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Differentiating the contributions of NMDA receptor-mediated synaptic plasticity in basal and lateral nuclei of the amygdala during Pavlovian fear conditioning

Author

Sterlace, Sarah Rose

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Differentiating the contributions of NMDA receptor-mediated synaptic plasticity in basal and lateral nuclei of the amygdala during Pavlovian fear conditioning

A dissertation submitted in partial satisfaction of the

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Doctor of Philosophy in Psychology

by

Sarah Rose Sterlace

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ABSTRACT OF THE DISSERTATION

Differentiating the contributions of NMDA receptor-mediated synaptic plasticity in basal and lateral nuclei of the amygdala during Pavlovian fear conditioning

By

Sarah Rose Sterlace

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2014

Professor Michael S. Fanselow, Committee Chair

Fear and the development of conditional fear are critical for survival. However, mal-adaptations in the fear system lead to psychiatric disorders such as Post-Traumatic Stress Disorder and anxiety disorders, such as specific phobias. Pavlovian fear conditioning in rodents allows for the study of the neural circuitry and biological mechanisms the underlie fear learning and memory.

The basolateral amygdala complex, containing the lateral (LA) and basal (BA) nuclei, are critical for cued and contextual fear learning and memory formation through

mechanisms that include *N*-methyl-D-aspartate receptor (NMDAR)-mediated synaptic plasticity. However, the relative contribution of NMDAR-mediated plasticity in the BA and LA is unknown because the pharmacological techniques previously used to implicate NMDAR have limited anatomical specificity. While lesion studies can be more anatomically precise, lesions affect far more than synaptic plasticity.

My thesis work has been focused on the role of the NMDA receptors in learning and memory, with a principle focus on using cellular manipulations of the *N*-methyl-D-aspartate receptor (NMDAR) on the BA and LA nuclei of the amygdala to measure the significance of NMDAR-mediated synaptic plasticity on auditory and contextual Pavlovian fear conditioning. This was achieved through temporary inactivation, the use of an shRNA virus targeted at depleting the Grin1 gene, and the use to transgenic mice to specifically isolate and dissociate the LA and BA nuclei. The behavioral effects of the manipulations were assessed with Pavlovian auditory and contextual fear conditioning.

Specifically, Chapter 2 concerns the role of selective NMDA subunit GluN2B antagonist on fear learning and retention. Chapter 3 utilizes the shRNA virus to look at NMDA-mediated plasticity in the lateral amygdala. Chapter 4 uses transgenic mice to address the role of NMDAR-mediated synaptic plasticity in both the LA and BA nuclei. Chapter 4 also addresses specific analyses that utilize the data to extract more information from viral infusion studies. Additionally, results from Chapter 4 suggest that the LA is a relay site for the convergence of the discrete CS and US to form an association, but that the information from the LA is projected to the BA and that is where NMDAR-mediated plasticity critical for auditory fear conditioning. The data that I

presented in Chapter 4, support the implication that the basal amygdala is important for contextual and auditory fear learning and memory in an intact animal. Importantly, my results show that in normal functioning animals, the NMDAR-mediated synaptic plasticity in the BA, as compared to the LA, is what is critical for driving the fear response during auditory fear conditioning. In Chapter 5 these findings will be synthesized into a model that can explain the role of NMDAR- mediated plasticity in fear learning and memory.

This research reformats how the fear circuitry functions to create enduring memories. Currently, most models of fear learning involve a serial circuit, emphasizing very few sites of synaptic plasticity. Since the models involve a straightforward prediction, disruption of the circuit either prior to learning or after learning should disrupt the fear response equally. My data suggests circuitry within the amygdala is adaptive. The neuro-architecture that creates fear memories should be a dynamic network, versus a serial circuit, in order to increase the chances of survival if damage occurs to the primary pathway.

The dissertation of Sarah Rose Sterlace is approved

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Thomas Minor

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Hugh Tad Blair

_____

Thomas O'Dell

_____

Michael S. Fanselow, Committee Chair

University of California, Los Angeles

2014

DEDICATION

To my parents,

who taught me to be just tall enough

so that my feet touch the floor.

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VITA

2005	B.A., Psychology University of California, Los Angeles
2010	M.A., Psychology, Behavioral Neuroscience University of California, Los Angeles
2012	Candidate of Philosophy, Psychology University of California, Los Angeles
2008-2014	Graduate Student Researcher Department of Psychology University of California, Los Angeles
2008-2014	Teaching Associate Department of Psychology University of California, Los Angeles

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Fanselow, M. S., and **Sterlace, S. R.,** (2014). Pavlovian Fear Conditioning: Function, Cause and Treatment. In McSweeney (Eds.), Handbook of Classical and Operant Conditioning. New Jersey: Wiley-Blackwell

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CHAPTER ONE

Introduction

Introduction

A fear response is an adaptation that is required to stay alive. However, mal-adaptations in the fear system lead to psychiatric disorders such as Post-Traumatic Stress disorder and anxiety disorders, such as specific phobias. Pavlovian fear conditioning in rodents allows for the study of the neural circuitry and cellular mechanisms the underlie fear learning and memory in mammals.

My thesis work has been focused on the role of the NMDA receptors in fear learning and memory, with a principle focus on using cellular manipulations of the *N*-methyl-D-aspartate receptor (NMDAR) on basolateral nuclei of the amygdala (BLA) to measure the significance of NMDAR-mediated synaptic plasticity on auditory and contextual Pavlovian fear conditioning. This was achieved through temporary inactivation, the use of a shRNA virus targeted at depleting the Grin1 gene, and the use to transgenic mice to specifically isolate and dissociate nuclei within the BLA, specifically the lateral nucleus (LA) and basal nucleus (BA). The behavioral effects of the manipulations were assessed with Pavlovian auditory and contextual fear conditioning.

In the following section, I will review Pavlovian fear conditioning, the neural circuitry that supports fear learning and memory, and the NMDA receptor and function. In addition, a set of experiments designed manipulate the NMDAR- related synaptic plasticity in BLA nuclei during auditory and contextual Pavlovian fear conditioning and the implications for the primary neural pathway projecting auditory fear associations, as

well as the pathway projecting contextual fear associations, to produce a fear response will be presented.

Behavioral Level: Pavlovian Fear Conditioning:

Fear conditioning is a type of Pavlovian learning produced when an aversive unconditional stimulus (US) such as a shock, becomes associated with a previously neutral, or conditional, stimulus (CS). Learning is indicated by a conditional fear response (CR), for example freezing, to the CS. This robust type of learning can cause fear memory after just one trial (Fanselow, 1990) . An example of Pavlovian conditioning, a neutral stimulus, such as a tone, light or the environment that the subject is conditioned in, becomes paired with an aversive shock.

When the CS is a tone, it is considered *auditory fear conditioning*, which is a type of *cued fear conditioning.* Cued fear conditioning refers to a brief presentation of a discrete cue, followed contiguously with an aversive US presentation. As you pair a discrete CS with a US conditioning occurs to the discrete CS (e.g., tone, or light), but also to the context that the subject received the pairings. When the CS is the context, or environment that the subject was placed in during acquisition, it is called *contextual fear conditioning*. Manipulating the parameters of cued conditioning will enhance fear to the discrete cue and reduce fear to the context, or a discrete cue can be omitted, producing maximal fear to the context (Fanselow, 1980; Fanselow, 2010; Wiltgen et al., 2006). In either case, the CS becomes to elicit fear after it is paired with shock. When a threat is presented, fear provokes a variety of species-specific defense reactions that enable the

subject to adaptively respond to the threat in order to increase chances of survival (Bolles, 1970; Bolles & Fanselow, 1980; Fanselow & Lester, 1988). In rodents, a reliable and easily quantifiable reaction to threat is suppression of movement, or freezing (Bolles & Collier, 1976; Fanselow, 1980).

Systems Level: **Neural Circuitry**

The Amygdala

The amygdala is an almond-shaped structure deep within the temporal lobe that was first identified by Burdach in the 19th century (Burdach, 1819). Since then, the examination of the amygdala has revealed increasingly structural differentiation, and the extent of its border and subdivision remain controversial. For the purposes of my research, I will follow closely with the Swanson version of anatomical location based on embryological, connectional, and functional data (Swanson & Petrovich, 1998) using Paxinos neurotransmitter allocation for histological purposes (Emson et al., 1979; Watson, Paxinos, & Tokuno, 2010).

The basolateral amygdala complex (BLA) is clearly implicated in forming the association between a previously neutral stimulus (conditional stimulus; CS) and an aversive stimulus, such as a shock (unconditional stimulus; US). Lesions to the BLA, consisting of both the lateral nucleus (LA) and the basal nucleus (BA), have been shown to eliminate fear, including innate fear of cats and prior to Pavlovian fear conditioning learning in both humans and non-humans (Blanchard & Blanchard, 1972; Bechara et al., 1995; Hitchcock & Davis, 1986; Gale et al., 2004; Lee et al., 1993; Phillips and LeDoux,

1992). Also, post training lesions or temporary inactivation of the BLA result in a pronounced deficit in fear (Maren, Aharonov, & Fanselow, 1996; Ponnusamy, Poulos, & Fanselow, 2007). Furthermore, temporarily inhibiting neural activity in the BLA disrupts both learning and expression of conditional fear (Helmstetter, 1992). Single-unit electrophysiological activity recordings in anesthetized rats revealed that neurons in the dorsal LA responded to both CS (tone) and US (footshock) stimuli (Romanski, et al., 1993). Additionally, a molecular imaging technique that utilizes the immediate early gene *Arc* known as cellular compartment analysis of temporal activity by fluorescent in-situ hybridization (catFISH) showed convergence of contextual information and shock in the BLA as well (for catFISH methodology see Guzowsky & Worley, 2001; Barot, et al., 2008). These studies further establish that BLA neurons are critical for mediating Pavlovian fear conditioning. However, the BLA receives information about the cue and context from different sources. Gainfully, the BLA is a cortex-like structure that receives highly processed information from several cortical and some thalamic regions (Swanson & Petrovich, 1998).

Amygdala Afferents

The BLA receives information about the cue and context from different sources. In auditory fear conditioning, the BLA receives highly processed information from cortical and thalamic regions, (Swanson & Petrovich, 1998) auditory information from the medial geniculate nucleus of the thalamus and the auditory cortex. These regions

converge with aversive stimuli onto the BLA during Pavlovian fear conditioning (LeDoux, 1993; Li, Stutzmann; & LeDoux, 1996).

CS Convergence: Auditory

Pre-training lesions to the LA produce a deficit in fear learning. LeDoux et al., (1990) lesioned the LA and then looked at mean arterial pressure as well as freezing and found a lower autonomic response and freezing deficits as opposed to controls. Additionally, the LA is implicated as the site where convergence of auditory signaling information (tone) and reinforcement (shock) result in the synaptic plasticity necessary to produce conditioned fear (Romanski et al., 1993; LeDoux, 2000; Blair et al. 2001; Paré, Quirk, & LeDoux, 2004; Davis, 2006; Sigurdsson et al., 2007; Ploski et al., 2010). Auditory cues from both the auditory thalamus (medial geniculate nucleus) and auditory cortex can acquire fear conditioning, inducing long-lasting changes in the LA (Boatman and Kim, 2006; Romanski and LeDoux, 1992; Clugnet & LeDoux, 1990; Doyère, Schafe, Sigurdsson, & LeDoux, 2003). As with tone-shock associations, the evidence on context-shock association formation also points to the basolateral amygdala (Phillips & LeDoux, 1992, Onishi & Xavier, 2010; Goosens & Maren, 2001; Humeau et al., 2007).

CS Convergence: Contextual

Contextual fear conditioning is a bit more complex because it requires hippocampal activation during the time of training. This activation is limited to the acquisition of contextual, but not auditory fear. Hippocampal lesions made just after

training block context fear, but not auditory fear (Kim & Fanselow, 1992). However, hippocampal activation follows a temporal gradient. If the lesion occurs after one week, a significant amount of fear is maintained (Kim & Fanselow, 1992). Pre-training hippocampal lesions do not have an effect on fear memory (Maren, Aharonov, & Fanselow, 1997). Therefore the hippocampus is thought to be involved in the formation of an integrated, gestalt-type configural representation of an environment as well as its temporary storage (Fanselow, 2000; Kim & Fanselow, 1992, Anagnostaras, Maren, & Fanselow, 1999). Contextual memories are then transferred to the cortex within thirty days for permanent storage (Frankland et al., 2004). As with cued fear conditioning, context-shock associations converge at the BLA. The dorsal hippocampus forms the configural representation of the context, then must pass through the ventral hippocampus before it arrives at the amygdala via the ventral angular bundle, and lesions within this pathway attenuate contextual, but not auditory fear conditioning (Anagnostaras, Maren, & Fanselow, 1999; Maren & Fanselow 1995). While the hippocampus is important for forming the contextual representation the amygdala is critical for the context-shock association. Interestingly, it has been shown that the BA is important for contextual, but not auditory, fear conditioning (Onishi & Xavier, 2010). However, electrolytic lesions or loss of GluA1 in the BA is shown to disrupt both contextual and auditory fear conditioning (Goosens & Maren, 2001; Humeau et al., 2007).

Amygdala Efferents

The amygdala receives and interprets information from the medial pre-frontal cortex, hippocampus, and thalamus, and undergoes synaptic plasticity during fear learning (Schafe et al., 2001; Clugnet & LeDoux, 1990; Maren & Fanselow, 1995; Mahan & Ressler, 2012). Once the BLA processes the association between the CS and the US, fear responses are elicited via the medial central nucleus of the amygdala (CEAm) and bed nuclei of the stria terminalis (BST). The CEAm contains projection neurons to downstream structures that generate fear responses including analgesia, autonomic and respiration changes; potentiated startle, and freezing, as well as to the BST (Fendt & Fanselow, 1999; LeDoux, 2000; Maren & Fanselow, 1995; Nagy & Paré, 2008). The BLA nuclei and the CEA are separated by paracapsular intercalated cells (ITC; Millhouse, 1986). There are multiple pathways connecting the nuclei within the BLA to the CEAm that are important for fear expression, although it is unclear if one pathway is more efficient, or relied upon, as the primary pathway.

The cortex-like BLA consists of excitatory glutamatergic projection neurons and inhibitory interneurons. The CEA is striatal-like and so its projection neurons release the inhibitory transmitter γ-Aminobutyric acid (GABA; Swanson & Petrovich, 1998). The PICs are also GABAergic (Nitecka & Ben-Ari, 1987).

Within the BLA complex, the BA nucleus projects directly to the CEAm, while the LA has no known direct connections to the CEAm (Carlsen, 1989; Paré, Quirk, & LeDoux, 2004; Pitkänen & Amaral, 1991). Since the LA is implicated in processing auditory fear conditioning, the pathway to elicit fear responses via the CEAm have been explored (Haubensak et al., 2010; Paré, Quirk, & LeDoux, 2004; Nader et al., 2001). A

pathway of interest involves the projection from the LA to the BA, and then the BA to the CEAm (Pitkänen & Amaral, 1991). A second indirect connection comprises the LA projections to the lateral central nucleus of the amygdala (CEAl), which possesses reciprocal inhibitory γ-Aminobutyric acid (GABA) neurons regulating CEAm output (Haubensak et al., 2010). Still, a third pathway leading information from the LA to the CEAm is via the intercalated cells. The LA sends projections to the GABAergic ITC neurons, which then disinhibits neurons in the CEAm, which then executes fear responses (Paré, Quirk, & LeDoux, 2004).

Molecular Level

NMDA Receptors

The NMDA receptor is constructed from four subunits to form a heteromeric pentamer with a variety of physiological and pharmacological properties that depend on the assemblage with the obligatory GluN1 subunit (Cull-Candy & Leszkiewicz, 2004; Traynelis et al., 2010). Embryonic synaptic NMDA receptors predominantly have subunits GluN1/GluN2B diheteromers and once synaptic connections are formed, activity results in a triheteromer NMDA receptor type containing GluN1/GluN2A/GluN2B, or diheteromers GluN1/GluN2A and GluN1/GluN2B (Tovar & Westbrook, 1999; Endele et al., 2010). During postnatal development in rats, a developmental shift from GluN2B-containg receptors to an increase GluN2A expression is shown in thalamic and cortical neurons, and suggests that synaptic GluN2B is displaced to extrasynaptic sites after the insertion of the GluN2A (Tovar & Westbrook, 1999; Kew et al., 1998; Liu, Murray, &

Jones, 2004). In adult cortex and hippocampus, GluN2A and GluN2B are predominant subunits and determine distinct functional responses to NMDARs. GluN2B is important for synaptic plasticity, which is critical for learning and memory formation (Kirkwood, Rioult, & Bear, 1996; Cull-Candy, Brickley, & Farrant, 2001). GluN2B is particularly crucial in long-term depression in the adult hippocampus, with a loss of GluN2B sufficient to cause learning deficits and an overexpression of GluN2B enhancing synaptic plasticity and learning (Clayton et al., 2002; Zhao et al., 2005; Brigman et al., 2010; Tang et al., 1999).

NMDA receptors are coincidence detectors because it requires both pre-synaptic and post-synaptic activity (Mayer, Westbrook & Guthrie, 1984; Nowak et al., 1984). NMDAR have voltage-gated channels requiring a decrease overall voltage, via an EPSC, on the membrane in order to release the magnesium ion that is stuck in the receptor (Mayer, Westbrook & Guthrie, 1984). Additionally, pre-synaptic activity is required, which is the ligand binding of glutamate to the NMDAR to allow both sodium and calcium to enter the cell (Fukunaga et al., 1993). Calcium entering the cell triggers multiple responses including phosphorylation of protein kinases and secondary messenger systems and ultimately result in learning induced changes at the synaptic level, which include increased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptors (AMPAR) and retrograde messengers such as nitric oxide.

NMDA receptors are important mediators of synaptic plasticity and learning at most excitatory synapses in the central nervous system. The amygdala receives and interprets information from the medial pre-frontal cortex, hippocampus, and thalamus,

and undergoes synaptic plasticity during fear learning (Schafe et al., 2001; Clugnet & LeDoux, 1990; Maren & Fanselow, 1995; Mahan & Ressler, 2012). NMDA-dependent synaptic plasticity is an essential component for memory formation of aversive conditioning, creating permanent changes within cells (Maren, 1996; Guzowski et al, 2000; Blair et al., 2001). The majority of studies examining the role of NMDAR in synaptic plasticity have examined long-term potentiation (LTP) induced by stimulation of Schafer collaterals onto CA1 pyramidal cells of the hippocampus Davies & Collinridge, 1989). Although this has also been shown in neurons within the BLA important for CS-US convergence (Rodriguez & Schafe, 2004). Here NMDAR are critical for induction but not expression of LTP or do they play a critical role in cell firing. Consistent with this, the classic NMDAR antagonist, 2-amino-5-phosphonovaleric acid (APV) prevents acquisition of hippocampus-dependent memory but does not prevent the expression of an already formed memory (Morris et al., 1996; Kim et al., 1991). The selective effect of APV on acquisition but not expression of learned behavior is critical to isolate the role for NMDA receptors in memory formation. The interpretation of studies with intra-BLA administration of APV is less straightforward. Pre-training infusions of APV into the BLA blocks acquisition of both auditory and contextual fear (Fanselow & Kim, 1994; Miserendino et al., 1990). However, pretesting intra-BLA infusion has also been found to block expression of an already acquired fear memory (Maren , Aharonov, & Fanselow, 1996; Fendt, 2001). Additionally, intra-BLA APV not only prevents the induction of LTP in the BLA, it also reduces neuronal spiking (Maren & Fanselow, 1995). Such findings

make it difficult to attribute intra-BLA APV's actions on fear conditioning specifically to a prevention of LTP.

NMDA-dependent synaptic plasticity is an essential component for memory formation of aversive conditioning, creating permanent changes within cells (see below, and Maren, 1996; Guzowski et al, 2000; Blair et al., 2001). Processed information is then transmitted to the CeA, which sends projections to various structures including the periaqueductal gray (freezing response), modulatory systems (arousal), and nuclei within the hypothalamus to regulate stress hormone release (LeDoux et al., 1998; LeDoux, 1993). Sensory and contextual information are thought to project both directly and indirectly to the LA and BA. The inputs to the amygdala, and within the amygdala itself are responsible for fear learning, with the CeA responsible for the output to regulatory systems as described above.

Dissertation Objective

My thesis work has been focused on the role of the *N*-methyl-D-aspartate receptor (NMDAR) in learning and memory, with a principle focus on using cellular manipulations of the NMDAR on basolateral nuclei of the amygdala (BLA) to measure the significance of NMDAR-mediated synaptic plasticity on auditory and contextual Pavlovian fear conditioning. This was achieved through temporary inactivation, the use of a shRNA virus targeted at depleting the Grin1 gene, and the use to transgenic mice to specifically isolate and dissociate the LA and BA nuclei. The behavioral effects of the manipulations were assessed with Pavlovian auditory and contextual fear conditioning. In

the following sections, I will explain a set of experiments designed manipulate the NMDAR- related synaptic plasticity in BLA nuclei during auditory and contextual Pavlovian fear conditioning and implications for the primary pathway projecting auditory fear associations from the LA to produce a fear response. Specifically, Chapter 2 concerns the role of selective GluN2B antagonist on fear learning and retention. Chapter 3 utilizes the shRNA virus to look at NMDA-mediated plasticity in the lateral amygdala. Chapter 4 uses transgenic mice to address the role of NMDAR-mediated synaptic plasticity in both the LA and BA nuclei. Chapter 4 also addresses specific analyses that utilize the data to extract more information from viral infusion studies, and implicates the primary pathway for projecting auditory fear associations, and a pathway to project contextual fear associations, to produce a fear response. Explicitly, my data support the NMDAR-mediated plasticity in the BA as being imperative for auditory and contextual fear conditioning. In Chapter 5 these findings will be synthesized into a model that can explain the role of NMDAR- mediated plasticity in fear learning and memory.

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CHAPTER TWO

The NMDA subunit GluN2B in the basolateral amygdala is critical for the acquisition of contextual fear

Abstract

Previous studies have shown that neural mechanisms of fear learning involve the amygdala. N-methyl-D-aspartate receptors (NMDAR), and especially the GluN2B subunit of the NMDAR, have been implicated in the mechanism of long-term potentiation, a neuronal substrate for fear learning. Here, we show that by temporarily inactivating the GluN2B subunit of the NMDAR in the basolateral amygdala (BLA) a deficit in contextual fear learning is produced. Unlike previous studies using intra-BLA application of the classic NMDA receptor antagonist d-amino-phosphonovalerate (APV) the effects of intra-BLA ifenprodil could not be attributed to state-dependent learning, interference with working memory or suppression of performance.

Introduction

Fear conditioning is a type of Pavlovian learning produced when an aversive unconditional stimulus (US), such as a shock, becomes associated with a neutral stimulus because the two co-occurred in a conditional or dependent relationship (Pavlov, 1927; Rescorla, 1967). This associative learning transforms the neutral stimulus into what Pavlov termed a conditional stimulus (CS) and is reflected by the emergence of a new or conditional response (CR) to the CS. In the typical preparation a brief cue (e.g., a tone) is presented immediately prior to the US and both the cue and the context (conditioning chamber) become CSs.

The amygdala, specifically the basolateral complex (BLA) consisting of the basolateral, basomedial, and lateral nuclei of the amygdala, is essential for fear learning to both the cue and the context (Maren et al., 1996; Gale et al., 2004; Lee et al., 1993; Phillips & LeDoux, 1992). Lesions or temporary inactivation of the BLA produces impairments of both cued and contextual fear acquisition and expression (Blanchard & Blanchard, 1972; Helmstetter, 1992; Helmstetter & Bellgowan, 1994; Hitchcock & Davis, 1986; Muller et al., 1997; Maren et al., 1996a). However, the BLA receives information about the cue and context from different sources. Auditory cues arrive via the auditory thalamus and cortex (Boatman & Kim, 2006; Romanski & LeDoux, 1992); while contextual information relies on projections between the hippocampal formation and BLA (Kim & Fanselow, 1992a; Maren & Fanselow, 1995).

NMDA receptors are important mediators of synaptic plasticity and learning at most excitatory synapses in the central nervous system. The NMDA receptor is

constructed from four subunits to form a heteromeric pentamer with a variety of physiological and pharmacological properties depending on the assemblage with the obligatory GluN1 subunit (Cull-Candy & Leszkiewicz, 2004; Traynelis et al., 2010). Embryonic synaptic NMDA receptors are predominantly GluN1/GluN2B diheteromers and once synaptic connections are formed, activity results in a triheteromer NMDA receptor type containing GluN1/GluN2A/GluN2B, or diheteromers GluN1/GluN2A and GluN1/GluN2B (Tovar & Westbrook, 1999; Endele et al., 2010).

There is a developmental shift during postnatal development from GluN2B-containing receptors to an increase GluN2A expression in thalamic and cortical neurons, and suggests that synaptic GluN2B is displaced to extrasynaptic sites after the insertion of the GluN2A (Kew et al., 1998; Tovar & Westbrook, 1999; Liu, Murray, & Jones, 2004). In adult cortex and hippocampus, GluN2A and GluN2B are predominant subunits and determine distinct functional responses to NMDARs. GluN2B is important for synaptic plasticity, which is critical for learning and memory formation (Kirkwood et al., 1996; Cull-Candy et al., 2001; Quinlan et al., 2004). GluN2B is particularly crucial in long-term depression in the adult hippocampus, with a loss of GluN2B sufficient to cause learning deficits and an overexpression of GluN2B enhancing LTP and learning (Clayton et al., 2002; Zhao et al., 2005; Brigman et al., 2010; Tang et al., 1999).

Acquisition of fear requires N-methyl-D-Aspartate receptor (NMDAR)-mediated synaptic plasticity in the BLA (Fanselow & LeDoux, 1999). The majority of studies examining the role of NMDAR in synaptic plasticity have examined long-term potentiation (LTP) induced by stimulation of Schafer collaterals onto CA1 pyramidal

cells of the hippocampus (Davies & Collingridge, 1989). Here NMDAR are critical for induction but not expression of LTP or do they play a critical role in cell firing. Consistent with this, the classic NMDAR antagonist, 2-amino-5-phosphonovaleric acid (APV) prevents acquisition of hippocampus-dependent memory but does not prevent the expression of an already formed memory (Morris et al., 1986; Kim et al., 1991). The selective effect of APV on acquisition but not expression of learned behavior is critical to isolate a role for NMDA receptors in memory formation (Rudy &Keith, 1990).

Furthermore, Rudy and Keith (1990) point out that studies that block NMDAR only at the time of training are insufficient in implicating NMDAR in the acquisition of memory because they allow for a state-dependent decrement in performance. Drug states may act as cues for memory retrieval and a change in drug state between training and testing may cause a loss of performance simply because the cues have changed between training and testing (Overton, 1968). State-dependent effects of drugs on learning are best addressed by factorially manipulating the presence or absence of the drug during both training and testing within the same experiment. Using such a design, Kim et al., (1991) ruled out an effect of APV on hippocampus-dependent learning because administering the drug at the time of acquisition blocked performance equivalently regardless of drug state at the time of test.

The interpretation of studies with intra-BLA administration of APV is less straightforward. As in the hippocampus, pre-training infusions of APV into the BLA blocks acquisition of both cued and contextual fear (Fanselow &Kim, 1994; Miserendino et al., 1990). However, pretesting intra-BLA infusion has also been found to block

expression of an already acquired fear memory (Fendt, 2001; Lee et al., 2001; Maren et al, 1996b). Additionally, intra-BLA APV not only prevents the induction of LTP in the BLA, it also reduces neuronal spiking (Maren &Fanselow, 1995). Such findings make it difficult to attribute intra-BLA APV's actions on fear conditioning specifically to a prevention of LTP.

LeDoux and colleagues turned to ifenprodil, a selective noncompetitive NMDA antagonist, that specifically binds to the GluN2B subunit by acting on its polyamine site, stabilizing the inactivated form of the ion channel (Bauer et al., 2002); Blair et al., 2005; Rodrigues et al., 2001). This drug is able to bind to the magnesium-gated channel inhibiting the channels activation. Previous studies have shown that ifenprodil maximally blocks GluN1/GluN2B receptors without having an effect on GluN1/GluN2A receptors (Williams, 1993; Legendre & Westbrook, 1991). Like APV, intra-BLA ifenprodil blocked acquisition of fear learning. However, unlike intra-BLA APV, ifenprodil did not block expression of a previously learned fear response (Bauer et al., 2002).

The purpose of the present experiment was to rule out state-dependent generalization decrement as the source of ifenprodil's action on fear memory. This was accomplished by factorially manipulating pre-training and pretesting drug state. An account of drug action specifically on memory formation requires an effect on the pre-training drug condition with no effect on the pretesting condition and no interaction between the two factors. A state-dependent account predicts a reliable interaction between the two factors.

In addition we examined the effect of pre-training ifenprodil on freezing during

acquisition. Unlike APV directed at the hippocampus (Kim et al., 1991; Quinn et al., 2005), intra-BLA APV severely reduces freezing during training (Maren et al., 1996b). Freezing during training reflects working memory for the context-shock association (Fanselow, 1980; Kim et al., 1992b) and a loss of behavior during training makes attribution of the drugs effects to memory inconclusive.

Materials and Methods

All experiments were conducted in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Subjects

Forty-one male Long-Evans rats initially weighing 250–280 g were obtained from a commercial supplier (Harlan, Indianapolis, IN, USA). After arrival, rats were housed individually in standard stainless-steel cages on a 12/-h light/dark cycle and were provided free access to food and tap water. After being housed, the rats were handled daily (60–90 s per rat) for five days to acclimate them to the experimenter. The number of animals used was the minimum required to ensure reliability of the results, and every effort was made to minimize animal suffering.

Surgery

Under aseptic conditions, animals were given atropine methyl nitrate (0.04 mg/kg, i.p.), anesthetized using isofluorine and mounted into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The scalp was incised and retracted, and head position was adjusted to place bregma and lambda in the same horizontal plane. Small holes were drilled in the skull to implant 26-gauge guide cannula (Plastics One, Roanoke, VA, USA) bilaterally into the amygdala (from bregma: anteroposterior, −3.1 mm; mediolateral, ±5.3 mm; dorsoventral, −7.6 mm). Implanted cannulae were then cemented to the skull using three anchoring screws to stabilize the dental acrylic. After surgery the cannulae were kept clean and free of infection by inserting dummy cannula. These dummies were replaced daily with clean ones. This adapted the rat to handling during the 12–13 day recovery period making it easy to insert the injectors in awake animals at the time of ifenprodil (IFEN) or artificial cerebro-spinal fluid (ACSF) infusion without agitating the animals. For drug infusion, 33-g injectors were inserted and extended 1 mm below the guide cannula.

Infusion

Ifenprodil tartrate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ACSF (0.4 mg/mL, pH 7.4) or vehicle (ACSF with 0.1% tartaric acid pH, 7.4) was micro infused bilaterally into the BLA using 33-gauge infusion cannula attached to polyethylene tubing connected to 5uL Hamilton syringes (Hamilton Company, Reno, NV, USA). The infusion cannula protruded 1mm beyond guide cannula and injected

0.25 uL per side at a rate of 0.1 uL per minute. Injectors remained for two minutes post-infusion to minimize backflow.

Apparatus

The context consisted of a chamber with aluminum sidewalls and Plexiglas in the front, back, and top (28x21x22 cm; Lafayette Instruments, Lafayette, IN, USA). The floor of each chamber had 18 stainless steel rods (4mm diameter, 1.5mm apart from center) connected to a shock scrambler, and computer controlled (Med-Associates, St. Albans, VT, USA). The chambers were scented with 10% Simple Green and cleaned with 70% isopropyl alcohol. Locomotor activity was recorded and hand-scored by a blind observer for two seconds every eight seconds. The number of observations were summed and converted to a percentage of number of freezing observations over total number of observations. During the training phase, freezing was measured during the first three minutes and after the termination of each shock. During testing, freezing was measured for the entire duration of the test.

Procedure

Thirty-six animals were placed into one of four groups. Animals received either a pre-training injection of ifenprodil (IFEN) or artificial cerebro-spinal fluid (ACSF) fifteen minutes before contextual training on day 1. On day 2, pre-testing injections of IFEN or ACSF were given fifteen minutes prior to the context test. These injections were properly counter-balanced (see Table 1). On day 1, subjects were in the context for a total of 8.4

minutes. They were able to explore the context for the first three minutes. After the initial exploratory period, they were shocked five times with each shock consisting of .9mA for a duration of two seconds every 64 seconds. One minute after the last shock, they were removed. On day 2, the subjects were put back in the same context for an 8-minute context test to measure fear learning. Our measure of fear was the freezing response, which is defined as lack of movement except for respiration (Fanselow, 1980).

Histology

At the end of the experiment, subjects were euthanized with sodium pentobarbital and perfused intracardially with PBS followed by 4% buffered paraformaldehyde. The brains were removed and stored in 4% buffered paraformaldehyde for five days before being cryoprotected using 30% sucrose in PBS mixture for 48 hours prior to being frozen. Coronal sections at 50 microns were taken and stained using the Nissl procedure to verify the cannulation placement (Paxinos &Watson, 1998). Stained sections were examined using a light microscope (Zeiss, Oberkochen, Germany).

Results

Cannula placement verification

As illustrated in Figure 1, all cannula placements were determined to be within the boundaries of the BLA.

Fear Acquisition

Freezing scores taken during training and testing were subjected to appropriate ANOVA. Inspection of Figure 2, which displays the training data, reveals a significant increase in freezing between shock trials indicating acquisition of context fear $F(4,31) = 22.8$, $p < 0.001$, but no interaction between trial and pre-training injections of IFEN (N=18) versus ACSF (N=18) during acquisition $F(4,31) < 1$. All subjects were able to show freezing to the context following five aversive shocks.

Context Test

The test data are shown in Figure 3. Pre-training injections of IFEN produced a significant impairment compared to pre-training injections of ACSF controls regardless of posttest injections. A 2x2 ANOVA indicated a reliable main effect for pre-training injections $F(1,32) = 24.949$, $p < 0.001$. There was no effect of posttest infusions $F(1,32) = 1.472$, $p > 0.05$, indicating that ifenprodil did not impair expression of freezing behavior. Importantly, the lack of an interaction of the pretesting and pre-training drug factors indicates that the results cannot be interpreted in terms of a drug state dependent effect $F(1,32) < 1$.

Discussion

Consistent with previous reports (Rodriguez et al., 2001), when the selective GluN2 antagonist, ifenprodil, was given prior to Pavlovian fear conditioning, fear was reduced during a later test of long-term contextual fear memory. Additionally, when the

drug was only given during testing, expression of a previously acquired fear remained intact. Importantly, fear was blocked when the animals were trained and tested in the presence of the drug, arguing against a state-dependent interpretation of the drug's action on behavior.

We also assessed ifenprodil's effects during the acquisition session. Freezing during this time reflects a working memory for the context-US association (Fanselow 1980; Kim et al., 1992). The drug had no effect on behavior at this time suggesting that ifenprodil did not affect context (CS) or shock (US) processing. Thus the most likely account of ifenprodil's effect is that it blocked long-term memory formation. Such an effect on associative learning is consistent with the drug's known action on NMDAR-mediated plasticity.

The overall pattern of effects found on contextual fear conditioning with intra-amygdala ifenprodil was very similar to that found with NMDA antagonist, APV, when it is administered directly to the hippocampus or intra-cerebro-ventricularly (Kim et al., 1991; Quinn et al., 2005). However, in the hippocampus NMDAR contributes to learning about the context per se, while in the BLA, NMDAR play a role in associating the context with shock (Matus-Amat et al; 2007). Surprisingly, intra-BLA ifenprodil had very different effects from intra-amygdala APV. Both NMDAR antagonists block later memory expression when given prior to training, although APV seems more effective (Fanselow &Kim, 1994, Maren et al., 1996b). However, ifenprodil seems to have a more "pure" effect on long-term memory formation, as it did not interfere with working

memory or expression of a long-term memory acquired in a drug-free state. Furthermore, ifenprodil's effects were not caused by drug-state cuing of memory.

Functional NMDAR are composed of an obligatory GluN1 subunit that is usually paired with either a GluN2A or a GluN2B subunit. The differential effects of APV and ifenprodil suggests that GluN2B containing NMDAR play a somewhat exclusive role in supporting long-term memory formation while GluN2A containing NMDAR have a more general role in synaptic transmission in the BLA (Maren & Fanselow, 1995; Maren et al., 1996). Further elucidation of the role of GluN2A and GluN2B subunits in the BLA may provide keys to more selective modulation of fear and anxiety disorders.

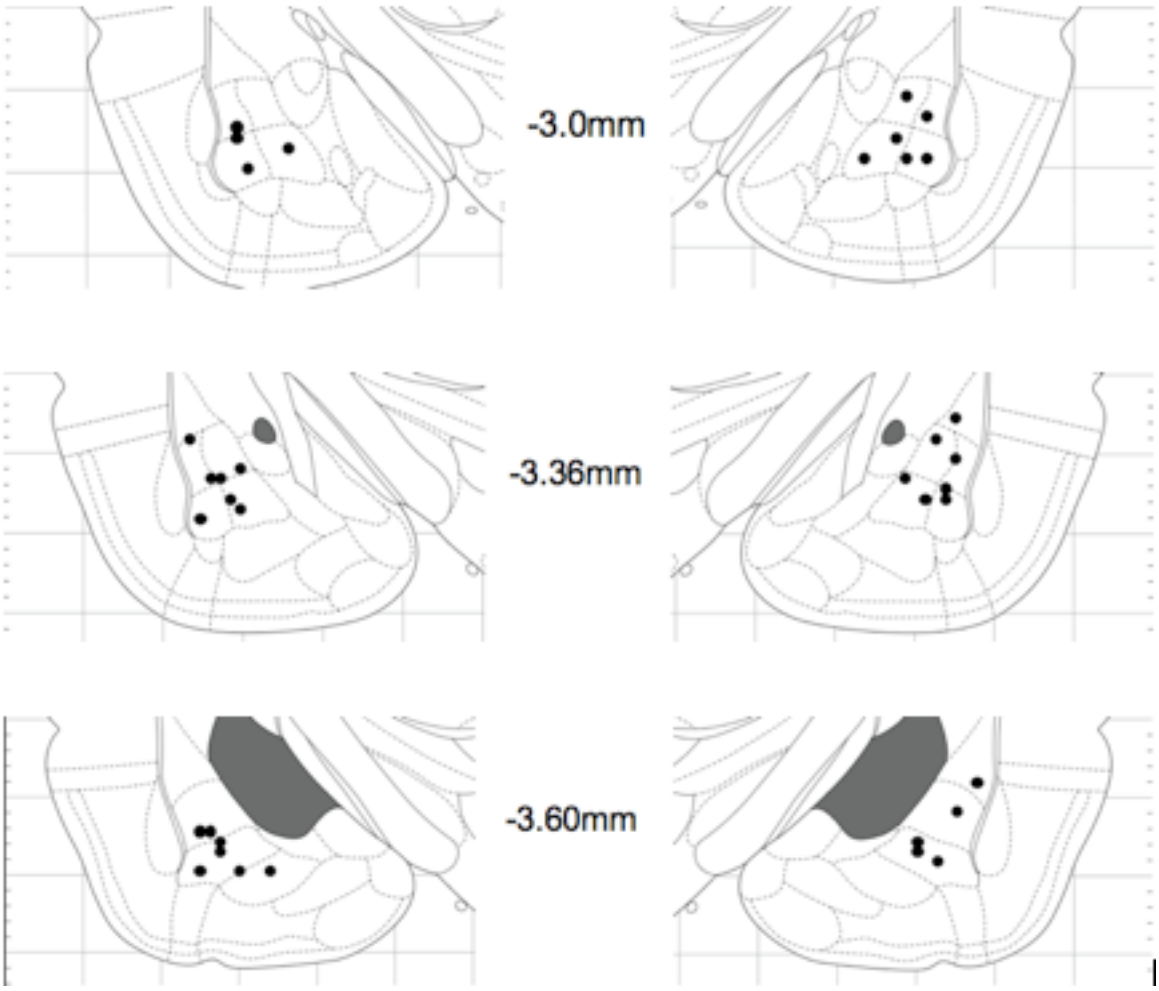

Figure 1. Histological analysis of BLA cannula implantations. Reconstruction of the cannula tip placement in the region of the BLA (AP 2.6-3.3mm). Coronal section images adapted from Paxinos and Watson, 2007.

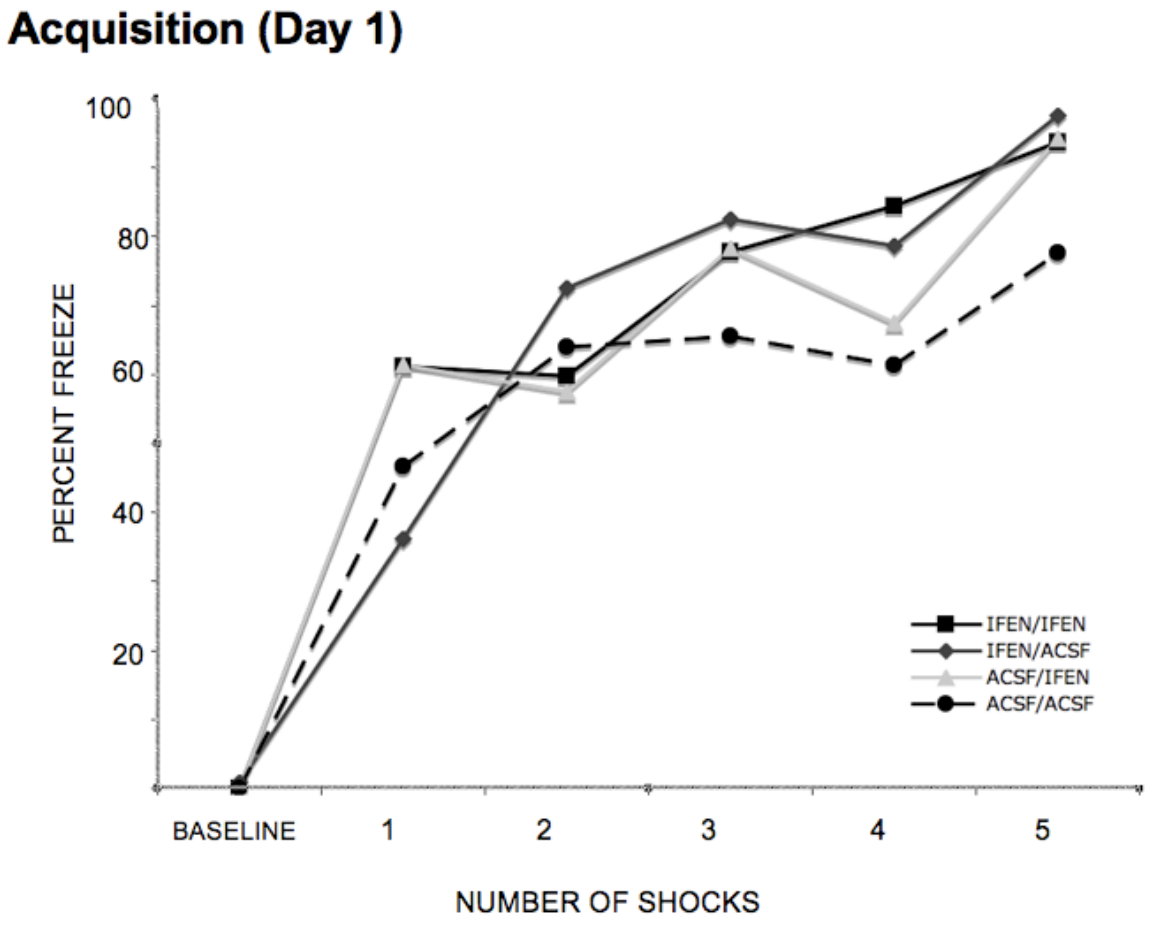

Figure 2: Effect of intra-amygdala infusions of IFEN on contextual fear conditioning. Mean percentage of freezing during acquisition in rats infused with either intra-amygdala IFEN (N=18) or ACSF (N=18) 15 minutes prior to training. There was no interaction between trial and drug $F(4,31) < 1$.

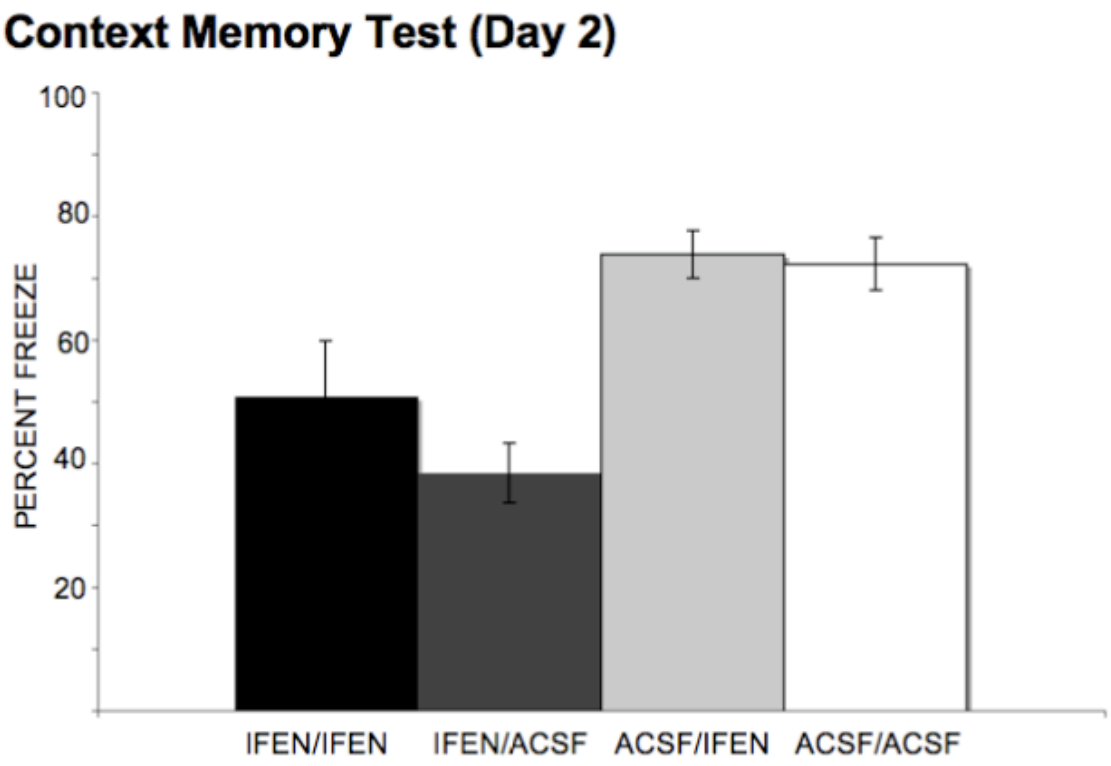

Figure 3: Effect of intra-amygdala infusions of IFEN on long-term memory for contextual conditioning. Mean +/- S.E.M during the 8-minute shock-free context-fear memory test in rats with either IFEN or ACSF infused into the amygdala 15 minutes prior to training and/or IFEN or ACSF 15 minutes prior to testing. IFEN prior to training significantly retarded learning fear to the context as compared to ACSF prior to training regardless of the posttest infusion drug $p < 0.001$.

Table 1. Contextual Fear Conditioning Groups

Group	Day 1: Context A	Day 2: Context A	animals
1.	IFEN/ 5 shocks	IFEN/ Test	8
2.	IFEN/ 5 shocks	ACSF/ Test	10
3.	ACSF/ 5 shocks	IFEN/ Test	10
4.	ACSF/ 5 shocks	ACSF/ Test	8

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CHAPTER THREE

shRNA-induced knockdown of NMDA receptors in the lateral amygdala causes a deficit in auditory fear conditioning

Abstract

The lateral amygdala (LA) is implicated as the site where convergence of auditory signaling information (tone) and reinforcement (shock) result in the synaptic plasticity necessary to produce conditioned fear. Synaptic plasticity that mediates the formation of fear memories is thought to be dependent upon N-methyl-D-aspartate receptors (NMDAR). Techniques previously used to implicate NMDAR, such as pharmacological antagonists, have been limited in addressing specificity of anatomical location and the particular receptors they bind to. Additionally, the lack of promoters specific to the LA limits the genetic mutation approach (e.g., knockout mice). The GluN1 subunit is obligatory for NMDA receptors to assemble. Therefore, we used an shRNA construct directed at the GluN1 subunit to interfere with NMDA receptor expression. Prior to training, rats were given bilateral infusions of a lentivirus containing either the shRNA or a scrambled sequence, to knockdown the GluN1 subunit in the LA. Four weeks after infection, rats received a five tone-shock Pavlovian fear conditioning procedure. It was found that depletion of the GluN1 subunit in the LA caused a deficit in auditory, but not contextual fear conditioning.

Introduction

Pavlovian fear conditioning signifies to the learning of associations between neutral stimuli (conditional stimulus; CS) and painful stimuli, such as a footshock (unconditional stimulus; US). The major components of the neural circuit mediating from environmental stimulation to fear behavior are well established (Fanselow & Poulos, 2005; Kim & Jung, 2006; Paré, Quirk, & LeDoux, 2004). The amygdala, specifically the basolateral complex (BLA) consisting of the basolateral, basomedial, and lateral nuclei of the amygdala, is shown to essential for fear learning to both the cue and the context (Maren, Aharonov, & Fanselow, 1996; Gale et al., 2004; Lee et al., 1993; Phillips and LeDoux, 1992). Lesions or temporary inactivation of the BLA produces impairments of both cued and contextual fear acquisition and expression (Blanchard and Blanchard, 1972; Helmstetter, 1992; Helmstetter and Bellgowan, 1994; Hitchcock and Davis, 1986; Muller et al., 1997; Maren, Aharonov, & Fanselow, 1996). However, the BLA receives information about the cue and context from different sources. Conveniently, the BLA is a cortex-like structure that receives highly processed information from several cortical and some thalamic regions (Swanson & Petrovich, 1998).

The lateral amygdala (LA) is implicated as the site where convergence of auditory signaling information (tone) and reinforcement (shock) result in the synaptic plasticity necessary to produce conditioned fear (Romanski et al., 1993; LeDoux, 2000; Blair et al. 2001; Pare, Quirk, & LeDoux, 2004; Davis, 2006; Sigurdsson et al., 2007; Ploski et al., 2010). Auditory cues from both the auditory thalamus (medial geniculate

nucleus) and auditory cortex can acquire fear conditioning, inducing long-lasting changes in the LA (Boatman and Kim, 2006; Romanski and LeDoux, 1992; Clugnet & LeDoux, 1990; Doyère, Schafe, Sigurdsson, & LeDoux, 2003).

Synaptic plasticity that mediates the formation of fear memories is thought to be dependent upon N-methyl-D-aspartate receptors (NMDAR; Hitchcock and Davis, 1991; Maren, 1999; Ressler et al., 2002). The NMDAR is constructed from four subunits to form a heteromeric pentamer with a variety of physiological and pharmacological properties depending on the assemblage with the obligatory GluN1 subunit (Cull-Candy & Leszkiewicz, 2004; Traynelis et al., 2010). The majority of studies examining the role of NMDAR in synaptic plasticity have examined long-term potentiation (LTP) induced by stimulation of Schafer collaterals onto CA1 pyramidal cells of the hippocampus (Davies and Collingridge, 1989). Here NMDARs are critical for induction but not expression of LTP or do they play a critical role in cell firing. Consistent with this, the classic NMDAR antagonist, 2-amino-5-phosphonovaleric acid (APV) prevents acquisition of hippocampus-dependent memory but does not prevent the expression of an already formed memory (Morris et al., 1986; Kim et al., 1992). The selective effect of APV on acquisition but not expression of learned behavior is critical to isolate a role for NMDA receptors in memory formation (Rudy and Keith, 1990). The interpretation of studies with intra-BLA administration of APV is less straightforward. As in the hippocampus, pre-training infusions of APV into the BLA blocks acquisition of both cued and contextual fear (Fanselow and Kim, 1994; Miserendino et al., 1990). However, pretesting intra-BLA infusion has also been found to block expression of an already

acquired fear memory (Fendt, 2001; Lee et al., 2001; Maren et al, 1996). Additionally, intra-BLA APV not only prevents the induction of LTP in the BLA, it also reduces neuronal spiking (Maren and Fanselow, 1995). Such findings make it difficult to attribute intra-BLA APV's actions on fear conditioning.

Techniques previously used to implicate NMDAR, such as pharmacological antagonists, have established the importance of amygdalar NMDA receptors in the acquisition of fear conditioning (Boulis et al., 1990; Fanselow & Kim, 1994; Rodrigues, Schafe, & LeDoux, 2001; Goosens & Maren, 2004). However, these procedures are somewhat limited in terms of anatomical and pharmacological specificity. Additionally, the lack of promoters specific to the LA has limited the genetic mutation approach (Bardgett et al., 2004; Sprengel & Single, 1991). NMDARs are important mediators of synaptic plasticity and learning at most excitatory synapses in the central nervous system.

The purpose of the present experiment was to examine the role of NMDA receptors in the lateral amygdala during auditory fear conditioning using a small hairpin RNA (shRNA) lentivirus that targeted the Grin1 gene. To target rat Grin1 (GluN1 subunit of the NMDAR) for RNA interference, shRNA sequences were chosen based on the ability to down-regulate Grin1 expression while minimizing off target effects that might interfere with the expression of other related proteins. Based on previous data as described above, our predictions were that a reduction of NMDA receptors in the lateral amygdala would an impairment in auditory fear memory, and possible a deficit in contextual fear memory if the LA is important for mediating contextual memory as well. This is a novel approach using techniques that allow for anatomical specificity without

the disruption of information passing through the region.

Materials and Methods

All experiments were conducted in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Subjects

Twenty-eight male Long Evans rats initially weighing 250–280 g were obtained from a commercial supplier (Harlan, Indianapolis, IN, USA). After arrival, rats were housed individually in standard stainless-steel cages on a 12-hour light/dark cycle and were provided free access to food and water. Animals were handled daily (one-two minutes per rat) for at least one week prior to the start of surgery and behavioral training to acclimate them to the experimenter. The number of animals used was the minimum required to ensure reliability of the results, and every effort was made to minimize animal suffering.

Grin1 shRNA design and virus production

To target rat Grin1 (GluN1 subunit of the NMDAR) for RNA interference, shRNA sequences (from Alvarez et al.) were chosen based on the ability to down-regulate Grin1 expression, while minimizing off target effects that might interfere with

the expression of other related proteins. DNA oligonucleotides encoding the shRNAs were cloned into the lentiviral vector pLL3.7 (as described in Lasek et. al., 2007) and tested for the ability to down-regulate Grin1 expression in 293FT cells. Cells were co-transfected with the Grin1 shRNA lentiviral plasmids and a Grin1 cDNA expression plasmid. The most effective sequence, shGrin1-1173 (corresponding to shRNA sequence "*a*" published by Alvarez et. al., 2007), reduced Grin1 expression by 85% in 293FT cells. Lentivirus was produced from shGrin1-1173 and a control shRNA plasmid (shScr) in 293FT cells (as described in Lasek et. al.). Viral titers were approximately 5 x 10^7 pg p24 antigen per mL

Lentiviral shRNA Injection

Under aseptic conditions, animals were given carprofen (1 mg/kg, s.c.), anesthetized using isofluorine and mounted into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The scalp was incised and retracted, and head position was adjusted to place bregma and lambda in the same horizontal plane. Small holes were drilled bilaterally into the amygdala. Male Long-Evans rats were randomly assigned to either the shRNA virus (n=10), the scramble virus (n=9), or a control group (n=9). shRNA or a scrambled sequence control viral infusions were made bilaterally targeting the LA at -3.0mm from Bregma, -/+ 5.2 lateral to midline, and -7.6 below the skull surface. Injection cannulae (33 gauge) were attached to a 5µl microsyringe (Hamilton Instruments) via polyethylene tubing (PE20) and inserted into guide cannulae (28 gauge) attached to the arms of the stereotax. Microsyringes were mounted into a syringe pump

(Harvard Apparatus, South Natick, MA) for controlled microinfusions. Cannulae were lowered and infusions of .75 µl viral injections were made across eight minutes (0.1 µl /min rate). Cannulae remained in place for an additional ten minutes to allow for adequate diffusion and reduction of backflow. Following infusions, incisions were closed with stainless steel wound clips and animals were given i.p. injections of the analgesic/anti-inflammatory carprofen (1 mg/kg) and placed on heating pads until they recovered from anesthesia. Carprofen injections were continued for an additional two days post-surgery. In addition, rats were given the antibiotic trimethoprim sulfa (TMS) in their drinking water, weighed, monitored and handled for one-week following surgery. Rats were allowed a total of 28 days of recovery prior to behavioral training.

Apparatus

All behavioral training was performed using two different sets of four identical conditioning chambers. The context consisted of a chamber with aluminum sidewalls and Plexiglas in the front, back, and top (28x21x22 cm; Lafayette Instruments, Lafayette, IN, USA). The contexts were surrounded with a sound-attenuating chamber, and each set of chambers was in a unique spatial location. Animals received fear conditioning and contextual testing in one context. A novel context was used to test generalization as well as auditory fear conditioning. The two context types were differentiated by lighting, scent, cleaning solution, background noise, context shape, and different transport to and from the contexts. One context contained a brightly lit chamber, a standard grid floor which had18 stainless steel rods (4mm diameter, 1.5mm apart from center) connected to

a shock scrambler, and computer controlled (Med-Associates, St. Albans, VT, USA). The chambers were scented with 10% Simple Green and cleaned with 70% isopropyl alcohol in between each subject training. Animals were transported into this context using a portable cart where their cages hung individually from racks mounted to the cart. The alternate context had no lights on and contained a plastic triangle to change the shape of the context. The grid flooring consisted of 18 steel rods (4mm diameter, 1.5mm apart from center) that were staggered to create uneven flooring. The chambers were scented and cleaned with 1% acetic acid solution. Animals were placed in a dark black box that had clean bedding at the floor for transport into this context.

Procedure

Subjects were given one-month recovery prior to training and were handled for one minute per day for one week prior to the beginning of training. During fear conditioning, rats were able to explore the context for the first three minutes. After the initial exploratory period, subjects were given five tone-shock pairings. This consisted of a 30 second 2800Hz tone that co-terminated with a two second .9mA shock, followed by an inter-trail interval of 60 seconds prior to the next tone presentation. One minute after the last shock, they were removed. The following day, subjects were put back in the same context for an 8-minute context test to measure fear learning. On Day 3, a tone test was given in a novel context that consisted of the same time frame and duration as training, but without the shock administration. These tests were counterbalanced across subjects.

Histology

At the end of the experiment, subjects were euthanized with sodium pentobarbital and perfused intracardially with PBS followed by 4% buffered paraformaldehyde. The brains were removed and stored in 4% buffered paraformaldehyde for five days before being cryoprotected using 10% sucrose in PBS mixture for 24 hours, then 20% sucrose in PBS mixture for 24 hours, and finally, 30% sucrose in PBS mixture for 24 hours prior to being frozen. Coronal sections at 40 microns were taken and immunohistochemistry was preformed. We looked at expression of GFP tagged viral infusion with the addition of DAPI to stain nuclei. Stained sections were examined using a light microscope (Zeiss, Oberkochen, Germany).

Statistical Analysis

Fear was indexed by defensive freezing behavior, as defined by the absence of all movement except for those necessitated by respiration (Fanselow, 1980). Behavior was recorded using an automated near infrared (NIR) video tracking equipment and computer software (VideoFreeze, Med-Associates Inc.). Video was recorded at 30 frames per second and the software calculated the noise (standard deviation) for each pixel in a frame by comparing its grayscale value to previous and subsequent frames. This produced an "activity unit" score for each frame. Based on previous validation with hand scoring (correlation of $r > 0.9$ between automated system and highly trained human observers) freezing was defined as sub-threshold activity (when the motion threshold was

held at 50 activity units) for longer than 1 second. Average freezing was scored for the baseline period in all phases and the first 28 s of each tone used for conditioning (prior to US onset). For presentation purposes, the 30 tone presentations during extinction sessions were blocked into six bins of five tone presentations and freezing was averaged within each bin. Freezing data were statistically analyzed using between-subjects analyses of variance (ANOVAs) and repeated measures (trial) ANOVAs where appropriate. Post-hoc comparisons were performed following significant findings and a Bonferroni correction was applied to control for the number of comparisons made. The level of significance used for all analyses was $p < .05$.

Results

shRNA infection in the Lateral Amygdala

Figure 1 represents the target area of the virus and a representative image of the viral infusion at a 40x magnification in the lateral amygdala. The extent of the shRNA virus in the lateral amygdala was consistent with previous research involving excitotoxic lesions, electrolytic lesions, and cannula placement within the LA.

Animals with shRNA viral infusions in the LA acquire fear, maintain contextual fear recent memory, but have a deficit in tone fear memory

Fear Acquisition

Mean freezing (+/- SEM) to each 30s tone of five conditioning trials are not shown. Rats were split by infusion (shRNA virus, Scramble virus, control) resulting in

three groups. All animals displayed <1% baseline freezing to the context during the initial three-minute exploratory period, suggesting that the surgery alone did not generate inappropriate freezing behavior. A repeated measures ANOVA by trial revealed significant main effect for tone fear acquisition ($F_{(3,27)} = 132.2$, $p = 0.0001$), with no significant main effect of group ($F_{(3,27)} < 1$) or significant interaction of trial by group ($F_{(3,27)} < 1$) indicating that shRNA injections into the LA did not impair fear acquisition.

Context Test

Figure 2 presents mean freezing (+/- SEM) across the eight-minute context test. An ANOVA revealed no significant main effect of context freezing between the groups ($F_{(3,27)} < 1$) indicating that shRNA injections into the LA did not impair expression of freezing behavior to the context.

Tone Test

Figure 3 presents Mean freezing (+/- SEM) to each 30s tone of five tone-test trials. The apparent decrease in percent freezing as a function of type of viral infusion that are indicated in Figure 3 was confirmed by a one-way ANOVA performed on the data, which revealed a significant main effect of type of viral infusion on percent freezing $F(2,26) = 4.302$, $p = 0.025$.

To test prediction that an shRNA virus would produce a severe deficit in freezing behavior to the tone than either the scramble virus or controls, and that the scramble virus would produce a similar deficit in freezing to the tone compared to controls, post-hoc

multiple comparisons were conducted to compare the individual condition means. The comparison of shRNA virus versus controls revealed that the average percent freezing of the shRNA viral animals was significantly lower than the percent freezing of the controls, $p = 0.025$. The comparison of shRNA versus the scramble virus revealed that the average percent freezing to the tone of the shRNA viral animals were not significantly different than the percent freezing to the controls, $p > .05$. Additionally, the comparison of the scramble virus versus the control animals revealed that the average percent freezing of the scramble viral animals were not significantly different than the percent freezing to the controls, $p > .05$.

Discussion

The lateral amygdala is hypothesized to be the site where convergence of auditory signaling information (tone) and reinforcement (shock) cause the NMDA-dependent synaptic plasticity that mediates the formation of fear memories (Blair et al. 2001; Sigurdsson et al., 2007; Ploski et al., 2010). This data support the hypothesis that the LA is important for mediating fear memories, but was inconclusive on whether NMDAR-related plasticity was the cause for the deficit in fear memory, since the scramble virus also cause a deficit in tone fear. The LA is an important site where convergence of auditory signaling information (tone) and reinforcement (shock) produce conditioned fear (Romanski et al., 1993; LeDoux, 2000; Pare, Quirk, & LeDoux, 2004; Davis, 2006). Selective knockdown of GluN1 subunit of the NMDA receptor in the LA caused a

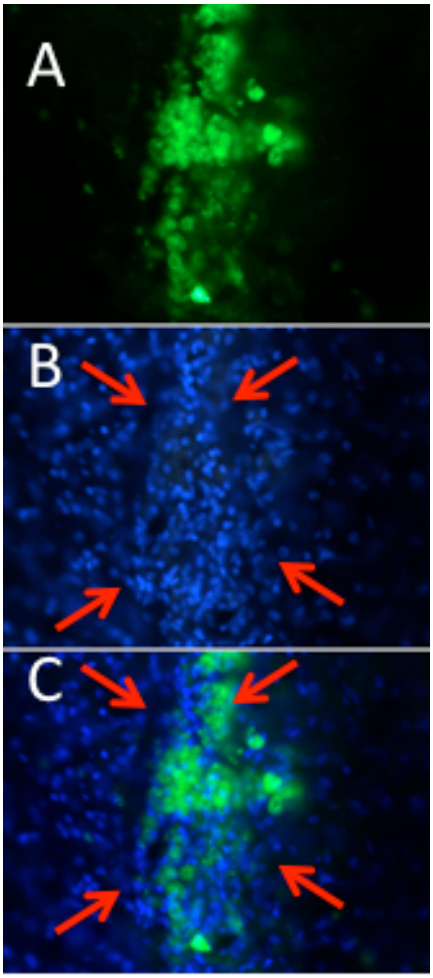
specific impairment of auditory fear learning when compared to control animals. Surprisingly, the scramble virus caused a minor deficit to tone freezing as well, and did not reach a statistical significance difference when compared to the shRNA viral animals.

The data also suggests that contextual fear memory may not be reliant on the LA. There were no significant differences in freezing between any of the groups during the context. This indicates that LA dysfunction is not sufficient to disrupt contextual fear. As with tone-shock associations, the evidence on context-shock association formation also points to the amygdala (Phillips & LeDoux, 1992). Hippocampal lesions will attenuate context conditioning but leave cued conditioning intact, even though both types of associative learning occurred at the same time (Phillips & LeDoux, 1992). The study hints that the BA might be a potential region of interest for exploring the relationship between the hippocampus and the NMDAR- related synaptic plasticity in specific nuclei within the BLA.

Some limitations to this study included the vector transport of interest. A lentiviral vector has a large vesicle and makes it more difficult to transfect a large population of neurons. Consequently, a higher volume of virus must be infused into the region of interest. The data suggest that the sheer volume of the infusion alone into the LA was enough to produce a disruption in LA functioning to produce a deficit in tone memory. This was supported by the fact that the scramble virus, which did not affect any gene expression, caused a deficit in freezing during the tone test.

The purpose of the present experiment was to examine the role of NMDA receptors in the lateral amygdala during auditory fear conditioning using a small hairpin

RNA (shRNA) lentivirus that targeted the Grin1 gene. Based on previous data our predictions were that a reduction of NMDA receptors in the lateral amygdala would cause an impairment in auditory fear memory, and possibly an impairment in contextual fear memory, if the LA was a critical site for the association between contextual information and aversive stimuli. This was a novel approach using techniques that allow for anatomical specificity without the disruption of information passing through the region. Although the results indicate problems with the transfection rate of the specific viral vector (i.e. lentivirus), this study provides insight for the potential of using RNA interference as a powerful strategy for analyzing memory formation and amnesia.



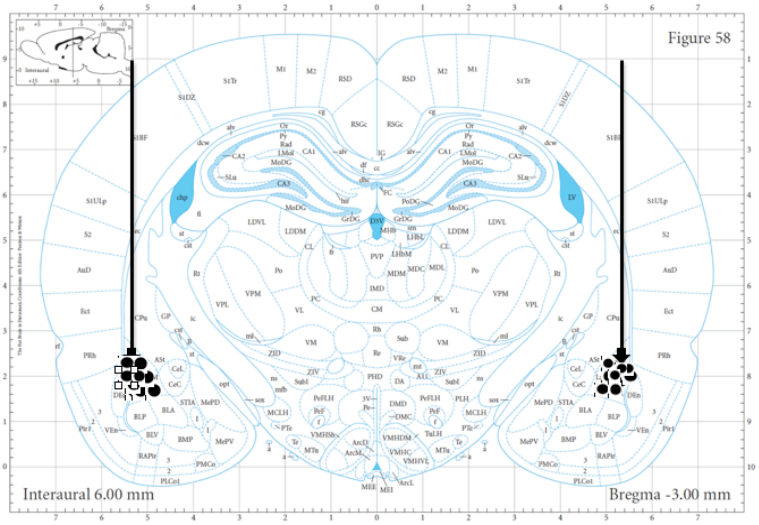


Figure 1: Virus Injection Site (40x) and a diagram of infusion region. The site of the GFP tagged viral infusion in green (A), DAPI-stained nuclei are blue and the lateral nucleus is indicated with red arrows (B). The merged figure (C) shows successful targeting of the virus at the dorsal portion of the lateral nucleus.

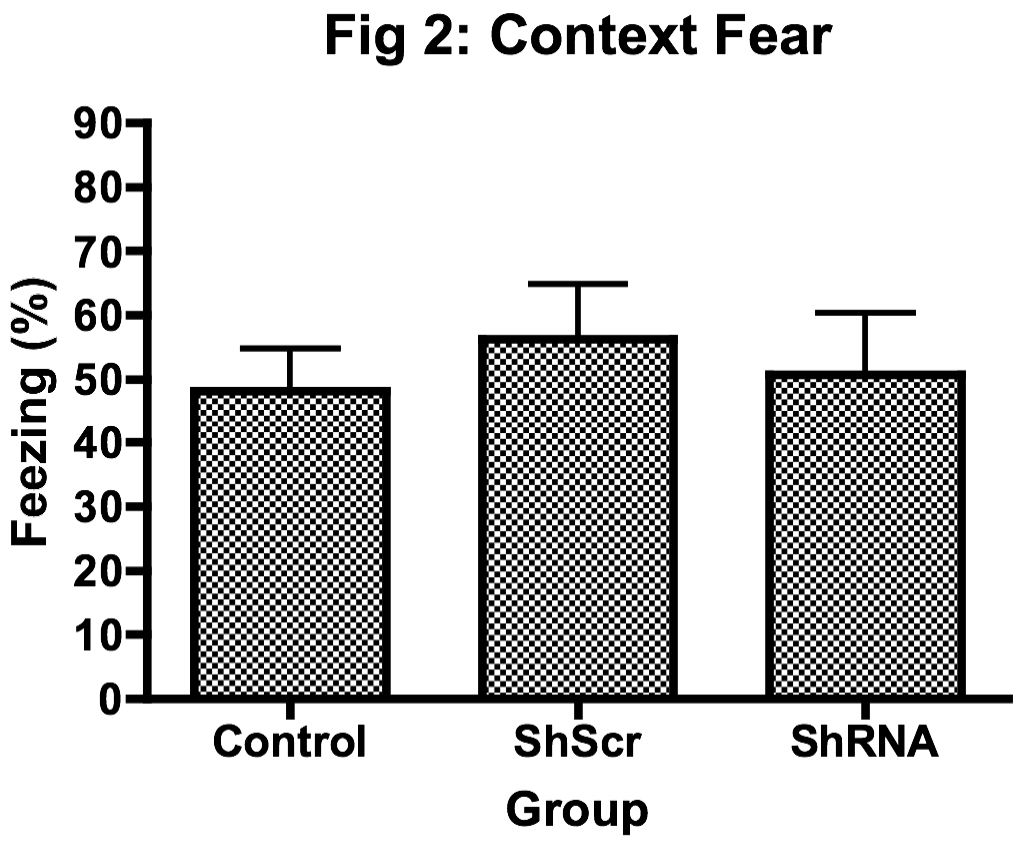


Figure 2: Effect of Intra-amygdalar injections of shRNA or shScr lentivirus on long-term memory for contextual conditioning. Data from the context test are shown. There was no effect of infusions into the lateral nucleus of the amygdala on freezing to context.

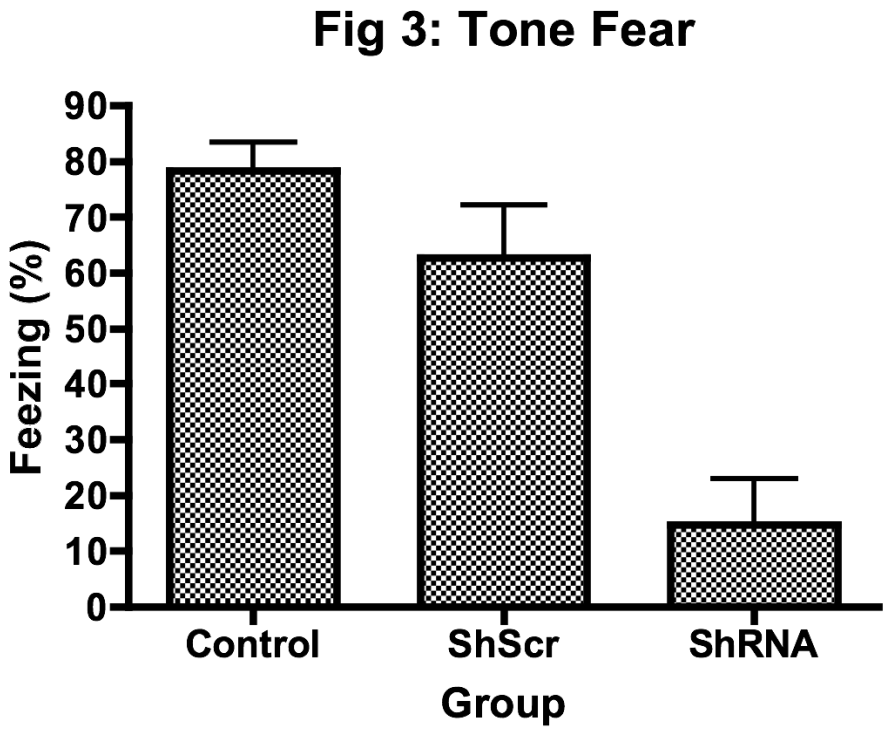


Figure 3: **Effect of Intra-amygdalar injections of shRNA or shScr lentivirus on long-term memory for auditory fear conditioning.** The data from tone testing on Day 3 are shown. Relative to unmanipulated controls, the scrambled construct (ShScr) produced a small reduction in freezing that did not approach statistical reliability ($p<0.1$). The interfering sequence (ShRNA) produced a large reduction in freezing relative to both the unmanipulated controls ($p<.0001$) and the and control virus ($p<.005$).

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CHAPTER FOUR

Dissociating the relative contribution of NMDA receptors in the basal and lateral amygdala in supporting auditory and contextual fear learning

Abstract

The basolateral amygdala complex, containing the lateral (LA) and basal (BA) nuclei, are critical for cued and contextual fear learning and memory formation through mechanisms that include *N*-methyl-D-aspartate receptor (NMDAR)-mediated synaptic plasticity. However, the relative contribution of NMDAR-mediated plasticity in the BA and LA is unknown because the pharmacological techniques previously used to implicate NMDAR have limited anatomical specificity. While lesion studies can be more anatomically precise, lesions affect far more than synaptic plasticity. To overcome these limitations we used Grin1^{flox} mice combined with stereotaxic delivery of an AAV2/1 virus coding for Cre-recombinase (Cre-GFP or GFP-only control) targeted at either the BA or LA. The virus will delete Grin1 from infected cells causing a loss of GluN1, which is necessary for a functional NMDAR. One-month after viral infusion, mice received fear conditioning, which consisted of five tone-shock pairings. Following training, mice were given both a context test and a tone test. Using an ANOVA separating groups by their nominal target infection region of either the LA or BA, we found that targeting the LA produced a deficit in tone fear, while targeting the BA produced a deficit in both tone and contextual fear. Since the viral constructs used were tagged with a green fluorescent protein, we were able to identify the spread of the virus and found that regardless of intended target there was often some infection of neighboring nuclei. Therefore, we performed immunohistochemistry to identify the amount of GluN1 per region, and used GluN1 levels as predictors in a linear regression model. Our model indicated that GluN1 levels significantly predicted time spent freezing during both the context and tone tests.

Depletion of GluN1 in the BA was a strong predictor for a deficit in both contextual and auditory fear conditioning over and above the effect of GluN1 levels in the LA. The relationship between auditory fear conditioning and GluN1 levels in the LA fell just short of statistical significance. These results indicate that NMDARs in the BA are important for both auditory and contextual fear conditioning. They also illustrate how the combination of genetic and viral techniques, whose effectiveness can be directly assessed with immunohistochemistry and then correlated with behavior, allows far greater precision than previous methodologies.

Introduction

Fear and the development of conditional fear are critical for survival. When faced with an immediate threat, the fear response is an adaptation that is required for survival. However, mal-adaptations in the fear system lead to psychiatric disorders such as Post-Traumatic Stress Disorder and anxiety disorders, such as specific phobias. Pavlovian fear conditioning in rodents allows for the study of the neural circuitry and cellular mechanisms the underlie fear learning and memory in mammals.

The basolateral amygdala complex (BLA) is clearly implicated in forming the association between a previously neutral stimulus (conditional stimulus; CS) and an aversive stimulus, such as a shock (unconditional stimulus; US). Lesions to the BLA, consisting of both the lateral nucleus (LA) and the basal nucleus (BA), during Pavlovian fear conditioning result in a pronounced deficit in fear learning in both humans and non-humans (Blanchard & Blanchard, 1972; Bechara et al., 1995; Hitchcock & Davis, 1986; Maren, Aharonov, & Fanselow, 1996; Gale et al., 2004; Lee et al., 1993; Phillips and LeDoux, 1992). Furthermore, temporarily inhibiting neural activity in the BLA disrupts both learning and expression of conditional fear (Helmstetter, 1992). Single-unit electrophysiological activity recordings in anesthetized rats revealed that neurons in the dorsal LA responded to both CS (tone) and US (footshock) stimuli (Romanski, et al., 1993). Additionally, a molecular imaging technique that utilizes the immediate early gene *Arc* known as cellular compartment analysis of temporal activity by fluorescent in-situ hybridization (catFISH) showed convergence of contextual information and shock in the BLA as well (for catFISH methodology see Guzowsky & Worley, 2001; Barot, et al.,

2008). Using a variety of techniques, these studies further establish that BLA neurons are critical for mediating Pavlovian fear conditioning. However, the BLA receives information about the cue and context from different sources. Gainfully, the BLA is a cortex-like structure that receives highly processed information from several cortical and some thalamic regions (Swanson & Petrovich, 1998).

Pre-training lesions to the LA produce a deficit in fear learning. LeDoux (1990) lesioned the LA and then looked at mean arterial pressure and found a lower autonomic response and freezing deficits as opposed to controls. Additionally, the LA is implicated as the site where convergence of auditory signaling information (tone) and reinforcement (shock) result in the synaptic plasticity necessary to produce conditioned fear (Romanski et al., 1993; LeDoux, 2000; Blair et al. 2001; Pare, Quirk, & LeDoux, 2004; Davis, 2006; Sigurdsson et al., 2007; Ploski et al., 2010). Auditory cues from both the auditory thalamus (medial geniculate nucleus) and auditory cortex can acquire fear conditioning, inducing long-lasting changes in the LA (Boatman and Kim, 2006; Romanski and LeDoux, 1992; Clugnet & LeDoux, 1990; Doyère, Schafe, Sigurdsson, & LeDoux, 2003). As with tone-shock associations, the evidence on context-shock association formation also points to the amygdala (Phillips & LeDoux, 1992).

Contextual fear conditioning is a bit more complex because it requires hippocampal activation during the time of training. This activation is limited to the acquisition of contextual, but not auditory fear. Hippocampal lesions made just after training block context fear, but not auditory fear (Kim & Fanselow, 1992). However, hippocampal activation follows a temporal gradient. If the lesion occurs after one week,

a significant amount of fear is maintained (Kim & Fanselow, 1992). Pre-training hippocampal lesions do not have an effect on fear memory (Maren, Aharonov, & Fanselow, 1997). Therefore the hippocampus is thought to be involved in the formation of an integrated, gestalt-type configural representation of an environment as well as its temporary storage (Fanselow, 2000; Kim & Fanselow, 1992, Anagnostaras, Maren, & Fanselow, 1999). Contextual memories are then transferred to the cortex within thirty days for permanent storage (Frankland et al., 2004). As with cued fear conditioning, context-shock associations converge at the BLA. Information about the context arrives at the amygdala via the ventral angular bundle and lesions within this pathway attenuate contextual, but not auditory fear conditioning (Anagnostaras, Maren, & Fanselow, 1999; Maren & Fanselow 1995). While the hippocampus is important for forming the contextual representation the amygdala is critical for the context-shock association. Interestingly, the BA is important for contextual, but not auditory, fear conditioning (Onishi & Xavier, 2010). Although some studies found impairment to the BA via electrolytic lesions, or the absence of GluA1 caused a deficit in both context and auditory fear learning (Goosens & Maren, 2001; Humeau et al., 2007).

Once the BLA processes the association between the CS and the US, fear responses are elicited via the medial central nucleus of the amygdala (CEAm) and bed nuclei of the stria terminalis (BST). The CEAm contains inhibitory projection neurons to downstream structures that generate fear responses including analgesia, autonomic and respiration changes; potentiated startle, and freezing, as well as to the BST (see Figure 1; Fendt & Fanselow, 1999; LeDoux, 2000; Maren & Fanselow, 1996; Nagy & Paré, 2008).

The BLA nuclei and the CEA are separated by inhibitory γ-Aminobutyric acid (GABA) paracapsular intercalated cells (ITC; Millhouse, 1986). There are multiple pathways connecting the nuclei within the BLA to the CEAm that are important for fear expression, although it is unclear if one pathway is more efficient, or relied upon, as the primary pathway.

As shown in Figure 1, within the BLA complex, the BA nucleus projects excitatory neurons directly to the CEAm, while the LA has no known direct connections to the CEAm (Carlsen, 1989; Paré, Quirk, & LeDoux, 2004; Pitkänen & Amaral, 1991). Since the LA is implicated in processing auditory fear conditioning, the pathway to elicit fear responses via the CEAm have been explored (Haubensak et al., 2010; Paré, Quirk, & LeDoux, 2004; Nader et al., 2001). A pathway of interest involves the projection from the LA to the BA, and then from the BA to the CEAm (Pitkänen & Amaral, 1991). A second indirect connection comprises the LA projections to the lateral central nucleus of the amygdala (CEAl), which possesses reciprocal inhibitory GABA neurons regulating CEAm output (Haubensak et al., 2010). Still, a third pathway leading information from the LA to the CEAm is via the intercalated cells. The LA sends projections to the GABAergic ITC neurons, which then disinhibits neurons in the CEAm, which then executes fear responses (Paré, Quirk, & LeDoux, 2004).

The amygdala receives and interprets information from the medial pre-frontal cortex, hippocampus, and thalamus, and undergoes synaptic plasticity during fear learning (Schafe et al., 2001; Clugnet & LeDoux, 1990; Maren & Fanselow, 1995; Mahan & Ressler, 2012). N-methyl-*D*-aspartate (NMDA) is known to be important for

encoding fear memories. NMDA receptors (NMDAR) are known as coincidence detectors because it requires both pre-synaptic and post-synaptic activity (Mayer, Westbrook & Guthrie, 1984; Nowak et al., 1984). NMDAR have voltage-gated channels requiring a decrease overall voltage, via an EPSC, on the membrane in order to release the magnesium ion that is stuck in the receptor (Mayer, Westbrook & Guthrie, 1984). Additionally, pre-synaptic activity is required, which is the ligand binding of glutamate to the NMDAR to allow both sodium and calcium to enter the cell (Fukunaga et al., 1993). Calcium entering the cell triggers multiple responses including phosphorylation of protein kinases and secondary messenger systems and ultimately result in learning induced changes at the synaptic level, which include increased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptors (AMPAR) and retrograde messengers such as nitric oxide.

NMDA-dependent synaptic plasticity is an essential component for memory formation of aversive conditioning, creating permanent changes within cells (Maren, 1996; Guzowski, et al., 2000; Blair et al., 2001). Processed information is then transmitted to the CeA, which sends projections to various structures including the periaqueductal gray (freezing response), modulatory systems (arousal), and nuclei within the hypothalamus to regulate stress hormone release (LeDoux, et al., 1988; LeDoux, 1993). As illustrated in Figure 1, sensory and contextual information are thought to project both directly and indirectly to the LA and BA. The inputs to the amygdala, and within the amygdala itself are responsible for fear learning, with the CeA responsible for the output to regulatory systems as described above.

Some limitations with previous research involving pharmacological manipulations, such as temporary inactivations through the use of agonists or antagonists (i.e muscimol, or APV) have been limited in addressing specificity of anatomical location due to unknown spread of the drug. Additionally, excitotoxic and electrolytic lesions, might disrupt the flow of information passing through a specific region. These set of experiments use Grin1^{flox} transgenic mice to decipher the significance of NMDAR-related synaptic plasticity BLA nuclei on auditory and contextual Pavlovian fear conditioning.

Materials and Methods

All experiments were conducted in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Subjects

Grin1^{flox} mice

All subjects were *Grin1^{flox}* mice, which possess *loxP* sites flanking the sequence of the Grin1 gene that encodes the entire transmembrane domain and C-terminal region. Mice that are homogenous for this allele do not show any behavioral abnormalities. The colony was backcrossed with C57BL/6J mice for multiple generations prior to receiving the strain.

All mice were bred and maintained in the Psychology Department vivarium at the University of California, Los Angeles and were housed on a 12h light/dark cycle in groups of 3-5 with free access to food and water. Experiments were performed on both female and male mice during the light phase of their cycle (n = 15-20 per group, for a total of 80 mice).

Surgery

Under aseptic conditions, animals were given carprofen (1 mg/kg, s.c.), anesthetized using isofluorine and mounted into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The scalp was incised and retracted, and head position was adjusted to place bregma and lambda in the same horizontal plane. Small holes were drilled bilaterally into the skull so that microsyringes could be placed in the amygdala. Grin1^{floxed} mice were randomly assigned to the AAV2/1-CAG-Cre-GFP virus (n=39; LA targeted=19, BA targeted = 20), the AAV2/1-CAG-GFP virus (n=30; LA targeted=15, BA targeted = 15), or a control group (n=11). Viral infusions were made bilaterally targeting the LA (-1.7mm from Bregma, -/+ 3.1 lateral to midline, and -4.3 below the skull surface) or BA (-1.31mm from Bregma, -/+ 2.8 lateral to midline, and -4.75 below the skull surface. Injection microsyringes (Hamilton Instruments) 33 gauge, were mounted into a syringe pump (David Kopf Instruments, Tujunga, CA, USA) for controlled microinfusions. Syringes were lowered and infusions of .15 µl viral injections were made across 3 min (50 nl/min rate). Microsyringes remained in place for an additional ten minutes to allow for adequate diffusion and reduction of backflow.

Following infusions, incisions were closed with stainless steel wound clips and animals were given i.p. injections of the analgesic/anti-inflammatory carprofen (9 mg/kg) and placed on heating pads until they recovered from anesthesia. Carprofen injections were continued for an additional two days post-surgery. In addition, mice were given the antibiotic trimethoprim sulfa (TMS) in their drinking water, weighed, monitored and handled for one-week following surgery. Mice were allowed a total of 28 days of recovery prior to behavioral training.

Apparatus

All behavioral training was performed using two different sets of four identical conditioning chambers. The context consisted of a chamber with aluminum sidewalls and Plexiglas in the front, back, and top (28x21x22 cm; Lafayette Instruments, Lafayette, IN, USA). The contexts were surrounded with a sound-attenuating chamber, and each set of chambers was in a unique spatial location. Animals received fear conditioning and contextual testing in one context. A novel context was used to test generalization as well as auditory fear conditioning. The two context types were differentiated by lighting, scent, cleaning solution, background noise, context shape, and different transport to and from the contexts. One context contained a brightly lit chamber, a standard grid floor which had18 stainless steel rods (4mm diameter, 1.5mm apart from center) connected to a shock scrambler, and computer controlled (Med-Associates, St. Albans, VT, USA). The chambers were scented with 10% Simple Green and cleaned with 70% isopropyl alcohol in between each subject training. Animals were transported into this context

using a portable cart where their cages hung individually from racks mounted to the cart. The alternate context had no lights on and contained a plastic triangle to change the shape of the context. The grid flooring consisted of 18 steel rods (4mm diameter, 1.5mm apart from center) that were staggered to create uneven flooring. The chambers were scented and cleaned with 1% acetic acid solution. Animals were placed in a dark black box that had clean bedding at the floor for transport into this context.

Procedure

Subjects were given one-month recovery prior to training and were handled for one minute per day for one week prior to the beginning of training. During fear conditioning, mice were able to explore the context for the first three minutes. After the initial exploratory period, subjects were given five tone-shock pairings. This consisted of a 30 second 2800Hz tone that co-terminated with a two second .9mA shock, followed by an inter-trail interval of 60 seconds prior to the next tone presentation. One minute after the last shock, they were removed. The following day, subjects were put back in the same context for an 8-minute context test to measure fear learning. On Day 3, a tone test was given in a novel context that consisted of the same time frame and duration as training, but without the shock administration. These tests were counterbalanced across subjects.

Histology

Ninety minutes after the start of testing, mice were perfused intra-cardially with KPBS and 4% paraformaldehyde. Brains were cryo-protected in (10%, 20%, and 30%

sucrose), frozen (-80^{o} C), and serial sections were taken at 40um. We looked at expression of GFP tagged viral infusion, NMDA, and AChE in every other slice. In alternate slices we looked at expression of GFP tagged viral infusion, c-Fos, and DAPI. Free-floating sections were washed in PBS and incubated in 10% normal goat serum + 10% normal donkey serum + 0.1% Triton-X 100 + PBS. They were then incubated in rabbit anti serum against $NMDA_{\varepsilon 1}$ (1:500; Santa Cruz #SC-9056) and goat antiserum against AChE (1:500; Santa Cruz #SC-6432) or rabbit anti serum against cFos (1:500; Santa Cruz #SC-52) and DAPI for 72 hours at 4^{o} C. Sections were the washed in PBS and incubated overnight in biotinylated goat anti-rabbit AlexaFluor 568 (1:250; Invitrogen) and donkey anti-goat AlexaFluor 350 (1:250; Invitrogen). The following day, sections were washed in PBS, mounted, and coverslipped with fluorescent-protective mounting medium (either with or without DAPI; Vectashield). Stained sections were examined using a light microscope (Zeiss, Oberkochen, Germany).

Image Analyses

Positivity for NMDAR was assessed at an excitation wavelength of 568nm with an emission wavelength of 602nm. Image analysis was performed using ImageJ and an automatic custom-made macro. We developed an ImageJ macro-command to process the images in three basic steps: (1) determining the amount of NMDAR on the image, (2) thresholding that against the background, (3) Analyzing the set measurements, and displaying the label. Once the macro was created, it was used for all slices.

Positivity for viral infusion tagged with GFP was assessed at an excitation wavelength of 488nm and an emission wavelength of 510nm. Image analysis was performed using ImageJ and an automatic custom-made macro. We developed an ImageJ macro-command to process the images and received guidance for maintaining processing integrity from ImageJ associates since the background for the GFP was high (see acknowledgments). The macro required a bandpass filter with large structures down to 200 pixels and small structures down to 0 pixels to help eliminate background. The image was then thresholded and converted to a mask. Particles were analyzed by setting the pixel size (^2) from 1000-infinity, with a circularity of 0.1-1.0 and then displaying the results. Once created, the macro was used for all slices.

Statistical Analysis

Fear was indexed by defensive freezing behavior, as defined by the absence of all movement except for those necessitated by respiration (Fanselow, 1980). Behavior was recorded using an automated near infrared (NIR) video tracking equipment and computer software (VideoFreeze, Med-Associates Inc.). Video was recorded at 30 frames per second and the software calculated the noise (standard deviation) for each pixel in a frame by comparing its grayscale value to previous and subsequent frames. This produced an "activity unit" score for each frame. Based on previous validation with hand scoring (correlation of $r > 0.9$ between automated system and highly trained human observers) freezing was defined as sub-threshold activity (when the motion threshold was held at 50 activity units) for longer than 1 sec. Average freezing was scored for the

baseline period in all phases and the first 28 s of each tone used for conditioning (prior to US onset). For presentation purposes, the 30 tone presentations during extinction sessions were blocked into six bins of five tone presentations and freezing was averaged within each bin.

Freezing data were statistically analyzed using between-subjects analyses of variance (ANOVAs) and repeated measures (trial) ANOVAs where appropriate. Post-hoc comparisons were performed following significant findings and a Bonferroni correction was applied to control for the number of comparisons made. The level of significance used for all analyses was $p < .05$.

Additionally, a linear regression model was used to predict the percent freezing in subjects during the context test or the tone test based on the percent of NMDAR in both the lateral amygdala and the basal amygdala. Since the viral vector had a GFP tag, we were able to precisely detect the amount of spread per infusion. After immunohistochemistry, we were able to determine the amount of NMDAR per nuclei. Given the ability to accurately describe the location and percentage of the virus, as well as the amount of NMDAR, we used a linear regression model to look at our data with finer detail and precision.

Results

AAV2/1 infection in the Lateral Amygdala and Basal Amygdala

Figure 2 represents the target area of the AAV2/1-CAG-Cre-GFP virus and a representative image of the viral infusion at a 40x magnification in the lateral amygdala and basal amygdala. Additionally, Figure 2 represents the target area of the AAV2/1-CAG-GFP control virus and a representative image of the control viral infusion at a 40x magnification in the lateral amygdala and basal amygdala.

Grin1^{flox} mice with a down-regulation of NMDAR in the basal amygdala acquire fear, but have a deficit contextual fear recent memory, and in tone fear memory. Grin1^{flox} mice with down-regulation of NMDAR in the lateral amygdala acquire fear, maintain contextual fear recent memory, but have a deficit in tone fear memory.

Analysis of Variance

Fear Acquisition

Mean freezing (+/- SEM) to each 30s tone of five conditioning trials are shown in Figure 3. Mice were split by viral injection type resulting in two Cre-expressing viral groups: AAV2/1-CAG-Cre-GFP virus targeted to the LA (Lateral; n= 19), AAV2/1-CAG-Cre-GFP virus targeted to the basal amygdala (Basal; n=20). The criterion for inclusion into either group was for the virus to be expressed at least 90% in the targeted region. The control groups consisted of three groups: the AAV2/1-CAG-GFP control virus targeted to the LA (n=15), the AAV2/1-CAG-GFP control virus targeted to the BA

(n=15), or a control group (n=11). There were no differences in the freezing behaviors of the three control groups, so the data was collapsed across all three groups (Control; n = 41). This revealed an end total of three groups (Lateral, Basal, and Control). All animals displayed low baseline freezing to the context during the initial three-minute exploratory period, suggesting that the surgery alone did not generate inappropriate freezing behavior. A repeated measures ANOVA by trial revealed significant main effect for tone fear acquisition ($F_{(3,27)} = 132.2$, $p = 0.0001$), with no significant main effect of group ($F_{(3,27)} < 1$) or significant interaction of trial by group ($F_{(3,27)} < 1$) indicating that AAV2/1 injections into the LA or BA did not impair fear acquisition.

Context Test

Figure 4 presents mean freezing (+/- SEM) across the eight-minute context test. The apparent decrease in percent context freezing as a function of down-regulation of NMDAR in particular brain regions, that are indicated in Figure 4 was confirmed by a one-way ANOVA performed on the data, which revealed a significant main effect of brain region on percent context freezing F (2,78) = 8.251, $p = 0.001$.

To test prediction that the down-regulation of NMDAR in the BA would produce a deficit in freezing during the context test than either the LA or controls, and that the LA group would produce a similar deficit in freezing compared to controls, post-hoc multiple comparisons were conducted to compare the individual condition means. The comparison of BA versus controls revealed that the average percent freezing to the context of the BA group was significantly lower than the percent context freezing of the

controls, $p = 0.001$. The comparison of the BA group versus LA group revealed that the average percent freezing to the context of the BA group was significantly lower than the percent context freezing of the LA group, $p = 0.012$. Additionally, the comparison of the LA group versus the control group revealed that the average percent context freezing of the LA group were not significantly different than the percent freezing to the controls, $p > .05$.

Tone Test

Figure 5 presents mean freezing (+/- SEM) across each 30s tone of five tone-test trials.. The apparent decrease in percent freezing as a function of down-regulation of NMDAR in particular brain regions, that are indicated in Figure 5 was confirmed by a one-way ANOVA performed on the data, which revealed a significant main effect of brain region on percent freezing $F(2,78) = 15.609$, $p = 0.001$.

To test prediction that the down-regulation of NMDAR in the BA group and LA group would produce a deficit in freezing during the tone test than the control group, and that the LA group would produce a similar deficit in tone freezing compared to the BA group, post-hoc multiple comparisons were conducted to compare the individual condition means. The comparison of BA versus controls revealed that the average percent freezing to the tone of the BA group was significantly lower than the percent tone freezing of the controls, $p < 0.001$. Additionally, the comparison of the LA group versus control group revealed that the average percent freezing to the tone of the LA group was

significantly lower than the percent tone freezing of the control group, $p = 0.004$. The comparison of the LA group versus the BA group revealed that the average percent tone freezing of the LA group were not significantly different than the percent freezing to the BA group, $p > .05$.

Grin1^{flox} mice with a down-regulation of NMDAR in the basal amygdala predict a deficit in freezing during the context test and tone test.

We modeled the amount of NMDAR based on immunohistochemistry to measure the amount of NMDAR per nuclei and determined parameters using a custom-made macro in ImageJ as described above. We then performed a linear regression on the data to make predictions on the amount of NMDAR per area's influence on percent freezing during the context and auditory test (see Figures 6 and 7, respectively).

Linear regression analysis

Context Test

The main purpose of the regression analysis was to predict the percent freezing in subjects during the context test based on the percent of NMDAR in both the lateral amygdala and the basal amygdala and is represented in Figure 6. The amount of NMDAR in the basal amygdala and lateral amygdala predicted 31.7% of the variability in the model (adjusted $R^2 = .244$) and was statistically significant $F(2,42) = 4.651$, $p = 0.022$ (see Figure 6 A and B). For every 1% decrease in NMDAR in the basal amygdala, there is a 0.639% decrease in context freezing over and above the effect of NMDAR in

the lateral amygdala $t(42) = 2.399$, $p = 0.026$ (see Figure 6C). For every 1% decrease in NMDAR in the lateral amygdala, there is a 0.323% decrease in context freezing over and above the effect of NMDAR in the basal amygdala $t(42) = 1.56$, $p = 0.135$ (see Figure 6D). The amount of NMDAR in the lateral amygdala is not shown to be making a significant contribution to the percent freezing during the context test, but the overall model is considered accurate according to the regression. The regression model for the context test is as follows:

$$\textbf{Freezing to Context} = 35.88 + (0.639)\ NMDA_{BA} + (0.323)\ NMDA_{LA}$$

Tone Test

The main purpose of the regression analysis was to predict the percent freezing in subjects during the tone test based on the percent of NMDAR in both the lateral amygdala and the basal amygdala and is represented in Figure 7. The amount of NMDAR in the basal amygdala and lateral amygdala predicted 47.7% of the variability in the model (adjusted $R^2 = .424$) and was statistically significant $F(2,42) = 9.113$, $p = 0.002$ (see Figure 7 A and B). For every 1% decrease in NMDAR in the basal amygdala, there is a 0.948% decrease in tone freezing over and above the effect of NMDAR in the lateral amygdala $t(42) = 4.093$, $p = 0.001$ (see Figure 7C). For every 1% decrease in NMDAR in the lateral amygdala, there is a 0.312% decrease in tone freezing over and above the effect of NMDAR in the basal amygdala $t(42) = 1.730$, $p = 0.099$ (see Figure 7D). The amount of NMDAR in the lateral amygdala is shown to be trending towards

making a significant contribution to the percent freezing during the tone test, but the overall model is considered accurate according to the regression. The regression model for the tone test is as follows:

$$\textbf{Freezing to Tone} = 52.09 + (0.948)\ NMDA_{BA} + (0.312)\ NMDA_{LA}$$

With new techniques and technologies, it is important to advance our data analyses to reflect our progress. As the precision to identify areas of infection increased with the use of the transgenic mice and a virus with a GFP tag, we utilized the available statistical analyses to extract as much information as possible from the data.

Discussion

Using an AAV2/1-CAG-Cre-GFP virus into the BA or LA of $Grin1^{flox}$ mice produced a depletion of NMDAR in their respective regions. This approach allowed for the evaluation of the impact of NMDAR-mediated plasticity in the BA or LA on acquisition and maintenance of both contextual and auditory fear conditioning. The results indicate NMDAR-mediated plasticity in the BA predicts a deficit in freezing behavior for both contextual and auditory fear conditioning and shows NMDAR-mediated plasticity in the BA is critical for fear expression during both contextual and auditory fear conditioning.

The results do not conflict with the popular view that normal function of the BLA is important for acquisition of Pavlovian fear conditioning (Fanselow & LeDoux, 1999; LeDoux, 1993; Maren, 1999; LeDoux, 2000). The data indicates that NMDAR-mediated plasticity in the BA is critical for contextual fear memory. NMDAR depletion in the BA produced a severe deficit in freezing as compared to NMDAR depletion in the LA or the control animals. The linear regression revealed NMDAR depletion in the BA accounted for the majority of the freezing deficit during fear learning when controlling for the LA. These data support studies that used excitotoxic lesions of the BA, which produced a deficit in contextual fear learning (Onishi & Xavier, 2010).

Additionally, the data also support the convergence of auditory and aversive stimuli in the LA. The lateral amygdala is hypothesized to be the site where convergence of auditory signaling information (tone) and reinforcement (shock) cause the NMDA-dependent synaptic plasticity that mediates the formation of fear memories (Blair et al. 2001; Sigurdsson et al., 2007; Ploski et al., 2010). This data support the hypothesis that the LA is the site where convergence of auditory signaling information (tone) and reinforcement (shock) produce conditioned fear (Romanski et al., 1993; LeDoux, 2000; Pare, Quirk, & LeDoux, 2004; Davis, 2006). However, it is the NMDAR-mediated plasticity in the BA that seems to be driving the fear expression. Selective knockdown of GluN1 subunit of the NMDA receptor in the LA caused a specific impairment of auditory fear learning when compared to control animals. Surprisingly, NMDAR depletion in both the BA and the LA caused a significant impairment in freezing during the tone test as compared to controls. The linear regression revealed NMDAR depletion in the BA

accounted for the majority of the freezing deficit during the tone test when controlling for the LA. The data supports previous findings that post-training electrolytic lesions of the BA abolish tone fear expression (Anglada-Figueroa & Quirk, 2005). The results suggest that the LA is a relay site for the convergence of the discrete CS and US to form an association, but that the information from the LA is projected to the basal amygdala (BA) and that is where NMDAR-mediated plasticity critical for auditory fear conditioning.

Contextual Fear Conditioning

NMDAR-mediated synaptic plasticity in the basal amygdala is critical for the contextual fear conditioning. Literature on the BLA's role in fear conditioning have focused on the LA as an essential site for the tone-shock association, but less focus has been given to the processes that underlie contextual fear learning in the BLA, although much research has been devoted to the role of the hippocampus in contextual fear learning.

The BLA has a strong anatomical connection with the hippocampus via the ventral angular bundle. While the majority of projections from the hippocampus synapse onto the BA, some also synapse onto the LA, although the BA is implicated as the site for hippocampal-dependent contextual-shock associations (Goosens & Maren, 2001; Pitkänen, Savander, & LeDoux, 1997). Information about the context arrives at the BLA via the ventral angular bundle and lesions within this pathway attenuate contextual, but not auditory fear conditioning (Anagnostaras, Maren, & Fanselow, 1999; Maren & Fanselow 1995). Additionally, the BA is shown to undergo associative plasticity during

fear learning (Humeau et al., 2007). Furthermore, the BA is important for contextual fear conditioning, with ibotenic or electrolytic lesions of the BA cause an impairment in freezing during the context test (Onishi & Xavier, 2010; Goosens & Maren, 2001).

These results support previous data showing the BA is required for acquisition of contextual fear conditioning. These data further this idea by stating NMDAR-mediated synaptic plasticity in the basal amygdala is critical for contextual fear conditioning.

Auditory Fear Conditioning

NMDAR-mediated plasticity in the BA is more important for producing an auditory fear response than the LA during auditory fear conditioning. The LA forms an association between a discrete cue and an aversive stimulus (Romanski et al., 1993; LeDoux, 2000; Blair et al. 2001; Pare, Quirk, & LeDoux, 2004; Davis, 2006; Sigurdsson et al., 2007; Ploski et al., 2010). The data supports this notion, since depletion of the NMDAR in the LA caused an impairment in freezing during the auditory fear test. More interesting, a similar deficit was seen when the depletion of NMDAR was in the BA. The linear regression revealed the amount of NMDAR in the BA was the main predictor of the freezing deficit seen in auditory fear conditioning over and above the effect of the NMDAR depletion in the LA, suggesting NMDAR in the BA is critical for auditory fear expression.

Literature on the BLA's role in auditory fear conditioning focuses on the LA as an essential site for the tone-shock association, but less focus is given to the involvement of the BA during Pavlovian fear conditioning. Interestingly, the studies that have focused

on the BA mostly dealt with pre-training lesions and did not see a deficit in training. Pre-training electrolytic or excitotoxic lesions to the LA or the CEA, but not BA, disrupt freezing behavior elicited by the tone (Onishi & Xavier, 2010; Nader et al., 2001). These results indicate that when the BA is not present, auditory fear learning can occur. However, when the BA is functioning normally, post-training lesions of the BA ablate fear expression to the previously trained auditory cue, suggesting that the BA is an important component in auditory fear learning in intact animals (Anglada-Figueroa & Quirk, 2005). Further, Humeau et al. (2007) also reported that the loss of synaptic plasticity in the BA of GluR1$^{-/-}$ mice impaired freezing to both discrete cues and to the context. Additionally, Anglada-Figueroa & Quirk (2005), re-trained animals that had received BA lesions and those animals were able to re-acquire auditory fear.

This indicates that when the BA is absent prior to training, the LA uses alternate routes, with less straightforward means, to connect to the CEA via two different pathways to compensate, but that when the BA is intact and functioning normally, this seems to be the primary pathway. Specifically, when the BA is not functioning, the tone-shock association that is formed in the LA can send projections to the lateral central nucleus of the amygdala (CEAl), which possesses reciprocal inhibitory GABA neurons regulating CEAm output (Haubensak et al., 2010). Still, another viable pathway leading information from the LA to the CEAm is via the intercalated cells. The LA sends projections to the GABAergic ITC neurons, which then connect to the CEAm to execute fear responses (Paré, Quirk, & LeDoux, 2004).

These results support previous data showing the LA is required for the tone-shock association during auditory fear conditioning. More interesting, the data reveals that the NMDAR-mediated synaptic plasticity in the BA is critical for auditory fear conditioning and is responsible for driving the fear response. Specifically, the results suggest that the LA is a relay site for the convergence of the discrete CS and US to form an association, but that the information from the LA is projected to the BA and that is where NMDAR-mediated plasticity critical for auditory fear conditioning. When the BA is functioning normally, post-training lesions of the BA ablate fear expression to the previously trained auditory cue (Anglada-Figueroa & Quirk, 2005). In intact animals, the NMDAR-mediated receptor plasticity in the BA is what is essential to produce an auditory fear response.

The Fear Circuit

The present study sought to elucidate the role of NMDAR-mediated plasticity within the BLA on auditory and contextual fear conditioning. However, in doing so, we also discovered an important component in the fear conditioning circuitry. Specifically, that NMDAR-mediated plasticity in the BA plays a much more important role in discrete cued and contextual fear conditioning than previously expected.

The pattern of results is congruent with previous literature on contextual fear conditioning such that the BA is critical for contextual fear conditioning and extends this finding to describe the importance of NMDAR-mediated plasticity in the BA as essential for contextual fear learning. Similarly, the data is consistent with literature on auditory

fear conditioning wherein the LA forms an association between a discrete cue and an aversive shock, however extends these findings to reveal NMDAR-mediated synaptic plasticity in the BA is what is necessary to modulate fear expression.

By uniting these results with the established data on contextual and auditory fear conditioning, a more defined fear circuit emerges. First, information about the context forms a configural representation in the hippocampus and project to the BLA via the ventral angular bundle (Anagnostaras, Maren, & Fanselow, 1999; Maren & Fanselow 1995). Although these projections synapse onto the BA and LA (Goosens & Maren, 2001; Pitkänen, Savander, & LeDoux, 1997), the NMDAR- mediated plasticity in the BA seems to be responsible for driving the context-shock associations. From there, information travels from the BA to the CEAm to produce a fear response, including analgesia, autonomic and respiration changes; potentiated startle, and freezing (Fendt & Fanselow, 1999; LeDoux, 2000; Maren & Fanselow, 1996; Nagy & Paré, 2008).

Likewise, information about a discrete cue, such as a tone projects from the medial geniculate nucleus of the thalamus via thalamo- and cortico- pathways to the LA. Neurons in the LA are responsible for forming a tone-shock association and undergo NMDAR-mediated plasticity (Romanski et al., 1993; LeDoux, 2000; Blair et al. 2001). From there, neurons project to the BA and the NMDAR-mediated synaptic plasticity in this region is what is critical for auditory fear expression. Similar to contextual fear, information flows from the BA to the CEAm to generate fear responses.

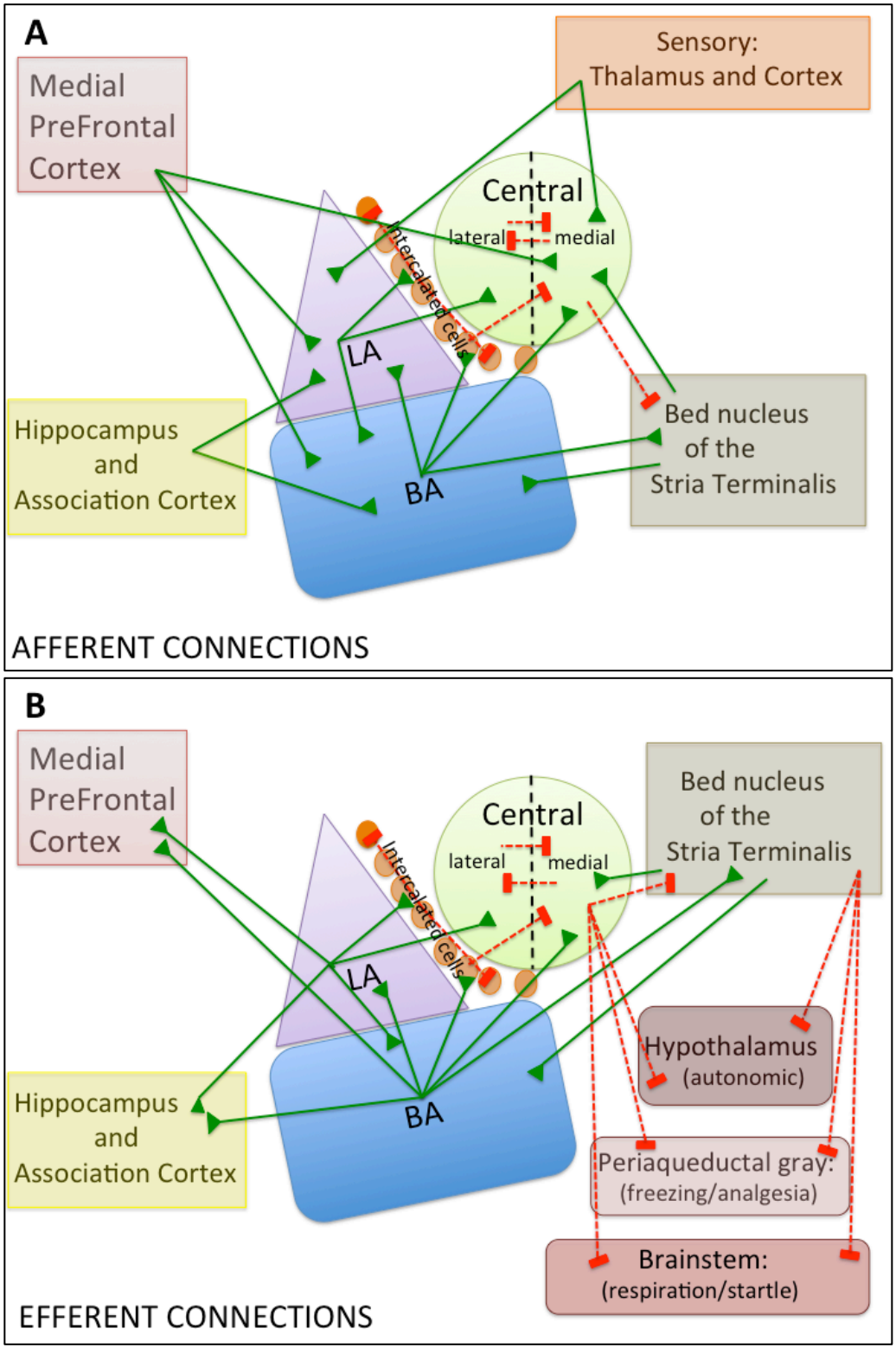
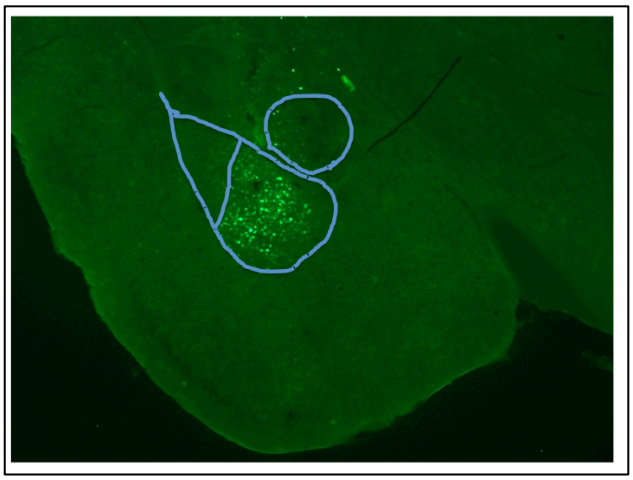
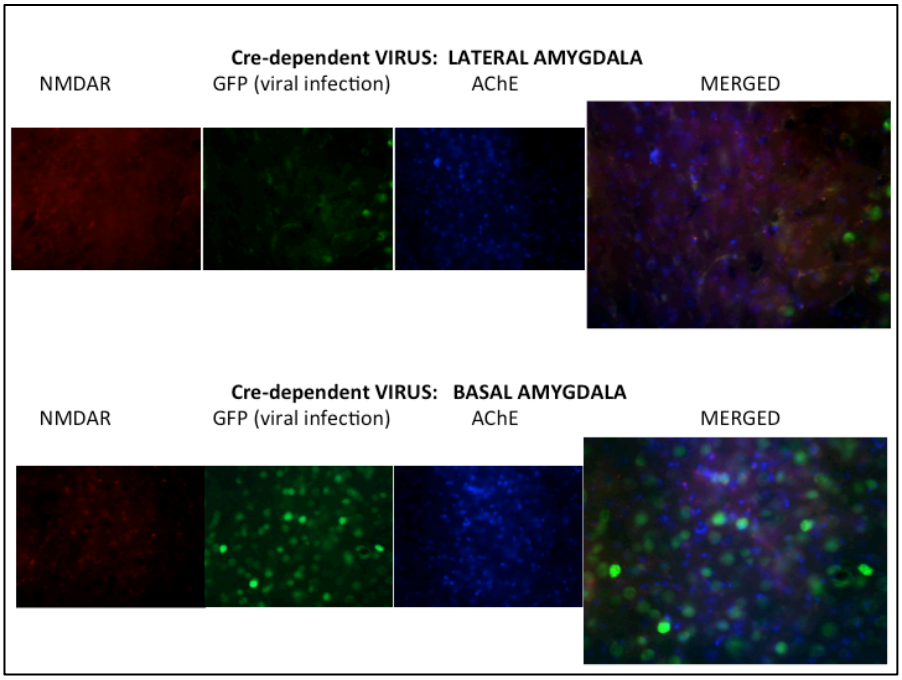

A
Medial PreFrontal Cortex
Sensory: Thalamus and Cortex
Central
lateral
medial
Intercalated cells
LA
BA
Hippocampus and Association Cortex
Bed nucleus of the Stria Terminalis
AFFERENT CONNECTIONS
B
Medial PreFrontal Cortex
Central
lateral
medial
Intercalated cells
LA
BA
Bed nucleus of the Stria Terminalis
Hypothalamus (autonomic)
Hippocampus and Association Cortex
Periaqueductal gray: (freezing/analgesia)
Brainstem: (respiration/startle)
EFFERENT CONNECTIONS

Figure 1: Connections within and between the basolateral amygdaloid complex. (A) Schematic representing the afferent inputs between and within the amygdala including the lateral amygdala (LA), basal amygdala (BA), and central nucleus of the amygdala. Green represents excitatory Glutamatergic neurons and red represents inhibitory GABA neurons. **(B)** Schematic representing the efferent outputs between and within amygdala. (Note: For simplification, no neuromodulatory inputs were included.

A



B


Cre-dependent VIRUS: LATERAL AMYGDALA
NMDAR
GFP (viral infection)
AChE
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C

Cre-dependent VIRUS: BASAL AMYGDALA
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GFP (viral infection)
AChE
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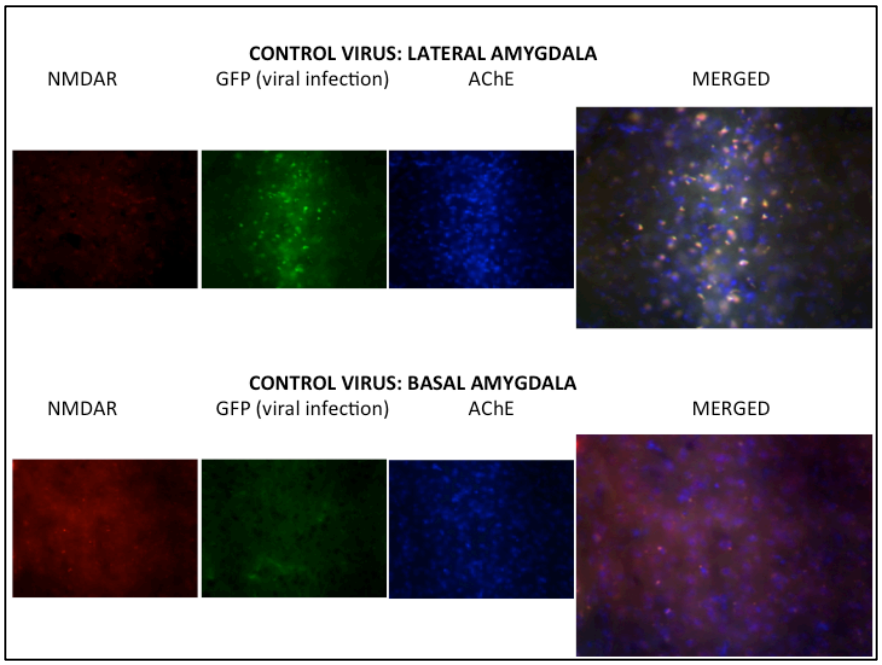


Figure 2: Virus Injection Site and a diagram of infusion region. (A)The site of the nuclear GFP tagged viral infusion in green at 4X. (B), NMDAR stain on the processes are red, nuclear GFP tagged Cre-expressing virus is green and AChE stained nuclei are blue along with the merged image in the lateral nucleus. (C) shows the same figures as B, but in the basal nucleus. Similarly, (D) represents NMDAR stain on the processes are red, nuclear GFP tagged control virus is green and AChE stained nuclei are blue along with the merged image in the lateral nucleus. (E) shows the same figures as D, but in the basal nucleus.

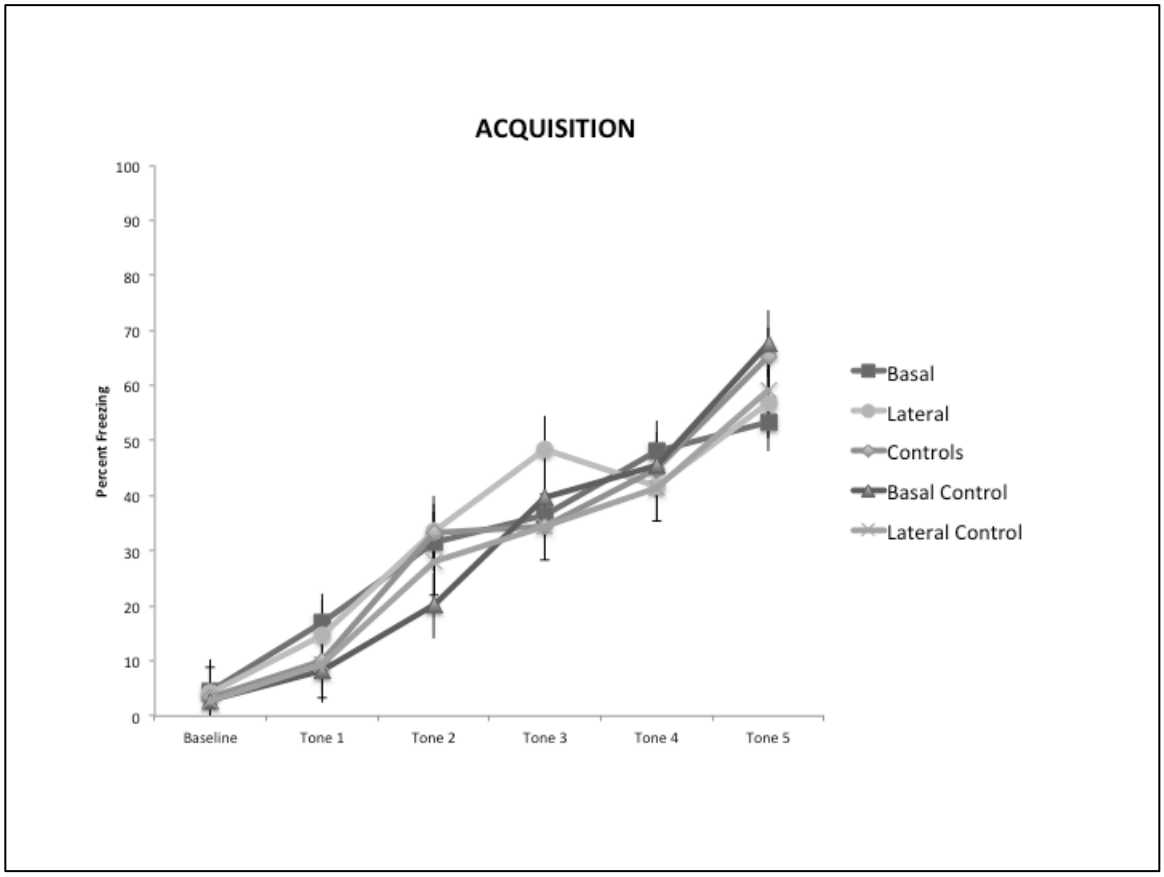


Figure 3: Effect of Intra-amygdalar injections of AAV2/1-CAG-Cre-GFP or AAV2/1-CAG-GFP on acquisition during contextual and auditory fear conditioning

Data from the acquisition are shown. This figure presents mean freezing (+/- SEM) during each 30s. tone period prior to the shock in Grin1^{flox} mice. Mice were split by viral injection type resulting in two Cre-expressing viral groups: AAV2/1-CAG-Cre-GFP virus targeted to the lateral amygdala (Lateral; n= 19), AAV2/1-CAG-Cre-GFP virus targeted to the basal amygdala (Basal; n=20). The criterion for inclusion into either group was for the virus to be expressed at least 90% in the targeted region. Two control virus groups: AAV2/1-CAG-Cre-GFP virus targeted to the lateral amygdala (Lateral Control; n=15), AAV2/1-CAG-Cre-GFP virus targeted to the basal amygdala (Basal Control; n=15), and

a Control group (n=11). All animals displayed low baseline freezing to the context during the initial three-minute exploratory period, suggesting that the surgery alone did not generate inappropriate freezing behavior. A repeated measures ANOVA by trial revealed significant main effect for tone fear acquisition ($F_{(3,27)} = 132.2$, $p = 0.0001$), with no significant main effect of group ($F_{(3,27)} < 1$) or significant interaction of trial by group ($F_{(3,27)} < 1$) indicating that AAV2/1 injections into the LA or BA did not impair fear acquisition.

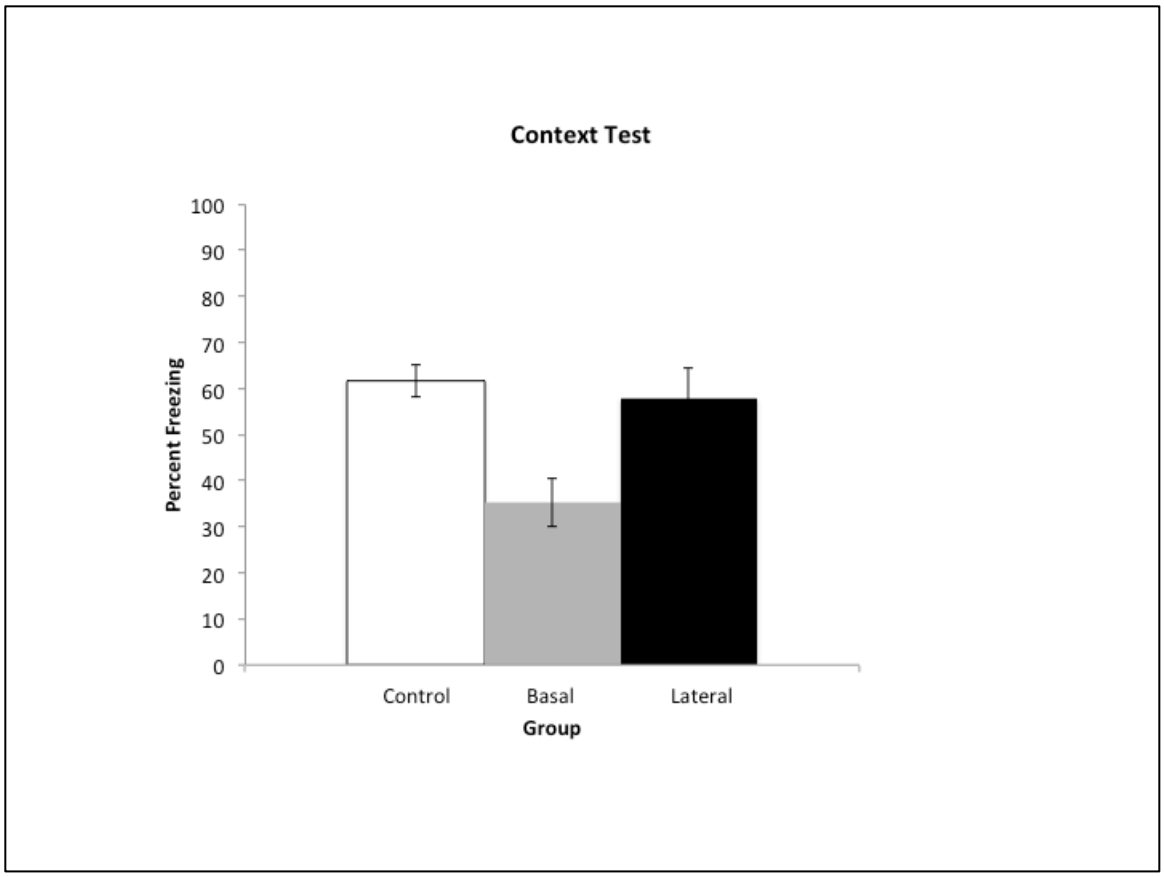


Figure 4: Effect of Intra-amygdalar injections of AAV2/1-CAG-Cre-GFP or AAV2/1-CAG-GFP on contextual fear memory. Data from the context test are shown. This figure presents mean freezing (+/- SEM) across the eight-minute context test. The apparent decrease in percent context freezing as a function of down-regulation of NMDAR in particular brain regions was confirmed by a one-way ANOVA performed on the data, which revealed a significant main effect of brain region on percent context freezing F (2,78) = 8.251, p = 0.001. Percent freezing for the Basal group was significantly lower than the percent context freezing of the Controls, (p = 0.001) and the Lateral group (p = 0.012). Additionally, the comparison between the Lateral group and the Control group was not significantly different (p > .05).

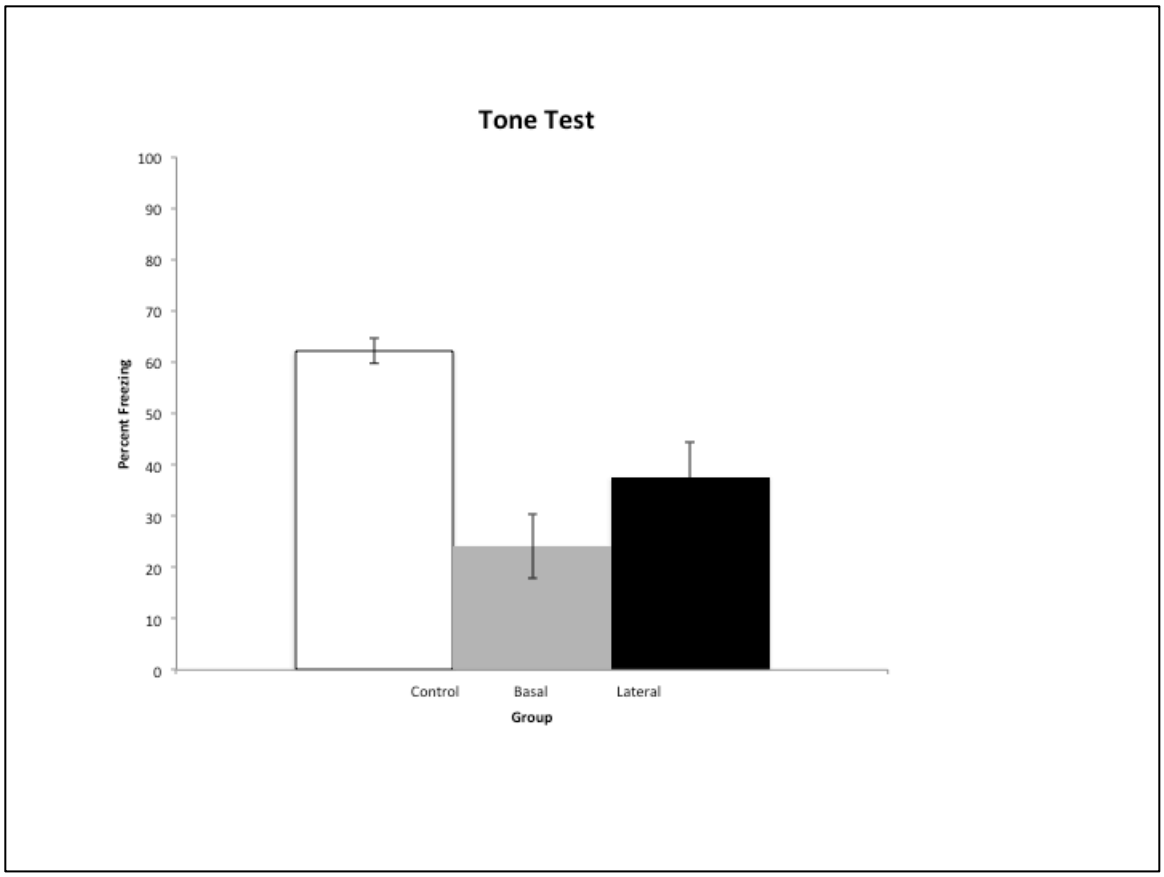
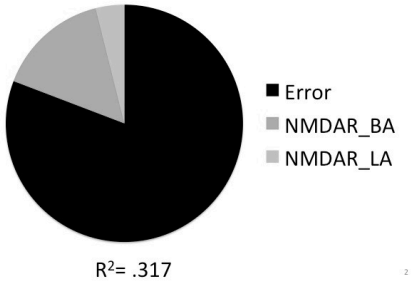


Figure 5: Effect of Intra-amygdalar injections of AAV2/1-CAG-Cre-GFP or AAV2/1-CAG-GFP on auditory fear memory. Data from the tone test are shown. This figure presents mean freezing (+/- SEM) across each 30s tone of five tone-test trials. The apparent decrease in percent freezing as a function of down-regulation of NMDAR in particular brain regions, that are indicated in was confirmed by a one-way ANOVA performed on the data, revealed a significant main effect of brain region on percent freezing F (2,78) = 15.609, p = 0.001. Percent freezing for the Basal was significantly lower than the percent context freezing of the Controls, $p < 0.001$. Additionally, freezing during the tone in the Lateral group was significantly lower than Control group, p = 0.004. The comparison of the Lateral group versus the Basal group revealed that the

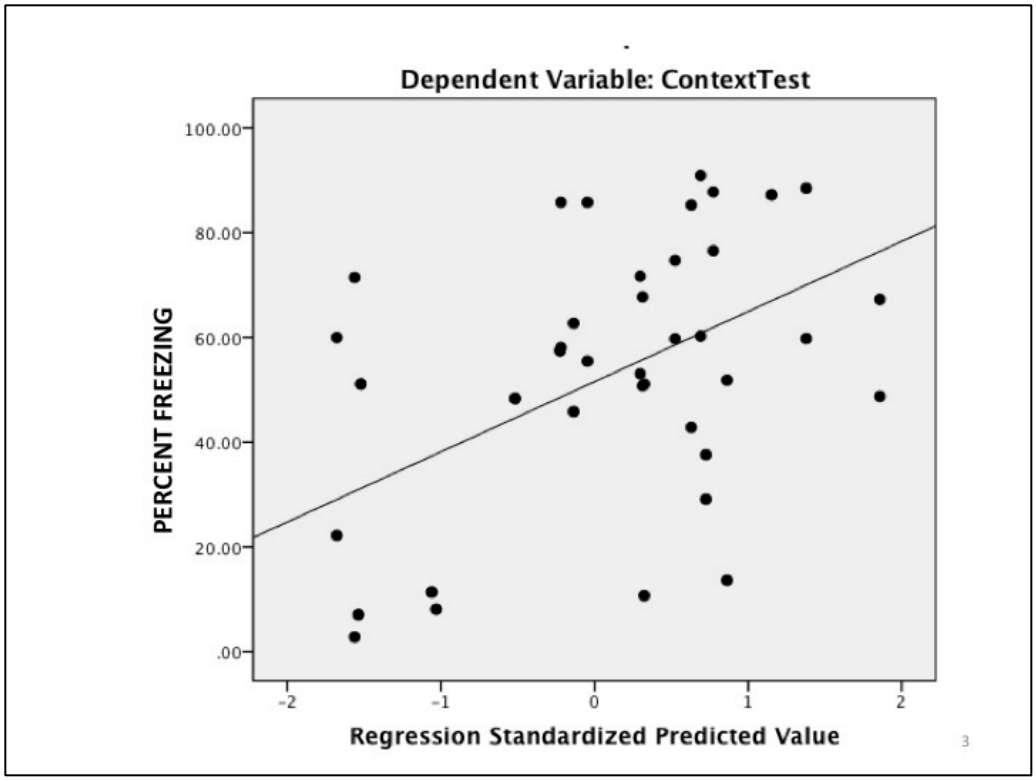
average percent tone freezing of the Lateral group were not significantly different than the percent freezing to the Basal group, $p > .05$.

A

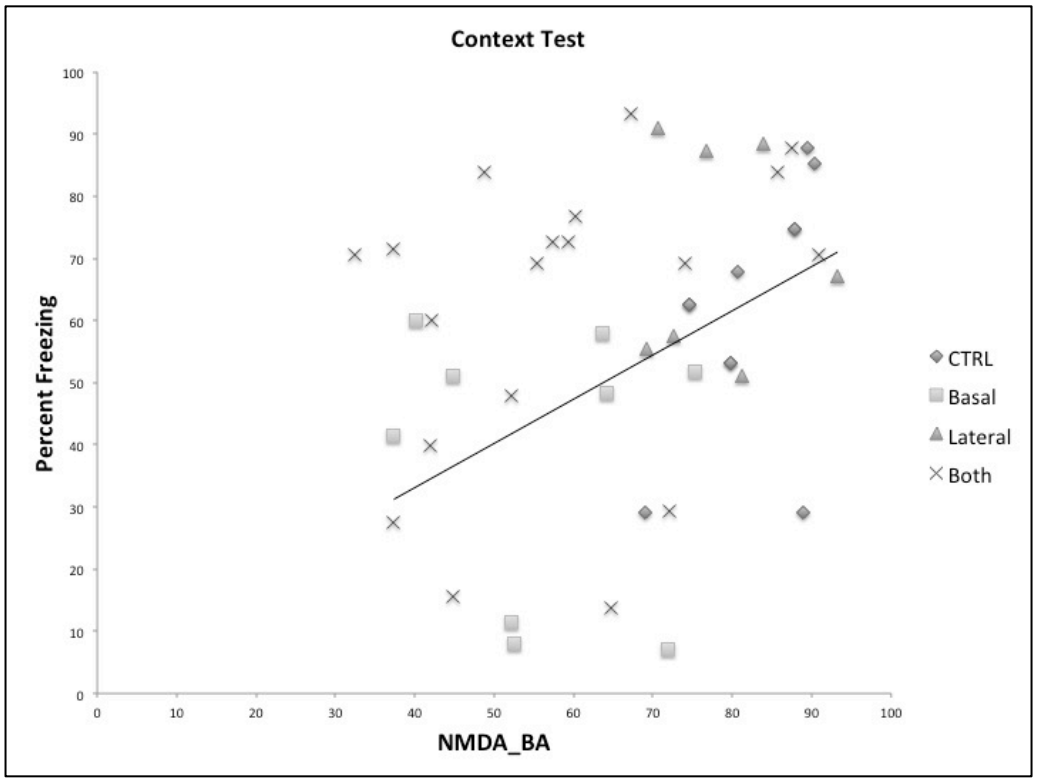

Error
NMDAR_BA
NMDAR_LA

R^2= .317

B


Dependent Variable: ContextTest
PERCENT FREEZING
100.00
80.00
60.00
40.00
20.00
.00
-2
-1
0
1
2
Regression Standardized Predicted Value

C


Context Test
Percent Freezing
NMDA_BA
CTRL
Basal
Lateral
Both

D

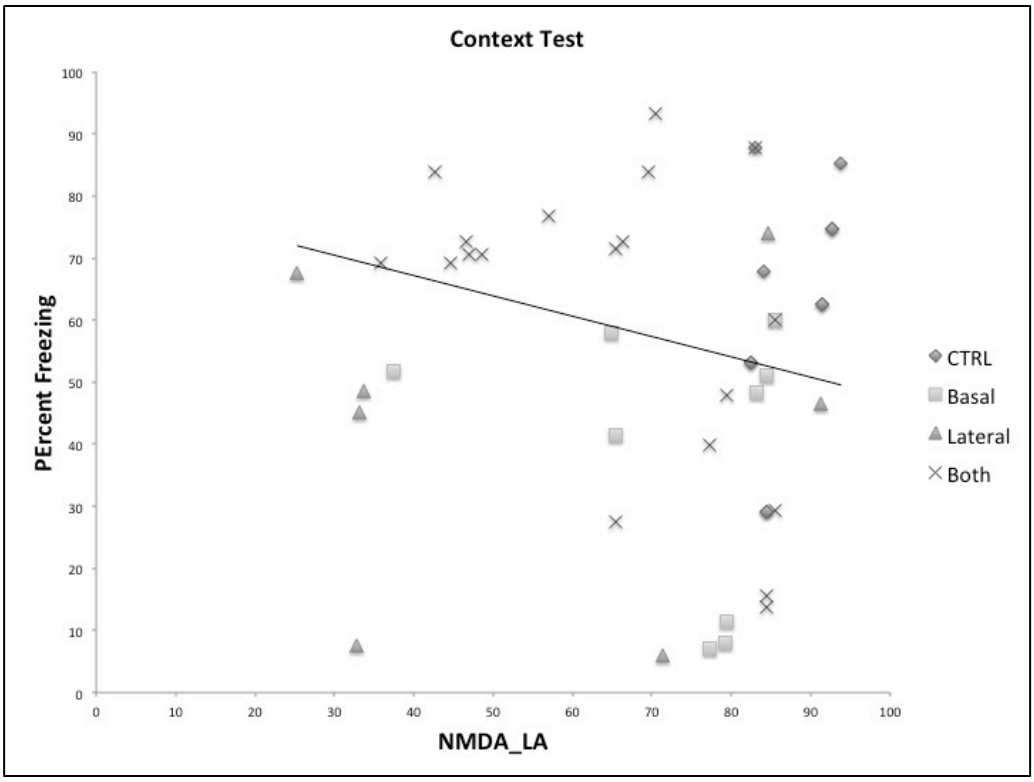
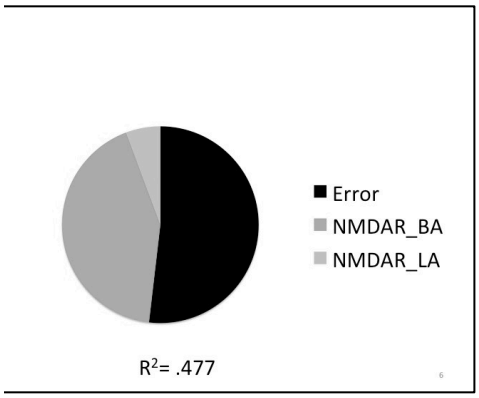
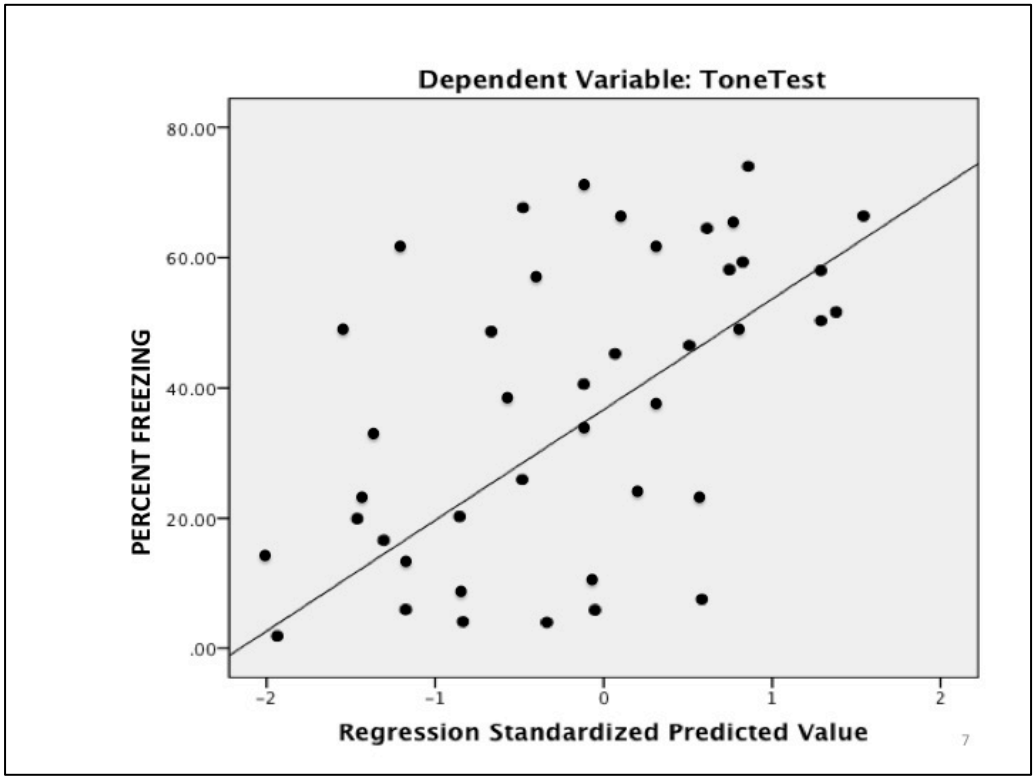

Context Test
PErcent Freezing
NMDA_LA
CTRL
Basal
Lateral
Both

Figure 6: Using a linear regression to predict the effect of NMDAR depletion in the BA and LA on contextual fear memory. (A) Using the amount of NMDAR in the BA and the amount of NMDAR in the LA as predictors of contextual freezing in a linear regression, the model accounted for 31.7% of the variance in freezing during the context test. (B) This scatterplot shows context freezing on the Y-axis and that predicted value based on the amount of NMDAR in both the LA and BA amygdala on the x-axis. (C) This scatterplot has the amount of NMDAR in the BA on the x-axis and the context freezing on the Y-axis. The regression showed that for every 1% decrease in NMDAR in the BA, there was a .639% decrease in freezing during the context test over and above the amount of NMDAR in the LA, which was a statistically significant predictor. (D) This scatterplot has the amount of NMDAR in the LA on the x-axis and the context freezing on the Y-axis. The regression showed that for every 1% decrease in NMDAR in the LA, there was a .323 increase in freezing during the context test over and above the amount of NMDAR in the BA, but this was not a statistically significant predictor in the model.

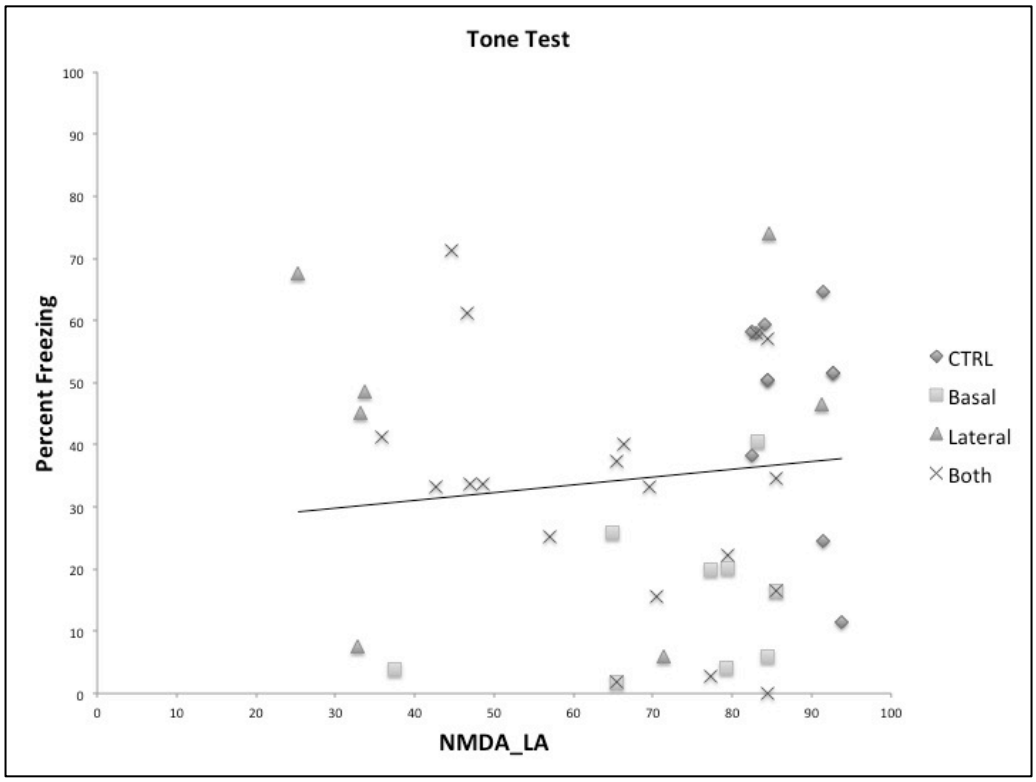
A


Error
NMDAR_BA
NMDAR_LA
R2= .477

B


Dependent Variable: ToneTest
PERCENT FREEZING
80.00
60.00
40.00
20.00
.00
-2
-1
0
1
2
Regression Standardized Predicted Value

C


Tone Test
Percent Freezing
NMDA_LA
CTRL
Basal
Lateral
Both

D

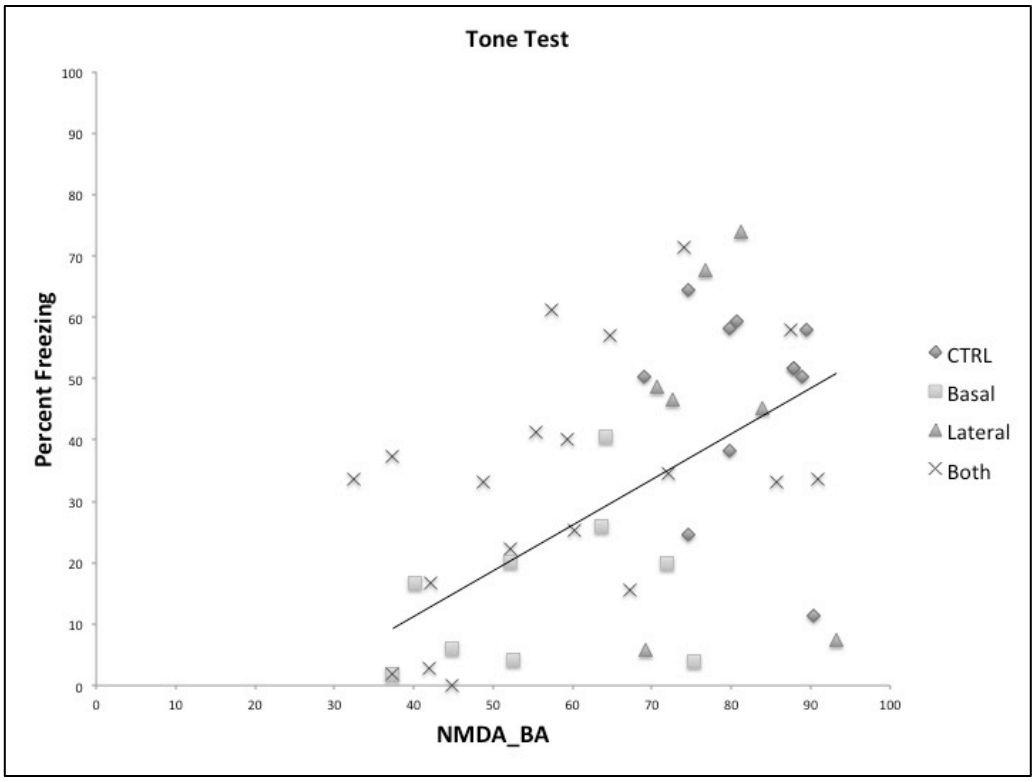

Tone Test
Percent Freezing
NMDA_BA
CTRL
Basal
Lateral
Both

Figure 7: Using a linear regression to predict the effect of NMDAR depletion in the BA and LA on tone fear memory. (A) Using the amount of NMDAR in the BA and the amount of NMDAR in the LA as predictors of tone freezing in a linear regression, the model accounted for 47.7% of the variance in freezing during the tone test. (B) This scatterplot shows tone freezing on the Y-axis and that predicted value based on the amount of NMDAR in both the LA and BA amygdala on the x-axis. (C) This scatterplot has the amount of NMDAR in the BA on the x-axis and the tone freezing on the Y-axis. The regression showed that for every 1% decrease in NMDAR in the BA, there was a .948% decrease in freezing during the tone test over and above the amount of NMDAR in the LA, which was a statistically significant predictor. (D) This scatterplot has the amount of NMDAR in the LA on the x-axis and the tone freezing on the Y-axis. The regression showed that for every 1% decrease in NMDAR in the LA, there was a .312increase in freezing during the tone test over and above the amount of NMDAR in the BA, but this was not a statistically significant predictor in the model.

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CHAPTER FIVE

Summary: The role of NMDAR- mediated plasticity in the basal amygdala and lateral amygdala on Pavlovian contextual and auditory fear conditioning

Discussion

Anxiety disorders are highly prevalent and affect 18% of Americans per year, with 4.1% being diagnosed as severe (Kessler et al., 2005). In order to find advancements for treatments, an animal model is necessary to research the underlying behavior, circuitry, and cellular mechanisms arising from the foundation of the disorder, including mal-adaptive fear.

Fear is highly conserved across species, and Pavlovian fear conditioning in rodents allows for the study of the neural circuitry and cellular mechanisms the underlie fear learning and memory in mammals. For example, a rodent will make an association between a previously neutral stimulus (conditional stimulus; CS) that is paired with an aversive stimulus (unconditional stimulus; US) and will develop a fear response to the conditional stimulus (conditional response, CR). When tested 16 months later, the rat will still show a significant conditional fear response to the CS (i.e. freezing; Gale et al., 2004).

My thesis work has been focused on the role of the *N*-methyl-D-aspartate receptor (NMDAR) in fear learning and memory, with a principle focus on using cellular manipulations of the NMDAR on basolateral nuclei of the amygdala (BLA) to measure the significance of NMDAR-mediated synaptic plasticity on auditory and contextual Pavlovian fear conditioning. This was achieved through temporary inactivation, the use of a shRNA virus targeted at depleting the Grin1 gene, and the use to transgenic mice to specifically isolate and dissociate nuclei within the BLA, specifically the lateral nucleus

(LA) and basal nucleus (BA). The behavioral effects of the manipulations were assessed with Pavlovian auditory and contextual fear conditioning.

The experiments in this dissertation were designed to manipulate the NMDAR-related synaptic plasticity in BLA nuclei during auditory and contextual Pavlovian fear conditioning. Specifically, Chapter 2 concerns the role of selective $GluN_{2B}$ antagonist on fear learning and retention. Chapter 3 utilizes the shRNA virus to look at NMDA-mediated plasticity in the lateral amygdala. Chapter 4 uses transgenic mice to address the role of NMDAR-mediated synaptic plasticity in both the LA and BA nuclei. More importantly, the results from Chapter 4 explores the primary pathways projecting contextual and auditory fear associations, as well as reveals the critical role of NMDAR-mediated synaptic plasticity in the BA to drive a fear response.

The NMDA subunit GluN2B in the basolateral amygdala is critical for the acquisition of contextual fear

The GluN2B subunit of the NMDAR is implicated in the mechanism of long-term potentiation, a neuronal substrate for fear learning. This experiment was designed to show that by temporarily inactivating the GluN2B subunit of the NMDAR in the basolateral amygdala (BLA) a deficit in contextual fear learning is produced. Unlike previous studies using intra-BLA application of the classic NMDA receptor antagonist d-amino-phosphonovalerate (APV) the effects of intra-BLA ifenprodil could not be attributed to state-dependent learning, interference with working memory or suppression

of performance. Pretesting intra-BLA infusion has also been found to block expression of an already acquired fear memory (Fendt, 2001; Lee et al., 2001; Maren et al, 1996b). Additionally, intra-BLA APV not only prevents the induction of LTP in the BLA, it also reduces neuronal spiking (Maren & Fanselow, 1995). Such findings make it difficult to attribute intra-BLA APV's actions on fear conditioning specifically to a prevention of LTP.

Freezing scores taken during training and testing revealed a significant increase in freezing between shock trials indicating acquisition of context fear but no interaction between trial and pre-training injections of ifenprodil versus artificial cerebrospinal fluid (ACSF) during acquisition (**see Chapter 2, Figure 2**). Freezing during acquisition reflects a working memory for the context-US association (Fanselow 1980; Kim et al., 1992). The drug had no effect on behavior at this time suggesting that ifenprodil did not affect context (CS) or shock (US) processing. Thus the most likely account of ifenprodil's effect is that it blocked long-term memory formation. Such an effect on associative learning is consistent with the drug's known action on NMDAR-mediated plasticity.

Additionally, context test data (**see Chapter 2, Figure 3)** indicated pre-training injections of ifenprodil produced a significant impairment compared to pre-training injections of ACSF controls regardless of posttest injections. There was no effect of posttest indicating that ifenprodil did not impair expression of freezing behavior. Importantly, the lack of an interaction of the pretesting and pre-training drug factors indicates that the results cannot be interpreted in terms of a drug state dependent effect.

Consistent with previous reports (Rodriguez, Schafe, & LeDoux, 2001), when the selective GluN2B antagonist, ifenprodil, was given prior to Pavlovian fear conditioning, fear was reduced during a later test of long-term contextual fear memory. Additionally, when the drug was only given during testing, expression of a previously acquired fear remained intact.

Functional NMDAR are composed of an obligatory GluN1 subunit that is usually paired with either a GluN2A or a GluN2B subunit. The differential effects of APV and ifenprodil suggests that GluN2B containing NMDAR play a somewhat exclusive role in supporting long-term memory formation while GluN2A containing NMDAR have a more general role in synaptic transmission in the BLA (Maren & Fanselow, 1995; Maren et al., 1996). Further elucidation of the role of GluN2A and GluN2B subunits in the BLA may provide keys to more selective modulation of fear and anxiety disorders.

shRNA-induced knockdown of NMDA receptors in the lateral amygdala causes a deficit in auditory fear conditioning.

The lateral amygdala (LA) is implicated as the site where convergence of auditory signaling information (tone) and reinforcement (shock) result in the synaptic plasticity necessary to produce conditioned fear (Romanski et al., 1993; LeDoux, 2000; Blair et al. 2001; Pare, Quirk, & LeDoux, 2004; Davis, 2006; Sigurdsson et al., 2007; Ploski et al., 2010). Auditory cues from both the auditory thalamus (medial geniculate nucleus) and auditory cortex can acquire fear conditioning, inducing long-lasting changes

in the LA (Boatman and Kim, 2006; Romanski and LeDoux, 1992; Clugnet & LeDoux, 1990; Doyère, et al., 2003). Synaptic plasticity that mediates the formation of fear memories is thought to be dependent upon N-methyl-D-aspartate receptors (NMDAR; Hitchcock and Davis, 1991; Maren, 1999; Ressler et al., 2002).

This experiment was designed to show that injection of an shRNA lentivirus containing a construct directed at the GluN1 subunit to interfere with NMDA receptor expression in the LA, will produce a deficit in auditory fear conditioning.

The results from this study indicate shRNA or scramble virus injections into the LA did not impair fear acquisition. However, the data during the tone test demonstrated injection of either the shRNA virus or the scramble virus produced a deficit in freezing during the tone test (**Chapter 3, Figure 3**). This data support the hypothesis that the LA is important for mediating fear memories, but was inconclusive on whether NMDAR-related plasticity was the cause for the deficit in fear memory, since the scramble virus also cause a deficit in tone fear. The LA is an important site where convergence of auditory signaling information (tone) and reinforcement (shock) produce conditioned fear. Selective knockdown of GluN1 subunit of the NMDA receptor in the LA caused a specific impairment of auditory fear learning when compared to control animals. Surprisingly, the scramble virus caused a minor deficit to tone freezing as well, and did not reach a statistical significance difference when compared to the shRNA viral animals or the control viral animals. This could be due to the fact that infusing the lentivirus itself disrupted the functionality of the LA, regardless of the sequence (e.g. shRNA or scrambled virus).

The data also suggests that contextual fear memory may not be reliant on the LA. There were no significant differences in freezing between any of the groups during the context (**Chapter 3, Figure 2**). This indicates that LA dysfunction is not sufficient to disrupt contextual fear. As with tone-shock associations, the evidence on context-shock association formation also points to the amygdala (Phillips & LeDoux, 1992). Hippocampal lesions will attenuate context conditioning but leave cued conditioning intact, even though both types of associative learning occurred at the same time (Phillips & LeDoux, 1992). The study hints that the BA might be a potential region of interest for exploring the relationship between the hippocampus and the NMDAR- related synaptic plasticity in specific nuclei within the BLA.

Some limitations to this study included the vector transport of interest. A lentiviral vector has a large vesicle and makes it more difficult to transfect a large population of neurons. Consequently, a higher volume of virus must be infused into the region of interest. The data suggest that the sheer volume of the infusion alone into the LA was enough to produce a disruption in LA functioning to produce a deficit in tone memory. This was supported by the fact that the scramble virus, which did not affect any gene expression, also caused a deficit in freezing during the tone test. This was a novel approach using techniques that allow for anatomical specificity without the disruption of information passing through the region. Although the results indicate problems with the transfection rate of the specific viral vector (i.e. lentivirus), this study provides insight for the potential of using RNA interference as a powerful strategy for analyzing memory formation and amnesia.

Dissociating the relative contribution of NMDA receptors in the basal and lateral amygdala in supporting auditory and contextual fear learning.

These set of experiments use Grin1^{flox} transgenic mice to decipher the significance of NMDAR-related synaptic plasticity BLA nuclei on auditory and contextual Pavlovian fear conditioning. With new techniques and technologies, it is important to advance our data analyses to reflect our progress. As the precision to identify areas of infection increased with the use of the transgenic mice and a virus with a GFP tag, we utilized multiple statistical analyses to extract as much information as possible from the data.

The results from this study indicate AAV2/1-CAG-Cre-GFP shRNA or AAV2/1-CAG-GFP injections into the LA or BA did not impair fear acquisition. However, Grin1^{flox} mice with a down-regulation of NMDAR in the basal amygdala acquire fear, but have a deficit contextual fear memory, and in tone fear memory. Grin1^{flox} mice with down-regulation of NMDAR in the lateral amygdala acquire fear, maintain contextual fear recent memory, but have a deficit in tone fear memory. Furthermore, Grin1^{flox} mice with a down-regulation of NMDAR in the basal amygdala predict a deficit in freezing during the context test and tone test. NMDAR- mediated synaptic plasticity in the BA is critical for expressing a fear response in both contextual and auditory fear conditioning.

Contextual Fear Conditioning

The context test revealed that the average percent freezing to the context in animals with a depletion of NDMAR in the BA was significantly lower than the percent context freezing of the controls, and the group with a depletion of NDMAR in the LA. Additionally, there was no significant freezing between the LA group and the control group (**Chapter 4, Figure 4**).

We modeled the amount of NMDAR based on immunohistochemistry to measure the amount of NMDAR per nuclei and determined parameters using a custom-made macro in ImageJ as described in Chapter 4. We then performed a linear regression on the data to make predictions on the amount of NMDAR per area's influence on percent freezing during the context test (**Chapter 4, Figure 6**).

The amount of NMDAR in the basal amygdala and lateral amygdala significantly predicted the variance in freezing during the context test. For every 1% decrease in NMDAR in the basal amygdala, there is a 0.639% decrease in context freezing over and above the effect of NMDAR in the lateral amygdala. For every 1% decrease in NMDAR in the lateral amygdala, there is a 0.323% decrease in context freezing over and above the effect of NMDAR in the basal amygdala.

NMDAR-mediated synaptic plasticity in the basal amygdala is critical for the contextual fear conditioning. Literature on the BLA's role in fear conditioning have focused on the LA as an essential site for the tone-shock association, but less focus is given to the processes that underlie contextual fear learning in the BLA, although research has been devoted to the role of the hippocampus in contextual fear learning.

The BLA has a strong anatomical connection with the hippocampus via the

ventral angular bundle. While the majority of projections from the hippocampus synapse onto the BA, some also synapse onto the LA, although the BA is implicated as the site for hippocampal-dependent contextual-shock associations (Goosens & Maren, 2001; Pitkänen, Savander, & LeDoux, 1997). Information about the context arrives at the BLA via the ventral angular bundle and lesions within this pathway attenuate contextual, but not auditory fear conditioning (Anagnostaras, Maren, & Fanselow, 1999; Maren & Fanselow 1995). Additionally, the BA undergoes associative plasticity during fear learning (Humeau et al., 2007). Furthermore, the BA is important for contextual fear conditioning, with ibotenic or electrolytic lesions of the BA cause an impairment in freezing during the context test (Onishi & Xavier, 2010; Goosens & Maren, 2001). Projections from the basal amygdala are sent to the CEAm in order to express the fear response (see Figure 1A; Pitkänen & Amaral, 1991).

The results support previous data showing the BA is required for acquisition of contextual fear conditioning. The data further this idea by stating NMDAR-mediated synaptic plasticity in the basal amygdala is critical for contextual fear conditioning. Figure 1A outlines the micro circuitry within the amygdala for the contextual fear conditioning.

Auditory Fear Conditioning

The tone test revealed that the average percent freezing to the tone in animals with a depletion of NDMAR in the LA and the BA was significantly lower than the percent

tone freezing of the control group (**Chapter 4, Figure 5**). There was no significant difference in freezing levels between the LA and BA groups during the tone test.

We modeled the amount of NMDAR based on immunohistochemistry to measure the amount of NMDAR per nuclei and determined parameters using a custom-made macro in ImageJ as described in Chapter 4. We then performed a linear regression on the data to make predictions on the amount of NMDAR per area's influence on percent freezing during the tone test (**Chapter 4, Figure 7)**.

Using a linear regression analysis we were able to predict the percent freezing in subjects during the tone test based on the percent of NMDAR in both the lateral amygdala and the basal amygdala. The amount of NMDAR in the basal amygdala and lateral amygdala significantly predicted the variance in freezing during the tone test. For every 1% decrease in NMDAR in the basal amygdala, there is a 0.948% decrease in tone freezing over and above the effect of NMDAR in the lateral amygdala. For every 1% decrease in NMDAR in the lateral amygdala, there is a 0.312% decrease in tone freezing over and above the effect of NMDAR in the basal amygdala. These results indicate that NMDAR mediated synaptic plasticity in the BA is more critical than NMDAR mediated synaptic plasticity in the LA for driving the auditory fear response.

NMDAR-mediated plasticity in the BA is more important for producing an auditory fear response than NMDAR-mediated plasticity in the LA during auditory fear conditioning. The LA forms an association between a discrete cue and an aversive stimulus (Romanski et al., 1993; LeDoux, 2000; Blair et al. 2001; Pare, Quirk, & LeDoux, 2004; Davis, 2006; Sigurdsson et al., 2007; Ploski et al., 2010). The data

supports this notion, since depletion of the NMDAR in the LA caused an impairment in freezing during the auditory fear test. More interesting, a similar deficit was seen when the depletion of NMDAR was in the BA. The linear regression revealed the amount of NMDAR in the BA was the main predictor of the freezing deficit seen in auditory fear conditioning over and above the effect of the NMDAR depletion in the LA, suggesting NMDAR in the BA is critical for auditory fear expression, as compared to NMDAR in the LA.

Literature on the BLA's role in auditory fear conditioning have focused on the LA as an essential site for the tone-shock association, but less focus is given to the involvement of the BA during Pavlovian fear conditioning. Interestingly, the studies that have focused on the BA mostly dealt with pre-training lesions and did not see a deficit in training. Pre-training electrolytic or excitotoxic lesions to the LA or the CEA, but not BA, disrupt freezing behavior elicited by the tone (Onishi & Xavier, 2010; Nader et al., 2001). These results indicate that when the BA is not present, auditory fear learning can occur.

However, when the BA is functioning normally, post-training lesions of the BA ablate fear expression to the previously trained auditory cue, suggesting that the BA is an important component in auditory fear learning in intact animals (Anglada-Figueroa & Quirk, 2005). Further, Humeau et al. (2007) also reported that the loss of synaptic plasticity in the BA of GluR1$^{-/-}$ mice impaired freezing to both discrete cues and to the context. Additionally, Anglada-Figueroa & Quirk (2005), re-trained animals that had received BA lesions and those animals were able to re-acquire auditory fear.

This indicates that when the BA is absent prior to training, the LA uses alternate routes, with less straightforward means, to connect to the CEA via two different pathways to compensate, but that when the BA is intact functioning normally, this seems to be the primary pathway. Specifically, when the BA is active, the tone-shock association is formed in the LA, which then sends projections to the BA. The BA then sends inputs to the CEAm to express the fear response (see Figure 1B; Pitkänen & Amaral, 1991). When the BA is not functioning, the tone-shock association is formed in the LA, which then sends projections to the lateral central nucleus of the amygdala (CEAl), which possesses reciprocal inhibitory GABA neurons regulating CEAm output (see Figure 1C; Haubensak et al., 2010). Still, another viable pathway leading information from the LA to the CEAm when the BA is not intact, is via the intercalated cells (ITC). The LA sends projections to the GABAergic ITC neurons, which then connect to the CEAm to execute fear responses (see Figure 1D; Paré, Quirk, & LeDoux, 2004).

The results support previous data showing the LA is required for the tone-shock association during auditory fear conditioning. More interesting, the data reveals that NMDAR-mediated synaptic plasticity in the BA is critical for auditory fear conditioning and is responsible for driving the fear response. Specifically, the results suggest that the LA is a relay site for the convergence of the discrete CS and US to form an association, but that the information from the LA is projected to the BA and that is where NMDAR-mediated plasticity critical for auditory fear conditioning. When the BA is functioning normally, post-training lesions of the BA ablate fear expression to the previously trained

auditory cue (Anglada-Figueroa & Quirk, 2005). In intact animals, the NMDAR-mediated receptor plasticity in the BA is what is essential to produce an auditory fear response.

The Fear Circuit

Chapter 4's study sought to elucidate the role of NMDAR-mediated plasticity within the BLA on auditory and contextual fear conditioning. However, in doing so, we also discovered an important component in the fear conditioning circuitry. Specifically, that NMDAR-mediated plasticity in the BA plays a much more important role in discrete cued and contextual fear conditioning than previously expected.

The pattern of results is congruent with previous literature on contextual fear conditioning such that the BA is critical for contextual fear conditioning and extends this finding to describe the importance of NMDAR-mediated plasticity in the BA as essential for contextual fear learning. Similarly, the data is consistent with literature on auditory fear conditioning wherein the LA is needed for the discrete cue- aversive shock association, however these findings reveal the significance of NMDAR-mediated synaptic plasticity in the BA to modulate fear expression.

By uniting these results with the established data on contextual and auditory fear conditioning, a more defined fear circuit emerges. First, information about the context forms a configural representation in the hippocampus and project to the BLA via the ventral angular bundle (Anagnostaras, Maren, & Fanselow, 1999; Maren & Fanselow 1995). Although these projections synapse onto the BA and LA (Goosens & Maren,

2001; Pitkänen, Savander, & LeDoux, 1997), the NMDAR- mediated plasticity in the BA seems to be responsible for driving the context-shock associations. From there, information travels from the BA to the CEAm to produce a fear response, including analgesia, autonomic and respiration changes; potentiated startle, and freezing (see Figure 1A; Fendt & Fanselow, 1999; LeDoux, 2000; Maren & Fanselow, 1996; Nagy & Paré, 2008).

Likewise, information about a discrete cue, such as a tone projects from the medial geniculate nucleus of the thalamus via thalamo- and cortico- pathways to the LA. Neurons in the LA are responsible for forming a tone-shock association and undergo NMDAR-mediated plasticity (Romanski et al., 1993; LeDoux, 2000; Blair et al. 2001). From there, neurons project to the BA and the NMDAR-mediated synaptic plasticity in this region is what is critical for auditory fear expression. Similar to contextual fear, information flows from the BA to the CEAm to generate fear responses (see Figure 1B; Pitkänen & Amaral, 1991). Figure 1 outlines the micro circuitry within the amygdala for the primary pathway for auditory fear conditioning. Additionally, Figure 1, details the two alternate pathways that can support auditory fear conditioning in the absence of the basal amygdala.

Conceptual Significance

This research has led us to rethink how the fear circuitry functions to create enduring memories. Currently, most models of fear learning involve a serial circuit,

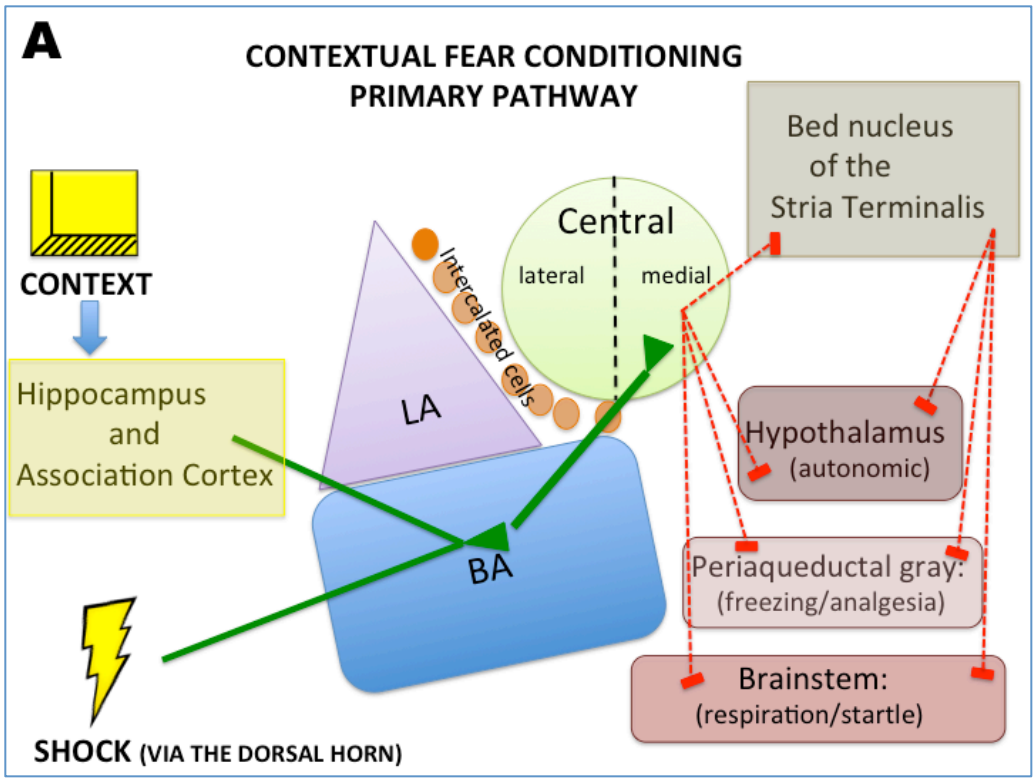
emphasizing very few sites of synaptic plasticity. Since the models involve a straightforward prediction, disruption of the circuit either prior to learning or after learning should disrupt the fear response equally. However, research involving the hippocampus and contextual fear conditioning disconfirmed this prediction by showing pre-training lesions to the hippocampus produced little deficit compared to post-training lesions to the hippocampus (Maren, Aharonov, & Fanselow, 1997; Kim & Fanselow, 1992). Further more that the post-training lesions were time dependent (Kim & Fanselow, 1992). The approach adopted was to think about the circuit in terms of primary and alternate pathways, versus a serial circuit, with an interconnected network to support fear learning when one area is compromised (Fanselow, 2000).

Similar to the hippocampal studies, experiments have shown that without the BLA, fear learning can still occur, but that the learning was inefficient and could not be maintained when tested one week later (Ponnusamy, Poulos, & Fanselow, 2007; Poulos et al., 2009; Poulos et al., 2010). Additionally, pre-testing inactivations of the BLA revealed that when learning occurs when the BLA is intact, an alternate pathway is not sufficient to acquire and maintain the fear memory (Ponnusamy, Poulos, & Fanselow, 2007; Anglada & Quirk, 2005). Thus, an alternate pathway can support learning when the primary pathway is not online during training.

The data that I presented in Chapter 4, support the implication that the basal amygdala is important for contextual and auditory fear learning and memory in an intact animal. Anglada & Quirk (2005), present data supporting this option, since post-training lesions of the basal amygdala abolish auditory fear conditioning. Additionally, Humeau

et al., (2007) supported this idea with the lack of GluA1 in the BA also produced a deficit in auditory and contextual fear learning. Importantly, my results show that in normal functioning animals, the NMDAR-mediated synaptic plasticity in the BA, as compared to the LA, is what is critical for driving the fear response during auditory fear conditioning.

Hebb recognized that in order for a cell assembly to form, there must by a network consisting of multiple neurobiological representations in order to support meaningful memories (Hebb, 1949). The neuro-architecture that creates fear memories should be a dynamic network, versus a serial circuit, in order to increase the chances of survival if damage occurs to the primary pathway.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PRIMARY PATHWAY
CONTEXT
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LA
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Central
lateral
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of the
Stria Terminalis
Hypothalamus
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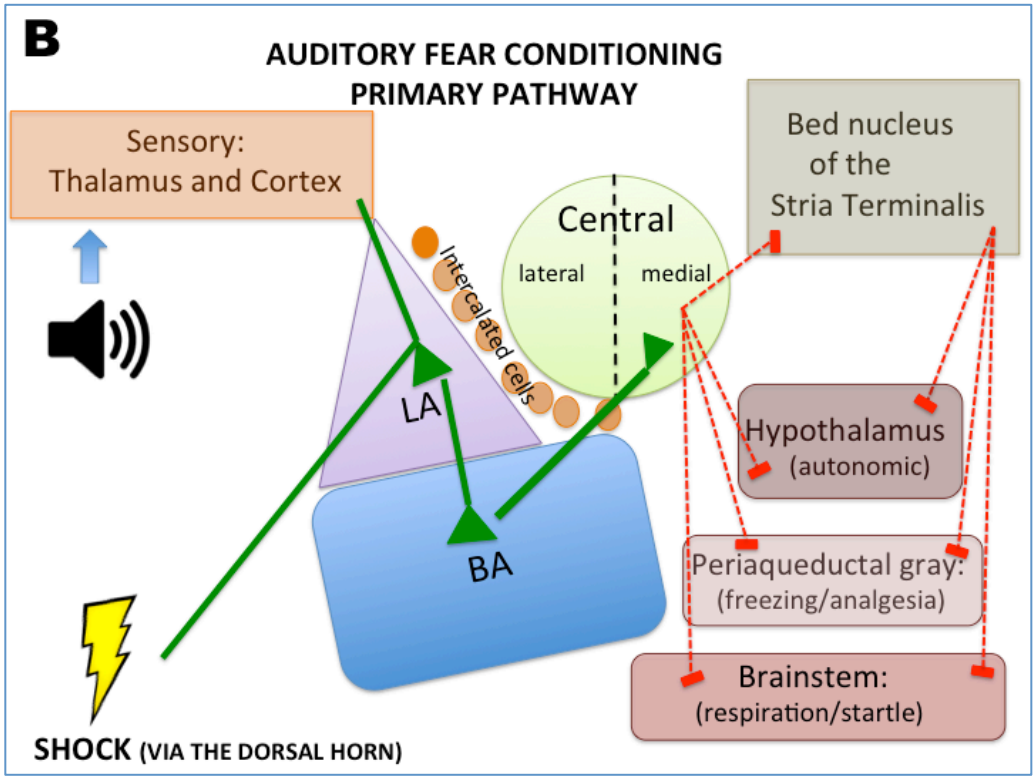
Periaqueductal gray:
(freezing/analgesia)
Brainstem:
(respiration/startle)
SHOCK (VIA THE DORSAL HORN)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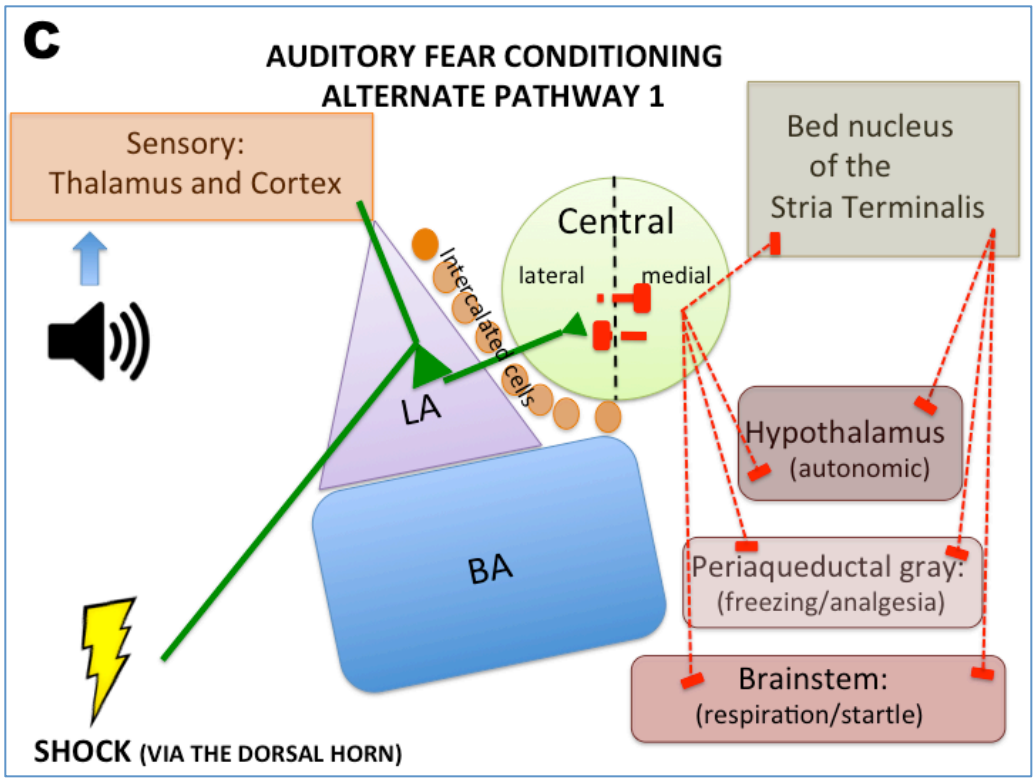
Periaqueductal gray:
(freezing/analgesia)
Brainstem:
(respiration/startle)
SHOCK (VIA THE DORSAL HORN)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ALTERNATE PATHWAY 1
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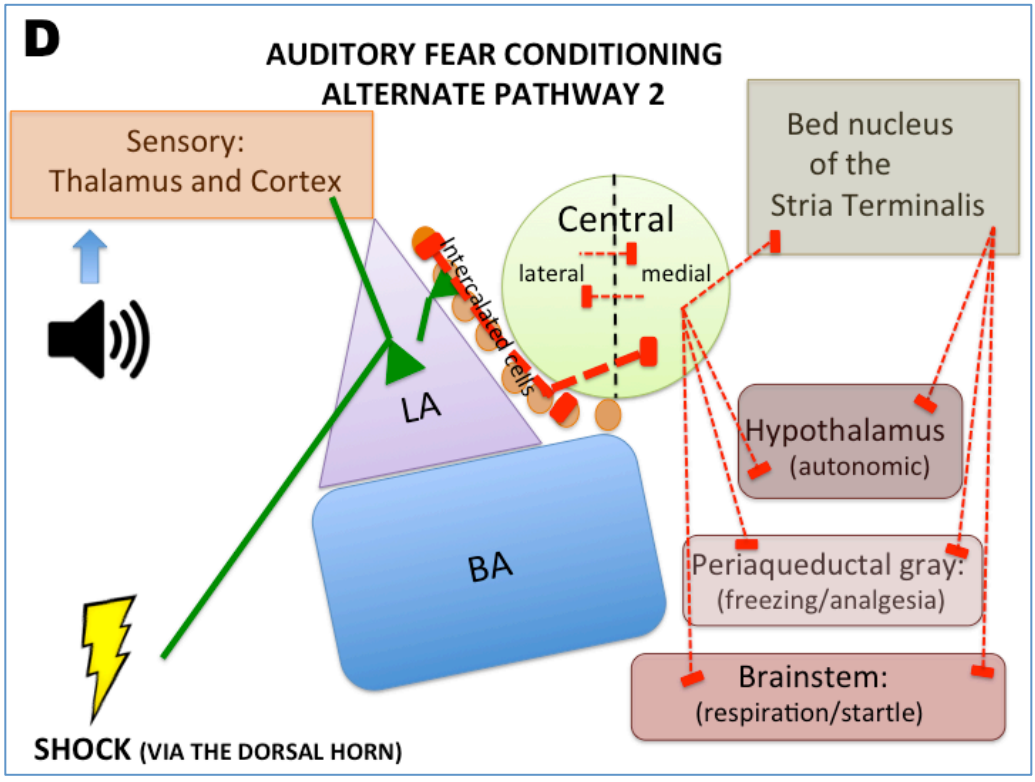
LA
BA
Hypothalamus
(autonomic)
Periaqueductal gray:
(freezing/analgesia)
Brainstem:
(respiration/startle)
SHOCK (VIA THE DORSAL HORN)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ALTERNATE PATHWAY 2
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Hypothalamus
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Periaqueductal gray:
(freezing/analgesia)
Brainstem:
(respiration/startle)
SHOCK (VIA THE DORSAL HORN)

Figure 1: Primary and alternate pathways with and the between the basolateral complex during contextual and auditory fear conditioning. Schematic representing the afferent and efferent connections between and within the amygdala including the lateral amygdala (LA), basal amygdala (BA), and central nucleus of the amygdala. Green represents excitatory Glutamatergic neurons and red represents inhibitory GABA neurons. (Note: For simplification, no neuromodulatory inputs were included. **(A)** Primary pathway for contextual fear conditioning. **(B)** Primary pathway for auditory fear conditioning. **(C-D)** Alternate pathways for auditory fear conditioning when the basal amygdala is damaged prior to training.

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