ROLE OF POSTSYNAPTIC DENSITY PROTEIN 95 (PSD95) AND NEURONAL NITRIC OXIDE SYNTHASE (NNOS) INTERACTION IN THE REGULATION OF CONDITIONED FEAR

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ROLE OF POSTSYNAPTIC DENSITY PROTEIN 95 (PSD95) AND NEURONAL NITRIC OXIDE SYNTHASE (NNOS) INTERACTION IN THE REGULATION OF CONDITIONED FEAR

Stimulation of N-methyl-D-aspartic acid receptors (NMDARs) and the resulting activation of neuronal nitric oxide synthase (nNOS) are critical for fear memory formation. A variety of previously studied NMDAR antagonists and NOS inhibitors can disrupt fear memory, but they also affect many other CNS functions. Following NMDAR stimulation, efficient activation of nNOS requires linking nNOS to a scaffolding protein, the postsynaptic density protein 95 (PSD95). We hypothesized that PSD95-nNOS interaction in critical limbic regions (such as amygdala and hippocampus) during fear conditioning is important in regulating fear memory formation, and disruption of this protein-protein binding may cause impairments in conditioned fear memory.

Utilizing co-immunoprecipitation, electrophysiology and behavioral paradigms, we first showed that fear conditioning results in significant increases in PSD95-nNOS binding within the basolateral amygdala (BLA) and the ventral hippocampus (vHP) in a time-dependent manner, but not in the medial prefrontal cortex (mPFC). Secondly, by using ZL006, a small molecule disruptor of PSD95-nNOS interaction, it was found that systemic and intra-BLA disruption of PSD95-nNOS interaction by ZL006 impaired the consolidation of cue-induced fear. In

contrast, disruption of PSD95-nNOS interaction within the vHP did not affect the consolidation of cue-induced fear, but significantly impaired the consolidation of context-induced fear. At the cellular level, disruption of PSD95-nNOS interaction with ZL006 was found to impair long-term potentiation (LTP) in the BLA neurons. Finally, unlike NMDAR antagonist MK-801, ZL006 is devoid of adverse effects on many other CNS functions, such as motor function, social activity, cognitive functions in tasks of object recognition memory and spatial memory.

These findings collectively demonstrated that PSD95-nNOS interaction within the conditioned fear network appears to be a key molecular step in regulating synaptic plasticity and the consolidation of conditioned fear. Disruption of PSD95-nNOS interaction holds promise as a novel treatment strategy for fear-motivated disorders, such as post-traumatic stress disorder and phobias.

Philip L. Johnson, Ph.D., Chair

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AC	Adenyl cyclase
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
Arc	Activity-regulated cytoskeletal-associated protein
BA	Basal nucleus of amygdala
BDNF	Brain derived neurotrophic factor
BH4	Tetrahydrobiopterin
BLA	Basolateral amygdala
β-ARs	β-adrenergic receptors
CaM	Calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CeA	Central nucleus of amygdala
CFC	Contextual fear conditioning
cGMP	Cyclic guanosine monophosphate
Co-IP	Co-immunoprecipitation
c-PITO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CPP	Conditioned place preference
CR	Conditioned response
CRE	cAMP response element
CREB	cAMP response element binding protein
CS	Conditioned stimulus
DEGs	Differentially expressed genes

dHP	Dorsal hippocampus
EDRF	Endothelial-derived relaxation factor
EPSCs	Excitatory post-synaptic currents
eEPSPs	Evoked excitatory post-synaptic potentials
EGR-1	Early growth response gene 1
eNOS	Endothelial NOS
ERK	Extracellular regulated kinases
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
$GABA_ARs$	Gamma-aminobutyric acid type A receptors
HP	Hippocampus
HPS	High frequency stimulation
IC87201	2-((1H-benzo (d) triazol-5-ylamino) methyl)-4,6-dichlorophenol
IGF-II	Insulin-like growth factor 2
IL	Infralimbic prefrontal cortex
iNOS	Inducible nitric oxide synthase
IP3	Inositol 1,4,5-triphosphate
L-LTP	Late phase of LTP
LA	Lateral nucleus of amygdala
LTM	Long-term memory
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MGB	Medial geniculate body

mGluRs	Metabotropic glutamate receptors
mPFC	Medial prefrontal cortex
NAPDH	Nicotinamide adenine dinucleotide phosphate
NMDARs	N-methyl-d-aspartate receptors
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NORT	Novel object recognition test
NSF	N-ethylmaleimide sensitive factor
PDZ	Post-synaptic density/Discs-large/ZO-1
PI3-K	Phosphstidylinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PL	Prelimbic prefrontal cortex
PSD95	Postsynaptic density protein 95
PTSD	Post-traumatic stress disorder
sGC	Soluble guanylyl cyclase
SMTC	S-methyl-L-thiocitrulline
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
STM	Short-term memory
TrkB	Tyrosine kinase receptor B
US	Unconditioned stimulus
vHP	Ventral hippocampus

vmPFC	Ventral medial prefrontal cortex
VTA	Ventral tegmental area
ZL006	4-(3,5-Dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid

CHAPTER 1

Fear doesn't exist anywhere except the mind – Dale Carnegie

Introduction

1.1 Innate Fear and Conditioned Fear

Fear is an adaptive emotional response to environmental threats. It motivates and organizes autonomic and endocrine changes supporting defensive behaviors necessary for survival. Fear responses can be triggered by a variety of stimuli that may cause treats to personal safety, including predators, pain and heights. Defensive behaviors induced by these types of stimuli do not depend on a learning process where a valence of danger is assigned to the threat. This type of fear has been referred to as 'innate fear' or 'unlearned fear' (Blanchard & Blanchard 1989). When experiencing innate fear, an association between the innate fear-inducing stimulus and a neutral stimulus, such as the context related to the threat, will be established. This associative form of memory, where a neutral stimulus acquires the ability to elicit fear responses, has been referred to as 'conditioned fear' or 'learned fear'. Conditioned fear has been the focus of scientific research to understand the neural basis of fear (Izquierdo et al 2016, LeDoux 2014).

1.2 The Fear Conditioning Paradigm

In the laboratory, conditioned fear or learned fear is modeled by Pavlovian fear conditioning paradigm, in which a neutral stimulus (conditioned stimulus,

CS), is paired with an aversive stimulus (unconditioned stimulus, US). After several CS-US pairings, the subject learns that the presentation of CS is predictive of the US. Once learned, the CS acquires the ability to induce fear responses termed conditioned response (CR), regardless of the presentation of US (Johansen et al 2011).

In rodents, a typical fear conditioning procedure involves an auditory CS (such as a tone) that co-terminated with an aversive US (usually a foot shock). Upon repeated CS-US pairings, the subject would develop fear behavior to the CS alone. Besides, the context in which the subject received fear conditioning will also induce fear responses due to context-US association. Expression of fear can be measured using the species-specific CRs, such as behavioral freezing (Blanchard & Blanchard 1969). Pavlovian fear conditioning is a well-established laboratory model of fear learning to cues and contextual stimuli. It is often used to study the neural circuits and the molecular mechanisms of fear learning and memory (Johansen et al 2011). In the laboratory, conditioned fear can be attenuated through a procedure in which a tone previously paired with a foot shock is delivered repeatedly in the absence of foot shock. This procedure results in a gradual decrease in fear responses that is attributed to a process called fear extinction (Myers & Davis 2007).

1.3 Brain Systems Encoding Fear Learning and Memory

Animal studies using fear conditioning models have revealed that there is a distributed network of brain regions that are involved in the acquisition, consolidation and extinction of conditioned fear. Among those involved brain regions, the amygdala, the hippocampus and the medial prefrontal cortex (mPFC) have received the most intensive attention, and these three different brain structures regulate, in concert, the different aspects of a conditioned fear response. Figure 1 shows a schematic representation of the major neural circuitry in cued and contextual fear memory. Briefly, tone (CS) and shock (US) inputs from the thalamus (LeDoux et al 1990) and contextual inputs (CS) from the hippocampus (Pitkänen et al 2000) are transmitted to the amygdala, which serves as the primary site at which information related to the CS and the US converge. The amygdala sends projections to a variety of structures in the brain stem, which regulate fear responses, such as freezing (LeDoux et al 1988). mPFC innervates amygdala and plays a critical role in the extinction of conditioned fear (Cho et al 2013). The roles of amygdala, hippocampus and mPFC in fear memory are described in greater details in the following three subsections.

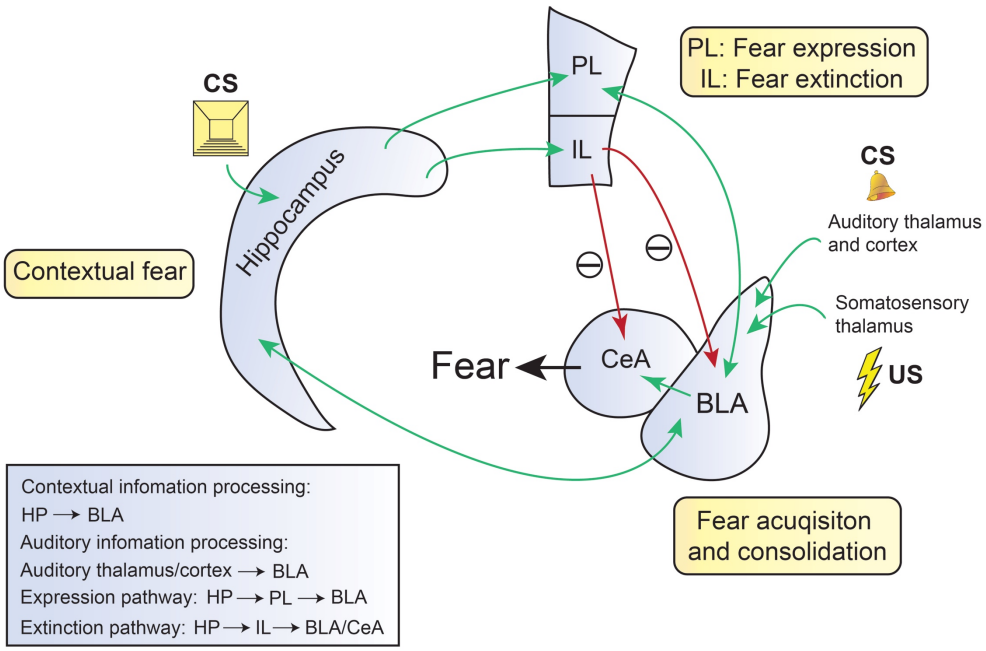

Figure 1. Neural circuits modulating fear memory

Amygdala is the critical structure regulating fear acquisition and consolidation. Auditory cue (CS) input from auditory thalamus/auditory cortex and foot shock (US) inputs from somatosensory thalamus converge in the basolateral amygdala (BLA). The BLA projects to the central nucleus of the amygdala (CeA), which is the major output structure of amygdala. The CeA projects to brain stem and control fear response, such as freezing. Hippocampus (HP) plays a crucial role in regulating contextual fear conditioning where the context is associated with shock. Configural representation of several contextual elements (e.g. lighting, olfactory cues, spatial cues and floor texture) is formed in the HP and then transmitted to the BLA where the associative connections between the hippocampal representation of context and the aversive stimulus (such as a foot shock) is established. HP also modulates fear responses through an indirect projection to the BLA via the medial prefrontal cortex (specifically, the prelimbic cortex (PL) and the infralimbic cortex (IL)). PL innervates the BLA and plays a role in mediating fear expression, whereas IL exerts feed-forward inhibition of neurons in the BLA and CeA, thereby suppressing the expression of fear in response to the conditioned stimulus that was previously extinguished. Figure 1 was modified from Maren and Quirk (2004).

1.3.1 Role of the amygdala

The amygdala is an almond-shaped structure located in the anterior portion of the temporal lobe. It consists of several functionally and anatomically distinct nuclei, including the lateral nucleus (LA), the basal nucleus (BA) (together referred to as the basolateral amygdala, BLA) and the central nucleus (CeA) (LeDoux 2003). The BLA is the sensory gateway of the amygdala; it receives inputs from the auditory thalamus and auditory cortex, somatosensory thalamus and hippocampus, allowing different information of fear memory to converge (LeDoux 2003). The CeA serves as the primary output area of the amygdala; it projects to various structures in the brain stem and mediates conditioned fear responses (CR) (LeDoux et al 1988). A series of inactivation and lesion studies using fear conditioning paradigm has demonstrated that the amygdala is one of the critical brain structures for fear acquisition and consolidation. Selective inactivation of BLA using gamma-aminobutyric acid type A receptors ($GABA_AR$s) agonist muscimol before fear conditioning significantly attenuate the acquisition of conditioned fear response (Maren et al 2001, Muller et al 1997, Wilensky et al 1999). Animals with excitotoxic lesions of BLA displayed a slow acquisition of conditioned fear and a substantial forgetting when remote memory was tested (Poulos et al 2009). Animals that received intra-BLA infusion of muscimol prior to test also showed low level of freezing to the CS (Muller et al 1997). As a result, BLA is thought to be the primary storage site of the association between CS and US and also plays an essential role in the fear memory expression.

Similar to the BLA, the CeA has also been shown to be essential for fear acquisition and expression. Lesions in CeA block or attenuate the expression of conditioned fear responses to both auditory and contextual cues (Campeau & Davis 1995, Goosens & Maren 2001, Zimmerman et al 2007). A study using a combination of *in vivo* electrophysiological, pharmacological and optogenetic techniques further demonstrated that the lateral subdivision of the CeA is required for the acquisition of fear, whereas the medial subdivision of the CeA is required for driving conditioned fear responses (Ciocchi et al 2010).

1.3.2 Role of the hippocampus

Following auditory cue associated fear conditioning that is induced after several tone/shock pairings, animals act fearful the moment they are put into the conditioning box, i.e., even before the delivery of conditioned stimulus (tones). This phenomenon of contextual fear conditioning (CFC) indicates that animals learn to associate the general context of the conditioning box with the noxious foot shock. By using cued fear conditioning and contextual fear conditioning where the context (the conditioning box) is paired with shock, many studies have demonstrated a critical role of the hippocampus in processing the contextual information of fear memories.

Studies with electrolytic lesions to the dorsal hippocampus (dHP) have shown an impaired acquisition of conditioned fear responses to the context (Phillips & LeDoux 1992, Phillips & LeDoux 1994). Similarly, inactivation of the

ventral hippocampus (vHP) with muscimol also reduced contextual fear memory (Rudy & Matus-Amat 2005). In addition, post-training lesions of either dHP or vHP can significantly disrupt contextual fear conditioning (Frankland et al 1998, Maren & Holt 2004). The functional role of the hippocampus in contextual fear conditioning is to assemble several independent contextual elements (e.g. lighting, olfactory cues, spatial cues, floor texture) into a unified, configured representation in order to use them as a CS (Young et al 1994). This view was further supported by a recent study using a targeted optogenetic approach in mice (Ramirez et al 2013). The authors have found that the hippocampal neurons that have been previously activated in a context can serve as functional CS in a different context when optically reactivated during the delivery of US. As a result, animals showed increases freezing responses in the original context, in which a US (foot shock) was never delivered (Ramirez et al 2013). More interestingly, the role of the hippocampus in contextual fear conditioning seemed to be time-limited as contextual fear deficits were only found when hippocampal lesions were made a couple of days (1 to 14 days) after conditioning. Animals for which the interval between conditioning and hippocampal lesions was longer (weeks) retained significant contextual fear memory (Anagnostaras et al 1999, Kim & Fanselow 1992, Maren et al 1997). These findings suggested that the integrated representation of the context was initially developed in the hippocampus but was subsequently stored elsewhere.

The hippocampus transmits the contextual information to the amygdala via the ventral angular bundle (VAB) which arises from the CA1 area, the ventral subiculum and the lateral entorhinal cortex (Canteras & Swanson 1992, Ottersen 1982). In the BLA, the associative connections between the hippocampal representation of context and the aversive stimulus (such as a foot shock) are established (Fanselow & Poulos 2005, LeDoux 2003). Therefore, the HP-BLA circuit is critical in the regulation of contextual fear conditioning. Animal studies have shown that lesions in the hippocampal regions that project to the BLA (ventral subiculum and lateral entorhinal cortex) resulted in an impaired contextual fear conditioning (Maren & Fanselow 1995); Similarly, lesions in the BLA also produced deficits in contextual fear conditioning (Maren & Fanselow 1995).

1.3.3 Role of the medial prefrontal cortex

The medial prefrontal cortex (mPFC) is usually defined as a collection of brain regions lying along the medial wall of the frontal lobe, including anterior cingulate, infralimbic (IL), prelimbic (PL) and medial orbito-frontal cortex (Heidbreder et al 2003). mPFC has been implicated in the extinction of fear memory in a variety of studies (Herry & Garcia 2002, Herry & Mons 2004, Morgan & LeDoux 1995, Morgan et al 1993, Santini et al 2004). Later research with manipulations restricted to subregions of mPFC has pinpointed the IL as the critical area involved in fear extinction. For example, electrophysiological studies found that IL neurons displayed increased activity to the CS after extinction

training (Milad & Quirk 2002). Furthermore, the increase in the IL responses to the CS was inversely correlated with freezing responses (Milad & Quirk 2002). In support of the view that IL is involved in fear extinction, studies using pharmacological or electrical stimulation techniques showed that intra-IL infusion of $GABA_A$ receptor agonist muscimol significantly impaired fear extinction (Laurent et al 2009), whereas microstimulation of IL enhanced fear extinction (Maroun et al 2012, Milad et al 2004, Vidal-Gonzalez et al 2006). Consistent with these findings, a more recent study using optogenetics demonstrated that activation of IL neurons during extinction training via channelrhodopsin 2 (ChR2) stimulation reduced fear responses and enhanced extinction memory the following day, whereas inactivation of IL neurons via halorhodopsin stimulation impaired the memory of fear extinction (Do-Monte et al 2015). In contrast to the fear-suppressing role of IL, PL region of mPFC has been shown to be involved in the expression of fear. For example, microstimulation of PL significantly enhanced the freezing responses to CS (Vidal-Gonzalez et al 2006), whereas inactivation of PL using either sodium channel blocker tetrodotoxin (Corcoran & Quirk 2007) or $GABA_A$ receptor agonist muscimol (Sierra-Mercado et al 2011) resulted in impaired fear responses.

Both IL and PL have bidirectional connections with the amygdala. IL projects to the LA, lateral division of central nucleus (CeL) as well as intercalated cell mass (ITC), a region between the BLA and CeA (McDonald et al 1996, McDonald 1998). In contrast, PL primarily targets BA (McDonald et al 1996,

McDonald 1998). In return, both IL and PL receive projections from the BLA (Hoover & Vertes 2007, Jin & Maren 2015). It has been thought that PL and IL exert effects on fear memory via their interactions with the amygdala (Arruda-Carvalho & Clem 2015, Marek et al 2013). Both PL and IL also receive substantial inputs from the vHP with sparse inputs from the dHP (Cenquizca & Swanson 2007, Hoover & Vertes 2007). HP-IL pathway has been thought to play a key role in mediating context-dependent modulation of fear extinction (for reviews see (Maren et al 2013, Rozeske et al 2015)).

1.4 Cellular and Molecular Mechanisms within the Amygdala Underlying Fear Learning and Memory

With the substantial evidence for the critical role of the amygdala in fear learning and expression, intensive research has been conducted to elucidate the cellular and molecular mechanisms in the amygdala (specifically in the BLA) underlying fear conditioning. Considerable evidence indicates that long-term potentiation (LTP) of synaptic transmission in the BLA neurons underlies fear conditioning.

LTP was first discovered in the hippocampus (Bliss & Collingridge 1993a, Bliss & Lømo 1973). Subsequently, it was found in a large number of brain structures, including various cortical areas (Artola & Singer 1987, Hirsch & Crepel 1992, Iriki et al 1989), the amygdala (Chapman et al 1990, Clugnet & LeDoux 1990) and the midbrain structures (Liu et al 2005, Overton et al 1999, Pu

et al 2006), paved the way for a widely accepted concept of synaptic plasticity. Traditionally, LTP is induced by high frequency stimulation (HFS) of presynaptic afferents or by pairing presynaptic activity with a sufficient level of postsynaptic depolarization. In both cases, the NMDA (N-methyl-d-aspartate) receptors with bound glutamate are allowed to be relieved from Mg^{2+} blockade, facilitating a large Ca^{2+} influx into postsynaptic compartments, resulting in a cascade of molecular changes, and thereby strengthening the synaptic efficacy for prolonged periods, referred to as LTP (Caporale & Dan 2008).

In this section, I will first briefly introduce the evidence supporting the notion that increased synaptic efficacy in the BLA is the basis of fear conditioning and then review the molecular processes underlying fear conditioning. Upon fear conditioning, fear memory is newly formed (or acquired) and undergoes a further strengthening process, referred to as 'consolidation', where it becomes stabilized and resilient to rapid decay or disruption (McGaugh 2000). Considerable work has been done to understand the molecular mechanisms by which the transient/labile synaptic changes become stabilized during the consolidation process. At the end of this section, the molecular processes in the amygdala underlying consolidation are briefly discussed (for a thorough review, see (Johansen et al 2011)).

1.4.1 Cellular mechanism underlying fear conditioning: synaptic plasticity in the amygdala

There are some neurons in the BLA that display responses to both auditory and somatosensory stimuli (Romanski et al 1993), leading to a notion that the BLA may be responsible for linking information about the CS and the US (Maren & Quirk 2004). Indeed, it has been shown that fear conditioning induces changes in the electrophysiological responses of the BLA neurons. The responses of BLA neurons to the CS^+ (tones that were paired with shock), but not to the CS^- (tones that did not paired with shock) were enhanced following training (Collins & Paré 2000, Ghosh & Chattarji 2015, McKernan & Shinnick-Gallagher 1997, Quirk et al 1995, Rogan et al 1997).

Parallel work has revealed that LTP exists at the thalamic afferent to the BLA. For example, in a study utilized HFS protocol demonstrated that by stimulation of the medial geniculate body (MGB, a part of the auditory thalamus), LTP could be induced in the BLA (Clugnet & LeDoux 1990). In a more recent study, Kwon and Choi showed that contingent pairings of MGB stimulation (CS) and a foot shock (US) can also induce LTP-like increases in the evoked field potentials in the BLA (Kwon & Choi 2009). More importantly, by concurrently measuring CS-evoked field potential in the amygdala and CS-evoked freezing behavior, LeDoux and colleagues showed that fear conditioning alters CS-evoked responses in the same way as LTP induction, and the changes in CS-evoked responses reflect the processes underlying behavioral fear responses

(Rogan et al 1997). LTP has also been shown to occur at the cortical input to the BLA (Doyère et al 2003, Schroeder & Shinnick-Gallagher 2004, Schroeder & Shinnick-Gallagher 2005). However, LTP at cortical inputs are quite different from those at thalamic input in terms of magnitude and duration. Studies have shown that the magnitude of cortico-amygdala LTP was robust within 24 hours and gradually faded within 3 days. In contrast, LTP at thalamic input, although initially smaller than cortical LTP, can remain stable for a longer time (6 days) (Doyère et al 2003). These findings indicated that thalamic and cortical inputs may play different roles in the acquisition and consolidation of fear memory (Doyère et al 2003).

One of the essential features of LTP is input-specificity, which means LTP is only induced at activated synapses rather than all synapses on the same neuron (Bliss & Collingridge 1993b, Nishiyama et al 2000). Although considerable work has been done to explore the relationship between amygdalar LTP and fear conditioning, it is still unclear whether input-specific LTP in the amygdala is associated with memory in fear conditioning. By using a combination of behavioral labeling approach, electrophysiology and behavioral paradigm, a recent study performed by Kim and Cho showed that discriminative fear conditioning induces LTP in the CS^+ pathway but not in the CS^- pathway to the LA (Kim & Cho 2017). Furthermore, by employing dual behavioral labeling approach, they were able to show that upon photostimulation of CS^+-responding auditory cortex/thalamus axons (labeled with ChR2-eYFP), the AMPA/NMDA

EPSC ratio was only increased in tdTomato-positive LA neurons labeled by fear conditioning, but not in non-labeled neurons (Kim & Cho 2017). Collectively, these findings showed that fear conditioning is associated with synapse-specific or input-specific LTP in the amygdala.

The electrophysiological studies described above collectively showed that BLA neurons display synaptic plasticity during auditory fear conditioning. While these studies provide correlational rather than causal evidence for fear conditioning, additional studies showed that disruption of synaptic plasticity in the BLA during fear conditioning prevented memory formation of fear conditioning. For example, application of NMDA receptor antagonists APV to the amygdala slices impaired HFS-induced LTP at the thalamic inputs to the BLA (Bauer et al 2002); and intra-BLA infusion of APV blocked the acquisition of conditioned fear (Campeau et al 1992, Gewirtz & Davis 1997, Kim et al 1991). NMDA receptor is a heteromeric complex comprised of two different subunits: NR1 subunit, which is required for the ion pore, and NR2 subunit, which consists of proteins NR2A-2D and has been suggested to modulate the biophysics of channels (Monyer et al 1992). NMDA receptors with NR2B subunit display longer excitatory post-synaptic potentials (EPSPs) (Monyer et al 1994), indicating NR2B-containing NMDA receptors might be particularly well suited for the coincidence detection, and thus promoting synaptic plasticity and fear conditioning. Indeed, intra-amygdala infusion of ifenprodil, a selective NR2B receptor antagonist also blocks LTP at thalamic inputs to BLA in vitro (Bauer et al 2002) and the acquisition of

conditioned fear in vivo (Rodrigues et al 2001). Recently, by employing advanced techniques, Nabavi and his colleagues showed that optogenetically induced depotentiation in the auditory input to the LA prevents the conditioned fear responses to the shock (Nabavi et al 2014). This is further supported by a more recent study performed by Kim and Cho, showing that dampening LTP at CS^+ pathway to the LA significantly reduced CS-elicited freezing behavior (Kim & Cho 2017).

1.4.2 Molecular mechanisms for fear acquisition and consolidation

The overall rise in the intracellular Ca^{2+} concentration upon NMDA receptors activation during fear conditioning triggers various downstream signaling pathways, including CaMKII signaling, protein kinases and nNOS-NO-PKG signaling, etc. These signaling pathways are mutually interconnected and are eventually transduced to the nuclei to regulate protein synthesis, which is essential for the stabilization of synaptic structures and the consolidation of fear memory. A schematic graph summarizing the molecular processes in the amygdala mediating fear acquisition and consolidation is shown in Figure 2 and are further discussed in the following sections.

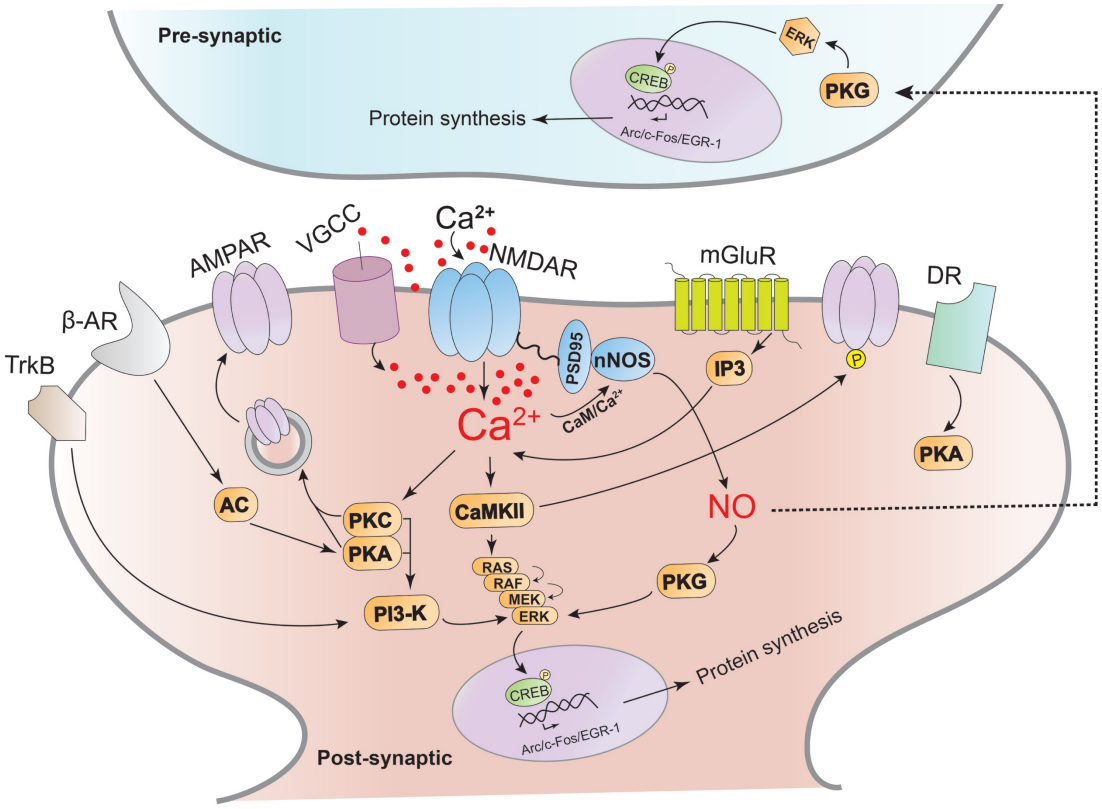

Figure 2. A working model of molecular cascades in the amygdala mediating acquisition and consolidation of fear memory

A postsynaptic increase in intracellular Ca^{2+} concentration, mediated through Ca^{2+} influx via NMDARs, voltage-gated calcium channels (VGCCs) and through the release from intracellular calcium stores upon activation of metabotropic glutamate receptors (mGluRs), triggers various downstream signaling cascades. Three major signaling pathways that are mutually interconnected are Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII), the protein kinase (PK) family, and nNOS pathways. Signaling mediated by activated CaMKII and PKA/PKC include phosphorylation of NMDARs as well as Ser845 site of GluR1, which could promote AMPARs insertion to the synapses. In addition, activation of the dopamine receptor (DR) and β-adrenergic receptor (β-AR) could also modulate the insertion of AMPARs through the activation of PKA. These molecular cascades are thought to be involved in the acquisition of fear memory. Protein kinases signals converge on the extracellular regulated kinases (ERK) signaling transduction pathway. ERK translocates into the nucleus and phosphorylates transcription factors, such as cAMP response element binding protein (CREB), which in turn triggers mRNA transcription and protein synthesis that is critical for the stabilization of synaptic structures and the consolidation of fear memory. BDNF (brain-derived neurotrophic factor)-TrkB signaling pathway could also regulate protein synthesis via activation of ERK. The role of PSD95/nNOS-NO-PKG signaling in the memory consolidation is of particular

research interest as it regulates ERK-triggered transcriptional changes not only in the post-synaptic neurons but also in the pre-synaptic neurons via diffusion of NO into the pre-synapses; in addition to the transcriptional effects, NO also cause effects on protein functions at both pre- and post-synaptic neurons via a post-translational modification process called S-nitrosylation (this part will be further discussed in the following section). CaM, Calmodulin; IP3, inositol 1,4,5-triphosphate; AC, adenyl cyclase; PI3-K, phosphatidylinositol-3 kinase; EGR-1, early growth response gene 1; Arc, activity-regulated cytoskeletal-associated protein

1.4.2.1 Molecular processes in the amygdala that underlie fear acquisition

***CaMKII*:** Upon activation by increased intracellular Ca^{2+} signaling through NMDARs, CaMKII undergoes autophosphorylation on a specific threonine residue (Thr^{286}) which allows the kinase to remain activated even after the intracellular Ca^{2+} concentration fall to the baseline level (Miller & Kennedy 1986, Yang & Schulman 1999). Autophosphorylation of CaMKII is essential for memory formation in various types of learning models (Silva 2003). By using Pavlovian fear conditioning, studies found an increased level of autophosphorylated (active) form of CaMKII in BLA spines 15 min following fear conditioning (Rodrigues et al 2004). Furthermore, intra-amygdala infusion of a CaMKII inhibitor, KN-62 prior to conditioning can dose-dependently impair fear acquisition (Rodrigues et al 2004). Autophosphorylation of CaMKII can then engage a variety of downstream molecular events in the BLA, which participate in the formation of fear memories (Lisman et al 2002).

***AMPA receptors*:** The role of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the fear conditioning was revealed by a series of behavioral studies in a combination of molecular, genetic and electrophysiological techniques. It has been shown that fear conditioning induces increased synaptic membrane insertion of GluR1 subunit-containing AMPA receptors in the BLA, which is required for fear memory formation (Humeau et al 2007, Rumpel et al 2005, Yeh et al 2005). In animals with genetically knock out of GluR1, acquisition of auditory fear conditioning and LTP at thalamo-BLA

spines was significantly impaired (Humeau et al 2007). This finding was consistent with an earlier study in rats showing that AMPA receptors trafficking into the synapses of LA (part of the BLA) is essential for fear conditioning in a wild-type background (Rumpel et al 2005). In this study, the ability to learn fear was tested in animals that received bilateral intra-LA injections of virus encoding the C-terminal of GluR1 fused with GFP. This recombinant protein acts as a dominant-negative construct to prevent endogenous GluR1-AMPA receptors from trafficking to the synapses. Remarkably, animals, in which this recombinant protein was expressed in as few as 10%-20% of LA neurons, showed a significant impairment in fear conditioning (Rumpel et al 2005).

***Monoamine neurotransmitters*:** Previous studies support the idea that the monoamine neurotransmitters such as norepinephrine (NE) and dopamine (DA) released in the emotional state contributes to the synaptic plasticity and fear conditioning. Aversive stimulus (foot shock) activates neurons in the locus coeruleus (LC) and ventral tegmental area (VTA), which result in increased NE and DA content, respectively, to the amygdala (Brischoux et al 2009, Chen & Sara 2007, Galvez et al 1996, Yokoyama et al 2005). These findings implicated that NE and DA acting through their respective receptors in the amygdala may modulate the acquisition of fear conditioning. Indeed, infusion of NE β-adrenergic receptors (β-AR receptors) antagonist, propranolol into the amygdala prior to training impaired acquisition of fear conditioning (Bush et al 2010).

***mGluRs*:** Behavioral and pharmacological studies indicate that metabotropic glutamate receptors (mGluRs) are important for the acquisition of fear memory. Intra-amygdala infusion of 2-methyl-6-(phenyl-ethynyl)-pyridine (MPEP), a specific mGluR5 antagonist, dose-dependently impairs the acquisition, but not consolidation of fear memory (Rodrigues et al 2002). In contrast, intra-amygdala infusion of (R.S.)-3,5-dihydroxyphenylglycine (DPHG), a group 1 mGluR agonist enhances fear responses typically produced by weak foot shock (Rudy & Matus-Amat 2009). Furthermore, LTP at thalamo-BLA synapses is impaired by bath application of MPEP in brain slices (Rodrigues et al 2002).

***Summary of acquisition*:** Evidence from previous studies suggests that NMDA receptors, especially those containing NR2B subunits, as well as AMPA receptors and CaMKII are involved in the acquisition of conditioned fear. In addition, neuromodulators, such as NE, DA and mGluRs may also regulate the initial formation of fear memory.

1.4.2.2 Molecular processes in the amygdala that underlie fear consolidation

Consolidation is the process by which short-lasting memory is stabilized into persistent memory. Unlike covalent modification of existing synaptic proteins, such as phosphorylation of glutamate receptors that are involved in the acquisition of conditioned fear responses, consolidation process often engages activation of second messengers, including CREB, ERK and NO. Second messenger signaling are eventually transduced into the nuclei and regulate gene

transcription and protein synthesis, which may lead to structural changes at the synapses (e.g., morphological changes in dendritic spines) (Lamprecht & LeDoux 2004).

***Gene transcription and protein synthesis*:** Considerable evidence indicates that both gene transcription and protein synthesis in the amygdala are required for the consolidation of fear memory. For example, intra-BLA infusion of actinomycin-D (a mRNA synthesis inhibitor) before training disrupted fear retention. However, fear responses to the CS-US pairings in the initial training session remained intact (Bailey et al 1999). This finding was supported by a later study using two different mRNA synthesis blockers, α-Amanitin and DRB. Intra-amygdala infusion of these blockers dose-dependently impairs memory retention of conditioned fear tested 24 hours after acquisition, but leaves the short-term memory (tested 4 hours after acquisition) intact (Duvarci et al 2008). Similarly, intra-BLA infusion of anisomycin, a protein synthesis inhibitor, dose-dependently attenuates fear memory retention 24 hours after training, but the short-term memory tested 4 hours after training remain intact (Schafe & LeDoux 2000).

***Protein kinases*:** Protein kinases, such as PKA (protein kinase A), PKC (protein kinase C) and MAPK (mitogen-activated protein kinase), have been shown to be involved in the consolidation of fear memory. They may exert their effects on consolidation via phosphorylating CREB (cAMP response element (CRE) binding protein). Activation of CREB triggers transcriptions of numerous

plasticity-related genes through binding with CRE during the consolidation process (Alberini 2009). Behavioral studies with pharmacological and genetic techniques have shown that both PKA and PKC are required in the BLA for the consolidation of fear memory (Goosens et al 2000, Schafe & LeDoux 2000, Weeber et al 2000). PKA and PKC signals are known to converge on the MAPK signaling pathways (Adams & Sweatt 2002). MAPK, originally called ERK (extracellular regulated kinase), has been widely implicated in the long-term synaptic plasticity and memory consolidation (Kandel 2001, Thomas & Huganir 2004). Studies have shown that MAPK is transiently activated/phosphorylated in the BLA following fear conditioning and intra-amygdala blockade of MAPK with its inhibitor U0126 impairs the consolidation of fear memory (Schafe et al 2000). In support of the important role of MAPK in fear memory consolidation, intra-amygdala inhibition of PI3-K (phosphatidylinositol-3 kinase), an upstream of MAPK, also blocked consolidation of fear memory (Lin et al 2001).

***BDNF signaling*:** Brain-derived neurotrophic factor (BDNF) signaling via tyrosine kinase receptor B (TrkB) has also been shown to be involved in the consolidation of fear memory (Bekinschtein et al 2008, Cowansage et al 2010). Several studies found an enhanced BDNF signaling in the BLA after fear conditioning, as evidenced by increased BDNF mRNA and protein levels as well as activation of TrkB receptors (Ou & Gean 2006, Ou & Gean 2007, Ou et al 2010, Rattiner et al 2004a, Rattiner et al 2004b). Furthermore, intra-amygdala inhibition of BDNF-TrkB signaling via infusion of a TrkB ligand scavenger (TrkB-

IgG) or Trk receptor inhibitor (K252a) attenuates memory consolidation of conditioned fear (Ou et al 2010). In addition, stimulation of TrkB receptors induces activation of PI3-K and MAPK, indicating that BDNF signaling may exert its effects on fear consolidation through activation of PI3-K and MAPK (Ou & Gean 2006).

***nNOS-NO-PKG signaling*:** In response to NMDA receptors-mediated Ca^{2+} signaling, activation of the enzyme neuronal nitric oxide synthase (nNOS) results in a production of the signaling molecule nitric oxide (NO). NO is a gas neurotransmitter that is known to modulate synaptic plasticity and fear memory via both pre- and post-synaptic mechanisms in the amygdala. Studies have shown that during fear conditioning, NO signaling in the BLA coordinately regulates MAPK-driven transcriptional changes in both auditory thalamus and BLA neurons that serve to regulate pre- and post-synaptic changes at thalamo-BLA synapses, respectively. In addition to the transcriptional effects, NO also exerts effects on the functions of several plasticity-related proteins via a posttranslational modification called S-nitrosylation. Disruption of nNOS-NO-PKG signaling via pharmacological and genetic means impairs the consolidation of fear memory. This topic will be more intensively discussed in the following text in section 1.5.

***Structural changes at the synapses*:** A previous study reported that fear conditioning produces an increase in synapse size in the amygdala (Ostroff et al

2010), and this increase may be in part due to rearrangement of cytoskeletal filaments (Lamprecht & LeDoux 2004). Studies have shown that fear conditioning induces movement of profilin, an actin-polymerization regulatory protein, into the spines in the amygdala and profilin-positive spines display enlarged sizes (Lamprecht et al 2006). Fear conditioning does not only affect the size of spines, but also has been shown to affect the spine density in the amygdala. Previous work using immunostaining or Golgi-Cox staining showed that the spine density in the amygdala was significantly increased after fear conditioning (Heinrichs et al 2013, Radley et al 2006). Alternations in the spine number and morphology have been thought to contribute to the endurance of synaptic changes and the long-term consolidation of memory (Bonhoeffer & Caroni 2016, Lamprecht & LeDoux 2004).

***Summary of consolidation*:** Ca^{2+} influx through NMDA receptors during CS-US pairings recruits a variety of protein kinases, including PKA, PKC and MAPK, which in turn, activate downstream substrates in the nucleus, including CREB. These nuclear substrates, in turn, trigger mRNA transcription and new protein synthesis that may lead to structural modifications of synapses and thereby contribute to the consolidation of fear memory. Also, neurotrophin signaling, especially BDNF-TrkB pathway, facilitates memory stabilization via activation of MAPK and PI3-K. The role of nNOS-NO-PKG signaling in the memory consolidation is of particular research interest as it regulates transcriptional changes as well as post-translational changes not only in the post-

synaptic neurons but also in the pre-synaptic neurons via a NO-driven retrograde signaling mechanism.

1.5 The Role of nNOS-NO Signaling in Synaptic Plasticity and Fear Memory

Nitric Oxide (NO) is a highly soluble gas generated by the conversion of the amino acid L-arginine to L-citrulline by the members of the nitric oxide synthase (NOS) family of enzymes. There are three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Förstermann & Sessa 2011). nNOS is constitutively expressed in brain areas such as the cortex, the amygdala, the hippocampus and etc. It has been widely implicated in the regulation of synaptic signaling and plasticity. eNOS is expressed primarily in the endothelial cells and regulates vascular function. Activities of nNOS and eNOS are primarily regulated by an increase in the intracellular Ca^{2+}, which activate nNOS and eNOS through calmodulin binding (Bredt & Snyder 1990). iNOS is expressed in many cell types throughout the body in responses to pro-inflammatory cytokines or endotoxins, thus playing a role in the regulation of immune responses (Förstermann & Sessa 2011). Unlike the Ca^{2+}- dependence feature of nNOS and eNOS activity, the activity of iNOS is not regulated by Ca^{2+} signaling (Galea et al 1992) .

In this section, I will first discuss the possible mechanisms through which nNOS-NO signaling may affect synaptic plasticity, and then summarize the findings from the behavioral studies using fear conditioning paradigm that

support the important role of nNOS-NO signaling in conditioned fear memory. A schematic graph summarizing the key molecular processes involved in synaptic plasticity that are affected by nNOS-NO signaling is shown in Figure 3 and are further discussed in the following sections.

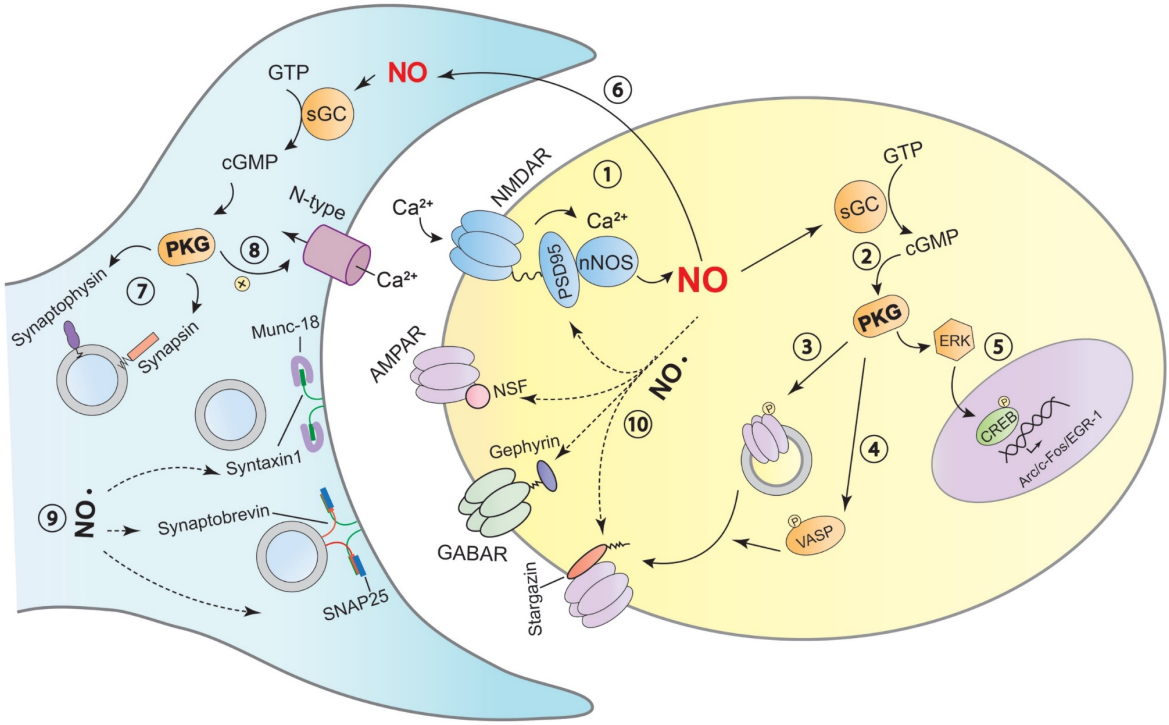

Figure 3. The effects of nNOS-NO signaling on both pre- and post- synaptic molecular processes involved in synaptic plasticity

Upon the activation of nNOS by Ca^{2+}/calmodulin, NO is synthesized (①) and acts on a wide array of targets mainly through the following two mechanisms: **1) NO-cGMP-PKG signaling mechanism.** In the post-synapse, activation of soluble guanyl cyclase (sGC) by NO produces cyclic GMP (cGMP), which in turn activates a variety of downstream signaling, including cGMP-dependent protein kinase (PKG) (②). Activated PKG has been found to form a complex with GluR1 and directly phosphorylate GluR1 (③), which promotes synaptic insertion of AMPA receptors. Activated PKG also phosphorylates vasodilator-stimulated phosphoprotein (VASP), which may, in turn, contribute to the clustering of GluR1 (④). By triggering the phosphorylation of the transcription factor CREB, PKG affects the transcription of a variety of genes that are required for the maintenance of LTP (⑤). Upon its synthesis, NO freely diffuse from the post-synapse to the pre-synapse (⑥), where it activates sGC-cGMP-PKG signaling pathway. Activated PKG in the pre-synapse may increase the clustering of vesicle related proteins, such as synaptophysin and synapsin (⑦). In addition, presynaptic N-type Ca^{2+} channel activities have been found to be affected by PKG activity (⑧). **2) Post-translational modification mechanism by S-nitrosylation.** In the presynapse, S-nitrosylation of syntaxin 1, an essential component of vesicle release/fusion machinery, releases syntaxin 1 from

interaction with Munc 18-1, thereby facilitating the interaction of syntaxin 1 with vesicular fusion machinery and the resulted vesicle release. Synaptobrevin and SNAP25, the other two essential components of vesicle release machinery have also been shown to be S-nitrosylated (⑨). In the post-synapse, receptors like NR2A subunit of NMDARs and scaffolding proteins, such as PSD95, NSF (N-ethylmaleimide sensitive factor), gephyrin and stargazin can be nitrosylated by NO, which in turn produce various synaptic outcomes (⑩).

1.5.1 NMDA-nNOS-NO signaling in the modulation of synaptic plasticity

The first evidence supporting a role for NO as a neurotransmitter was reported by Garthwaite et al., who observed that by acting on NMDARs in the cerebellum, glutamate induces the release of a diffusible molecule with similar properties to endothelial-derived relaxation factor (EDRF) (Garthwaite et al 1988). Before this study was published, research has shown that the biological activity and chemical properties of NO were indistinguishable from those of EDRF, suggesting that NO and EDRF are identical (Ignarro et al 1987, Palmer et al 1987). Subsequently, a larger number of studies have shown that NO acts as a neurotransmitter and modulates synaptic activities at many synapses throughout CNS.

Upon the activation of nNOS by Ca^{2+}/calmodulin, NO is synthesized and freely diffuses from cell to cell. After synthesis, NO acts on a wide array of targets, among which soluble guanyl cyclase (sGC) is the most prominent one. Activation of sGC by NO produces cyclic GMP (cGMP), which in turn activates a variety of downstream signaling, including cGMP-dependent protein kinase (PKG). It has been established that NO-cGMP-PKG signaling pathway plays a role in the synaptic plasticity in various brain regions. In addition to the cGMP-mediated signaling, NO also exerts synaptic effects via a posttranslational modification mechanism. By covalently binding to the cysteines of proteins (S-nitrosylation), NO affects the activities of key proteins involved in synaptic transmission and vesicle release.

1.5.1.1 Role of NO-cGMP-PKG signaling in synaptic plasticity

As discussed above in section 1.4.2, activation of NMDA receptors induces clustering of AMPA receptors, especially GluR1 subunit to the post-synaptic membrane, which plays a critical role in the formation of LTP. Studies have found that inhibitors of nNOS or PKG can block both the synaptic potentiation and the increase in GluR1 cluster induced by glutamate in hippocampal neurons (Wang et al 2005). Moreover, the blockade of GluR1 clustering can be rescued by 8-pCPT-cGMP, a potent cGMP analog which activates PKG (Wang et al 2005). Vasodilator-stimulated phosphoprotein (VASP) is a synaptic protein that has been implicated in the stabilization of actin following glutamate stimulation. Further experiments found that during potentiation, activated PKG phosphorylated VASP, which may, in turn, contribute to the clustering of GluR1 by regulating the dynamics of actin (Wang et al 2005). In addition, in a separate study, Serulle et al. found that activated PKG induced by NO donor application forms a complex with GluR1 and in this complex, PKG phosphorylates GluR1 at S845, which promotes the priming of AMPA receptors for synaptic insertion (Serulle et al 2007). Overall, these findings clearly suggested involvement of NO-cGMP-PKG signaling pathway in the modulation of synaptic plasticity, and the effects of this pathway on plasticity may act through the mechanisms regulating AMPARs trafficking.

Previous studies have also indicated a different mechanism by which NO-cGMP-PKG signaling pathway regulates synaptic potentiation, especially the

maintenance of potentiation. This mechanism involves the phosphorylation of the transcription factor CREB (cAMP response element binding protein) and the expression of a variety of genes. Studies have shown that during the late phase of LTP (L-LTP), which persisted beyond one hour, phosphorylation of CREB was enhanced in the hippocampus (Lu et al 1999). Furthermore, both increased phospho-CREB and L-LTP can be blocked by either direct PKG inhibition or inhibition of the PKG downstream target, the ryanodine receptors (Lu & Hawkins 2002). Upon the phosphorylation of CREB, the transcriptional program is initiated and is thought to produce new RNA and protein synthesis that are required for the maintenance of LTP (Benito & Barco 2010).

Earlier studies have found that hippocampal LTP can also be impaired by hemoglobin and oxymyoglobin which bind NO and cannot be taken up by cells (O'dell et al 1991, Schuman & Madison 1991). On the contrary, LTP can be facilitated by NO donor when it was injected into the presynaptic neurons (Arancio et al 1995, Arancio et al 1996). Increased cGMP was also found in the presynaptic neurons after NO donor treatment (Southam & Garthwaite 1993). These findings suggested that NO might act as a retrograde messenger that induces cGMP formation in the pre-synaptic terminals, thus modulating cellular processes which are required for LTP. In support of this view, a subsequent study found an increase in clusters of presynaptic protein synaptophysin during potentiation and this increase was accompanied by NO-mediated increase in cGMP (Wang et al 2005). Furthermore, increased synaptophysin cluster can be

reduced by PKG inhibitor, Rp-8-Br-cGMPS (Wang et al 2005). Evidence from other synapses also supports a presynaptic site of action of NO-cGMP-PKG signaling. At cerebellar mossy fiber-granule cell synapses, NO induces presynaptic current changes during LTP induction (Maffei et al 2003). In rostral ventrolateral medulla neurons, the NO donor spermine NONOate was found to significantly promote glutamate release through enhanced presynaptic N-type Ca^{2+} channel activities that mediated by cGMP-PKG signaling (Huang et al 2003).

1.5.1.2 Role of S-nitrosylation in synaptic plasticity

In addition to the NO-cGMP-PKG signaling pathway discussed above, NO has also been implicated in other pathways, including posttranslational modification. NO can lead to nitrosylation of the thiol side chain of cysteine termed S-nitrosylation (–SNO, the addition of a NO molecule to a thiol group). S-nitrosylation can cause effects on protein activities, protein structures and protein-protein interactions. Increasing evidence shows that critical proteins which mediate synaptic transmission and plasticity can undergo S-nitrosylation at both pre-synaptic and post-synaptic sites.

***At the pre-synaptic site*:** There is a large protein complex regulating vesicular release of neurotransmitters termed as SNARE proteins (an acronym derived from soluble N-ethylmaleimide sensitive factor attachment protein receptor). Vesicular SNAREs (vSNAREs, located on synaptic vesicles), such as

synaptobrevin and target SNAREs (tSNAREs, located on the presynaptic membrane), such as SNAP-25 and syntaxin are essential components of vesicle release/fusion machinery. A previous study has found that NO donor caused widespread S-nitrosylation of proteins in purified synaptic vesicle fractions, including above-mentioned SNAREs (Prior & Clague 2000). Later studies identified specific sites in some of SNARE proteins to be S-nitrosylated. For example, a study demonstrated that Cys^{145} of syntaxin 1 can be S-nitrosylated by NO donor treatment in rat brain homogenates and S-nitrosylation at this site acts as a molecular switch, turning off the closed state of syntaxin 1 by releasing it from interaction with Munc 18-1, thereby facilitating the interaction of syntaxin 1 with vesicular fusion machinery and the resulted vesicle release (Palmer et al 2008). Expression of nitrosomimetic syntaxin 1 (a mutated syntaxin 1 with a mutation that approximate an S-nitrosylated cysteine residue) in living cells disrupts Munc 18-1 interaction with syntaxin and alters exocytosis release kinetic and quantal size (Palmer et al 2008).

***At the post-synaptic sites*:** S-nitrosylation modifies the functions and structures of a variety of receptors and scaffolding proteins. Studies have reported a critical cysteine residue (Cys 399) in NR2A subunit of NMDA receptors undergoes S-nitrosylation by both exogenous and endogenous NO. When this single cysteine was substituted by alanine (an amino acid that cannot be S-nitrosylated), inhibition of NMDA-evoked currents by endogenous NO was significantly abolished (Choi et al 2000). NMDA receptors are located within the

post-synaptic density regions which contains a principal scaffolding protein post-synaptic density-95 (PSD95) that influence synaptic plasticity. Compelling evidence has shown that NO physiologically nitrosylated PSD95 at Cys3 and Cys5, and thus inhibiting synaptic PSD95 clustering (Ho et al 2011). Another post-synaptic receptor that undergoes S-nitrosylation is AMPA receptor. Previous work has shown that the GluR1 subunit of AMPA receptor is physiologically S-nitrosylated under basal conditions and upon the stimulation of NMDA receptors, S-nitrosylation of GluR1 became increased (Selvakumar et al 2013). Moreover, S-nitrosylation of GluR1 has been found to play an important role in regulating NMDARs-dependent phosphorylation of GluR1 (Selvakumar et al 2013). A family of small transmembrane AMPA receptor regulatory proteins is auxiliary subunits of AMPARs that control both AMPARs trafficking and channel gating. Stargazin and NSF (N-ethylmaleimide sensitive factor) are two of these auxiliary subunits that have been best characterized (Nicoll et al 2006). It has been reported that stargazin also undergoes S-nitrosylation under physiological condition. Nitrosylated stargazin has a higher binding affinity with GluR1, causing increased surface expression of AMPAR (Selvakumar et al 2009). Similarly, it has been shown that NO production upon NMDARs stimulation elicits S-nitrosylation of NSF whose binding with GluR2 is thereby enhanced, thus facilitating the surface insertion of AMPARs (Huang et al 2005). Gephyrin is one of the key scaffolding protein at inhibitory synapses that is essential for post-synaptic clustering of $GABA_A$Rs. A series of studies found that gephyrin interacts with nNOS and that gephyrin is S-nitrosylated in vivo. Pharmacological inhibition of nNOS caused a

loss of S-nitrosylation of gephyrin and a larger cluster of gephyrin at synaptic sites, ultimately increasing the surface expression of $GABA_ARs$ and enhancing inhibitory transmission (Dejanovic & Schwarz 2014).

1.5.2 Evidence for the role of nNOS-NO signaling in conditioned fear from animal studies

The critical role of NO signaling in the formation of conditioned fear has been revealed by a series of studies utilizing pharmacological and genetic techniques in a combination of animal models of fear conditioning. A study performed by Schafe et al. showed that intra-amygdala infusion of NOS inhibitor 7-Nitroindazole (7-Ni) impairs the consolidation of fear memory using a rat model of auditory fear conditioning (Schafe et al 2005). This fear-reducing effect of NOS inhibition was later shown to act through NO-cGMP-PKG pathway (Ota et al 2008). Intra-amygdala infusion of either PKG inhibitor Rp-8-Br-PET-cGMPS or PKG agonist 8-Br-cGMP dose-dependently attenuates or improves fear memory consolidation, respectively (Ota et al 2008). Consistent with this finding, PKG-deficient animals also exhibit impaired cued fear memory retention (Paul et al 2008). Interestingly, the consolidation of cued fear memory can also be disrupted by intra-amygdala infusion of NO scavenger c-PTIO (Schafe et al 2005). In addition, bath application of either 7-Ni or c-PTIO effectively blocked LTP at thalamic inputs to the BLA (Schafe et al 2005). Considering that c-PTIO is membrane-impermeable, these findings strongly suggested that both nNOS activation and NO diffusion into extracellular spaces are required for synaptic

plasticity and fear memory formation. Animal studies with genetic manipulations also supported the important role of nNOS-NO signaling in conditioned fear memory. Mice with nNOS gene knock out exhibit impairments in both cued fear learning and contextual fear learning (Kelley et al 2009).

Auditory fear conditioning has been shown to be associated with significant increases in the expressions of several synaptic proteins in the amygdala, such as GluR1 at the postsynaptic membrane, synapsin and synaptophysin at the presynaptic membrane (Ota et al 2010). Studies have shown that these pre- and post-synaptic changes are regulated by NO-cGMP-PKG signaling pathway. Intra-amygdala infusion of either 7-Ni or Rp-8-Br-PET-cGMPS significantly reduces fear conditioning induced expression of GluR1, synapsin and synaptophysin in the LA, whereas animals received intra-amygdala infusion of 8-Br-cGMP displayed increased expression of these proteins in the LA (Ota et al 2010). However, animals infused with NO scavenger c-PITO only showed reduced expression of synapsin and synaptophysin in the LA, but no impairment in the expression of GluR1 (Ota et al 2010). These results suggested that NO-cGMP-PKG signaling pathway regulates both pre- and post-synaptic alternations in the LA synapses, thus facilitating fear memory consolidation.

1.6 PSD95-nNOS Interaction as A Key Factor in The Regulation of nNOS Activity

nNOS is preferentially activated by the Ca^{2+} influx through the NMDA receptors but not through non-NMDA receptors that also generate Ca^{2+} influx (Kiedrowski et al 1992, Sattler et al 1999), suggesting a specific link between NMDA receptors mediated Ca^{2+} signaling and nNOS. Studies have identified a ternary complex containing NMDA receptors, PSD95 and nNOS (Christopherson et al 1999). PSD95 in this complex serves as a linker that brings nNOS in the proximity of the NMDA receptors, where nNOS can be efficiently activated by Ca^{2+} infux through NMDA receptors (Christopherson et al 1999). In the following sections, I will describe the structures of nNOS enzyme, especially the PDZ domain of nNOS that interact with PSD95, followed by an introduction of small molecules that disrupt PSD95-nNOS interaction.

1.6.1 Structure and isoforms of nNOS enzyme

The structure of nNOS monomer consists of two domains: oxygenase domain (N-terminal) and reductase domain (C-terminal), which are separated by a calmodulin (CaM) binding motif. The oxygenase domain is responsible for the binding of the substrate L-arginine. It also contains binding sites for tetrahydrobiopterin (BH4) and haem. The reductase domain binds the reduced adenine dinucleotide phosphate (NAPDH) and contains binding sites for flavin mononucleotide (FMN) and Flavin adenine dinucleotide (FAD) (Figure 4A). nNOS monomer is capable of transferring electrons (e^-) provided by NADPH to

FDA and FMN and has a limited capacity to catalyze NO production. Therefore, nNOS displays limited activity as monomer. Upon binding of cofactor BH4, haem and L-arginine, nNOS became a functional dimer, coupling haem and O_2 reduction to the synthesis of NO **(**Figure 4B). Binding of CaM facilitates the flow of NADPH-derived electrons in the reductase domain to the location of haem in the oxygenase domain, thus enhancing nNOS activity (Abu-Soud & Stuehr 1993, Noguchi et al 2001, Roman & Masters 2006, Sagami et al 2001, Szaciłowski et al 2005).

Due to alternations in mRNA splicing, there are five isoforms of nNOS: nNOSα, nNOSβ, nNOSγ, nNOSμ and nNOS-2 (Luo & Zhu 2011). Only nNOSα and nNOSμ have an N-terminal PDZ domain (post-synaptic density/Discs-large/ZO-1) that participates in the dimerization of nNOS and interacts with other proteins via PDZ-PDZ interactions, including PSD95 (Luo & Zhu 2011). nNOSα is the full-length form of nNOS with a predicted molecular weight of 160 KDa. It is the predominant isoform in the brain that contributes significantly to NO production (Alderton et al 2001). nNOSμ is a muscle-specific isoform of nNOS with a unique 34-amino acid insertion between the FMN and Calmodulin domains (Silvagno et al 1996). In vitro experiments with purified nNOSμ demonstrated that nNOSμ has a similar catalytic activity to that of nNOSα, and the dependence of its activity on Ca^{2+}/CaM is also very similar to that of nNOSα (Silvagno et al 1996). Due to the lacking of PDZ domain, nNOSβ and nNOSγ are thought to be localized to the cytosolic fraction and might not be responsive to NMDARs

stimulation (Brenman et al 1996). Catalytic assays in COS cells with overexpression of nNOSβ or nNOSγ indicated that nNOSγ displayed limited activity which was ~3% that of nNOSα, whereas nNOSβ had activity comparable to nNOSα (~80% of nNOSα) (Brenman et al 1996). Previous studies using in situ hybridization and immunostaining showed that nNOSβ also accounts for NO production in the brain, especially in the cortex and striatum (Eliasson et al 1997). nNOS-2 has been detected in mouse brain (Ogura et al 1993) and human neuroblastoma cell lines (Fujisawa et al 1994). It has a deletion of 105 amino acids in the L-arginine binding domain, leading to speculation that nNOS-2 may be catalytically inactive and negatively regulate the activity of nNOS (Brenman et al 1997). The functional role of nNOS-2 in the brain remains unclear.

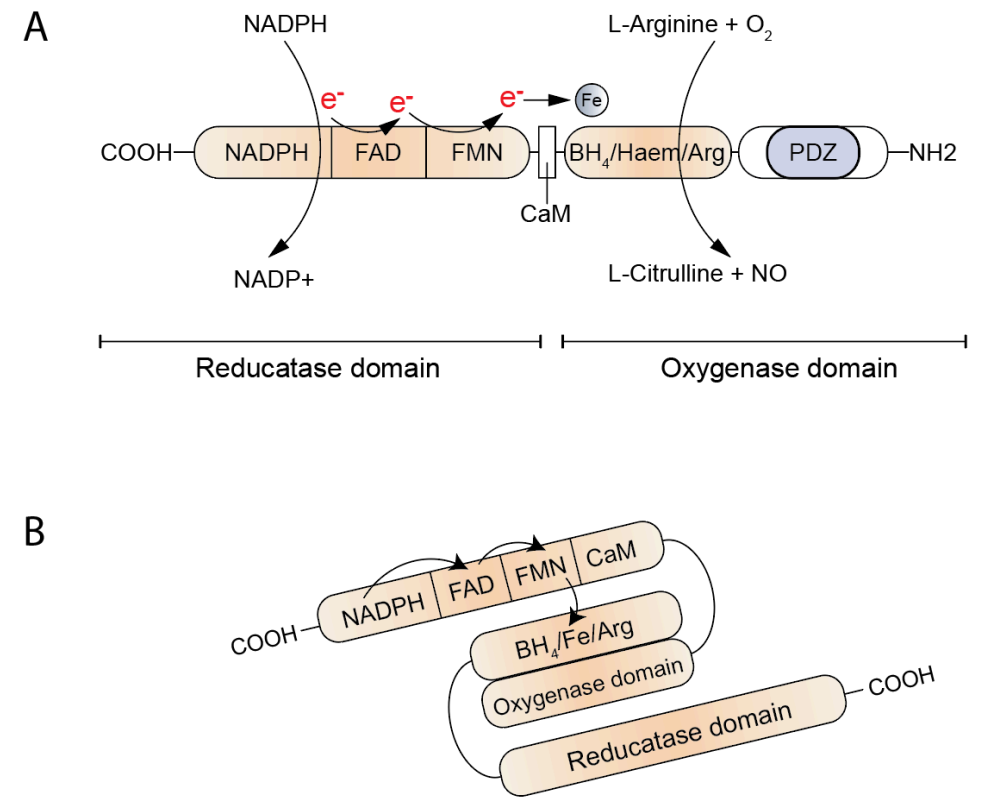

Figure 4. Schematic representation of nNOS protein structure

(A) The structure of nNOS consists of two domains: The N-terminal oxygenase domain and the C-terminal reductase domain, which are linked by a calmodulin (CaM) binding motif. The reductase domain contains binding sites for nicotinamide adenine dinucleotide phosphate (NAPDH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The oxygenase domain binds the substrate L-arginine and contains binding sites for tetrahydrobiopterin (BH4) and haem. Electrons transfers from the reductase domain to the oxygenase domain. Adapted from Zhou & Zhu, 2005 (B) Scheme of nNOS dimer. Arrows indicate electron transfer pathways. Dimerization of two oxygenase domains allows NADPH-derived electrons to transfer from FAD and FMN to hame irons; Electron transfer between reductase and oxygenase domains on the same subunit does not occur. Adapted from Szaciłowski et al 2005.

1.6.2 Mechanisms for PSD95/nNOS PDZ dimer formation

The nNOS PDZ domain terminates with a ~30 residue amino acids, which forms the β finger peptide. Previous studies have shown that the β finger peptide is required for PSD95/nNOS PDZ dimer formation (Christopherson et al 1999, Tochio et al 2000). Furthermore, point mutational analysis indicated that the salt bridge between Arg121 in the β finger peptide and Asp62 in the canonical PDZ domain of nNOS is critical for the binding of β finger peptide with PSD95 PDZ2. Disruption of this salt bridge by Arg121Gln mutation melts the β finger structure, therefore preventing nNOS PDZ from binding to PSD PDZ2 (Tochio et al 2000) (Figure 5).

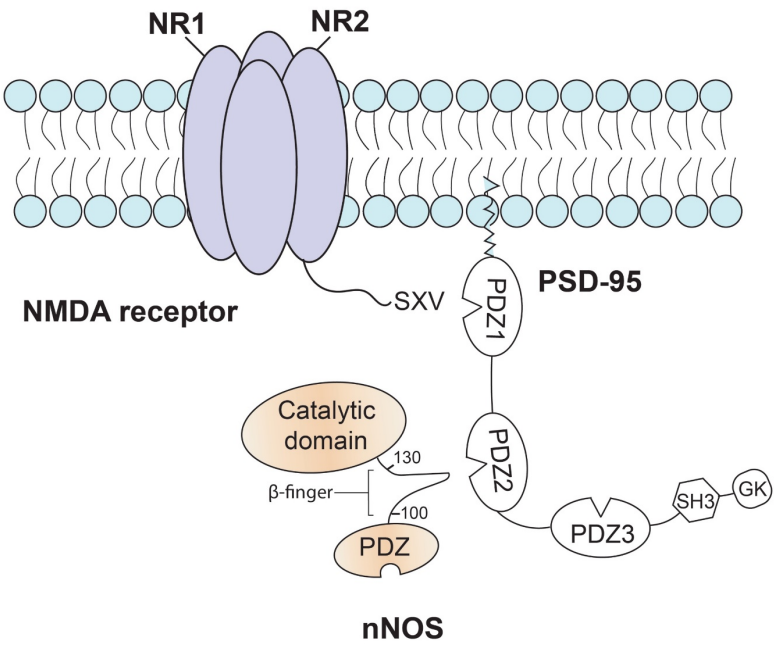

Figure 5. The ternary complex containing NMDA receptors, PSD-95 and nNOS

The NR2B subunit of NMDA receptors interacts with both the first and second PDZ domain of PSD-95 via its C-terminus (tSXV motif) (Kornau et al 1995). nNOS interact with the second PDZ domain of PSD-95 via the β finger peptide, which is a 30-amino acid extension beyond the canonical nNOS PDZ domain. PSD-95 serves as a scaffolding protein that links nNOS to the proximity of the NMDA receptors, where nNOS can be efficiently activated by the stimulation of NMDA receptors to produce NO. Adapted from Christopherson et al 1999.

1.6.3 Small molecules disrupting PSD95/nNOS interaction

Disruption of PSD95/nNOS interaction has been achieved with a group of unique small molecules. 2-((1H-benzo {d} {1,2,3} triazol-5-ylamino) methyl)-4,6-dichlorophenol (IC87201) was first identified in a high throughput screen using a PSD95/nNOS binding assay with an IC50 of 31 μM (Florio et al 2009). IC87201 inhibited NMDARs dependent cGMP production (an indirect measurement of NO production) in neuronal cultures and it attenuates nerve-injury induced mechanical allodynia (Florio et al 2009) as well as formalin-evoked nociceptive behavior in rats (Carey et al 2017). An analog related to IC87201, 4-(3,5-Dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid (ZL006) was synthesized based on the molecular determinants required for PSD95 and nNOS interaction (Zhou et al 2010). ZL006 was designed to interact with several residues in the nNOS PDZ, including Arg121, resulting in a disrupted salt bridge which is critical for the binding of nNOS PDZ to PSD95 PDZ2. ZL006 has been verified to selectively inhibit PSD95/nNOS interaction without affecting PSD95 interactions with other proteins (Lee et al 2015, Zhou et al 2010). In rodents, ZL006 crosses the blood brain barrier and demonstrates neuroprotection (Zhou et al 2010).

1.7 Hypothesis and Significance

My central hypothesis is that activation of the NMDA receptor associated PSD95-nNOS complex in critical limbic regions such as the amygdala and hippocampus during fear conditioning is a key molecular step in regulating the

development of conditioned fear, and disruption of this protein-protein binding may cause impairments in conditioned fear memory.

1.7.1 Fear conditioning associated PSD95-nNOS interaction in critical brain regions, and the effects of small molecule mediated disruption of PSD95-nNOS interaction

Chapter 2 describes how I determined the distribution and dynamics of PSD95-nNOS interaction within the conditioned fear network, including the BLA, the vHP and the mPFC following fear conditioning. I showed that fear conditioning results in significant increases in PSD95-nNOS binding within the BLA and the vHP in a time-dependent manner, but not in the mPFC. In addition, systemic treatment with a small molecule, ZL006 that disrupts PSD95-nNOS binding shortly after fear conditioning prevented the increases in PSD95-nNOS complex in both BLA and vHP. These results suggest that amygdalar and hippocampal PSD95-nNOS interaction are temporally regulated molecular processes subsequent to fear conditioning, indicating a role for this protein-protein interaction in the consolidation of fear memory.

1.7.2 The effects of disruption of PSD95-nNOS interaction on conditioned fear responses and other non-fear related behaviors

In chapter 3, I first determined the effects of systemic and regional disruption of PSD95-nNOS binding on the consolidation of conditioned fear responses. By utilizing a rat model of auditory fear conditioning, I showed that

systemic injection of ZL006, a small molecule that disrupts PSD95-nNOS binding, impairs cue-induced fear memory. Disrupting PSD95-nNOS binding directly within the BLA also attenuates cue-induced fear memory. In contrast, disrupting PSD95-nNOS binding within the vHP has no effects on cue-induced fear memory, but leads to an impaired context-induced fear memory. These results indicate that PSD95-nNOS complex in different brain regions mediate different aspects (cue- or context-related) of conditioned fear responses. Next, it was shown that disruption of PSD95-nNOS interaction by ZL006 has no effects on locomotion, social activity, object recognition memory and spatial memory. These findings indicate that the disrupting PSD95-nNOS interactions with ZL006 may be specific to fear-related behaviors. Disruption of PSD95-nNOS interaction may represent a novel therapeutic approach for reducing conditioned fear responses without eliciting motor deficits and adverse cognitive effects.

1.7.3 The synaptic mechanism in the amygdala associated with conditioned fear and the effects of disruption of PSD95-nNOS interaction

In chapter 4, the cellular and network mechanism underlying the effects of ZL006 on conditioned fear memory was investigated. By utilizing electrophysiological recording in amygdalar slices, it was shown that disruption of PSD95-nNOS interaction by ZL006 application blocks the long-term potentiation (LTP), a cellular model of memory in the BLA neurons.

CHAPTER 2

Fear Conditioning Associated PSD95-nNOS Interaction in Critical Brain Regions, and The Effects of Small Molecule Mediated Disruption of PSD95-nNOS interaction

2.1 Introduction

PSD95 is a scaffolding protein that interacts with both nNOS and NMDA receptors at excitatory synapses and assembles them into a ternary signaling complex (Christopherson et al 1999, Sattler et al 1999). Efficient activation of nNOS requires its interaction with PSD95, which brings it to the proximity of NMDA receptors and thus the NMDAR-mediated calcium influx (Zhou & Zhu 2009). Therefore, the interaction between PSD95 and nNOS serves as a key factor in the regulation of nNOS activity upon NMDARs stimulation. It is known that nNOS signaling is one of the critical molecular cascades triggered by the activation of NMDA receptors that underlie memory formation of conditioned fear (Ota et al 2010, Ota et al 2008). Inhibition of nNOS, similar to NMDARs antagonism, has been shown to disrupt fear memory in multiple animal models of fear conditioning (Kelley et al 2009, Pavesi et al 2013, Schafe et al 2005). Despite of the involvement of nNOS signaling in fear conditioning, little is known about the distribution and dynamics of PSD95-nNOS complex within the conditioned fear network during the processes of fear memory formation. In this chapter, by using co-immunoprecipitation technique, I tested if fear conditioning can time-dependently induce significant increases in PSD95-nNOS binding within

the CNS circuit implicated in conditioned fear that includes the amygdala, the hippocampus and the mPFC; and, if pretreatment of ZL006, a small molecule that disrupts PSD95-nNOS interaction can prevent the increases in PSD95-nNOS complex within the conditioned fear network.

2.2 Results

Fear conditioning induced robust increases in PSD95-nNOS interaction within the BLA and the vHP, but not in the mPFC

In the first experiment, by using co-immunoprecipitation, I determined the PSD95-nNOS complex levels within the conditioned fear network at several time points following conditioned fear training. Animals who underwent fear conditioning training were sacrificed and the levels of PSD95/nNOS binding in the BLA, the vHP and the mPFC were quantified with co-immunoprecipitation (Co-IP) with nNOS antibody followed by immunoblotting with nNOS and PSD95 antibodies. Previous studies showed that molecular changes usually occur within several hours (< 6 h) following fear conditioning (Igaz et al 2002, Schafe & LeDoux 2000), therefore Co-IP experiments were conducted at multiple time points following fear conditioning ranging from 0.5 to 6 h to determine the time course for PSD95-nNOS interaction (Figure 6). As a control, a separate group of rats which received the same procedure but without shock were sacrificed 0.5 h following no shock training ('Tone only') (Figure 6). Significantly increased interaction between PSD95 and nNOS were observed at 1 h and 2 h after fear conditioning in both BLA and vHP, when compared with 'Tone only' controls

(Figure 7A and B). The increased interaction between PSD95 and nNOS recovered to baseline level by 6h for both regions (Figure 7A and B). However, no significant changes of the interaction were observed at any time points following fear conditioning in the mPFC (Figure 7C). To exclude the possibility that the observed differences found in PSD95-nNOS interaction among the groups were due to different abilities of fear learning, behavioral performances during fear conditioning were analyzed for each animal. It was found that all of the fear conditioned groups showed comparable freezing responses during fear training (Figure 8A and B).

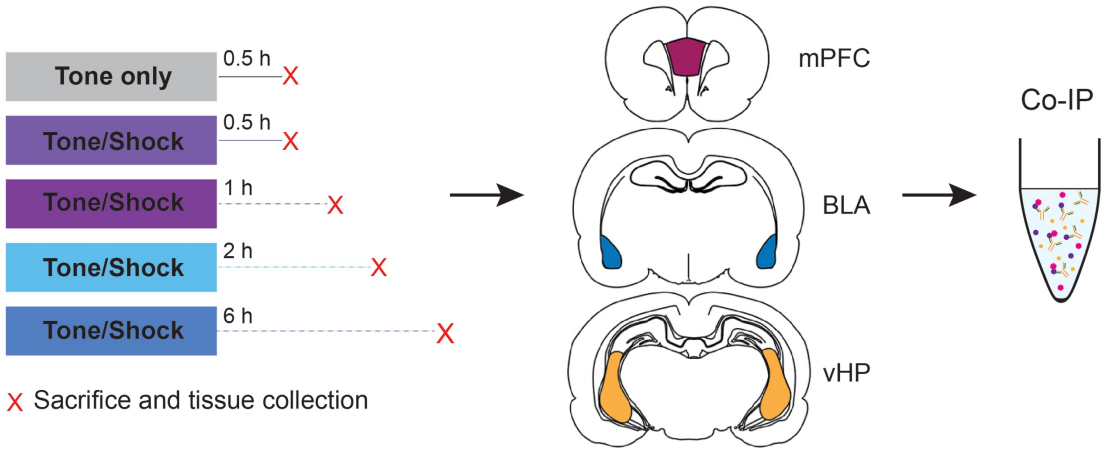

Figure 6. Schematic protocol of the Co-IP experiment in different brain regions following fear conditioning

Fear conditioned rats received a training of 3 tone/shock pairings and were sacrificed either 0.5, 1, 2 or 6 hours after conditioning. The 'Tone only' group received the same training but without shock pairing and were sacrificed 0.5 h after the non-shock training. Tissue punches from the BLA, the vHP and the mPFC were prepared for Co-IP experiment.

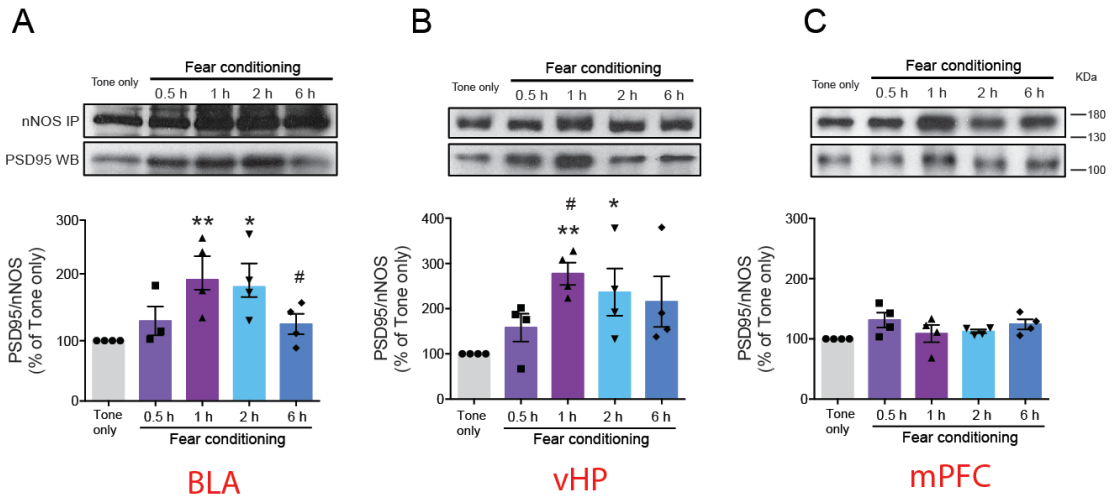

Figure 7. Fear conditioning induces dynamic changes in PSD95/nNOS binding within the BLA and the vHP, but not in the mPFC

Protein lysates from the BLA (A), the vHP (B) and the mPFC (C) were immunoprecipitated with nNOS antibody and the PSD95-nNOS complex was then probed with PSD95 antibody and nNOS antibody (tops, representative blots). Levels of PSD95/nNOS ratio were expressed as a percentage of those in 'Tone only' controls (bottoms, for the BLA, n = 3 or 4, $F_{4, 14} = 3.526$, $P < 0.05$; for the vHP, n = 4, $F_{4, 15} = 3.262$, $P < 0.05$; for the mPFC, n = 4, $F_{4, 15} = 1.792$, $P > 0.05$). * $P < 0.05$, ** $P < 0.01$ relative to 'Tone only' group; # in (A) $P < 0.05$ relative to 1 h group; # in (B) $P < 0.05$ relative to 0.5 h group

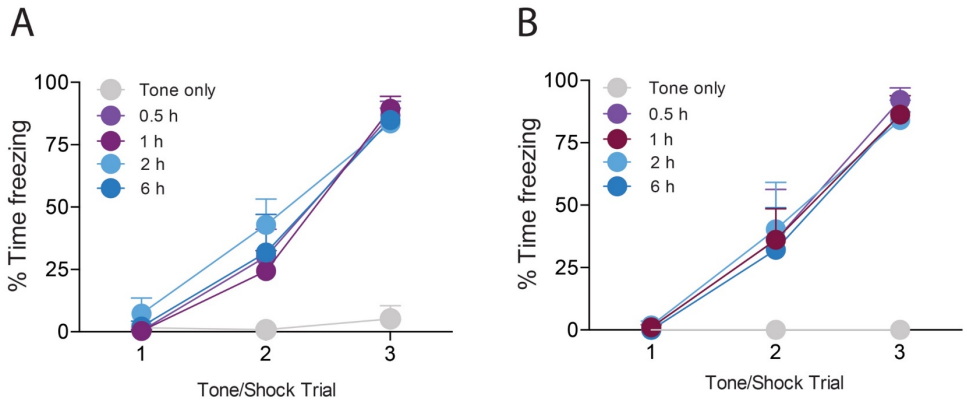

Figure 8. Acquisition of conditioned fear in animals used for Co-IP experiments

Animals in fear conditioned groups acquired fear normally and equivalently (for graph A: n = 3-4, Trial: $F_{2, 22} = 237.6$, $P < 0.0001$; Treatment: $F_{3, 11} = 0.2241$, $P > 0.05$; Treatment x Trial: $F_{6, 22} = 0.9237$, $P > 0.05$; for graph B: n = 4, Trial: $F_{2, 24} = 82.95$, $P < 0.0001$; Treatment: $F_{3, 12} = 0.04454$, $P > 0.05$; Treatment x Trial: $F_{6, 24} = 0.091$, $P > 0.05$). Animals in 'Tone only' group showed no freezing responses to the tone. After fear conditioning, animals were sacrificed at different time points as indicated in the graphs. BLA punches were obtained from animals in (A) and vHP and mPFC punches were obtained from animals in (B); these punches were further processed for Co-IP experiments that was described in Figure 6.

The expression levels of PSD95 and nNOS were not altered over time following fear conditioning in the vHP and the mPFC

Increased or decreased interaction between PSD95 and nNOS that were observed in the above Co-IP experiments may result from up-regulation or down-regulation of protein expressions. To this end, the expression levels of PSD95 and nNOS at various time points were probed. Results from western blot experiments showed that neither the expression level of PSD95 or that of nNOS was changed over time following fear conditioning within the vHP (Figure 9A and B). This data indicated that the dynamic changes (increased or decreased) in the PSD95-nNOS complex within the vHP following fear conditioning were not due to altered expression levels of PSD95 or nNOS, but simply resulted from enhanced or weakened interaction between these two proteins. The preliminary study in our laboratory also showed similar results in the BLA that the expression levels of PSD95 and nNOS were not changed over time following fear conditioning (data not shown). In the mPFC, consistent with the Co-IP experiment, the expressions of PSD95 and nNOS remained at similar levels over time following fear conditioning (Figure 9C and D).

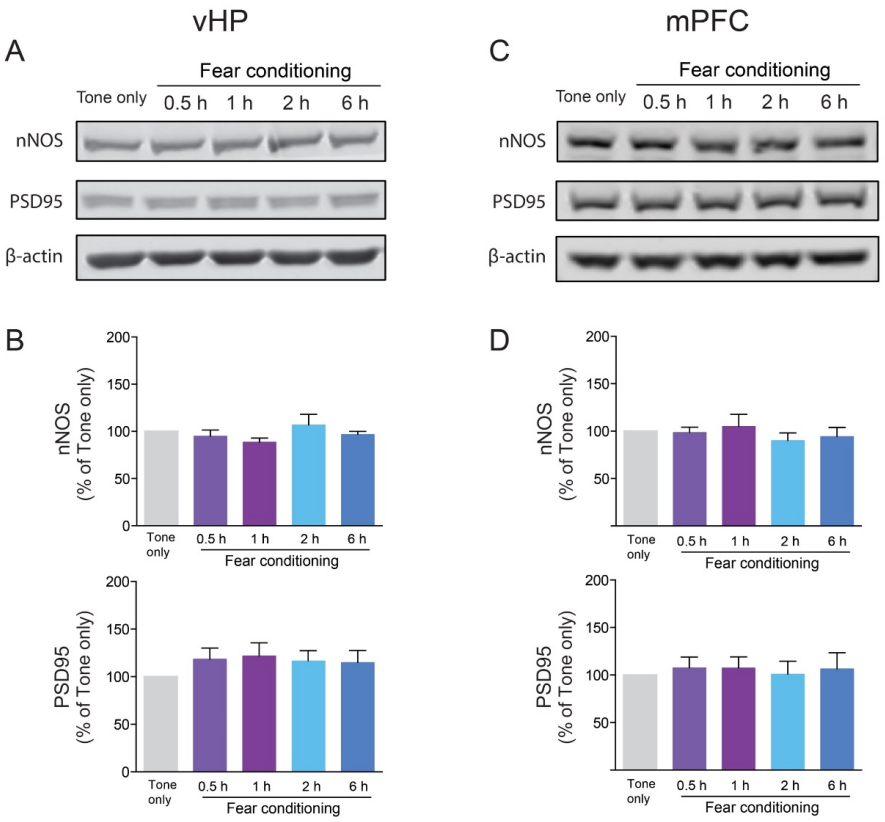

Figure 9. The expression levels of PSD95 and nNOS were not altered over time following fear conditioning in the vHP and the mPFC

(A, C) Representative western blots of nNOS and PSD95 in vHP (A) and mPFC (C). β-actin was used for normalization purpose. (B, D) Quantification graphs of nNOS (Top) and PSD95 (Bottom) levels in the vHP (B) and mPFC (D). The results are presented as a percentage change relative to the 'Tone only' group, which was assigned a value of 100 (n = 4, P > 0.05 for all graphs).

Fear conditioning-induced increased PSD95-nNOS binding within the BLA and the vHP can be prevented by a pretreatment of ZL006

Next, I tested if the fear conditioning-induced increases in PSD95-nNOS complex could be blocked by pre-treatment with ZL006, a small molecule that disrupts PSD95-nNOS binding. Animals that were subjected to conditioned fear training (tone-shock pairings) were administrated with an i.p. injection of either vehicle or 10 mg/kg ZL006 immediately following conditioning (Figure 10). The dose of 10 mg/kg was chosen based on previous data (Carey et al 2017, Lee et al 2015) and the current data (discussed in Chapter 3) indicating ZL006 at this dose was effective in disrupting behaviors related with pain and fear in rats. The levels of PSD95/nNOS complex within the BLA and the vHP were quantified with Co-IP 1 h after conditioned fear training, the time point when the PSD95/nNOS interaction peaked following fear conditioning as previously described (Figure 10). Once again, the levels of PSD95-nNOS complex within the BLA and vHP were significantly increased at 1 h following fear conditioning and these robust increases were blocked in the animals treated with ZL006 (Figure 11A and B). Animals that were used in these Co-IP experiments showed comparable conditioned freezing response during fear conditioning training (Figure 12A and B).

In addition, I tested if pre-treatment of ZL006 had effects on the expression of PSD95 and/or nNOS. Results from western blot experiments demonstrated that pre-treatment of ZL006 did not alter the expression levels of

PSD95 and nNOS in the vHP (Figure 13). Preliminary data also showed similar expression levels of PSD95 and nNOS in the BLA from animals with or without ZL006 treatment (data not shown). In summary, these results indicated that fear conditioning-induced enhanced interaction between PSD95 and nNOS can be blocked by a pre-treatment of ZL006.

Locations of the punches from different brain regions that used for Co-IP experiments and western blotting

A schematic graph in Figure 14 depicts the locations of the punches from different brain regions that used for Co-IP experiments and western blotting experiments.

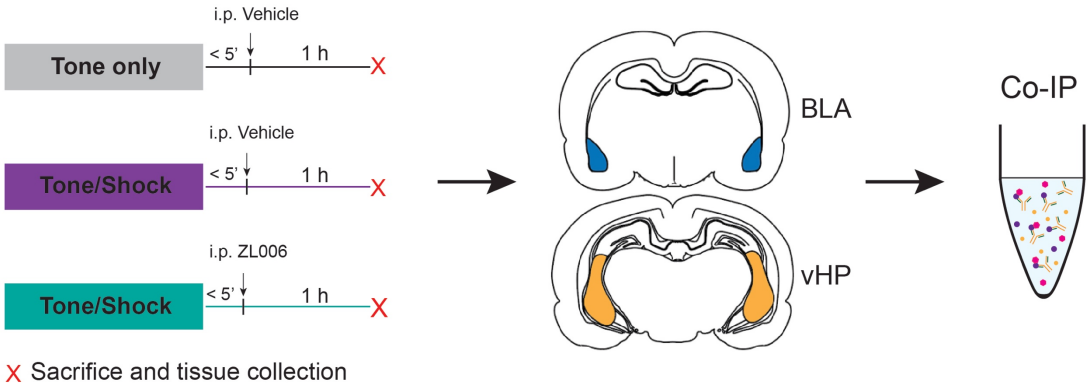

Figure 10. Schematic protocol of the Co-IP experiment in animals treated with ZL006

Immediately (less than 5 minutes) following fear conditioning, rats were treated with i.p. injections of either vehicle or 10 mg/kg ZL006. Animals in the 'Tone only' group received a non-shock training during fear conditioning and were treated with vehicle. All animals were sacrificed 1 h following fear conditioning. Tissue punches from the BLA and the vHP were collected for Co-IP experiment.

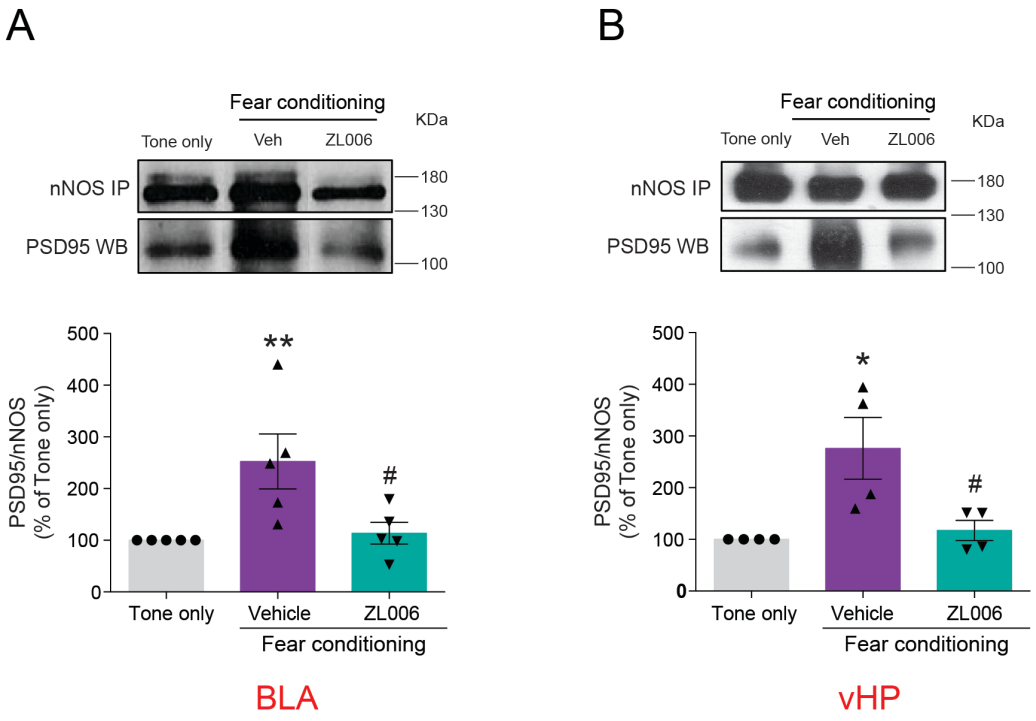

Figure 11. Fear conditioning-induced increases in the PSD95-nNOS complex within the BLA and the vHP can be prevented by pre-treatment of ZL006

Protein lysates from the BLA (A) and the vHP (B) were immunoprecipitated with nNOS antibody and then immunoblotted with PSD95 antibody (tops, representative blots). Levels of PSD95/nNOS ratio were expressed as a percentage of those in 'Tone only' controls (bottoms, n = 5, $F_{2, 12} = 5.895$, $P < 0.05$ for BLA; n = 4, $F_{2, 9} = 7.149$, $P < 0.05$ for vHP). * $P < 0.05$, ** $P < 0.01$ relative to 'Tone only' group; # $P < 0.05$ relative to vehicle group.

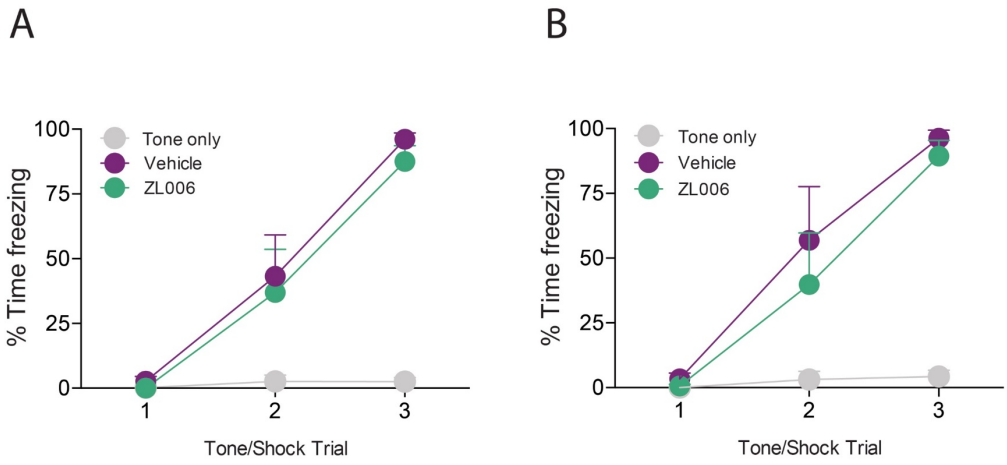

Figure 12. Acquisition of conditioned fear in animals used for Co-IP experiments

Fear conditioned animals in the vehicle and ZL006 group acquired fear normally and no difference was found in the freezing level across trials between groups (for graph A: n = 5; Trial: $F_{2, 16} = 55.77$, $P < 0.0001$; Treatment: $F_{1, 8} = 0.3638$, $P > 0.05$; Treatment x Trial: $F_{2, 16} = 0.0598$, $P > 0.05$; for graph B: n = 4; Trial: $F_{2, 12} = 34.24$, $P < 0.0001$; Treatment: $F_{1, 6} = 0.5950$, $P > 0.05$; Treatment x Trial: $F_{2, 12} = 0.2274$, $P > 0.05$). Animals in 'Tone only' group showed no freezing responses to the tone. After fear conditioning, BLA punches and vHP punches were obtained from animals in (A) and animals in (B) respectively, and were further processed for Co-IP experiments that was described in Figure 9.

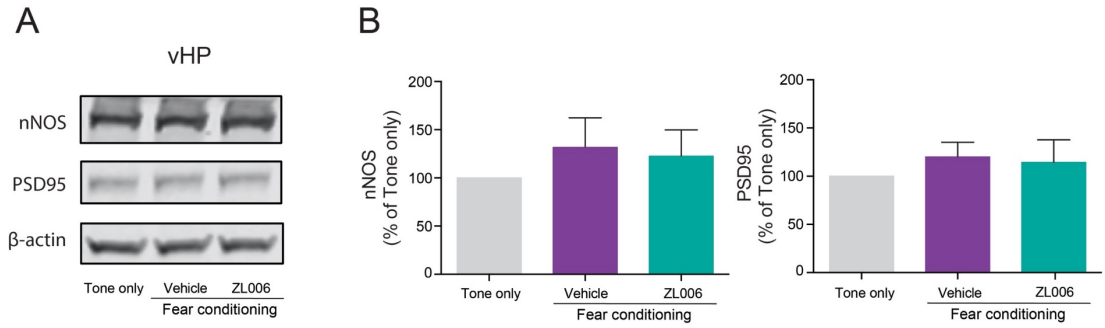

Figure 13. Pre-treatment of ZL006 did not alter the expression levels of PSD95 and nNOS in the vHP

(A) Representative western blots of nNOS and PSD95 in the vHP. β-actin was used for normalization purpose. (B) Quantification graphs of nNOS (left) and PSD95 (Right) levels. The results are presented as a percentage change relative to the 'Tone only' group, which was assigned a value of 100 ($n = 4$, $P > 0.05$ for both graphs).

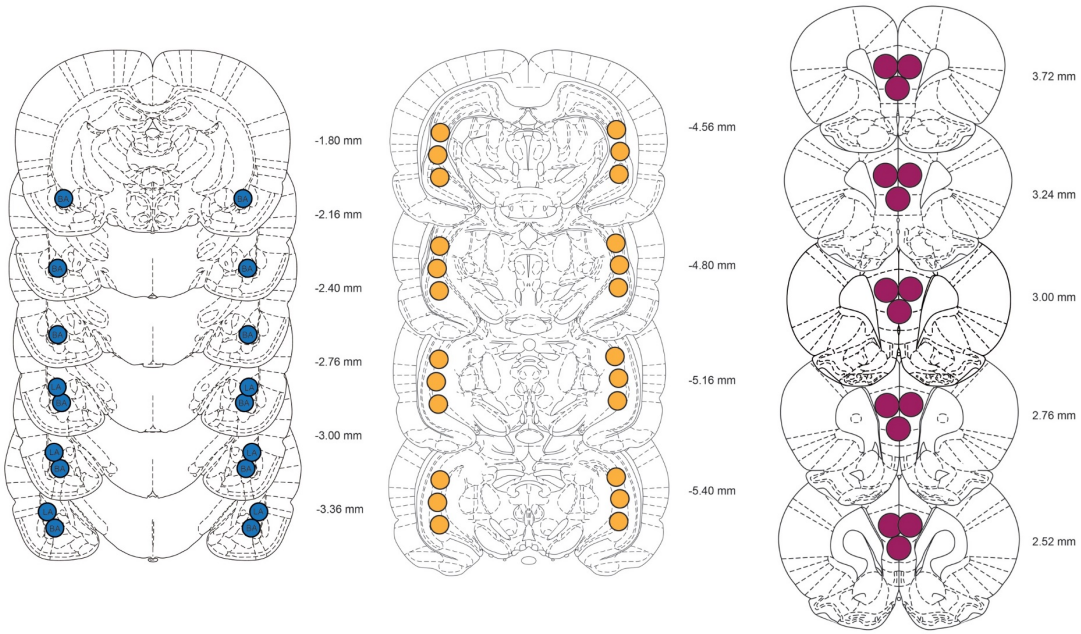

Figure 14. Schematic image depicting the locations of the punches from different regions that used for Co-IP experiments and western blotting

The blue, yellow and red circles delimit the tissue punches from the BLA, the vHP and the mPFC respectively. LA: lateral nucleus of the amygdala; BA: basal nucleus of the amygdala. Drawings are adapted from an atlas (Paxinos and Watson 2005). Numbers indicate the distance from bregma (in mm).

2.3 Discussion

The role of PSD95-nNOS interaction has been investigated in various NMDARs-dependent neurological and neuropsychiatric disorders, such as stroke (Zhou et al 2010), chronic pain (Florio et al 2009, Lee et al 2015), Parkinson's disease (Hu et al 2014) and depression (Doucet et al 2013, Sherwin et al 2017). It has been shown that ischemia can cause a significant increase in the PSD95-nNOS complex within the cortex 24 hours following reperfusion and block of the interaction reduces ischemic injury following stroke in mice (Zhou et al 2010). The PSD95-nNOS complex has also been found in the spinal cord of rats who developed chronic pain by receiving treatments of paclitaxel (Carey et al 2017). Small molecules that disrupt PSD95-nNOS interaction, such as ZL006 has been shown to demonstrate pain-reducing effects in distinct models of NMDARs-mediated hyperalgesia and allodynia (Florio et al 2009, Lee et al 2015). In this chapter, I extended the investigation of PSD95-nNOS interaction to auditory fear conditioning.

Pavlovian fear conditioning is acquired when a previously neutral event is paired with an aversive event. Numerous studies have shown that fear conditioning processes are dependent on the activation of NMDARs and its various downstream molecular changes that result in neuroplasticity within the fear network. One such downstream effect following NMDARs stimulation involves activation of nNOS, which is coupled to the scaffolding protein PSD95. In this chapter, by utilizing Co-IP assay, I first determined the levels of PSD95-

nNOS binding as a function of time following cue-induced fear conditioning within the amygdala, the hippocampus and the mPFC, three critical regions involved in fear regulation. The results showed that amygdalar PSD95-nNOS interaction appeared to begin to increase by 30 minutes following fear conditioning, peaked at 1 h, and remained increased until 6 hours after fear conditioning. Systemic treatment of ZL006 by i.p. injection immediately after fear conditioning blocked the increases in PSD95-nNOS interaction measured at 1 h following conditioning. These results collectively suggested that the increased PSD95-nNOS interaction within the BLA is involved in the subsequent consolidation process following fear conditioning and that ZL006 is an effective tool for the disruption of PSD95-nNOS interaction.

The brain tissues used for the Co-IP experiments were from animals which underwent cue-induced (auditory) fear conditioning, where an association between the phasic CS (a tone) and the US (foot shock) is established by delivering multiple pairings of tone and shock. In the meanwhile, the association also formed between the context (overall environment of the conditioning box) and the US, although the context-US association is usually weaker than the CS-US association (Phillips & LeDoux 1994). It is widely believed that the hippocampus plays a vital role in the contextual processing and conditioning (Maren et al 2013). The results from Co-IP experiments showed that auditory fear conditioning also induced an enhancement of PSD95-nNOS interaction within the vHP in a time-dependent manner that was seen within the amygdala.

The maximal level of PSD95-nNOS interaction in the vHP was observed at 1 h after fear conditioning, and this binding was prevented by treating the animals with ZL006 immediately after training. These results indicated an involvement of hippocampal PSD95-nNOS complex in the formation of the context-US association and disrupting this complex may interfere with contextual fear conditioning and/or contextual fear expression.

In contrast to the enhanced PSD95-nNOS interaction found in the amygdala and the hippocampus, the interaction of these two proteins in the mPFC remained at baseline levels even up to 6 hours following fear conditioning, suggesting that PSD95-nNOS interaction in the mPFC may play a minimal role in the processes immediately following fear conditioning. This is not surprising as considerable literature has demonstrated a critical role for mPFC in the regulation of fear expression and extinction rather than in the regulation of consolidation (see section 1.3.3). A previous study showed that the activation of nNOS signaling in the mPFC plays an important role in the expression of contextual fear conditioning (Moraes Resstel et al 2007). Bilateral administration of nNOS inhibitor into the ventral medial prefrontal cortex (vmPFC) shortly before the testing of contextual fear significantly reduced the freezing responses to the conditioned context (Moraes Resstel et al 2007). This finding indicates that nNOS may undergo activation during the expression of contextual fear. Considering the critical role of PSD95-nNOS interaction in the regulation of

nNOS activation, it is speculated that the interaction between PSD95 and nNOS could be enhanced during contextual fear expression.

Overall, these results provided evidence that the interaction between PSD95 and nNOS within both amygdala and hippocampus are part of the molecular processes subsequent to auditory fear conditioning. These temporally regulated binding of PSD95-nNOS in the BLA and vHP following fear conditioning further suggests the possibility that amygdalar PSD95-nNOS interaction may play a role in the formation of the cue-US association, whereas PSD95-nNOS interaction in the hippocampus may contribute to the fear responses to the context. Although the increases of PSD95-nNOS interaction within the mPFC were not observed following auditory fear conditioning, it does not exclude the possibility that enhanced PSD95-nNOS interaction in the mPFC may be seen in the expression and/or extinction of fear conditioning. The results also showed that the increases in the amygdalar and hipppcampal PSD95-nNOS interaction can be prevented by pretreatment of ZL006, providing us a useful tool to test the effects of disrupting this protein-protein interaction on the conditioned fear responses. The effects of ZL006 at the behavioral level will be discussed in the following chapter.

CHAPTER 3

The Effects of Disruption of PSD95-nNOS Interaction on Conditioned Fear Responses and Other Non-Fear Related Behaviors

3.1 Introduction

The results from Chapter 2 demonstrate that PSD95-nNOS interaction in the BLA and vHP is temporally regulated molecular processes subsequent to fear conditioning (e.g. consolidation). Here, I hypothesized that disruption of this PSD95-nNOS interactions could reduce fear, similar to NMDAR antagonists. Due to the different roles the amygdala and hippocampus play in the fear conditioning, it is further hypothesized that disruption of PSD95-nNOS interaction within the amygdala and the hippocampus would more likely disrupt cued-induced fear and context-induced fear, respectively. To this end, a series of behavioral studies utilizing auditory fear conditioning with tests of cued fear and contextual fear were performed.

Although a number of studies have demonstrated fear-reducing effects of NMDAR antagonists when administered to animals undergoing fear conditioning (Campeau et al 1992, Fendt 2001, Lee et al 2001, Miserendino et al 1990, Rodrigues et al 2001, Zhang et al 2008), NMDAR antagonists also affect many other important physiological processes (Krystal et al 1994, Olney & Wang 1991). Therefore, NMDA antagonists have limitations in their potential as therapeutic avenues for treating fear-related disease, such as post-traumatic

stress disorder (PTSD). Disrupting PSD95-nNOS interaction would allow for a specific inhibition of a sub-pathway downstream of NMDAR activation, i.e., NMDARs-nNOS-NO signaling, therefore circumventing undesirable effects on many other CNS functions mediated by NMDA receptors. To test this idea, a battery of behavioral assays was utilized to establish and compare the behavioral effects profile for NMDAR antagonist MK-801 against the PSD95-nNOS interaction disruptor ZL006.

3.2 Results

Systemic disruption of PSD95-nNOS interaction and inhibition of NMDARs impaired cue-induced conditioned fear memory

To investigate if disruption of PSD95-nNOS interaction could impair conditioned fear memory, rats were treated with i.p. injections of different doses of a small molecule disruptor of PSD95-nNOS interaction, ZL006 (1 mg/kg, 3 mg/kg or 10 mg/kg) immediately following fear conditioning. Their cue-induced fear memories were tested 24 hours after fear conditioning by presenting 10 tones alone without foot shock (Figure 15A). In these tests, a group of animals that received the same training procedure but without shock pairing ('Tone only') were utilized as controls to make sure the fear response was induced by the tone/shock pairing, not by the tone itself. During the fear conditioning training, animals who acquired fear froze during the presentation of auditory cues (tones), whereas 'Tone only' controls do not freeze to the tone. When given systemically immediately after fear training, animals in the vehicle control group showed robust conditioned fear responses (freezing) during the fear expression tests,

whereas rats treated with 10 mg/kg ZL006 displayed significantly reduced freezing responses to the tones (multiple comparisons *post hoc* Fisher's LSD test, $P < 0.05$) (Figure 15A and B). Lower doses of ZL006 (1 mg/kg and 3 mg/kg) failed to impair freezing responses in these tests. As the first 3 to 5 tones in the fear expression test are usually used to measure the consolidation of memory, the averaged freezing time across the first 4 tones was also analyzed among all of the groups. Rats treated with 10 mg/kg ZL006 displayed significantly lower averaged freezing time in the first 4 tones than vehicle treated groups; Rats treated with 1 mg/kg or 3 mg/kg ZL006 displayed comparable averaged freezing time in the first 4 tones than vehicle controls (Figure 15C). ZL007 is a close structural analog of ZL006 but is inactive in disrupting PSD95-nNOS interaction. It was utilized as a negative control in these tests. Animals who treated with 10 mg/kg ZL007 showed comparable freezing responses as vehicle controls (Figure 15B and C). Similar to ZL006 (10 mg/kg), post-training systemic administration of NMDAR antagonist MK-801 (0.1 mg/kg) also significantly impaired cue-induced freezing responses in the fear expression test (Figure 16A, B and C). Collectively, these findings showed that similar to NMDAR antagonist MK-801, disruption of PSD95/nNOS interaction can impair the consolidation of cue-induced conditioned fear memory.

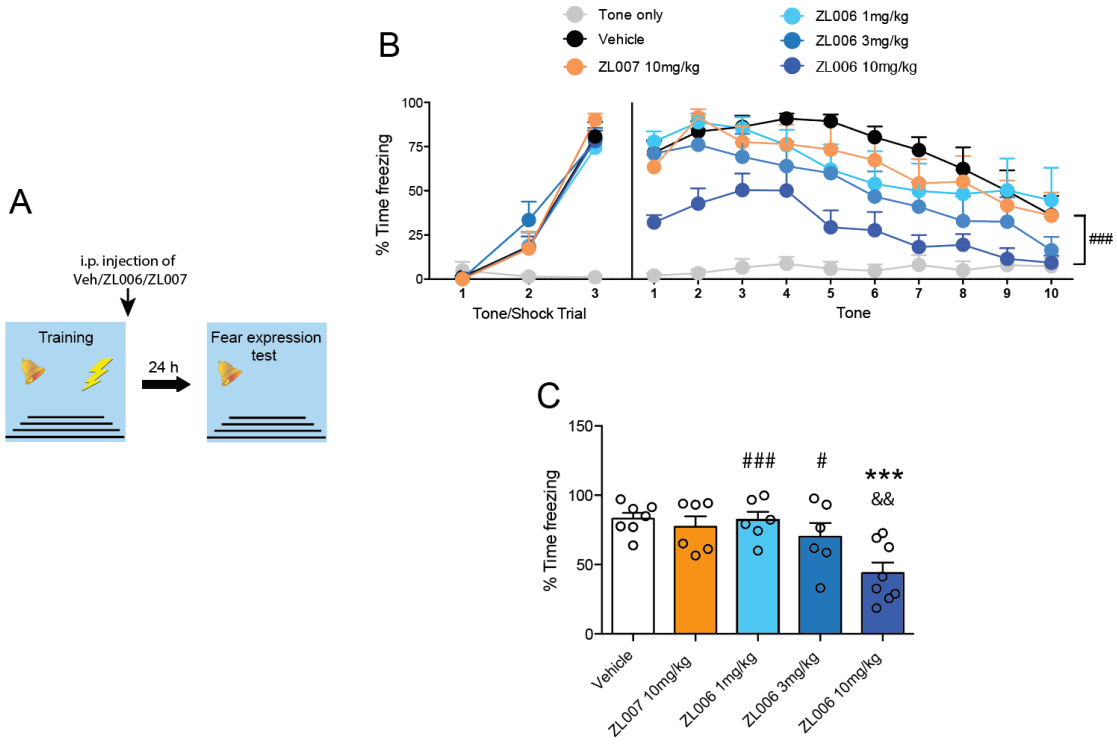

Figure 15. Systemic disruption of PSD95-nNOS interaction by ZL006 impaired the consolidation of cue-induced conditioned fear

(A) Schematic of the behavioral protocol. Immediately following fear conditioning, rats were treated with i.p. injections of indicated treatments. Cue-induced conditioned fear memory was tested 24 hours later. (B) The five groups of animals that were trained with tone/shock pairings displayed normal fear acquisition (Trial: $F_{2, 56} = 299.4$, $P < 0.0001$; Treatment: $F_{4, 28} = 0.3566$, $P > 0.05$; Treatment x Trial: $F_{8, 56} = 1.009$, $P > 0.05$). In the test of fear expression, animals treated with ZL006 at 10 mg/kg (n = 8) showed significantly reduced freezing responses when compared with vehicle controls (n = 7) (*post hoc* Fisher's LSD test: t = 3.77, DF = 28, $P < 0.001$). $^{###}$ $P < 0.001$ ZL006 10 mg/kg Vs. vehicle; Groups with lower doses of ZL006 (1 mg/kg and 3 mg/kg, n = 6) and ZL007 group (n= 6) showed comparable freezing responses during fear expression test compared to the vehicle group ($P > 0.05$). Rats in the 'Tone only' group (n = 7) did not freeze to the tones during neither fear training nor fear expression test. (C) Averaged freezing time across the first 4 tones of the expression test in different groups. ($F_{4, 28} = 5.764$, $P < 0.01$). *** $P < 0.001$ relative to vehicle group; $^{&&}$ $P < 0.01$ relative to ZL007 group; $^{#}$ $P < 0.05$, $^{##}$ $P < 0.01$ relative to ZL006 10 mg/kg group.

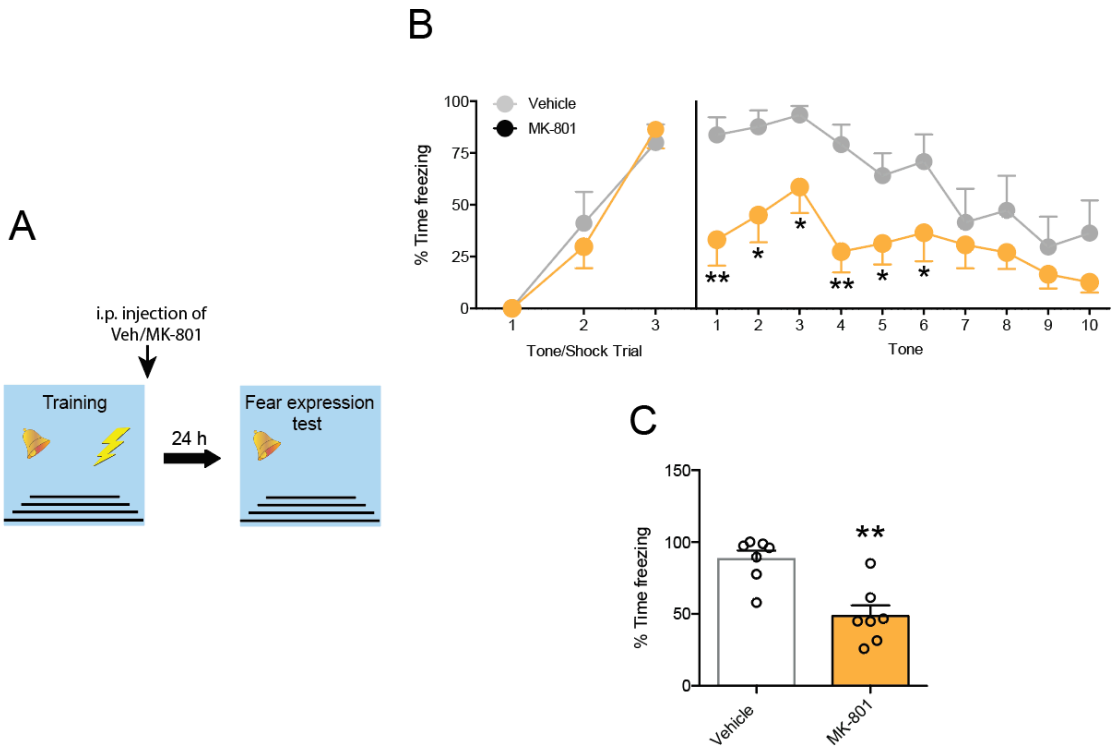

Figure 16. Systemic inhibition of NMDARs by MK-801 impaired consolidation of cue-induced conditioned fear

(A) Schematic of the behavioral protocol. Rats were given i.p. injections of either vehicle (N=7) or 0.1 mg/kg MK-801 (N=7) immediately after fear conditioning. Cue-induced conditioned fear memory was tested 24 hours later. (B) Both groups of animals acquired fear normally (Trial: $F_{2, 24} = 59.62$, $P < 0.0001$; Treatment: $F_{1, 12} = 0.03817$, $P > 0.05$; Treatment x Trial: $F_{2, 24} = 0.6815$, $P > 0.05$). However, MK-801 treated animals displayed significantly reduced freezing responses in cue-induced fear expression test (Treatment: $F_{1, 12} = 8.550$, $P < 0.05$; Trial: $F_{9, 108} = 9.593$, $P < 0.0001$; Treatment x Trial: $F_{9, 108} = 0.9450$, $P > 0.05$). (C) Averaged freezing time across the first 4 tones of the expression test in vehicle and MK-801 treated groups ($t = 4.163$, $DF = 12$, $P < 0.05$). *$P < 0.05$, ** $P < 0.01$ relative to vehicle group.

Inhibiting PSD95-nNOS interaction directly in the amygdala also reduces cue-induced conditioned fear memory

Next, it was tested if local injections of PSD95-nNOS interaction disruptor ZL006 directly into the BLA could impair cue-induced memory formation of conditioned fear. Seven days before fear conditioning, rats were implanted with guide cannulas into the BLA. Intra-BLA infusions of vehicle or ZL006 at 10 μM were applied immediately following fear conditioning and the conditioned fear memory for each rat was tested 24 hours after conditioning (Figure 17A). During fear conditioning, all groups of animals showed comparable freezing responses to the tone (Figure 17B). However, in the fear expression test, animals that received intra-BLA infusions of ZL006 displayed significantly decreased freezing responses compared with vehicle controls (Figure 17B). The averaged freezing time across the first 4 tones in the expression test was also reduced in ZL006 treated animals (Figure 17C). Thus, similar to systemic disruption of PSD95/nNOS interaction, intra-BLA treatment of ZL006 immediately following fear conditioning also impairs the consolidation of cue-induced conditioned fear memory. In 3 additional rats, the injection sites were outside the BLA boundaries (anterior to the BLA); ZL006 injections at these sites had no effects (data not shown) and these animals were removed from data analysis.

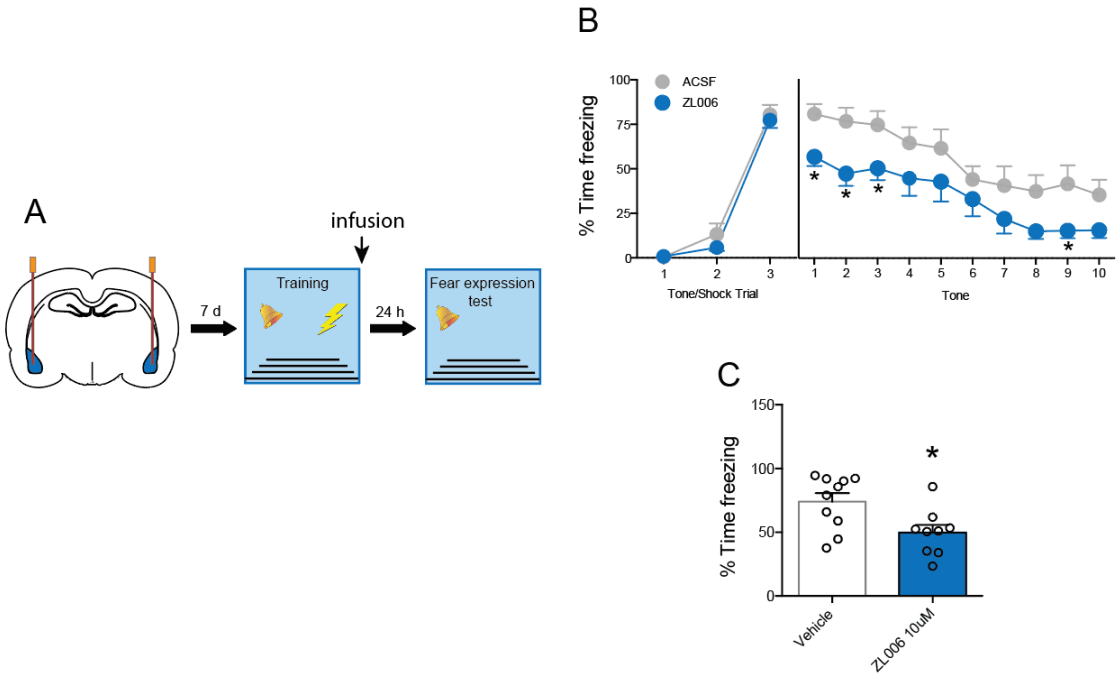

Figure 17. Intra-BLA disruption of PSD95-nNOS interaction by ZL006 also impaired the consolidation of cue-induced conditioned fear

(A) Schematic of the behavioral protocol. Immediately after fear conditioning, rats were administrated with intra-BLA infusion of ACSF (n = 10) or 10 μM ZL006 (n = 9) and the retention of cue-induced fear memory was tested 24 hours later. Pre-assigned vehicle and ZL006 groups of animals acquired cue-induced fear normally and equivalently (Trial: $F_{2, 34} = 308.8$, $P < 0.0001$; Treatment: $F_{1, 17} = 0.6920$, $P > 0.05$; Treatment x Trial: $F_{2, 34} = 0.6287$, $P > 0.05$) (B, Left). Animals with ZL006 treatment displayed significantly decreased freezing responses in the fear expression test (Treatment: $F_{1, 17} = 4.974$, $P < 0.05$; Trial: $F_{9, 153} = 23.27$, $P < 0.0001$; Treatment x Trial: $F_{9, 153} = 0.5252$, $P > 0.05$) (B, Right). (C) Averaged freezing time across the first 4 tones of the expression test in vehicle and ZL006 treated groups (unpaired *t* test, t = 2.687, DF = 17, $P < 0.05$). * $P < 0.05$ relative to vehicle group.

Inhibiting PSD95-nNOS interaction directly in the vHP has no effects on cue-induced conditioned fear, but leads to an impaired context-induced conditioned fear

In this experiment, a modified paradigm was utilized in order to test both cue- and context- induced conditioned fear. Animals were placed in Box A to receive fear conditioning training. 24 hours later, context-induced conditioned fear was tested in the same box for a total of 5 minutes where animals were only exposed to the context without any tone presentation. Cue-induced conditioned fear was tested 5 minutes after contextual fear test in another box (Box B) where animals received multiple tone presentations while being exposed to a new context (Figure 18A). To investigate the effects of intra-vHP disruption of PSD95-nNOS binding on both cue- and context- induced conditioned fear, I implanted rats with guide cannulas into the vHP and performed fear conditioning seven days after recovery from the surgery of implantation. Intra-vHP infusions of vehicle or 10 μM ZL006 (either 250 nl/side or 500 nl/side) were given immediately following fear conditioning. During conditioning, all groups of animals acquired fear normally and equivalently (Figure 18B). In the contextual fear test that was performed 24 hours after conditioning, animals receiving a higher volume (500 nl/side) of intra-vHP infusion of 10 μM ZL006 displayed significantly reduced freezing responses to the context over time (multiple comparisons *post hoc* Fisher's LSD test, $P < 0.05$); The total amount of freezing time during this test in the 500 nl/side group was significantly less than the other two groups (Figure 18C). The lower volume (250 nl/side) group failed to show reduced fear

responses in this test. In contrast, in the cue-induced fear test, animals received intra-vHP infusion of ZL006 (250 nl or 500 nl per side) displayed comparable freezing responses as animals treated with vehicle ($P > 0.05$) (Figure 18D *left*) and the averaged freezing time across the first 4 tones in all these groups are comparable (Figure 18D *right*). Taken together, these results indicate that PSD95-nNOS complex in different brain regions mediate different aspects of conditioned fear, with amygdalar PSD95-nNOS complex mediating the consolidation of cue-induced fear and hippocampal PSD95-nNOS complex mediating the consolidation of context-induced fear.

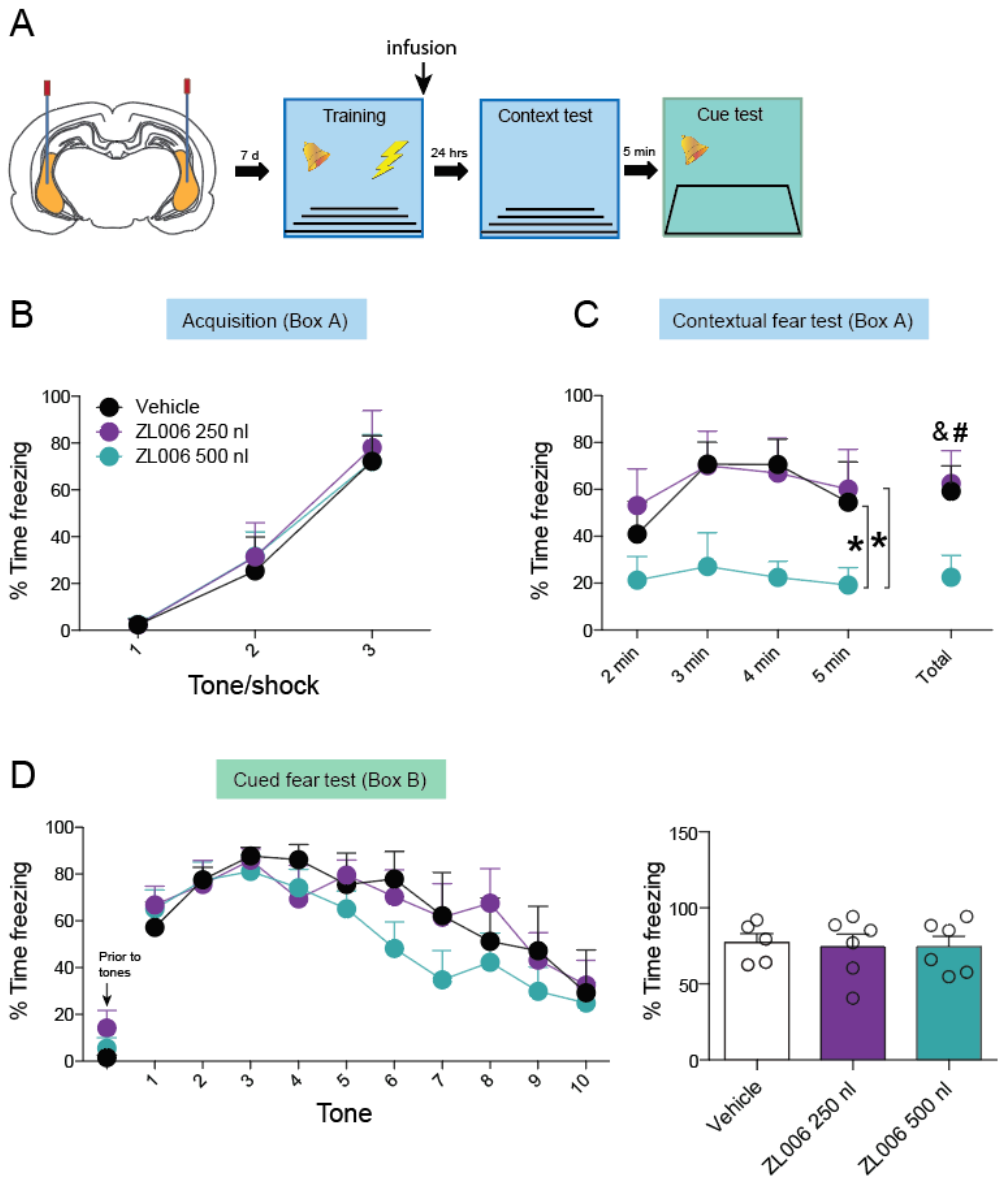

Figure 18. Intra-vHP disruption of PSD95-nNOS interaction by ZL006 did not affect cue-induced fear memory, but significantly impaired context-induced fear memory

(A) Schematic of the behavioral protocol. Rats were given intra-vHP infusion of ACSF (n = 5, 250 or 500 nl/side), ZL006 250 nl/side (n = 6) or ZL006 500 nl/side (n = 6) immediately after fear conditioning. The retention of context-induced fear memory was tested 24 hours later in the same box where fear conditioning was performed. The cue-induced fear memory was tested 5 min after the contextual retention test in a different box. (B) All of the animals acquired cue-induced fear normally and equivalently (Trial: $F_{2, 28} = 46.55$, $P < 0.0001$; Treatment: $F_{2, 14} = 0.05826$, $P > 0.05$; Treatment x Trial: $F_{4, 28} = 0.0766$, $P > 0.05$). (C) In contextual fear test, animals received 500 nl per side of ZL006 showed impaired freezing

responses compared with animals treated with vehicle or 250 per side of ZL006 (Treatment: $F_{2, 14} = 3.751$, $P < 0.05$, Trial: $F_{3, 42} = 3.892$, $P < 0.05$; Treatment x Trial: $F_{6, 42} = 0.7694$, $P > 0.05$). The total amount of freezing time during the test in the 500 nl group was significantly less than the other two groups. * $P < 0.05$; & $P < 0.05$ vehicle Vs 500 nl; # $P < 0.05$ 250 nl Vs 500 nl (D) In the cue-induced fear test, animals with ZL006 treatments (250 nl or 500 nl per side) displayed comparable freezing responses as animals treated with vehicle (Treatment: $F_{2, 14} = 0.5126$, $P > 0.05$; Trial: $F_{9, 126} = 16.93$, $P < 0.0001$; Treatment x Trial: $F_{18, 126} = 1.039$, $P > 0.05$) (D *left*); The averaged freezing time across the first 4 tones in all groups were also comparable ($F_{2, 14} = 0.044$, $P > 0.05$) (D *right*).

Cannula placements within the BLA and vHP

A schematic graph in Figure 19 depicts the cannula tips within the BLA and the vHP. Cannula tips were observed throughout the BLA or vHP at various rostro-caudal levels. Only rats with cannula tips within the boundaries of the BLA or the vHP were included in the data analysis. In the intra-BLA infusion experiment, a total of 22 rats were used, of which three (one from vehicle group, two from ZL006 group) were with the injection sites anterior to the BLA and were removed from the data analysis. In the intra-vHP experiment, a total of 18 rats were used, of which one vehicle rat was removed from the data analysis due to improper injection site.

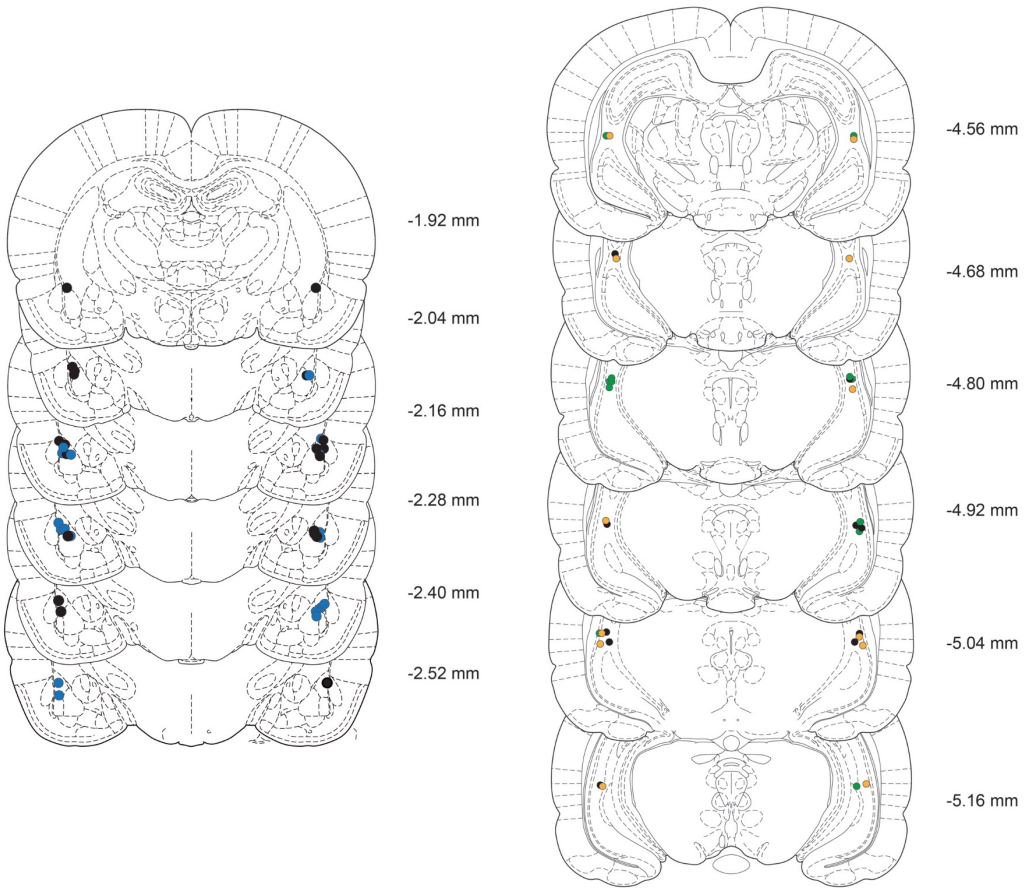

Figure 19. Schematic image depicting the cannula placements within the BLA and the vHP

Left: The symbols represent the injection sites in vehicle (black circle, N = 10) and ZL006 (blue circle, N = 9) treated animals that were included in the intra-BLA infusion experiment. Right: The symbols represent the injection sites in vehicle (black circle, N = 5), ZL006 250 nl/side (green circles, N = 6) and ZL006 500 nl/side (orange circles, N = 6) treated animals that were included in the intra-vHP infusion experiment. Sections are based on the atlas of Paxinos and Watson (2005). Numbers indicate the distance from bregma (in mm).

The effects of disruption of PSD95-nNOS interaction on other non-fear related behaviors

To determine if the effects of disruption of PSD95-nNOS interaction were specific to fear-related behaviors or would be generalized to a boarder range of behaviors, the following series of behavioral tests were performed to examine the effects of systemic ZL006 on motor activity, social activity, novel object recognition memory and spatial memory. The effects of MK-801 on these behaviors were also tested as a comparison. These tests were conducted utilizing ZL006 (10 mg/kg i.p.) and MK-801 (0.1 mg/kg i.p), both at the doses that were previously found effective in reducing fear memory. In the open field (OF) test, the total distances traveled in the open-field arena after ZL006 injections were comparable to the vehicle group, whereas animals treated with MK-801 traveled further than the other two groups (Figure 20B). In the social interaction (SI) test, while ZL006 treated animals displayed comparable interaction activity with the vehicle controls, MK-801 treated animals showed significantly less interaction time (Figure 20C). In the novel object recognition test (NORT), analysis on the discrimination index demonstrated that ZL006 treatment had no effects on the discrimination behavior when compared with the vehicle controls, whereas rats treated with MK-801 displayed impaired discrimination as they were unable to discriminate between the familiar and the novel object (Figure 21B). Y-maze with a two-trial test was utilized to test the spatial memory. It was found that both vehicle controls and ZL006 treated animals displayed intact spatial recognition memories: these animals showed a higher frequency of visits

(Vehicle: $F_{2, 15} = 4.892$, $P < 0.05$; ZL006: $F_{2, 15} = 6.628$, $P < 0.01$) (Figure 22B) and longer durations (Vehicle: $F_{2, 15} = 10.56$, $P < 0.01$; ZL006: $F_{2, 15} = 7.965$, $P < 0.01$) (Figure 22C) within the novel arm than in the other arms. In contrast, the rats treated with MK-801 showed impaired spatial recognition memories: they visited the novel arm less than the other arms ($F_{2, 15} = 3.896$, $P < 0.05$) (Figure 22B) and spent same amount of time in all of the three arms ($F_{2, 15} = 0.3418$, $P > 0.05$) (Figure 22C).

In summary, these results suggested that disrupting PSD95-nNOS binding by the small molecule ZL006 does not elicit the acute effects seen with NMDA receptor antagonists MK-801, such as hyper-locomotion, impaired social interaction, and disrupted memories in object recognition and spatial recognition.

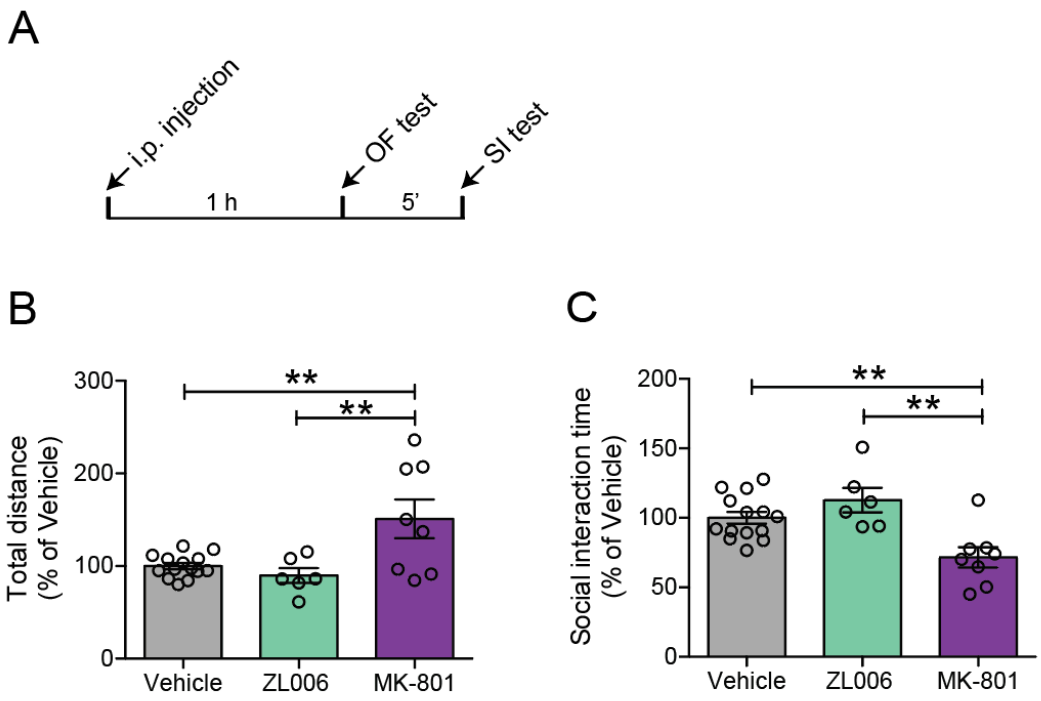

Figure 20. The effects of ZL006 and MK-801 on locomotion and social activity

(A) Schematic of the behavioral protocol. Animals received i.p. injections of vehicle or treatments 1 h prior to the OF Test. SI was performed 5 min after the OF test. (B) In the OF test, MK-801 treated animals displayed increased locomotor activity ($F_{2, 25} = 7.562$, $P < 0.01$) whereas ZL006 treatment has no effects on locomotor activity ($P > 0.05$). (C) In the SI test, MK-801 treated animals showed decreased social activity ($F_{2, 25} = 9.548$, $P < 0.001$) whereas ZL006 treatment has no effects on social activity ($P > 0.05$). n = 14, 6 and 8 for control, ZL006 and MK-801 respectively. ** $P < 0.01$; OF, open field; SI, social interaction

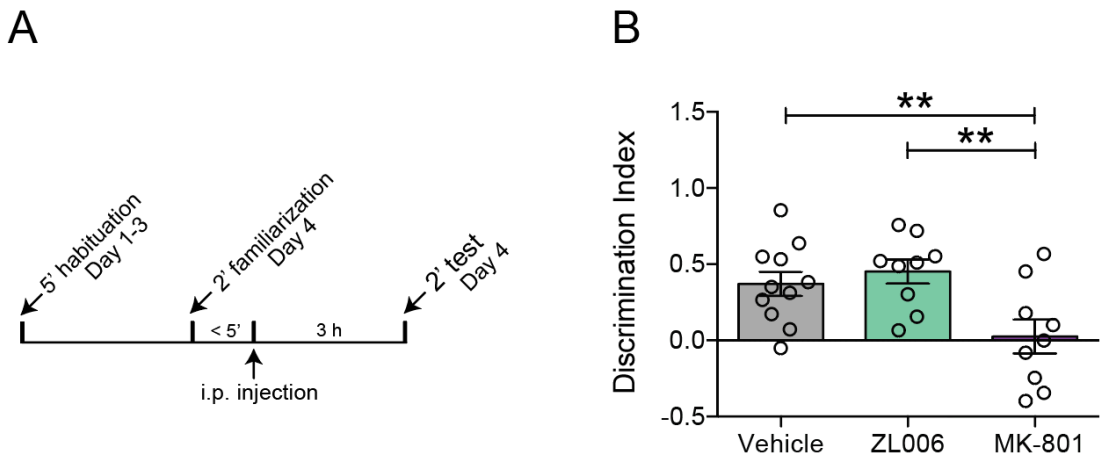

Figure 21. The effects of ZL006 and MK-801 on novel recognition task

(A) Schematic of the behavioral protocol. Prior to NORT, animals were allowed to explore the apparatus for 5 min per day for 3 consecutive days. On day 4, animals received i.p. injections of vehicle or treatments immediately (< 5 min) after the familiarization trial and the test trial was conducted after a 3 h ITI. (B) Discrimination index for different groups of animals during the test trial demonstrated that MK-801 treated rats showed deficits in the discrimination behavioral (n = 11, 9 and 9 for control, ZL006 and MK-801 respectively; $F_{2, 26}$ = 5.993, P < 0.01). However, the recognition memory in the ZL006 treated rats was intact. ** P < 0.01; ITI, inter-trial interval; NORT, novel object recognition

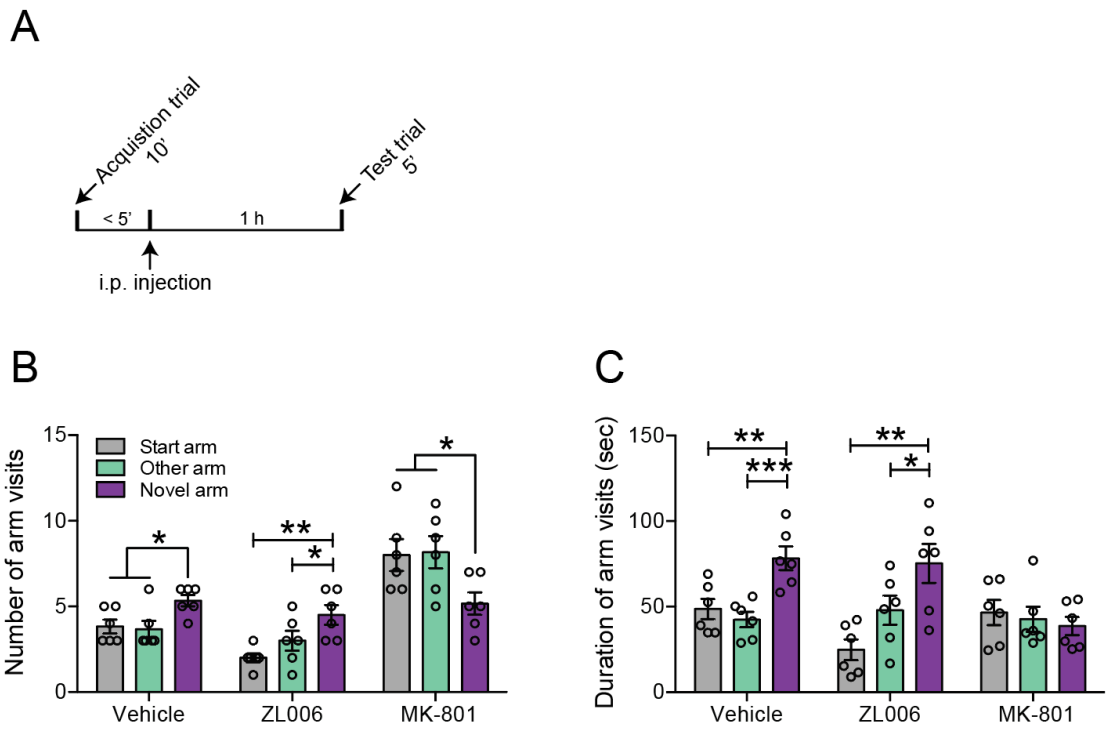

Figure 22. The effects of ZL006 and MK-801 on Y-maze task

(A) Schematic of the behavioral protocol. In the Y-maze test, animals received i.p. injections of vehicle or treatments immediately (< 5 min) after the acquisition trial and the test trial was conducted after a 1 h ITI. n = 6 for each group. (B) Both vehicle and ZL006 treated animals visited novel arm more than the other two arms ($P < 0.05$); however, animals with MK-801 treatment visited novel arm less that the other arms ($P < 0.05$). (C) Both vehicle and ZL006 treated rats spent more time in the novel arm than the other arms ($P < 0.01$); no arm difference was found in the rats treated with MK-801 ($P > 0.05$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$; ITI, inter-trial interval

3.3 Discussion

The major findings from this current study are that disrupting PSD95-nNOS interaction by ZL006 within the amygdala and within the hippocampus, respectively, impaired the consolidation of cue-induced and context-induced conditioned fear. This is consistent with a number of previous studies with pharmacological inhibition of nNOS and gene deletion of nNOS (Itzhak et al 2012, Kelley et al 2009, Pavesi et al 2013, Schafe et al 2005). Similar to ZL006, impairment of conditioned fear was also observed with NMDA receptor antagonist MK-801. However, unlike MK-801, ZL006 does not appear to affect locomotion, social activity and other short-term memory performances. Collectively, these results suggest that PSD95-nNOS interaction appears to be a selective downstream molecular step in the regulation of conditioned fear consolidation and that disruption of PSD95-nNOS interaction could be a more targeted approach to reduce fear without affecting other NMDAR-dependent signaling pathways.

The important role of NMDARs and nNOS activity in fear memory formation have been validated in a number of studies where animals received applications of either NMDARs antagonists or NOS inhibitors prior to conditioning and displayed impaired conditioned fear (Campeau et al 1992, Miserendino et al 1990, Rodrigues et al 2001, Schafe et al 2005, Zhang et al 2008). However, these studies did not exclude the possible explanation of the drug effects that these drugs may alter shock-related pain sensitivity and thus affecting the

acquisition of fear. In the present study, this issue was circumvented by applying the treatment shortly after training, and found that both MK-801 and ZL006 still attenuated fear memory when tested 24 h after conditioning.

Results from the current study showed that ZL006, unlike MK-801, selectively disrupted conditioned fear memory without affecting many other CNS functions, such as locomotion and social activity. It was found that systemic administration of MK-801 at the dose effective in reducing fear memory (0.1 mg/kg) caused hyperactivity in rats in an OF test. Reduced locomotor activity was reported in animals treated with 7-Ni, a non-selective inhibitor of NOS (Harkin et al 2003, Maren 1998). In a SI test, animals received treatments of MK-801 displayed impaired social activities, which was in agreement with a previous study suggesting a defective social interaction by MK-801 in a dose-dependent manner (Rung et al 2005). 7-Ni has been reported to have anxiolytic effect in the SI test (Volke et al 1997), our experiment failed to observe any anxiolytic effect of ZL006 in the SI test.

Due to a number of studies demonstrating learning deficits caused by NMDAR antagonists (Bannerman et al 1995, Butelman 1989, de Lima et al 2005, Shapiro & Caramanos 1990) and non-selective NOS inhibitors (Akar et al 2009, Hölscher et al 1996, Mutlu et al 2011, Zou et al 1998) in several hippocampal memory tests, I investigated whether ZL006 affects hippocampus-dependent memories by utilizing NORT and Y-maze test. In consistency with previous

research (Boultadakis & Pitsikas 2010, Cunha et al 2008, de Lima et al 2005), it was found that post-training i.p. injection of MK-801 disrupted animals' performance in both of the tests. However, ZL006 application did not cause deficits in these tests. Overall, these results are consistent with the previous findings that systemic ZL006 does not affect locomotor function (Lee et al 2015, Smith et al 2016), spatial memory (Zhou et al 2010) or source memory (Smith et al 2016) in rodents.

In agreement with the effects of ZL006 action on NORT and Y-maze test, a previous study demonstrated that performances in NORT and Morris water maze test remained intact after administering TRIM, a NOS inhibitor preferentially inhibit nNOS over eNOS (Mutlu et al 2011). However, those performances became disrupted when both nNOS and eNOS were inhibited with 7-Ni, a non-selective NOS inhibitor (Mutlu et al 2011). Together, these findings indicate that recognition memory and spatial memory engage both eNOS and nNOS activities, and eNOS activation may be compensating for the disruption of nNOS activity. Therefore, tests with NORT and Y-maze are insensitive to ZL006 treatment. In support for this view, an electrophysiological study showed that LTP in the CA1 region of the hippocampus from nNOS knockout mice and eNOS knockout mice were intact, but LTP was significantly impaired in nNOS and eNOS double knockout mice (Son et al 1996).

In the present study, I showed that disruption of PSD95-nNOS interaction by ZL006 impaired hippocampus-dependent context-induced fear conditioning. However, recognition memory and spatial memory, other two forms of hippocampus-dependent memory were not significantly affected by ZL006 as measured by NORT and Y-maze behaviors. These findings suggested that fear-related learning and non-fear related learning, although both are believed to be dependent on the hippocampus, are differentially sensitive to inhibition of PSD95-nNOS interaction.

Several factors might account for the differential sensitivities. Fear conditioning is a task based on a stressful stimulus (foot shock) and requires high cognitive function. Animals subjected to contextual fear conditioning usually display high emotionality and stress responses which include the release of corticosterone (Kelley et al 2009) and norepinephrine (Feenstra et al 1999), two primary stress hormones. The altered emotional states may interfere with cognitive function in the stressful fear conditioning test but not in the NORT or Y-maze tests which induce minimal stress. This view is supported by a previous study showing that nNOS knock out mice displayed impaired spatial performance in a stressful water maze, but display no deficits in the less stressful T-maze test (Weitzdoerfer et al 2004). Although the mechanism through which nNOS inhibition affects cognitive functions only under stressful conditions is not clear. The authors in this study suggested that alternations in the serotonergic system might be involved in the mechanisms, considering the intensive interaction of

nNOS signaling pathway with serotonergic pathway (Chanrion et al 2007, Chiavegatto et al 2001, Chiavegatto & Nelson 2003, Kaehler et al 1999).

In summary, the results show that disrupting PSD95-nNOS interaction with the small molecule ZL006 attenuates cue-induced fear and context-induced fear when given locally into the amygdala and the hippocampus, respectively. Unlike NMDAR antagonist MK-801, systemic ZL006 is devoid of effects on locomotor function, social activity, and acute effects on non-fear related memories, indicating that disrupting PSD95/nNOS interaction represents a novel therapeutic approach for reducing conditioned fear without eliciting adverse effects.

CHAPTER 4

The Synaptic Mechanism in the Amygdala Associated with Conditioned Fear and The Effects of Disruption of PSD95-nNOS Interaction

4.1 Introduction

The results from Chapter 3 have demonstrated that disruption of PSD95-nNOS interaction shortly after fear conditioning with either systemic treatment of ZL006 or intra-amygdala infusion of ZL006 could impair the consolidation of cue-induced fear memory. In this chapter, experiments were designed to investigate the mechanism in the amygdala mediating the effects of disruption of PSD95-nNOS interaction on cue-induced fear. LTP is an enduring form of synaptic plasticity that mediates learning and memory in mammals (Maren 1999a). LTP in the amygdala has been thought to underlie the induction and expression of cue-induced fear memory (Rogan et al 1997). To investigate the mechanism of ZL006 action in impairing the consolidation of cue-induced fear memory, it was examined whether local application of ZL006 would alter long-term synaptic plasticity in the BLA slice preparations. To this end, high frequency stimulation (HFS) protocol and whole-cell patch clamp technique were employed to induce and record LTP in the BLA projection neurons; and the amplitude of EPSP in the ZL006 treated neurons were compared to neurons treated with vehicle or ZL007, an inactive isomer of ZL006.

4.2 Results

Disrupting PSD95-nNOS interaction by ZL006 prevents long-term potentiation of BLA neurons

At resting conditions, perfusion of BLA slices with 10 μM ZL006 had no significant effects on evoked excitatory post-synaptic potentials (eEPSPs) or input resistance during recordings of BLA neurons. After establishing basal responses to positive current injection, it was observed that eEPSPs of BLA neurons in both vehicle and 10 μM ZL007 control conditions underwent a sustained potentiation upon HFS (183.4 ± 7.8 and 185.6 ± 8.4 percent of baseline at 1 h for vehicle group and ZL007 group, respectively). However, eEPSPs of ZL006 treated BLA neurons gradually returned to baseline levels (98.0 ± 3.9 percent of baseline at 1 h) following short-term potentiation, and were statistically different from the eEPSPs of control neurons at all time points $t > 16$ min (Figure 23B and C). The negative current was injected once per minute to test whether changes to eEPSP amplitude resulted from changes to membrane resistance and no differences were detected between conditions or over time (Figure 23D). Collectively, these findings indicated that disrupting PSD95/nNOS interaction by ZL006 impairs LTP, a cellular signature of synaptic plasticity in BLA neurons.

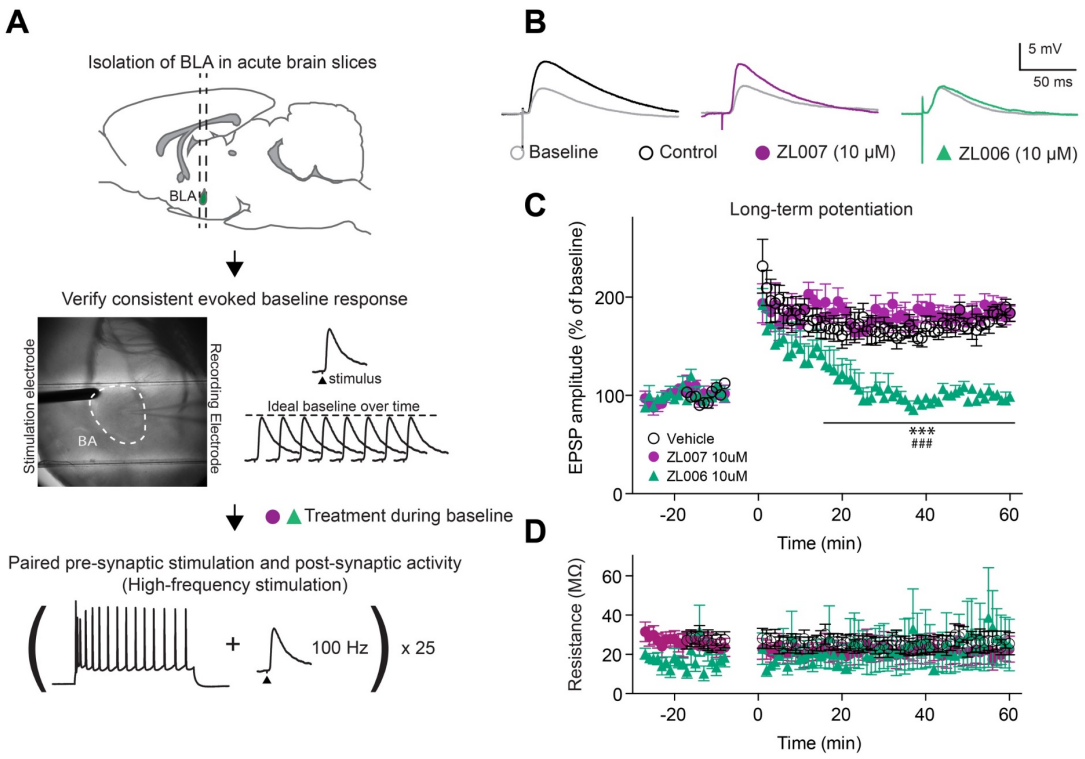

Figure 23. Disrupting PSD95/nNOS binding by ZL006 prevents HFS induced LTP of BLA neurons

(A) Schematic graphs show the experimental protocol of LTP induction (B) Representative traces show EPSP responses before and 1 h after HFS under different conditions (C) LTP produced in the ZL006 treated cells following HFS was significantly impaired compared with vehicle and ZL007 treated cells (Treatment: $F_{2, 1243} = 434.0$, $p < 0.0001$). Multiple comparisons post hoc analysis revealed that EPSP responses in ZL006 treated cells were different from vehicle or ZL007 treated cells at all time point t > 16 minutes. *** P < 0.001 ZL006 Vs. ZL007, ### P < 0.001 ZL006 Vs. Control (n = 10, 6 and 6 for vehicle, ZL007 and ZL006 respectively). (D) All groups of cells displayed similar membrane resistance over time (n = 8, 6 and 5 for vehicle, ZL007 and ZL006 respectively). Arrow indicates initiation of high frequency stimulation (HFS). (Data courtesy of Erik Dustrude).

4.3 Discussion

In the electrophysiological experiments, the effects of disruption of PSD95-nNOS interaction via ZL006 on amygdala LTP was examined using a high frequency stimulation protocol. The findings showed that bath application of ZL006 effectively impaired LTP in the BLA neurons, without causing effects on baseline EPSPs alone. However, ZL007, an inactive isomer of ZL006 had no such effects on amygdala LTP. These results suggested an involvement of PSD95-nNOS interaction in the amygdala LTP and that blocking PSD95-nNOS interaction and resultant NO signaling leads to impaired LTP in the amygdala. This is consistent with a previous study which showed that bath application of either the NOS inhibitor 7-Ni or the NO scavenger c-PTIO effectively impaired LTP in the BLA neurons (Schafe et al 2005).

It was found that the impairing effect of ZL006 on amygdala LTP was not shown until minutes after HFS, as the eEPSPs of BLA neurons treated with ZL006 underwent a short-term potentiation (lasts for 16 minutes after HFS) before gradually decaying to the baseline level. This observation indicated that unlike the sensitivity of the later maintenance phase of LTP to ZL006 treatment, the initial induction phase of LTP was not affected by ZL006 treatment at this dose. In light of this delayed block of LTP by ZL006, it would be of interest to test the potential mechanisms of the early versus later stages of LTP induced by HFS, and elucidate the role of PSD95-nNOS interactions in the two stages of fear: acquisition versus consolidation. Indeed, a previous study has shown that

animals with intra-amygdala infusion of the NOS inhibitor 7-Ni or the NO scavenger c-PTIO prior to training displayed no deficits in the acquisition of conditioned fear, but showed impaired consolidation of conditioned fear (Schafe et al 2005).

It has been generally recognized that postsynaptic changes in AMPA receptor function contribute to the expression of LTP (Sweatt 1999). Considering the compelling evidences showing the important role of NO signaling in the modulation of AMPA receptor function (Huang et al 2005, Selvakumar et al 2009, Selvakumar et al 2013, Serulle et al 2007, Wang et al 2005), it was speculated that ZL006 may impair amygdala LTP through a mechanism altering surface AMPA receptor availability and function. Additional electrophysiological studies conducted in our lab have found that fear-conditioning induced enhanced AMPAR-mediated excitatory post-synaptic currents (EPSCs) can be blocked by ZL006 treatment (unpublished data), indicating that ZL006 does affect AMPA receptor function. Further studies are needed to investigate the detailed mechanisms by which ZL006 affect AMPA receptor function. These mechanisms include: modulating AMPA receptor trafficking to the synaptic sites, regulating membrane expression level of AMPA receptors and altering AMPA receptors properties (further discussed in section 5.3.1).

To summarize, the electrophysiological studies using amygdala slices and HFS protocol showed that disruption of PSD95-nNOS interaction via ZL006

blocked LTP in the BLA neurons. The mechanisms underlying ZL006 effect on amygdala LTP may be associated with altered AMPA receptor function. Additional experiments are needed to investigate the mechanisms further.

CHAPTER 5

Summary, Discussion, and Perspectives

5.1 Summary

The studies in this dissertation have demonstrated a critical role of PSD95-nNOS interaction within key limbic regions in the regulation of conditioned fear. First, I determined the spatial and temporal dynamics of PSD95-nNOS interaction within the amygdala, the hippocampus, and the mPFC, three key regions involved in conditioned fear (Figure 7). The data showed that PSD95-nNOS interaction within the BLA and the vHP began to increase by 30 min following auditory fear conditioning, peaked at 1 h, and remained increased until 6 h after conditioning, suggesting involvement of amygdalar and hippocampal PSD95-nNOS interaction in the subsequent consolidation process following fear conditioning. Furthermore, the robust increases in PSD95-nNOS interaction in both regions could be prevented by pretreatment of ZL006, a small molecule disruptor of PSD95-nNOS interaction. Unlike the BLA and the vHP, PSD95-nNOS interaction within the mPFC remained at the basal level after fear conditioning. Second, I determined that systemic and intra-BLA disruption of PSD95-nNOS interaction by ZL006 impaired the consolidation of cue-induced fear (Figure 15 and 17). In contrast, disruption of PSD95-nNOS interaction within the hippocampus did not affect the consolidation of cue-induced fear, but significantly impair the consolidation of context-induced fear (Figure 18). At the

cellular level, disruption of amygdalar PSD95-nNOS interaction with ZL006 was found to impair LTP in the BLA neurons (Figure 23).

Similar to ZL006, impairment in conditioned fear was also observed with systemic application of NMDA receptor antagonist MK-801 (Figure 16), which is in agreement with previous studies (Fendt 2001, Lee et al 2001). However, unlike the NMDA receptor antagonist, ZL006 selectively disrupts conditioned fear without affecting many other CNS functions, such as locomotor function (Figure 20B), social activity (Figure 20C) and cognitive functions in NORT (Figure 21) and Y-maze tests (Figure 22), indicating that disrupting PSD95-nNOS interaction may represent a novel therapeutic approach for reducing conditioned fear without eliciting adverse effects.

Collectively, the current discoveries presented in this dissertation demonstrated that PSD95-nNOS interaction within the fear network appears to be a critical molecular step in regulating synaptic plasticity and the consolidation of conditioned fear. Disruption of this protein-protein interaction attenuates fear consolidation and has no effects on motor function, social activity and cognitive functions. Therefore, PSD95-nNOS interaction holds promise as a novel therapeutic target for fear-motivated disorders with minimal side effects. These studies have made contributions to the fields of NMDA-NO signaling and fear memory research and have opened up several lines of research that will be discussed in section 5.3.

5.2 Discussion

In this section, the role of PSD95-nNOS interaction in cued and contextual fear memory is further discussed. The translational impact of this study is also discussed.

5.2.1 Amygdalar PSD95-nNOS interaction and cue-induced fear memory

Experiments in this present study have shown that the systemic disruption of PSD95-nNOS interaction by ZL006 shortly after fear conditioning produced an impairing effect on the long-term memory (LTM) of cue-induced fear when assessed the following day (~24 hours after fear conditioning) (Figure 15); and the effect could be recapitulated by local application of ZL006 into the amygdala (Figure 17). These results indicated that the amygdala might be the locus mediating ZL006 action on cue-induced fear responses. Consistent with this idea, a study by Schafe et al. showed that intra-amygdala infusions of either NOS inhibitor 7-Ni or NO scavenger c-PTIO prior to fear conditioning also significantly impaired the LTM of cue-induced fear (Schafe et al 2005). In addition to the LTM assessed the following day, Schafe et al. also investigated short-term memory (STM) that was examined 1 hour and 3 hours after fear conditioning. Interestingly, it was found that STM was not affected by pre-conditioning treatment of either 7-Ni or c-PTIO (Schafe et al 2005). These results, together with those described in this thesis indicated that NO signaling in the amygdala is required for the long-term retention (or consolidation) of cue-induced fear memory, rather than the initial acquisition of conditioned fear memory. This idea

was further supported by the later studies from the Schafe group showing that intra-amygdala manipulations of several downstream molecules of NO signaling, namely sGC, cGMP, PKG and ERK also affect the consolidation of cued fear memory (Ota et al 2008). In support of the role of NO signaling in the consolidation of conditioned fear, both inhibition of the upstream of NO signaling, PSD95-nNOS interaction, by ZL006 and inhibition of the downstream components of NO signaling by sGC inhibitor and PKG inhibitor impaired LTP in the amygdalar neurons (Ota et al 2008).

It could be argued that disruption of NO signaling by ZL006 causes effects on the locomotor function which may confound with results of fear conditioning. The results of the current study and those of others (Doucet et al 2013, Tillmann et al 2017) have shown that i.p. injection of ZL006 at 10 mg/kg, the effective dose for reducing fear has no acute effect on the locomotor activity 1 hour after drug administration (Figure 20). Although I did not directly test the chronic effect of NO signaling inhibition by ZL006 on the locomotor function, a previous study showed that i.p. injection of NOS inhibitor SMTC (S-methyl-L-thiocitrulline) did not affect locomotion 24 hours after drug administration (Kelley et al 2010), a time point that was used to test the LTM of conditioned fear. Also, results from chronic treatments (7 days) with ZL006 at 10 mg/kg in sham TBI model mice showed no effects on baseline locomotor activity and fine motor functions (Xiao-Ming Xu et al., MS under review). These findings collectively suggested that disruption of NO signaling by ZL006 has specific effects on LTM of cue-induced fear.

The finding that the systemic injection of ZL006 following fear conditioning impaired the consolidation of cued fear memory was different from a previous study showing that immediate post-conditioning administration of a preferential nNOS inhibitor, SMTC at 20-200 mg/kg, did not cause effects on the consolidation of cued fear (Kelley et al 2010). The author reasoned that the lack of effects of SMTC may due to its fast pharmacokinetics. As measured with brain associated nitrite and nitrate, two markers of NO production, similar i.p. doses of SMTC has a very short duration of action (< 1hour) that may prevent it from affecting the consolidation of conditioned fear (Kelley et al 2010). This is probably the case when considering the results from the Co-IP experiments (Figure 7). The Co-IP experiments suggested a robust increase of PSD95-nNOS interaction at 1 hour and 2 hours after conditioning, and this increase appeared to be remained until 6 hours after conditioning, indicating that NO signaling was continuously activated during the time period from 1 hour to 2 hours (and potentially up to 6 hours) following conditioning, and NO signaling within this time period was associated with the molecular processes underlying memory consolidation. Therefore, drugs with a longer duration of action that affect NO signaling within this critical period of time may influence the consolidation of fear memory. It would be of interest to test if multiple doses of SMTC following fear conditioning could elongate the duration of action and therefore affect the consolidation of cued fear memory.

In summary, the results suggested that PSD95-nNOS interaction plays a critical role in regulating synaptic strengthening, LTP and cue-induced fear memory consolidation within the amygdala. Further studies are needed to investigate the specific molecular mechanisms underlying ZL006 action on LTP and fear behaviors.

5.2.2 Hippocampal PSD95-nNOS interaction and context-induced fear memory

The first study investigating the role of NO signaling in the contextual fear conditioning reported that systemic administration of NOS inhibitor 7-Ni did not have effects on contextual fear conditioning (Maren 1998). However, the results may have been influenced by the decreased locomotor activity produced by 7-Ni (Maren 1998). A later study employed several control experiments demonstrated that pharmacological inhibition or enhancement of NO signaling can respectively impair or improve the consolidation of contextual fear and these effects on contextual fear cannot be explained by drug effects on locomotor activity (Kelley et al 2010). In agreement with these findings, contextual fear learning and expression was severely impaired in mice lacking the nNOS gene (Kelley et al 2009). Overall, these findings confirmed an involvement of NO signaling in the regulation of contextual fear memory. However, at present, few studies have investigated the neural substrates mediating the effects of NO signaling inhibition on contextual fear.

In the current study, this question was examined by focusing on the ventral hippocampus (vHP), as a wealth of behavioral and anatomical data have suggested a critical role of vHP in contextual fear. For example, ventral hippocampal lesions impair the acquisition and expression of contextual fear (Maren 1999b, Trivedi & Coover 2004). In addition, intra-vHP infusions of $GABA_A$ receptor agonist muscimol (Bast et al 2001) and NMDA receptor antagonist MK-801 (Zhang et al 2001) also cause deficits in the contextual fear. Anatomically, the ventral hippocampus has intimate reciprocal connections with the amygdala (Pitkänen et al 2000), a key region where the information of CS and US converge, indicating an important role of vHP-amygdala interaction in contextual modulation of fear. In the current study, by utilizing a Co-IP assay, a robust increase of PSD95-nNOS interaction in the vHP was observed at 1 hour and 2 hours after fear conditioning, and this increase probably remained until at least 6 hours after conditioning (Figure 7). This finding suggested an involvement of ventral hippocampal PSD95-nNOS interaction in the subsequent consolidation process following contextual fear conditioning. We next showed that post-training infusion of PSD95-nNOS interaction inhibitor ZL006 into the vHP significantly disrupted the conditioned fear responses to the context when tested one day later (Figure 18), further supporting a role of ventral hippocampal PSD95-nNOS interaction in the regulation of contextual fear memory consolidation.

NO signaling in the vHP has been implicated in the modulation of anxiety-related behavior. For example, intra-vHP inhibition of NO signaling by 7-Ni

produces anxiolytic effects assessed by elevated T-maze test (Calixto et al 2010). Hence, it could be argued that modulation of NO signaling by ZL006 may influence anxiety-related behavior (e.g., anxiolytic like behavior), which can confound the measurements of fear responses (behavioral freezing). However, data from the current study and those from others suggested that this is not the case. I found that intra-vHP infusion of ZL006 selectively affected fear responses to the context but not to the tone. If intra-vHP ZL006 produced anxiolytic effect, then it would be expected that both contextual and cued fear memory would be affected by the ZL006 treatment. In addition, unlike direct inhibition of the enzyme nNOS, disruption of PSD95-nNOS interaction appears to have no effect on anxiety/anxiolytics-related behavior assessed by multiple tests, such as social interaction test (Figure 20), light-dark box and elevated plus maze (Doucet et al 2013). Collectively, these data suggested that the effect of intra-vHP ZL006 is best interpreted as an effect on the memory function of ventral hippocampal PSD95-nNOS interaction.

One might worry that the effect of ZL006 treatment was due to its spreading outside the vHP, such as the dorsal hippocampus (dHP). dHP has also been shown to be involved in the modulation of contextual fear conditioning (Holt & Maren 1999, Maren et al 1997, Maren & Fanselow 1997, Matus-Amat et al 2004, Matus-Amat et al 2007, Phillips & LeDoux 1992). Thus, it is possible that the results from the intra-vHP infusion experiment were due to ZL006 diffusing into the dHP. To address this possibility, future studies can be done using a more

ventral infusion site in the vHP, which would reduce the likelihood of ZL006 diffusion into the dHP. If the results from our experiment were due to ZL006 diffusion into the dHP, it would be expected to see no effect in this future experiment; However, if ventral hippocampal ZL006 does play a role in the regulation of contextual fear, similar effects would be observed.

In summary, the current study showed that intra-vHP infusion of ZL006 significantly reduced the freezing responses to the contextual CS; However, the freezing responses to the auditory CS remained unaffected. This specificity has also been observed in an earlier study with intra-vHP infusion of NMDA receptor antagonist MK-801 (Zhang et al 2001). Together, these findings indicate that NMDA-NO mediated mechanisms in the vHP are critical in the memory formation of contextual but not cued fear conditioning. These mechanisms might be required for contextual fear memory storage in the vHP. To further address this issue, electrophysiological studies can be utilized to investigate the effects of MK-801 and ZL006 on the LTP in area CA3-CA1 of the vHP. These experiments are currently being run in our laboratory.

5.2.3 Non-specific effect profile of small-molecule inhibitors of PSD95-nNOS interaction

Despite the important role of NMDA receptors in disrupting fear memory formation, the clinical use of NMDA receptor antagonists is hampered as they also produce adverse side effects, including cognitive deficits and motor

impairments (Krystal et al 1994, Pal et al 2002). Consistent with the previous studies (Rung et al 2005), I found that i.p. injection of the NMDA receptor antagonist MK-801 at the dose effective for reducing fear (0.1 mg/kg) resulted in hyper-locomotion and impaired social activity in rats assessed by open field test and social interaction test respectively (Figure 20). In addition, in agreement with previous research (Boultadakis & Pitsikas 2010, Cunha et al 2008, de Lima et al 2005), the discrimination memory in NORT (Figure 21) and the spatial memory in Y-maze test (Figure 22) were also impaired in animals treated with MK-801. However, I found that ZL006, unlike MK-801, can selectively impair fear memory without affecting locomotor function (Figure 20B), social activity (Figure 20C) and the short-term memory in NORT and Y-maze test (Figure 21 and Figure 22). This lack of acute motor and cognitive effects of ZL006 has significant clinical implications. NMDA receptor antagonists, specifically ketamine, appears to be an effective treatment for reducing PTSD symptoms (Feder et al 2014). However, a significant limitation of drugs like ketamine is their acute effects on cognition and mental state that could last several hours to days after an initial administration. Based on this current study, PSD95-nNOS interaction appears to represent a novel therapeutic target that could reduce conditioned fear but without eliciting such acute CNS adverse effects. Future studies are needed to further investigate the molecular mechanisms underlying ZL006 action (further discussed in section 5.3) and support the development of PSD95-nNOS interaction-based treatment approach for fear-related disorders, such as PTSD and phobias.

5.3 Limitations and Future Directions

The work presented in this dissertation demonstrated a critical role of PSD95-nNOS interaction in regulating synaptic plasticity and the consolidation of conditioned fear. However, there are some aspects of the underlying mechanism that I did not fully elucidate and were outside the scope of my current project. In this section, I will explain the limitations of this study (5.3.1) and discuss the future directions (5.3.2~5.3.4) in further understanding the mechanisms and developing PSD95-nNOS interaction based novel therapeutic target for fear-related disorders.

5.3.1 Limitations and Caveats

The Co-IP experiments described in Chapter 2 demonstrated a robust increase in PSD95-nNOS interaction in both BLA and vHP at 1 h and 2 h following fear conditioning (Figure 7), indicating increased nNOS activity at these time points in both regions. In support of this view, a previous study using in vivo microdialysis with Griess test found that the NO production in the amygdala began to rise at 1 h and maintained increased until 3 h after fear conditioning, as measured by the levels of nitrite/nitrate (NO metabolites) (Sato et al 2006). However, the levels of NO metabolites following fear conditioning in the BLA and vHP were not directly tested in this current study. Future experiments with Griess assay can be done to monitor nitrite/nitrate levels in both regions at different time points following fear conditioning. Co-IP experiment in the current study also demonstrated that ZL006 treatment is effective in disrupt PSD95-nNOS

interaction in the BLA and vHP (Figure 11). However, whether ZL006 can decrease nNOS activity and/or NO production in both regions was not determined. Previous studies have verified the ability of ZL006 to reduce NO production in hippocampal neurons (Smith et al 2016) and cerebellum slices (Tillmann et al 2017) by the measurement of cGMP or nitrite/nitrate levels. I therefore reasoned that ZL006 treatment in this current study should also reduce cGMP levels and/or nitrite/nitrate concentrations in both BLA and vHP. Additional experiments using cGMP assay and Griess assay can be done in tissue homogenates of BLA and vHP to confirm the NO-decreasing effect of ZL006. This series of experiments will enable a more complete argument that ZL006 action on fear memory was mediated via NO signaling-based mechanisms.

Behavioral experiments in the current study showed that intra-BLA and intra-vHP injection of ZL006 can respectively impair the consolidation of cue-induced and context-induced fear memory (Figure 17 and 18). A limitation of these behavioral studies is that I did not determine if nNOS, rather than iNOS or eNOS, was required for the effects seen with ZL006. To define the primacy of nNOS protein for the behavioral effects of ZL006, the same set of behavioral experiments can be done in animals with local anti-nNOS shRNA treatment in the BLA or vHP. We anticipate that the fear-reducing effect of ZL006 treatment will not be observed in animals that have the expression of the key protein nNOS silenced.

5.3.2 Molecular mechanisms in the amygdala associated with cued fear consolidation and the effects of disruption of PSD95-nNOS interaction

The results from the present study and those from others (Ota et al 2008, Paul et al 2008, Schafe et al 2005) clearly demonstrated a critical role of amygdalar NO signaling in modulating the consolidation of auditory fear conditioning. Consolidation is the process in which temporary stimulus-fear association via CS/US pairing is stabilized into a persistent memory. Previous research has suggested that the consolidation of cued fear is characterized by enduring enhanced synaptic efficacy in the BLA synapses, which is in part attributed to enhanced AMPA receptor function (e.g. increased receptor affinity, slowing receptor deactivation) and/or increased expression of synaptic AMPARs (e.g. increased AMPARs insertion/trafficking to the post-synaptic membrane). Electrophysiological studies with amygdala slices from my laboratory (unpublished) found enhanced AMPAR-mediated EPSCs (AMPA-EPSCs) at both the early- and the late-phase of consolidation (~3.5 hours and ~24 hours following auditory fear conditioning, respectively). Interestingly, the enhanced AMPA-EPSCs at both time points could be blocked by pretreatment of ZL006, indicating a role of NO signaling in the regulation of AMPARs properties and/or synaptic expression of AMPARs. However, the precise molecular mechanisms by which NO signaling mediates the AMPARs properties and synaptic insertion remain unknown and represent a fruitful area for further inquiry.

Here, I will discuss the potential mechanisms based on the previous research and recent findings in our laboratory. Proposed experiments that could be used to test these mechanisms are also discussed. A proposed model of NO-signaling in the BLA neurons mediating AMPARs properties and expression is shown in Figure 24.

5.3.2.1 NO signaling and AMPARs in early consolidation

Previous research has shown that activated PKG via NMDAR-nNOS-NO-sGC signaling pathway binds the C-terminal domain of GluR1, and that in this complex, PKG can phosphorylate S845 of GluR1 and increase AMPAR level in the plasma membrane of hippocampal neurons (Serulle et al 2007). In addition, the increased surface AMPARs can be blocked by pretreatment of nNOS inhibitor and sGC inhibitor (Serulle et al 2007). This function of PKG provides a mechanism by which NO signaling regulates AMPARs trafficking in the hippocampus. However, whether this mechanism can be generalized to the amygdala and underlies the NO signaling-mediated effects on fear consolidation in not clear. To address this issue, synaptosome extracted from the amygdala of fear-conditioned and non-conditioned animals with or without ZL006 treatment can be utilized to examine the levels of PKG-GluR1 interaction and GluR1 phosphorylation. It is expected to see enhanced PKG-GluR1 interaction and GluR1 phosphorylation in the amgydalar synaptosome from fear-conditioned animals when compared with those from non-conditioned animals, and the

increased expression of PKG-GluR1 complex and phosphorylated GluR1 can be blocked in animals treated with ZL006.

In addition to the PKG-dependent mechanism, NO signaling can also influence AMPARs trafficking via a post-translational mechanism called S-nitrosylation. It has been reported that NO production upon NMDARs stimulation elicits S-nitrosylation of stargazin and NSF (N-ethylmaleimide sensitive factor), two AMPA receptor regulatory proteins. While nitrosylated stargazin causes an increased surface expression of AMPARs by enhancing stargazin binding with GluR1 (Selvakumar et al 2009), nitrosylated NSF facilitates the surface insertion of AMPARs by enhancing NSF binding with GluR2 (Huang et al 2005). Additional experiments with biotin-switch assay will allow us to examine if fear conditioning induces S-nitrosylation of stargazin and NSF and if pretreatment of ZL006 could block the increase of nitrosylated stargazin and NSF.

While affecting the membrane expression of AMPARs, NO has also been implicated in the modulation of AMPARs properties. Receptor binding studies with brain sections from different regions reported that NO appears to be able to increase the affinity of AMPARs, thus increasing the synaptic responses (Dev & Morris 1994). The underlying mechanisms could be cGMP-dependent or cGMP-independent (Dev & Morris 1994). Additional experiments are required to test if NO can increase AMPARs affinity in the amygdala slices and if the increased

affinity of AMPA binding site can be decreased by disruption of PSD95-nNOS interaction by ZL006.

5.3.2.2 NO signaling and AMPARs in late consolidation

Transcriptional profiling studies in my laboratory (unpublished) have revealed a set of differentially expressed genes (DEGs) in the amygdala 24 hours following auditory fear conditioning. Some of the DEGs, such as *igf2 (insulin-like growth factor 2)* and *itgb6* (*integrin beta 6)* can be rescued by ZL006 treatment. Recent studies have shown that both IGF-II and integrins can regulate surface expression of AMPARs via endocytosis (Alberini & Chen 2012, Chen et al 2011, Cingolani et al 2008, Pozo et al 2012). Thus, IGF-II-dependent and/or integrin-dependent endocytosis of AMPARs may represent one of the mechanisms underlying the increased AMPA-EPSCs and the effects of ZL006 that were observed at the late-stage of consolidation. Confirmatory studies, such as intra-amygdala inhibition/activation of gene expression or protein function, will be needed to definitively conclude the role of these genes in the regulation of AMPARs expression and the consolidation of fear memory. The effects of disruption of PSD95-nNOS interaction on the expression of IGF-II and integrins can be further confirmed with PCR and western blotting experiments.

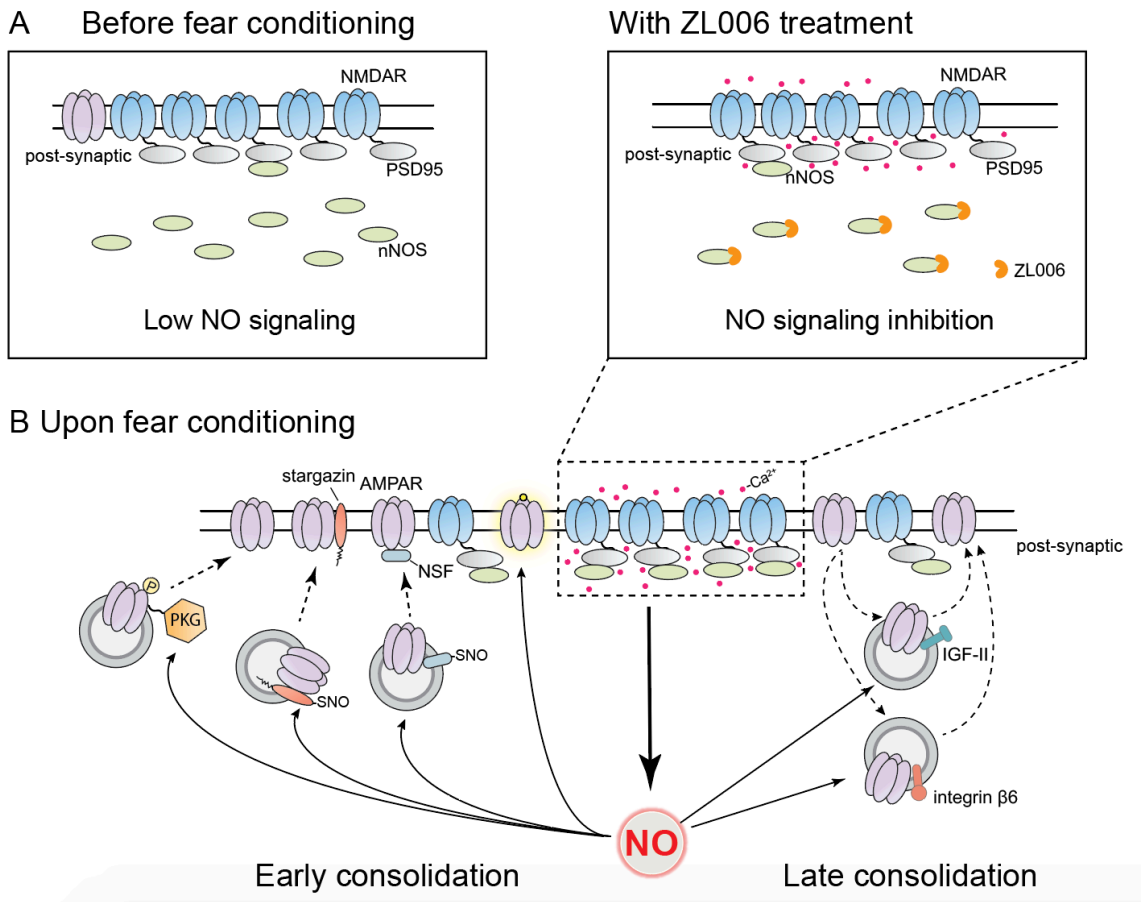

Figure 24. A proposed model of NO-signaling in the BLA neurons mediating AMPARs properties and expression which underlies the consolidation of fear

(A) Before fear conditioning, nNOS enzyme displays minimum activity due to a lack of interaction with the scaffold protein PSD95; therefore, NO signaling is largely inhibited. (B) Upon fear conditioning, nNOS translocates from the cytosol to membrane via PSD95-nNOS interaction, which is critical for the efficient activation of nNOS by the Ca^{2+} influx through the NMDARs. Upon the activation of nNOS, NO is synthesized and acts on multiple targets that are potentially involved in the regulation of AMPARs properties and expression. During the early phase of consolidation (~ 3.5 h following fear conditioning), increased NO promotes membrane expression of AMPARs either via activation of NO-cGMP-PKG pathway or through modifying the regulatory proteins of AMPARs by S-nitrosylation. Specifically, activated PKG forms a complex with GluR1 and directly phosphorylated GluR1, which facilitating synaptic insertion of AMPARs; NO-induced nitrosylated stargazin and NSF (N-ethylmaleimide sensitive factor) can both enhance their binding with AMPARs and thus promoting membrane expression of AMPARs. In addition, NO appears to be able to increase the affinity of AMPARs through an unclear mechanism. During late consolidation (~ 24 h after fear conditioning), NO associated signaling pathway downregulated

the gene expression of *igf2 (insulin-like growth factor 2)* and *itgb6* (*integrin beta 6)* via unknown mechanisms. IGF-II- and integrin β3-dependent endocytosis of AMPARs are therefore reduced and membrane expression of AMPARs is correspondently increased. In animals treated with ZL006, the interaction between PSD95 and nNOS is significantly disrupted, leaving nNOS activation at a minimal level. Therefore, NO signaling and the resultant various synaptic outcomes are impaired/prevented.

5.3.3 PSD95-nNOS interaction in different stages of fear memory

Although the current study and studies from others have clearly shown an important role of NO signaling in fear memory consolidation, it remains unclear if NO signaling has a consistent role in other aspects of fear memory, namely, reconsolidation and extinction. Specifically, whether inhibition of NO signaling by disrupting PSD95-nNOS interaction consistently impair fear memory across conditioning, reactivation and extinction remains unknown. Future studies addressing these issues will provide additional insights into the field of NMDA-nNOS-NO signaling and fear memory research.

5.3.3.1 NO signaling and reconsolidation of fear memory

Although fear memories are consolidated following conditioning, they can be labile (destabilized) after its retrieval through a process that has been termed reconsolidation. Memory retrieval or reactivation is usually done by a single presentation of CS that was used to signal shock during acquisition. Once reactivated, memories enter into a labile state and require reconsolidation processes in order to be retained (Alberini 2005, Johansen et al 2011, Tronson & Taylor 2007).

Ample evidence suggests that reconsolidation and consolidation of fear memories may share some key features in terms of the intracellular cascade of events triggered by NMDARs activation (Alberini 2005, Johansen et al 2011, Tronson & Taylor 2007). Indeed, NO signaling, as a downstream of NMDARs

activation, has been implicated in the reconsolidation of associative memories using various conditioning paradigms. For example, post-retrieval administration of either NMDARs antagonist MK-801 or NOS inhibitor 7-Ni impaired the memory of cocaine-induced conditioned place preference (CPP) in mice (Itzhak 2008); additionally, administration of NO donor molsidomine following retrieval of place preference enhanced reconsolidation and improved CPP expression in nNOS knockout mice (Itzhak & Anderson 2007). Similarly, by using inhibitory avoidance task, inhibition of NO signaling has also been shown to impair the reconsolidation of the original learning (Baratti et al 2008).

Despite the critical role of NO signaling in memory reconsolidation in CPP and inhibitory avoidance tasks, few studies have investigated the role of NO signaling in the reconsolidation of fear memory using fear conditioning paradigms. At present, only one study directly addressed this question by infusing 7-Ni into the amygdala of fear conditioned rats (Schafe et al 2005). However, in this study, the authors failed to observe any effects of intra-amygdala 7-Ni on the reconsolidation of auditory fear conditioning. Several factors may account for the negative results. First, the effects of NO signaling inhibition on memory reconsolidation may be dependent on the timing of drug administration. In CPP and inhibitory avoidance tasks, drugs were given immediately after memory retrieval (reactivation), in contrast, in the auditory fear conditioning task, drugs were administrated 30 minutes prior to memory retrieval. Second, it is possible that other brain regions, such as hippocampus and mPFC

may be the neural locus mediating the effects of NO signaling in the fear memory reconsolidation. Clearly, additional experiments are required to further determine the role of NO signaling in the regulation of memory reconsolidation of fear conditioning.

5.3.3.2 NO signaling and extinction of fear memory

Fear extinction is a form of inhibitory learning after repeated presentation of the CS, without the US, which results in a gradual reduction of previously acquired conditioned fear responses (Myers & Davis 2007). There is increasing interest in the research elucidating the neural circuits and molecular mechanisms supporting fear extinction due to its clinical relevance in fear-related disorder, such as PTSD. Previous work has identified a facilitatory role of BDNF-TrkB signaling in fear extinction (Andero & Ressler 2012). A recent study revealed that PSD95-nNOS interaction may be a novel fear extinction modulator that acts through BDNF-TrkB signaling (Cai et al 2018). The authors showed that intra-hippocampal disruption of PSD95-nNOS interaction by repeated infusion of small molecule ZL006 or peptide Tat-nNOS$_{1\text{-}133}$ following fear recall can significantly facilitate the extinction of contextual fear memory (Cai et al 2018). Mechanistically, disruption of PSD95-nNOS association in CA3 of the hippocampus upregulated BDNF expression and the coupling of TrkB with PSD95 in this area; and the extinction-enhancing effects of disruption of PSD95-nNOS interaction could be abolished by BDNF scavenger and TrkB receptor antagonist (Cai et al 2018). Since fear extinction is a complex phenomenon

which primarily involves not only the hippocampus but also the amygdala and the mPFC (Bredy et al 2007, Chhatwal et al 2006, Peters et al 2010, Rosas-Vidal et al 2014), it is tempting to examine if the up-regulatory role of disruption of PSD95-nNOS interaction in BDNF signaling can be generalized to the other two brain regions. Also, it is of interest to investigate if disruption of PSD95-nNOS interaction facilitates the extinction of cue-induced fear memory, which is more dependent on the amygdala.

5.3.4 NO signaling in conditioned fear: Relevance to PTSD

Normal fear learning and memory is essential to animals' survival as it allows animals to predict and avoid physical dangers. However, dysregulated fear learning and memory following traumatic events can lead to symptoms of syndromes, such as post-traumatic stress disorder (PTSD). Numerous clinical studies of PTSD demonstrated that repetitive recall of traumatic memories, avoidance symptoms, and chronic hyperarousal are the defining features of PTSD (Sherin & Nemeroff 2011). PTSD is frequently accompanied by other comorbid psychiatric illness along with high rates of functional disability (Galatzer-Levy et al 2013). While PTSD is recognized as a significant health challenge, there is no highly effective treatments for it (Berg et al 2007). The current first-line medication for treating PTSD symptoms is serotonin reuptake inhibitors (SRIs, such as fluoxetine, sertraline, and venlafaxine), which produce only partial effectiveness (Berg et al 2007). Additionally, a substantial proportion of patients are resistant to this treatment (Stein et al 2006).

PTSD is considered as a fear pathology in which the memories of learned fear are likely to sustain, generalized, and resistant to extinction (Elzinga & Bremner 2002). Therefore, understanding the neural basis underlying fear memory itself is critical for identifying the etiology of PTSD and for developing better drugs. Pavlovian fear conditioning is a leading animal model for studying fear learning and memory (Johansen et al 2011). By using fear conditioning, studies have revealed a distributed network of brain regions that are involved in fear learning and extinction. Three brain regions that have received the most intensive research are amygdala, hippocampus, and medial prefrontal cortex. These three different brain structures regulate, in concert, the various aspects of fear memory (see Chapter 1). The roles of these brain regions in fear memory were validated in human fear conditioning studies using functional magnetic resonance imaging (fMRI). It has been shown that the activity of amygdala was increased during the acquisition of both cue- and context-induced fear conditioning (Alvarez et al 2008, Delgado et al 2006, LaBar et al 1998). Hippocampus is significantly activated during contextual fear conditioning (Alvarez et al 2008). Activation of mPFC was observed during fear extinction (Milad et al 2007, Phelps et al 2004). Furthermore, these brain regions have been shown to be dysregulated in patients with PTSD. Several functional neuroimaging studies demonstrated that PTSD patients displayed greater activity in the amygdala in response to trauma-related stimuli (e.g. combat sounds, emotional words and faces) when compared with control subjects (Etkin & Wager 2007, Liberzon et al 1999). Functional imaging studies in the hippocampus have

yielded mixed findings. Some studies reported increased activation in the hippocampus in PTSD patients (Thomaes et al 2011, Werner et al 2009); Other studies found a failure of hippocampal activation in patients with PTSD (Bremner et al 2003). Dorsal anterior cingulate (dACC) and ventral mPFC (vmPFC) are thought to be the putative human homologues of the PL and IL in rodents, respectively (Milad & Quirk 2012). Studies have shown increased dACC activity during fear conditioning (Rougemont-Bücking et al 2011) and decreased dACC activity during extinction (Bremner et al 2005) in PTSD patients relative to controls. Deficits in the recall of extinction memory in subjects with PTSD were found to be correlated with lower activity in vmPFC (Milad et al 2009). These findings from human studies, especially those studies in PTSD patients, underscore the translational validity of rodent models of fear conditioning.

By utilizing fear conditioning model in rodents, numerous studies have been done to investigate the molecular and cellular mechanisms underlying fear learning and memory. Studies described in this thesis and those from others collectively suggested a critical role of PSD95-nNOS interaction in different stages of fear memory, namely consolidation, reconsolidation and extinction, supporting PSD95-nNOS interaction as a promising target for reducing fear memories in animals and eventually PTSD patients. Importantly, disruption of PSD95-nNOS interaction with small molecules elicits minimal adverse effects while reducing fear responses (Figure 20, 21 and 22).

Despite the mounting evidence regarding the beneficial effects of disruption of PSD95-nNOS interaction on reducing fear, to my knowledge, previous studies were only undertaken in normal animals exposed to fear conditioning alone, which is insufficient to produce PTSD phenotype (Pitman et al 1993). To elucidate the role of PSD95-nNOS interaction in the pathophysiology of PTSD and to further support the development of PSD95-nNOS interaction-based avenues for PTSD treatment, it is essential to conduct investigations using valid animal models of PTSD. Although inducing PTSD-like symptoms in animals is challenging, there are several animal models that have been proven to resemble clinical situation with better face validity (how well the model captures the symptomatology of diseases) and construct validity (how well the model reflects the underlying theory) than fear conditioning model (Daskalakis et al 2013). One of these models is predator exposure/predator scent exposure (PredEX). In this model, exposed animals usually develop sustained behavioral manifestation of anxiety, which mimics persistent anxiety phenotype seen with PTSD patients (Adamec et al 1993, Zoladz et al 2008). Interestingly, a recent study in humans demonstrated a critical role of a functional nNOS gene variation in the regulation of anxiety process (Kuhn et al 2016). nNOS gene with a variation in the exon 1f (NOS1 ex1f-VNTR, short alleles) has been shown to be associated with increased expression of nNOS mRNA in human amygdala tissues (Weber et al 2015). Subjects with this variation displayed enhanced anxiety traits (Kuhn et al 2016). It is tempting to investigate whether inhibition of nNOS by disrupting PSD95-nNOS interaction with ZL006 could alleviate anxiety-

like behaviors in the PredEX model of PTSD. Other PTSD models include exposure to single prolonged stress (SPS) and exposure to foot shock with additional stressors, in which exposed animals would display increased acoustic startle response, a feature symptom of PTSD (Pitman et al 2012). Future studies investigating the role of PSD95-nNOS interaction with the use of animal models of PTSD will provide powerful insight on the mechanisms underlying abnormal fear responses in PTSD and thus further facilitating the development of PSD95-nNOS interaction based treatment for PTSD symptoms, such as re-experiencing traumatic events.

CHAPTER 6

Materials and Methods

6.1 Materials

6.1.1 Animals

Adult male Sprague-Dawley rats (250-300g, Harlan, IN, USA) were utilized for the behavioral and biochemical experiments. Rats were allowed to acclimate to housing for at least 3 days following delivery; and they were housed singly on a regular 12-hour light/dark cycle (lights on at 0700 hours) in a temperature-controlled room (22˚C), with free access to food and water. Each rat was handled daily for a minimum of 3 days before any behavioral experiment. Animal care procedures were conducted under the NIH Guidelines for the Care and Use of Laboratory Animals, 8th Edition and approved by the IUPUI Institutional Animal Care and Use Committee.

6.1.2 Drugs and chemicals

To perform i.p. injections of drugs, ZL007 and ZL006 were dissolved in a vehicle of 10% DMSO (Sigma Aldrich, St. Louis, MO, USA), with the remaining 90% consisting of 100% ethanol (Fisher Scientific, Pittsburgh, PA, USA), emulphor (Alkamuls EL-620, Solvay, Brussels, Belgium) and sterilized saline at a ratio of 1:1:8, respectively. ZL007 was synthesized in the laboratory of Dr. Ganesh Thakur at the Northeastern University Center for Drug Discovery

(Boston, MA, USA). ZL006 was purchased from Sigma. MK-801 was dissolved in sterilized saline and was also purchased from Sigma. The volume for an i.p. injection was 1mg/kg and control animals were injected with an equal volume of vehicle. To perform local infusions, ZL006 was diluted from ZL006 stock solution (dissolved in 100% DMSO) at 1:1000 in ACSF (artificial cerebrospinal fluid) to yield a final concentration of 10 μM. ACSF containing in mM: 130 NaCl, 3.5 KCl, 1.1 KH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 30 $NaHCO_3$, 10 glucose (~ 315 mOsm, 7.4 pH).

6.2 Methods

6.2.1 Behavioral tests

6.2.1.1 Fear conditioning and fear expression tests

Rats were habituated to the conditioning box measuring L 25.5 × W 25.5 × H 39.5 cm for 10 minutes the day before conditioning. The conditioning box (Box A) was situated in a larger sound-attenuated chamber, which was illuminated with a white 15-Lux light. The floor of Box A was constructed of parallel stainless-steel bars and connected to a scrambled shock generator (Stoelting Co., Wood Dale, IL, USA). In the rear wall of the chamber, a speaker was mounted and operating to provide white noise during all experimental sessions. Before conducting fear conditioning for each animal, both the chamber and the conditioning box were thoroughly cleaned with 70% ethanol (Fisher Scientific) to remove olfactory cues. During fear conditioning, rats were trained with 3

tone/shock pairings, with each pairing consisting of a 20 s, 4 kHz, 80 dB tone that co-terminated with a 0.5 s, 0.8 mA footshock. The inter-trial interval (ITI) was 120 s. Each rat was allowed to explore the conditioning box and the chamber for 100 s before fear conditioning began and remained in the conditioning box for 60 s after the last tone/shock trial. Animals in the 'Tone only' group were placed in the conditioning box and exposed to 3 tones only (20 s, 4kHz, 80 dB) without receiving footshocks. Shortly after fear conditioning (< 5 min), animals were treated with drugs or vehicle. Cue-induced conditioned fear memory was tested in the same conditioning box (Box A) with a presentation of 10 tones (4 kHz, 80 dB, 20 s, ITI 60 s) 24 hours after fear conditioning. Total time of freezing during the tone presentations were recorded and scored manually by blinded raters. Data was expressed as a percentage of the total tone duration.

In the experiment with intra-vHP infusions where both context-induced and cue-induced fear were tested, animals were first placed into the conditioning box (Box A) for contextual fear test and then into Box B for cued fear test. For context-induced fear test, rats were exposed to the context of the box without tone or shock for a total of 5 minutes. Total time freezing during the last 4 minutes were recorded and scored for each rat. The cue-induced fear test was the same as described above, excepted that it was tested in Box B, a novel box. The context of Box B is different from Box A in that the floor of the Box B was constructed of a smooth black plastic board and the four walls of the box were decorated with black/white checked paper sheets. When tested in Box B, the

olfactory cues between animals were cleared by using 1% acetic acid (Fisher Scientific). Freezing was defined as the absence of all movement except for normal breathing.

6.2.1.2 Open field test

The open field apparatus consisted of a Plexiglas open-topped chamber measuring L 91.5 × W 91.5 × H 30.5 cm, a ceiling-mounted CCD camera and a 25 W red light bulb placed 2 meters above the center of the chamber. Rats were gently placed in the center of the chamber 1 hour following treatments of vehicle or drugs and allowed to freely explore the chamber 5 min while being tracked by an automated tracking system (ANY-MAZE, Stoelting Co., Wood Dale, IL, USA). Total distance traveled was used to measure locomotor activity and results were normalized to vehicle controls.

6.2.1.3 Social interaction test

Social interaction test was performed 5 min after open field test in the same apparatus. The protocol used for the social interaction test has been described previously (Sanders & Shekhar 1995a, Sanders & Shekhar 1995b). Briefly, the ‘experimental’ rat and the ‘partner’ rat were simultaneously placed into the chamber and were allowed to move for a total of 5 min freely. The age, sex and weight of the ‘partner’ rats were matched to the ‘experimental’ rats. Social interaction time (in seconds) for each pair of rats was measured as time spent by the ‘experimental’ rat engaging in non-aggressive physical investigation

of the ‘partner’ rat; This is defined by the ‘experimental’ rat sniffing, climbing over and crawling under the ‘partner’ rat, mutual grooming, genital investigation or following and walking around the partner. All tests were video recorded from above and then manually scored by blinded raters using ODlog for Mac OS X version 2.6.1.

6.2.1.4 Novel object recognition test

NORT was performed as previously described (Pitsikas et al 2006) with minor modifications. Prior to testing, animals were placed into an open field box (L 100 × W 100 × H 20 cm) and allowed to freely explore the box for 5 min per day for 3 consecutive days with no objects present. The experiment is consisted of two 2 min trials: familiarization trial and test trial. During the familiarization trial, rats were placed into the box containing two identical objects (plastic cylinders 6 cm in diameter and 12 cm tall in white and red) in two opposite corners. The rats were released against the center of the opposite wall with its back to the objects. According to previous reports, this was done to prevent coercion to explore the objects (Reger et al 2009). The rats were considered to be exploring when they were facing, sniffing or biting the object with nose and/or forepaws. Shortly following familiarization, rats were treated with vehicle or drugs systemically, and were returned to their home cages. Rats were allowed to stay in their home cages for a period of 3 hours (ITI = 3 h) before they were placed back into the box for the test trials. During this trial, a novel object (plastic building block in yellow or green, L 7 × W 3.5 × H 9 cm) replaced one of the familiar objects used

in the familiarization trial. The box and the objects were thoroughly cleaned with 70% of ethanol after each trial. The time spent in exploring each object during both trials were recorded manually by using a stopwatch. Discrimination index (DI) used to measure the object recognition memory was calculated as the difference in exploration time for the novel (T_N) versus familiar objects (T_F), then dividing this value by the total time spent exploring the two objects in the test trial: $DI = T_N - T_F / T_N + T_F$ (Cavoy & Delacour 1993).

6.2.1.5 Y-maze test

Y-maze task was performed as previously described (Conrad et al 1996) with minor modifications. The apparatus employed for the Y-maze test was constructed of acrylic plexiglass with 'Y' shaped arms; each of the three arms measured L 34 × W 8 × H 14.5 cm. Visual cues (paper cuts in different colors and shapes) were placed on the walls of the maze. The maze was located in a room with a light of 350 Lux brightness. Several distal cues (tables, chairs, computers, and multiple different small objects) were around the Y-maze and were kept constant during the entire behavioral testing period. The floors and walls of the maze were cleaned thoroughly with 70% ethanol between trials to remove olfactory cues. The three arms were randomly designated as start arm, in which the animals started to explore (always open), novel arm, which was blocked during the 1st trial, but open during the 2nd trial, and other arm (always open). There are two trials in the Y-maze test: acquisition trial and test trial. During the 10-min acquisition trial, the rat was allowed to freely explore the start

arm and the other arm only, with no access to the novel arm. Shortly after the familiarization trial, animals were treated with vehicle or drugs and placed back to their home cages. The test trail was performed after 1 h waiting period (ITI = 1 h) in the home cages. During this trial, the novel arm was opened and the animals were allowed to freely explore all three arms for 5 min. All trial were recorded by a ceiling-mounted CCD camera. Video recordings were later analyzed by a blinded rater to determine the number of entries and the time spent in each arm for each rat.

6.2.2 Co-immunoprecipitation analysis

Following fear conditioning, rats were sacrificed at different time points by decapitation under isoflurane and the brains were immediately removed and frozen in iso-pentane (Thermo Fisher Scientific) on dry ice. Brain samples were stored at -80˚C until processed. Punches containing the mPFC or BLA or vHP were obtained using a 1 mm diameter Harris micro-punch (Electron Microscopy Sciences, Hatfield, PA, USA) from 300 μm thick sections taken on a freezing microtome (see Figure 14 for locations of micropunches). Punches were immediately transferred into 100 ul (for mPFC and BLA punches) or 150 ul (for vHP punches) of ice-cold lysis buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 5% glycerol, 1% NP-40, PH 7.4) supplemented with Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA) and then lysed on ice for 15 min by dounce homogenization. Samples were then centrifuged at 13,000g/4 ˚C for 15 min. The supernatants

were pre-incubated with control agarose resin (25 ul, Thermo Fisher Scientific) for 1 hour at 4 ˚C and then centrifuged to remove proteins that bind nonspecifically to the resin. The supernatants were then incubated overnight (> 16 hours) at 4 ˚C with nNOS antibody at 2 ug per 100 ug total protein. Protein A/G Agarose (Thermo Fisher Scientific) was added to the antibody/lysate sample the next day and incubated for 1 hour at 4 ˚C. Immune complexes were precipitated by centrifugation and washed 5 times with lysis buffer. The bound proteins were then eluted by heating at 95 ˚C in loading buffer for 10 min. After cooling down, the protein samples were then used for western blot. Samples were loaded to acrylamide denaturing gels (10%) and transferred to nitrocellulose membranes (Amersham, Pittsburgh, PA, USA). Membranes were then blocked for an hour with 5% milk in TBST buffer (50 mM Tris-Cl, pH 7.6; 150 mM NaCl; 0.1% Tween 20) prior to overnight incubation with primary nNOS and PSD95 antibodies. On the next day, the membranes were incubated with appropriate secondary antibodies. Protein bands were visualized by adding chemoluminescent buffer (Millipore, Billerica, MA, USA) to the blots. Films were scanned and densitometry of protein band was performed using ImageJ 1.48 software (NIH). Antibodies used in Co-IP and the following western blotting are listed in Table 1.

6.2.3 Western blot analysis

The expression levels of PSD95 and nNOS were determined by immunoblotting analysis. Punches were obtained and lysed as described above.

Protein concentrations were determined by BCA assay (Pierce BCA protein assay kit, Thermo Fisher Scientific). Protein samples were treated with Laemmli 2X loading buffer (Bio-Rad, Hercules, CA, USA) and heated at 95 ˚C for 5 minutes. Equal amount of proteins were electrophoresed and transferred to nitrocellulose membranes. Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE, USA) and then incubated with mouse nNOS antibody at 1:1000, (Santa Cruz), mouse PSD95 antibody at 1:1000 (Invitrogen) and mouse β-actin antibody at 1:10000 (Santa Cruz). Subsequently, membranes were incubated with IRDye 800CW secondary antibody from LI-COR. Protein band signals were detected by using Odyssey CLx Imaging System (LI-COR) and quantified by Image studio Lite software (LI-COR). The total protein levels were normalized to β-actin protein levels. Antibodies used in western blotting are listed in Table 1.

Table 1. Antibodies used in Co-IP and Western blotting

Co-immunoprecipitation			
Primary antibodies	Host species	Catalog # and Manufacturer	Dilution
nNOS (A-11)	Mouse	sc-5302; Santa Cruz Biotechnology, Dallas, TX	2 µg per 100 µg protein for pull down
nNOS (A-11)	Mouse	Sc-5302; Santa Cruz Biotechnology, Dallas, TX	1: 1000
PSD95 (7E3-1B8)	Mouse	MA1-046; Invitrogen, Rockford, IL	1: 2000
β-actin (C4)	Mouse	Sc-47778; Santa Cruz Biotechnology, Dallas, TX	1: 10000
Secondary antibody	Host species	Manufacturer	Dilution
anti-mouse IgG (HRP)	Goat	Sc-2005; Santa Cruz Biotechnology, Dallas, TX	1: 2000 for nNOS; 1: 5000 for PSD95; 1: 10000 for β-actin
Western blotting			
Primary antibodies	Host species	Manufacturer	Dilution
β-actin (C4)	Mouse	Sc-47778; Santa Cruz Biotechnology, Dallas, TX	1: 10000
PSD95(7E3-1B8)	Mouse	MA1-046; Invitrogen, Rockford, IL	1: 1000
nNOS (A-11)	Mouse	Sc-5302; Santa Cruz Biotechnology, Dallas, TX	1: 1000
Secondary antibody	Host species	Manufacturer	Dilution
anti-mouse IgG (IRDye 800CW)	Goat	LI-COR Biosciences, Lincoln, NE	1: 10000

6.2.4 Surgery

Prior to surgery, rats were anesthetized in a closed plastic box connected to an Isoflurane system (MGX Research Machine, Vetamac, Rossville, IN, USA). Following anesthesia in the box, the animals were secured on a stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA). Anesthesia was maintained through a nose cone, which allowed for a flow of isoflurane at constant rate (2–3% by volume) throughout the surgery. After making an incision in the scalp and cleaning the skull, two stainless-steel guide cannulas (26 gauge, Plastics One, Roanoke, VA, USA) were implanted bilaterally into the BLA (anterior, -2.3 mm; lateral, ±4.9 mm; and ventral, -7.4 mm) or into the ventral hippocampus (vHP) (anterior, -5.1 mm; lateral, ±5.0 mm; and ventral, -5.2 mm) with the guidance of the brain atlas of Paxinos and Watson (Fifth edition). The guide cannulas were secured into place using three 2.4mm screws anchored into the skull along with cranioplastic cement. To prevent occlusions,dummy cannulas (Plastics One) with lengths matching the guide cannulas were placed inside the guide cannulas. All rats were treated with pain medication (buprenorphine, Indiana University School of Medicine Laboratory Animal Resources) following surgery and were allowed to recover in their home cages for 7 days before any behavioral test. During the recovery period, rats were gently handled every day for a minimum of 2 min.

6.2.5 Intracranial Injections

To execute local infusions into BLA or vHP, the dummy cannulas were quickly removed from the guide cannulas and were replaced by injection

cannulas which are extended 1.0 mm beyond the guide cannulas (Plastics One). The injection cannulas were connected via polyethylene tubing to 10 µl microsyringes (Hamilton, Reno, NV, USA). Injections were performed using a Harvard PHD 2000 (Harvard Apparatus, Inc., South Natick, MA, USA) syringe pump at a rate of 0.1 µl/min. After drug infusion, injection cannulas remained in the guide cannulas for 1 min to allow diffusion of the drug from the tip. After behavioral tests, rats were euthanized by an overdose of isoflurane and perfused with 4% Paraformaldehyde (PFA) (Fisher Scientific). Neutral red staining and light microscopy were used to verify the locations of the cannula tips within the BLA and the vHP.

6.2.6 Slice Electrophysiology

Electrophysiological recordings were conducted as previously described (Rainnie 1999). Rats (150-200 g) were sacrificed under isoflurane by decapitation. Brains were then rapidly removed and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF, solution in mM: 130 NaCl, 3.5 KCl, 1.1 KH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 30 $NaHCO_3$, 10 glucose, 315 mOsm, 7.4 pH). Coronal slices (350 µM) containing the BLA (BLA, ~ -2.3 mm from bregma) were prepared. To improve cell viability, slices were first incubated in 30 °C ACSF for 30 min and then in room temperature ACSF until recording. Oxygenated ACSF was warmed to 30 °C and perfused at a rate of 2-3 ml/min during recording on the platform of a Nikon E600FN Eclipse microscope (Nikon Instruments, Melville, NY, USA). Borosilicate glass electrodes (WPI, Sarasota,

FL, USA) (resistances 3-6 MΩ) were filled with potassium gluconate based recording solution containing in mM: 130 K-Gluconate, 3 KCl, 3 MgCl2, 2 K-ATP, 0.2 Na-GTP, 10 HEPES, 5 phosphocreatine, 0.05 picrotoxin (Sigma) and whole-cell patch clamp recordings in current clamp mode were obtained by standard techniques using Multiclamp700B amplifier and Digidata1440 digitizer (Molecular Devices, Sunnyvale, CA, USA). Pyramidal neurons in the BLA were identified according to their characteristic size and shape and further validated by basic electrophysiological property of input resistance with ~35 MΩ (McDonald et al 2005). GABA-B receptor antagonist CGP52432 (Tocris, Minneapolis, MN, USA) at 1μM and ZL006/ZL007 compounds at 10 μM were added directly to the ACSF solution. Cell holding potential was maintained at -70 mV and Master8 pulse stimulator (A.M.P.I, Jerusalem, Israel) was utilized to provide stimulation as previously described (Li et al 2011). eEPSPs were generated by electrical stimulation with a concentric, platinum/iridium, bipolar electrode (FHC, Bowdoin, ME, USA) placed ~1 mm from the recorded neurons, within the BLA and directly medial to the external capsule. A 10-min baseline period was recorded at the beginning of each experiment to verify consistent cell properties of resistance and evoked response amplitude. For conditions where ZL006 or ZL007 were tested, an extended baseline period (20 min) was recorded to determine if the drug treatments had any effect on amplitude of eEPSPs. The injecting current for evoked responses was adjusted for each neuron to produce roughly 5 mV depolarization. HFS was applied to neurons that demonstrated a consistent baseline to induce potentiation of eEPSPs as previously described (Molosh et al

2014). When bursts of 100 pulses at 100 Hz was delivered once every 20 sec (20 bursts total), it was able to produce short-term and long-term potentiation. Cells that did not display short-term potentiation were removed from analysis.

6.3 Statistical Analysis

The effects of treatment and time across the trials in the fear-related behavioral experiments were compared using two-way repeated ANOVA with *post hoc* Fisher's LSD test. Differences between treatments and overtime in the slice electrophysiology experiment were analyzed using two-way ANOVA followed by *post hoc* Fisher's LSD tests. In other experiments where more than two groups are compared, statistical differences were calculated by one-way ANOVA followed *by post hoc* Fisher's LSD tests. When compare means between two groups, unpaired two-tailed t-test was utilized. The Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. Data were expressed as Mean ± SEM. The sample size was determined based on power calculations from previous reports and/or pilot studies in my laboratory. GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) was utilized for data analysis, and a P value < 0.05 was taken to indicate significant differences.

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Education

2019	Ph.D., Major: Medical Neuroscience; Minor: Life Sciences, Indiana University, Indianapolis, IN
2012	M.S., Neurobiology, Southern Medical University, Guangzhou, China
2009	B.S., Medical Imaging, Southern Medical University, Guangzhou, China

Awards and Fellowships

2017	Graduate Student Travel Fellowship, Indiana University (IU)
2017	Graduate Student Travel Grant, Indiana University School of Medicine (IUSM)
2017	MedNeuro Travel Award, Stark Neurosciences Research Institute, IUSM
2017	The Graduate and Professional Educational Grant, IU
2013	MedNeuro Travel Award, Stark Neurosciences Research Institute, IUSM
2012	Outstanding Graduate of Southern Medical University (SMU)
2009	Recommended Graduate Candidate of SMU: Exempted from Graduate Admission Examination (awarded to Top 1% students)

2007	Top-prize Scholarship for Outstanding College Student of China
2007	Top-prize Scholarship for Excellent Medical Student of SMU
2006	Excellent Student Leadership Award of SMU

Publications

1. **L-P Li**, ET Dustrude, MM Haulcomb, AR Abreu, SD Fitz, PL Johnson, GA Thakur, AI Molosh, Y Lai, A Shekhar (2018), PSD95 and nNOS interaction as a novel molecular target to modulate conditioned fear: relevance to PTSD, *Translational psychiatry*, 14;8(1):155.

2. X Cao, **L-P Li**, Q Wang, Q Wu, H-H Hu, M Zhang, Y-Y Fang, J Zhang, S-J Li, W-C Xiong, H-C Yan, Y-B Gao, J-H Liu, X-W Li, L-R Sun, Y-N Zeng, X-H Zhu & T-M Gao (2013), Astrocyte-derived ATP modulates depressive-like behaviors, *Nature Medicine*, 19, 773-777.

3. X Cao, **L-P Li**, X-H Qin, S-J Li, M Zhang, Q Wang, H-H Hu, Y-Y Fang, Y-B Gao, X-W Li, L-R Sun, W-C Xiong, T-M Gao, X-H Zhu (2013), Astrocytic adenosine 5'-triphosphate release regulates the proliferation of neural stem cells in the adult hippocampus, *Stem Cells*, 31(8):1633-43.

4. Y-B Gao, **L-P Li**, X-H Zhu, T-M Gao (2012), Recent progress in neurobiological mechanisms of depression, *Chin J of Physiology*, 64(4):475-80.

5. **L-P Li**, Q Wang, Y-L Pan, Y-H Zhang, J-Y Wei, Y-H Chen, T-M Gao (2012), Behavioral manifestations of C57BL/6J and NIH Swiss mice in animal models of depression, *Chin J Biomed Eng*,, Vol. 18, No.3.

Manuscripts in preparation/under review

1. J Patel, **L-P Li**, ET Dustrude, AR Abreu, MM Haulcomb, AI Molosh, A Shekhar, Disruption of PSD95/nNOS interaction alters gene expression profiles in the basolateral amygdala after fear conditioning (Co-first author, In preparation).

2. AR Abreu, PL Johnson, SD Fitz, **L-P Li**, CS Bernabe, IF Caliman, LT Mesquita, AA Souza, SJ Yan, HX Ren, AI Molosh, TA Zimmers, DA. Chianca Jr., RC. de Menezes, A Shekhar, Effects of high-fat diet during adolescence on orexin system and the development of hypertension, anxiety and panic-like responses in adult rats (revised manuscript under review at *The Journal of Physiology*).

Abstracts and Poster Presentations

1. ET Dustrude, **L-P Li**, SD Fitz, AI Molosh, Y Lai, A Shekhar (2018), Network dynamics of PSD95 and nNOS interaction in the acquisition of conditioned fear, The Society for Neuroscience Annual Meeting, San Diego, CA

2. **L-P Li**, ET Dustrude, MM Haulcomb, AR Abreu, SD Fitz, X Rao, GA Thakur, Y Liu, AI Molosh, Y Lai, A Shekhar (2018), The role of PSD95 and nNOS interaction in the regulation of conditioned fear: a novel target for treatment of PTSD?, Society of Biological Psychiatry Annual Meeting, New York, NY

3. **L-P Li**, ET Dustrude, MM Haulcomb, AR Abreu, SD Fitz, GA Thakur, AI Molosh, Y Lai, A Shekhar (2017), PSD95 and nNOS interaction in the regulation

of conditioned fear: a novel molecular target for PTSD, The Society for Neuroscience Annual Meeting, Washington DC

4. **L-P Li**, ET Dustrude, MM Haulcomb, AR Abreu, SD Fitz, S Florio, AI Molosh, Y Lai, A Shekhar (2017), Inhibition at PSD95-nNOS binding in the amygdala blocks LTP and reduces fear, Gill Symposium, Bloomington, IN

5. **L-P Li**, ET Dustrude, MM Haulcomb, SD Fitz, AI Molosh, Y Lai, A Shekhar (2016), Inhibition at PSD95-nNOS interface has fear-reducing properties in rats, Indianapolis Society for Neuroscience Annual Meeting, Indianapolis, IN

6. AR Abreu, CS Bernabe, SD Fitz, **L-P Li**, IF Caliman, PL Johnson, LT Mesquita, AA Souza, SJ Yan, HX Ren, AI Molosh, TA Zimmers, DA. Chianca Jr., RC. de Menezes, A Shekhar (2016), High fat diet obese rats have increase anxiety and enhanced panic-associated cardiovascular responses to CO_2 challenge, FASEB Science Research Conferences, Saxtons River, VT

7. **L-P Li**, D Arendt, C Bernabe, LM Federici, R Patibandla, SD Fitz, ZA Rodd, G Deehan, PL Johnson, A Shekhar (2013), Pharmacological depletion of serotonin in the basolateral amygdala reduces anxiety and disrupts fear conditioning, The Society for Neuroscience Annual Meeting, San Diego, CA

Teaching and Service Activities

2014	Student Ambassador Volunteer, IUSM graduate program campus recruits
2011	Lecturer for Animal Behavioral Tests in Neuroscience Course, SMU

2006 – 2009	Volunteer, Red Cross Society of SMU
2005 – 2007	Vice President, The Students' Union of SMU

Professional Affiliations

2013 – Present	The Society for Neuroscience
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Certificates

2019	Good Clinical Practice (GCP), CITI Program
2019	Good Laboratory Practice (GLP), CITI Program
2017	Certificate of Completion in Making Medicines: The Process of Drug Development Course, Eli Lilly