly exposes the patient to a feared object or situation, but involves no hierarchy. Instead, the patient is exposed to their worst possible fear (within realistic safety limitations) and are prevented from escaping the situation until the fear is eliminated (Moulds & Nixon 2006). Overshadowing and latent inhibition are two conditioning concepts that are sometimes used in addition to systematic desensitization to treat ANV (Figueroa-Moseley *et al.* 2007, Miller & Kearney 2004, Stockhorst *et al.* 1993). The magnitude of aversion to the environmental cues present during chemotherapy sessions can be reduced by presenting a novel salient cue such as flavored drink during the chemotherapy session, or overshadowing the environmental cues with the novel salient cue. Latent inhibition is a process by which previous exposure to a stimulus prevents conditioned associations from being formed with that stimulus. Anticipatory nausea and vomiting can be reduced by pre-exposing patients to the environmental cues that will be present during the chemotherapy session (Hall & Symonds 2006). More and better therapies await greater understanding of the

neurobiology of fear conditioning and fear extinction. In particular, understanding the molecular mechanisms of fear conditioning and extinction may provide therapeutic targets for drugs that can be used as adjuncts to the therapies described above.

The neurobiology of fear conditioning and fear extinction has been studied extensively. As this knowledge grows, one can begin to form and test hypotheses about the mechanisms that might be altered in anxiety disorders and develop interventions for those with anxiety disorders or others that suffer symptoms resulting from forming an association with aversive events (e.g., AVN). Currently, the neurobiological literature on fear conditioning and fear extinction includes work conducted at multiple levels of analysis and aimed at understanding the anatomical substrates as well as the cellular and molecular mechanisms that contribute to these processes.

**The anatomy of fear conditioning—amygdala and hippocampus:**

The amygdala is the hub of fear memory circuitry. Within the amygdala, there are 2 core nuclear groups: the basolateral amygdala (BLA; includes lateral nucleus, basolateral and basomedial nucleus) and central nucleus of the amygdala (CE). The BLA is the primary sensory input station of the amygdala (LeDoux *et al.* 1990a). CS information from the thalamus, hippocampus, and several cortical regions reach the BLA via glutamatergic projections.

The best-characterized pathway carrying information about the CS into the BLA is the thalamic pathway. The thalamic pathway contains fibers from the medial geniculate nucleus (MGN), the auditory relay nucleus of the thalamus. The MGN receives auditory information from the inferior colliculus, and lesions to either the MGN or inferior colliculus abolish fear conditioning to a simple auditory cue suggesting their importance in processing auditory CS information (LeDoux *et al.* 1984). Downstream recipients of MGN projections include the auditory cortex and many subcortical structures, including the amygdala. Whereas lesions to the amygdala disrupt conditioning to an simple auditory cue (Iwata *et al.* 1986), lesions to the subcortical structures to which the MGN projects or to the auditory cortex do not affect conditioning to simple auditory cues (LeDoux et al. 1984). Together, these studies suggest that auditory information critical for fear conditioning to simple auditory cues passes from the inferior colliculus to the MGN and then projects via the thalamic pathway to the BLA. The thalamic pathway is sufficient for *delay* fear conditioning to simple auditory cues such as a tone. “Delay” fear conditioning refers to conditioning in which the CS onset occurs briefly before the US onset and terminates at either the time of US onset or US termination. However, when the CS is more complex, the thalamic pathway is insufficient.

More complex CSs require greater processing and polysynaptic routes through cortical regions are necessary before CS information arrives at the BLA via the cortical pathway. In these cases, information about the environment in which the

shock is received during fear conditioning (i.e., contextual information) is more complex than a simple auditory tone and thus requires processing by the hippocampus and entorhinal cortex (Maren & Fanselow 1995, Anagnostaras *et al.* 1999) and perirhinal and postrhinal cortex (Amaral *et al.* 1992) before arriving at the BLA via the cortical pathway. Lesions to the ventral angular bundle (part of the cortical pathway from the hippocampus and entorhinal cortex), perirhinal cortex, and postrhinal cortex impair fear conditioning to context, but not simple auditiory cues (Bucci *et al.* 2000, Burwell *et al.* 2004, Maren & Fanselow 1995). When the ultrasonic distress call of a rat, rather than a simple tone, is used as the CS, information about the CS arrives at the BLA only after processing in the perirhinal cortex. Lesions to the perirhinal cortex impair conditioning to ultrasonic distress calls, but not simple auditory cues, further suggesting that complexity of the CS is a key determinant of the pathway by which CS information reaches the BLA (Lindquist *et al.* 2004). Another way to increase the complexity of the CS is to separate the CS from the US in time. That is, using a training protocol in which the CS terminates briefly before the US onset. Such a procedure is called “trace” conditioning (in contrast to “delay” conditioning described above). CS information in trace fear conditioning to a simple auditory cue requires processing by the hippocampus and anterior cingulate cortex. Lesions to the hippocampus disrupt trace fear conditioning to a simple auditory cue (McEchron *et al.* 1998). Lesions to the anterior cingulate cortex also disrupt trace fear conditioning to a simple auditory cue but spare context fear conditioning (Han *et al.* 2003).

Pain information representing the US (footshock) can arrive at the BLA from the posterior thalamus or insular cortex (Brunzell & Kim 2001, Shi & Davis 1999). Either of these routes is sufficient, but neither is necessary for fear conditioning. Combined lesions of both of these pathways impairs cue conditioning to tone, but context conditioning remains intact suggesting that there are alternative routes for US information to reach the BLA (Brunzell & Kim 2001).

Though the BLA is the major site of CS and US input to the amygdala, the CE also receives sensory input. An early tracing study suggested that the auditory portions of the thalamus project only to the BLA (LeDoux *et al.* 1990b). A more recent tracing study suggests that the CE may also receive projections from the auditory portion of the thalamus (Linke *et al.* 2000). Pain information representing the US (footshock) arrives at the CE from subcortical structures such as the parabrachial nucleus, nucleus of the solitary tract, and the dorsal horn of the spine (Benarroch 2001, Rami Burstein 1993, Gauriau & Bernard 2002). In addition to receiving some sensory inputs, the CE is the major output station of the amygdala. The CE projects to areas of the brainstem and hypothalamus that control the expression of fear responses. Lesions to the CE interfere with behavioral responses (freezing), autonomic (sympathetic and parasympathetic) responses (Kapp *et al.* 1979, LeDoux *et al.* 1988), stress hormone (ACTH and glucocorticoid) release (Feldman & Weidenfeld 1997), potentiation of somatic reflexes (Canli & Braown 1996, Rosen *et al.* 1991), and changes in pain reactivity elicited by a CS (Helmstetter 1992, Watkins *et al.* 1993).

The classic model of fear conditioning circuitry in the amygdala proposes a serial circuit. In this model, it is posited that CS and US information arrives in the BLA where it undergoes association via a long-term potentiation-like mechanism. Then, the associative signal is relayed to the CE, where the expression of fear behavior is directed. In this model, the BLA is the site of convergence and critical plasticity. Evidence for this model is substantial. First, the BLA receives sensory information about both the CS and US. In fact, Romanski and LeDoux (1993) showed that individual neurons in the BLA fire in response to presentation of both CS (clicks) and US (footshock). Second, lesions and temporary inactivation of the BLA during conditioning interferes with acquisition of conditioned fear (Phillips & LeDoux 1992, Maren *et al.* 2001, Wilensky *et al.* 1999). Third, BLA principal neurons exhibit LTP during fear conditioning (Rogan *et al.* 1997). Finally, disruption of molecular signaling mechanisms in the BLA prevents formation of long-term fear memories (Schafe *et al.* 2001). Key to this model is the assumption that there are direct projections from BLA to CE to brainstem. Unfortunately, there is no evidence that such projections actually exist (Pitkänen *et al.* 1997). Indeed, it is this fact that has led to the proposal of a new model, one in which the BLA and CE process information in parallel (Pare *et al.* 2004).

In this emerging model, the intercalated cell masses (ICMs) play a central role. ICMs are dense clusters of GABAergic neurons located between the BLA and CE (Millhouse 1986). ICM neurons receive glutamatergic inputs from the BLA

and generate feed-forward inhibition in the CE (Royer *et al.* 1999a). The end result is the facilitation of CE output by the BLA. In this way, the BLA disinhibits CE output neurons, enabling synaptic plasticity in the thalamic inputs into CE. This model posits that distributed plasticity in thalamic afferents to both the BLA and CE is required for fear conditioning. Like the earlier model, there is substantial evidence for this model. First, as described above, the CE likely receives information about both the CS and US. Second, blocking NMDA receptors (Gossens & Maren 2003) in the CE, or temporarily inactivating the CE during acquisition, blocks formation of long-term fear memories (Wilensky *et al.* 2000). Finally, CE is capable of mediating fear conditioning on its own. This is illustrated in a study in which rats with BLA lesions can still acquire conditioned fear after extensive overtraining (Maren 1999b), mediated by the CE (Zimmerman *et al.* 2007). This observation cannot be explained by the serial model of CS and US processing in the amygdala, but rather supports the parallel processing model where CS and US information is processed in both the BLA and CE.

In addition to the amygdala, the hippocampus is necessary for Pavlovian fear conditioning when the CS is stimuli that happen to be present when the fearful stimulus is experienced (contextual conditioning), The hippocampus is not necessary when simple, discreet CSs like a tone or light are used (Kim & Fanselow 1992, Phillips & LeDoux 1992). This is consistent with the view that the hippocampus plays an important role in situations in which interrelations among

multiple stimuli are important (O'Keefe & Nadel 1978). It has been suggested that the hippocampus is required for assembling the elemental cues within a particular training context into a configural representation that then comes into association with the foot shock in the amygdala (Fanselow 2000). Evidence in support of this idea comes from a study in which hippocampus lesion-induced deficits in contextual fear conditioning can be eliminated if pre-exposure to the context occurs one month prior to lesioning and conditioning (Young *et al.* 1994). Presumably, formation of a context-US association and contextual conditioning proceeds normally in this case because the contextual representation was encoded and consolidated prior to the hippocampal damage. Context was the CS used throughout the studies presented in this dissertation since one of the mouse models employed ($Ca_v1.3$ knockout mice) cannot hear and the use of auditory CSs would have been impossible in these mice (Platzer *et al.* 2000).

**Long-term potentiation—a cellular mechanism for fear conditioning:**

It is widely believed that memories are established by alterations of synaptic connections in the brain. How the brain accomplishes these changes is not well understood and is highly-debated. The leading candidate mechanism for fear memories is long-term potentiation (LTP) in the amygdala (Blair *et al.* 2001, Ki A. Goosens 2002, Maren 1999a). LTP is a persistent, activity-dependent increase in synaptic transmission. Two characteristics of LTP that make it an attractive mechanism for learning and memory are (1) co-operativity and (2) associativity. Co-operativity refers to the fact that a neuron must be sufficiently depoloarized

before LTP can be induced in its afferents (McNaughton *et al.* 1978). Co-operativity ensures that not every stimulus in the environment will lead to the formation of a memory trace. Associativity refers to the observation that pairing stimulation of a weak pathway with simulation of a strong pathway results in facilitation of synaptic transmission in both pathways (Kelso 1986). Associativity makes LTP suitable for encoding associations in the external world like that between CS and US. LTP was first described the hippocampus by Bliss and Lomo (Bliss & Lomo 1973). It has since been described in a number of brain regions, including the BLA (Bauer *et al.* 2002b, Chapman & Bellavance 1992, Chapman *et al.* 1990, Drephal *et al.* 2006, Maren & Fanselow 1995, Weisskopf *et al.* 1999) and CE (Samson & Pare 2005). According to the current hypothesis, fear conditioning is mediated by an increase in the strength of synapses that transmit CS information to the neurons that are the site of convergence with information about the US. This site of convergence would be the BLA in the case of the serial model (described above) of CS and US processing in the amygdala or both the BLA and CE in the case of the parallel model (described above). The hypothesis assumes that prior to conditioning, the CS inputs to these neurons of convergence are weak and unable to elicit a fear response. US inputs to these neurons of convergence, however, are stronger and capable of eliciting robust responses. During fear conditioning, the weak CS inputs arrive at the neurons of convergence while they are being strongly depolarized by the US inputs to the same neurons. As a result, the CS inputs become stronger and more capable of

driving the neurons of convergence and their downstream effectors such that the CS can elicit fear responses on its own.

**Changes in neuronal excitability—another hypothesized neurobiological substrate for learning and memory:**

Though it is widely believed that LTP is a mechanism by which fear memories are encoded and stored, other mechanisms in addition to LTP may also play a role. It has been hypothesized that changes in neuronal excitability are a mechanism that may participate in the enccoding and storing memories (Zhang & Linden 2003). Though no studies have addressed learning-induced changes in neuronal excitability in the amygdala, it has been studied extensively in other brain structures. In Chapter 4 of this dissertation, I measure neuronal excitability in the BLA of a mouse model that is impaired in the ability to consolidate fear memories ($Ca_v1.3$ knockout mice).

Experimentally, the afterhyperpolarization (AHP) and spike accommodation are used as measures of neuronal excitability. The AHP is a hyperpolarizing voltage deflection that follows a burst of action potentials and serves to limit firing to a sustained depolarizing input (Alger & Nicoll 1980, Hotson & Prince 1980, Madison & Nicoll 1984). The AHP is often described as having 3 components: a fast, medium and a slow AHP. The fast AHP (fAHP) occurs immediately after individual action potentials and lasts only 1-5 ms. The medium AHP (mAHP) is typically observed after a burst of action potentials and has a decay constant of approximately 100 ms. The slow AHP (sAHP) has a time constant of 1-5 seconds

and is voltage-independent (Faber & Sah 2007). Spike accommodation is a phenomena in which the firing frequencies of successive action potentials to a sustained depolarization decays to a steady state, in some cases the neuron may even stop firing (Disterhoft & Oh 2007). Learning-induced changes in neuronal excitability have been studied extensively in the hippocampus and, to a lesser extent, cortex. For example, training in hippocampal-dependent tasks increases excitability (reduced AHP and spike accommodation) of neurons in the CA1 region of the hippocampus in multiple species, including rabbits, rats, and mice (Moyer Jr *et al.* 1996, Oh *et al.* 2003, Ohno *et al.* 2006). Similarly, increased excitability of neurons in the piriform cortex is observed following odorant-discrimination learning (Saar *et al.* 1998) and neurons in the infralimbic prefrontal cortex exhibit decreased excitability (larger AHP and increased spike accommodation) after fear conditioning, an effect that is reversed by fear extinction (Santini *et al.* 2008). Additionally, neuronal excitability of CA1 neurons of the hippocampus decreases with age and correlates with age-related cognitive decline in performance on hippocampal-dependent learning and memory tasks (Disterhoft & Oh 2007). Manipulations that reverse the age-related decrease in neuronal excitability (Murphy *et al.* 2004, Oh *et al.* 1999) act to ameliorate the associated age-related cognitive impairments. Future study may demonstrate that this mechanism also functions in the amygdala to mediate fear memory.

**The molecular mechanisms of fear conditioning:**

Calcium influx seems to be a critical first step in the molecular signaling mechanism that mediates fear conditioning. Blockade of either NMDARs (Miserendino *et al.* 1990, Maren *et al.* 1996) or L-type voltage-gated calcium channels (L-VGCCs) (Bauer *et al.* 2002a) in the BLA dramatically impairs fear conditioning. The combined calcium signal provided by these two sources triggers multiple intracellular events including activation of protein kinase second messenger pathways.

The cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), calcium-calmoduliin-dependent kinase (CaMKII), and extracellular signal-regulated kinase/mitogen-activated protein (ERK/MAP) kinase have all been implicated in fear conditioning. Administration of PKA inhibitors to the cerebral ventricles (Bourtchouladze *et al.* 1998) or BLA (Schafe & LeDoux 2000) blocks fear conditioning. Transgenic mice that overexpress (R(AB)), and inhibitory isoform of PKA, exhibit deficits in fear conditioning (Abel *et al.* 1997). Infusion of a PKA/PKC inhibitor into the BLA, but not CE, impairs fear conditioning (Goosens *et al.* 2000). Transgenic mice expressing a mutant form of CaMKII exhibit fear conditioning deficits (Mayford *et al.* 1996). PKA, PKC, and CaMKII are all known to converge on extracellular signal-regulated kinase/mitogen-activated protein (ERK/MAP) kinase signaling pathway (Adams & Sweatt 2002). Not surprisingly, ERK/MAP pathway activation is required for acquisition of fear memory (Schafe *et al.* 2000).

Activated kinases translocate to the nucleus, where they phosphorylate cAMP response element-binding protein (CREB). CREB binds to cAMP-response elements (CREs) in regulatory regions of target genes and stimulates transcription. The final step of this cascade is transient activation of genes involved in synthesis of proteins essential for modification of preexisting synapses and formation of new ones. Mice with disruption of the $\alpha$ and $\delta$ isoforms of CREB exhibit robust impairment in contextual and auditory fear conditioning (Bourtchouladze *et al.* 1994). It is interesting that many of the molecules that mediate fear conditioning also mediate BLA-LTP.

**The anatomy of fear extinction: basolateral amygdala and prefrontal cortex:**

Compared with fear conditioning, much less is known about the neurobiology of fear extinction, but knowledge in this area is rapidly expanding. The anatomy of fear extinction is one area in which considerable progress has been made. Many structures have been identified which seem to play some role in fear extinction, including sensory cortex (Armony *et al.* 1998, Falls & Davis 1993, Quirk *et al.* 1997), periaqueductal gray (McNally 2005, McNally & Westbrook 2003), inferior colliculus (Heldt & Falls 2003), lateral septum (Yadin & Thomas 1996, Yadin & Thomas 1981, Yadin *et al.* 1993), bed nucleus of stria terminalis (Waddell *et al.* 2006), and ventral and dorsal striatum (Josselyn *et al.* 2004). Three structures are particularly well studied: the amygdala, hippocampus, and prefrontal cortex. I will review the role of these three structures in fear extinction.

Evidence suggesting a role for the amygdala in fear extinction comes from infusion and *in vivo* electrophysiology studies. Lesion studies examining the contribution of the amygdala to extinction are problematic because, as described above, lesions of the lateral amygdala or central nuclei eliminate expression of conditioned fear. Clearly, it is impossible to study the extinction of fear responses that are no longer expressed.

The first data implicating the BLA in fear extinction came from a study in which the NMDA antagonist 2-amino-5-phophonopentanoic acid (APV) was infused into the BLA in rats prior to fear extinction training (Falls *et al.* 1992). When retention of fear extinction was tested off-drug one day later, fear extinction was blocked in a dose-dependent manner. In a related experiment, the NMDA agonist d-cycloserine infused to the BLA prior to fear extinction training facilitated fear extinction (Walker *et al.* 2002). These experiments, however, did not assess within-session extinction and therefore did not establish whether the effect of NMDA receptor manipulation was on acquisition or consolidation of extinction. Subsequently, local blockade of NMDA receptors in the BLA was shown to impair acquisition of extinction (Sotres-Bayon *et al.* 2007). Blockade of other molecules in the BLA, including MAPK (Cyril Herry 2006) and metabotropic glutamate receptors (Kim *et al.* 2007), interfere with acquisition of extinction. Consolidation of extinction is dependent on protein synthesis in the BLA (Lin *et al.* 2003b) and interference with the kinases MAPK (Lu *et al.* 2001) and PI-3 kinase, (Lin et al.

2003b) orthe phosphatase calcineurin (Lin *et al.* 2003a)—all in the BLA—prevents consolidation of extinction.

*In vivo* electrophysiology studies also implicate the BLA in fear extinction. In one study, short latency (< 20 ms after tone onset) neuron spiking in the BLA was increased by fear conditioning and eliminated by fear extinction (Quirk *et al.* 1995). A follow up study by the same group showed that there may be two groups of neurons in the BLA: one that exhibits CS-related activity at the beginning of extinction training and then stops, and one that continues to fire, though at a decreased level, throughout extinction training (Repa *et al.* 2001). Hobin et al. (2003) recorded BLA neurons several days after extinction. They found that when extinction retention was tested in the same context in which extinction training was performed, BLA neurons exhibited very little CS-related firing. However, when retention testing was done in another context, there was robust CS-related firing. These results highlight the context dependency of extinction and represent a cellular correlate of renewal. Together, the infusion study data and *in vivo* electrophysiology studies made a strong argument for the role of the BLA in fear extinction.

The hippocampus also seems to be important for fear extinction. As fear extinction is a context-dependent phenomenon, this is not surprising. As mentioned above, fear responses can be uncovered following extinction by presenting unsignaled USs in the same context in which training occurred

(reinstatement) (Rescorla & Heth 1975), or by altering the test context (renewal) (Bouton & Bolles 1979). The context modulation of fear extinction is dependent on the hippocampus. For example, lesions to the hippocampus or fimbria-fornix prior to fear conditioning abolish reinstatement (Wilson *et al.* 1995, Frohardt *et al.* 2000).

The role of the hippocampus in renewal is more complex. Permanent lesions to the hippocampus or fimbria-fornix prior to fear conditioning have produced inconsistent effects (Ji & Maren 2005, Wilson et al. 1995, Frohardt et al. 2000), but temporary inactivation of the hippocampus with the $GABA_A$ receptor agonist muscimol yields more consistent results. When the renewal context is different from the context in which extinction training occurred (i.e., AAB or ABC design) and muscimol is infused before the retention test, no renewal is observed. That is, freezing does not return in any context. This observation has been interpreted as meaning that the inactivation of the hippocampus prior to the retention test frees extinction from contextual gating. However, when the renewal context is the same as the conditioning context (i.e., an ABA design), muscimol infusions to the hippocampus prior to the retention test have no effect (Corcoran *et al.* 2005). That is, renewal is observed. The findings suggest that the mechanism for ABA renewal is different from that of AAB and ABC renewal.

Temporary inactivation of the hippocampus with muscimol during extinction training leads to high levels of CS-associated freezing during the retention test no

matter which context is used during the retention test (Corcoran *et al.* 2005). There are three possible interpretations of this observation. The first and most interesting is that the high levels of freezing during the retention test represent renewal. That is, because the hippocampus was unable to encode the context during extinction training, it recognizes any context as a different context when hippocampus function is restored and renewal results as with any context shift. Second, it may reflect state dependency of the extinction effect since training occurred “on-drug” and the retention test occurred “off-drug.” Finally, the high levels of freezing to CS in all contexts may simply reflect a disruption of extinction consolidation by muscimol.

A number of studies have implicated the medial prefrontal cortex (mPFC), generally, and the infralimbic (IL) cortex sub-region, specifically, in retention and/or expression of fear extinction. Evidence for a role for the mPFC in fear extinction is manifold and includes the following. First, fear extinction induces LTP in the mPFC and interference with this LTP impairs fear extinction (Farinelli *et al.* 2006, Sandrine Hugues 2006, Garcia *et al.* 2006, Herry & Garcia 2002, Herry & Garcia 2003, Herry *et al.* 1999). Second, unit recordings show increased CS-related activity during fear extinction (Milad & Quirk 2002). Third, microstimulation of the mPFC during extinction training reduces conditioned freezing and facilitates later retention of extinction (Milad *et al.* 2004). Fourth, immunocytochemistry shows increased expression of immediate early genes within mPFC following extinction training (Herry & Mons 2004, Santini *et al.*

2004). Finally, infusion of a protein synthesis inhibitor into the mPFC before extinction training (Santini et al. 2004) or a MAPK inhibitor immediately after extinction (Hugues *et al.* 2004) impairs extinction retention tested later in the absence of the drug. Despite all this evidence, there are studies that did not find a deficit in fear extinction after making lesions to the mPFC (Gewirtz *et al.* 1997, Garcia et al. 2006).

Evidence suggests that the mPFC may mediate its effect on fear extinction by modulating amygdala activity. The IL cortex projects to BLA, CE, and the ICMs .(Sesack *et al.* 1989, Robert 2004) Stimulation of mPFC inhibits CE unit reponses to afferent stimulation (Quirk *et al.* 2003). The effect of mPFC stimulation upon BLA activity seems to be primarily inhibitory (Rosenkranz & Grace 2002, Rosenkranz *et al.* 2003), but one group reports that it is excitatory (Likhtik *et al.* 2005). The ICMs appear to be activated upon stimulation of the mPFC stimulation. This has not been ascertained directly, but activation of ICM is suggested by increased ICM expression of Fos following injections of a $GABA_A$ receptor antagonist into the IL cortex (Berretta *et al.* 2005). As ICMs are primarily GABA-ergic (L. Nitecka 1987), receive projections from the BLA and project to the CE (Millhouse 1986, Pare & Smith 1993), modulate CE excitability (Royer *et al.* 1999b), and exhibit synaptic plasticity (Royer et al. 1999a), it has been proposed that mPFC may modulate ICM activity either directly or indirectly (via the BLA) and thereby gate amygdala throughput and suppress triggering of fear responses in extinction (Pare et al. 2004).

**LTP and neuronal excitability in mediating fear extinction:**

Evidence suggests, as with fear conditioning, that LTP is part of the mechanism of fear extinction. Above, I mentioned the appearance of LTP in the mPFC after fear extinction and that disrupting LTP impairs fear extinction. I described how LTP of afferents to the ICMs might participate in fear extinction. Additionally, it has recently been reported that changes in neuronal excitability occur in the IL cortex after Pavlovian fear conditioning and fear extinction (Santini et al. 2008). Neurons in the IL cortex become less excitable after fear conditioning, an effect that is reversed by fear extinction. The roles of LTP and neuronal excitability in mediating fear extinction are not well understood, but these early data suggest both processes might be important.

**The molecular mechanisms of fear extinction:**

Much of what is known about the molecular mechanisms of fear extinction was discussed above in the section that describes infusion studies in the BLA. There is evidence that endocannabinoids are involved in fear extinction, but they were not discussed because their role has not been localized to the BLA. Cannabinoid receptor type 1 (CB1 receptor)-deficient mice are impaired in both short-term and long-term extinction of cue-conditionied fear. Systemic injection of the CB1 receptor antagonist SR14176 blocks both short-term and long-term extinction of cue- (Marsicano *et al.* 2002) and contextually–conditioned fear (Suzuki *et al.* 2004) . Importantly, neither CB1 receptor-deficient mice nor the CB1 receptor

antagonist- treated mice are impaired with respect to acquisition or consolidation of conditioned fear.

Another class of molecules for which there is evidence for a role in fear extinction is L-VGCCs. L-VGCCs were not discussed in the earlier section because their role has not been localized to the BLA. Previous studies implicating L-VGCCs in fear extinction have relied on systemic administration of L-VGCC antagonists. The L-VGCC antagonists nifedipine and nimodipine block both short-term, within-session extinction (i.e., acquisition of extinction) and long-term fear extinction (i.e., retention of extinction) when administered systemically prior to extinction training (Cain *et al.* 2002, Suzuki et al. 2004). Together with the evidence above for the role of NMDA receptors in fear extinction, this data suggests that calcium influx is critical for fear extinction. It is likely that this calcium influx is the first step in a signaling cascade. Indeed, as described above, the inhibition of a number of signaling molecules downstream of calcium influx blocks fear extinction.

**A molecular genetic approach to understanding L-VGCCs in fear conditioning, fear extinction, and amygdala function:**

On initial review of the literature relevant to fear conditioning and fear extinction, I was intrigued that L-VGCCs had been implicated in both the consolidation phase of fear conditioning and fear extinction. Absent from this literature was an information regarding which of the brain-expressed L-VGCC sub-types mediate these phenomena, because all previous investigations used L-VGCC antagonists that block all subtypes of L-VGCCs. I thought that a molecular

genetic approach could provide subtype-specific information on the role of L-VGCCs in fear conditioning and fear extinction. This dissertation describes my investigation into the specific roles of the two brain-expressed L-VGCCs, $Ca_v1.2$ and $Ca_v1.3$, in fear conditioning and fear extinction using genetically modified mice. I also address the role of $Ca_v1.3$ in neurophysiological function of the BLA, a region critical for both fear conditioning and fear extinction.

To assess $Ca_v1.3$ function, I used $Ca_v1.3$ knockout (KO) mice. In these mice, the gene for the pore-forming subunit of the $Ca_v1.3$ calcium channel ($\alpha_{1D}$) has been deleted by insertion of a neomycin cassette into exon 2 which results in a complete null mutation in all cells throughout development and in adulthood (Platzer et al. 2000). These mice have been demonstrated to have cardiac, endocrine, and hearing deficits (Platzer et al. 2000, Namkung *et al.* 2001). The cardiac phenotype of $Ca_v1.3$ knockout mice includes resting bradycardia and arrhythmia (Platzer et al. 2000). Body weight in these mice is decreased relative to their wild-type littermates, likely reflecting impaired pancreatic function. Beta-cell death and a decreased number of pancreatic islets of Langerhan's is observed along with hypoinsulinemia and impaired glucose tolerance in adult $Ca_v1.3$ knockout mice (Namkung et al. 2001). Additionally, these mice appear to be deaf due to inner hair cell dysfunction and cochlear sensory cell degeneration (Platzer et al. 2000). Neurological function in $Ca_v1.3$ knockout mice, however, has been shown to be largely normal. Thermal and mechanical nocioceptive thresholds are unaltered in these mice, as is the ability to exhibit NMDA-receptor

dependent and NMDA-receptor independent long-term potentiation in the CA1 region of the hippocampus (Clark *et al.* 2003).

Because $Ca_v1.2$ is critical for mouse development, mice in which the gene for $Ca_v1.2$ is deleted in all cells throughout development and adulthood could not be used to assess $Ca_v1.2$ function. Instead, mice in which the gene for $Ca_v1.2$ was deleted postnatally in alpha calcium calmodulin kinase II ($\alpha$CaMKII) expressing neurons of the forebrain was used to assess $Ca_v1.2$ function. These mice were the generated using the loxP-Cre recombination system. In this system, DNA *flanked* by *loxP* sites (aka 'floxed') is deleted by the enzyme Cre recombinase. This system, has been used to generate mice with mutations limited to certain cell types (tissue-specific knockout) or mice with mutations that can be activated by drug administration (inducible knockout) (Sauer 1998). Experimental mice were generated by crossing mice heterozygous for the floxed $Ca_V1.2$ gene ($Ca_V1.2^{f/+}$ mice) with transgenic mice that expressed Cre-recombinase (Chen et al. 2006) driven by the $\alpha$CaMKII promoter. Alpha-CaMKII reaches peak expression levels postnatally (Sugiura & Yamauchi 1992, Kelly *et al.* 1987) and is restricted glutamatergic neurons of the forebrain (Chen et al. 2006). Offspring from this cross that were heterozygous floxed and Cre positive (i.e. $Ca_V1.2^{f/+}$, CaMK-$Cre^{Cre/+}$) were then intercrossed (non-sibling) with mice heterozygous floxed and Cre negative (i.e. $Ca_V1.2^{f/+}$, CaMK-$Cre^{+/+}$) to achieve the following genotypes: conditional knockout mice ($Ca_V1.2^{f/f}$, CaMK-$Cre^{Cre/+}$); wild-type mice ($Ca_V1.2^{+/+}$, CaMK-$Cre^{+/+}$); floxed controls ($Ca_V1.2^{f/f}$, CaMK-$Cre^{+/+}$) and Cre

controls ($Ca_V1.2^{+/+}$, CaMK-$Cre^{Cre/+}$) all on an F2 129Sve:C57Bl/6 hybrid background. For ease of reading, conditional knockout mice ($Ca_V1.2^{f/f}$, CaMK-$Cre^{Cre/+}$) are referred to as $Ca_V1.2^{cKO}$ throughout the remainder of the dissertation. These mice have been previously been demonstrated to be impaired in the ability to consolidate remote spatial memories (White *et al.* 2008). Using a strain of mice in which the gene for $Ca_v1.2$ was homozygously deleted in the forebrain and heterozygously deleted in the rest of the body, Moosmang et al. (2005) found that $Ca_v1.2$ is important for hippocampus function. These mice were impaired on two different hippocampus-dependent learning tasks and NMDA receptor-independent LTP in the CA1 region of the hippocampus. The MAPK signaling pathway and CREB-mediated transcription were also disrupted in CA1 pyramidal neurons isolated from these mice (Moosmang *et al.* 2005).

I demonstrate that $Ca_v1.3$ knockout mice mice are impaired with respect to consolidation of contextually-conditioned fear. However, they can extinguish contextually-conditioned fear as effectively as wild-type mice and exhibit no other neurological abnormalities. Results are presented in Chapter 2 and were published in *Learning and Memory* (McKinney & Murphy 2006).

Next, I studied $Ca_V1.2^{cKO}$ mice. Surprisingly, I found that these mice were capable of consolidating and extinguishing conditioned fear as effectively as wild-type mice. I concluded that neither of the brain-expressed L-VGCCs are required for fear extinction. On further investigation, I demonstrated that previous studies

were likely confounded by an aversive state induced by L-VGCC antagonists. These findings appear in Chapter 3 and were published in *Learning and Memory* (McKinney *et al.* 2008).

Finally, I assessed field potential LTP in the BLA following high-frequency stimulation of external capsule fibers and used whole-cell patch clamp to assess excitability of BLA principal neurons by measuring the AHP and spike accommodation. I found that $Ca_v1.3$ KO mice exhibit impaired LTP and enhanced neuronal excitability. These data are presented in Chapter 4 and are currently in preparation for journal submission. It is my hope that these findings have contributed to the understanding of the neurobiology of fear, fear conditioning, and fear extinction. Ultimately, I hope these findings will lead to a better understanding of the neurobiological substrates that underlie anxiety disorders, and contribute to improved treatments for those that suffer from them.

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## Chapter II

## The L-type voltage-gated calcium channel $Ca_v1.3$ mediates consolidation, but not extinction, of contextually-conditioned fear in mice

**Abstract:**

Using pharmacological techniques, it has been demonstrated that both consolidation and extinction of Pavlovian fear conditioning are dependent to some extent upon L-type voltage gated calcium channels (LVGCCs). Although these studies have successfully implicated LVGCCs in Pavlovian fear conditioning, they do not provide information about the specific LVGCC isoform involved. Both of the major LVGCC subtypes found in the brain ($Ca_v1.2$ and $Ca_v1.3$) are targets of the pharmacological manipulations used in earlier work. In this study, we employed mice in which the gene for the pore-forming subunit ($\alpha_{1D}$) of $Ca_v1.3$ was deleted ($Ca_v1.3$ knockout mice) to elucidate its contribution to consolidation and extinction of conditioned fear. We find that the $Ca_v1.3$ knockout mice exhibit significant impairments in consolidation of contextual fear conditioning. However, once sufficiently over-trained, the $Ca_v1.3$ knockout mice exhibit rates of extinction that are identical to that observed in wild-type mice. We also find that $Ca_v1.3$ knockout mice

perform as well as wild-type mice on the hidden platform version of the Morris water maze, suggesting that the consolidation deficit in conditioned fear observed in the $Ca_v1.3$ knockout mice is not likely the result of an inability to encode the context, but may reflect an inability to make the association between the context and the unconditioned stimulus.

**Introduction:**

In Pavlovian fear conditioning, pairing a conditional stimulus (CS) with an aversive unconditional stimulus (US) results in a conditioned fear response. A fear response is said to be contextually-conditioned when it is elicited by the context in which the US was delivered. In this case, the context serves as the CS (for recent review see (Fanselow & Poulos 2005)). After contextual fear conditioning, extended exposure to the context in the absence of the US results in reduced probability and amplitude—or extinction—of the conditioned response. Both consolidation and extinction of conditioned fear have been demonstrated to be critically dependent on the amygdala (for review see (Maren 2003).

Recently, many studies have investigated the molecular basis of fear conditioning and its extinction in rodents. Molecules involved in synaptic plasticity within the amygdala have been of particular interest. In light of evidence that a form of long-term potentiation (LTP) that depends on L-type voltage-gated calcium channels (LVGCCs) exists in the amygdala (Weisskopf *et*

*al.* 1999), multiple groups have explored the role of LVGCCs in fear conditioning and its extinction. In rats, blockade of LVGCCs in the lateral amygdala impairs consolidation of auditory conditioned fear (Bauer *et al.* 2002). Systemic blockade of LVGCCs in mice, however, does not impair the acquisition, consolidation, or expression of conditioned fear, but rather its extinction (Cain *et al.* 2002, Suzuki *et al.* 2004). Demonstration that infusions into the basolateral amygdala of an LVGCC antagonists block, whereas infusions of an LVGCC agonist facilitate, extinction of conditioned fear in mice provides additional support for the importance of LVGCCs in extinction of conditioned fear (Barad 2005).

Thus far, studies of the role of LVGCCs in fear conditioning and its extinction have relied solely on pharmacological techniques. Though these techniques are powerful in elucidating a prominent role for LVGCCs in conditioned fear, they do not allow for identification of the specific LVGCCs involved. There are two major subtypes of brain LVGCCs, $Ca_v1.2$ and $Ca_v1.3$, both of which are the targets of pharmacological manipulations. In this study, we employ mice in which the gene for the pore-forming subunit ($\alpha_{1D}$) of the $Ca_v1.3$ L-VSCC subtype has been deleted ($Ca_v1.3$ knockout mice; a generous gift from D. James Surmeier, Northwestern University) to elucidate its contribution to consolidation and extinction of contextually-contextual conditioned fear.

## Results:

### *General Neurological Screen*

It has been previously reported $Ca_V1.3$ homozygous knockout mice are by and large neurologically normal (Clark *et al.* 2003). Similarly, we found that deletion of $Ca_V1.3$ does not result in significant impairments in vestibular function, spontaneous locomotor activity, or anxiety-like behavior. $Ca_V1.3$ KO mice (n = 6) were not significantly different when compared to WT (n = 6) mice on the rotarod **(Figure 2-1A)** as both groups exhibited significant improvement in performance as training progressed ($F_{(1,10)} = 27.1$, $p < 0.05$ for effect of training) and there was no significant effect of genotype ($F_{(1,10)} = 2.35$, $p > 0.05$). We did not find any significant difference between the $Ca_V1.3$ KO mice (n = 6) and their WT littermates (n = 5) in the open field **(Figure 2-1B)**. The total distance traveled by the $Ca_V1.3$ KO mice (702 ± 215 cm) was not significantly different when compared to WT mice (612 ± 118 cm; $t_{(9)} = .35$, $p > 0.05$). In addition, we quantified the ratio of distance spent in the center of the open field as a function of the total distance traveled **(Figure 2-1C)** as a measure of anxiety-like behavior (Crawley 1999). The ratio of center distance to total distance traveled in the open field did not differ between $Ca_V1.3$ KO mice (center: total ratio 0.426 ± 0.159) and WT mice (0.351 ± 0.127; $t_{(9)} = 0.36$, $p > 0.05$) suggesting that deletion of $Ca_V1.3$ did not affect anxiety-like behavior. However in contrast to the earlier report by Clark et al, we did not find any significant difference in body weight in the $Ca_V1.3$ KO mice (N = 7; 18.3 ± 0.83 g) when compared to WT littermates (N = 6; 19.3 ± 0.72; $t_{(9)} = 0.82$, $p > 0.05$) **(Figure 2-1D).**

***$Ca_V$1.3 KO mice are impaired on consolidation of contextually-conditioned fear***

As outlined above, pharmacological blockade of L-type calcium channels disrupts consolidation of conditioned fear (Bauer et al. 2002); therefore we sought to determine the relative contribution of the $Ca_V$1.3 isoform during the consolidation of conditioned fear. For these experiments three separate groups of $Ca_V$1.3 KO mice and their WT littermate controls were conditioned with a single unsignaled footshock (see Methods and Materials). Results are shown in **Figure 2-2**. During conditioning, neither $Ca_V$1.3 KO nor WT mice in any of the three groups froze significantly during the 3 minutes preceding the shock. Each group was returned to the conditioning chambers at a different time point after conditioning. Mice returned to the conditioning chambers 1 hour (N = 8 for KO and WT) or 6 hours (N = 8 for KO and WT) after conditioning exhibited significant freezing compared to before conditioning ($F_{(1,14)}$ = 27.8, $p$ < 0.05 and $F_{(1,14)}$ = 25.4, $p$ < 0.05 respectively for effect of conditioning), however there was no effect of genotype at either the 1 hour ($F_{(1,14)}$ = 2.2, $p$ > 0.05; **Figure 2-2A**) or 6 hour ($F_{(1,14)}$ = 1.4, $p$ > 0.05; **Figure 2-2B**) time point. Similarly, mice returned to the conditioning chambers 24 hours (N = 8 & 11 for KO and WT respectively) after conditioning exhibited significant freezing ($F_{(1,17)}$ = 64.5, $p$ < 0.05 for effect of conditioning) compared to before conditioning. However, at the 24 hour time point, the $Ca_V$1.3 KO mice exhibited significantly less freezing (14.9 ± 4.2 %) when compared to their WT littermates (37.9 ± 4.6 %; **Figure 2-2C**) with a significant main effect of genotype ($F_{(1,17)}$ = 12.2, $p$ < 0.05) and training x

genotype interaction ($F_{(1,17)}$ = 13.1, $p < 0.05$). It is important to note that deletion of $Ca_V1.3$ had no effect on postshock freezing measured for 30 seconds after the footshock in the 24 hour group. Both KO and WT mice exhibited substantial post shock freezing (20.9 ± 9.2% for KO and 26.7 ± 6.3% for WT; $F_{(1,17)}$ = 19.0, $p < 0.05$ for effect of training), however, there was no main effect of genotype on post-shock freezing ($F_{(1,17)}$ = 0.2, $p > 0.05$) and the effect of the shock did not interact with genotype ($F_{(1,17)}$ = 0.3, $p > 0.05$) suggesting that foot shock sensitivity and US processing were not disrupted in KO mice.

The absence of an effect of genotype immediately following the shock or at 1 hour and 6 hours after training suggests that acquisition of contextually-conditioned fear is intact in $Ca_V1.3$ KO mice. However, that an effect of genotype arises 24 hours after training argues that $Ca_V1.3$ KO mice are impaired with respect to consolidation of contextually-conditioned fear.

We also obtained similar results with mice in a C57BL/6 genetic background, however the deficit was somewhat more pronounced. After 2 days of context conditioning (1 unsignaled shock per day, as above) both the $Ca_V1.3$ KO (n = 8) and WT mice (n = 11) exhibited an increase in conditioned fear on day 3 ($F_{(2,34)}$ = 25.9, $p < 0.05$ for the effect of training). However, the average percent time freezing in the $Ca_V1.3$ KO mice after 2 days of training (13.8 ± 2.8 %) was significantly less than that observed for the WT mice (33.5 ± 7.4 %). A repeated measures ANOVA revealed a significant effect of genotype ($F_{(1,17)}$ = 5.0, $p <$

0.05) and a significant interaction between genotype and training ($F_{(2,34)} = 4.2$, $p < 0.05$). Thus in 2 different genetic backgrounds, deletion of the $Ca_V1.3$ pore-forming subunit results in disrupted contextual fear conditioning. These results are consistent with previous experiments demonstrating that L-type calcium channel antagonists disrupt consolidation of conditioned when infused directly into the lateral amygdala (Bauer et al. 2002).

***Extinction of contextually-conditioned fear is normal in $Ca_v1.3$ KO mice***

Our preliminary experiments suggested that the impairment in consolidation of contextually-conditioned fear exhibited by the $Ca_V1.3$ KO mice in the F2 hybrid background could be overcome by additional training. Thus, we used a separate group of mice to test extinction of contextually-conditioned fear (**Figure 2-3**). For this experiment, $Ca_V1.3$ KO mice (N = 17) and WT mice (N = 20) were conditioned as before, but an additional day of training was administered so that extinction learning could be examined (see Methods and Materials). On the following day approximately one half of the mice (9 KO mice and 12 WT mice) were returned to the conditioning chambers for a 30 minute extinction session and contextually-conditioned fear was assessed by measuring freezing throughout the session. The remaining mice (8 KO mice and 8 WT mice) were not re-exposed to the context (retention control groups). Neither $Ca_V1.3$ KO nor WT littermates froze significantly during the 3 minutes prior to the shock on day

one (**Figure 2-3A**). Consistent with our previous results, $Ca_V1.3$ KO exhibited significantly less freezing than WT littermates on day 2, 24 hours following a single training trial ($F_{(1,35)} = 4.3$, $p < 0.05$; Fig. 3A). However, $Ca_V1.3$ KO and WT littermates exhibited similar levels of freezing during the first 3 minutes of context exposure on day 3, 24 hours after a second training trial ($F_{(1,19)} = 0.05$, $p > 0.05$; Fig. 3A). This confirmed that the additional training was sufficient for $Ca_V1.3$ KO mice to overcome their impaired ability to consolidate contextually-conditioned fear after a single training trial. Across the 30 minute extinction session, both the $Ca_V1.3$ KO and WT mice exhibited significant extinction of contextually-conditioned fear ($F_{(1,19)} =53.2$, $p < 0.05$ for the effect of CS exposure) with the $Ca_V1.3$ KO mice freezing on average 21.1 ± 4.0 % during the last 10 minutes of CS exposure while the WT mice similarly froze on average 23.3 ± 5.6% during the same time interval (**Figure 2-3B**). This observation coupled with the fact that there was no significant difference between genotypes during the session ($F_{(1,19)} = 0.51$, $p > 0.05$) demonstrates that the $Ca_V1.3$ L-type calcium channel isoform is not required for short-term extinction of conditioned fear. Twenty-four hours later, all of the mice were returned to the conditioning chambers for a 5-minute exposure to the context (**Figure 2-3C**). $Ca_V1.3$ KO mice in the retention control group exhibited robust freezing (46.4%) as did their wild-type littermates (48.9%). In contrast, all mice in the extinction group froze significantly less with the $Ca_V1.3$ KO mice freezing only 15.4 % of the time, while the WT mice froze slightly more at 26.4 %. A two-way ANOVA revealed a significant effect of group ($F_{(1,33)} = 16.9$ $p < 0.05$) with no significant effect of genotype ($F_{(1,33)} = 1.1$ $p >$

0.05). Taken collectively, these data demonstrate that $Ca_V1.3$ is not involved in short or long-term extinction of conditioned fear.

***$Ca_V$1.3 KO mice are not impaired on the hippocampus-dependent version of the Morris water maze***

There are numerous reports demonstrating that the dorsal hippocampus is critically involved in contextually conditioned fear (e.g. (Daumas *et al.* 2005, Kim & Fanselow 1992)) whereas cued conditioning (where the CS is a tone for example) is thought to be critically dependent on the amygdala but less dependent on the hippocampus (see (Fanselow & Poulos 2005) for recent review). Because the $Ca_V1.3$ KO mice are deaf (Platzer *et al.* 2000), tone-cued fear conditioning could not be used as a means to dissociate which anatomical location was impacted by the deletion of $Ca_V1.3$. Instead, we sought to assess hippocampal function in the $Ca_V1.3$ KO mice by examining their ability to encode spatial information in the Morris water maze (MWM).

Both $Ca_V1.3$ KO mice (N = 7) and WT littermates (N = 9) were trained 2 trials a day for 6 days on the hidden-platform version of the MWM. Acquisition data is shown in **Figure 2-4A**. The latency to reach the platform in both groups decreased as training progressed reaching an average escape latency of 17.5 ± 3.2 sec for the $Ca_V1.3$ KO mice and 21.6 ± 3.5 sec for the wild-types. There was a main effect of training day on the latency to find the hidden platform ($F_{(5,70)}$ = 10.9 $p < 0.05$) however there was no main effect of genotype on latency ($F_{(1,14)}$ =

0.13; $p > 0.05$) and no interaction between training day and genotype ($F_{(5,70)} = 0.77$; $p > 0.05$). These data suggest that $Ca_V1.3$ KO and WT mice acquire the hidden-platform version of the MWM equally well.

In addition to measuring latency to platform during the training trails a probe trial was conducted 24 hours after completion of the last training trial on day 6. Probe trial data are shown in **Figure 2-4B**. During the probe trial, the $Ca_V1.3$ KO mice spent significantly more time (45 ± 5.5%) in the quadrant where the platform was previously located (training quadrant TQ in **Figure 2-4B**) than would be predicted by chance ($t_{(8)} = 3.7$ $p < 0.05$ single group t-test compared to 25%). Similarly, the WT mice spent the majority of the probe trial (39.9 ± 4.1%) selectively searching in the training quadrant ($t_{(8)} = 3.6$ $p < 0.05$ single group t-test compared to 25%). However there was no significant difference in the amount of time that the $Ca_V1.3$ KO mice spent in the training quadrant compared to their WT littermates ($t_{(14)} = 0.8$; $p > 0.05$ unpaired t-test). In addition, these same mice were tested in a non-spatial, non-hippocampal dependent version of the Morris water maze in which the escape platform was clearly marked with a small flag. When the platform was marked in this manner, both groups found the platform with minimal latencies and exhibited comparable swim speeds (**Figure 2-4C**). These results demonstrate that the hippocampal function in the $Ca_V1.3$ KO mice is not overtly disrupted and therefore suggest that the deficits observed in the fear conditioning experiments are likely the result of impaired functioning in the amygdala.

**Discussion:**

The principal finding of the present study is that $Ca_v1.3$ KO mice are impaired in their ability to consolidate contextually-conditioned fear. In addition, we find that deletion of the $Ca_V1.3$ gene does not alter extinction of contextually-conditioned fear in these mice. These results indicate that the L-type calcium channel $Ca_v1.3$ is critical for normal consolidation, but not extinction, of contextually conditioned fear. These results are to our knowledge the first demonstration of an isoform specific role for L-type calcium channels in Pavlovian conditioned fear. In our hands these mice appear to have normal weight gain and normal performance on the rota-rod and in the open field. In addition, we found no significant difference between $Ca_V1.3$ KO mice and their wild-type littermates in the visible platform version of the Morris water maze. Taken collectively, these data are consistent with a previous study (Clark et al. 2003) which found that the $Ca_V1.3$ KO mice were neurologically normal and strongly suggest that the deficit in consolidation of contextually-conditioned fear is not the result of gross neurological impairment.

Consistent with the results of the present study is the demonstration in rats that blockade of LVGCCs in the lateral amygdala impairs acquisition of long-term auditory conditioned fear (Bauer et al. 2002). The results of this study, however, are inconsistent with those from a study in which systemic blockade of LVGCCs did not block consolidation of conditioned fear (Cain et al. 2002). There are a few

issues that may account for the inconsistency. First, it is difficult know the degree to which LVGCC activity in general, and $Ca_v1.3$ activity in particular, were blocked by the various LVGCC antagonists used in Cain et al. 2002. In heterologous expression systems, dihydropyridine antagonists like nifedipine and nimodipine are significantly less efficient at blocking $Ca_v1.3$ currents than $Ca_v1.2$ currents (Koschak *et al.* 2001, Xu & Lipscombe 2001). It is possible that the residual activity resulting from incomplete blockade of $Ca_v1.3$ would be sufficient to allow for consolidation of conditioned fear. Second, differences in training protocol may account for these apparent inconsistencies. The training protocol used by Cain and colleagues (Cain et al. 2002) involved one session in which 5 shocks were administered and thus was quite intense. This is in contrast to the single shock used in the present study. Our conditioning protocol produces a more gradual learning curve and may be more sensitive in revealing subtle changes in learning produced by $Ca_v1.3$ deletion. Others have used similar conditioning protocols to examine subtle effects on acquisition of conditioned fear (Young & Fanselow 1992). The number of conditioning trials seems particularly important in light of the fact that, in our experiments, additional training trials are sufficient to overcome the impairments in acquisition of conditioned fear observed after a single training trial. Further supporting the importance of training trials is the observation that overtraining can mitigate the detrimental effects of basolateral amygdala lesions on acquisition of contextual fear conditioning in rats (Maren 1998, Maren 1999).

Acquisition of contextually-conditioned fear is thought to be dependent on both the amygdala and hippocampus (Blanchard *et al.* 1970, Kim & Fanselow 1992, Maren *et al.* 1997, Phillips & LeDoux 1992). Because the knockout mice used in the present experiments lack $Ca_V1.3$ in both of these brain regions it is not possible at this point to definitively know if the hippocampus, amygdala or both areas are impacted by the loss of $Ca_V1.3$. However, it is widely thought that during context conditioning, the hippocampus acts to integrate the many elements of the context in to a functional representation (Rudy *et al.* 2002). Thus, the observation that the $Ca_V1.3$ KO mice exhibited no impairments in the hidden platform version of the Morris water maze, leads us to conclude that the $Ca_V1.3$ KO mice can, in fact, form a spatial representation and that hippocampus function is intact in these mice. Therefore it seems likely that the deficits observed in the $Ca_V1.3$ KO mice are the result of a disruption of neuronal function within the amygdala proper. This is of particular interest given that long-term potentiation (LTP), a form of synaptic plasticity thought to be a cellular substrate of learning and memory, is L-type calcium channel dependent in the amygdala (Bauer et al. 2002). Impaired consolidation of contextually-conditioned fear in $Ca_V1.3$ KO mice may represent a disruption of this putative cellular substrate of learning and memory in the amygdala and thus serve as an excellent model for studying the relationship between LTP in the amygdala and contextual fear conditioning. Interestingly, it was recently reported that LTP of synapses in the lateral amygdala, whose presynaptic neurons arise in the cortex and hippocampus, can be specifically blocked by the L-type calcium channel

antagonist nifedipine (Drephal *et al.* 2006). This is important as it demonstrates an L-type calcium channel dependent communication between two brain structures known to be critical for contextually-conditioned fear.

Despite impaired consolidation of contextually-conditioned fear, $Ca_V1.3$ KO mice extinguish contextually-conditioned fear normally. In light of previous experiments demonstrating that systemic injections of nifedipine/nimodipine can block extinction of context fear (Cain et al. 2002) our results would suggest that $Ca_V1.3$ is not the key LVGCC isoform involved in this process and instead point to $Ca_V1.2$ as the likely candidate. Indeed it has recently been reported that $Ca_V1.2$ is found in abundance within the basolateral amygdala and is primarily found on the in soma and dendrites of pyramidal neurons but is occasionally found on inhibitory interneurons (Pinard *et al.* 2005). While similar immunohistochemical data regarding the cellular and subcellular distribution for $Ca_V1.3$ within the amygdala is lacking, these data open up the intriguing possibility that two different isoforms of the same calcium channel sub-type might subserve two fundamentally different forms of learning. These data also suggest that any therapeutic interventions designed to treat emotional disorders in humans by pharmacologically manipulating LVGCCs will likely need to achieve sufficient specificity to distinguish the $Ca_V1.2$ and $Ca_V1.3$ isoforms.

## Materials and Methods:

### *Mice*

For all experiments, both male and female $Ca_v1.3$ knockout (KO) mice and their wild-type littermates (WT) were used. All mice were between 2-4 months old at the time of testing. In the KO mice, the gene for the pore-forming subunit of the $Ca_v1.3$ calcium channel ($\alpha_{1D}$) has been deleted by insertion of a neomycin cassette into exon 2 which results in a complete null mutation (Platzer et al. 2000). Mice were maintained on a C57BL/6 background by successively crossing heterozygous offspring with C57BL/6 wild-type mice purchased from Taconic Farms (Hudson, NY). The majority of the experiments were conducted on mice on a C57BL/6:129Sve F2 Hybrid background. We also did a small subset of fear conditioning experiments using mice on a C57BL/6 genetic background as noted in the results section.

***Open field***

The open field chamber was a white acrylic box (71 x 71 x 30 cm) in room lit by indirect white light. Mice were placed singly in the center of the chamber and allowed to explore for 5 minutes. The open field was divided into a center zone and a peripheral zone. Total distance traveled and center distance: total distance ratio were calculated.

***Rotarod***

Mice were placed on the rotating drum of an accelerating rotarod (UGO Basile Accelerating Rotarod) and the time each mouse was able to walk on top of the drum was measured. The speed of the rotarod accelerated from 4 to 40 rpm

over a 5-minute period. Mice were given 1 trial/day for 5 days with a maximum time of 300 seconds (5 minutes).

***Pavlovian Fear Conditioning***

Each of the 4 conditioning chambers was equipped with a stainless steel grid floor designed for mice (Med Associates: St Albans, VT). The grid floor was positioned over a stainless steel drop-pan which was lightly cleaned with 95% ethyl alcohol to provide a background odor. The front, top, and back of the chamber were made of clear acrylic and the two sides made of modular aluminum. The conditioning chambers were arranged in a 2 × 2 configuration on a steel rack. The rack was positioned in an isolated room lit by overhead fluorescent lighting. Each chamber was connected to a solid-state shock scrambler and each scrambler was connected to an electronic constant-current shock source which was controlled via an interface connected to a Dell Windows XP computer running Actimetrics FreezeFrame software (Actimetrics; Evanston IL). Four cameras were mounted (one above each chamber) to the steel rack, and video signals were sent to the same computer. Freezing was assessed using the Actimetrics FreezeFrame software which digitizes the video signal at 4 Hz and compares movement frame by frame to determine the amount of freezing.

*Contextual fear conditioning*: Mice were transferred from their home cages into the conditioning chambers individually in groups of 4 at one time. During conditioning, mice were placed in the chamber for three minutes prior to the onset of the US (2 sec 0.50 mA foot shock). Thirty seconds after the footshock

mice were removed from the chambers and returned to their home cages. Contextual conditioning was assessed in different groups of mice 1 hour, 6 hours, and 24 hours after conditioning by returning mice to the same chambers and measuring freezing during a 3 minute shock-free test session.

*Extinction of contextually-conditioned fear:* Behavioral training proceeded in three phases: fear acquisition, fear extinction, and testing. During the acquisition phase, mice were conditioned as described above, However, because $Ca_V1.3$ KO mice exhibited impairments with respect to contextually-conditioned fear following a single training trial (see results); an additional training trial was administered by delivering a single shock immediately following the 3-minute shock-free test session on the 2$^{nd}$ day. As with the first training trial, mice were removed from the chambers 30 seconds after the shock. The following day (the 3$^{rd}$ day), mice were returned to the same chambers and contextual conditioning was again assessed by measuring freezing during a 3-minute shock-free test session. Because contextually-conditioned fear does not differ significantly between KO and WT mice on the 3$^{rd}$ day, following two training trials, mice were left in the chambers for an additional 27 minutes following the 3-minute test session on the 3$^{rd}$ day (30 minute total) to extinguish contextually-conditioned fear. An additional control group of $Ca_V1.3$ KO and WT mice (the retention control group) was included in this experiment. These mice received the exact same training as described above but were not given the 30 minute extinction

trail. On the following day, all mice were returned to the same chambers for a 5 minute test session.

***Morris Water Maze***

The Morris water maze (MWM) used in these experiments consisted of a 1.2 meter diameter pool filled with water which was made opaque with white non-toxic paint. Water temperature was maintained at 25 ± 2 C° throughout the experiment.

Every training trial began with the mouse on the platform for 15 seconds. The mouse was then placed into the water facing the wall of the pool and allowed to search for the platform. The trial ended either when the mouse climbed onto the platform or when 60 seconds had elapsed. At the end of each trial the mouse was allowed to rest on the platform for 15 seconds. Mice were given 2 trials per day for 6 days, with the starting position chosen pseudo-randomly among 6 start positions. Probe trials were conducted 24 hours after the last training trail. During the probe trial, the escape platform was removed and mice were placed in the pool at the start location directly opposite of the platform and allowed to swim for 60 seconds. Mice were run in the visible-platform version of the water maze 24 hours following the probe trial. The visible-platform version consisted of a single day of training with 6 trials during which the platform was moved to a

new quadrant (excluding the target quadrant from the hidden-platform version) every 2 trials and marked with a distinct local cue.

### *Data Analysis*

Statistical comparisons for single measures across genotype were made using a two-tailed unpaired student's t-test. In addition, a single group t-test was used within genotypes to compare Morris water maze probe trial performance with respect to chance (25%). All other comparisons were made using analysis of variance (ANOVA) with post hoc comparisons being made using Fisher's PLSD.

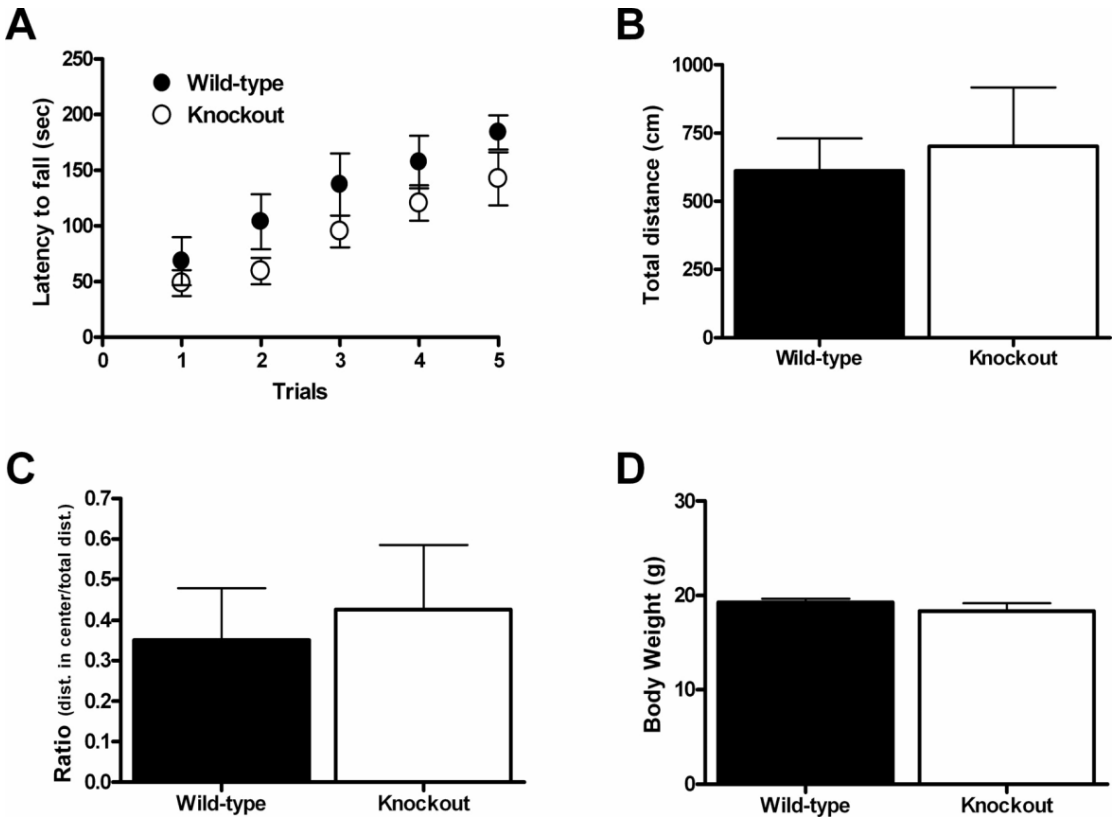


**Figure 2-1.** $Ca_V1.3$ knockout mice are neurologically normal. **(A)** Mice were placed on the accelerating rota-rod for a maximum of 300 seconds once a day for 5 days and the latency to fall was recorded for $Ca_V1.3$ knockout mice and wild-type littermates. The latency to fall for knockout mice was not significantly different from wild-type animals. **(B & C)** Exploratory behavior as measured in the open field was similar in $Ca_V1.3$ knockout mice and wild-type littermates both in the overall distance traveled (panel B) and in the ratio between the amount of time spent in the center divided by the total distance traveled (panel C). **(D)** Average body weight was not significantly different between wild-type mice and the $Ca_V1.3$ knockout mice. All data are presented as mean ± S.E.M.

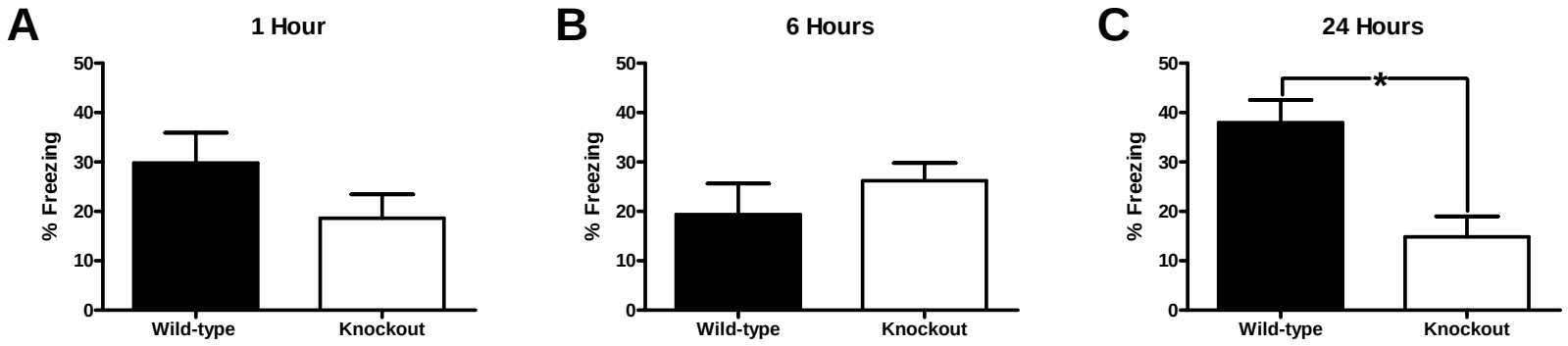


**Figure 2-2.** Deletion of $Ca_V1.3$ disrupts normal consolidation, but not acquisition, of contextual fear conditioning. **(A)** $Ca_V1.3$ knockout mice and wild-type littermates exhibit similar levels of freezing 1 hour following a trial of contextual fear conditioning. **(B)** $Ca_V1.3$ knockout mice and wild-type littermates exhibit similar levels of freezing 6 hours following a trial of contextual fear conditioning. **(C)** $Ca_V1.3$ knockout mice exhibit significantly less freezing when compared to their wild-type littermates 24 hours after a conditioning trial. (*) $p < 0.05$ for post hoc comparison between genotypes. All data are presented as mean ± S.E.M.

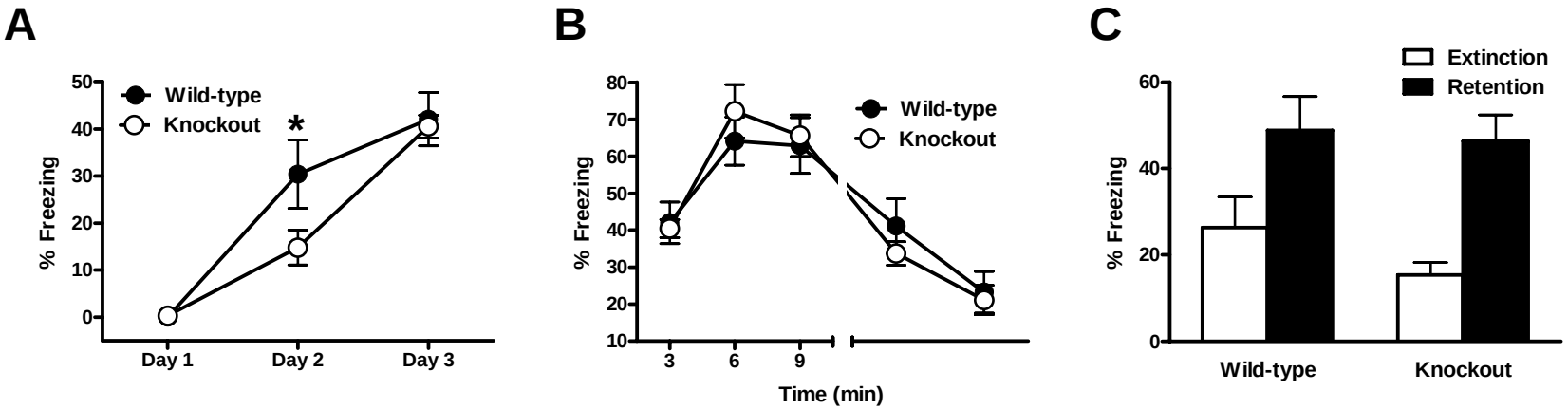


**Figure 2-3.** $Ca_V1.3$ knockout mice exhibit normal short- and long-term extinction. **(A)** $Ca_V1.3$ knockout freeze to a similar degree as their wild-type littermates at the beginning of testing on day 3, overcoming the impairment exhibited on day 2. **(B**) Short-term extinction in $Ca_V1.3$ knockout mice was not significantly different from wild-type mice with both groups freezing significantly less by the end of the 30 minute exposure to the context (10 minute bins). **(C)** Twenty-four hours after the extinction training, mice were re-exposed to the same context for five minutes to measure long-term extinction. There was no difference between $Ca_V1.3$ knockout and wild-type mice in levels of freezing ($p > 0.1$) after extinction. However, both groups (knockouts and wild-types) showed significant reductions in freezing when compared to mice of the same genotype that had not been re-exposed to the context ($p < 0.5$ and $p < 0.005$ for wild-type and knockouts versus retention control group). All data are presented as mean ± S.E.M.

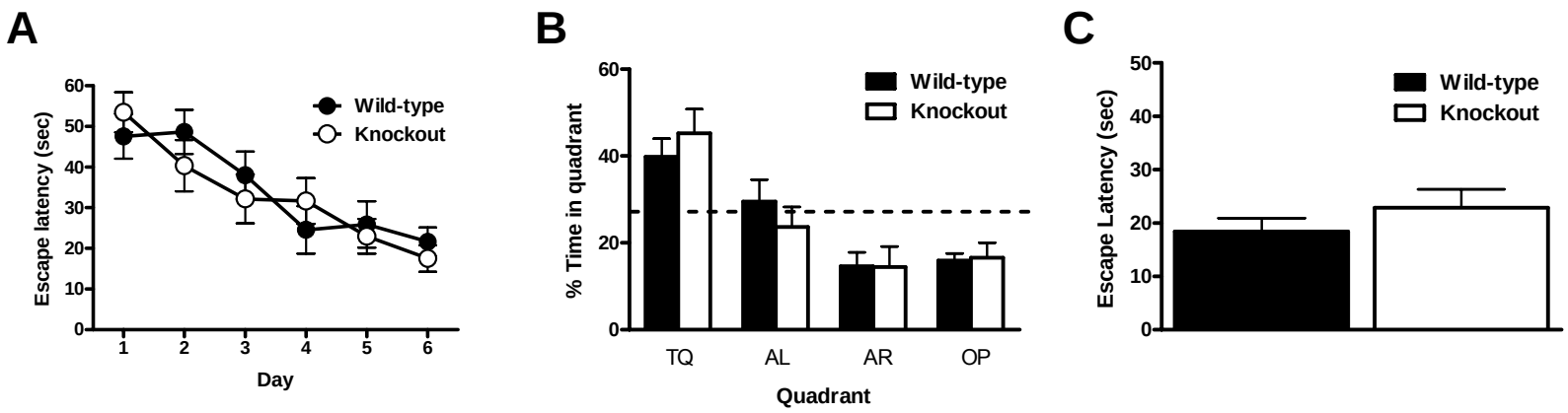


**Figure 2-4.** $Ca_V1.3$ knockout mice are not impaired in the Morris water maze. **(A)** Mice were trained for 2 trials a day for 6 days. The time to reach the hidden platform (escape latency) was not significantly different for $Ca_V1.3$ knockout mice when compared to wild-type littermate control mice. **(B)** A 60 second probe trial completed 24 hours after the last training trail (trial 12; day 6) reveals that both $Ca_V1.3$ knockout and wild type mice spend the a significant amount of time during the trial searching in the quadrant where the platform was previously located (TQ; training quadrant) but there was no significant difference between the genotypes. The dashed line (25%) represents random or "chance" performance. (AR, AL, OP abbreviated for Adjacent Right, Adjacent Left & OPposite, respectively). **(C)** Average escape latencies for $Ca_V1.3$ knockout mice recorded during the visible platform version of the Morris water maze were not significantly different when compared to wild-type littermate controls. All data are presented as mean ± S.E.M

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## Chapter III

## L-type voltage-gated calcium channels in conditioned fear: a genetic and pharmacological analysis

**Abstract:**

Using pharmacological approaches, others have suggested that L-type voltage-gated calcium channels (L-VGCCs) mediate both consolidation and extinction of conditioned fear. In the absence of L-VGCC isoform specific antagonists we have begun to investigate the subtype-specific role of LVGCCs in consolidation and extinction of conditioned fear using a molecular genetics approach. Previously, we used this approach to demonstrate that the $Ca_v1.3$ isoform mediates consolidation, but not extinction, of contextually-conditioned fear. Here, we used mice in which the gene for the L-VGCC pore-forming subunit $Ca_v1.2$ was conditionally deleted in forebrain excitatory neurons ($Ca_v1.2^{cKO}$ mice) to address the role of $Ca_v1.2$ in consolidation and extinction of conditioned fear. We demonstrate that $Ca_v1.2^{cKO}$ mice consolidate and extinguish conditioned fear as well as control littermates. These data suggest that $Ca_v1.2$ is not critical for these processes and together with our previous data argue against a role for either of the brain-expressed L-VGCCs ($Ca_v1.2$ or $Ca_v1.3$) in extinction of conditioned fear. Additionally, we present data demonstrating that the L-VGCC antagonist nifedipine, which has been used in previous conditioned fear extinction studies,

impairs locomotion and induces an aversive state. We further demonstrate that this aversive state can enter into associations with conditioned stimuli that are present at the time that it is experienced, suggesting that previous studies using nifedipine were likely confounded by drug toxicity. Taken together, our genetic and pharmacological data argue against a role for $Ca_v1.2$ in consolidation of conditioned fear as well as a role for L-VGCCs in extinction of conditioned fear.

## Introduction:

Pavlovian fear conditioning is a popular paradigm for both the study of associative learning (Fanselow & Poulos 2005) and modeling anxiety disorders (Delgado *et al.* 2006, Hofmann 2007). In this paradigm, an association between a conditional stimulus (CS) and an aversive unconditional stimulus (US) is acquired through pairing the CS with the US. Learning of this association is identified by the emergence of new responses to the CS, termed conditioned fear responses. The process by which this learning is transformed into a stable long-term memory with the passage of time is referred to as consolidation. A fear response is said to be contextually-conditioned when it is elicited by the context in which the US was delivered. In this case, the context is the CS. Once the CS-US association is established, responding to the CS can be diminished by repeatedly presenting the CS in the absence of the US—this process is referred to as extinction. In the case of contextually-conditioned fear responses, extinction can be accomplished through extended exposure to the conditioning context in the absence of the US.

Previous work has suggested that L-type voltage-gated calcium channels (L-VGCCs) mediate both consolidation (Bauer *et al.* 2002) and extinction (Cain *et al.* 2002, Cain *et al.* 2005, Suzuki *et al.* 2004) of Pavlovian conditioned fear. All of these studies have used pharmacological antagonists to demonstrate a role for L-VGCCs (Bauer et al. 2002, Cain et al. 2002, Cain et al. 2005, Suzuki et al. 2004). One limitation of these studies is that the L-VGCC antagonists used target all L-VGCCS and do not allow for the identification of the specific brain-expressed L-VGCC, $Ca_v1.2$ or $Ca_v1.3$, involved. Therefore, to investigate the subtype specific role of LVGCCs in consolidation and extinction of conditioned fear, we have adopted a molecular-genetics approach. Previously, we used this approach to demonstrate that deletion of the $Ca_v1.3$ subtype disrupted consolidation, but not extinction, of contextually-conditioned fear (McKinney & Murphy 2006).

In the present study, the role of $Ca_v1.2$ in consolidation and extinction of conditioned fear was examined using mice in which the gene for the L-VGCC pore-forming subunit $Ca_v1.2$ was conditionally deleted in the fore-brain ($Ca_v1.2^{cKO}$ mice). We have previously demonstrated that these mice have disrupted remote spatial memories (White *et al.* 2008a). Here we demonstrate that conditional deletion of $Ca_v1.2$ does not disrupt consolidation of conditioned fear. In addition, we find that deletion of $Ca_v1.2$ does not significantly impact the extinction of conditioned fear. Taken together with our previous finding that

$Ca_v1.3$ was not required for extinction, these results suggest that L-VGCCs do not play a significant role in extinction of conditioned fear. Because these results were at odds with previous findings (Cain et al. 2002, Cain et al. 2005, Suzuki et al. 2004), we replicated the studies that implicated L-VGCCs in extinction of conditioned fear by blocking extinction of conditioned fear with systemic administration of the L-VGCC antagonist nifedipine. However, we find that the apparent extinction impairment in mice treated with nifedipine is likely the result of nifedipine's ability to decrease locomotion and induce an aversive state in mice.

**Results:**

***Conditional deletion of $Ca_v1.2$ in the basolateral amygdaloid complex of $Ca_v1.2^{cKO}$ mice.*** Conditional deletion of $Ca_v1.2$ was achieved by crossing mice in which exon 2 of $Ca_v1.2$ was flanked by two *loxP* sites with mice in which expression of Cre-recombinase is driven by the calcium-calmodulin-dependent kinase II$\alpha$ (CaMKII$\alpha$) promoter [termed *CaMK-Cre* here; (Chen *et al.* 2006)]. The conditional deletion of $Ca_v1.2$ in the neocortex and hippocampus of $Ca_v1.2^{cKO}$ mice, two brain regions critically involved in fear conditioning and its extinction (Maren 2001, Myers & Davis 2006) has previously been described (White *et al.* 2008b). We have subsequently conducted a series of experiments to examine the expression pattern of cre-recombinase and to determine the extent of the deletion of $Ca_v1.2$ in the basolateral amygdaloid complex (BLA; comprised of the lateral, basolateral, and basomedial amygdaloid nuclei).

To assess the pattern of cre-recombinase expression pattern, we crossed hemizygous *CaMK-Cre* mice with homozygous R26R reporter mice, which carry the *loxP-STOP-loxP-lacZ* cassette targeted into the ubiquitously expressed ROSA26 locus (Soriano 1999). Brains from bigenic animals were stained for for β-galactosidase activity. β-galactosidase activity and thus cre recombinase expression was present in much of the forebrain of these mice (**Figure 3-1A**), including the BLA (**Figure 3-1A, inset**). To determine the extent of $Ca_v1.2$ deletion within the BLA, we subjected tissue punches from the BLA of $Ca_v1.2^{cKO}$ mice to Western blot analysis using anti-$Ca_v1.2$ specific antibodies. **Figure 3-1B** shows a representative section of mouse brain from which a BLA tissue punch was obtained. Western blot analysis of brain tissue isolated from BLA tissue punches is presented in **Figure 3-1C**. Consistent with our previous findings in the hippocampus and cortex (White et al. 2008b), deletion of exon 2 of $Ca_v1.2$ resulted in complete loss of immunoreactivity within the BLA.

***Conditional deletion of $Ca_v1.2$ does not disrupt consolidation of contextual conditioned fear memories.*** As described above, others have implicated L-VGCCs in consolidation of conditioned fear (Bauer et al. 2002) and we have previously demonstrated that $Ca_v1.3$ mediates consolidation of contextually-conditioned fear (McKinney & Murphy 2006). To determine if $Ca_v1.2$ might also contribute to consolidation of contextually-conditioned fear, we conditioned $Ca_v1.2^{cKO}$ mice (n = 28) and control littermates (n = 39) over the course of two

days in which mice received one unsignaled foot shock per day. The experimental protocol for these experiments is outlined in **Figure 3-2A**. Prior to delivery of the first footshock, mice in both groups exhibited negligible freezing but as training progressed the percent time spent freezing dramatically increased for both groups ($F_{(1,65)} = 150.3.2$, $P < 0.0001$; **Figure 3-2B**), however, there was no difference between genotypes ($F_{(1,65)} = 1.7$, $P > 0.05$; **Figure 3-2B**) and no training-genotype interaction ($F_{(1,65)} = 1.3$, $P > 0.05$; **Figure 3-2B**) demonstrating that conditional deletion of $Ca_v1.2$ does not significantly impact acquisition of contextually conditioned fear in mice. After training, mice were separated into four groups [control-retention (N=17), control-extinction (N=22), $Ca_v1.2^{cKO}$-retention (N=11), $Ca_v1.2^{cKO}$–extinction (N=17)] based on similar day 2 post-shock freezing. On day 3 (24 hrs after the last day of training), mice in the extinction groups were returned to the fear conditioning chambers for a 3-minute session. During this session, $Ca_v1.2^{cKO}$ and control mice froze similarly ($t_{1,37)} = 0.5$, $P > 0.05$; **Figure 3-2C**). These data demonstrate that $Ca_v1.2^{cKO}$ mice are not impaired with respect to consolidation of contextually-conditioned fear and argue against a role for $Ca_v1.2$ in consolidation of conditioned fear.

***$Ca_v1.2^{cKO}$ mice extinguish contextually-conditioned fear as well as control mice.*** Studies using antagonists for L-VGCCs have implicated a role for L-VGCCs in extinction of conditioned fear (Cain et al. 2002, Cain et al. 2005, Suzuki et al. 2004). Previously, we demonstrated that $Ca_v1.3$ KO mice are able to extinguish contextually-conditioned fear as well as WT mice (McKinney &

Murphy 2006) suggesting that $Ca_v1.3$ does not mediate extinction of contextually-conditioned fear. Based on these data, we hypothesized that $Ca_v1.2$ likely plays a role in the extinction of conditioned fear. To test this hypothesis, we exposed the mice that had been conditioned to extinction training by leaving them in the fear conditioning chambers for an additional 57 minutes after retention was assessed on day 3 (**Figure 3-2A)**. Short-term extinction was measured by assessing freezing for the first 30 minutes of this 1-hour extinction session. Both $Ca_v1.2^{cKO}$ (N=17) and control mice (N=22) exhibited significant decreases in freezing across the first 30 minutes of training ($F_{(2,74)} = 63.2$, $P < 0.0001$; **Figure 3-2D**), however, there was no difference between genotypes ($F_{(1,37)} = 0.4$, $P > 0.05$; **Figure 3-2D**) and no training-genotype interaction ($F_{(2,74)} = 0.08$, $P > 0.05$; **Figure 3-2D**) demonstrating that conditional deletion of $Ca_v1.2$ does not disrupt short-term, with-in session, extinction of contextually-conditioned fear.

To explore the possibility that $Ca_v1.2$ is involved in long-term, across-session, extinction of contextually-conditioned fear, mice were returned to the conditioning chambers twenty-four hours after extinction training (on day 4). In addition to the mice that were subjected to extinction training, a retention control group was also tested on day 4. This group consisted of $Ca_v1.2^{cKO}$ (N=10) and control mice (N=17) that were conditioned identically as the group that received extinction training, but were not subjected to extinction training themselves (**Figure 3-2A**). When mice were returned to the conditioning chambers on day 4 to access long-term retention of extinction, mice that were subjected to extinction training froze

significantly less than those in the retention control group as revealed by a two-way ANOVA effect of training group (extinction training vs. retention control; $F_{(1,62)} = 14.1$, $P < 0.001$; **Figure 3-2E**). However, there was no significant effect of genotype ($F_{(1,62)} = 0.4$, $P > 0.05$; **Figure 3-3E**) or training group X genotype interaction ($F_{(1,62)} = 0.03$, $P > 0.05$; **Figure 3-3E**) on day 4 freezing. From these experiments we conclude that conditional deletion of $Ca_V1.2$ does not disrupt long-term extinction of conditioned fear.

Taken collectively, the above data demonstrate conditional deletion of $Ca_v1.2$ does not disrupt either short-term or long-term extinction of contextually-conditioned fear. Together with our previous work showing that $Ca_v1.3$ KO mice are able to extinguish contextually-conditioned fear as well as WT mice, these data argue against a role for L-VGCCs in the extinction of conditioned fear. This conclusion, however, is at odds with work using pharmacological antagonists for L-VGCCs that suggest a role for L-VGCCs in extinction of conditioned fear. As a first step towards addressing the discrepancy between results of extinction experiments performed using genetic mouse models and those that employed pharmacological antagonists we tested our ability to block extinction of conditioned fear with the L-VGCC antagonist nifedipine using our protocol in WT mice with a similar genetic background as the $Ca_v1.2^{cKO}$ and $Ca_v1.3$ KO mice used in our experiments.

***Consistent with previous reports, the L-VGCC antagonist nifedipine appears to block extinction of contextually-conditioned fear.*** The L-VGCCs antagonists nifedipine and nimodipine have been used previously to implicate L-VGCCs in the extinction of conditioned fear. Here, we sought to test our ability to reproduce the findings of Cain et al. (2005, 2002) using conditions similar to those used in our genetic studies of the extinction of conditioned fear. The experimental design is presented in **Figure 3-3A**. Like the mice in the above experiment, wild-type mice (N = 21) mice were conditioned once daily for 2 days with a single unsignaled footshock. Data from the first two days is presented in **Figure 3-3B**. Training resulted in a significant increase in freezing as measured on day 2 ($F_{(1,40)} = 135$; $P < 0.0001$ for main effect of training).

Twenty-four hours after the last training trial (on day 3), mice were assigned to four matched treatment groups based on day 2 post-shock freezing. Two of the groups were returned to the conditioning chamber for 1-hour of extinction training and the other two groups were left in their home cages to serve as retention controls. Of the mice that were returned to the conditioning chambers for extinction training (N=10), mice in one group (N=6) were injected with nifedipine and the mice in the other group (N=4) were injected with saline 50 minutes before the beginning of extinction training. Similarly, of the two groups that were returned to their home cages to serve as retention controls, one group (N=5) was injected with nifedipine and mice in the other group (N=6) was injected with saline (N=6).

Injection of nifedipine before extinction training had no effect on initial levels of freezing as measured during the first 3 minutes of the extinction training ($t_{(8)}$ = 0.126; **Figure 3-3C**). However, mice that were injected with saline before extinction training exhibited a significant decrease in freezing as the extinction training proceeded, whereas those that were injected with nifedipine did not show a similar decrease in freezing (**Figure 3-3D**). A repeated-measures ANOVA revealed that there was not a significant effect of extinction training on freezing across the session ($F_{(5,40)}$ = 1.06, $P > 0.05$; **Figure 3-3D**), but that there was an effect of treatment ($F_{(1,40)}$ = 7.36, $P < 0.05$; **Figure 3-3D**) as well as a extinction training X treatment interaction ($F_{(5,40)}$ = 7.87, $P < 0.0001$; **Figure 3-3D**) suggesting that nifedipine treatment blocks short-term, with-in session, extinction of contextually-conditioned fear. Twenty-four hours following extinction training (day 4), all mice were returned to the conditioning chambers for a five-minute test session. A two-way ANOVA of freezing on day 4 revealed a significant effect of treatment (nifedipine vs. saline; $F_{(1,17)}$ = 15.8, $P < 0.001$; **Figure 3-3E**), but not training group (extinction training vs. retention control; $F_{(1,17)}$ = 3.15, $P > 0.05$; **Figure 3-3E**). However, there was a treatment X training group interaction ($F_{(1,17)}$ = 5.86, $P < 0.05$; **Figure 3-3E**). Planned post-hoc comparisons (unpaired t-test) revealed that mice injected with saline and subjected to extinction training froze significantly less than retention control mice ($t_{(1,8)}$ = 2.49, $P < 0.05$; **Figure 3-3E**) but those that were injected with nifedipine and subjected to extinction training froze no less than retention control mice ($t_{(1,9)}$ = 0.56, $P < 0.05$; **Figure 3-3E**) suggesting that nifedipine blocks long-term extinction of contextually-conditioned

fear. These results confirm previous reports that treatment with L-VGCC antagonists appears to block both short-term and long-term extinction of conditioned fear.

While conducting these experiments it became apparent that mice injected with nifedipine were generally less active and more lethargic than mice injected with saline. It occurred to us that such an effect may complicate the use of nifedipine to study extinction and lead to results that could be misinterpreted. To explore this possibility, we proceeded to examine the effect of nifedipine on locomotor activity and freezing behavior.

***Nifedipine decreases locomotor activity to an extent interpretable as freezing.*** We first explored the effect of nifedipine on locomotor behavior using the open-field test. Mice were injected with nifedipine (N = 6) or saline (N = 6) fifty minutes prior to the open-field session. Mice were placed at the edge of the open field and allowed to explore for 5 minutes, total distance traveled was measured. Nifedipine-treated mice traveled a significantly shorter distance (1369.2 ± 455.4 cm) in the open field than saline-treated mice (3091.3 ± 427.78; $t_{(1,10)} = 2.76$, $P < 0.05$) confirming our suspicion that nifedipine decreases locomotor activity.

We next explored if the decrease in locomotor activity induced by nifedipine could be misinterpreted as freezing. To do this, we repeated the conditioning and

extinction protocol in the absence of foot shocks (**Figure 3-4A**). On days 1 and 2, mice were placed in the conditioning chambers for 4 minutes without shocking. On day 3, mice were injected with either saline (N = 6) or nifedipine (N = 8) and returned to the conditioning chambers 50 minutes later for 1 hour to simulate extinction training. On day 4, mice were returned to the chamber for 5 minutes. The saline- and nifedipine-treated mice both exhibited minimal freezing behavior on days 1 and 2 and there was no difference between groups ($t_{(1,12)} = 0.55$ and 0.19, respectively, $P > 0.05$; data not shown) . The nifedipine-treated group exhibited significant freezing on day 3. A two-way ANOVA revealed that there was a significant effect of treatment (saline vs. nifedipine; $F_{(1,72)} = 204.6$, $P < 0.0001$; **Figure 3-4B**) and time-in-chamber ($F_{(5,72)} = 6.67$, $P < 0.001$; **Figure 3-4B**) on freezing as well as a treatment-time-in-chamber interaction ($F_{(5,72)} = 4.21$, $P < 0.05$; **Figure 3-4B**). Nifedipine-treated mice exhibited more freezing during the day 3 session than saline-treated mice suggesting that nifedipine treatment can affect locomotor activity to the extent that it can be measured as freezing and may explain why treatment with nifedipine appears to block short-term extinction of conditioned fear. However, the acute affect of nifedipine on locomotor activity and freezing behavior would not explain the previously-demonstrated effect of nifedipine on blocking long-term extinction of conditioned fear. Interestingly, when mice were returned to the conditioning chambers and tested off-drug on day 4, mice that had experienced the conditioning chambers under the influence of nifedipine on day 3 froze significantly more than those injected with saline before day 3 training ($t_{(1,12)} = 2.77$, $P < 0.05$; **Figure 3-4C**). These results suggest

that treatment with nifedipine has long-term effects on freezing behavior. To us, these results resembled previous work suggesting that cues present during an aversive state can elicit species-specific defensive behaviors (e.g., freezing) upon subsequent presentation of the cues (McNally & Akil 2001).

***Long-term effect of nifedipine on freezing is context-specific.*** If the long-term effect of nifedipine on freezing is the result of an association between the conditioning context and nifedipine-induced sickness, then the freezing observed when mice are tested 24 hours after nifedipine treatment should be eliminated if testing is carried out in an alternate context, that is, in the absence of the CS. To explore if the long-term effects of nifedipine on freezing behavior are context-specific, we conducted an experiment similar to the one described immediately above with a minor modification—half of the mice were placed in the conditioning context during the long-term test (day 4) and the other half were placed in a reconfigured context during the long-term test (day 4; **Figure 3-5A**). As with the previous experiment, both the saline-treated mice (N = 16) and nifedipine-treated mice (N = 16) exhibited minimal freezing behavior on days 1 and 2 and there was no difference between groups ($t_{(1,30)}$ = 0.25 and 0.85, respectively, $P > 0.05$; data not shown) . The nifedipine-treated group exhibited freezing on day 3. A two-way ANOVA revealed that there was a significant effect of treatment (saline vs. nifedipine; $F_{(1,180)} = 99.3$, $P < 0.0001$; **Figure 3-5B**) and time-in-chamber ($F_{(5,180)} = 21.7$, $P < 0.001$; **Figure 3-5B**) on freezing as well as a treatment-time-in-chamber interaction ($F_{(5,180)} = 3.65$, $P < 0.05$; **Figure 3-5B**). On day 4, approximately half

of the mice returned to the same chamber in which they were placed on day 3 and the rest were placed in a reconfigured chamber and freezing was measured for 5 minutes. A two-way ANOVA revealed a significant effect of treatment ($F_{(1,28)} = 9.48$, $P < 0.01$; **Figure 3-5C**) and context ($F_{(1,28)} = 27.8$, $P < 0.0001$; **Figure 3-5C**) on day 4 freezing as well as a treatment-context interaction ($F_{(1,28)} = 0.73$, $P > 0.05$; **Figure 3-5C**). Planned post-hoc comparisons (unpaired t-test) reveal that nifedipine-treated mice freeze more than saline-treated mice when tested in the training context ($t_{(1,14)} = 2.90$, $P < 0.05$; **Figure 3-5C**), but not in the reconfigured context ($t_{(1,14)} = 1.05$, $P > 0.05$; **Figure 3-5C**). Mice exhibited less freezing in the novel environment than in the training context independent of whether they were treated with nifedipine ($t_{(1,14)} = 4.40$, $P < 0.05$; **Figure 3-5C)** or saline ($t_{(1,14)} = 2.97$, $P < 0.05$; **Figure 3-5C)**, likely reflecting the phenomenon of novelty-induced exploration (van Abeelen 1989).

***Nifedipine-induced nausea associates with taste to produce conditioned taste avoidance.*** In the conditioned taste avoidance paradigm, mice learn to avoid drinking fluid with a specific taste after it has been paired with a nausea-inducing stimulus such as lithium chloride. To confirm that nifedipine was inducing an aversive state and could serve as an unconditioned stimulus and enter into an association with a cue, we paired saccharin with nifedipine (N = 6) or saline (N = 5) in wild type mice and measured their avoidance of saccharin (avoidance index = [(grams of water)/(grams of water and saccharin)] X 100%) 24 hours later. An unpaired t-test reveals that mice in which saccharin was paired

with nifedipine avoided saccharin significantly more than mice in which saccharin was paired with saline (avoidance indices = 71.17 ± 7.67 and 48.77 ± 5.25, respectively; $t_{(1,9)}$ = 2.37, $P < 0.05$; **Figure 3-6**). These data suggest that nifedipine can act as an unconditioned stimulus and thus supports our conclusion that the apparent blocking of long-term extinction of contextually-conditioned fear by nifedipine is actually the elicitation of defensive freezing by the context in which extinction training was performed and thus illness experienced.

**Discussion:**

In this study, we show that conditional deletion of $Ca_v1.2$ does not significantly impact consolidation or extinction of contextually-conditioned fear. Previously we have demonstrated that deletion of $Ca_v1.3$, the alternate L-VGCC subtype, disrupts consolidation but not extinction of contextually conditioned fear.

Taken collectively these data suggest that L-VGCCs likely do not play a role in extinction of conditioned fear. However, consistent with previous reports by others and contradictory to our own genetic studies, we show that systemic treatment of wild type mice with the L-VGCC antagonist nifedipine before extinction training appears to block both short-term and long-term extinction. However, upon further investigation, we found that the apparent ability of nifedipine to block extinction of conditioned fear does not reflect nifedipine's effect on extinction learning, but instead is a product of nifedipine-induced aversive state. Specifically, we show that mice that are under the influence of

nifedipine are less active than saline-treated mice and that in the fear conditioning chambers this decreased activity can be interpreted as freezing behavior. Interestingly, mice that were treated with nifedipine and placed in the fear conditioning chambers continue to exhibit freezing when they are returned to the same fear conditioning chambers when they are tested off drug 24 hours later. We have demonstrated that this “off-drug” freezing is context-dependent which suggests that the long-term freezing in nifedipine-treated mice is the result of association being formed between the conditioning context and nifedipine-induced aversive state. Further demonstrating that nifedipine can induce and aversive state and enter into an association with a cue, we show that nifedipine can be used as the unconditioned stimulus to induce conditioned avoidance of saccharin in a standard conditioned taste avoidance paradigm.

Our conclusion that L-VGCCs likely do not play a role in extinction of conditioned fear appears to be at odds with previous studies. We suggest that this discrepancy is a result of the different approaches used: pharmacological versus genetic. In light of the data presented here regarding the apparent toxicity of nifedipine, it could be agued that if L-VGCCs do mediate extinction of conditioned fear it is unlikely that their putative role could be uncovered with this approach.

We arrive at our conclusion that L-VGCCs likely do not play a role in extinction of conditioned fear by a process of elimination: global deletion of $Ca_v1.3$ disrupts consolidation but not extinction of contextually conditioned fear while conditional

deletion of $Ca_v1.2$ does not disrupt either process. However we must consider at least four other alternate explanations that would still feature a role for L-VGCCs in the extinction of contextual fear conditioning.

The first explanation is that both brain-expressed L-VGCCs need to be blocked to realize impaired extinction of conditioned fear. Setting aside the toxicity issue discussed above for the moment—this would explain why treatment with L-VGCC antagonists and not genetic deletion of individual L-VGCC subtypes leads to impaired extinction of conditioned fear. Such an explanation would suggest that the two brain-expressed L-VGCCs ($Ca_v1.2$ and $Ca_v1.3$) are functionally similar and one can compensate for the other in its absence. In our hands, however, there is no change in $Ca_v1.3$ expression levels upon deletion of the gene for $Ca_v1.2$ or vice versa (unpublished results). Further, $Ca_v1.2$ and $Ca_v1.3$ have quite different neuronal localization (Hell *et al.* 1993) and biophysical characteristics (Helton *et al.* 2005, Xu & Lipscombe 2001) making them unlikely functional substitutes for each other. Still, the possibility of a synergistic effect of simultaneous inactivation of both $Ca_v1.2$ and $Ca_v1.3$ cannot be dismissed.

The second explanation relates to the conditional nature of the deletion of $Ca_V1.2$ in the present study. Because the gene for $Ca_v1.2$ was deleted primarily in the forebrain of $Ca_v1.2^{cKO}$ mice, the lack of an effect on the ability to extinguish conditioned fear may have resulted from the gene for $Ca_v1.2$ not being deleted in a critical region for extinction of conditioned fear. However, this seems unlikely

given that $Ca_v1.2$ in the $Ca_v1.2^{cKO}$ mice is deleted in most of the brain regions that are critical for extinction of conditioned fear including the cortex, hippocampus (White et al. 2008b) as well as the amygdala. This, of course, does not eliminate the possibility that $Ca_v1.2$ is still expressed in a currently unrecognized area of the brain critical for extinction of conditioned fear.

Third, because the cre-recombinase expression in $Ca_v1.2^{cKO}$ mice was driven the calcium-calmodulin-dependent kinase II alpha promoter, the gene for $Ca_v1.2$ was only deleted in excitatory, but not inhibitory, neurons of the forebrain (Kelly 1991). If $Ca_v1.2$ activity in inhibitory neurons is critical for the successful extinction of conditioned fear, then we would not have observed impaired extinction of conditioned fear in $Ca_v1.2^{cKO}$ mice as these mice presumably express the gene for $Ca_v1.2$ in inhibitory neurons. If this were in fact the case, it would seem likely that $Ca_v1.2$ is exerting its influence upon inhibitory neurons outside of the amygdala, since almost all of the $Ca_V1.2$ immunoreactivity observed in the BLA is found in CaMKII$\alpha$ positive pyramidal neurons (Pinard *et al.* 2005).

And finally, the extinction protocol used here may not have been suitable for detecting $Ca_v1.2$-mediated deficits in conditioned fear extinction. We demonstrated successful extinction of contextually-conditioned fear using a single 1-hour context exposure. Though this protocol did not uncover an effect of genotype on extinction, it is possible that protocols that utilized longer exposure

periods or different training styles (e.g., spaced extinction training) may have been more successful. We were, however, able to successfully reproduce the previously-demonstrated effect of nifedipine on extinction using this protocol and thus it seems likely that this protocol would be sufficient to parse out any contribution of $Ca_v1.2$ to the nifedipine effect. Our experiments, of course, do not rule out the contribution of $Ca_v1.2$ to extinction in other paradigms (e.g., cue fear conditioning, Morris water maze).

Using a molecular genetics approach, we have begun to investigate the role that specific L-VGCCs isoforms play in Pavlovian fear conditioning. Based on the current study and our previous work and with the above alternate explanations withstanding, we conclude that $Ca_V1.3$ plays a critical role in the consolidation of contextually conditioned fear and that L-VGCCs do not mediate extinction of contextual conditioned fear memories.

## Materials and Methods:

### *Mice.*

Genetic studies were performed on mice in which the gene for the pore-forming subunit $Ca_v1.2$ L-VGCC subtype was conditionally deleted in excitatory neurons of the mouse forebrain. To generate experimental mice, heterozygous floxed $Ca_V1.2$ mice ($Ca_V1.2^{f/+}$ mice) which were maintained on a 129SvEv genetic background (White et al. 2008b) were crossed with transgenic mice that expressed Cre recombinase under the control of the calcium calmodulin kinase

IIα (CaMKIIα) promoter (Chen et al. 2006). These mice (termed here as the CaMK-Cre mice) which express Cre recombinase in excitatory neurons of the forebrain (Chen et al. 2006) were propagated and maintained on a C57BL/6NTac genetic background (10+ generations).

Offspring from the $Ca_V1.2^{f/+}$ X CaMK-$Cre^{Cre/+}$ cross (the F1 generation) that were heterozygous floxed and Cre positive (i.e. $Ca_V1.2^{f/+}$, CaMK-$Cre^{Cre/+}$) were then intercrossed (non-sibling) to achieve the following genotypes: conditional knockout mice ($Ca_V1.2^{f/f}$, CaMK-$Cre^{Cre/+}$); wild-type mice ($Ca_V1.2^{+/+}$, CaMK-$Cre^{+/+}$); floxed controls ($Ca_V1.2^{f/f}$, CaMK-$Cre^{+/+}$) and Cre controls ($Ca_V1.2^{+/+}$, CaMK-$Cre^{Cre/+}$) all on a 129S6B6F2/Tac hybrid genetic background. For ease of reading, conditional knockout mice ($Ca_V1.2^{f/f}$, CaMK-$Cre^{Cre/+}$) are referred to as $Ca_V1.2^{cKO}$ throughout the text. For the fear conditioning experiments, all four genotypes were used. A post-hoc repeated measures ANOVA of the three control genotypes ($Ca_V1.2^{+/+}$, CaMK-$Cre^{+/+}$; $Ca_V1.2^{f/f}$, CaMK-$Cre^{+/+}$ and $Ca_V1.2^{+/+}$, CaMK-$Cre^{Cre/+}$) revealed no significant difference in freezing during training ($F_{(2,36)} = 2.6$; $P > 0.05$, for the effect of genotype) or during extinction training ($F_{(2,19)} = 2.6$; $P > 0.05$, for the effect of genotype), therefore the three genotypes are presented in aggregate (labeled as “Controls”).

All pharmacological studies were done on WT mice with a 129S6B6F2/Tac hybrid genetic background. To obtain these mice, 129S6B6F1/Tac hybrid mice were generated first by crossing C57BL/6NTac mice purchased from Taconic Farms (Hudson, NY) with 129S6/SvEvTac mice that were similarly obtained.

129S6B6F1/Tac mice were subsequently intercrossed to obtain experimental mice.

Mice were housed under uniform conditions including a 12h-12h light-dark cycle with lights on at 6 AM, average temperature of 22$^{o}$C and *ad libitum* food and water. Mice were housed together in groups of 3-5 with same-sex siblings. Mice were between 2-6 months of age at testing and approximately equal numbers of male and female mice were used in all experiments. Behaviorally naïve mice were used in each experiment. All experiments were conducted according to NIH guidelines for animal care and were approved by the University Committee on Use and Care of Animals of the University of Michigan.

***X-gal Staining.***

Cre expression in CaMK-Cre mice was localized with the aid of R26R reporter mice which carry the *loxP-STOP-loxP-lacZ* cassette targeted into the ubiquitously expressed ROSA26 locus (Soriano 1999), a generous gift from Dr. Miriam Meisler.

Brain sections from mice carrying both the *CaMK-Cre* and *loxP-STOP-loxP-lacZ* transgenes were stained for β-galactosidase activity. Mice were anesthetized, decapitated, and their brains were removed and immediately frozen in isopentane (-30$^{o}$C). Brains were then sectioned (40 µm), mounted on microscope slides, and stained overnight at 37$^{o}$ C with X-gal staining solution [1 mg/ml X-gal, 50 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ in 100 mM phosphate buffer, pH 7.5]. After staining, sections were washed with PBS, fixed in 4% paraformaldehyde, cover-slipped, and imaged.

***Immunoblotting.***

Immunoblotting was carried out as previously described (White et al. 2008b) using basolateral amygdala (BLA) samples from $Ca_v1.2^{cKO}$ mice and control ($Ca_V1.2^{+/+}$, CaMK-$Cre^{+/+}$) littermates. Mice were anesthetized, decapitated, and their brains were removed and placed in HSE buffer (10 mM HEPES, 350 mM Sucrose, and 5 mM EDTA, pH = 7.4) containing Complete Protease Inhibitor (Roche Diagnostics, Mannheim Germany). Brains were then sectioned and the BLA removed with a 0.5 mm diameter sample corer (Fine Science Tools). The BLA samples were then homogenized and the homogenate centrifuged for 5 minutes at 2,000 X g at 4° C. The supernatant was removed and protein content was determined by Bradford assay (Bio-Rad, Hercules, CA), using bovine serum albumin (BSA) as a standard. BLA protein samples (50 µg) were solubilized in Laemelli buffer separated on a 7.5% SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad, Hercules, CA) overnight. Blots were probed with an anti-$Ca_V1.2$ antibody (1:200; Alomone Labs, Jerusalem, Israel product # ACC-022, Lot# AN-03) whose epitope is located in the N-terminus of the protein (residues 2-15). Blots were also probed with an NrCAM specific antibody (1:40000; ABCAM, Cambridge, UK) which served as the loading control. Incubation with the primary antibody was followed by washing with PBS-Tween and incubation with a horseradish peroxidase-conjugated anti-rabbit secondary (Bio-Rad, 1:5000). Blots were washed and immunoreactivity was visualized with an enhanced chemiluminescence detection system (ECL Plus, Amersham, UK).

***Drug.***

The LVGCC antagonist nifedipine (40 mg/kg; Sigma, St. Louis) was sonicated into 100% Cremophor (Sigma, St. Louis). Saline was added to make the final vehicle 10% Cremophor/90% saline. This dose of nifedipine was a partial suspension and thus care was taken to thoroughly mix prior to injecting. Mice were injected 50 minutes prior to behavioral testing (10 mL/kg) in all experiments. Drug dose and pretreatment time were based on previous studies of nifedipine and conditioned fear (Cain et al. 2005, Cain et al. 2002).

***Pavlovian Fear Conditioning.***

*Conditioning Apparatus:* Pavlovian fear conditioning was performed as previously described (McKinney & Murphy 2006, McKinney *et al.* 2007). Each of the 4 conditioning chambers was equipped with and a stainless steel grid floor designed for mice (Med Associates: St Albans, VT). The grid floor was positioned over a stainless steel drop-pan which was lightly cleaned with 95% ethyl alcohol to provide a background odor. The front, top, and back of the chamber were made of clear acrylic and the two sides made of modular aluminum. The conditioning chambers were arranged in a 2 × 2 configuration on a steel rack. The rack was positioned in an isolated room lit by overhead fluorescent lighting. Each chamber was connected to a solid-state shock scrambler and each scrambler was connected to an electronic constant-current shock source which was controlled via an interface connected to a Dell Windows XP computer running Actimetrics FreezeFrame software (Actimetrics; Wilmette, IL). Four cameras were mounted (one above each chamber) to the steel rack, and video

signals were sent to the same computer. Freezing was defined as the absence of movement except that associated with respiration and was measured by subjecting the video signal to a sensitive global motion-detection algorithm (Freezeframe and Freezeview software; Actimetrics, Wilmette, IL).

*Contextual fear conditioning*: Mice received 2 training trials (1 trial per day) in a 3 minute stimulus-free interval was followed by a 2 second, 0.50 mA foot shock delivered via the grid floor. Fifty-eight seconds after the foot shock mice were removed from the chambers and returned to their home cages. Twenty-four hours after the second day of training, mice were returned to the fear conditioning chambers for a 3 minute retention test.

*Extinction of contextually-conditioned fear:* Mice which received extinction training were first conditioned as described above. Twenty-four hours after the second conditioning trial (on day 3), mice were returned to the same chambers and extinction training commenced. Extinction training consisted of a single 1-hour exposure to the training context in the absence of foot shock. Retention controls were included in extinction experiments. These mice were treated identically to those that received extinction training except that they were not subjected to the 1-hour extinction session. On the following day, all mice were returned to the same chambers for a 5 minute test session.

***Open field.***

Locomotor activity was assessed in an open field as described in (McKinney et al. 2007, McKinney & Murphy 2006). On day 1 of open-field testing, mice were

injected with nifedipine or saline 50 minutes before being placed in the open field. Mice were placed at the edge of the open field and allowed to explore for 5 minutes. Distance traveled was measured using Actimetrics software.

***Unconditioned freezing behavior in fear conditioning chambers.***

Mice were placed in the fear conditioning chambers for 4 minutes a day for two days. On day 3, mice were injected with nifedipine or saline 50 minutes before being returned to the fear conditioning chambers for 1 hour. On day 4, mice were returned to the same fear conditioning chambers or reconfigured fear conditioning chambers for 5 minutes. This protocol was designed to be identical to the fear conditioning/extinction protocol described above except for no shocks were delivered and the fear conditioning chambers were reconfigured in a subset of experiments. Freezing was measured on all four days. Freezing was defined as the absence of movement except that associated with respiration and was measured by subjecting the video signal to a sensitive global motion-detection algorithm (Freezeframe and Freezeview software; Actimetrics, Wilmette, IL).

***Conditioned taste avoidance.***

Conditioned taste avoidance (CTA) was performed as in Josselyn et al.(Josselyn *et al.* 2004). Mice were singly-housed in cages with food, but no water 20 hours before the experiment. During a 5-day habituation period, mice were given access to two drinking bottles filled with water for decreasing periods of time each day (4 hours, 2 hours, 1 hour, 30 minute, 30 minute drinking period). On

day 6, mice were presented with a single bottle filled with 2mM saccharin for 30 minutes. Thirty minutes after the drinking session, mice were injected with 40 mg/kg nifedipine or 0.15M LiCl at a dose of 2% body weight. Twenty four hours later, mice were given a choice test in which they were presented with two bottles for 30 minutes; one containing 2mM saccharin and the other containing water. Liquid consumed from each bottle was measured and an aversion index (AI) calculated as follows: [(grams of water)/(grams of water and saccharin)] X 100%.

**Statistical Analysis**.

All data are presented as mean ± SEM. Fear conditioning acquisition/consolidation and extinction training data were analyzed using repeated-measures ANOVA with a between-subject factor for genotype or treatment group and a repeated measure for training day or time-in-chamber. Unpaired t-tests between genotypes were used to analyze data from the final test session of acquisition/consolidation training. Two-way ANOVAs with genotype or treatment group and training group (extinction vs. retention) or testing context (training vs. novel) were used to analyze data from the long-term extinction test sessions. Unpaired t-tests were used for post-hoc comparisons when there was a significant interaction between factors. Open field and conditioned taste avoidance data were analyzed using unpaired t-tests between treatment or pairing groups, respectively. Results were considered significantly different when $P < 0.05$.

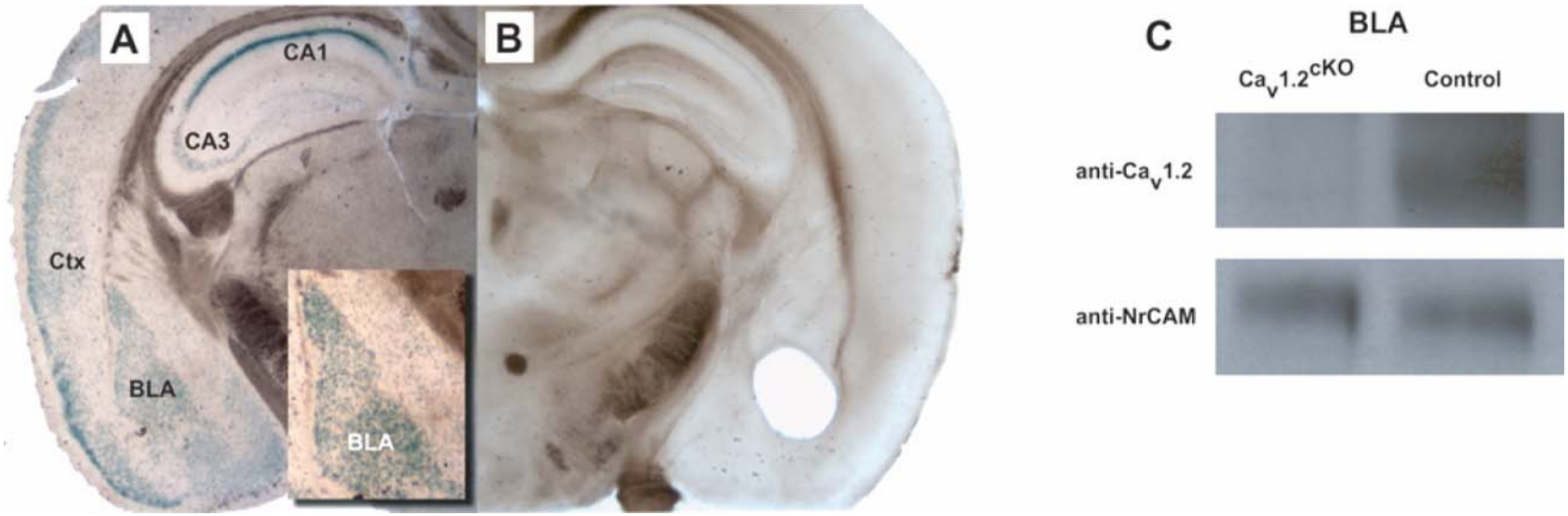


**Figure 3-1.** $Ca_v1.2$ is deleted from the basolateral amygdala (BLA) of $Ca_v1.2^{cKO}$ mice. **(A)** β-galactosidase activity is detected in regions of the brain critical for consolidation and extinction of conditioned fear including the cortex (Ctx), CA1 and CA3 regions of the hippocampus, and BLA of mice carrying both the *CaMK-Cre* and *ROSA26* transgenes. **(B)** Representative brain section showing from where tissue was harvested for BLA immunoblotting. **(C)** Immunoreactivity for $Ca_v1.2$ is present in BLA tissue from control, but not $Ca_v1.2^{cKO}$ mice. Similar immunoreactivity for the loading control (NrCAM) is present in BLA tissue from both $Ca_v1.2^{cKO}$ and control mice.

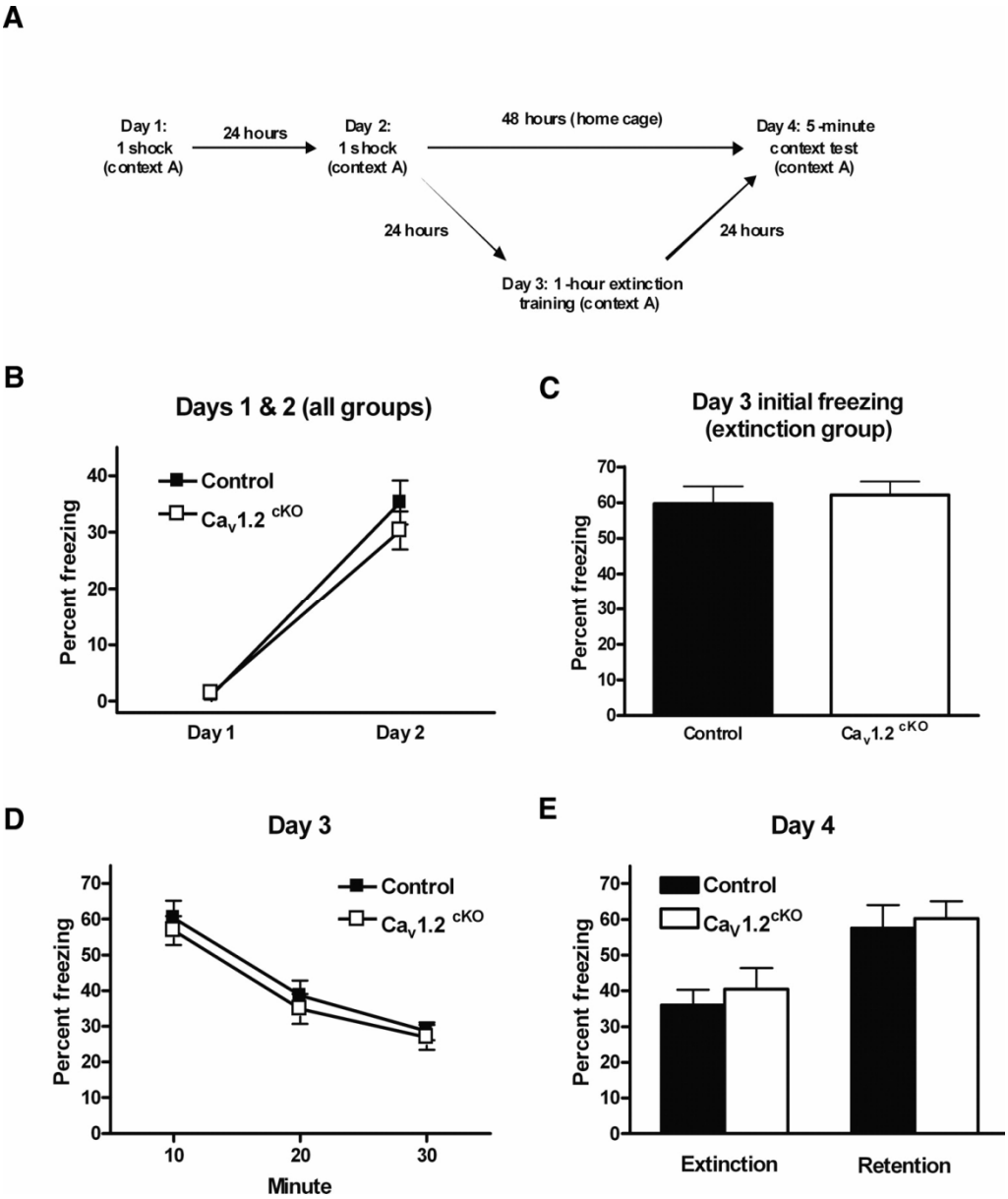


**Figure 3-2.** $Ca_v1.2^{cKO}$ mice consolidate and extinguish contextually-conditioned fear as well as their control littermates. **(A)** Training protocol, conditioning and extinction training. **(B)** $Ca_v1.2^{cKO}$ and control mice exhibit similar levels of freezing prior to conditioning (day 1) and 24 hours after the first conditioning trail (day 2). **(C)** $Ca_v1.2^{cKO}$ and control mice exhibit similar levels of freezing 24 hours after the last conditioning trial. **(D)** $Ca_v1.2^{cKO}$ and control mice exhibit similar rates of extinction across the first 30 minutes of a 1-hour extinction training session (day 3). **(E)** Twenty-four hours after extinction training (day 4), mice were again exposed to the conditioning chambers and both $Ca_v1.2^{cKO}$ mice and control mice exhibited significant reductions in freezing compared to mice of the same genotype that did not undergo extinction training (retention group). All data are presented as mean ± S.E.M.

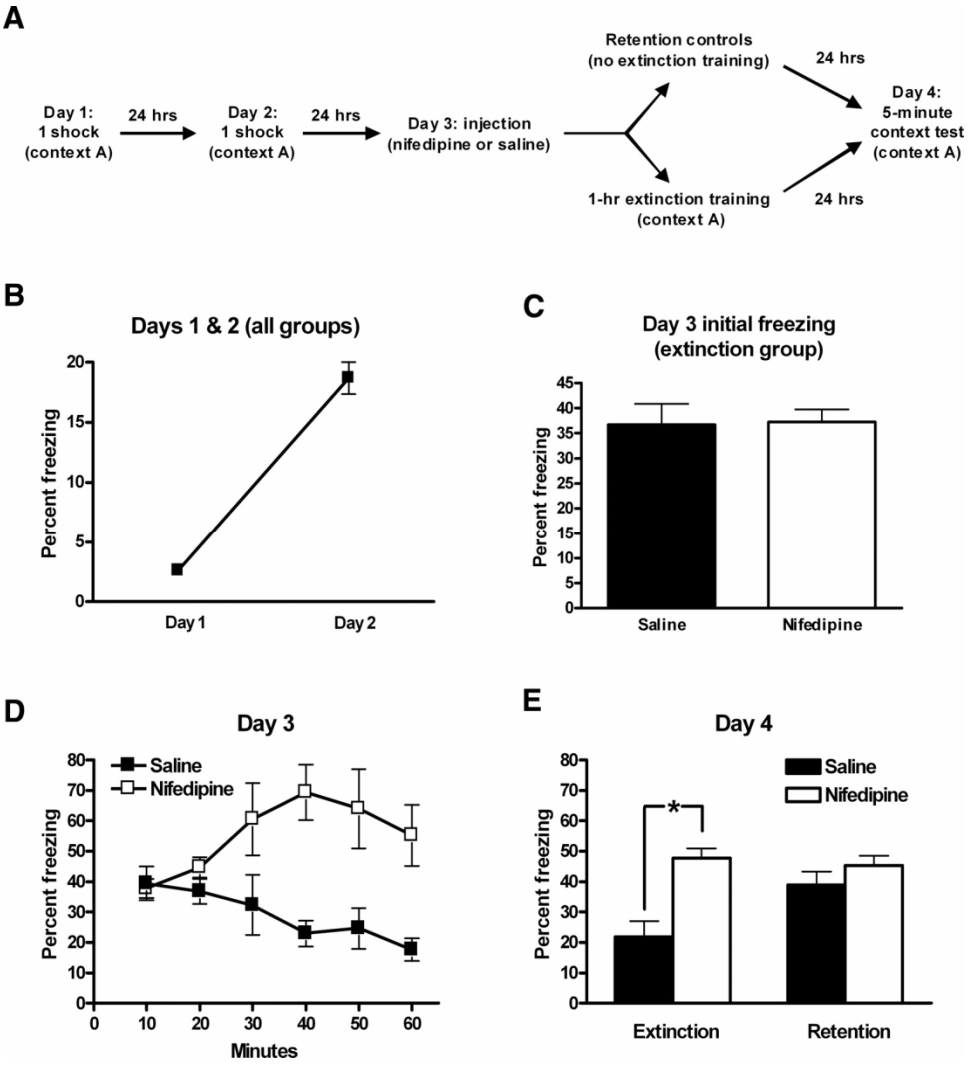


**Figure 3-3.** Consistent with previous reports, the L-VGCC antagonist nifedipine appears to block extinction of contextually-conditioned fear. **(A)** Training protocol, conditioning and extinction training with nifedipine treatment. **(B)** Wild type mice were conditioned with one shock daily for two days and exhibited significant freezing 24 hours after the first conditioning trail. **(C)** Wild type mice were separated into four groups based on day 2 post-shock freezing. Two of these groups were returned to the conditioning chambers 24 hours after the second training trial and 50 minutes after treatment with nifedipine or saline. There was no difference in freezing between these two groups during the first 3 minutes of day 3. **(D)** Saline-treated mice exhibited significant reductions in freezing across a 1-hour extinction training session (day 3) however, nifedipine-treated did not exhibit a similar reduction. **(E)** Twenty-four hours after extinction training (day 4), mice were again exposed to the conditioning chambers. Saline-treated exhibited significant reductions in freezing compared to similarly-treated mice that did not undergo extinction training (retention group). Nifedipine-treated mice, however, exhibited similar levels of freezing as nifedipine-treated mice in the retention group. (*) $p < 0.05$. All data are presented as mean ± S.E.M.

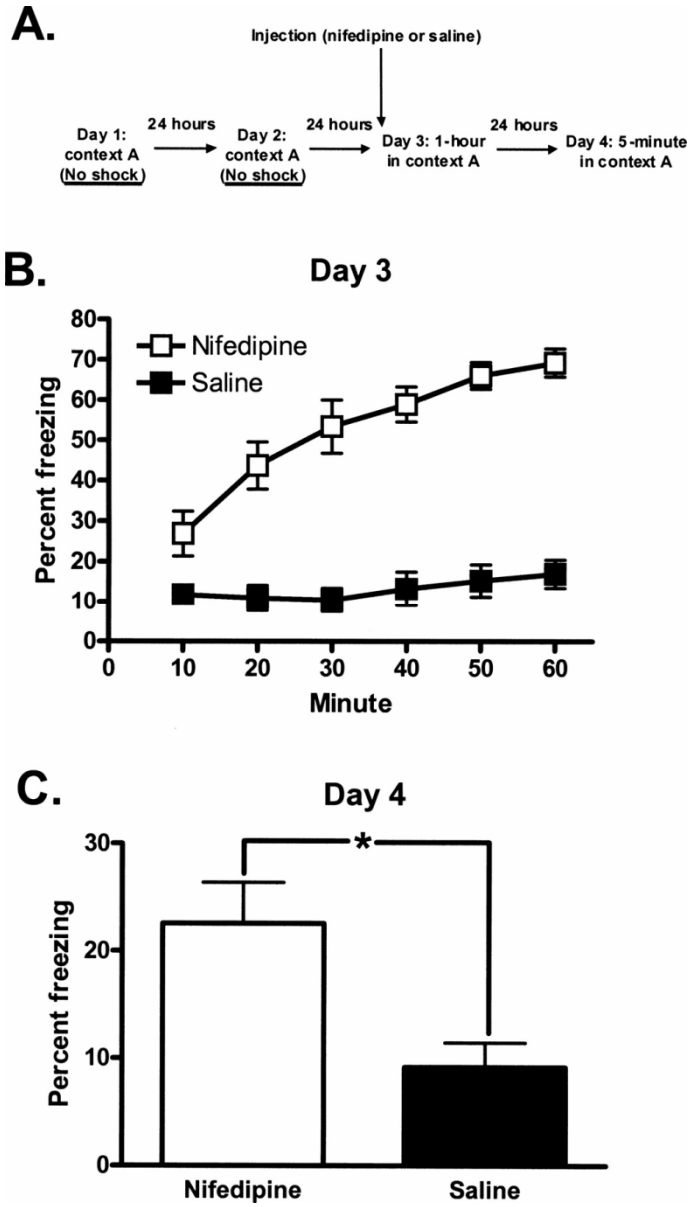


**Figure 3-4.** Nifedipine induces freezing that persists upon return to the conditioning chambers 24 hours after treatment. **(A)** Training protocol, unconditioned freezing. **(B)** Fifty minutes following treatment with nifedipine, mice freeze more than saline-treated mice when placed in the conditioning chambers for 1 hour. **(C)** Nifedipine-treated mice freeze more than saline treated mice when returned to the conditioning chambers 24 hours after treatment. (*) $p < 0.05$. All data are presented as mean ± S.E.M.

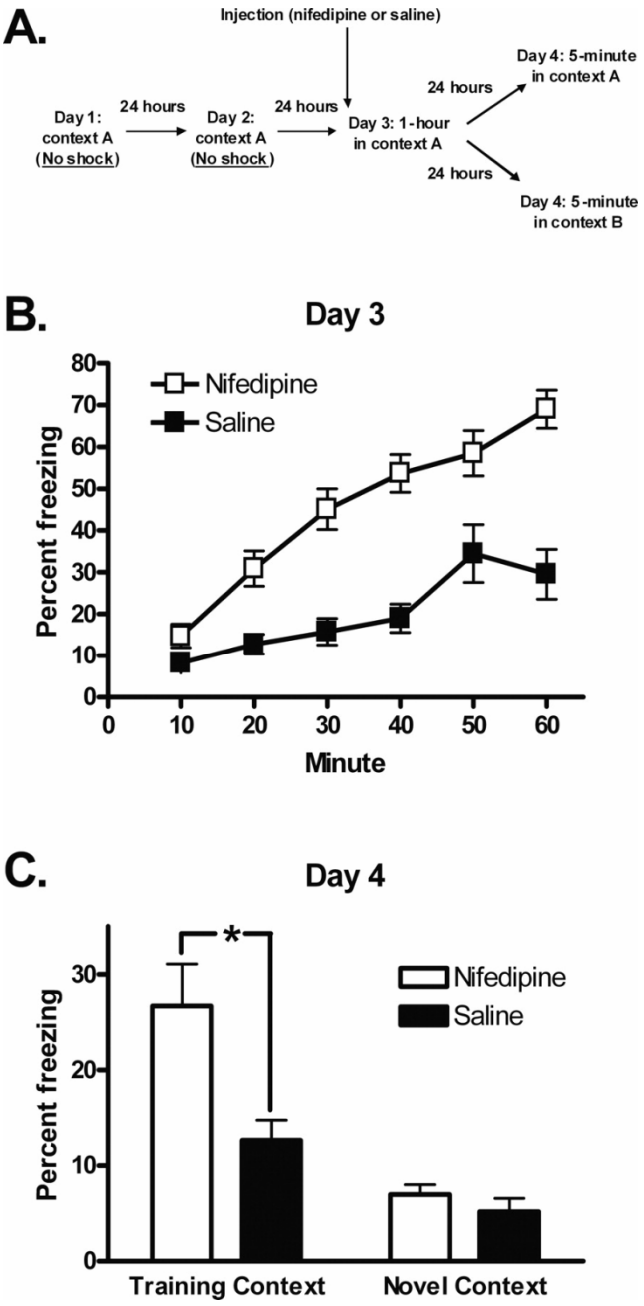


**Figure 3-5.** The long-term effect of nifedipine on freezing is context-specific. **(A)** Training protocol. **(B)** Fifty minutes following treatment with nifedipine, mice freeze more than saline-treated mice when placed in the conditioning chambers for 1 hour. **(C)** Nifedipine-treated mice freeze more than saline treated mice when returned to the conditioning chambers (training context) 24 hours after treatment. When the conditioning chambers are reconfigured as a novel context, freezing does not differ between nifedipine- and saline-treated mice. (*) $p < 0.05$. All data are presented as mean ± S.E.M

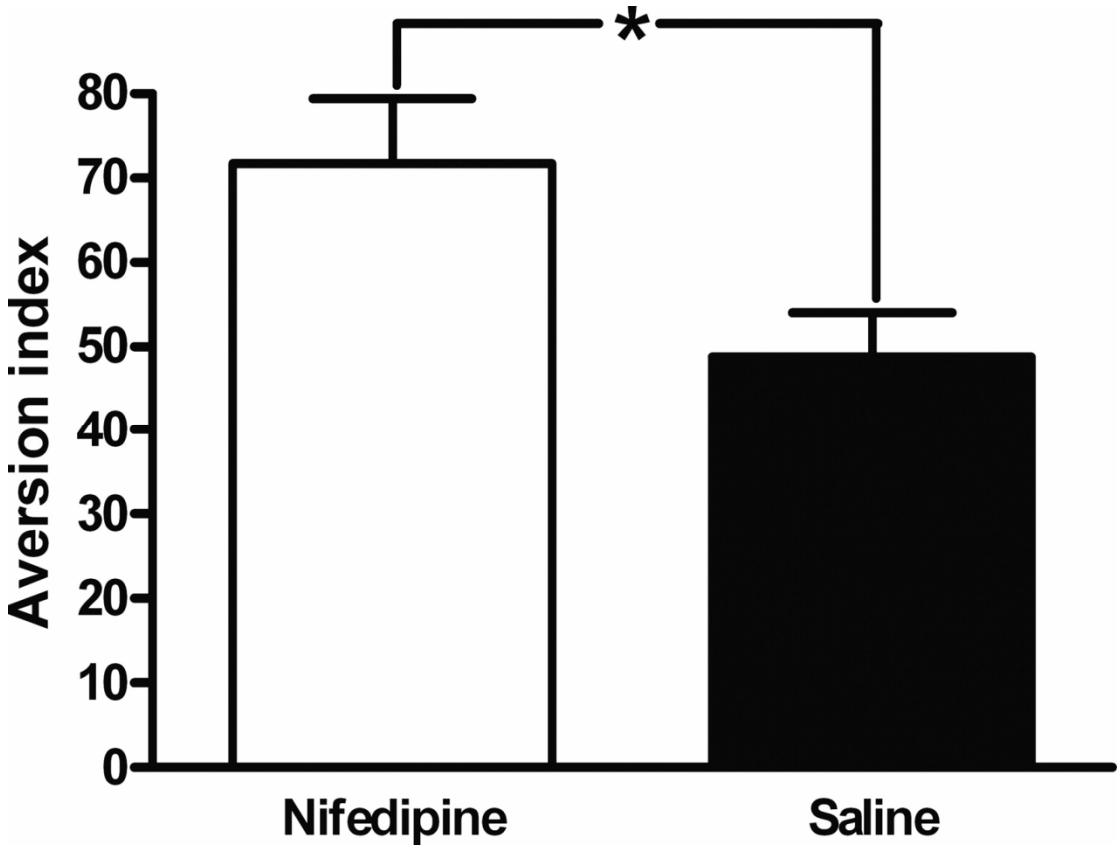


**Figure 3-6.** Nifedipine associates with taste to produce conditioned taste avoidance. WT mice learn to avoid saccharin following a saccharin-nifedipine pairing. (*) $p < 0.05$. All data are presented as mean ± S.E.M.

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## Chapter IV

## Impaired long-term potentiation and enhanced neuronal excitability in the basolateral amygdala of $Ca_V1.3$ knockout mice

**Abstract:**

Having previously shown that mice in which the gene for the L-type voltage-gated calcium channel (L-VGCC) $Ca_V1.3$ is deleted ($Ca_V1.3$ knockout mice) exhibit an impaired ability to consolidate contextually-conditioned fear, a type of learning and memory critically dependent on the basolateral amygdala (BLA), we were interested in the mechanisms by which $Ca_V1.3$ contributes to BLA neurophysiology. Others have shown that some forms of long-term potentiation (LTP) and neuronal excitability as measured by the afterhyperpolarization (AHP) are dependent on L-VGCCs. These studies, however, used L-VGCC antagonists that target both brain-expressed L-VGCCs ($Ca_V1.2$ and $Ca_V1.3$) and thus could not determine the relative contribution of each of the subunits to these phenomena. In this study, we used $Ca_V1.3$ knockout mice to explore the role of $Ca_V1.3$ in LTP and neuronal excitability in the BLA. We find that LTP in the BLA, induced by high-frequency stimulation of the external capsule, was greatly impaired in $Ca_V1.3$ knockout mice. Additionally, we found that BLA principal neurons from $Ca_V1.3$ knockout mice exhibited enhanced excitability as measured

by a reduced post-burst AHP and spike accommodation. Together, these results demonstrated altered neuronal function in the BLA of $Ca_V1.3$ knockout mice which may account for the impaired ability of these mice to consolidate contextually-conditioned fear.

**Introduction:**

Recently, we demonstrated that mice in which the gene for the L-type voltage-gated calcium channel (L-VGCC) $Ca_V1.3$ is deleted ($Ca_V1.3$ knockout mice) exhibit an impaired ability to consolidate contextually-conditioned fear (McKinney & Murphy 2006). As fear conditioning is known to be critically dependent on the basolateral amygdala (BLA) (Maren *et al.* 1996, Phillips & LeDoux 1992), the neurophysiological function of $Ca_V1.3$ in this structure is of interest. Long-term potentiation (LTP) and neuronal excitability are two neurophysiological phenomena that have been shown previously to be dependent on L-VGCCs, however, the specific L-VGCC subtype(s) mediating each has not previously been addressed

Long-term potentiation is a sustained, activity-dependent increase in synaptic strength that can be induced *in vitro* in brain slice preparations using several different stimulation patterns. Two patterns of stimulation commonly used are paired stimulation and high-frequency stimulation (HFS). Paired stimulation is the pairing of action potentials in the post-synaptic neuron, induced by somatic or

dendritic current injections, with stimulation of afferents to that same neuron. Delivery of a train of high-frequency stimulations to the afferents of a neuron or group of neurons causes the summation of synaptic potentials such that there is significant depolarization of the postsynaptic neuron. There are two major afferent pathways to the BLA, the thalamic pathway and the cortical pathway (the fibers of the cortical pathway travel within the external capsule (EC)). Stimulation of either of these pathways with a pairing protocol (Bauer *et al.* 2002, Weisskopf *et al.* 1999) or HFS protocol (Drephal *et al.* 2006, Chapman & Bellavance 1992, Chapman *et al.* 1990, Huang *et al.* 2000) leads to LTP at the synapses between these afferents and BLA neurons (BLA-LTP). Interestingly, induction of BLA-LTP by both paired stimulation of thalamic afferents (Bauer et al. 2002) and HFS of the EC have been shown to require L-VGCCs using pharmacological antagonists (Drephal et al. 2006, Chapman et al. 1990). It is interesting to note that the EC contains afferents from brain structures implicated in processing contextual information (Drephal et al. 2006, von Bohlen und Halbach & Albrecht 2002), information of the type with which $Ca_V1.3$ knockout mice have difficulty forming associations.

In addition to LTP, a number of groups have implicated L-VGCCs in neuronal excitability (Lima & Marrion 2007, Marrion & Tavalin 1998, Power *et al.* 2002, Shah & Haylett 2000). Neuronal excitability is often assessed experimentally by measuring the afterhyperpolarization (AHP). The AHP is a hyperpolarizing

voltage deflection that follows a burst of action potentials and serves to limit firing to a sustained depolarizing input (Alger & Nicoll 1980, Hotson & Prince 1980, Madison & Nicoll 1984). The AHP is often described as having 3 components: a fast, medium and a slow AHP. The fast AHP (fAHP) occurs immediately after individual action potentials and lasts only 1-5 ms. The medium AHP (mAHP) is typically observed after a burst of action potentials and has a decay constant of approximately 100 ms. The slow AHP (sAHP) has a time constant of 1-5 seconds and is voltage-independent (Faber & Sah 2007). Several studies using L-VGCC antagonists have shown that blockade of these channels leads to a significant reduction in the currents underlying the AHP, suggesting that the AHP is generated by calcium influx via L-VGCCs (Power *et al.* 2002, Shah & Haylett 2000, Lima & Marrion 2007, Marrion & Tavalin 1998) . In addition, an increase in expression of L-VGCCs in the hippocampus occurs with aging, and application of L-VGCC antagonists results in a reduction of the AHP in neurons from aging animals (Thibault *et al.* 2001, Thibault & Landfield 1996, Veng *et al.* 2003). Spike accommodation is another way to measure neuronal excitability. Studies showing that spike accommodation correlates with age-related increases in L-VGCC expression and activity suggest that this phenomena might also be L-VGCC-dependent (Disterhoft & Oh 2007).

In the present study, we investigated the role of $Ca_V1.3$ in BLA-LTP induced by HFS of the cortical pathway and the excitability of principal neurons of the BLA

using $Ca_V1.3$ knockout mice. We found that $Ca_V1.3$ is critical for BLA-LTP induced in this manner and for generation of the afterhyperpolarization (AHP). Spike accommodation is also reduced in BLA principal neurons of these mice. Together, these results suggest that neuronal function is impaired in the BLA of $Ca_V1.3$ knockout mice and this may account for the impaired ability of these mice to consolidate contextually-conditioned fear.

**Materials and Methods:**

*Mice:* The $Ca_v1.3$ knockout mice were generated by introducing a neomycin cassette into exon 2 of the gene for the pore-forming subunit of the $Ca_v1.3$ calcium channel (Platzer *et al.* 2000). The null allele was maintained on a C57BL/6NTac background by successively crossing (> 12 generations) heterozygous null offspring with C57BL/6NTac wild-type mice purchased from Taconic Farms (Hudson, NY). Experimental mice were bred onto a 129S6B6F2/Tac hybrid genetic background. To obtain experimental mice, 129S6B6F1/Tac hybrid mice were generated first by crossing heterozygous null C57BL/6NTac mice from the maintenance cross with wild-type 129S6/SvEvTac mice purchased from Taconic Farms (Hudson, NY). The $Ca_v1.3$ heterozygous null 129S6B6F1/Tac mice were subsequently intercrossed to obtain experimental mice. For all experiments approximately equal numbers of young (2-6 mos) male and female mice were used. All comparisons were made between $Ca_v1.3$ knockout mice and wild-type littermates and the experimenter was kept blind as

to the genotype throughout the experiment. All experiments were conducted in accordance with the guidelines set forth by the University of Michigan Committee on Use and Care of Animals.

*Slice Preparation:* Mice were anesthetized with isoflurane and decapitated. The brain was removed and horizontal slices were prepared as described in Drephal et al. (2006) and Stoop and Pralong (2000). Briefly, the brain was divided at the midline and each hemisphere was positioned flat on its medial surface. The olfactory bulb, the cerebellum, and the brain stem were subsequently removed and the dorsal side of the brain was cut along a plane orthogonal to the sagittal place, which was tilted at a $10^{o}$ posterosuperior-anteroinferior angle of a line passing between the lateral olfactory tract and the base of the brain stem. The exposed dorsal side of the brain was glued onto a cutting block, the brain was covered with ice-cold (<1°C) oxygenated sucrose-based cutting solution containing the following (in mM): 2.8 KCl, 1 $MgCl_2$, 2 $MgSO_4$, 1.25 $NaH_2PO_4$, 1 $CaCl_2$, 206 sucrose, 26 $NaHCO_3$, 10 D-glucose, 0.40 ascorbic acid and horizontal slices (400 μm) were prepared with a vibratome. These slices contained the ventral hippocampus, the medial and lateral divisions of the entorhinal cortex, the perirhinal cortex, and, the BLA. Slices were transferred to a holding chamber filled with oxygenated artificial cerebrospinal fluid (aCSF) containing the following (in mM):124 NaCl, 2.8 KCl, 2 $MgSO_4$, 1.25 $NaH_2PO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 D-glucose, 0.40 ascorbic acid at room temperature and

remained there for at least 1 hour before being individually transferred to a submersion chamber and continuously perfused (~1.5 ml/min) with oxygenated aCSF heated to 31°C.

*Electrophysiology:* Field potentials were recorded using glass-pipettes made from Clark Borosilicate Standard Wall glass (Warner Instruments), pulled using a P-97 Flaming-Brown pipette puller (Sutter Instruments), filled with ACSF (tip resistances ~ 1 MΩ), and placed in the BLA. Bipolar (Pt/Ir) stimulation electrodes were used to stimulate the external capsule (EC) which contains fibers from the perirhinal and entorhinal cortex (von Bohlen und Halbach & Albrecht 2002, Drephal et al. 2006). Stimulation of the EC results in a compound waveform that contains the summation of both EPSPs and synchronized action potentials (Doyere *et al.* 2003, Watanabe *et al.* 1995). Because the slope measure of this waveform in the lateral amygdala is more sensitive to variability and noise in the (Doyere et al. 2003), we analyzed the amplitude of field potentials in the present study.

Basal synaptic transmission was assessed by examining the input-output relationship within the BLA. Input-output response curves were constructed by varying the intensity of single-pulse stimulation, and averaging five responses to each intensity. For experiments that examined LTP, the stimulus intensity that evoked a field potential amplitude equal to ~50% of the maximal response was then used for high-frequency stimulation (HFS) as well as single-pulse

stimulations that preceded and followed the HFS. A stable baseline of responses was obtained for at least 5 minutes and then a HFS consisting of two trains at 100 Hz (1 s duration; 30 s apart) was administered. Subsequent responses to single stimuli were recorded for 60 min, and their amplitude quantified as change in percentage with respect to baseline. Stimulus frequency pre- and post-HFS was 0.067 Hz.

To investigate changes in neuronal excitability, whole-cell recordings were made from BLA principal neurons using a Dagan BVC-700A amplifier in bridge mode using the "blind" method (Blanton *et al.* 1989). Patch-pipettes were made from Clark Borosilicate Standard Wall glass (Warner Instruments), pulled using a P-97 Flaming-Brown pipette puller (Sutter Instruments) with resistances of 3-6 MΩ, and filled with the following internal solution (in mM): 120 potassium methylsulfate, 20 KCl, 10 HEPES, 4 $Na_2$-ATP, 2 $MgCl_2$, 0.3 GTP, 0.2 EGTA, 7 phosphocreatine. Seal resistances of >1 GΩ were achieved prior to rupturing into whole-cell mode. A neuron was considered to be principal neuron (Washburn & Moises 1992) and acceptable for data collection (i.e., healthy) if (1) it had a resting membrane potential more negative than -58 mV, (2) it exhibited action potentials that overshot > 0 mV, (3) exhibited spike accommodation to a sustained depolarizing current pulse, and (4) exhibited a "sag" current in response to a strong hyperpolarizing current pulse. Action potentials were measured from rest and were analyzed for spike threshold, amplitude and width.

Spike width was measured at ½ of the action potential amplitude. Neurons were held at 5 mV below action potential threshold and the AHP was studied using a 100 ms current step sufficient to elicit 5 action potentials. AHP measurements were made from the average of 10 successive traces from each neuron. Spike accommodation was studied at rest in these neurons using a series of 500 ms current injection of increasing intensity (-0.05 nA to 0.35nA, .05 nA steps).

Data were acquired and analyzed using pClamp 10.0 (Axon Instruments). Sample sizes refer to number of cells or slices (data was collected from a maximum of 2 cells or slices per mouse) and all values are expressed as mean ± SEM. Statistical analysis was performed using student t-tests or ANOVA using post hoc student t-tests where appropriate.

## Results:

**$Ca_V$1.3 knockout mice exhibit intact synaptic transmission, but impaired LTP.** An input-output response curve was constructed to assess synaptic transmission in $Ca_V$1.3 knockout mice (**Figure 4-1**). BLA field potential amplitudes increased with EC stimulus intensity ($F_{(6, 150)} = 90.3$, $p < 0.05$; **Figure 4-1**), but did not differ between wild-type (N = 15) and $Ca_V$1.3 knockout (N = 12) mice ($F_{(1, 25)} = 0.16$, $p > 0.05$; **Figure 4-1**)

After establishing that that synaptic transmission was intact in $Ca_V1.3$ knockout mice, we investigated the ability of $Ca_V1.3$ knockout mice to exhibit BLA-LTP following HFS delivered via the EC. **Figure 4-2** shows the effect of HFS on field potential amplitude in the BLA. Paired t-tests reveal that average field potential 55-60 minutes post-HFS was significantly potentiated over the average field potential during the five-minute baseline in wild-type (N=13; $t_{(1, 11)} = 5.2$, $p < 0.05$; **Figure 4-2**) and $Ca_V1.3$ knockout mice (N=11; $t_{(1, 9)} = 2.8$, $p < 0.05$; **Figure 4-2**). However, an unpaired t-test between genotypes showed that field potential amplitudes were significantly less potentiated in $Ca_V1.3$ knockout mice (N=11; 112.7 ± 4.6 %) than wild-type mice (N=13; 144.3 ± 8.6 %). This suggests that $Ca_V1.3$ knockout mice are impaired in the ability to exhibit BLA-LTP following HFS to the EC.

**$Ca_V1.3$ knockout mice exhibit enhanced excitability: reduced AHPs and spike accommodation.** $Ca_V1.3$ knockout mice did not differ from wild-type mice with respect to membrane properties such as resting membrane potential, input resistance, action potential threshold, width, or peak amplitude (unpaired t-tests, $p > 0.05$; **Table 4-1**).

While deletion of $Ca_V1.3$ did not significantly alter membrane properties at rest or during a single action potential, loss of $Ca_V1.3$ resulted in substantial changes in

firing properties in response to more prolonged depolarization. The AHP that resulted after 5 action potentials was significantly smaller in the $Ca_V1.3$ knockout mice in overall area (N = 14; 5552.8 ± 762.2 mV ● ms) when compared with the AHP recorded in wild-type mice (N = 16; 10003.8 ± 1249.6.2 mV ● ms; $t_{(1, 28)}$ = 2.9, $p < 0.05$; **Figure 4-3**). A change in the overall area of the AHP could be the result of alterations in either the mAHP or sAHP. To determine whether the mAHP was affected in $Ca_V1.3$ knockout mice, we measured the amplitude of the AHP at its peak (~60 ms after current stimulus offset) and 200 ms after current stimulus offset. The peak amplitude of the AHP did not differ between wild-type (N = 16; -9.9 ± 0.6 mV) and $Ca_V1.3$ knockout mice (N = 14; -9.6 ± 0.5 mV; $t_{(1, 28)}$ = 0.6, $p > 0.05$; **Figure 4-4A**). There was a strong, but statistically non-significant, trend for a smaller AHP amplitude at 200 ms after current stimulus offset in $Ca_V1.3$ knockout mice (N = 14; -4.8 ± 0.4 mV; **Figure 4-4B)** compared with wild-type mice (N = 16; -6.0 ± 0.5 mV; $t_{(1, 28)}$ = 2.0 p = 0.06; **Figure 4-4B)**. To assess if the sAHP was affected in $Ca_V1.3$ knockout mice, we measured the amplitude of the AHP at 1000 ms after current stimulus offset. The AHP amplitude at 1000 ms was significantly smaller in $Ca_V1.3$ knockout mice (N = 14; -1.7 ± 0.3 mV; **Figure 4-4C)** than wild-type mice (N = 16; -2.9 ± 0.3 mV; $t_{(1, 28)}$ = 2.4, $p < 0.05$; **Figure 4-4C)** suggesting that deletion of $Ca_V1.3$ selectively impacts the generation of the sAHP. Additionally, AHP duration is significantly shorter in $Ca_V1.3$ knockout mice (N = 14; 4.4 ± 0.7 seconds; **Figure 4-4D**) than in wild-type mice (N = 16; 6.7 ± 0.6 seconds, $t_{(1, 28)}$ = 2.5; $p < 0.05$, **Figure 4-4D**).

In addition to altering the sAHP, deletion of $Ca_V1.3$ also resulted in a decrease in spike accommodation (**Figure 4-5**). Prolonged current steps (500 ms) consistently generated more spikes in BLA principal neurons from $Ca_V1.3$ knockout mice (N = 14; **Figure 4-5A**) when compared with wild-type mice (N = 16; $F_{(6, 168)} = 4.6$; $p < 0.05$, **Figure 4-5A)**. Instantaneous firing frequency of the first four spikes generated by a 500 ms, 0.35 nA current injection were also calculated, $Ca_V1.3$ knockout mice (N = 14) were found to exhibit significantly higher frequencies than wild-type mice (N = 16) for the third (45.8 ± 3.7 vs. 31. 9 ± 4.0 seconds$^{-1}$; $t_{(1, 28)} = 2.5$; $p < 0.05$, **Figure 4-5B**) and fourth (37.5 ± 3.0 vs. 25.0 ± 2.8 seconds$^{-1}$; $t_{(1, 28)} = 3.0$; $p < 0.05$, **Figure 4-5B**) spikes further pointing to reduced spike accommodation in $Ca_V1.3$ knockout mice.

## Discussion:

We demonstrated altered neuronal function in the BLA of $Ca_V1.3$ knockout mice. Deletion of $Ca_V1.3$ results in reduced HFS-induced LTP in the cortical pathway (via stimulation of the EC) to the BLA (BLA-LTP). Principal neurons in the BLA of $Ca_V1.3$ knockout mice exhibit enhanced excitability as measured by the AHP and spike accommodation. These findings suggest a critical role for $Ca_V1.3$ in BLA-LTP induced in this manner and in principal neuron excitability in the BLA.

Several studies of BLA-LTP have demonstrated L-VGCC-dependence (Bauer et al. 2002, Chapman et al. 1990, Drephal et al. 2006). The dependence of BLA-LTP on L-VGCCs seems dependent on which afferents are stimulated and the stimulation protocol. The major afferent pathways to the amygdala include the thalamic pathway carrying auditory afferents and the cortical pathway traveling in the EC and carrying afferents from higher-order sensory cortices (de Olmos *et al.* 1985, Pitkänen *et al.* 1997). In the thalamic pathway, BLA-LTP induced by pairing presynaptic stimulation with postsynaptic depolarization is L-VGCC-dependent, whereas HFS stimulation that produces prolonged postsynaptic depolarization but not spikes is L-VGCC-independent (Bauer et al. 2002, Weisskopf et al. 1999). BLA-LTP induced by HFS of the EC is L-VGCC-independent in the coronal slice preparation, but strongly L-VGCC-dependent in the horizontal slice preparation (Drephal et al. 2006, Huang et al. 2000). For this reason as well as the fact that the EC of the horizontal slice preparation contains intact afferents from brain regions (hippocampus, perirhinal and entorhinal cortices) critical for encoding contextual information of the type with which $Ca_V1.3$ knockout mice are impaired in forming associations (von Bohlen und Halbach & Albrecht 1998, von Bohlen und Halbach & Albrecht 2002), we chose to work with the horizontal slice preparation in this study. Additionally, it is advantageous that LTP can be induced in the horizontal slice preparation without blocking inhibitory transmission as is the case with other preparations (Drephal et al. 2006). To date, all studies that have explored the L-VGCC dependence of BLA-LTP have relied on the use of L-VGCC antagonists. Because all currently available L-

VGCC antagonists block both brain-expressed L-VGCCs, $Ca_v1.2$ and $Ca_v1.3$, the pharmacological approach does not allow for the elucidation of their relative contribution to phenomenon like LTP. Using mice in which the genes for $Ca_v1.2$ and $Ca_v1.3$ have been deleted, it is possible to dissect the contributions of each brain-expressed L-VGCCs. When the horizontal slice preparation is treated with L-VGCC antagonists, HFS stimulation of the EC does not lead to potentiation of the field potential 1 hour after HFS. In this study, we show, using $Ca_V1.3$ knockout mice, that $Ca_V1.3$ is responsible for much of the potentiation observed following HFS of the EC. In fact, $Ca_V1.3$ knockout mice exhibit only ~25% as much field potential potentiation as wild-type mice 1 hour after HFS, suggesting that $Ca_V1.3$ mediates ~75% of the LTP blocked by L-VGCC antagonists and $Ca_V1.2$ likely mediates the remaining ~25%. It is interesting to note that a previous study found that L-VGCC-dependent LTP in the hippocampus of $Ca_V1.3$ knockout mice was not impaired (Clark *et al.* 2003). This suggests that different L-VGCC subtypes subserve L-VGCC-dependent LTP in different brain regions. Alternatively, the discrepancy between our LTP data and that from the hippocampus of $Ca_V1.3$ knockout mice may reflect differences in the stimulation protocols used.

A limitation to the genetic approach presented here and the pharmacological approach used by others is the inability to determine which cell population is responsible for the electrophysiological observation. Because the $Ca_V1.3$

knockout mice lack $Ca_V1.3$ in all cell types and pharmacologically L-VGCC antagonists are not cell-type specific, neither approach can elucidate which population of cells is responsible for the effect observed. In the future, it will be of interest to know in which cell population(s) $Ca_V1.3$ function is critical. This could be addressed by generation of a mouse line in which the gene for $Ca_V1.3$ is floxed so that cell line specific deletion can produced by mating with mouse lines that express cre-recombinase in cell specific manner.

Like BLA-LTP, neuronal excitability measured by the AHP is L-VGCC dependent. Several studies using L-VGCC antagonists have shown that blockade of these channels leads to a significant reduction in the AHP (Shah & Haylett 2000, Lima & Marrion 2007, Marrion & Tavalin 1998, Power et al. 2002). Increased activity and expression of L-VGCCs in the hippocampus occurs with aging and is correlated with an increase in the AHP and spike accommodation, an effect that is reversed with the application of L-VGCC antagonist (Moyer *et al.* 2000, Thibault *et al.* 2001, Thibault & Landfield 1996, Veng & Browning 2002). Previously, using mice in which the genes for $Ca_V1.2$ or $Ca_V1.3$ were deleted, we demonstrated that $Ca_V1.3$ mediates the sAHP in the CA1 region of the hippocampus but does not affect spike accommodation. Deletion of $Ca_V1.2$ does not have this effect on the sAHP (Kuo et al., submitted). Here, as in the CA1 region of the hippocampus, we show that $Ca_V1.3$ mediates the AHP in the principal neurons of the lateral amygdala. Unlike in the hippocampus, however,

principal BLA neurons exhibited significantly less spike accommodation than wild-type mice. These effects on the AHP and spike accommodation in principal BLA neurons from $Ca_V1.3$ knockout mice were seen in the absence of any effects on basic membrane properties. Though this difference between the spike accommodation data may represent a different role for $Ca_V1.3$ in amygdala than in the hippocampus, we feel it more likely reflects a difference the absolute size of AHPs recorded in each study. AHPs in this study were 2-3 times larger than those recorded in Kuo et. al (submitted) and thus more likely to affect spike accommodation. The difference in size of the AHP between the two studies as well as the spike accommodation effect likely reflects a difference in the recording methods used (visualized vs. “blind”) since recordings from CA1 pyramidal neurons using the “blind” method closely resemble those from the BLA with respect to AHP size and spike accommodation (McKinney et al, unpublished results). Because the AHP amplitude is significantly reduced at 1000 ms after current stimulus offset, but not 200 ms or at the peak of the AHP (~60 ms after current stimulus offset) in $Ca_V1.3$ knockout mice, it seems that $Ca_V1.3$ is selectively involved in the sAHP. This is supported by the observation that AHP duration is significantly shorter in $Ca_V1.3$ knockout mice than in wild-type mice as the sAHP is the only component of the AHP with a time constant on the order of seconds (Storm 1990) and thus capable of affecting the duration so dramatically. It is worth noting that though not statistically significant, there is a strong trend toward a reduction in the AHP amplitude at 200 ms after current stimulus offset, suggesting that $Ca_V1.3$ may mediate the mAHP to a modest extent. Though

significantly reduced, a residual sAHP is present in the principal BLA neurons of $Ca_V1.3$ knockout mice. This is consistent with previous studies demonstrating the lack of complete abolition of the sAHP using pharmacological blockade of L-VGCCs (Marrion & Tavalin 1998, Lima & Marrion 2007, Shah & Haylett 2000), and suggests that additional calcium sources contribute to the generation of the sAHP. One possibility is that the calcium source for the residual sAHP is calcium influx through the other brain-expressed L-VGCC, $Ca_V1.2$. This seems somewhat unlikely in light of our previous demonstration that the sAHP in mice lacking the gene for $Ca_V1.2$ in CA1 neurons of the hippocampus is similar to that of wild-type mice (Kuo et al, submitted), however brain-region specific differences in sAHP generation cannot be ruled out.

It is also possible that calcium from intracellular calcium stores can also mediate the residual sAHP. Indeed, disruption of calcium-induced calcium release (CICR) from intracellular calcium stores has been shown to reduce the currents that underly the sAHP (Shah & Haylett 2000, Torres *et al.* 1996, Borde *et al.* 2000). One study even found a reduced sAHP after inhibition of CICR (Shah & Haylett 2000, Torres *et al.* 1996). It is important to note, however, that studies in which CICR was implicated in generation of the sAHP used unclamped calcium spikes (Borde et al. 2000, Torres et al. 1996) or prolonged spike trains (Shah & Haylett 2000) to activate the sAHP. In contrast, in the present study the AHP was elicited with only 5 action potentials. Interestingly, CICR blockade reverses the age-

related increase in the sAHP, but has no effect on the sAHP in young animals (Gant *et al.* 2006). Whether this age-dependent role for CICR in sAHP generation reflects increase in calcium release from intracellular stores or an increase in activation of CICR in response to an age-related increase in L-VGCC channel density is not known. If CICR is responsible for the residual sAHP, our data would suggest that the source of calcium that triggers the calcium release from internal stores does not require $Ca_V1.3$.

A final explanation for the residual sAHP may lie in the action of other voltage-gated calcium channels. The residual sAHP may be mediated by P-, Q-, or N-type voltage-gated calcium channels. In support of this possibility is the observation that the N-type calcium channel blocker ω-conotoxin-GIVA significantly reduces $I_{sAHP}$ in cultured rat pyramidal neurons (Shah & Haylett 2000).

The fact that BLA-LTP and neuronal excitability, two hypothesized neurobiological substrates for learning and memory, are altered in $Ca_v1.3$ knockout mice makes it tempting to speculate that one or both of the abnormalities leads to the impaired ability of $Ca_v1.3$ knockout mice to consolidate contextual fear learning. Of the two major brain structures known to be necessary for context fear learning, the hippocampus and BLA, the BLA seemed more likely

as we previously demonstrated that the hippocampus in $Ca_v1.3$ knockout mice is capable of encoding spatial information (McKinney 2006) and LTP in the hippocampus of $Ca_V1.3$ knockout mice is intact (Clark et al. 2003). Within the BLA, a strong case can be made that LTP is necessary for fear learning. Sensory information about many potential conditioned and unconditioned stimuli, including auditory, contextual, and somatosensory information, converge on the BLA (Romanski *et al.* 1993, von Bohlen und Halbach & Albrecht 1998, von Bohlen und Halbach & Albrecht 2002) and LTP has been demonstrated in each of these afferent pathways (Bauer et al. 2002, Drephal et al. 2006, Huang et al. 2000, Paul F. Chapman 1990, Rogan *et al.* 1997, Weisskopf et al. 1999). Additionally, fear learning has been shown to modify synaptic strength of afferents on o BLA neurons in a way similar to experimentally-induced LTP (McKernan & Shinnick-Gallagher 1997). Further, fear learning and BLA-LTP share similar stimulus contingencies (Bauer *et al.* 2001) and molecular mechanisms (Rodriques *et al.* 2004). Together, these observations suggest that an LTP-like mechanism underlies fear learning in the BLA. An example of the shared molecular mechanisms of fear learning and BLA-LTP which is consistent with our findings comes from a study in which Bauer et al (2002) demonstrated that the L-VGCC antagonist blocks BLA-LTP as well as long-term fear memory when a L-VGCC antagonist was infused into the BLA. Based on these studies it would be logically consistent to hypothesize that the deficits in contextual fear conditioning consolidation observed in the $Ca_v1.3$ knockout mice is due to a reduction in BLA-LTP, however this hypothesis may be simplistic. There is a significant disparity in

time course between the BLA-LTP experiments and the deficits observed in the $Ca_v1.3$ knockout mice with regard to the consolidation of contextual fear conditioning (see figure 2 in McKinney and Murphy 2006). This disparity has been reported frequently in the literature. Many manipulations (genetic and pharmacological) that impair long-term but not short-term memory block LTP within minutes after induction. The work of Bauer et al. (2002) provides a relevant example. Application of the L-VGCC antagonist verapamil impaired BLA-LTP induced by paired stimulation of the EC immediately, whereas intra-amygdala infusion of the same L-VGCC antagonist before fear learning resulted in impairment performance 24 hours later, but not at earlier time points. Some have argued that this disparity in time course may reflect a difference in the way in which LTP is engaged naturally *in vivo* from the way it is studied *in vitro.* These differences include patterns of stimulation and modulation by other areas of the brain (Schafe *et al.* 2001). Experiments using *in vivo* electrophysiological methods will be necessary to resolve this question. The LTP underlying fear memories may not be induced during training, but rather at a later time point, for example, during sleep. There is evidence that consolidation of memories may occur via the replaying of newly acquired patterns of activity during sleep (Axmacher *et al.* 2006). Replaying of specific patterns of neuronal activity during sleep may induce LTP.

Like LTP, changes in neuronal excitability have been proposed as a neurobiological substrate of learning. It has been suggested that enhanced excitability is not part of the encoding of learning per se, but rather serves a permissive function for synaptic modification (Zhang & Linden 2003). It is proposed that when a neuron is more excitable, the threshold for LTP is lower, thus information encoding is facilitated. Following this line of reasoning, one would expect animals with more excitable neurons to be better capable at encoding information and thus more efficient learners. Our results suggest this is not the case in $Ca_v1.3$ knockout mice. That is, though $Ca_v1.3$ knockout mice exhibit enhanced excitability in principal neurons of the BLA (the present study) and in CA1 neurons of the hippocampus (Kuo et al., submitted; McKinney et al., unpublished results), neither hippocampus-dependent learning (Morris water maze) nor BLA-dependent learning (contextual fear conditioning) is enhanced (McKinney & Murphy 2006). It could be that enhanced excitability does facilitate learning, but $Ca_v1.3$ knockout mice have other deficits that interfere with learning (e.g., impaired BLA-LTP). Further behavioral characterization of $Ca_v1.3$ knockout mice may reveal that there are some learning tasks on which they perform better than wild-type mice. Our data, however, are consistent with other data in which manipulations that enhance excitability rescue impaired learning and memory in aged animals (Murphy *et al.* 2005, Disterhoft *et al.* 1996), but not young animals (Giese *et al.* 1998, Disterhoft et al. 1996).

L-VGCCs have previously been implicated in amygdala neurophysiology, including LTP and neuronal excitability, but these data are the first to implicate $Ca_v1.3$ specifically. The role of the other brain-expressed L-VGCC, $Ca_v1.2$, in these processes should be explored in future studies. Though it is tempting to speculate that changes in BLA-LTP and neuronal excitability could account for the impaired ability of $Ca_v1.3$ knockout mice to consolidate conditioned fear, thorough understanding of the mechanism by which this learning deficit arises awaits further study.

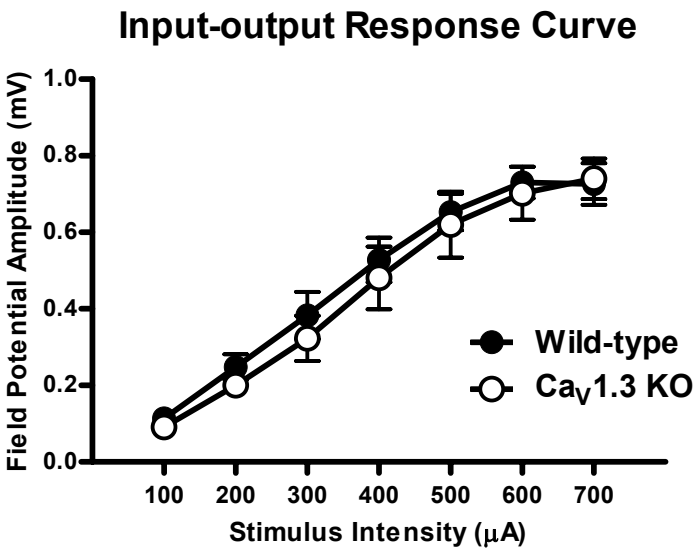


**Figure 4-1.** Synaptic transmission in the EC-LA pathway does not differ between $Ca_V1.3$ knockout (N=12) and wild-type (N=15) mice. Field potential amplitude in the LA increases and plateaus with increasing stimulation of the EC in both $Ca_V1.3$ knockout and wild-type mice and there are no differences in this response between the genotypes. All data are presented as mean ± S.E.M.

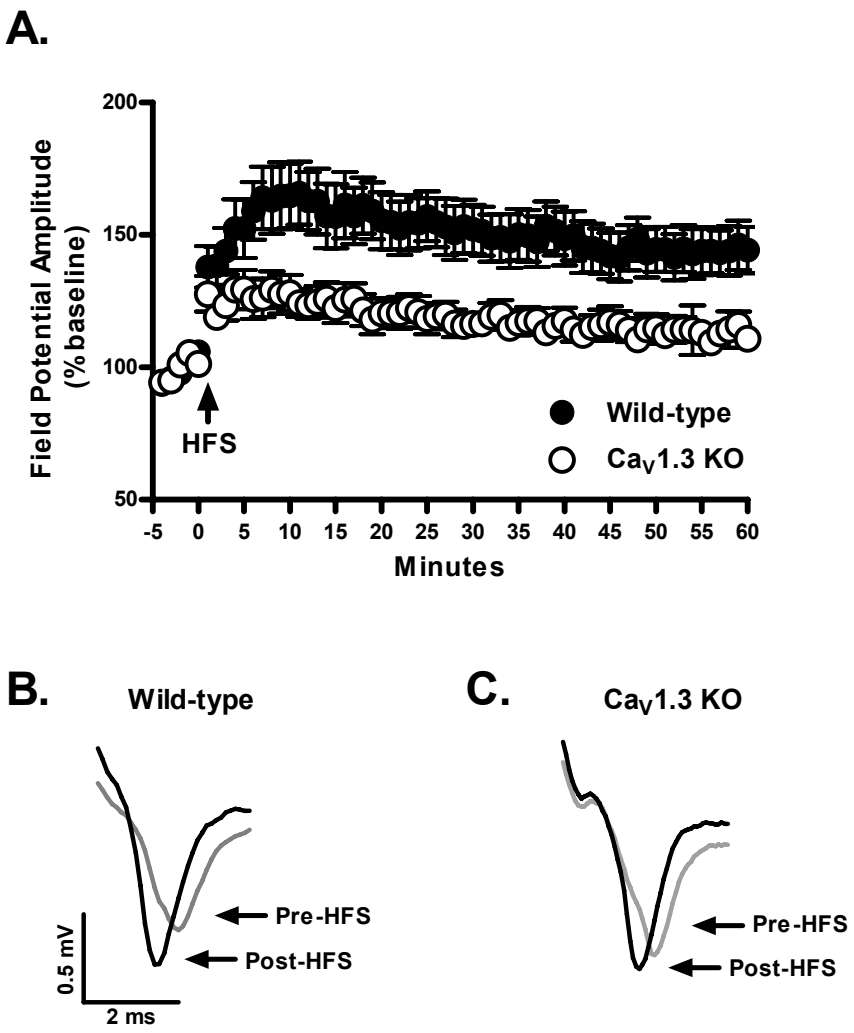


**Figure 4-2.** Long-term potentiation of BLA field potentials (BLA-LTP) induced by high-frequency stimulation (HFS) of the EC is impaired in $Ca_V1.3$ knockout mice. **(A)** Significant BLA-LTP is induced by HFS in both wild-type (N=13 slices) $Ca_V1.3$ knockout (N=11 slices), but BLA-LTP in $Ca_V1.3$ knockout mice is significantly smaller than in wild-type mice. Data points represent averaged amplitudes normalized with respect to baseline. All data are presented as mean ± S.E.M. **(B)** Representative traces of field potentials evoked by EC stimulation before and after HFS stimulation in wild-type mice. **(C)** Representative traces of field potentials evoked by EC stimulation before and after HFS stimulation in $Ca_V1.3$ knockout mice.

| | RMP (mV) | IR (mΩ) | AP Threshold (mV) | AP Width (ms) | AP Height (mV) |
|---|---|---|---|---|---|
| Wild-type | -65.4 ± 1.5 | 173.3± 10.3 | 48.4 ± 1.7 | 1.2 ± 0.05 | 90.9 ± 2.4 |
| Knockout | -63.6 ± 1.2 | 147.1± 13.9 | 47.2 ± 0.9 | 1.4 ± 0.08 | 86.6 ± 2.1 |

**Table 4-1.** Biophysical properties of BLA principal neurons are similar in wild-type (N=16 neurons) and $Ca_V1.3$ knockout (N=14 neurons) mice. Abbreviations: RMP, resting membrane potential, IR, input resistance, AP, action potential. All data is presented as mean ± S.E.M.

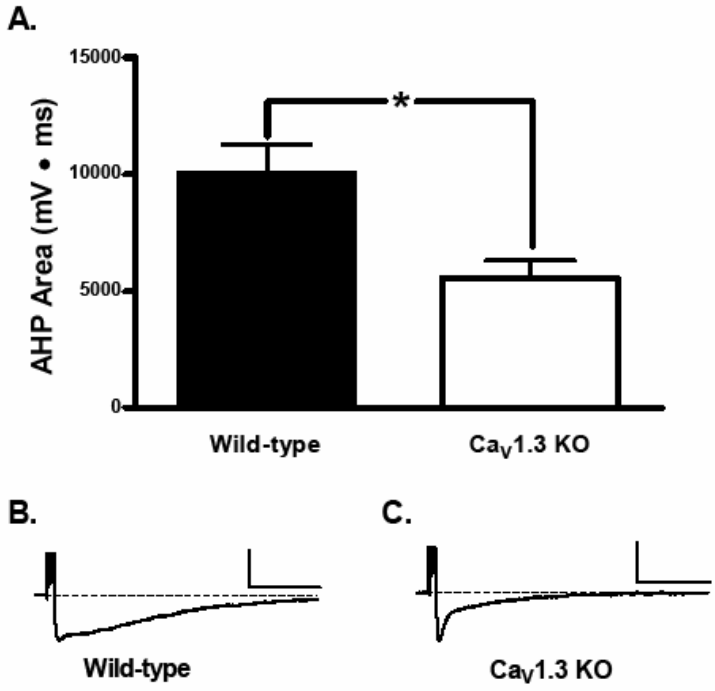


**Figure 4-3.** Area of the AHP is significantly smaller in $Ca_V1.3$ knockout mice. **(A)** Group data exhibiting that the area of the AHP in $Ca_V1.3$ knockout mice (N=14 neurons) is ~50% of that in wild-type mice (N=16 neurons). **(B)** Representative trace of AHP from a neuron of a wild-type mouse. **(C)** Representative trace of AHP from a neuron of a $Ca_V1.3$ knockout mouse. (*) $p < 0.05$. All data are presented as mean ± S.E.M.

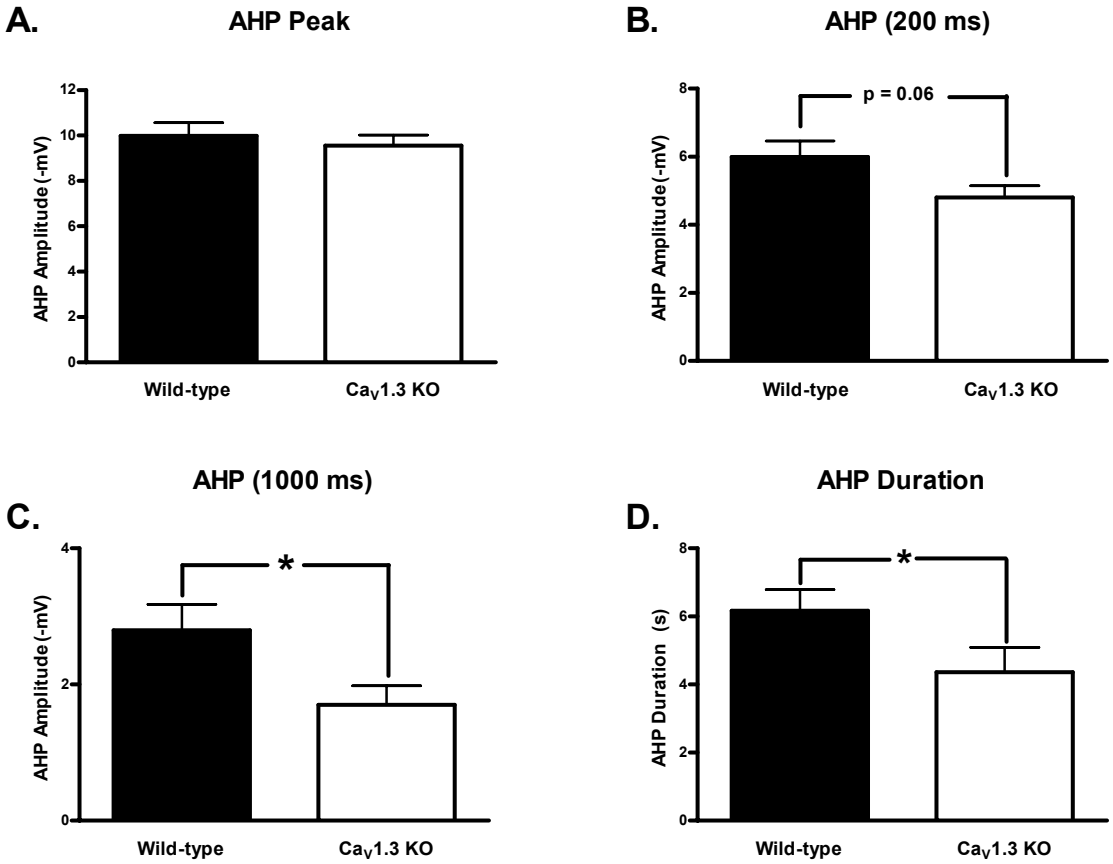


**Figure 4-4.** AHP parameters in which sAHP is major component are selectively affected in $Ca_V1.3$ knockout mice. **(A)** AHP peak amplitude does not differ between wild-type (N=16 neurons) and $Ca_V1.3$ knockout (N=14 neurons) mice. **(B)** There is a strong, but statistically insignificant, trend for a smaller AHP amplitude at 200 ms after stimulus offset in $Ca_V1.3$ knockout than wild-type mice. **(C)** The AHP amplitude at 1000 ms after stimulus offset is significantly smaller in $Ca_V1.3$ knockout mice than wild-type mice. **(D)** The duration of the AHP is significantly shorter in $Ca_V1.3$ knockout mice than wild-type mice. (*) $p < 0.05$. All data are presented as mean ± S.E.M.

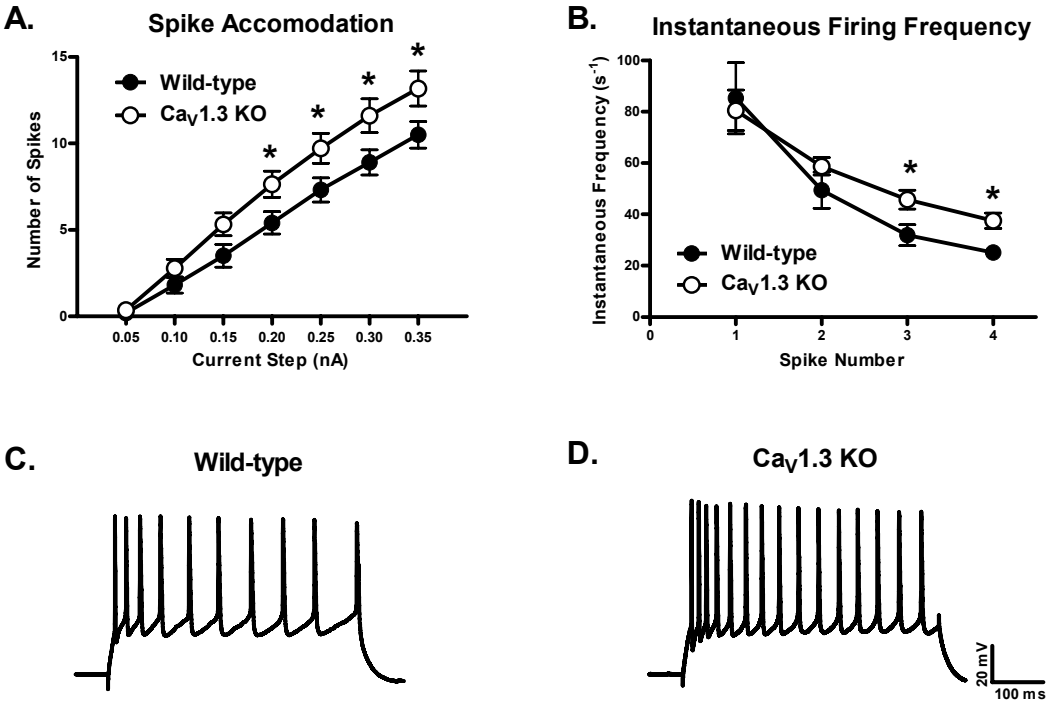


**Figure 4-5.** BLA principal neurons from $Ca_V1.3$ knockout (N = 14 neurons) mice exhibit greater excitability than neurons from wild-type (N= 16 neurons) mice. **(A)** Spike accommodation is less pronounced in neurons from $Ca_V1.3$ knockout mice than wild-type neurons. **(B)** Instantaneous fire frequency is significantly greater in neurons from $Ca_V1.3$ knockout mice than it is in neurons from wild-type mice. **(C)** Representative trace from a wild-type neuron during a 500 ms, 0.35 nA current injection. **(D)** Representative trace from a $Ca_V1.3$ knockout neuron during a 500 ms, 0.35 nA current injection. (*) $p < 0.05$. All data are presented as mean ± S.E.M.

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# Chapter V

# Conclusion

## Overview of rationale and findings:

Fear conditioning and fear extinction are popular models for studying the neurobiology of fear, associative and inhibitory learning, and fear disorders. Recently, the molecular mechanisms underlying fear conditioning and fear extinction have been a particularly active subject for research. Intense interest and active investigation into this area has led to a greater understanding of the molecular mechanisms of fear conditioning and fear extinction. It is interesting that many of the same molecules found to be important for fear conditioning are also implicated in fear extinction. In this dissertation, I focused my studies on one such class of molecules, the L-type voltage-gated calcium channels (L-VGCCs). Previously, others used L-VGCC antagonists to demonstrate a role for L-VGCCs in consolidation and extinction of conditioned fear. Though L-VGCC antagonists are powerful in elucidating a prominent role for LVGCCs in conditioned fear, they do not permit identification of the specific LVGCCs involved. There are two major subtypes of brain LVGCCs, $Ca_v1.2$ and $Ca_v1.3$, both of which are the targets of currently-available L-VGCC antagonists. As $Ca_v1.2$ and $Ca_v1.3$ have different cellular and sub-cellular distributions (Hell *et al.* 1993b), exhibit different biophysical properties

(Helton *et al.* 2005, Xu 2001), and have been implicated in quite different physiological processes (Cui *et al.* 2007, Nitert *et al.* 2008), it seemed to me that an understanding of the specific subtype(s) involved in fear conditioning and fear extinction would be valuable for understanding fear, associative and inhibitory learning, and could have implications for human health. To address the question of subunit-specific involvement of L-VGCCs in fear conditioning and fear extinction, I characterized the ability of mice in which the gene for either of the brain-expressed L-VGCCs was deleted ($Ca_v1.2^{cKO}$ and $Ca_v1.3$ knockout mice) to consolidate and extinguish conditioned fear. As is detailed in chapters 2 and 3 of this dissertation, I demonstrated that $Ca_v1.3$, but not $Ca_v1.2$, mediates consolidation of conditioned fear and neither $Ca_v1.2$ nor $Ca_v1.3$ alone plays a role in extinction of conditioned fear. I go on to show that previous studies suggesting a role for L-VGCCs in extinction of conditioned fear were likely confounded by an aversive state produced by the L-VGCC antagonists used in those studies. In chapter 4 of this dissertation, I explore the role of $Ca_v1.3$ in basolateral amygdala (BLA) neurophysiology, a brain structure implicated in both fear conditioning and fear extinction. I show that $Ca_v1.3$ mediates long-term potentiation (LTP) and neuronal excitability in this structure.

**<u>$Ca_v1.3$ mediates consolidation of conditioned fear:</u>**

In 1949, D. O. Hebb introduced the “dual trace” hypothesis of memory formation. Hebb proposed that reverberation of activity within assemblies of neurons was the essence of short-term memory and that if maintained long enough some

growth processes at the level of the synapse could lead to long-term memory (Hebb 2002). This hypothesis about the cellular basis of memory has been the dominant one since Hebb's time and has driven much of the research in the field. Interestingly, much of what we know now about the cellular and molecular mechanisms of memory formation look very much like what Hebb proposed.

Currently, initial acquisition of information (i.e., learning) and its maintenance in the short-term is thought to occur on the cellular and molecular level via post-translational modifications to existing proteins and is thus protein synthesis independent. Following learning, consolidation takes place. Consolidation is the process by which information acquired during learning is transformed from a labile state into long-term stable memories and stored in the brain. The cellular events thought to underlie consolidation include activation of second messenger systems, new gene transcription, and *de novo* synthesis of proteins important for creating new synapses or modifying existing synapses. Consolidation is measured by the period of time when memory is sensitive to the inhibition of protein synthesis, usually on the order of hours following initial learning (Dudai 2004). Some types of memories, particularly hippocampus-dependent declarative-type memories, are thought to undergo further systems-level consolidation (Dudai 2004, Frankland & Bontempi 2005). Systems-level consolidation can take weeks, months, and even years to be completed. The duration of systems-level consolidation is measured by the time it takes for memory to become independent of the hippocampus (Dudai 2004).

Suppressing the transcription of new mRNA (Bailey *et al.* 1999) or preventing the translation of new protein from mRNA in the amygdala disrupts long-term fear memory assessed at 24 hours, but not earlier time points (Parsons *et al.* 2006, Schafe *et al.* 2000). Therefore, according to the definition of consolidation presented above, fear memories assessed 24 hours after fear conditioning can be used as a measure of consolidation. Bauer et al. (2002) first showed that consolidation of fear conditioning was mediated by L-VGCCs using an approach in which they infused the L-VGCC antagonist verapamil into the BLA of rats prior to fear conditioning and then testing them at various times after conditioning. Rats infused with verapamil prior to conditioning performed as well as rats infused with vehicle when tested 1, 3, and 6 hours after conditioning, however, verapamil-infused rats exhibited severely impaired fear memory 24 hours after conditioning. Similarly, I demonstrated that that $Ca_v1.3$ knockout mice exhibit significantly impaired fear memory 24 hours after conditioning, but were not impaired at 1 and 6 hours after conditioning. $Ca_v1.2^{cKO}$ mice performed as well as wild-type mice with respect to consolidation of conditioned fear. Together, these data suggest that $Ca_v1.3$, but not $Ca_v1.2$, mediates consolidation of conditioned fear. The infusion data suggests that the BLA is a locus at which $Ca_v1.3$ is critical for the mediation consolidation of conditioned fear. However, it is not understood how $Ca_v1.3$ might function in the BLA to bring about this deficit. Also, it cannot be ruled out that $Ca_v1.3$ functions to mediate consolidation of conditioned fear in other brain areas as well.

**Future directions for unraveling the role of $Ca_v1.3$ in consolidation of conditioned fear—location, location, location:**

Location at which $Ca_v1.3$ functions with respect to consolidation of conditioned fear is one issue not addressed well by my studies. Here, I use "location" to include brain structures, cell types, and point in time. These issues were not addressed in my experiments because of my choice of approach, namely, the use of a mouse model in which the gene for $Ca_v1.3$ is deleted in all cells at all times. I have relied on what is known about the neurobiology of fear conditioning to hypothesize the likely brain structures and cells in which $Ca_v1.3$ is critical to mediate consolidation of conditioned fear. For example, I have focused my investigations on the BLA because (1) it has been repeatedly demonstrated to be necessary for conditioned fear (Phillips & LeDoux 1992, Maren *et al.* 1996, Maren 1999, Iwata *et al.* 1986, Wilensky *et al.* 1999) and (2) LTP in this structure is dependent on L-VGCCs (Drephal *et al.* 2006, Bauer et al. 2002). For my study of neuronal excitability, I chose to focus on BLA principal neurons because *in vivo* electrophysiology after fear conditioning suggest that these neurons participate in encoding fear memories (Rogan *et al.* 1997). Though my choices of brain structure and neurons to study were logical given the information available, an advancement in our understanding of the impairment observed in $Ca_v1.3$ knockout mice and thus the molecular mechanisms of consolidation of conditioned fear awaits the unraveling of the question of where and when $Ca_v1.3$ functions should be one of the primary focuses of this project's future.

With respect to identifying brain structures in which $Ca_v1.3$ is critical, a number of possible experiments can be envisioned. All of the best options depend on the development of new tools. Local infusion of L-VGCC antagonists in specific brain regions either before or shortly after fear conditioning is one approach to addressing brain structures in which $Ca_v1.3$ is critical for consolidation of conditioned fear. The study by Bauer et al. (2002) used such an approach to demonstrate the importance of L-VGCCs in the BLA for mediating consolidation of conditioned fear. However, this study was limited to only one brain structure. It is certainly possible, if not likely, that L-VGCCs in other brain structures are critical. The major limitation of such an approach, however, is the absence of any subunit specific L-VGCC antagonists. All currently-available L-VGCC antagonists target both $Ca_v1.2$ and $Ca_v1.3$. The advent of subunit-specific antagonists would be necessary for this approach to be maximally effective.

Another approach for delineating the brain structures in which $Ca_v1.3$ is important for consolidation of conditioned fear would require the use of Cre-loxP recombination (Metzger & Chambon 2001). One can “flox” a gene and thus render it capable of being conditionally deleted. “Floxing” refers to the introduction of two loxP sites into the gene of interest. The loxP sites are 34 base-pair stretches of DNA that are recognized for cleavage by cre-recombinase and placement of a loxP site on each side of a critical piece of a gene will mark that segment of DNA for removal by cre-recombinase and render the gene non-

functional. Cre-recominase, however, is not normally present in mammalian cells and its introduction by the experimenter dictates when the gene of interest is deleted. The development of a mouse model in which the gene for $Ca_v1.3$ is floxed would allow for some powerful experiments in the effort to uncover the function of $Ca_v1.3$ in the consolidation of fear memories. If such a mouse model existed, one such application would be to use local infusion of viruses expressing cre-recombinase into specific brain structures so that the gene for $Ca_v1.3$ could be regionally deleted and the effects of these deletions on the consolidation of conditioned fear investigated.

Though similar to the pharmacological approach, the use of Cre-loxP recombination offers different advantages and poses different problems. First, the development of such a mouse model and the requisite viruses is probably technically less challenging and more realistic in the short term than developing subunit specific antagonists. This approach also allows for the engineering of a method to determine more specifically in which cells the gene was deleted. A disadvantage of using L-VGCC antagonists as suggested above is the inability to know the extent of diffusion and thus the area of L-VGCC inactivation. A floxed gene could be engineered to express a marker upon cleavage by cre-recombinase allowing the cells in which the gene is deleted to be visualized. Disadvantages of this approach include the lack of temporal resolution and inability to reverse the effect. That is, because this approach targets the gene, the delay until $Ca_v1.3$ is no longer present will be dependent on the half-life of the

protein. Also, once the gene is excised from the genome, the genes function cannot be recovered.

In addition to identifying brain structures in which $Ca_v1.3$ function is critical for conditioned fear, the types of neurons in which $Ca_v1.3$ mediates its function with respect to conditioned fear are of interest. Addressing the cells in which $Ca_v1.3$ function is important would benefit from an approach utilizing cre-loxP recombination. Mice carrying a floxed version of the gene for $Ca_v1.3$ could be crossed with mice carrying the *cre* gene driven by various cell-specific promoters to generate mice in which the gene for $Ca_v1.3$ is deleted specifically in those cells expressing cre. This is the same approach that I used for the experiments presented in Chapter 3 of this dissertation to assess the role of $Ca_v1.2$ in conditioned fear. The structures of the brain have complicated neural circuitries and are composed of many different types of cells with differing neurotransmitters and connectivity. A primary dysfunction in any one group of neurons could lead to impaired function of any of the neurons to which it is functionally connected. Within the BLA, there are numerous types of neurons (Faber *et al.* 2001). A logical first step to addressing the types of cells in which $Ca_v1.3$ functions during consolidation of conditioned fear would be to use cre-lox recombination to delete the gene for $Ca_v1.3$ in the two most broad classes of neurons: inhibitory and excitatory neurons. Mouse models expressing cre-recombinase under the control of the appropriate promoters for such an approach currently exist (Stenman *et al.* 2003, Chen *et al.* 2006). Once the broad

class of neurons in which $Ca_v1.3$ is important is established, a narrowing of the neurons involved can be achieved by using mice in which cre expression is driven by more specific promoters.

Location in time is another parameter that is important to understand with respect to the role of $Ca_v1.3$. The current data sets with respect to the role of $Ca_v1.3$ in consolidation of conditioned fear do not tell us a lot about when $Ca_v1.3$ is important. Bauer et al. (2002) infused an L-VGCC antagonist into the BLA prior to fear conditioning. It is likely that L-VGCCs were blocked for much longer than just the period of fear conditioning. As discussed above, learning and memory formation proceeds in phases. By infusing the L-VGCC before fear conditioning, Bauer et al. (2002) likely blocked L-VGCCs across multiple phases. At a minimum, using this approach blocked L-VGCCs during acquisition and early consolidation. The use of $Ca_v1.3$ knockout mice in my experiments did not allow for temporal resolution as there were never any functioning $Ca_v1.3$ channels at any stage of memory formation.

Infusion studies are likely the best tool for addressing the temporal phase during which $Ca_v1.3$ is important. Of course, subunit-specific antagonists would be ideal. Genetic approaches, including inducible deletions, would not provide the necessary temporal resolution as most memory phases last minutes to hours and the half-lives of most proteins are hours to days. If $Ca_v1.3$ is important for mediating consolidation of conditioned fear and not acquisition as current data

suggests, infusions of L-VGCC antagonists immediately after fear conditioning should block consolidation of conditioned fear as well as those prior to fear conditioning. It would be interesting to know how long after fear conditioning L-VGCC antagonists could be delivered and still disrupt consolidation.

Understanding the "location" at which $Ca_v1.3$ mediates its effect with respect to fear conditioning will be a significant step forward for the understanding of $Ca_v1.3$ and fear conditioning generally. These experiments, however, will not be easy, or inexpensive. The lines of research that I have proposed will likely require development of new technology that will be costly in terms of both money and manpower. This raises the question: "what is the value of understanding fear conditioning?"

**The significance of understanding how fear memories are formed:**

Evolutionarily, the ability of humans to form fear memories has likely been quite important to our survival. Even today this ability serves us in learning about, and avoiding, dangers in our environment for which we do not have innate fear. For example, one might learn to avoid red stove-top burners after touching one and suffering a burn. There is no doubt that understanding how such memories are formed is important for comprehending ourselves as human beings and fear conditioning is a valuable tool for developing such an understanding. However, I am particularly interested in how understanding fear conditioning might be harnessed to serve human health and reduce suffering. Understanding the

molecular mechanisms underlying fear conditioning, including the role of $Ca_v1.3$, is an important part of this growing knowledge especially because molecules are the targets of pharmacotherapy.

One situation in which a greater understanding of how fear memories are formed might benefit human health and comfort is during the administration of chronic cancer chemotherapy. Many forms of cancer chemotherapy induce nausea and vomiting. Interestingly, stimuli surrounding the administration cancer chemotherapy (CS) (e.g., health care personnel, drug delivery equipment, doctor's office or hospital) can enter into association with the chemotherapy-induced nausea and vomiting (US) (Stockhorst *et al.* 2007). Subsequent encounters with these stimuli leads to the experience of nausea and vomiting in many patients even in the absence of additional chemotherapy administration. In fact, 20-30% of cancer patients receiving chemotherapy report nausea and vomiting when being re-exposed to the stimuli that usually signal the chemotherapy session and drug infusion. This phenomenon is referred to as anticipatory nausea and vomiting (ANV). It is not uncommon for ANV to lead to drop out from cancer treatment (Miller & Kearney 2004).

If the molecular mechanisms that mediate the formation of such aversive associations were better understood, it is possible that pharmacological inhibitors of this process might be successful in preventing ANV and subsequent drop out from chemotherapy treatment. Recently, a rat model of ANV has been

established in which nausea-inducing lithium chloride is paired with a particular context such that subsequent exposure to that context elicits conditioned gaping (a marker for nausea in rats) (Limebeer *et al.* 2006). This model will likely be valuable for assessing potential ANV therapeutics. It would be interesting to investigate whether any of the same manipulations that disrupt fear conditioning are also able to interfere with conditioned gaping given their obvious similarities. In particular, I would like to know if $Ca_v1.3$ mediates conditioned gaping. There would be challenges to assessing $Ca_v1.3$ knockout mice in the conditioned gaping paradigm since mice, unlike rats, do not show any clear signs of nausea (Welzl *et al.* 2001). Using L-VGCC antagonists in rats might be another approach for addressing this issue. In actuality, I may have inadvertently addressed this issue while performing the experiments that I present in Chapter 3 of this dissertation to assess whether the acute effects induced by nifedipine injection were able to enter into association with the context in which it was experienced. In those experiments, I paired nifedipine with a fear conditioning chamber and showed that subsequent exposure to that chamber elicited the same response as acute nifedipine injection (i.e. immobility). Because a high dose of nifedipine was used as the US in those studies, it seems reasonable to assume that both $Ca_v1.2$ and $Ca_v1.3$ were effectively blocked. The fact that mice were still able to form the association between the acute effects of nifedipine and the context in which they were experienced suggests to me that L-VGCCs are not critical for forming this association. While it is unclear how mice perceive the acute effects of nifedipine, it seems that L-VGCCs do not mediate the association of internal perception with

their surroundings and this may imply that L-VGCCs do not participate in the formation nausea-context associations. If this is the case, it does not necessarily mean that other molecules that mediate fear conditioning do not participate in conditioned gaping or ANV and thus are potentially valuable therapeutic targets.

Additionally, as discussed in this dissertation, the development of some fear disorders may proceed via a process like fear conditioning. An excellent example is the development of panic disorder. A panic attack is the sudden onset of intense apprehension, fear, or terror, often associated with feelings of impending doom. Individuals with panic disorders experience unexpected panic attacks and develop anxiety focused on the next potential attack (Barlow *et al.* 1994). The conditioning theory of development of panic disorder posits that exposure to panic attacks causes the conditioning of anxiety to cues present at the time of the attack, including one's internal state. Anxiety and panic differ in that anxiety is an anticipatory emotional state, whereas panic is an emotional state that is experienced when a traumatic event is in progress. The presence of conditioned anxiety potentiates the next panic, which begins the individual's spiral into panic disorder (Bouton *et al.* 2001, Wolpe & Rowan 1988). In a extension of this theory, if previous attacks have occurred at a public places (e.g., shopping mall or sporting arena), an individual might then come to avoid these places by not leaving the safety of their home and thus develop agoraphobia (Klein & Gorman 1987). Like ANV in cancer chemotherapy treatment, individuals with panic disorder may benefit from interventions that impair the formation of aversive

associative memories.

**$Ca_v$1.3 in BLA neurophysiology:**

After establishing that $Ca_v$1.3 *is* involved in consolidation of conditioned fear, I wanted to know *how* it is involved. As a first step towards addressing how $Ca_v$1.3 might be involved in consolidation of conditioned fear, I sought to understand the role of $Ca_v$1.3 in BLA neurophysiology, I investigated long-term potentiation (LTP) and neuronal excitability in the BLA of $Ca_v$1.3 knockout mice. Though it has been demonstrated that L-VGCCs are important for both LTP and neuronal excitability, the contribution of $Ca_v$1.3 to these phenomena has not been assessed. I focused on these two phenomena because there is strong evidence that both are neurobiological substrates of learning and memory. I reasoned that understanding the role of $Ca_v$1.3 in these phenomena in this brain structure that is required for fear conditioning might provide insight into the mechanism responsible for the impaired ability of $Ca_v$1.3 knockout mice to consolidate contextually-conditioned fear. It should be noted that the purpose of these experiments was not necessarily to demonstrate causation of the fear conditioning deficit, but rather to understand the role of $Ca_v$1.3 in the BLA and generate hypotheses about how the fear conditioning deficit in $Ca_v$1.3 knockout mice might be mediated.

Interestingly, I found that that $Ca_v$1.3 knockout mice exhibit only ~25% as much LTP in the BLA (BLA-LTP) as wild-type mice upon high-frequency stimulation of

the cortical pathway afferents traveling in the external capsule. Based on a great deal of evidence, it has been suggested that BLA-LTP is the substrate of fear conditioning (Blair *et al.* 2001, Sah *et al.* 2008, Goosens & Maren 2002). Evidence for BLA-LTP as a neurobiological substrate for Pavlovian fear conditioning comes from studies showing that fear conditioning can produce LTP-like changes in synaptic strength in the BLA (McKernan & Shinnick-Gallagher 1997, Rogan et al. 1997). BLA-LTP and fear learning are sensitive to the same stimulus contingencies (Bauer *et al.* 2001) and share similar molecular mechanisms (Rodriques *et al.* 2004). In light of the hypothesized role for BLA-LTP as a substrate for fear conditioning, it is tempting to speculate that the impaired ability of $Ca_v1.3$ knockout mice to consolidate contextually conditioned fear is explained by their impaired ability to exhibit robust BLA-LTP. One issue that makes it difficult to draw this connection is the discrepancy in time course of the two impairments. That is, while the BLA-LTP deficit develops as soon as 10-15 minutes after induction, the consolidation deficit does not present itself until somewhere between 6 and 24 hours after conditioning. This discrepancy between LTP and learning deficit is not unique to $Ca_v1.3$ knockout mice, in fact, it is common to both pharmacological and molecular genetic studies of LTP and learning. One explanation for this discrepancy may be the way in which the processes are engaged *in vivo*. That is, *in vitro* studies employ artificial patterns of electrical stimulation to induce LTP which are unlikely to reproduce the natural activity patterns that occur in the BLA of behaving animals during fear conditioning. Further, brain slice preparation disrupts the connections between

BLA neurons and modulatory inputs that are normally present *in vivo*. Additionally, it might be that the LTP underlying fear memories is not induced during training, but rather occurs at a later time point, for example, during sleep. Indeed, there is evidence that consolidation of memories may occur via the replaying of newly acquired patterns of activity during sleep (Axmacher *et al.* 2006). It seems possible that such replaying of specific patterns of neuronal activity during sleep may induce LTP. These factors may explain the discrepancy between the time course of LTP deficits and learning deficits, generally, and also the discrepancy between the BLA-LTP deficit we observe in horizontal brain slices from $Ca_v1.3$ knockout mice and the fear conditioning deficit observed in these mice. Studies using *in vivo* LTP recording techniques and natural patterns of stimulation may provide insight into this discrepancy. Nevertheless, these data suggest that the capacity for $Ca_v1.3$ knockout mice to exhibit normal plasticity is impaired, and this may have unknown implications for their impaired inability to consolidate conditioned fear.

I also found that principal neurons in the BLA of $Ca_v1.3$ knockout mice exhibit enhanced neuronal excitability as measured by a decreased afterhyperpolarization (AHP) and increased number of spikes in response to a depolarizing current injection (i.e., reduced spike accommodation). The observation that $Ca_v1.3$ mediates the AHP and neuronal excitability in BLA principal neurons is consistent with our laboratory's data from CA1 pyramidal neurons in the hippocampus (Kuo *et al.* Submitted). Though these data suggest

that the role of $Ca_v1.3$ in generation of the AHP is a generic mechanism and not specific to the CA1 pyramidal neurons of the hippocampus, it is difficult to imagine that this abnormality explains the learning impairment observed in $Ca_v1.3$ KO mice. In fact, in the hippocampus, reversing age-related decreases in excitability can rescue the corresponding learning deficits (Deyo *et al.* 1989a, Deyo *et al.* 1989b). Thus, one might expect enhanced neuronal excitability to facilitate learning and memory, however, this is not the case: $Ca_v1.3$ KO mice perform as well as wild-type mice in the hippocampus-dependent Morris water maze and exhibit an impaired ability to consolidate contextually-conditioned fear. The fact that increased neuronal excitability did not enhance learning and memory in these mice be accounted for by the fact that manipulations that increase excitability tend to improve learning and memory in aged animals (Murphy *et al.* 2004, Disterhoft *et al.* 1996) but not young animals (Giese *et al.* 1998, Disterhoft et al. 1996). It is interesting to note, however, that I did not observe a difference in performance between aged $Ca_v1.3$ KO and wild-type mice in the Morris water maze (McKinney *et al.* In Preparation).

That the change in excitability may be too small to influenc information processing in these brain regions and may therefore have no effect on learning and memory. Indeed, in CA1 pyramidal neurons of the hippocampus, the area of the AHP is only decreased by ~30% in $Ca_v1.3$ KO mice relative to wild-type mice and spike accommodation is not affected at all (Kuo et al. Submitted). In BLA principal neurons, the effect is slightly bigger: the area of AHP is ~50% of that in

wild-type mice and spike accommodation is significantly reduced in $Ca_v1.3$ KO mice. In both the hippocampus and amygdala of $Ca_v1.3$ KO mice, a significant portion of the AHP remains, and even in the amygdala, the effect on spike accommodation is minimal and only manifests at strong, prolonged current injections suggesting that the change may not be large enough to be physiologically relevant.

Though unlikely, it is possible that the excitability changes observed in $Ca_v1.3$ KO mice do contribute to the learning impairment that these mice exhibit. Most reports of learning-induced changes in neuronal excitability describe excitability varying directly with learning. These studies, however, have been performed in a limited number of brain structures and none have been done in the amygdala. It seems possible that the direction of excitability change of a neuron after learning is dependent on its place in the circuit mediating learning. For example, its was demonstrated recently that the excitability of neurons in the infralimbic (IL) cortex of rats decreased after fear conditioning and returned to baseline when the fear memory was extinguished (Santini *et al.* 2008). IL cortex neurons project to the amygdala (BLA, CE, and intercalated cell masses) (Sesack *et al.* 1989, Robert 2004). The net effect of these projections on amygdala output seems to be primarily inhibition (Likhtik *et al.* 2005, Quirk *et al.* 2003, Rosenkranz & Grace 2002, Rosenkranz *et al.* 2003). It is in this way that excitability of IL cortical neurons may regulate fear expression. Decreased excitability of these neurons may lead to less inhibition of the amygdala, creating a permissive state for fear

conditioning-related plasticity. If the IL cortex neurons exhibited enhanced excitability, like the neurons of the hippocampus and amygdala of $Ca_v1.3$ KO mice, the amygdala may not be disinhibited and a permissive state for plasticity may not be established, thus preventing plasticity and impairing fear learning. If the role of $Ca_v1.3$ in AHP generation is a general mechanism across all brain regions, then IL cortex neurons in $Ca_v1.3$ KO mice may be more excitable and thus prevent the plasticity in the amygdala necessary to support fear conditioning. Alternatively, the hyper-excitability of BLA principal neurons might disrupt the circuitry of the amygdala in a way that leads to impaired fear memory.

Participation in signaling to the nucleus is the final mechanism by which $Ca_v1.3$ may mediate consolidation of contextually-conditioned fear. Many agree that the series of events described below is involved in memory formation in a variety of paradigms and brain structures (Kandel 2001, Lamprecht & LeDoux 2004, Silva 2003, Thomas & Huganir 2004). Input signals carrying new information act on neurons resulting in calcium influx. Calcium initiates an intracellular signal transduction cascade: the activation of adenyl cyclase, the increased synthesis of cAMP, and the activation of several protein kinases. Activated kinases are translocated to the neuron nucleus, where they phosphorylate the cAMP response element-binding protein (CREB). CREB binds to the cAMP-response elements (CRE) in regulatory regions of target genes and stimulates their transcription. The final step of this cascade is the transient activation of genes

involved in the synthesis of proteins essential both for modification of preexisting synapses and formation of new ones.

L-VGCCs may be one of the sources of the calcium influx that initiates the series of events described above. Experiments with neuronal cell cultures have shown that calcium influx via L-VGCCs can efficiently activate extracellular signal-related kinases (ERKs) (Dolmetsch *et al.* 2001, Rosen *et al.* 1994, Hardingham *et al.* 2001). In hippocampal slices from mice in which the gene for $Ca_v1.2$ is deleted, ERK phosphorylation and nuclear translocation is impaired following activation (Moosmang *et al.* 2005). Activation of ERK may contribute to memory formation by stimulating many events, including transcription of memory-related genes (Sweatt 2004). The downstream effector of ERK, CREB, is a transcription factor that has been shown to be critical for memory (Silva *et al.* 1998). Activation of CREB and CRE-dependent gene expression is achieved by phosphorylation of $Ser^{133}$ (Brindle & Montminy 1992). Activation of hippocampal slices from mice in which the gene for $Ca_v1.2$ is deleted fails to induce CREB phosphorylation and nuclear translocation as strongly as in hippocampal slices from control mice (Moosmang et al. 2005).

In light of the evidence implicating L-VGCCs and $Ca_v1.2$ as one source of calcium influx for memory-related cascades, I hypothesize that $Ca_v1.3$ is also important for signaling to the nucleus and transcription of memory related genes. In preliminary studies, I treated hippocampal slices and BLA punches from

$Ca_v1.3$ knockout mice and wild-type mice with tetraethylammonium (TEA) and assessed phosphorylation of CREB thirty minutes later. TEA is a non-specific potassium channel blocker, the application of which greatly increases membrane excitability, calcium influx, CREB phosphorylation, and LTP (Aniksztejn & Ben-Ari 1991). Initial results suggested that TEA treatment induced robust CREB phosphorylation in tissue from wild-type, but not $Ca_v1.3$ knockout mice. Unfortunately, these results were inconclusive due to small samples size and high variability. However, this line of research should be continued and if $Ca_v1.3$ is important for new gene transcription, it will be important to determine which genes are transcribed. I hypothesize that such genes might encode structural proteins important for synapse formation and modification.

Though data supporting a role for $Ca_v1.3$ in signaling to the nucleus and gene transcription is currently tenuous, it is my belief that further investigation will be fruitful in uncovering important memory-related genes and gene products. Since consolidation of learning into long-term memories is thought to be dependent on new gene transcription and *de novo* protein synthesis, deficient signaling to the nucleus is more likely to explain impairments like the one observed in $Ca_v1.3$ knockout mice than impaired LTP or altered neuronal excitability. A transcriptional mechanism is also more consistent with the time course of the impairment than dependence on LTP. Early-phase LTP that I measured in my studies is dependent on modification of already existing proteins and thus is more relevant to maintaining memories immediately post training. In contrast,

signaling to the nucleus, new gene transcription, *de novo* protein synthesis, and integration of new proteins into the synaptic structure likely takes hours and is more suited for mediating consolidation of learning into long-term memories.

**L-VGCCs probably do not mediate extinction of conditioned fear:**

In addition to a role for L-VGCCs in consolidation of fear conditioning, early reports suggested that L-VGCCs were critical for both short-term (within session) and long-term (24 hours after extinction training) extinction (Suzuki *et al.* 2004, Cain *et al.* 2002). Using $Ca_v1.2^{cKO}$ mice and $Ca_v1.3$ knockout mice, I demonstrated that neither $Ca_v1.2$ or $Ca_v1.3$ alone are critical for either short-term or long-term extinction. I then showed that the L-VGCC antagonist nifedipine, used in the original experiments implicating L-VGCCs in extinction of conditioned fear, had effects that may have confounded the interpretation of extinction experiments in which it was used. First, nifedipine seemed to impair locomotion acutely. The locomotor impairment was so profound that when mice were treated systemically with nifedipine and then placed in fear conditioning chambers, they appeared to be exhibiting freezing behavior even though they had never been conditioned. This behavior, when superimposed upon actual conditioned freezing behavior during extinction training, presents as an inability to extinguish conditioned freezing in the short term. In addition to the acute effect of nifedipine, I found that the nifedipine apparently induces an aversive state that can enter into association with the context used for extinction training. Upon retesting in the same context, the context appears to serve as a CS and leads to freezing in mice

treated with nifedipine previously. The sum of this freezing and the freezing to context resulting from the original conditioning is quite large during retesting and could be misinterpreted as impaired long-term extinction in mice. This type of misinterpretation is what appears likely to have happened in the earlier studies that implicated L-VGCCs in extinction of conditioned fear.

Recently, two other groups have presented data that sheds light on the apparent blockade of fear extinction by L-VGCC antagonists (Busquet *et al.* 2008, Waltereit *et al.* 2008). Busquet et al. (2008) showed that the apparent nifedipine-induced blockade of fear extinction was abolished in a mouse model expressing DHP-insensitive $Ca_v1.2$ L-VGCCs (these $Ca_v1.2$ channels are not blocked by nefedipine) suggesting that the effect of systemically administered nifedipine on fear extinction is mediated through $Ca_v1.2$ rather than $Ca_v1.3$. Interestingly, they showed that the effects of nifedipine on fear extinction are likely mediated via peripheral $Ca_v1.2$ channels as intracerebroventricular infusion of nifedipine does not affect fear extinction. Further, in a test of activity in the open field, they show that mice given systemic injection of nifedipine exhibit marked reductions in exploratory behavior.

Another group, however, suggests another mechanism by which L-VGCC antagonists may act to influence fear extinction (Waltereit et al. 2008). In their experiments, Waltereit et al. systematically vary the timing of systemic injections of nifedipine relative to the onset of extinction training. They find that

intraperitoneal injection (i.p.) or sub-cutaneous (s.c). injections of nifedipine impair fear extinction as many as 2 or 4 hours prior to extinction training, respectively, despite the fact that nifedipine peaks in blood serum as quickly as 30 minutes after administration and is nearly undetectable by 2 hours following i.p. injection and by 4 hours following s.c. injection. It is, therefore, highly unlikely that blockade of L-VGCCs in the brain is responsible for the effects of nifedipine on fear extinction when administered at these intervals. Further, they show that both i.p. and s.c. injections of nifedipine are associated with significant elevations in plasma corticosterone at either 2 or 4 hours following injection, respectively. Thus, Waltereit et al. suggest that nifedipine induces protracted stress response following systemic administration and this stress response likely accounts for its observed effects on fear extinction. Such a suggestion is consistent with the observation that both acute and chronic stress impair fear extinction (Izquierdo *et al.* 2006, Miracle *et al.* 2006), while leaving fear acquisition intact. Waltereit et al. also observed decreases in locomotor activity and a complete loss of rearing behavior that lasts up to 4 hours after s.c. administration of nifedipine.

Combining the findings from these two studies (Waltereit et al. 2008, Busquet et al. 2008) and my study of fear extinction (McKinney *et al.* 2008), I propose the following mechanism for the effect of systemic injection of nifedipine on fear extinction. With regard to the apparent effect of nifedipine on short-term extinction, I propose that nifedipine induces a hypotensive state through interaction with $Ca_v1.2$ channels on the peripheral vasculature and that this state

is perceived as aversive and leads to stress hormone release and malaise. This idea is consistent with the observation of decreased locomotion/exploratory behavior in my experiments and those of the other two groups as well as peripheral distribution of $Ca_v1.2$ channels and the documented actions of L-VGCCs on peripheral vasodilation and hypotension (Kubo *et al.* 1981, Barrett *et al.* 1988). Addtionally, as I referred to earlier in the discussion of this data, I propose that the aversive state induced by nifedipine administration enters into association with cues surrounding extinction training and that these cues serve as CSs that cause mice to re-experience the acute effects of nifedipine when they are presented with these cues at a later time. Such a mechanism is not without precedence. Studies in rats of Pavlovian conditioning processes in morphine withdrawal suggest that exposure to contextual or olfactory cues previously paired with morphine withdrawal provoke the species-typical defense response of freezing (McNally & Akil 2001). Further, rats that receive a nausea-inducing stimulus, experience nausea again when they are placed back into the context in which the originally received the nausea-inducing stimulus (Limebeer et al. 2006).

Though my findings and the two new studies above have led to a rethinking of the role of L-VGCCs in fear extinction (Schafe 2008), other factors may contribute and should be considered.

**Alternative explanations for the apparent lack of a role for L-VGCCs in extinction of conditioned fear:**

First, it may be necessary to block both brain-expressed L-VGCCs to impair extinction of conditioned fear. If one disregards the complications presented by nifedipine, this would explain why treatment with L-VGCC antagonists and not genetic deletion of individual L-VGCC subtypes leads to impaired extinction of conditioned fear. Such an explanation would suggest that the two brain-expressed L-VGCCs ($Ca_v1.2$ and $Ca_v1.3$) are functionally similar and one can compensate for the other in its absence. In our hands, however, there is no change in $Ca_v1.3$ protein expression levels upon deletion of the gene for $Ca_v1.2$ or vice versa (unpublished results). Further, $Ca_v1.2$ and $Ca_v1.3$ have quite different neuronal localization (Hell *et al.* 1993a) and biophysical characteristics (Helton et al. 2005, Xu & Lipscombe 2001) making them unlikely functional substitutes for each other. Also, if functional compensation is at play, it appears surprisingly specific for extinction given the consolidation deficit described in $Ca_v1.3$ knockout mice. Still, the possibility of a synergistic effect of simultaneous inactivation of both $Ca_v1.2$ and $Ca_v1.3$ cannot be dismissed.

The second explanation relates to the conditional nature of the deletion of $Ca_V1.2$ in the present study. Because the gene for $Ca_v1.2$ was deleted primarily in the forebrain of $Ca_v1.2^{cKO}$ mice, the lack of an effect on the ability to extinguish conditioned fear may have resulted from the gene for $Ca_v1.2$ not being deleted in a critical region for extinction of conditioned fear. However, this seems unlikely

given that $Ca_v1.2$ in the $Ca_v1.2^{cKO}$ mice is deleted in most of the brain regions that are critical for extinction of conditioned fear including the cortex, hippocampus (White *et al.* 2008) as well as the amygdala (McKinney *et al.* 2008). This, of course, does not eliminate the possibility that $Ca_v1.2$ is still expressed in a currently unrecognized area of the brain critical for extinction of conditioned fear. Also, in light of the reports of Busquet et al. (2008) and Waltereit et al. (2008), it is possible that deletion of $Ca_v1.2$ in the peripheral vasculature might have been required.

Third, because the cre-recombinase expression in $Ca_v1.2^{cKO}$ mice was driven by the calcium-calmodulin-dependent kinase II alpha (CaMKII$\alpha$) promoter, the gene for $Ca_v1.2$ was only deleted in excitatory, but not inhibitory, neurons of the forebrain (Kelly 1991). If $Ca_v1.2$ activity in inhibitory neurons is critical for the successful extinction of conditioned fear, then we would not have observed impaired extinction of conditioned fear in $Ca_v1.2^{cKO}$ mice, since these mice presumably express the gene for $Ca_v1.2$ in inhibitory neurons. $Ca_v1.2$ may exert its influence in inhibitory neurons outside of the amygdala, since almost all of the $Ca_V1.2$ immunoreactivity observed in the BLA is found in CaMKII$\alpha$ positive pyramidal neurons (Pinard *et al.* 2005). This explanation seems less likely in light of the recent evidence that nifedipine's effect on fear extinction is mediated peripherally (Busquet et al. 2008).

Finally, the extinction protocol used here may not have been suitable for detecting $Ca_v1.2$-mediated deficits in conditioned fear extinction. We demonstrated successful extinction of contextually-conditioned fear using a single 1-hour context exposure. Though this protocol did not uncover an effect of genotype on extinction, it is possible that protocols that utilized longer exposure periods or different training styles (e.g., spaced extinction training) may have been more successful. We were, however, able to successfully reproduce the previously-demonstrated effect of nifedipine on extinction using this protocol and thus it seems likely that this protocol would be sufficient to parse out any contribution of $Ca_v1.2$ to the nifedipine effect.

Despite the apparent lack of importance of $Ca_v1.2$ in fear conditioning and fear extinction, recent studies using mice in which the gene for $Ca_v1.2$ is conditionally deleted have demonstrated a role for $Ca_v1.2$ in other forms of memory. Using the same strain of mice that I used in my studies, White et al. (2008) showed that $Ca_v1.2$ is dispensable for short- and long-term spatial memory formation in the Morris water maze, but critical for remote memories. Using a strain of mice in which the gene for $Ca_v1.2$ was homozygously deleted in the forebrain and heterozygously deleted in the rest of the body, Moosmang et al. (2005) showed that $Ca_v1.2$ is important for hippocampus function. These mice were impaired on two different hippocampus-dependent learning tasks and NMDA receptor-independent LTP in the CA1 region of the hippocampus. The MAPK signaling pathway and CREB-mediated transcription were disrupted in CA1 pyramidal

neurons isolated from these mice. Together, this data suggests that the role for $Ca_v1.2$ in learning and memory is specific for certain types of learning and memory.

**The positive implication of a negative finding on the role of L-VGCCs in fear extinction:**

Negative findings can make a positive contribution. My initial findings regarding the role of L-VGCCs in fear extinction were negative. That is, I demonstrated that neither $Ca_v1.2$ nor $Ca_v1.3$ alone mediates extinction of conditioned fear. Had these results been the first on the subject, they may be less interesting. However, because these data were at odds with the claims of two other groups at the time, they were of great interest. Upon trying to explain the discrepancy among my data set and those published, I found that previous findings regarding L-VGCCs in fear extinction were likely misinterpreted as the result of an aversive state induced by the L-VGCC antagonists used. Perhaps the greatest implication of these findings is that future studies will not be based on the idea that L-VGCCs mediate fear extinction. Given the excitement surrounding the initial reports of a role for L-VGCCs in fear extinction, these studies may have been extensive and costly. Further, my findings highlight the power of using a combined approach when investigating molecular mechanisms of biological processes. Genetically modified mouse models and pharmacological approaches each have advantages and disadvantages. The major advantage of using genetically modified mice is specificity. That is, one specific gene product can be targeted for study without the possibility that observed phenotypes are the by-products of non-specific

effects that often accompanies drug studies. However, when using genetically modified mice, it is possible that the organism may compensate for the absence of the gene of interest in a way that obscures its normal biological role. Further, genetically modified mice provide poor temporal regulation. The drawback of poor temporal resolution was discussed earlier with regard to the timing of $Ca_v1.3$ involvement in consolidation of contextual fear. Mice in which the gene of interest has been deleted since early development, like $Ca_v1.3$ knockout mice, offer no temporal resolution. Even mice in which the gene can be deleted experimentally offer temporal resolution that is only as short as the half-life of the protein product. Pharmacological approaches offer much better temporal resolution as well as the possibility for anatomical resolution if the drug is infused into a particular anatomical region. Pharmacology, however, is often criticized for being non-specific. With respect to L-VGCC antagonists, none of those that are currently available are able to distinguish among the various subtypes of L-VGCCs. Also, there is always the risk that drugs may interfere with the function of molecules for which they were not designed. A combined approach allows for the advantages of both genetically modified mice and pharmacology to be exploited.

**A subtype-specific understanding of L-VGCCs in fear conditioning, fear extinction, and BLA neurophysiology:**

The studies presented in this dissertation represent the first attempt to understand the role of individual L-VGCCs subtypes in fear conditioning, fear extinction, and BLA neurophysiology. The findings of these studies suggest that

each L-VGCC subtype participates in a different subset of neurobiological processes. Understanding which subtype(s) participate in a particular neurobiological process provides a more complete understanding and directs future investigations. Additionally, it identifies more specific molecular targets for pharmacological manipulation of these neurobiological processes. Altogether, this work not only demonstrates the importance of particular L-VGCC subtypes, it also sets the stage for a subtype-specific understanding of L-VGCCs in learning and memory.

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