2010-12-15

The Roles of Nitric Oxide Signaling and cAMP Response Element Binding Protein in Fear Conditioning

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THE ROLES OF NITRIC OXIDE SIGNALING AND cAMP RESPONSE ELEMENT BINDING PROTEIN IN FEAR CONDITIONING

By

Jonathan Burke Kelley

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2010
THE ROLES OF NITRIC OXIDE SIGNALING AND cAMP RESPONSE ELEMENT BINDING PROTEIN IN FEAR CONDITIONING

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Pavlovian fear conditioning has face validity for acquired anxiety disorders such as posttraumatic stress disorder (PTSD) and specific phobias. Memory formation mediated by synaptic plasticity, including long-term potentiation (LTP), underlies fear conditioning. The established roles of nitric oxide (NO) signaling in synaptic plasticity and LTP suggest that NO signaling may be critical for fear conditioning. Therefore, my research objectives were to investigate the roles of NO signaling in the acquisition and consolidation of fear memory following fear conditioning. I hypothesized that genetic and pharmacological inhibitions of NO signaling will impair fear conditioning, and that pharmacological facilitation of NO signaling will improve fear conditioning. I found that in the absence of the neuronal nitric oxide synthase gene (nNOS knockout mice) there were complete impairments in contextual and visually cued fear conditioning and a partial impairment in auditory cued fear conditioning compared to wild-type (WT) counterparts. A positive correlation was shown between the magnitudes of the physiological (stress hormone) and behavioral (freezing) responses to conditioned fearful stimuli in both genotypes. Investigations of basal expressions of memory related proteins that are downstream of NO signaling revealed significant dysregulations in naïve nNOS
knockout mice compared to WT mice. Specifically, cyclic guanosine monophosphate (cGMP) was decreased while phosphorylated cyclic adenosine monophosphate binding protein (pCREB) was increased in the amygdala and hippocampus of nNOS knockout mice. Pharmacological inhibition of nNOS in WT mice resulted in impaired fear conditioning, and NO donor administration to nNOS knockout mice partially restored the fear conditioning deficits. Also, the nNOS inhibitor reduced expressions of cGMP and pCREB in WT mice, and NO donor administration partially restored the aberrant expressions of cGMP and pCREB in nNOS knockout mice. Thus, the behavioral and molecular results of the pharmacological experiments corroborated with the genetic experiments. It was also determined that multiple training sessions improved contextual and auditory, but not visually, cued fear conditioning in the nNOS knockout mice. Together, this line of investigations has revealed that the role of NO signaling in fear conditioning is dependent on a) the type of the conditioned stimulus, and b) the intensity of the training.
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<tbody>
<tr>
<td>7-NI</td>
<td>7-nitroindazole</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>BA</td>
<td>Basal nucleus of the amygdala</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CE</td>
<td>Central nucleus of amygdala</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CR</td>
<td>Conditioned response</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>E-LTP</td>
<td>Early-phase long term potentiation</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-related kinase (isoforms 1 and 2)</td>
</tr>
<tr>
<td>ir</td>
<td>Immunoreactive</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LA</td>
<td>Lateral nucleus of the amygdala</td>
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<tr>
<td>LGN</td>
<td>Lateral geniculate nucleus of the thalamus</td>
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<tr>
<td>L-LTP</td>
<td>Late-phase long term potentiation</td>
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<tr>
<td>LP</td>
<td>Lateral posterior nucleus of the thalamus</td>
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<tr>
<td>LTM</td>
<td>Long-term memory</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MGN</td>
<td>Medial geniculate nucleus of the thalamus</td>
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<tr>
<td>MOL</td>
<td>Molsidomine</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<td>PIN</td>
<td>Posterior intralaminar nucleus of the thalamus</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PTSD</td>
<td>Posttraumatic stress disorder</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
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<tr>
<td>SMTC</td>
<td>S-methyl-thiocitrulline</td>
</tr>
<tr>
<td>STM</td>
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<td>US</td>
<td>Unconditioned stimulus</td>
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<td>VAB</td>
<td>Ventral angular bundle</td>
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<td>WT</td>
<td>Wild-type</td>
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Chapter 1

Introduction

Epidemiology of acquired anxiety disorders

Anxiety disorders are a major public health concern associated with high prevalence, economic burden, and social consequences. In the United States, the lifetime prevalence of anxiety disorders is 28.8%, or about 86 million Americans, making them the most prevalent mental health disorder (Kessler et al., 2005a). The economic burdens are estimated up to $42 billion annually, or nearly one-third of the country’s total mental health bill (Greenberg et al., 1999). A contributing factor to the economic burden is an exceptionally high comorbidity rate reaching 80% with other psychiatric conditions including depression, substance abuse, and other types of anxiety (Brady, 1997). While the economic costs are substantial, the personal costs of prolonged suffering are incalculable.

Certain forms of anxiety disorders are acquired, including specific phobia (affecting 19 million Americans per year) and posttraumatic stress disorder (PTSD; 7.7 million Americans per year) (Kessler et al., 2005b). The criteria for diagnosis specified in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) include abnormal or inappropriate anxiety (i.e. flight-or-fight phenomenon) which disrupts an individual’s daily routine, self-esteem, and personal relationships (American Psychiatric Association, DSM-IV, 4th edition, 2000). Essentially, these disorders are characterized by irrational and intense fear reactions to certain stimuli or situations that do not warrant such reactions, and exposure to a traumatic event is an essential risk factor (Briere et al., 2005; Fyer, 1998). In addition to environmental risk factors, a complex combination of
genetic and personality risk factors are also known to play a role in their development (Mineka and Ohman, 2002).

Current treatment strategies utilize a combination of exposure-based psychotherapy and medication treatments including serotonin selective reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and benzodiazepines for treatment resistant cases (Blanco et al., 2003). It is believed that current pharmacotherapies reduce the severity of acute anxiety responses to certain cues or situations (Davis et al., 2006). Unfortunately, the anxiolytic effects of the drugs do not usually persist after discontinuation and also relapse among non-adherent patients is common (Blanco et al., 2003). New avenues for the development of medications that have persistent effects are an active and exciting area of research. Recently, dysfunctions of glutamatergic signaling have been implicated in the pathogenesis of PTSD (Garakani et al., 2006). The major goal of this thesis was to investigate the neurochemical mechanisms involved in the development of acquired “fear based” anxiety, so that the findings can be used to develop novel, effective, and long-lasting pharmacotherapies for anxiety disorders.

**The fear conditioning paradigm**

Our understanding of anxiety disorders has been vastly improved from research using the fear conditioning paradigm. Fear conditioning is based on Pavlovian conditioning in which an organism learns to predict aversive events based on associative learning. During fear conditioning, the presentation of a neutral stimulus (conditioned stimulus, CS) is temporally paired with the presentation of an aversive and painful stimulus (unconditioned stimulus, US). The subject learns that CS presentation is
predictive of the US after one or more CS-US pairings. Subsequently, presentation of the CS alone elicits similar physiological responses as the innate responses to the US. In addition, elements of the conditioning context become predictive of the US, and subsequent re-exposure to the context elicits a similar response as the US (Blanchard and Blanchard, 1969). Typical examples of neutral CS used for fear conditioning include tone, light, and gentle somatosensory stimulation while electric shock is the primary US used in human and animal studies.

The expression of learned fear (i.e. the conditioned response, CR) functions to prepare an organism for “fight-or-flight” responding. The conditioned fear response thus has the same function as the innate (unconditioned) fear response. In nature, fear-related CR serves a valuable evolutionary function, yet in pathological anxiety such as PTSD and phobias the responses to certain cues and situations become maladaptive. In rodents, the species specific CRs include defensive behaviors (behavioral freezing, potentiated startle reflexes), autonomic stimulation (increased heart rate, breathing), and endocrine responses (stress hormone release) (Blanchard and Blanchard, 1969). In human studies, the CRs commonly measured are galvanic skin response, increased heart rate, and verbal report (Rosen and Schulkin, 1998). The learned fear and CR in rodents following fear conditioning are considered analogous to the development and expression of the symptoms of PTSD in humans (Mineka and Oehlberg, 2008).

A major advantage of using the fear conditioning paradigm is the ability to investigate the contributions of various brain structures and neurochemical mechanisms in fear learning to cues and contextual stimuli. Discrimination of the roles of different components of fear conditioning, such as NO signaling, will advance our understanding
fear learning and certain anxiety disorders. In this thesis, the roles of various neuronal signaling molecules and training intensities for cued and contextual fear conditioning are extensively studied.

**Neuroanatomy of fear conditioning**

The neuroanatomical substrates of fear conditioning have been well characterized in animals and humans. Figure 1.1 shows a schematic representation of the major brain circuits involved in cued (Panel A) and contextual (Panel B) fear conditioning. Briefly, unimodal sensory information regarding the CS and US is transmitted first to the thalamus, and then to the amygdala via “direct” or “indirect” pathways (LeDoux et al., 1990; LeDoux et al., 1991). At the same time, polymodal information regarding the context is transmitted to the amygdala via the hippocampal formation (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Thus, the amygdala serves as the first brain substrate at which information related to the CS, US, and context of fear conditioning converges. Efferent projections from the amygdala to the brainstem, hypothalamus, and cortex regulate the physiological and emotional expressions of fear response (LeDoux et al., 1988). The following 4 subsections describe in greater detail the roles of the amygdala, hippocampus, and the US and CS processing pathways in fear conditioning.

**Amygdala:** As previously mentioned, the amygdala serves as the anatomical convergence site for sensory and contextual information related to fear conditioning (LeDoux et al., 1990; Phillips and LeDoux, 1992). The amygdaloid complex is a collection of 12-14 interconnected nuclei located in the medial temporal lobe. Three amygdalar nuclei are implicated in fear conditioning: the lateral nucleus (LA), basal
nucleus (BA), and central nucleus (CE) (LeDoux, 2000). Figure 1.2 shows the major nuclei and functional pathways within the amygdala for cued and contextual fear conditioning.

Neural tracing studies have revealed that the LA receives auditory, visual, gustatory, olfactory, and somatosensory information from the thalamus and cortex (LeDoux et al., 1990). Similar studies have shown that the BA receives hippocampal projections (Kishi et al., 2006). The proximity and topographic organization of the afferent projection synapses in the LA and BA led to the hypothesis that these nuclei are the loci of associative learning. Lesion studies confirmed the requirements of the LA for the acquisition of cued fear conditioning (LeDoux et al., 1990) and the BA for the acquisition of contextual fear conditioning (Maren et al., 1996). Human and non-human primate studies have confirmed roles for the LA and BA in the acquisition of fear conditioning, suggesting a conservation of function across species (Antoniadis et al., 2009; LaBar et al., 1998; Morris et al., 2001).

Profuse intra-amygdalar pathways consisting of local projection neurons originate in the LA and BA and synapse in the CE (Pitkanen et al., 1997). The CE is considered the major output nucleus of the amygdala because its activity and efferent projections modulate the autonomic, behavioral, and cognitive aspects of fear responses (LeDoux, 2000). Electrical and chemical (DL-homocysteic acid) stimulations of the CE elicited a wide range of fear responses (al Maskati and Zbrozyna, 1989), and conversely damage to the CE interferes with innate and conditioned fear responses (Gentile et al., 1986; Hitchcock and Davis, 1991; Killcross et al., 1997). A study in rats showed that electrical kindling of the amygdala enhanced behavioral CR and resulted in c-fos mRNA
expression (marker for neuronal activation) throughout the limbic system and neocortex (Rosen et al., 1996).

Direct observations of the functional coupling between the CE and its anatomical targets have helped elucidate each target’s specific contribution to the fear-related CR. Two major efferent pathways permit the CE to control the innate and conditioned fear responses: 1) the stria terminalis and 2) the ventral amygdalofugal pathway. Lesions to the target areas of these pathways abolished specific components of fear response (LeDoux et al., 1988), while damage to the CE generally disturbed a wide variety of defensive responses (Gentile et al., 1986; Hitchcock and Davis, 1991; Killcross et al., 1997). The study by LeDoux et al. (1988) found that the autonomic components of fear response were primarily mediated by the stria terminalis, while the ventral amygdalofugal pathway had greater influence over behavioral and cognitive components of fear response. The stria terminalis has projections to the lateral hypothalamus (blood pressure regulation, tachycardia, galvanic skin response), parabrachial nucleus (respiration), and the paraventricular nucleus of the hypothalamus (corticosteroid release) (Iwata et al., 1986). The ventral amygdalofugal pathway stimulates the central grey (defensive freezing) and the thalamic reticular nucleus (augmented startle reflexes) (LeDoux et al., 1998). Amygdalar influence on the cognitive and perceptive aspects of fear response are mediated by the ventral amygdalofugal pathway as well. First, the rostral cingulate gyrus and the orbitofrontal cortex, which are areas associated with emotional processing and decision making, receive CE projections. Second, CE projections can modulate the major neurotransmitter systems in the brain that are involved in arousal and vigilance: namely, the ventral tegmental area (dopamine), locus ceruleus (norepinephrine) and
laterodorsal tegmental nucleus (acetylcholine) (Davis, 1992; LeDoux, 2000; LeDoux et al., 1988).

**CS pathways:** The CS pathways involved in cued fear conditioning begin with the sensory organs, project to the sensory thalamus, and ultimately reach the LA via two pathways: the “direct” thalamo-amygdala or the “indirect” thalamo-cortico-amygdala circuits (Figure 1.1A) (Romanski and LeDoux, 1992). The direct pathway is entirely sub-cortical and involves monosynaptic projections from modality-specific thalamic nuclei to the LA. For auditory cued fear conditioning, the medial geniculate nucleus (MGN) and posterior intralaminar nucleus (PIN) of the thalamus are recruited (Quirk et al., 1997; Romanski and LeDoux, 1992). For visually cued fear conditioning, the lateral geniculate nucleus (LGN) and the lateral posterior (LP) nucleus of the thalamus are recruited (Shi and Davis, 2001). This direct pathway is believed to be a primitive mechanism of defense which has the function to quickly and subconsciously elicit fear responses (LeDoux et al., 1990).

The indirect pathway, which presumably evolved later, involves projections from the sensory thalamic nuclei to the cortex before reaching the LA (Figure 1.1A). The sequence of processing in the cortex begins with the primary sensory cortices, continues to the association cortices, and finally the limbic cortices may be involved (Yaniv et al., 2001). For auditory cued fear conditioning, the primary auditory cortex (A1) and auditory association cortex (TE3/TE1) are presynaptic to the LA (Romanski and LeDoux, 1992). For visually cued fear conditioning, the primary visual cortex (V1) and visual association cortex (TE2) are presynaptic to the LA (Shi and Davis, 2001). The perirhinal cortex (PR), which is a polymodal association area, has strong and reciprocal connections
with the amygdala and has been shown to play a role in auditory and visually cued fear conditioning (Shi and Davis, 2001; Suzuki, 1996). The functional role of the indirect pathway is believed to involve perceptual feedback and emotional processing of the cue before the signal is relayed to the LA (Yaniv et al., 2001).

There is some redundancy with the direct and indirect pathways because a) both pathways are activated during fear conditioning, and b) each pathway is sufficient by itself to support fear conditioning when damage has occurred to the other. These redundancies were discovered in rodents (Romanski and LeDoux, 1992; Shi and Davis, 2001) and they may hold true for humans as well. An interesting clinical case study documented a patient with complete bilateral cortical blindness (V1 occlusion) who successfully acquired fear conditioning to a visual cue (Hamm et al., 2003). This case demonstrates that cued fear learning can occur without cortical representation. It is important to note, however, that damage to both pathways prevents fear conditioning in rats (Romanski and LeDoux, 1992).

Electrophysiological and neuroimaging studies have demonstrated asymmetrical processing between the two pathways (Morris et al., 1999; Quirk et al., 1997). For instance, in vivo single-unit recording studies showed that the minimum response latency in the LA following cortical stimulation (A1) was 20 milliseconds, while direct thalamic stimulation evoked responses in the LA 12 milliseconds later (Quirk et al., 1997). An earlier study had shown that LA neurons exhibited potentiated auditory-evoked responses within 15 milliseconds of footshock (Quirk et al., 1995). These findings suggest that the direct pathway initiates plasticity in the LA antecedent to the direct pathway. Also, in the indirect pathway, posttraining auditory-evoked responses in the auditory association
cortex (TE3/TE1) were smaller and occurred temporally later than posttraining auditory-evoked responses in the LA (Quirk et al., 1997). This suggested that magnitude and rate of training-induced plasticity was reduced in the indirect pathway compared to the direct pathway. Evidence from functional magnetic resonance imaging (fMRI) studies in humans support the electrophysiological conclusions. Blood oxygen level dependent (BOLD) responses in the amygdala during fear conditioning correlated with the BOLD response in the thalamus but not the cortex (Morris et al., 1999). Overall, evidence suggests that plasticity in the indirect pathway is slower and more difficult to induce than plasticity in the direct pathway.

**US pathways:** The transmission of nociceptive information from the periphery (i.e. footshock US) to the forebrain occurs via the spinothalamic pain tract. This tract has terminations in the paraventricular nucleus (PVN) of the thalamus, which in turn has thick and monosynaptic projections with the LA and BA that comprise the direct US pathway (Figure 1.1A) (LeDoux, 2000). Interestingly, neurons in the dorsal subdivision of the LA were shown to be responsive to both nociceptive (US) and auditory stimuli (CS), confirming the tracing studies that suggested that multimodal information becomes integrated in the LA (LeDoux et al., 1990; Romanski et al., 1993). Similar to the CS pathways, an indirect pathway has been described for US processing (Figure 1.1A) (LeDoux, 2000). The spinothalamic pain tract has terminations in the ventral posterolateral nucleus (VPN) of the thalamus, which projects to the somatosensory cortex (McDonald, 1998). The somatosensory cortex has projections to the LA and BA through a cascade of higher order association areas of the cortex (McDonald, 1998). Importantly,
the US information is transmitted to both the LA and BA, which has significance for both
cued and contextual fear conditioning, respectively.

Hippocampus: While the amygdala has roles in both cued and contextual fear
conditioning, the hippocampal formation is required only for contextual fear conditioning
(Phillips and LeDoux, 1992). Preconditioning lesions of the dorsal hippocampus
selectively impaired contextual but not cued fear conditioning (Phillips and LeDoux,
1994). Selective ablations of the subfields of the hippocampus have revealed more
specific requirements. The CA3 and CA1 subfields in the dorsal portion of the
hippocampus were found to be required for the acquisition of contextual fear
conditioning, while the ventral portions of CA3 and CA1 were essential for the
expression of contextual fear (Hunsaker and Kesner, 2008). A time-dependent role of the
dorsal hippocampus in contextual fear conditioning has been shown by posttraining
lesion studies. Lesions which occurred 1 or 7 days posttraining produced retrograde
amnesia of previously learned contextual fear, while contextual fear was spared when the
lesions occurred 28 days posttraining (Anagnostaras et al., 1999; Kim and Fanselow,
1992; Maren et al., 1997). This suggests that the hippocampus has a time-limited role in
the formation of contextual fear memory acquisition and consolidation, and subsequently
the fear memory is permanently stored elsewhere.

The functional role of the hippocampus during fear memory acquisition is the
configuration and temporary maintenance of a unified representation of the contextual CS
(Figure 1.1B) (Anagnostaras et al., 2001; Phillips and LeDoux, 1992). This function is a
complex task due to the multimodal and temporally diffuse nature of the contextual
elements. Nevertheless, this function is beneficial to the organism because unification of
the individual elements of the context (e.g. floor texture, lighting, spatial parameters) becomes a more accurate (and stronger) predictor of the footshock than the individual contextual elements (Anagnostaras et al., 2001). Hippocampal long-term potentiation (LTP) in the Schaffer collateral pathway is thought to mediate this process (Phillips and LeDoux, 1992).

The hippocampal formation transmits the contextual information to the BA via the ventral angular bundle (VAB). This pathway includes connections originating in the CA1 and subiculum subregions of the hippocampus, the rostral entorhinal cortex, and areas 35 and 36 of the perirhinal cortex (Pitkanen et al., 2000). Pharmacological inhibition of glutamatergic signaling in the VAB was shown to impair contextual fear conditioning (Maren and Fanselow, 1995). In the same study, discrete lesions to either the subiculum, entorhinal cortex, or the BA impaired contextual fear conditioning (Maren and Fanselow, 1995). Similar to the role of the LA for cued fear conditioning, the BA supports the formation of context-US associations because it receives input from both the hippocampus and the US pathway (LeDoux, 2000; McDonald, 1998).

**Cellular mechanisms of fear conditioning: Synaptic plasticity**

It is widely accepted that synaptic plasticity in the LA, such as LTP and Hebbian learning, mediates cued fear conditioning (Figure 1.1A). LTP was previously known to occur in the LA following Ca$^{2+}$ influx through activated voltage gated calcium channels and the N-methyl-D-aspartate receptor (NMDAR) (Bauer et al., 2002; Chapman et al., 1990). Extracellular field potential recordings showed that stimulation of the MGN of the thalamus induced LTP in the LA in vivo (Clugnet and LeDoux, 1990; Rogan and LeDoux, 1995; Rogan et al., 1997). These studies used low-frequency electrical and
natural (auditory) stimulations to measure evoked potentials in the LA of rats, and found that they were larger after fear conditioning compared to preconditioning levels. Also, single-unit recording studies confirmed that auditory evoked responses in neurons of the LA, which were normally responsive to both auditory and somatosensory stimuli, are enhanced following pairing with an US (Quirk et al., 1997; Quirk et al., 1995). Later studies showed a positive correlation between the magnitude of LTP in the LA and the behavioral expression of CRs for cued fear conditioning (Blair et al., 2001; Sigurdsson et al., 2007). Pharmacological blockade of the glutamate binding site of NMDAR by APV (2R-amino-5-phosphonovaleric acid) in the LA impaired amygdalar LTP induced by thalamic stimulation in vitro and by fear conditioning in vivo (Bauer et al., 2002).

Studies have also shown a requirement for hippocampal LTP in contextual fear conditioning (Figure 1.1B). As previously mentioned, the assembly of a unified contextual representation is dependent on hippocampal LTP in the Schaffer collateral pathway (Phillips and LeDoux, 1992). Pharmacological experiments showed that NMDAR antagonism (APV) in the hippocampus blocked LTP and the acquisition of contextual fear conditioning (Young et al., 1994). Also, a positive correlation between the magnitude of hippocampal LTP and the rate of contextual fear conditioning was observed (Maren et al., 1994). In addition to a role of hippocampal LTP in contextual fear conditioning, amygdalar LTP and the NMDAR are also important for contextual fear conditioning. Similar to the findings in the LA for cued fear conditioning, NMDAR antagonism (APV) in the BA impaired contextual fear conditioning (Maren and Fanselow, 1995). Further, in vivo stimulation of the VAB elicited LTP in the BA (Maren
and Fanselow, 1995). Therefore, it has emerged that synaptic plasticity has a dual role in contextual fear conditioning: first in the hippocampus and the second in the BA.

Synaptic plasticity and LTP also occur in the indirect cortical pathways following fear conditioning. For instance, auditory fear conditioning induces plasticity in the auditory cortex (Quirk et al., 1997) and the MGN of the thalamus (Maren et al., 2001; Overeem et al., 2010; Quirk et al., 1995). Parallel to these observations, the visual fear conditioning pathway also exhibits synaptic plasticity (Shi and Davis, 2001). Further roles of synaptic plasticity in response to fear conditioning have been observed in the CE (Samson et al., 2005) and the cerebellum (Zhu et al., 2007). The requirement of these forms of plasticity for fear learning has not been confirmed, in contrast to the requirements of synaptic plasticity in the LA, BA, and hippocampus which have been confirmed.

The process of LTP consists of first, an increase in synaptic strength, and second, potentiation of the postsynaptic cell so that it is more likely to fire an action potential with a constant synaptic input (Bliss and Lomo, 1973). For fear conditioning, amygdalar LTP explains potentiated electrical responses of LA neurons to acoustic stimuli following auditory fear conditioning (Quirk et al., 1995). LTP is mediated by increased neurotransmitter release, transcription of new proteins (e.g. AMPA receptors), and morphological changes (Sweatt, 1999). LTP is subdivided into three sequential phases which generally correlate with the major stages of memory formation. First, initial LTP and short-term memory (STM) last about 30-45 min; the mechanisms are largely unknown but likely require transient changes in second messengers and enhanced synaptic vesicle release (Arancio et al., 1995; Little and Shaw, 1975; Roberson and
Sweatt, 1996). Early-phase LTP involves the persistent activation of protein kinases beginning about after 30 minutes and lasting 2-3 hours (Sweatt, 1999). Late-phase LTP involves changes in gene expression and lasts many hours (Sweatt, 1999). The latter two phases of LTP are associated with long-term memory (LTM) consolidation, during which STM is converted to relatively permanent LTM (McGaugh, 2000). The late-phase of LTP and consolidation of LTM require the transcription and translation of new proteins (Bailey and Chen, 1989; Davis and Squire, 1984; Kandel, 2001). It is understood that the consolidation of LTM for fear conditioning requires postsynaptic Ca\(^{2+}\) influx, which is in response to activation of amygdalar voltage-gated Ca\(^{2+}\) channels and NMDAR (Bauer et al., 2002). Also, postsynaptic Ca\(^{2+}\) influx occurs in the hippocampus and is a requirement for contextual fear conditioning (Maren and Fanselow, 1995).

**Molecular mechanisms of fear conditioning: Role of the NMDA receptor**

NMDAR activation allows intracellular Ca\(^{2+}\) increase, which is known to lead to LTP and LTM consolidation in many species (DeZazzo and Tully, 1995). A downstream mechanism of NMDAR activation is the production of second messenger signaling molecules such as cyclic adenosine monophosphate (cAMP) and activation of the cAMP response element binding protein (CREB) (Cammarota et al., 2000). The NMDAR→cAMP→CREB pathway is essential for synaptic plasticity and late-phase LTP (DeZazzo and Tully, 1995; Schulz et al., 1999). Activation of CREB leads to the transcription of genes with a CRE promoter sequence; a substantial subset of CRE genes have roles in LTP including the gene for neuronal nitric oxide synthase (nNOS) (Sasaki et al., 2000).
Several pharmacological studies have established the requirement of NMDAR-mediated signaling for fear conditioning. Pretraining administrations of NMDAR antagonist (APV) directly to the LA and BA prevented fear conditioning, and posttraining administrations had no effect (Fanselow and Kim, 1994; Miserendino et al., 1990; Walker and Davis, 2000). These studies also investigated the effects of NMDAR antagonism in the CE and did not observe any effects, suggesting that the NMDAR is critical for fear memory acquisition rather than expression. It was later shown that selective blockade of the NR2B subunit of the NMDAR with ifenprodil in the amygdala prevented cued and contextual fear conditioning, indicating that the NR2B subunit of the NMDAR has a specific role in fear learning (Rodrigues et al., 2001).

The effects of NMDAR antagonism (APV) in the hippocampus on contextual fear conditioning are similar to the effects described for the amygdala. Direct injection of APV to the ventrocaudal hippocampus blocked LTP and the acquisition of contextual fear conditioning (Maren and Fanselow, 1995). Recently, studies using CA3 restricted NMDAR knock-out mice suggested that NMDAR in the CA3 subfield of the hippocampus are required for the formation of unified context memory representations and the acquisition of contextual fear conditioning (Cravens et al., 2006; McHugh and Tonegawa, 2009). This is consistent with the role of NMDAR-mediated LTP in the Schaffer collateral pathway for contextual fear conditioning.

**Molecular mechanisms of fear conditioning: Role of ERK1/2**

Extracellular signal-related kinase (ERK isoforms 1 and 2) in mature neurons serves as a biochemical convergence substrate for multiple signaling pathways, including NO, that lead to gene transcription and LTM (Figure 1.6A) (Sweatt, 2001). ERK
activation, which is downstream of Ca\textsuperscript{2+} influx and NMDAR activation, is required for the full expression of late-phase LTP and CREB-dependent gene transcription in hippocampal neurons (Impey et al., 1998a). In vitro, NMDAR-mediated LTP in amygdalar and hippocampal slices has been shown to require ERK1/2 signaling (English and Sweatt, 1996; Impey et al., 1998a; Ota et al., 2008). Roles for ERK1/2 in vivo following cued (Schafe et al., 2000; Tarpley et al., 2009) and contextual (Atkins et al., 1998) fear conditioning have also been demonstrated. In the behavioral studies, ERK1/2 activation was significantly increased 1 hour after fear conditioning in the rat amygdala and hippocampus, while pharmacological inhibition of ERK led to long term fear learning impairments. Recently, a requirement for ERK 2 signaling in the dorsal hippocampus and entorhinal cortex was suggested for STM of fear conditioning (Igaz et al., 2006). It is hypothesized in this thesis that differences in the activation state of ERK, which is downstream of NO, will have a positive correlation with successful acquisition and LTM consolidation of fear conditioning.

**Molecular mechanisms of fear conditioning: Role of CREB**

Late-phase LTP and LTM formation are protein synthesis-dependent and require the dynamic regulation of nuclear transcription factors, including CREB (Kaang et al., 1993). CREB is a family of constitutively expressed transcription factors whose activity is regulated by the phosphorylation state of CREB at the serine-133 residue (Lamph et al., 1990). The phosphorylation of CREB (pCREB) can occur through a number of kinase signaling cascades including PKA, PKB, CaMKII, CaMKIV, and ERK1/2 (Silva et al., 1998). pCREB promotes the formation of a DNA binding complex with CREB binding protein and p300 (Chrivia et al., 1993). Studies in various species including mice
(Bourtchuladze et al., 1994), aplysia (Dash et al., 1990; Kaang et al., 1993), and
drosophila (Yin et al., 1994) have shown that disruptions of CREB function inhibit the
development of LTM for classical conditioning.

A requirement for CREB in fear conditioning was first shown when CREB
deficient mice exhibited deficits in LTM, but not STM, for cued and contextual fear
conditioning (Bourtchuladze et al., 1994). In the LA and CE of the amygdala, CREB
activation occurs following cued fear conditioning (Stanciu et al., 2001). Further, it was
shown that disruption of CREB by an inducible and reversible CREB repressor in the
hippocampus, amygdala, or prefrontal cortex during the consolidation of LTM (i.e.
posttraining) reduced the stability of LTM and the expression of subsequent fear response
(Kida et al., 2002). Several studies have shown that genetic and viral-mediated elevated
CREB expression facilitates the consolidation of auditory cued fear conditioning
(Josselyn et al., 2001; Viosca et al., 2009; Wallace et al., 2004).

In the CA1 and CA3 subfields of the hippocampus, CREB activation has been
observed during the consolidation of contextual fear conditioning (Ahi et al., 2004;
Impey et al., 1998b). It has been reported that in the hippocampus, CREB undergoes
biphasic activation with peaks at 1 hour and 12 hours (Ahi et al., 2004; Trifilieff et al.,
2006). In my preliminary studies in B6129S wild-type (WT) mice, increased pCREB
was found in the dorsal hippocampus 1 hour after contextual fear conditioning (Figures
1.3 and 1.4). In those studies, the effects of context exposure and footshock on pCREB
expression were compared using western blotting and stereological cell counts. The
results were consistent with the previous literature (Ahi et al., 2004; Trifilieff et al.,
2006), and lend support to the studies of this thesis by demonstrating that significant changes in pCREB occur in WT mice following contextual fear conditioning.

Based on the large body of evidence showing the involvement of CREB in fear-related LTM consolidation, the effects of the genetic and pharmacological manipulations of NO signaling on CREB activation are investigated in WT and nNOS KO mice. I hypothesize that inhibition of NO signaling will result in reduced pCREB expression and impaired fear conditioning.

**Hormonal response to fear conditioning: Role of corticosterone**

The CE initiates systemic changes in response to fear stimuli through its regulation of plasma glucocorticoid release (Marchand et al., 2007). The paraventricular nucleus (PVN) of the hypothalamus receives projections from the CE, which upon stimulation secretes the corticotropin-releasing hormone (CRH). CRH then stimulates the release of adrenocorticotropic hormone from the pituitary into the blood stream, which results in the secretion of glucocorticoids from the adrenal cortex (Rodrigues et al., 2009). Corticosterone, the main glucocorticoid in rodents, binds to high affinity mineralocorticoid receptors and low affinity glucocorticoid receptors that are present throughout the periphery and the CNS (Korte, 2001). Acute glucocorticoid signaling can affect the electrophysiological properties of neurons. For instance, bath application of corticosterone to LA and BA neurons in vitro, increases their excitability and reduces intrinsic responses to the inhibitory neurotransmitter GABA (Duvarci and Pare, 2007). In contrast, acute corticosterone has the opposite effect on hippocampal neurons through rapid inhibition of NMDAR-mediated currents via a PKA-dependent mechanism (Liu et al., 2007). Interestingly, selective agonists for the high-affinity mineralocorticoid
receptors in the dentate gyrus, CA3, and CA1 subfields of the hippocampus facilitated LTP, while selective agonists for low-affinity glucocorticoid receptors inhibited LTP (Pavlides and McEwen, 1999). These data suggest that acute and low-level increases of corticosterone are more likely to facilitate hippocampal LTP via high-affinity mineralocorticoid receptors in the hippocampus in a feed-forward manner. Therefore, acute and moderate corticosterone increases have similar effects of facilitating amygdalar and hippocampal LTP.

It has previously been reported that acute and systemic increases of corticosterone contribute to the consolidation of long term, tone-specific cued fear response (Hui et al., 2004; Marchand et al., 2007; Roozendaal et al., 2006) and contextual memory (Pugh et al., 1997; Thompson et al., 2004). In agreement with this, the effects of antagonism of glucocorticoid signaling in the amygdala and the hippocampus impaired LTM formation for cued and contextual fear conditioning (Donley et al., 2005). It is suggested that corticosterone plays a greater role in LTM consolidation than for STM because adrenalectomized rats exhibit intact post-shock freezing (STM) but impaired LTM (Pugh et al., 1997). In humans, high glucocorticoid levels (cortisol) after fear conditioning positively correlated with fear memory consolidation (Zorawski et al., 2006).

**Nitric oxide production in the CNS**

Nitric oxide (NO) is a retrograde messenger produced by three isoforms of nitric oxide synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Ogden and Moore, 1995). eNOS and nNOS are constitutively expressed, soluble cytosolic proteins which are Ca$^{2+}$-dependent, while iNOS is Ca$^{2+}$-independent as its activity is induced by cytokines (Bredt and Snyder, 1994). Figure 1.5 shows the steps
leading to nNOS activation and NO production in CNS neurons. nNOS becomes activated by Ca$^{2+}$-activated calmodulin which forms following Ca$^{2+}$ influx through the activated NMDAR (Brenman and Bredt, 1997). nNOS alpha, the predominant isoform of nNOS accounting for >95% of total nNOS, has a wide and uneven distribution throughout the mammalian brain including expression in the LA, BA, and hippocampus (Bredt et al., 1991; Kelley et al., 2009; Schafe et al., 2005). nNOS is associated with the NR2B subunit of the NMDAR through specialized PDZ binding domains with the postsynaptic density protein 95 (PSD-95) (Brenman et al., 1996). The biochemical association of nNOS with the NMDAR helps explain the link between NO production and NMDAR activation in the CNS (Garthwaite et al., 1989).

Following Ca$^{2+}$/calmodulin binding, nNOS dissociates from the PSD-95 complex and forms a soluble homodimer that consists of 686 amino acid residues. Each subunit of the homodimer maintains reductase ability, and conformational changes allow for electron transfer from NADPH to FAD to FMN to the heme region of the trans-oxygenase domain (Garcin et al., 2004). The production of NO is catalyzed by the conversion of L-arginine to L-citrulline, with NO formed as a byproduct. NO is a gaseous NO molecule that can diffuse in aqueous or lipid environments up to 0.3-0.4mm, which allows retrograde access to the presynaptic terminal (Lancaster, 1997; Wood and Garthwaite, 1994).

**NO signaling cascade for learning and memory**

The primary mechanism of NO signal transduction is activation of soluble guanylyl cyclase (sGC), which produces the second messenger cyclic 3’5’-guanosine monophosphate (cGMP) (Figure 1.6) (Denninger and Marletta, 1999). sGC is considered
the NO receptor and it is found in high abundance near the sources of NO production in
the postsynaptic cell as well as in the presynaptic terminal (Hawkins et al., 1994). In the
presynaptic terminal, increased cGMP production leads to the phosphorylation of
synaptic vesicle proteins which is important for vesicle mobilization, neurotransmitter
release, and STM (Hawkins et al., 1993). Direct injections of NO donors and cGMP into
the presynaptic terminal facilitated LTP in vitro (Arancio et al., 1995; Arancio et al.,
1996). Demonstrations of retrograde signaling have been shown by injections of non-
permeable NO scavengers into the extracellular space. These studies showed that NO
scavengers in the extracellular space prevent LTP in vitro (O'Dell et al., 1991) and
presynaptic gene transcription (Overeem et al., 2010). Thus, retrograde NO signaling is
important for presynaptic plastic events such as enhanced vesicle release, STM, LTP, and
presynaptic gene expression.

In the postsynaptic cell, cGMP-mediated signaling initiates a chain of kinase
activations beginning with protein kinase G (PKG) (Hawkins et al., 1994). PKG can
activate ERK1/2, which then translocates to the nucleus where it phosphorylates the
transcription factor CREB (Chien et al., 2003). Additional evidence suggests that the
NO-cGMP cascade activates adenylyl cyclase (AC), which initiates
cAMP→PKA→CREB signaling (Lu et al., 1999; Matsumoto et al., 2006). This
signaling pathway acts in parallel to the canonical NO→cGMP→CREB signaling
pathway and both promote CREB-mediated gene transcription in the postsynaptic neuron
(Lu et al., 1999). In addition, NO promotes CREB-DNA binding through the process of
S-nitrosylation of nuclear proteins, which facilitates CREB-mediated gene transcription
(Riccio et al., 2006). Figure 1.6A depicts a schematic diagram of the major NO signaling cascades involved in learning and memory.

In rodent amygdalar (Chien et al., 2003; Schafe et al., 2005) and hippocampal slices (Arancio et al., 2001; Lu et al., 1999; Puzzo et al., 2006; Zhuo et al., 1994), synaptic plasticity such as late phase LTP is dependent on NO$\rightarrow$cGMP$\rightarrow$CREB signaling. Behavioral studies have further supported a role for this pathway in learning and memory. Evidence from invertebrates suggest that NO has a major role in the consolidation of LTM (Kemenes et al., 2002; Lewin and Walters, 1999; Muller, 1996; Yin et al., 1994). In rodents, pharmacological inhibitors and facilitators of NO signaling have been shown to modulate spatial learning in the Morris Water Maze (Chien et al., 2005), inhibitory and passive avoidance learning (Bernabeu et al., 1995; Chien et al., 2008; Telegdy and Kokavszky, 1997), and object recognition learning (Furini et al., 2009). Interestingly, all of these tasks are hippocampus-dependent and all except for object recognition involve an aversive training procedure. I hypothesize in the current studies that NO signaling has a role in hippocampus-dependent contextual fear conditioning, which had not been previously shown.

**Nitric oxide signaling: Role in fear learning**

At present, few studies have investigated a role for NO signaling in fear conditioning. The first study investigated the effects of systemic administration of the nNOS inhibitor 7-nitroindazole (7-NI) and found that it did not prevent contextual fear conditioning (Maren, 1998). However, it was concluded by the author that the vehicle used for 7-NI administration caused hypomobility, which may have influenced the results. Another study used the nonspecific NOS inhibitor L-nitro-arginine methyl ester
(L-NAME) and this treatment did not have an effect for visually cued fear conditioning (Johnson et al., 2000). Recently it was shown that intra-amygdalar administrations of 7-NI and the NO scavenger 2-(4-carboxyphenyl)-4,4,5-tetramethylimidazoline-1-oxy-1-3-oxide (c-PTIO) impaired LTM for auditory cued fear conditioning (Schafe et al., 2005). In addition, it was shown that those treatments blocked LTP at thalamic inputs to the LA in vitro (Schafe et al., 2005). Recently, a study showed that intra-amygdalar infusions of inhibitors and activators of PKG, downstream of NO, caused dose-dependent impairments and enhancements, respectively, of auditory cued fear conditioning (Ota et al., 2008). Ota et al. (2008) also showed that the PKG modulators caused corresponding changes in ERK1/2 phosphorylation and LTP at thalamic inputs to the LA in vitro.

Another recent study showed that PKG-beta deficient mice have impaired auditory cued fear conditioning and LTP in the LA (Paul et al., 2008). Thus, a role of NO→cGMP→ERK signaling in the LA has emerged in the consolidation of LTM of auditory cued fear conditioning. In the current studies, the effects of global nNOS gene deletion and pharmacological modulations of NO signaling on the outcomes auditory and visually cued fear conditioning are investigated.

Considering the importance of NO signaling in other hippocampus-mediated learning tasks (Bernabeu et al., 1995; Chien et al., 2008; Chien et al., 2005; Telegdy and Kokavszky, 1997), it is surprising that so few studies have investigated a role for NO in contextual fear conditioning. In addition, nNOS-mediated phasic NO production is critical for hippocampal LTP, a process that is required for contextual fear conditioning (Figure 1.1B) (Hopper and Garthwaite, 2006). In the current studies, the effects of nNOS
gene deletion and pharmacological modulations of NO signaling on the outcome of contextual fear conditioning are investigated.

**Nitric oxide signaling: Previous studies in nNOS KO mice**

Mice with a targeted mutation of the nNOS gene were created by homologous recombination and were confirmed to have no major health, behavior, or histopathological abnormalities in the CNS (Huang et al., 1993). As might be expected, the nNOS KO mice have major deficits in hippocampal LTP which was recovered with high concentrations of exogenous NO donor (Hopper and Garthwaite, 2006).

Behavioral studies in nNOS KO mice have, for the most part, corroborated with findings following pharmacological inhibition of NO signaling. nNOS KO mice exhibit performance deficits in the Morris Water Maze task, in addition abnormalities in hippocampal protein expression (Kirchner et al., 2004; Weitzdoerfer et al., 2004). nNOS KO mice also exhibit impaired olfactory cued recognition memory, concomitant with olfactory bulb protein derangements (Juch et al., 2009). Studies using appetitive conditioning have shown that nNOS KO mice show a deficiency in conditioned place preference, which was shorter lived than in WT counterparts (Balda et al., 2006). It was also recently shown that nNOS KO mice had a complete deficiency in visual cue-dependent conditioned place preference (Itzhak et al., 2010).

**Research objectives and hypothesis**

LTM formation following traumatic events underlies the development of acquired anxiety disorders. Although evidence has implicated NO signaling in LTP and the formation of LTM, relatively little is known about the role of NO signaling in fear learning. It is expected that elucidation of how manipulations of NO signaling strengthen
or suppress fear learning will lead to a better understanding of how acquired anxiety disorders develop. This will advance the fields of NO signaling, fear learning, and medication development for certain anxiety disorders.

The goal of this thesis is to investigate the role of NO signaling in fear conditioning and the molecular implications of LTM formation in the absence of NO signaling. I hypothesize that LTM formation following fear conditioning is NO-dependent. The effects of nNOS gene deletion and inhibition on molecular determinants of learning and memory (i.e. cGMP, ERK, and CREB) are investigated, and it is discussed how these effects correlate with the behavioral outcomes. Understanding the role of NO signaling in fear learning in animal models will lead to improvements in targeted pharmacotherapy development and treatment strategies for anxiety disorders. I hypothesize that genetic and pharmacological inhibitions of NO signaling will reduce cGMP, pERK1/2, and pCREB expressions, while facilitation of NO signaling will increase the expression of the molecules.

In Chapter 2 of this thesis, the roles of the nNOS gene in contextual and auditory cued fear conditioning are investigated. Specifically, the effects of nNOS gene deletion on the behavioral and physiological responses to footshock-induced fear conditioning are investigated in male and female nNOS KO mice and their WT counterparts. Results from this chapter were published in the following paper:

In Chapter 3, the effects of pharmacological modulators of NO signaling on the acquisition and consolidation of fear conditioning are investigated in male WT and nNOS KO mice. Also, the effects of the NO modulators on cGMP production in the hippocampus and amygdala of naïve mice are determined. The results from this chapter were published in the following paper:


In Chapter 4, the role of the nNOS gene for visually cued fear conditioning is investigated in male mice. In addition, basal levels of ERK1/2 and CREB expression and activation are determined in naïve WT and nNOS KO mice. Next, the outcomes of the behaviorally effective doses of NO modulators on ERK1/2 and CREB in the amygdala and hippocampus are determined. The results of this chapter are included in a manuscript which was accepted for publication:


Finally, Chapter 5 is a general discussion and review of the central findings in this thesis. The significance and contributions of the findings to the fields of nitric oxide, fear conditioning, and memory formation are discussed. Lastly, future directions of research stemming from the current findings are explored.
Figure 1.1. Key neural circuits involved in cued and contextual fear conditioning (Panel A modified from Medina et al. 2002; Panel B modified from Maren et al. 2001).

For cued fear conditioning (Panel A), information regarding the sensory cue (visual, auditory CS) and footshock (US) are first relayed to modality-specific regions of the thalamus. From the thalamus, information reaches the lateral amygdala (LA) via two pathways. The direct pathway rapidly transmits information to the LA, and is thought to be important for the initiation of amygdaloid long-term potentiation (LTP) and the automatic (i.e. subconscious) responses to fear conditioning. The indirect pathway transmits information via primary sensory and association cortices to the LA. The indirect pathway is generally weaker and slower than the direct pathway. It plays a role in the cognitive and perceptual responses to fear conditioning. The LA projects to the central nucleus (CE), which is the major output structure of the amygdala. Projections from the CE reach the cingulate gyrus (CG), lateral hypothalamus (LH), and paraventricular nucleus (PVN), among other regions and mediate fear conditioned responses (CR). For contextual fear conditioning (Panel B), polymodal information regarding elements of the context is relayed to the hippocampus. The hippocampus is involved in the configuration of a unified representation of the contextual elements through an LTP-dependent process. The contextual representation is relayed to the basal amygdala (BA) where US information also converges. Amygdaloid LTP is required for context-US association. Also shown is the “weak” pathway which transmits context element information directly to the amygdala.
Figure 1.2. Amygdala structures involved in fear conditioning (modified from LeDoux 2000). The amygdala is a collection of 12-14 nuclei, three of which are relevant for fear conditioning: the lateral amygdala (LA), the basal amygdala nuclei (BA, here shown as B / AB for basal and accessory basal nucleus), and the central amygdala (CE). The piriform cortex (PIR) is lateral to the amygdala, and the caudate-putamen (CPU) is dorsal. Panel A shows a section stained for acetylcholinesterase which delineates the LA, B/AB, and CE. The major intra-amygdalar pathways connecting the nuclei of the amygdala are shown in Panel B. Afferent projections to the amygdala (IN) synapse in the LA and B/AB (not shown), which in turn project to the CE. The CE has efferent projections (OUT) via the stria terminalis and ventralamygdalofugal pathways. Conditioning to a tone CS involves projections from the auditory thalamus and cortex to the LA, and from the LA to the CE (Panel C). Contextual fear conditioning involves projections from the hippocampus to the B/AB, and from the basal nuclei to the CE (Panel D). The CE modulates the expression of fear responses.
Figure 1.3. Time-dependent increases of pCREB in the hippocampus of wild-type mice (B6129S). The effects of context and footshock exposures in WT mice on CREB phosphorylation were investigated in the hippocampus of WT mice. Western blotting analysis of tissue collected 30 minutes and 60 minutes following shock treatment showed a trend for time-dependent increases in pCREB (p=0.08; shocked 30 vs. 60 min). In context-only controls, pCREB was lower than in shocked mice (n=3/group).

Figure 1.4. Increased pCREB in the dorsal hippocampus following contextual fear conditioning. pCREB-immunoreactive (-ir) neurons are increased in shocked WT mice compared to context-only controls 60 minutes later. Panels A and C are representative images of coronal sections of the dorsal hippocampus exhibiting pCREB-ir staining in the CA1 and CA3 subregions (4x; scale bar = 100um). Panels B and D are the boxed areas in A and C, respectively, which showed pCREB-ir neurons the dentate gyrus (DG) (60x, scale bar = 40um). (E) Stereological estimates of the number of pCREB-ir neurons in 10 serial coronal sections of the dorsal hippocampus revealed greater number of pCREB-ir neurons in shocked mice versus context-only controls (*p<0.05; n=5-6/group). The findings are in agreement with literature in rats, which showed increased pCREB 1 hour after contextual fear conditioning.
Figure 1.5. Activation of neuronal nitric oxide synthase (nNOS) in the CNS. The excitatory neurotransmitter glutamate stimulates NMDAR and causes Ca\textsuperscript{2+} influx into the postsynaptic cell. Intracellular Ca\textsuperscript{2+} increase promotes the formation of Ca\textsuperscript{2+}/calmodulin (CaM) complex which interacts with nNOS. nNOS is associated with the post-synaptic density protein (PSD-95) and is located near the plasma membrane. Upon binding of the Ca\textsuperscript{2+}/CaM complex, in combination with BH\textsubscript{4}, nNOS dissociates from PSD-95 and translocates to the cytoplasm. The dephosphorylation of nNOS by calcineurin stimulates the production of nitric oxide (NO) through the enzymatic conversion of L-arginine to L-citrulline. NO positively regulates cyclic guanosine monophosphate (cGMP) production and stimulates cGMP-mediated signaling pathways which have roles in LTP and neurotransmission. nNOS is inactivated by phosphorylation by protein kinase A (PKA) or protein kinase C (PKC), whereupon it relocates to the plasma membrane and becomes associated with PSD-95. Reproduced from http://www.sigmaaldrich.com.
Figure 1.6. The nitric oxide signaling cascade for learning and memory.

NO produced in the postsynaptic cell following NMDAR activation stimulates soluble guanylyl cyclase (sGC), which is found abundantly in the presynaptic terminal and postsynaptic cell. NO can act as a retrograde signal by diffusing across the synaptic cleft. Stimulation of sGC causes the production of the second messenger, cyclic guanosine monophosphate (cGMP). In the presynaptic terminal, cGMP facilitates vesicle release which is important for the early phase of LTP (E-LTP) and short-term memory. cGMP activates protein kinase G (PKG), which phosphorylates (activates) extracellular signal-related kinase isoforms 1 and 2 (pERK1/2). pERK1/2 can translocate to the nucleus where it phosphorylates the transcription factor cyclic adenosine monophosphate response element binding protein (CREB). ERK1/2 and CREB-dependent gene transcription are required for the late phase LTP (L-LTP) and long-term memory. In addition, increased cGMP signaling stimulates adenylate cyclase (AC) to produce cAMP. cAMP activates PKA, which translocates to the nucleus and phosphorylates CREB.
Chapter 2

Impairments in fear conditioning in mice lacking the nNOS gene

Summary

The fear conditioning paradigm has been successfully used to investigate the roles of various genes, neurotransmitters and substrates in the formation of fear learning related to contextual and auditory cues. In the brain, nitric oxide (NO) produced by neuronal nitric oxide synthase (nNOS) functions as a retrograde neuronal messenger that facilitates synaptic plasticity including the late phase of long-term potentiation (LTP) and formation of long-term memory (LTM). Some evidence has implicated NO signaling in synaptic plasticity and LTM formation following fear conditioning, yet little is known about the role of the nNOS gene in fear learning. Using knockout (KO) mice with targeted mutation of the nNOS gene and their wild type (WT) counterparts, the role of NO signaling in fear conditioning was investigated. Plasma levels of the stress hormone corticosterone were also measured to determine the relationship between physiological and behavioral response to fear conditioning. LTM of contextual fear learning was severely impaired in male and female nNOS KO mice compared to WT counterparts; LTM of auditory cued fear learning was only slightly impaired in nNOS KO mice. Sex-dependent differences in both contextual and cued fear learning were not observed in either genotype. Deficits in contextual fear learning in nNOS KO mice were partially overcome by multiple training sessions. A relationship between increase in plasma corticosterone levels following footshock administration and the magnitude of contextual, but not auditory cued, freezing was also observed. Results suggest that the nNOS gene contributes more to optimal contextual fear learning than to cued fear learning, and
therefore inhibition of the nNOS enzyme may ameliorate context-dependent fear response.

**Background**

Anxiety disorders, such as posttraumatic stress disorder (PTSD), constitute the most prevalent mental illnesses in the United States costing nearly one-third of the country’s total health bill (Greenberg et al., 1999). The treatment of these disorders requires overcoming complications such as reluctance to seek mental health treatment and an extremely high comorbidity rate with other affective disorders, reaching 80% (Brady, 1997; Solomon and Davidson, 1997). Emerging evidence suggests that dysfunctions underlying acquired anxiety and PTSD include an abnormal reaction to stress which is mediated by specific neurochemical and neuroanatomical substrates (Adamec, 1997; Yehuda and McFarlane, 1995). Pharmacotherapies which target neuronal signaling molecules, such as NO, may play a role in the treatment of these disorders.

In the brain, N-methyl-D-aspartate receptor (NMDAR) activation and calcium influx into the cell activates the nNOS enzyme to produce NO, which has the role of retrograde messenger (Snyder, 1992). NO is involved in memory formation and synaptic plastic events such as late-phase long-term potentiation (LTP) (Arancio et al., 2001; Lu et al., 1999; Puzzo et al., 2006). Behavioral evidence in invertebrates (Kemenes et al., 2002; Lewin and Walters, 1999; Matsumoto et al., 2006; Muller, 2000) and vertebrates (Medina and Izquierdo, 1995; Rickard et al., 1998) suggest that NO has a major role in consolidation of LTM. Recently studies have shown that site-specific pharmacological blockade of NO signaling in rats impairs contextual (Resstel et al., 2008) and cued
(Schafe et al., 2005) fear learning. However, the role of the nNOS gene in fear conditioning has not been investigated.

In the present study, fear conditioning was investigated in homozygous nNOS knockout (KO) and wild type (WT) mice. In the fear conditioning paradigm, the association of a footshock (unconditioned stimulus; US), with a specific context and a neutral stimulus (auditory cue) results in learned fear. Re-exposure to the conditioning context and to the previously neutral auditory cue (conditioned stimulus; CS) elicits a freezing response in the absence of the aversive US. Thus, the fear conditioning paradigm includes both contextual and cued fear learning components which can be measured in separate tests. Fear conditioning recruits both the amygdala (emotional cue learning) and the hippocampus (spatial/contextual learning) (Goosens and Maren, 2004; Mei et al., 2005; Phillips and LeDoux, 1992). The involvement of these brain regions in fear learning and anxiety has been confirmed by animal and human imaging studies (LeDoux, 1998; Rauch et al., 2006).

We report that nNOS KO mice showed a severe deficiency in contextual fear learning and a less marked deficit in cued fear learning compared to WT mice after a single fear conditioning session. This deficiency was partially improved by multiple (four) fear conditioning sessions. In addition, we observed that plasma levels of corticosterone, the primary stress hormone in rodents, are related to contextual fear learning ability.
Materials and Methods

Animals

nNOS KO mice were generated on a mixed B6;129S genetic background; the targeted deletion of the α subunit of nNOS resulted in >95% reduction in brain nNOS catalytic activity (Huang et al., 1993). Adult male and female homozygous nNOS KO mice (B6;129S4-Nos1; 6-8 weeks old), and the parental strains of their hybrid WT counterparts (C57BL/6J and 129/SvImJ) were purchased from Jackson Laboratories (Bar Harbor, Maine). Our breeding colony, littermate selection, and animal care have been described earlier (Balda et al., 2006). Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and was approved by the University of Miami Animal Care and Use Committee. Adult (8-10 weeks old) WT and nNOS KO mice of both sexes were investigated.

Immunohistochemistry of nNOS

Adult male WT and nNOS KO mice (n=3/group) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), then perfused with sodium phosphate-buffered saline (PBS) followed by 4% p-formaldehyde. Serial coronal sections (50μm) were cut with a Vibratome 1000 (TPI Inc., St. Louis, MO), and treated as described previously (Balda et al., 2006). Sections were incubated (72h; 4°C) with a rabbit anti-nNOS polyclonal antibody (1:3000, Santa Cruz Biotechnology, CA), then further developed according to Vectastain Elite ABC kit directions (Vector Laboratories, Burlingame, CA), using diaminobenzidine (DAB) as a chromogen. nNOS-immunoreactive cells were detected with a compound light microscope (Olympus BX51;
C. Squared Co., USA) attached to a cooled monochrome camera (Retiga 2000R), using Image-Pro Plus software.

**Pain response thresholds in WT and nNOS KO mice**

To investigate genotype sensitivity to noxious stimuli two experiments were performed: a) the hotplate test and b) footshock-induced vocalization threshold.

**Hotplate test**

Male and female WT and KO mice (n=6-8/group) were placed individually inside a heated (55 ± 0.5 °C) glass cylinder (15cm diameter x 25cm high), and the time taken for mice to show the first sign of discomfort (licking paws, flinching or jumping) was recorded by two observers. A cut-off time of 30 seconds was used to prevent tissue damage.

**Vocalization thresholds**

Individual mice (n=6-8/group) were placed in the fear conditioning apparatus and electric footshocks (2 sec) of increasing intensity were delivered until an audible vocalization response was heard by two observers. The shock intensity began at 0.1 mA and was increased in increments of 0.05mA; two minutes elapsed between each shock.

**Fear conditioning**

**Apparatus**

Fear conditioning training and testing occurred in Plexiglas chambers (30.5 x 30.5 x 43.5 cm; Noldus Information Technology Inc., Leesburg, VA). Each chamber is equipped with a stainless-steel rod floor through which the electric shock was delivered, and an upper control panel containing a video camera, a sound emitter and a white light
illuminating one corner of the chamber. The chambers were housed in custom built sound-attenuating cubicles which gave the appearance of black walls to the chamber.

**Conditioning and testing**

A fear conditioning training session consisted of placement in the training context (context-A) and after 2 min an auditory cue (2.3 kHz; 70 dB) sounded for 30 sec which co-terminated with a 2 sec footshock (0.75mA). Mice were returned 30 seconds later to the Home cage. In the multiple trainings experiments, animals underwent 4 of the previously described training sessions with an intertrial interval (ITI) of 10-12 min, during which time mice were returned to Home cage. Contextual fear conditioning was measured in context-A, and consisted of digitally recording the animal’s percentage of total time spent “freezing” while in the chamber for 3 min; freezing, defined as a complete lack of movement besides respiration (<5% mobility), was calculated by EthoVision v3.1 software (Noldus Information Technology Inc., Leesburg, VA) and expressed as a percentage of total time in the chamber. Cued fear conditioning was measured in a different context (context-B), and the previously used tone sounded for 2 min after an initial habituation period of 3 min. Context-B utilized a smooth white foam floor covering the shock grid, four opaque white walls, and olfactory enrichment of pure orange extract affixed to the chamber ceiling. Hence visual, tactile, and olfactory cues were employed to differentiate context-A from context-B. Context- and auditory cue-dependent freezing were tested 1-2h after training for short-term memory (STM), and 24h and 7 days later for LTM. LTM tests for contextual and auditory cued freezing levels were determined in all subjects; the interval between context- and auditory cue-dependent freezing tests was 4h.
Measurements of plasma corticosterone

To investigate the acute effect of footshock on plasma corticosterone levels, blood samples (80ul) were drawn from the retroorbital venous plexus using heparinized microcapillary tubes on day 1, 15 min before and 15 min after the last cue-shock pairing. To investigate the physiological response to the conditioned stimuli (context and auditory cue) associated with the footshock, on day 7 mice were tested for context- or auditory cue-dependent freezing, and blood samples were drawn 15 min after re-exposure to the context (half of the subjects) and the auditory cue (half of the subjects). This schedule prevented multiple blood-drawings from the same mouse. Samples were treated according to instructions provided in a Corticosterone EIA kit (Immunodiagnostic Systems Ltd; Fountain Hills, AZ), and read on a spectrophotometer at 450nm.

Statistical analysis

Results of hotplate test and vocalization threshold were analyzed by two-way ANOVA with sex and genotype as between-subject factors. In the single trial fear conditioning experiments, differences in % freezing were analyzed by three-way ANOVA (sex x genotype x time; 1h, 24h and 7 days). Results of contextual STM tests following multiple trainings were analyzed by repeated measures ANOVA with the number of the training session as the repeated measure. Results of LTM tests were analyzed by four-way ANOVA (sex x genotype x time x type of test; context or cue). ANOVAs were routinely followed by Bonferroni post hoc analyses to compare between specific groups. In one case where auditory cued fear learning was analyzed (Figure 2.2B), overall ANOVA showed a significant genotype effect but Bonferroni post hoc test between pairs did not show significant difference between genotypes. Therefore other
post hoc tests were conducted: Scheffe, Sidak, Tukey and LSD. Comparisons of the results of the single and multiple training experiments were performed by two-tailed Student’s t-test. All results are shown as the mean and standard error of the mean. In all cases a p value of <0.05 was considered statistically significant.

Results

**nNOS immunoreactive neurons are found in the hippocampus and amygdala of WT but not nNOS KO mice**

In WT mice nNOS-immunoreactive neurons are present in the dentate gyrus region of the hippocampus (Figure 2.1A) and in the lateral, basal, and central nuclei of the amygdala (Figure 1C). nNOS-immunoreactive neurons were absent in KO mice (Figure 2.1B, 2.1D). Given the role of nNOS in the formation of LTM in the hippocampus and amygdala for contextual and cued fear learning, we assumed that the absence of nNOS in KO mice may influence fear learning.

**Pain response thresholds in WT and nNOS KO mice**

To determine if WT and nNOS KO mice display differences in response to painful stimuli that could influence the fear conditioned response, two tests were performed. First, nociceptive response was assessed using the hotplate test which involves supraspinal mechanisms of the nociceptive system. Second, the minimum footshock intensity required to elicit an audible vocalization response was determined. As shown in Table 2.1, hotplate test latencies did not differ significantly between the groups. Two-way ANOVA (sex x genotype) of the results revealed no sex-dependent effect ($F(1,37)=3.658; p=0.064$) and no genotype-dependent effect ($F(1,37)=0.376; p=0.543$); post hoc analysis revealed no significant differences between the groups. These findings
suggest that nNOS KO mice have no impairments in nociceptive response compared to WT mice. Results of vocalization threshold tests revealed no differences between sexes of each genotype but a higher threshold in the KO mice compared to WT counterparts (Table 2.1). A two-way ANOVA (sex x genotype) revealed no main effect of sex ($F_{(1,26)} = 0.24; p=0.878$) but a main effect of genotype ($F_{(1,26)} = 32.775; p<0.001$). The interaction of sex x genotype was not significant ($F_{(1,26)} = 0.599; p=0.446$). Post hoc tests revealed significant differences between WT and KO mice of both sexes ($p<0.01$). Importantly, the shock intensity used for fear conditioning (0.75mA) was much higher than the observed vocalization thresholds (0.31mA).

**Fear conditioning by a single training reveals fear learning impairments in nNOS KO mice**

Levels of pretraining (basal) freezing to the training context did not differ between genotypes and sexes ($16\pm2\%$) (Figure 2.2A). Posttraining contextual freezing (Figure 2A) and auditory cued freezing (Figure 2.2B) were analyzed separately by three-way ANOVA (sex x genotype x time). The time component of the analysis corresponded to 1h (STM), 24h and 7 day (LTM) used for testing. For contextual freezing, there was no significant sex-dependent effect ($F_{(1,108)}=2.32; p=0.13$), a significant genotype effect ($F_{(1,108)}=519.84; p<0.001$), and no significant time effect ($F_{(2,108)}=0.39; p=0.67$); interactions between all three variables were not significant ($F_{(2,108)}=0.22; p=0.82$). Post hoc tests showed a significant genotype effect in males ($t=15.2; p<0.001$) and females ($t=17.08; p<0.001$) at all three time points (Figure 2.2A). In the STM tests for contextual freezing, WT levels were $64\pm7\%$ for males and $58\pm6\%$ for females. For KO mice, freezing levels in the STM tests reached $22\pm4\%$ for males and $23\pm4\%$ for females.
Freezing levels in WT mice were maintained in the 24h (males: 68±5%; females: 57±6%) and 7 day (males: 62±5%; females: 55±7%) tests of LTM. KO mice displayed significantly lower percent freezing times in the respective 24 h and 7 day LTM tests, reaching 27±3% and 25±3% for males, and 25±3% and 26±3% for females (p<0.001) (Figure 2.2A). These results show that nNOS KO mice display reduced contextual freezing in both STM and LTM tests compared to WT counterparts.

Results of cued freezing were analyzed by three-way ANOVA (sex x genotype x time) which revealed no sex-dependent effect (F(1,133)=3.83; p=0.052), a significant genotype effect (F(1,133) = 57.9; p<0.001), and no significant time-effect; no interactions between variables were significant. Notably, Bonferroni post-hoc tests showed no significant differences between WT and KO mice of either sex in cued freezing levels for any of the time points investigated (Figure 2.2B; p>0.05). Because the ANOVA showed overall significance, and Bonferroni post hoc analysis did not show pair-wise significant differences, Scheffe, Sidak, and Tukey, and LSD post hoc tests were performed. Only the LSD post hoc test, which does not take into account multiple comparisons, showed significant differences between WT and KO mice at the three time points (Figure 2.2B; #p<0.05).

**Plasma corticosterone levels after a single training predicts fear learning ability**

The neuroendocrine response to trauma in humans and animals is known to effect fear learning ability (Charney et al., 1993). In order to determine if the blood drawing procedure influenced the learned freezing response, corticosterone levels were measured in half of the subjects (5-8) that had undergone fear conditioning. Results showed no differences in the magnitude of contextual and auditory cued freezing between subjects.
(male and female WT and KO mice) that underwent blood drawing and those that did not. Therefore, behavioral results of subjects that did and did not undergo blood drawing were combined (Figure 2.2). For day 7 tests (post-context and post-auditory cue), blood samples were drawn from different groups to avoid possible effects of repeated blood drawing.

The results of the corticosterone assays (Figure 2.3) were analyzed by three-way ANOVA (sex x genotype x time). There was no significant sex-dependent effect ($F_{(1,115)}=0.21; p=0.648$), a significant genotype effect ($F_{(1,115)}=8.948; p=0.003$), a significant time effect ($F_{(3,115)}=21.688; p<0.001$) and significant interaction between sex and genotype ($F_{(1,115)}=16.301; p<0.001$) and between genotype and time ($F_{(3,115)}=11.389; p<0.001$). Post hoc tests showed no significant differences between pretraining (basal) corticosterone levels across all groups (Figure 2.3). In WT males, significant increases in corticosterone levels from pretraining were observed on day 1 posttraining (15 min) and day 7 following re-exposure to the context and cue (15 min after each) ($p<0.001$) (Figure 2.3). In KO males, no significant differences between pretraining and posttraining corticosterone levels were observed ($p>0.05$); likewise, on day 7 context and auditory cue re-exposure had no significant effect on corticosterone (Figure 2.3). In WT females a significant increase in corticosterone levels was observed on day 1 posttraining ($p<0.01$), but not on day 7 following context or cue re-exposure (Figure 2.3). In KO females, like their male counterparts, a single fear conditioning session had no significant effect on corticosterone levels 15 min posttraining or 7 days later (Figure 2.3). The results suggest that a) increased corticosterone release following fear conditioning is related to successful contextual fear learning, and b) re-exposure to context and cue associated with
footshock causes marked increase in corticosterone long after the footshock experience in WT males but not females.

**Multiple trainings improves primarily contextual fear learning in nNOS KO mice**

To investigate whether a more robust training strategy would improve fear learning in nNOS KO mice, WT and KO mice underwent multiple (four) trainings with an ITI of 10-12min. Mice were tested for contextual STM (Figure 2.4); the percent freezing time was measured both pretraining and during a 2 min period in context-A (pre-shock) 10 min after the delivery of the first, second, and third footshock. Only contextual STM was investigated because of the magnitude of the learning deficit in nNOS KO mice. Results of both contextual and auditory cued LTM testing are shown in Figure 2.5.

**STM testing after multiple trainings**

Results of STM tests (Figure 2.4) for all groups were analyzed by a three-way ANOVA (sex x genotype x time). There was no significant sex-dependent effect ($F_{(1,120)} = 2.152; p=0.145$); a significant genotype-dependent effect ($F_{(1,120)} = 200.3; p<0.001$); a significant time-dependent effect ($F_{(1,120)} = 160.11; p<0.001$); and a significant interaction between genotype and time ($F_{(3,120)} = 12.939; p<0.001$). Results within groups were analyzed by repeated measures ANOVA to determine the effect of each training session on subsequent contextual freezing. Results for all groups showed an overall significant training effect ($p<0.001$). Post hoc tests were used to determine differences in % freezing after each training session. In WT males and females, there was a significant difference between pretraining and the first training tests ($p<0.001$) but no other significant differences were observed (Figure 2.4A and 2.4C). In KO males, a significant difference between pretraining and the first training session was observed ($p<0.001$) as well as
between the first (42±4%) and the third (65±6%) training sessions (p=0.004) (Figure 2.4B). In KO females, there were significant differences between pretraining and the second training (p<0.001) as well as between the first and the third (p=0.018) training sessions (Figure 2.4D). These results show that WT mice ceased improvements in contextual STM after a single training, while nNOS KO mice exhibited improvements after multiple trainings that exceeded freezing from a single training.

**LTM testing after multiple trainings**

Mice were tested for contextual and auditory cued freezing 24h and 7 days (LTM) after the multiple trainings (Figure 2.5). Results for the LTM tests were analyzed by four-way ANOVA: sex x genotype x time x test (contextual or cued freezing). There was no significant sex-dependent effect (F(1,124)=1.25; p=0.266), a significant genotype-dependent effect (F(1,124)=103.316; p<0.001), no significant time-dependent effect (F(1,124)=1.49; p=0.223) and a significant test-dependent effect (F(1,124)=62.877; p<0.001). The following significant interactions were observed: genotype x time (F(1,124)=4.927; p=0.028); genotype x test (F(1,124)=24.121; p<0.001); time x test (F(1,124)=11.707; p=0.001).

**Contextual LTM**

For both the 24h test (males: WT= 90±3%; KO= 53±6%) and the 7 day test (males: WT= 74±5%; KO= 29±4%) a significant genotype difference was observed (p<0.001) (Figure 2.5A and 2.5B). These results suggest that contextual fear learning in KO males remains impaired even after four training sessions. However, it should be noted that in the 24h test for KO males (53±6%; Figure 2.5B), contextual fear learning was two-fold higher than that observed after a single training (27±3%; Figure 2.2A;
p=0.001) and not significantly different from the % freezing of WT males 24h following a single training session (68±5%; Figure 2.2A; p>0.05). For WT and KO females, a similar trend was observed. KO females showed significant improvements in contextual LTM after the multiple trainings (42±6%, Figure 2.5D) compared to a single training (25±3%; Fig 2A; p=0.003) and not significantly different from % freezing of WT females 24h following a single training session (58±6%, Figure 2.2A; p>0.05). Thus, in nNOS KO mice the results from contextual LTM tests 24h after multiple trainings were similar to the results in WT mice after a single training.

For the 7 day tests in nNOS KO mice, contextual freezing after multiple trainings was significantly improved in females (p<0.05; Figure 2.5D) but not males (Figure 2.5B), suggesting that in males the improvement in contextual LTM was transient (24h but not 7 days).

**Auditory Cued LTM**

Post hoc comparisons for the magnitude of auditory cued freezing after the four training sessions revealed no significant differences between WT and KO males (Figure 2.5A and 5B), and WT and KO females (Figure 2.5C and 2.5D) at both time points. Comparisons between the results of cued freezing after the single and multiple trainings revealed significant improvements on the 7 day test in WT males (t=3.435; p<0.003) but not in KO males (p>0.05). This finding suggests that cued freezing in nNOS KO males following single and multiple trainings was similar. In nNOS KO females, but not in the WT counterparts, there was a significant difference between cued freezing following single and multiple trainings at the 24h and 7 day tests (p<0.05), suggesting that multiple trainings improved cued fear learning in nNOS KO females.
Multiple trainings resulted in increased corticosterone levels in nNOS KO

Corticosterone was measured in nNOS KO mice following multiple trainings in order to determine if levels would be predictive of contextual fear learning ability, as had been observed after a single training session (Figure 2.3). Only nNOS KO mice were tested because WT mice showed significant increases in corticosterone after a single training while nNOS KO mice did not (Figure 2.3). As before, corticosterone measurements were taken on day 1: 15 min before and after training, and 7 days later: 15 min post context or auditory cue re-exposure (Figure 2.6). Results were analyzed by two-way ANOVA (sex x time) and revealed a significant sex-dependent effect ($F_{(1,53)}=13.968; p<0.001$) a significant time effect ($F_{(3,53)}=24.984; p<0.001$), and a significant interaction between sex and time ($F_{(3,53)}=4.33; p=0.008$). Post hoc tests showed significant posttraining (day 1) elevations in corticosterone in male and female nNOS KO mice ($p<0.001$). On day 7, a significant increase in corticosterone was observed in male nNOS KO mice following cue re-exposure ($p<0.001$), and in females following both cue and context re-exposure ($p<0.001$). The results show that the multiple training sessions increased corticosterone levels in nNOS KO mice (Figure 2.6) which coincided with improvements in contextual freezing with this training strategy (Figure 2.5).

Discussion

Results of the present study show the following: a) The absence of the nNOS gene causes deficits in fear learning, a finding which supports pharmacological evidence implicating NO signaling in fear learning (Resstel et al., 2008; Schafe et al., 2005). b) Multiple trainings improved primarily contextual fear learning ability in nNOS KO mice. c) The physiological response to unconditioned and conditioned fearful stimulus is
similar, and appears to be related to optimal fear learning ability as determined by measuring plasma corticosterone levels.

**NO and fear conditioning**

At present, only a few studies have investigated the role of NO signaling in fear conditioning. Two studies have found that systemic administration of NOS inhibitors did not prevent fear learning in rodents (Johnson et al., 2000; Maren, 1998). However, recently it has been reported that blockade of NO signaling in the ventral portion of medial prefrontal cortex resulted in contextual fear learning deficits (Resstel et al., 2008) and that blockade of NO signaling in the lateral amygdala resulted in reduced LTP and impaired auditory cued fear learning in rats (Ota et al., 2008). Our findings show that global deletion of the nNOS gene in mice resulted in a more severe impairment in contextual than auditory cued fear learning. Fear conditioning is believed to recruit both the amygdala (emotional cued learning) and the hippocampus (spatial/contextual learning) (Goosens and Maren, 2004; Mei et al., 2005; Phillips and LeDoux, 1992). Given that nNOS-immunoreactive neurons are absent in both the hippocampus and amygdala of nNOS KO mice (Fig 1), it is unclear why contextual fear learning was more severely impaired than the auditory-cued learning. This may suggest that neuroadaptations in the nNOS KO mice in the amygdala—more so than in the hippocampus—resulted in NO-independent fear learning. Protein expression analysis of nNOS KO mice has revealed aberrant protein expression in the hippocampus (Kirchner et al., 2004) which may underlie the deficits in contextual fear learning observed in the present study as well as other cognitive deficits (Weitzdoerfer et al., 2004).
Pain response thresholds in WT and nNOS KO mice

The finding that nNOS KO mice showed deficits in conditioned fear learning raises the question of whether nNOS KO mice are less sensitive to painful stimulus. The results from the hotplate test suggest that nociceptive responses are intact in nNOS KO mice (Table 2.1) confirming an earlier report (Azad et al., 2001). We did observe a difference in vocalization thresholds between WT and nNOS KO mice where nNOS KO mice of both sexes required higher shock intensity before audible vocalization responses were made (Table 2.1). Whether the KO mice are less sensitive to the painful footshock or are less likely to vocalize in response to pain is not clear, however the intensity of the footshock during training was 2.5-fold higher than that required for vocalization. Further, KO mice exhibited normal cued fear learning (Figs. 2B, 5B and 5D).

Previous studies suggested that nNOS KO mice have blunted stress response and less anxiety-like behavior compared to WT counterparts following restraint (Bilbo et al., 2003). Hence, it may be argued that the absence of the nNOS gene contributes to an “anxiolytic effect” and consequently to impairment in fear conditioning. However, the finding that auditory cued fear learning in the absence of the nNOS gene was only slightly reduced (following single training) or near optimal (following multiple trainings) suggests that blunted stress response by itself cannot explain the severe impairment in contextual fear conditioning.

Multiple trainings

We investigated whether an increased training intensity (from one to four training sessions) would enable the nNOS KO mice to overcome the fear learning deficits. We found that four trainings improved the contextual learning ability of nNOS KO mice in
both STM and LTM tests. In KO mice, contextual STM improved after each training session (Figure 2.4B and 2.4D) while in WT mice contextual freezing was maximized by the first training session (Figure 2.4A and 2.4C). The four training sessions also increased LTM for contextual fear (24h) in KO mice (males: 53±6%; females: 42±4%) compared to results of a single training session (males: 27±3%; females: 25±3%). Nevertheless, results of LTM tests still showed that context re-exposure elicited less freezing in KO mice of both sexes compared to WT (Figure 2.5). Notably however, LTM of auditory cued fear learning after four training sessions was remarkably similar for both sexes in WT (82-88%) and KO (78-83%) mice (Figure 2.5) and was similar to the magnitude to after a single training (Figure 2.2B).

**Corticosterone**

In rodents, corticosterone has been implicated in LTM formation of contextual fear memory (Pugh et al., 1997; Thompson et al., 2004) and tone-specific cued fear memory (Hui et al., 2004; Marchand et al., 2007; Roozendaal et al., 2006). We aimed to investigate how nNOS gene deletion might affect the physiological response to fear conditioning. The results from the plasma corticosterone measurements implicated a relationship between plasma corticosterone and successful contextual fear learning for the following reasons. First, elevated corticosterone was observed 15 minutes after a single training session in WT mice (Figure 2.3) which exhibited successful STM and LTM of contextual fear learning (Figure 2.2), and not in KO mice (Figure 2.3) which did not exhibit successful contextual fear learning (Figure 2.2). Second, 15 minutes after the four trainings KO mice showed elevated corticosterone (Figure 2.6) concomitant with significant improvements in STM (Figure 2.4) and LTM (Figure 2.5) of contextual fear.
learning. Third, 7 days following the multiple trainings, male KO mice showed relatively low corticosterone release (Figure 2.6) following context re-exposure and a low magnitude of context-dependent freezing response (29±4%; Figure 2.5B). Conversely, female KO mice showed significantly elevated corticosterone (Figure 2.6) and a relatively higher context-dependent freezing response (40±3%; Figure 2.5D). Overall, it is notable that the context and the auditory cue associated with a painful stimulus elicited a physiological response (increase in plasma stress hormones) 7 days later which was similar in magnitude to that observed immediately following exposure to the painful stimulus (Figure 2.6). This finding suggests that measurements of the neuroendocrine response to cues associated with stressful stimulus may provide insight into the effectiveness of clinical treatments for anxiety disorders such as PTSD.

In summary, the present study demonstrates a role for the nNOS gene in contextual and auditory cued fear learning. Our results suggest that the nNOS gene deletion disrupts primarily contextual fear learning, which recruits the hippocampus, and to a lesser extent auditory cued fear learning which recruits the amygdala. Also, elevation in plasma stress hormones (e.g., corticosterone in rodents) may be predictive of optimal contextual fear learning. Hence, pharmacological inhibition of nNOS and drugs that block release or receptor binding sites of stress hormones may be potential therapeutics against contextual fear conditioning.
Table 2.1. Nociception and vocalization threshold in WT and nNOS KO mice. No genotypic differences were observed in hotplate latency. However, vocalization thresholds in male and female nNOS KO mice were slightly higher than in WT counterparts. The footshock intensity used in all fear conditioning studies was 0.75mA, which was over twice as high as the vocalization thresholds in WT and nNOS KO mice. (Taken from Kelley et al., 2009)

<table>
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<tr>
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<th>Wild type (WT) mice</th>
<th>nNOS knockout (KO) mice</th>
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<tr>
<td><strong>Hotplate latency (seconds)</strong></td>
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<tr>
<td>Male</td>
<td>5.3±0.6</td>
<td>4.1±0.9</td>
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<tr>
<td>Female</td>
<td>5.8±0.6</td>
<td>6.3±0.7</td>
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<tr>
<td><strong>Vocalization threshold (mA)</strong></td>
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<tr>
<td>Male</td>
<td>0.22±0.013</td>
<td>0.30±0.019*</td>
</tr>
<tr>
<td>Female</td>
<td>0.21±0.013</td>
<td>0.31±0.019*</td>
</tr>
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Data presented are averages (±S.E.). *p<0.01 for vocalization threshold in KO vs. WT mice.
Figure 2.1. Representative nNOS-immunoreactive neurons in the dentate gyrus and amygdala of male WT and nNOS KO mice. nNOS-immunoreactive neurons were present in the dentate gyrus of WT (A) but not nNOS KO mice (B); scale bar=100µm, magnification=20x. Likewise nNOS-immunoreactive neurons are present in the lateral, basal, and central nuclei of the amygdala of WT mice (C), but not KO mice (D). C and D scale bar=200µm, magnification=10x. The boxed region in C shows nNOS-immunoreactive neurons in the basal nucleus of the amygdala; scale bar= 60µm, magnification 60x. Schematic diagrams of representative brain regions from the mouse brain atlas (Paxinos and Franklin, 2001) show the approximate antero-posterior level (-1.43 to Bregma) at which select brain regions were analyzed and nNOS-immunoreactive neurons were found. (Taken from Kelley et al., 2009)
Figure 2.2. Short- and long-term memory (STM; LTM) of contextual and auditory cued fear conditioning in male and female WT and nNOS KO mice. Mice (n=10-16/group) underwent a single training; STM was determined 1h posttraining and LTM was determined 24h and 7 days posttraining. A. Results of contextual freezing are expressed as % of total time spent freezing to the training context. During pretraining (basal), no significant differences between genotypes in % freezing were observed. For the posttraining phase, Bonferroni post hoc tests showed significant genotype effect in males (t=15.2; *p<0.001) and females (t=17.08; *p<0.001) at all three time points, suggesting impairments in contextual fear learning in KO mice. B. Results of auditory cued freezing are expressed as % freezing during auditory cue re-exposure in a different context. Bonferroni multiple comparisons showed no significant differences between WT and KO mice at all three time points. Only the least restricted post hoc LSD test showed significant differences between WT and KO mice (#p<0.05). (Taken from Kelley et al., 2009)
Figure 2.3. **Corticosterone levels before and after a single training.** In WT males a significant increase in corticosterone levels was observed on day 1, 15min posttraining (*p<0.001), and on day 7 post context or auditory cue re-exposures (*p<0.05). In KO males no significant differences in corticosterone levels were observed. In WT females a significant increase in corticosterone was observed only on day 1, 15 min posttraining (*p<0.001). In KO females no significant differences between corticosterone levels pre- and posttraining were observed. (Taken from Kelley et al., 2009)
Figure 2.4. Contextual short-term memory (STM) following multiple trainings. A. Contextual STM in WT males (n=8); B. Contextual STM in KO males (n=9). C. Contextual STM in WT females (n=8). D. Contextual STM in KO females (n=10). Mice underwent four trainings (10-12 min intertrial interval); contextual freezing was measured pretraining and then 10min following the first, second and third training. Post hoc tests showed significant differences in % freezing between pretraining and the first posttraining tests for males of both genotypes and WT females (*p<0.001); KO females showed significant differences in % freezing between pretraining and the second posttraining test (*p<0.001). Significant differences between % freezing after the first and the third training session in KO mice were observed suggesting multiple trainings significantly increased contextual STM compared to a single training (#p<0.05). (Taken from Kelley et al., 2009)
Figure 2.5. Long-term memory (LTM) of contextual and auditory cued fear conditioning following multiple trainings. Mice that had been tested for STM of contextual fear (Figure 4) were tested after 24h and 7 days for contextual and auditory cued freezing. A. Contextual and auditory cued LTM in WT males (n=8). B. Contextual and auditory cued LTM in KO males (n=9). C. Contextual and auditory cued LTM in WT females (n=8). D. Contextual and auditory cued LTM in KO females (n=10). Contextual freezing: nNOS KO males (B) showed lower contextual freezing than their WT counterparts (A) in both the 24h and 7 day tests (*p<0.001). In KO males (B) there was a significant decrease in contextual freezing on day 7 compared to 24h posttraining (#p<0.05). nNOS KO females (D) showed lower contextual freezing than WT counterparts (C) on both the 24h and 7 day tests (*p<0.001). Auditory cued freezing: Bonferroni multiple comparison tests showed no significant differences in the level of auditory cued freezing among genotypes, sexes and time elapsed posttraining. (Taken from Kelley et al., 2009)
Figure 2.6. Corticosterone levels before and after multiple trainings in nNOS KO mice. In male KO mice, there was a significant increase in plasma corticosterone 15 min after the four training sessions, as well as on day 7 after re-exposure to the auditory cue (*p<0.001). In female KO mice, there was a significant increase in plasma corticosterone 15 min after the four training sessions, and on day 7 after re-exposure to context or auditory cue (*p<0.001). (Taken from Kelley et al., 2009)
Chapter 3

Pharmacological modulators of nitric oxide signaling and contextual fear conditioning

Summary

Nitric oxide (NO) produced by neuronal nitric oxide synthase (nNOS) is a retrograde neuronal messenger that participates in synaptic plasticity, including late-phase long-term potentiation (LTP) and long-term memory (LTM) formation. Our recent studies have shown that nNOS knockout (KO) mice have a severe deficit in contextual fear conditioning compared to wild type (WT) counterparts (Kelley et al., 2009). Given the role of the nNOS gene in fear conditioning, we investigated whether systemic administration of modulators of NO signaling affect the formation of contextual and cued fear memories and the effects of these modulators on cyclic 3’5’-guanosine monophosphate (cGMP) levels in the hippocampus and amygdala. The preferential nNOS inhibitor S-methyl-L-thiocitrulline (SMTC; 10-200 mg/kg) was administered (i.p.) to WT mice, and the NO donor molsidomine (10 mg/kg) was administered (i.p.) to nNOS KO mice either 30 minutes pretraining or immediately posttraining. Pretraining SMTC administration to WT mice impaired both short- and long-term memory of contextual (36% inhibition) but not cued fear conditioning. Pretraining molsidomine administration to nNOS KO mice improved their deficit in short- and long-term memory of contextual fear conditioning (46% increase). Posttraining drug administration had no effect on WT and nNOS KO mice. The systemic administration of SMTC dose-dependently decreased cGMP concentrations down to 25% of control, while molsidomine increased cGMP concentration (3- and 5-fold) in amygdala and hippocampus, respectively.
These findings suggest that neuronal NO and its downstream second messenger cGMP are important for acquisition and subsequent consolidation of LTM of contextual fear conditioning.

**Background**

Anxiety disorders are the most prevalent mental health disorders in the United States, affecting nearly 40 million adults in a given year (Kessler et al., 2005a). Research in animals and humans suggests that maladaptive Pavlovian learning underlies the development of acquired anxiety disorders such as posttraumatic stress disorder (PTSD) and specific phobias. In rodents, the learned fear and conditioned response following fear conditioning are considered analogous to the development and expression of the symptoms of PTSD (Mineka and Oehlberg, 2008). Understanding the mechanisms of fear memory acquisition and subsequent consolidation into long-term memory (LTM) will aid the development of pharmacotherapies for certain anxiety disorders.

In the fear conditioning paradigm, the presentation of a neutral stimulus such as a discreet context or sensory cue (conditioned stimulus; e.g. training context or auditory cue) is paired with an aversive stimulus (unconditioned stimulus), such as an electric footshock. After fear conditioning, re-exposure to the previously neutral stimulus elicits a learned fear response (behavioral freezing) in the absence of the aversive stimulus. Thus, the previously neutral stimulus has acquired affective properties of the unconditioned stimulus. Previous pharmacological investigations have shown the requirement of the N-methyl-D-aspartate receptor (NMDAR) in the acquisition of fear conditioning (Campeau et al., 1992; Kim et al., 1991; Miserendino et al., 1990; Rodrigues et al., 2001). The consolidation of LTM following fear conditioning is believed to involve long-term
potentiation (LTP) in the amygdala and the hippocampus (Abel et al., 1997; Blair et al., 2001; Maren et al., 1994; Sigurdsson et al., 2007).

Activation of the NMDAR stimulates neuronal nitric oxide synthase (nNOS) and the production of nitric oxide (NO) (Brenman and Bredt, 1997). In the brain, nitric oxide (NO) has the role of a retrograde neuronal messenger (Snyder and Bredt, 1992) which stimulates soluble guanylate cyclase (sCG) to produce the second messenger cyclic 3’5’-guanosine monophosphate (cGMP) (Denninger and Marletta, 1999). In vitro studies have implicated the NO→sGC→cGMP signaling pathway in LTP in slices of hippocampus (Arancio et al., 2001; Lu et al., 1999; Puzzo et al., 2006; Zhuo et al., 1994) and amygdala (Chien et al., 2003; Schafe et al., 2005). Recent studies have shown a role for NO signaling in the consolidation of auditory cued fear conditioning (Ota et al., 2008; Schafe et al., 2005) and the expression of contextual fear conditioning in rats (Resstel et al., 2008). We reported that mice lacking the nNOS gene (nNOS KO) displayed severe deficits in contextual fear conditioning and a relatively milder deficit in cued fear conditioning compared to WT counterparts (Kelley et al., 2009).

The current study was undertaken to investigate how pharmacological modulators of NO signaling affect fear conditioning. We investigated the effects of a) the preferential nNOS inhibitor S-methyl-L-thiocitrulline (SMTC) (WT mice) and b) the NO donor molsidomine (nNOS KO mice) on acquisition and consolidation of LTM for contextual and cued fear conditioning. Next, we investigated cGMP concentrations in hippocampus and amygdala of drug-treated mice to validate modulation of the NO signaling pathway. Results suggested that pharmacological and genetic modulation of NO signaling had similar outcomes on short-term memory (STM) and LTM of contextual fear conditioning.
Materials and methods

Animals

nNOS KO mice were generated via targeted mutation using J1 (129) embryonic stem (ES) cells and were maintained on a mixture of C57BL/6 and 129S genetic background (designated B6;129). The targeted deletion of the alpha subunit of nNOS resulted in >95% reduction in brain nNOS catalytic activity (Huang et al., 1993). Adult male homozygous nNOS KO mice (B6;129S4-Nos1; 6-8 weeks old), and the parental strains of their hybrid WT counterparts (C57BL/6J and 129S1/SvImJ; 6-8 weeks old) were purchased from Jackson Laboratories. Our breeding colony, littermate selection, and animal care have been described earlier (Balda et al., 2006); the WT and nNOS KO mice were reared under identical conditions. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and was approved by the University of Miami Animal Care and Use Committee. Adult (8-10 week old) male WT and nNOS KO mice were investigated.

Drug treatments

The preferential nNOS inhibitor S-methyl-L-thiocitrulline (SMTC) was administered to WT mice in doses of 10, 20, 50, 100, and 200 mg/kg. SMTC is considered a preferential nNOS inhibitor because it is 10-17 fold more sensitive for nNOS than to endothelial NOS (eNOS) (Furfine et al., 1994). The NO donor molsidomine (10 mg/kg) was administered to nNOS KO mice. Molsidomine (4mg/kg) has been shown to improve learning deficits due to nNOS inhibition in rats (Meyer et al., 1998). Because in our preliminary studies, administration of 4mg/kg molsidomine to nNOS KO mice had only minor effects on behavior and cGMP levels (data not shown),
higher doses (10 and 20 mg/kg) were investigated. SMTC was dissolved in distilled water and molsidomine was dissolved in saline; water and saline served as “vehicle” for control groups. Drugs were given intraperitoneally (i.p.) -either 30min pretraining or immediately posttraining in order to investigate drug effect on memory acquisition and consolidation, respectively. For cGMP assays, the drugs were administered 30 minutes prior to sacrifice.

**Measurement of motor behavior**

Measurements of motor behavior were determined in non-conditioned (no footshock) WT and nNOS KO mice that had received vehicle or drug (Table 1). The purpose of these experiments was to determine if the drugs tested affect motor behavior, which might have subsequently influenced the freezing response. WT mice received SMTC (100 mg/kg) and nNOS KO mice received molsidomine (10 mg/kg) and were placed in the training context (context A) 30 minutes later. The doses of SMTC and molsidomine that were tested correspond to the effective doses observed in the fear conditioning experiments. Recordings took place over 3 minutes during which the animals underwent a similar training as fear conditioned mice except that the footshock was omitted. Three measures of motor behavior were determined: 1) immobility (%), 2) locomotor activity (total distance traveled in cm), and 3) frequency of rearing events. A rearing event was detected when the body of the animal became vertically erect, and was determined as a >15% reduction in surface area measured from an overhead video camera. Detection and calculations of these parameters were performed by EthoVision v3.1 software. The same subjects were also tested for immobility 1 h and 24 h later as control (non-conditioned) subjects (Figures 3.1A and 3.1B).
Fear conditioning experiments were performed as previously described (Kelley et al., 2009). The fear conditioning training and testing occurred in Plexiglas chambers (30.5 x 30.5 x 43.5 cm; Noldus, Netherlands). Each chamber was equipped with a stainless steel rod floor through which the electric shock was delivered. An upper control panel housed the video camera, a sound emitter, a white light illuminating one corner of the chamber and a yellow light in the center. The chambers were contained in custom-built sound attenuating cubicles which gave the appearance of black walls to the chamber. The floors of the chambers were cleaned with a diluted soap solution (1% Alconox), wiped with water, and then dried after each animal during the training and tests.

During training, mice were placed in the training context (context A) and allowed to habituate for 120 seconds. Next, an auditory cue (2.3 kHz; 70dB) sounded for 30 seconds and co-terminated with a 2 second footshock (0.75mA). Mice were returned to the home cage 30 seconds later. A white light illuminating one corner was on during the training and testing in context A. In control experiments, mice underwent the same sequence of events except that the footshock was omitted (Table 3.1 and Figure 3.1). Contextual fear conditioning was determined in context A and consisted of digitally recording the animal’s percentage of time spent “freezing” while in the chamber for 2 minutes. Freezing was defined as a complete lack of movement besides respiration and was measured automatically with EthoVision 3.1 software (Noldus, Netherlands) using the following parameters: 6 samples/second, immobility threshold of 5% (Pham et al., 2009). Freezing is expressed as a percentage of the total time spent in the chamber.
Auditory cued fear conditioning was measured in a different context (context B) which utilized a smooth white foam pad floor that covered the shock grid, four opaque white walls, a central yellow light illuminating the cage, and an olfactory enrichment of pure orange extract that was affixed to the chamber ceiling. Thus visual, tactile, and olfactory cues were used to differentiate context A from context B. The tone used during training sounded for 2 minutes following a 2 minute habituation period (absence of cue) in context B. Freezing (%) during the 2 minute habituation period in context B was subtracted from freezing (%) during the 2 minute period of cue presentation to control for possible genotypic difference in motor activity (Table 1), which could interfere with the measurement of cued freezing. STM tests of contextual and auditory cued freezing were performed 1-2 h posttraining and LTM tests were performed 24 h posttraining; there was a 4 h interval between context and cue tests.

**Determination of cGMP**

Determination of cGMP concentrations provides a measure for changes in NO levels because soluble guanylate cyclase (sGC) is the NO-receptor. Mice were treated with vehicle or drug 30 minutes before sacrifice by cervical dislocation. Brains were immediately removed and placed in ice-cold saline. The hippocampus and amygdala were dissected, snap frozen on dry ice, and stored at -80°C until used. cGMP was quantified with a direct cGMP EIA kit (Assay Designs Inc, MI; CAT# 900-014) using the overnight acetylated version of the kit as described in the manufacturer’s protocol (sensitivity: 25 fmol/ml).
Statistics

Measurements of motor behavior shown in Table 1 were analyzed by two-way ANOVA (genotype x treatment; vehicle vs. drug) for each type of behavior. Control experiments (Figure 3.1) consisted of the following groups: 1) vehicle / conditioned, 2) vehicle / non-conditioned, 3) drug / non-conditioned and were analyzed by separate two-way ANOVA (group x time; STM and LTM) for contextual or auditory cued freezing. Results for the remaining behavioral experiments (Figures 3.2 and 3.3) were analyzed separate by two-way ANOVA (treatment x time; Basal, STM and LTM) for contextual or cued freezing. Results of cGMP assays for WT mice where multiple drug doses were investigated (Figure 3.4A) were analyzed by one-way ANOVA. Results of the cGMP assay for nNOS KO mice were analyzed by Student’s t-test (Figure 3.4B). The ANOVAs were followed by Bonferroni post-hoc analyses to compare between specific groups when the ANOVA showed significant interactions. Routinely, post-hoc tests were used to compare control vs. drug-treated groups. All results are shown as mean ± standard error of the mean. A $p$ value of <0.05 was considered statistically significant.

Results

Effects of the drug treatments on motor behavior and freezing response in the absence of associative learning

First, the acute effects of SMTC (100 mg/kg) and molsidomine (10 mg/kg) on motor behavior of non-conditioned WT and nNOS KO mice, respectively, were investigated (Table 1). There was no significant drug treatment effect on immobility, locomotion, and rearing behavior for either genotype, suggesting SMTC and molsidomine had no effect on motor behavior in the absence of associative learning.
However, the ANOVA revealed a significant genotype effect for immobility ($F_{(1,40)} = 31.952; \ p<0.001$), locomotion ($F_{(1,40)} = 30.244; \ p<0.001$) and rearing behavior ($F_{(1,40)} = 8.919; \ p=0.005$). Thus, nNOS KO mice were somewhat more active than their WT counterparts (Table 1).

Second, the effects of SMTC (100 mg/kg) and molsidomine (10 mg/kg) treatments on immobility were investigated 1 h and 24 h after drug administration to the non-conditioned control subjects (Table 1) and compared to conditioned vehicle-treated mice (Figure 3.1; STM = 1 h, LTM = 24 h). For contextual freezing in WT mice (Figure 3.1A) there was a significant group effect ($F_{(2,66)} = 98.616; \ p<0.001$) and a significant time effect ($F_{(1,66)} = 28.311; \ p<0.001$). As expected, the conditioned group expressed increased contextual freezing in the STM and LTM tests compared to the non-conditioned groups ($p<0.001$). Similarly, for auditory cued freezing in WT mice (Figure 3.1B) there was a significant group effect ($F_{(2,56)} = 33.375; \ p<0.001$) and a significant time effect ($F_{(1,56)} = 26.185; \ p<0.001$). As expected, the conditioned group expressed increased auditory cued freezing compared to the non-conditioned groups ($p<0.005$ for STM; $p<0.001$ for LTM). For both contextual and auditory cued freezing there were no differences between non-conditioned vehicle- and SMTC-treated groups. The significant time effects observed in Figure 3.1A and 3.1B are likely due to habituation in the non-conditioned groups following repeated exposures to the training context and to the auditory cue (i.e. immobility in the non-conditioned groups increased from $18\pm3\%$ for STM to $38\pm3\%$ for LTM; Figure 3.1A). Overall, the results of WT mice show that 1) a single fear conditioning session results in robust contextual and auditory cued freezing
response, and 2) in the absence of associative learning, the drug treatment had no effect on freezing behavior 1 h (STM test) and 24 h later (LTM test).

For contextual freezing in nNOS KO mice (Figure 3.1C), there was no significant group effect but a significant time effect \( (F_{(1,56)} = 10.235; p=0.002) \). The finding that contextual freezing in the conditioned mice is similar to non-conditioned mice suggests that a single fear conditioning session was insufficient to elicit contextual freezing in nNOS KO mice, and is in agreement with our previous studies (Kelley et al., 2009). The same group of conditioned nNOS KO mice expressed auditory cued freezing (Figure 3.1D); the ANOVA revealed a significant group effect \( (F_{(2,62)} = 61.678; p<0.001) \). For both contextual and auditory cued freezing there were no differences between non-conditioned vehicle- and molsidomine-treated groups. Overall, the results of nNOS KO mice suggest that 1) contextual fear conditioning is impaired, while auditory cued fear conditioning is intact, and 2) molsidomine treatment had no effect on freezing behavior 1 h (STM test) and 24 h later (LTM test) in the absence of associative learning.

**Effects of the nNOS inhibitor on fear conditioning of WT mice**

Two separate experiments were carried out to investigate the effect of the nNOS inhibitor SMTC on fear conditioning of WT mice. In the first experiment, SMTC (10-200 mg/kg) was administered 30 minutes pretraining to determine if the drug affects memory acquisition and subsequent consolidation. In the second experiment, SMTC (20-200 mg/kg) was administered immediately posttraining to determine if the drug has a specific effect on memory consolidation.
Pretraining administration of nNOS inhibitor to WT mice

Results of contextual and auditory cued freezing following pretraining SMTC treatments (10 – 200 mg/kg) are shown in Figure 3.2A and 3.2B, respectively. For contextual freezing, there was a significant treatment effect (F (5,166) = 11.268; p<0.001), a significant time effect (F (2,166) = 161.32; p<0.001) and a significant interaction between the variables (F (10,166) = 3.335; p<0.001). During the basal period (2 minutes; pre-shock), there were no differences between the levels of immobility among the various groups. However, post hoc tests revealed significantly reduced freezing for the SMTC 50 mg/kg group compared to vehicle in the STM test (26.4% reduction; t=4.185, p<0.001) and LTM test (22.4% reduction; t=3.45, p=0.004). Also, significantly reduced freezing was observed in the SMTC 100 mg/kg group for the STM test (27.2% reduction; t=4.548, p<0.001) and LTM test (35.5% reduction; t=6.123, p<0.001) compared to vehicle. Lower doses of SMTC (10 and 20 mg/kg) and the highest dose (200 mg/kg) had no significant effect on contextual freezing (Figure 3.2A). Thus, only the optimal doses of 50 mg/kg and 100 mg/kg impaired the development of contextual fear conditioning (Figure 3.2A). For cued freezing, none of the doses of SMTC administered pretraining had a significant effect on freezing (Figure 3.2B). The results suggest that pretraining administration of the preferential nNOS inhibitor impaired the acquisition (STM) and subsequent consolidation of LTM for contextual but not auditory cued fear conditioning.

Posttraining administration of nNOS inhibitor to WT mice

Results of contextual and auditory cued freezing in mice that received posttraining SMTC treatments (20 – 200 mg/kg) are shown in Figure 3.2C and 3.2D, respectively. The dose of 10 mg/kg SMTC was not tested because it had no effect in the
pretraining experiments. For contextual freezing, the ANOVA revealed a significant time effect ($F_{(2,105)} = 606.94; p <0.001$) which was due to increased freezing in the STM and LTM tests compared to basal freezing (Figure 3.2C; $p<0.001$ for all four doses of SMTC). However, no significant treatment effect was observed. For cued freezing, no significant effects were observed (Figure 3.2D). The results suggest that posttraining administration of the preferential nNOS inhibitor had no effect on consolidation of LTM for contextual and auditory cued fear conditioning.

**Effects of the NO donor on fear conditioning of nNOS KO mice**

Two separate experiments were carried out to investigate the effect of the NO donor molsidomine (10 mg/kg) on fear conditioning of nNOS KO mice. In the first experiment, molsidomine was administered 30 minutes pretraining to determine if the drug affected memory acquisition and subsequent consolidation. In the second experiment, molsidomine was administered immediately posttraining to determine if the drug has a specific effect on memory consolidation.

**Pretraining administration of molsidomine to nNOS KO mice**

Results of contextual and auditory cued freezing in nNOS KO mice that received pretraining molsidomine treatment (10 mg/kg) are shown in Figure 3.3A and 3.3B, respectively. For contextual freezing, the ANOVA showed a significant treatment effect ($F_{(1,42)} = 80.521 ; p<0.001$), a significant time effect ($F_{(2,42)} = 36.034 ; p<0.001$) and a significant interaction between both variables ($F_{(2,42)} = 17.586 ; p<0.001$). Post hoc tests revealed no differences between basal immobility. However, there was a significant increase in freezing in the molsidomine group compared to vehicle in the STM (31.6% increase; $t=6.074, p<0.001$) and LTM (46.1% increase; $t=8.855, p<0.001$) tests (Figure
The finding suggests that pretraining molsidomine administration to nNOS KO mice improved the acquisition (STM) and subsequent consolidation (LTM) of contextual fear conditioning. For cued freezing, the ANOVA revealed a significant treatment effect (F (1,27) = 9.271 ; p=0.005); cued freezing was slightly higher in the molsidomine-treated group for STM (14.2% increase) and LTM (10.8% increase) however this effect was not significant (Figure 3.3B). Overall, these results are similar to our previous findings showing that nNOS KO mice had a large deficit in contextual fear conditioning and a rather small deficit in auditory cued fear conditioning (Kelley et al., 2009). In accordance with those findings, the NO donor had a large effect on contextual but not auditory cued fear conditioning.

Posttraining administration of the NO donor to nNOS KO mice

Results of contextual and auditory cued freezing in nNOS KO mice that received posttraining molsidomine treatment (10 mg/kg) are shown in Figure 3.3C and 3.3D, respectively. For contextual freezing, there was no significant treatment effect but a significant time effect (F (2,66) = 15.167 ; p<0.001). Freezing was slightly increased from the magnitudes of basal freezing (vehicle: 10±2% and molsidomine: 12±1%) in vehicle-treated mice in the STM (22±2%) and LTM (24±3%) tests and in the LTM test in molsidomine-treated mice (20±3%). These findings confirmed that contextual freezing in vehicle-treated nNOS KO mice is very low, and that posttraining molsidomine administration had no significant effect (Figure 3.3C). For cued freezing, no significant effects were observed (Figure 3.3D). The results suggest that posttraining administration of the NO donor had no effect on the consolidation of contextual and auditory cued fear conditioning.
Effects of drug treatments on cGMP concentrations in WT and nNOS KO mice

Because the pretraining doses of 50 and 100 mg/kg SMTC proved to be the most efficacious in impairing contextual fear conditioning, the effects of the two doses on cGMP concentrations were determined in hippocampus and amygdala 30 minutes after drug administration in WT mice (Figure 3.4A). Results revealed a significant drug effect in both the hippocampus ($F_{(2,24)} = 50.583; p<0.001$) and the amygdala ($F_{(2,19)} = 17.449; p<0.001$). In the hippocampus, there was a 55% reduction in cGMP following the administration of 50 mg/kg SMTC ($t=6.435; p<0.001$) and a 75% reduction in cGMP following the administration of 100 mg/kg SMTC ($t=9.893; p<0.001$). In the amygdala, there was a 30% reduction in cGMP following the administration of 50 mg/kg SMTC ($t=2.489; p<0.05$) and a 56% reduction in cGMP following the administration of 100 mg/kg ($t=5.904; p<0.001$). The results show that systemic administration of SMTC produced dose-dependent decreases in cGMP concentrations, of which the largest decreases occurred in the hippocampus.

Following molsidomine (10mg/kg) administration in nNOS KO mice (Figure 3.4B), cGMP concentrations were found to be significantly elevated in the hippocampus (5.7-fold) and the amygdala (3.2-fold) compared to controls ($p<0.001$). Preliminary studies using a dose of 20 mg/kg produced higher elevations in cGMP. The results show that systemic administration of molsidomine markedly increased cGMP concentrations in the brain. Notably, the dose of 10 mg/kg molsidomine increased cGMP levels in the hippocampus almost twice as much as in the amygdala.
**Discussion**

The major findings of the present study are: 1) systemic administration of NO signaling modulators affected the acquisition of STM and LTM of contextual fear conditioning; 2) modulation of NO signaling has a direct effect on brain cGMP levels, and appears to be related to contextual fear learning ability; 3) results of the pharmacological modulation of NO signaling corroborate the results of fear conditioning in nNOS KO mice.

Several control experiments confirmed the specific effects of a) the footshock conditioning and b) the drug treatments on freezing response. First, WT mice that had undergone a single-footshock conditioning session developed robust contextual (Figure 3.1A) and auditory cued (Figure 3.1B) freezing responses compared to non-conditioned controls. Second, administration of the optimal dose of SMTC (100 mg/kg) to non-conditioned WT mice had no effect on a) mobility, locomotion, or rearing behavior 30 minutes later (Table 1), and b) freezing behavior 1 h or 24 h later (Figure 3.1A and 3.1B). Third, in agreement with our previous studies, nNOS KO mice that had undergone a single footshock conditioning session had impaired contextual freezing response (Figure 3.1C); however, auditory cued freezing response was relatively normal albeit to a slightly reduced magnitude than WT counterparts (Figure 3.1D) (Kelley et al., 2009). Fourth, administration of the NO donor molsidomine (10 mg/kg) to non-conditioned nNOS KO mice had no effect on motor behavior (Table 1) or freezing behavior 1 h and 24 h later (Figure 3.1C and 3.1D). The results suggest that, in the absence of associative learning, the drug treatments had no effect on motor behavior, either acutely or 1 h and 24 h after
drug administration. These findings support the premise that the drugs had specific
effects on STM and LTM.

It could be argued that modulation of NO signaling influences anxiety-like
behavior (Bilbo et al., 2003), and consequently the results of fear conditioning in nNOS
KO and WT mice may be different. However, several lines of evidence suggest that this
is not the case. First, nNOS KO mice, despite impairments in acquiring contextual fear
conditioning, acquire near optimal auditory cued fear conditioning after a single
footshock conditioning session (Figure 3.1D present study, and Kelley et al., 2009). Thus,
anxiolytic phenotype in nNOS KO mice does not explain the selective impairment in
contextual fear conditioning. Second, SMTC and molsidomine had selective effects on
contextual fear conditioning and no effects on auditory cued fear conditioning. If SMTC
was anxiolytic and molsidomine was anxiogenic, then it would be expected that both
contextual and auditory cued fear conditioning would be affected by the drugs. Third, a
recent study reported that nNOS KO mice exhibited hyperlocomotion and increased
exploratory behavior compared to WT mice in a battery of tests that are used to measure
anxiety-like behavior: open field test, light/dark transition test, and elevated plus maze
(Tanda et al., 2009). Results in Table 1 also suggest that nNOS KO mice exhibit
hyperlocomotion compared to WT mice. Tanda et al. (2009) concluded that nNOS KO
mice are not less anxious than WT controls. Instead, they present hyperlocomotion in
nNOS KO mice that is likely to produce false-positive results in animal models of anxiety
based on exploratory behavior. Together, these finding suggest that the role of NO
signaling in fear conditioning is not dependent on baseline anxiety or
anxiolytic/anxiogenic drug effects.
At present, few studies have investigated the effects of systemic modulation of NO signaling on contextual fear conditioning. In one study, pretraining administration (i.p.) of the nNOS inhibitor 7-nitroindazole had no effect on contextual fear conditioning in rats (Maren, 1998). However, it was concluded that the 7-nitroindazole reduced locomotor activity, which may have confounded the results. However, recent studies have shown that intra-medial prefrontal cortex administration of the nNOS inhibitor N-propyl-L-arginine inhibited the expression of contextual fear response in rats (Resstel et al., 2008).

Our previous study with nNOS KO mice implicated the nNOS gene primarily in hippocampus-mediated contextual fear conditioning (Kelley et al., 2009), therefore we hypothesized that pharmacological inhibition of nNOS in WT mice would impair contextual fear conditioning. The results showed that pretraining administration of the nNOS inhibitor SMTC (10-200 mg/kg) yielded in a U-shaped dose response curve of inhibition of contextual freezing (Figure 3.2A). The lowest doses of 10 and 20 mg/kg had no effect, while the highest (200 mg/kg) also had no effect. It is unclear why the highest dose of SMTC had no effect, but it has been reported that at high doses, non-selective NOS inhibitors can produce U-shaped dose-response curves, which may be due to interactions with eNOS (Esplugues, 2002) or conversion to L-arginine (Joca and Guimaraes, 2006). The results of posttraining administration of SMTC (50 and 100 mg/kg) suggest that optimal inhibition of nNOS in WT mice during the consolidation process alone did not impair contextual fear conditioning (Figure 3.2C and 3.2D). Rather, nNOS inhibition before the acquisition of fear conditioning is required for attenuation of short- and long-term contextual freezing response.
We also hypothesized that facilitation of NO signaling would improve contextual fear conditioning in nNOS KO mice. To test this hypothesis, molsidomine (10 mg/kg) was administered to nNOS KO mice in pretraining (Figure 3.3A and 3.3B) and posttraining (Figure 3.3C and 3.3D) experiments. Previous studies have shown that molsidomine improved learning impairments related to inhibition of NO signaling (Itzhak and Anderson, 2007; Meyer et al., 1998). The current results show that the pretraining molsidomine administration significantly improved contextual fear conditioning in nNOS KO mice, while posttraining drug administration had no effect. The results in nNOS KO mice are corroborative with the results from WT mice, and suggest a role for NO signaling primarily in the acquisition and subsequent consolidation of LTM for contextual fear conditioning. These conclusions may be in agreement with the finding that pretraining administration (10 minutes; i.p. and oral) of the NO signaling facilitator YC-1, a sGC activator, but not posttraining administration, resulted in improved learning in passive and active avoidance (footshock) learning tasks (Chien et al., 2005).

The pharmacokinetics of systemic administration of the drugs may have prevented their influence on memory consolidation in the posttraining experiments. We have measured NO byproducts (NO₂- and NO₃-) in the brain 1 h following similar doses of SMTC and found that the drug had <1 h duration of action (unpublished data). Some evidence suggests that molsidomine is also metabolized rather quickly. For instance, peak plasma levels were observed 0.5 h following oral molsidomine administration in rats; after 1 h the plasma contained only 24% of peak-level molsidomine and 37% of its active metabolite SIN-1 (Wilson et al., 1986). Future investigations utilizing site-specific drug administration may allow us further discrimination of the drugs’ effects on memory
consolidation, and whether selective modulation of hippocampal NO signaling has a similar effect on contextual fear conditioning.

A role for NO signaling in cued fear conditioning has been previously demonstrated where site-specific inhibition of nNOS in the lateral amygdala impaired LTM for auditory cued fear conditioning and LTP in rats (Schafe et al., 2005). Our results differ from that study because LTM of auditory cued fear conditioning was not affected by pretraining or posttraining administration of the nNOS inhibitor SMTC. This variation may be due to the different outcomes of global deletion of nNOS (Kelley et al., 2009) and the global inhibition of nNOS we performed in WT mice in the present study, compared to selective inhibition of amygdalar nNOS (Schafe et al., 2005). It appears that global deletion or inhibition of nNOS has a more profound effect on contextual fear conditioning than auditory cued fear conditioning, while selective inhibition of NO signaling in the lateral amygdala impaired auditory cued fear conditioning. It should be noted, however, that the study by Schafe et al. (2005) did not investigate the outcome of selective inhibition of NO signaling in the lateral amygdala on contextual fear conditioning; nor has the effect of selective inhibition of hippocampal nNOS on contextual fear conditioning been reported.

To confirm that the systemic administrations of SMTC and molsidomine modulated NO levels in the brain, cGMP concentrations were determined 30 minutes following drug administration to WT and nNOS KO mice. The hippocampus and amygdala were investigated because these substrates are critical for the acquisition and consolidation of LTM for contextual and cued fear conditioning (Phillips and LeDoux, 1992). In WT mice, the behaviorally effective doses of SMTC (50 and 100 mg/kg) dose-
dependently reduced cGMP concentrations in the hippocampus and amygdala (Figure 3.4A). In nNOS KO mice, molsidomine (10 mg/kg) markedly increased cGMP in the hippocampus and amygdala (Figure 3.4B). The results suggest that pharmacological modulation of cGMP concentrations via the NO signaling pathway correlates with the outcome of contextual fear conditioning. Also noteworthy is the finding that basal cGMP concentrations were markedly lower in nNOS KO mice compared to WT mice (Figure 3.4A and 3.4B). Together, these findings may implicate basal cGMP concentrations in the hippocampus and amygdala as predictive of contextual fear conditioning acquisition and consolidation.

The role for cGMP-mediated signaling in hippocampal LTP has been shown (Hopper and Garthwaite, 2006; Taqatqeh et al., 2009; Zhuo et al., 1994). Recently, it has been reported that cGMP-mediated signaling in the lateral amygdala is required for LTP and LTM consolidation (Ota et al., 2008; Paul et al., 2008). Site-specific inhibition of NO-cGMP-PKG signaling in the lateral amygdala blocked auditory cued fear conditioning (Ota et al., 2008). Also, mice deficient in cGMP-dependent protein kinase I (cGKI), but not cGKII had deficits in auditory cued fear conditioning (Paul et al., 2008). Our findings that a) nNOS KO mice did not show deficits in auditory cued fear conditioning (Kelley et al., 2009) and b) inhibition of nNOS by SMTC had no significant effect on auditory cued fear conditioning may seem to be in odds with the above studies. However, deletions of the nNOS gene and the cGKI gene may result in different outcomes. Also, we are not sure how deletion and inhibition of nNOS affects cGKI and cGKII. For instance, if in the absence of nNOS cGKII, but not cGKI activity, is modulated then it is expected that cued fear conditioning would not be affected (Paul et
al., 2008). Also, it should be noted that in the present study the NO signaling modulators, SMTC and molsidomine, had a greater effect on hippocampal cGMP levels than on amygdalar cGMP levels (Figure 3.4). This finding may explain in part the effect of the drugs on contextual (hippocampus-mediated) fear conditioning than on cued (amygdala-mediated) fear conditioning. Notably, modulation of cGMP signaling significantly altered hippocampus-mediated learning in social and object recognition tasks (Boess et al., 2004; Furini et al., 2009; Prickaerts et al., 2002).

In summary, the present study demonstrates the role for NO signaling in the acquisition and subsequent consolidation of LTM for contextual fear conditioning. The current findings are in agreement with our previous studies, which implicated the nNOS gene in contextual fear conditioning (Kelley et al., 2009). It appears that modulators of NO signaling have a direct effect on hippocampal and amygdalar cGMP, which implicates both NO and cGMP in memory acquisition and subsequent consolidation of LTM for contextual fear conditioning.
Table 3.1. Effects of drug treatments on motor behavior of non-conditioned WT and nNOS KO mice. No differences in immobility, locomotion, or rearing frequency were observed between vehicle- and SMTC-treated WT mice and between vehicle- and molsidomine-treated nNOS KO mice. This suggests that the NO modulators had no significant effects on activity which could have influenced fear conditioning. However, nNOS KO mice were found to be slightly more active than WT mice for immobility and locomotion.

<table>
<thead>
<tr>
<th>Group</th>
<th>Immobility (%)</th>
<th>Locomotion (cm)</th>
<th>Rearing (total frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>18.7±1.2</td>
<td>994±52</td>
<td>12.5±1.7</td>
</tr>
<tr>
<td>SMTC</td>
<td>20.2±2.4</td>
<td>890±71</td>
<td>12.0±2.0</td>
</tr>
<tr>
<td><strong>nNOS KO mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>11.9±1.1*</td>
<td>1441±106*</td>
<td>17.4±2.0</td>
</tr>
<tr>
<td>Molsidomine</td>
<td>9.8±1.0†</td>
<td>1334±86.0†</td>
<td>17.5±1.2</td>
</tr>
</tbody>
</table>

Data presented are mean ± SEM of the three behavioral parameters (n=13 mice/group). *p<0.05 for WT mice vehicle vs. nNOS KO mice vehicle; †p<0.05 for WT mice vehicle vs. nNOS KO mice molsidomine
Figure 3.1. Effects of vehicle (veh) and drug treatments on freezing behavior in non-conditioned WT and nNOS KO mice compared to freezing behavior in conditioned mice (n=10-12/group). The effect of SMTC (100 mg/kg) was tested in WT mice, and the effect of molsidomine (Mol, 10 mg/kg) was tested in nNOS KO mice. A. Contextual freezing in WT mice (*p<0.001). B. Auditory cued freezing in WT mice (*p<0.005). C. Contextual freezing in nNOS KO mice. D. Auditory cued freezing in nNOS KO mice (*p<0.001). (Taken from Kelley et al., 2010)
Figure 3.2. The effects of pretraining (30 min) and immediate posttraining administrations of SMTC (10 – 200 mg/kg) on contextual and auditory cued fear conditioning in WT mice (n=8-15/group). A. Contextual freezing following pretraining SMTC administration (*p<0.001). B. Auditory cued freezing following pretraining SMTC administration. C. Contextual freezing following posttraining SMTC administration. D. Auditory cued freezing following posttraining SMTC administration. (Taken from Kelley et al., 2010)
Figure 3.3. Effects of pretraining (30 min) and immediate posttraining administrations of molsidomine (10mg/kg) on contextual and auditory cued fear conditioning in nNOS KO mice (n=8-12/group). A. Contextual freezing following pretraining molsidomine administration (*p<0.001). B. Auditory cued freezing following pretraining molsidomine administration. C. Contextual freezing following posttraining molsidomine administration. D. Auditory cued freezing following posttraining molsidomine administration. (Taken from Kelley et al., 2010)
Figure 3.4. cGMP concentrations in the hippocampus and amygdala 30 minutes following systemic administration (i.p.) of NO signaling modulators. A. WT mice (n=6-10/group) received vehicle or SMTC (50 and 100 mg/kg) (*p<0.001; #p<0.05). The nNOS inhibitor dose-dependently reduced cGMP in the hippocampus and amygdala B. nNOS KO mice (n=2-6/group) received vehicle or the NO donor molsidomine (10 and 20 mg/kg) (*p<0.001). The NO donor increased cGMP in the hippocampus and amygdala.
Chapter 4

Long-term memory of visually cued fear conditioning: roles of the nNOS gene and CREB

Summary

Nitric oxide (NO) produced by neuronal nitric oxide synthase (nNOS) has a role in late-phase long-term potentiation (LTP) and long-term memory (LTM) formation. Our recent studies implicated NO signaling in contextual and auditory cued fear conditioning. The present study investigated the role of NO signaling in visually cued fear conditioning. First, visually cued fear conditioning was investigated in wild-type (WT) and nNOS knockout (KO) mice. Second, the effects of pharmacological modulators of NO signaling on the acquisition of visually cued fear conditioning were investigated. Third, plasma levels of corticosterone were measured to determine a relationship between physiological and behavioral responses to fear conditioning. Fourth, levels of ERK1/2 and CREB phosphorylation, downstream of NO signaling, were determined in the amygdala as potential correlates of fear learning. Mice underwent single or multiple (4) spaced trainings that consisted of a visual cue (blinking light) paired with footshock. WT mice acquired cued and contextual LTM following single and multiple trainings. nNOS KO mice acquired neither cued nor contextual LTM following a single training; however, multiple trainings improved contextual but not cued LTM. The selective nNOS inhibitor S-methyl-thiocitrulline (SMTC) impaired cued and contextual LTM in WT mice. The NO donor molsidomine recovered contextual LTM but had no effect on cued LTM in nNOS KO mice. Re-exposure to the visual cue 24h posttraining elicited freezing response and a marked increase in plasma corticosterone levels in WT but not nNOS KO
The expression of CREB phosphorylation (Ser-133) was significantly higher in naïve nNOS KO mice than in WT counterparts, and pharmacological modulators of NO had significant effects on levels of CREB phosphorylation and expression. These findings suggest that visual cue-dependent LTM is impaired in nNOS KO mice, and aberrant modulation of CREB in the absence of the nNOS gene may hinder cued and contextual LTM formation.

**Background**

Fear conditioning is an associative learning paradigm which is used to investigate cue and context-dependent long-term memory (LTM) formation. In the fear conditioning task, the presentation of an aversive unconditioned stimulus (US; foot shock) is temporally paired with the presentation of a neutral conditioned stimulus (CS; sensory cue) within a discrete context. The subject learns that the CS and the training context are predictive of the aversive US, and subsequent CS and context exposures elicit conditioned fear responses in the absence of the US. The fear response in rodents includes freezing behavior and the release of the stress hormone corticosterone (Rodrigues et al., 2009). The rodent responses to fear conditioning are considered analogous to the expression of the symptoms of posttraumatic stress disorder (PTSD) in humans (Mineka and Oehlberg, 2008). Understanding the mechanisms of LTM formation related to fear conditioning will facilitate the development of treatments for PTSD.

The neural pathways mediating cued and contextual fear conditioning have been extensively studied. Pharmacological and lesion studies suggest roles for the hippocampus and hippocampal long-term potentiation (LTP) in contextual fear conditioning (Ahi et al., 2004; Maren and Fanselow, 1995; Phillips and LeDoux, 1992).
For cued fear conditioning, direct thalamo-amgydala projections rapidly transmit sensory information regarding the CS and US to the basolateral amygdala where Hebbian LTP and LTM permit development of conditioned response (Bauer et al., 2001; Rogan et al., 1997). Before reaching the basolateral amygdala, retinal projections relay visual CS information (via the superior colliculus) to the lateral geniculate nucleus and lateral posterior nucleus of the thalamus (Doron and Ledoux, 1999; Shi and Davis, 2001). The auditory CS pathway includes the medial geniculate nucleus and posterior intralaminar nucleus of the thalamus (Doron and Ledoux, 1999; LeDoux, 2000). It was recently shown that for auditory fear conditioning, nitric oxide (NO) signaling in the basolateral amygdala regulated retrograde extracellular signal-related kinase (ERK1/2)-mediated gene transcription in the aforementioned auditory thalamic nuclei in rats (Overeem et al., 2010). This was the first study to demonstrate a role for retrograde NO signaling in the direct thalamo-amygdala pathway for fear conditioning. A role for NO signaling in visually cued fear conditioning has not been investigated.

NO in the brain is primarily produced by neuronal nitric oxide synthase (nNOS) and has the role of retrograde neurotransmitter. The NO signal transduction pathway facilitates synaptic plasticity and late-phase LTP in the amygdala (Chien et al., 2003; Schafe et al., 2005) and hippocampus (Arancio et al., 1996; Lu et al., 1999). NO stimulates pre- and post-synaptic cyclic nucleotide production leading to the activation ERK1/2 and subsequently the transcription factor cyclic adenosine monophosphate response element-binding protein (CREB) (Contestabile, 2008; Lu et al., 1999; Riccio et al., 2006). The nNOS gene and NO signaling have been implicated in contextual fear
conditioning (Kelley et al., 2010; Kelley et al., 2009; Resstel et al., 2008) and in auditory cued fear conditioning (Ota et al., 2008; Schafe et al., 2005).

The present study was undertaken to investigate the role of NO signaling in visually cued fear conditioning. First, visually cued fear conditioning was investigated in wild-type (WT) and nNOS knockout (KO) mice. Second, the effect of pharmacological modulators of NO signaling on acquisition of visual fear conditioning was investigated. Third, plasma levels of the stress hormone corticosterone were measured to determine the relationship between physiological and behavioral responses to fear conditioning. Fourth, levels of ERK1/2 and CREB phosphorylation, which are downstream of NO signaling, were determined in the amygdala and hippocampus as potential correlates of fear learning.

Materials and Methods

Subjects

Breeding of nNOS KO and WT mice was carried out in our facilities at the University of Miami, Miller School of Medicine, Miami, FL, as described previously (Balda et al., 2006). Adult male and female homozygote nNOS KO mice (B6;129S-Nos1; 6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). The targeted mutation of the nNOS gene in KO mice resulted in >95% inhibition of nNOS activity (Huang et al., 1993). The mice have not been further backcrossed onto any inbred strain, therefore the KO have approximately 1:1 ratio of the genetic backgrounds of the parental strains. Following arrival of the nNOS KO mice to the viral antibody free facilities at the University of Miami, Miller School of Medicine, animals were single-sex habituated to the new environment for 1 week before breeding. Each litter of newborn
nNOS KO mice (n=6-8) routinely contained about equal numbers of males and females. Mice were weaned on postnatal day (PD) 21 and were housed in single-sex groups of 4-5 per cage.

WT mice were generated from the breeding of C57BL/6J females with SV129 males. The F2 progeny are more appropriate controls than the F1 hybrids because the parental alleles of F1 mice are not segregating like those on a mixed B6;129S background. The F2 generation has a 1:1 proportion of the genetic backgrounds of C57BL/6J and SV129 strains, approximating the genetic background of nNOS KO mice (Jackson Laboratories, Bar Harbor, ME). Adult SV129 males and C57BL/6J females (6-7 weeks old) were purchased from Jackson Laboratories and bred in our facilities at the University of Miami, Miller School of Medicine. Following a 1 week habituation period, mice were bred to generate B6;129F1 progeny. After weaning (PD21), the mating of F1 x F1 offspring (>PD60) generated B6;129F2 progeny (8-12 per litter). This latter progeny is considered the WT counterpart to nNOS KO mice, as it is congenic to the KO mouse strain (Itzhak et al., 1998). WT mice were weaned on PD21 and segregated according to sex into groups of 5 per cage.

Adult (8-10 week) males were used for all experiments. Routinely, each experimental group of mice (n=5-14) contained subjects from 3 to 4 litters. As the KO and WT are considered inbred and hybrid, respectively, the concern of the “litter effect” is insignificant. Animals were housed in a temperature- (22±0.5 °C) and humidity- (50%) controlled room and maintained on a 12-h light/dark schedule. Food and water were available ad libitum. Animal care was in accordance with the Guide for the Care
Drug Treatments

The preferential nNOS inhibitor S-methyl-L-thiocitrulline (SMTC; 100mg/kg) was dissolved in water (vehicle) and administered to WT mice. The NO donor molsidomine (10 and 20 mg/kg) was dissolved in saline (vehicle) and administered to nNOS KO and WT mice. Drugs were given intraperitoneally (IP; volume of 0.1mL/10mg) 30 min pretraining or 30 min prior to sacrifice for the western blotting studies. We have shown that SMTC (100 mg/kg) and molsidomine (10 mg/kg) modulated a) the development of contextual fear conditioning, and b) the levels of cyclic guanosine monophosphate (cGMP) in the amygdala and hippocampus of WT and nNOS KO mice (Kelley et al., 2010). In the current study, two doses of molsidomine were investigated, 10 and 20 mg/kg.

Fear conditioning apparatus and procedure

The fear conditioning apparatus used was previously described (Kelley et al., 2009). Briefly, fear conditioning training and testing occurred in Plexiglas chambers (30.5 x 30.5 x 43.5 cm; Noldus Information Technology Inc., Leesburg, VA). Each chamber was equipped with a stainless steel floor through which the electric footshock was delivered. An upper control unit housed a white light illuminating one corner of the chamber; a video camera and yellow light stimulus were located in the center. The chambers were housed in custom built sound-attenuating chambers which gave the appearance of black walls. The floors of the chamber were cleaned with a diluted soap solution (1% Alconox), rinsed with water, and dried after each animal.
During training, mice were transferred in a covered carrier cage from the housing colony to the experimental room. Mice were placed in the training context (context A) and allowed to habituate to the novel environment for 134 s. Next, the corner white light was turned off and the center yellow light began blinking for 16 s (2 s on/off intervals). Pretraining mobility (%) in the context and during the visual cue was determined at this time as a measure of baseline activity which is depicted in the figures. The final 2 s blink of the yellow light (CS) co-terminated with a 2 s footshock (0.75 mA) and then mice were returned to the home cage 30 s later. In the multiple (4) training experiments, mice underwent a series of four of the previously described trainings with an intertrial interval (ITI) of 10-12 min, during which the mice were returned to home cage. Results of each experimental group were compared to a control group that underwent the same treatments in the absence of the footshock (referred to as “no shock” in the figures).

For testing, contextual fear conditioning was measured in context A (training context) and consisted of digitally recording the subject’s percentage of time spent “freezing” while in the chamber for 2 min. Freezing was defined as a complete lack of movement besides respiration and was measured automatically using EthoVision v3.1 software (Noldus Information Technology, Inc.) with the following parameters: 6 samples/s and immobility threshold of 5% (Pham et al., 2009). Freezing is expressed as a percentage of the total time for each test. Visually cued fear conditioning was measured in a different context (context B), which utilized a smooth white foam pad floor that covered the shock grid, four opaque white walls, and an olfactory enrichment of pure orange extract that was affixed to the chamber ceiling. To further distinguish the contextual and cued LTM tests, the subjects were transferred to the experimental room on
a wheeled cart and the lighting in the experimental room was dimmed. Thus, multiple sensory cues were changed to differentiate the contextual and cued LTM test conditions. After a 2 min habituation period in context B, the yellow light cue blinked for 2 min. Contextual and visually cued fear conditioning tests were separated by a 4 h interval and took place 24 h and 7 d posttraining. Results of the 24 h and 7 d tests following a single training are depicted in Figure 4.1. Since the results of the 24 h and 7 d tests following multiple trainings were similar, only the results of the 24 h tests are depicted in Figure 4.2.

**Plasma corticosterone measurements**

Corticosterone assays were performed as previously described (Kelley et al., 2009). On Day 1, 15 min before, and 15 min after fear conditioning training, blood samples (80µl) were drawn from the retroorbital venous plexus using heparinized microcapillary tubes. On Day 2, blood samples were drawn 15 min after the visual CS test. Samples were then treated according to instructions provided in a corticosterone EIA kit (Immunodiagnostic Systems, Inc.), and analyzed on a spectrophotometer at 450nm.

**Western blotting**

WT mice received injections of SMTC (100 mg/kg), molsidomine (20 mg/kg), or vehicle and nNOS KO mice received molsidomine (20 mg/kg) or vehicle 30 min before sacrifice by cervical dislocation. The amygdala and hippocampus were immediately microdissected according to The Mouse Brain Atlas (Paxinos and Franklin, 2001). Tissue was homogenized in ice-cold buffer (20mM Tris-HCl, pH 7.4, 0.32 sucrose, 1mM EDTA, 1mM EGTA, 1mM PMSF, 50mM NaF, phosphatase inhibitors (Cocktail II, Sigma)) and protease inhibitors (Complete Mini tablets; Roche Diagnostics). Lanes were
loaded with 50µg protein/well, separated using 10% SDS-PAGE, and transferred to PVDF membranes. Membranes were incubated first for 1h at room temperature (RT) in blocking buffer: TBS + 50mM NaF + 0.1% Tween-20 (TBS-T) + 0.4% I-BLOCK. Next, membranes were incubated with anti-phosphorylated CREB (Ser133) (1:1000) at 4°C for 72 h or anti-phosphorylated ERK1/2 (1:2000) at RT for 1 h (both Cell Signaling, Beverly, MA) diluted in TBS-T + 5% BSA. After incubation, membranes were washed 3x for 5 min with TBS-T and then incubated (1h at RT) with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit (dilutions of 1:1500 for pCREB; 1:2000 for pERK1/2) (Cell Signaling) and washed 3x for 5 min. The signal was visualized using an enhanced chemiluminescent (ECL) substrate for HRP enzyme (Pierce, Rockford, IL). Following development of the immunoblots for the phosphorylated protein, the immunoblots were stripped with Restore western blot stripping buffer (Pierce) for 15 min. After 3 quick rinses in TBS-T, the membranes were blocked again as above and then incubated with anti-total CREB (1:1000) or anti-total ERK1/2 (1:3000) antibodies (both Cell Signaling) for 1h at RT in TBS-T + 5% BSA. Next, membranes were washed and treated as described for pCREB and pERK1/2 except the secondary antibody was diluted at 1:3000. To control for protein loading membranes were stripped once more and probed for beta-tubulin. Quantification of the bands corresponding to changes in protein levels were calculated by scanned image densitometric analysis UN-SCAN-IT gel analysis software (v6.1, Silk Scientific Inc., Orem, UT). Relative density units are expressed as the phosphorylated/total isoform and total isoform/ beta-tubulin.
Statistical Analysis

Results of cued and contextual freezing following a single training were analyzed by two-way ANOVA (group x time; pretraining, 24 h or 7d) for each genotype. Results of cued and contextual freezing following multiple trainings were analyzed by two-way ANOVA (group x time; pretraining or 24 h) for each genotype. The corticosterone results were analyzed by three-way ANOVAs (genotype x group x time) for the single and multiple training experiments. A relationship between the magnitude of cued freezing and corticosterone levels following re-exposure to the visual cue 24 h posttraining was determined by Pearson product moment correlation. ANOVAs were followed by post hoc analysis using Bonferroni correction to determine differences between multiple groups. Protein levels in the Western blotting studies were compared using unpaired, two-tailed Student’s $t$ tests. All results are shown as mean ± standard error of the mean. A $p$ value of <0.05 was considered statistically significant.

Results

Fear conditioning in WT and nNOS KO mice following a single training

WT mice

WT mice acquired visually cued and contextual fear conditioning following a single training (Figure 4.1A, 4.1B). Pharmacological inhibition of nNOS impaired both types of learning (Figure 4.1A, 4.1B). For visually cued freezing, there were significant effects for group ($F_{(2,55)} = 20.002, p<0.001$), time ($F_{(2,55)} = 25.819, p<0.001$), and the interaction between the two variables ($F_{(4,55)} = 4.464, p<0.003$). Post hoc analysis of 24 h results revealed significantly higher freezing in the vehicle group (49±3%) than the SMTC group (28±4%, $p=0.006$) and the no shock group (26±4%, $p<0.001$). Analysis of
the 7 d results also showed significantly higher freezing for the vehicle group (56±4%) than the SMTC group (30±4%, p<0.001) and the no shock group (35±4%, p=0.01).

For contextual freezing, there were significant effects for group (F(2,55) = 37.720, p<0.001), time (F(2,55) = 72.107, p< 0.001), and the interaction between the two variables (F(4,55) = 9.314, p<0.001). Post hoc analysis of the 24 h results revealed significantly higher freezing in the vehicle group (70±4%) than the SMTC group (46±4%, p<0.001) and the no shock group (28±4%, p<0.001). Analysis of the 7 d results showed significantly higher freezing for the vehicle group (70±4%) than the SMTC group (47±4%, p=0.004) and the no shock group (35±4%, p<0.001). The results of the effects of SMTC on contextual fear conditioning are in agreement with our previous studies (Kelley et al., 2010).

**nNOS KO mice**

nNOS KO mice showed impairments in both visually cued and contextual fear conditioning (Figure 4.1C, 4.1D). The NO donor molsidomine improved contextual but not cued fear conditioning (Figure 4.1C, 4.1D). For visually cued freezing, the ANOVA revealed a significant time effect (F(2,69) = 23.148, p<0.001) and non-significant group effect. A trend of increased immobility on day 7 (20±3%) compared to pretraining (11±3%) and 24 h (16±3%) in the no shock group is likely due to habituation to the light cue. In rodents, habituation occurs following repeated exposure to a novel context in the absence of a biologically relevant consequence (i.e. no shock); as a result there is a decrease in exploratory behavior (Leussis and Bolivar, 2006). Decreased exploratory behavior resulted in a higher magnitude of immobility, or “freezing” as it is represented in the figures. Also, it was observed that pretraining, or baseline, immobility in the novel
context was reduced in nNOS KO compared to WT counterparts (WT: 17±1% vs. KO: 9±1%; Figures 4.1A, 4.1B). This suggested that nNOS KO mice are slightly more active and exhibited increased exploratory behavior in a novel context compared to WT, which we (Kelley et al., 2010) and others (Tanda et al., 2009) have previously reported. Importantly, in nNOS KO mice there was no significant group effect, suggesting that molsidomine at all doses tested had no effect on cued fear conditioning (Figure 4.1C).

Conversely, molsidomine dose-dependently improved contextual fear conditioning (Figure 4.1D). ANOVA revealed significant effects for group ($F_{(3,69)} = 36.397, p<0.001$), time ($F_{(3,69)} = 92.642, p<0.001$), and the interaction between the two variables ($F_{(6,69)} = 8.605, p<0.001$). For the molsidomine (10 mg/kg) group, contextual freezing after 24 h reached 40±3%, which was increased from the vehicle group (23±3%; $p=0.005$) and no shock group (22±3%; $p=0.004$). Freezing after 7 d in the molsidomine (10 mg/kg) group reached 39±4% which was increased from the vehicle group (22±3%, $p<0.05$) and the no shock group (22±3, $p<0.05$). For the molsidomine (20 mg/kg) group, contextual freezing reached 48±3% after 24 h and 62±3% after 7 d. The freezing in the molsidomine (20 mg/kg) group was significantly increased from all other groups ($p<0.001$ compared to vehicle and no shock groups; $p<0.05$ compared to molsidomine (10 mg/kg) group). The results of molsidomine effects on contextual freezing are similar to our previous studies where only a single dose of the NO donor was tested (10 mg/kg; Kelley et al., 2010).
Fear conditioning by multiple trainings improves LTM

Results of multiple (4) trainings of WT and nNOS KO mice are depicted in Figure 4.2. Only the results of the 24 h tests are presented for LTM because no significant differences between the results of 24 h and 7 d tests were observed.

WT mice

Mice underwent four spaced trainings with intertrial intervals of 10-12 min. WT mice acquired cued and contextual fear conditioning after multiple trainings (Figure 4.2A, 4.2B). For visually cued freezing there was a significant group effect (F(1,26) = 36.499, p<0.001), time effect (F(1,26) = 124.632, p<0.001), and interaction (F(1,26) = 34.714, p<0.001). Post hoc analysis revealed significantly higher freezing in the conditioned group (67±3%) compared to the no shock group (30±3%) during re-exposure to the visual CS (p<0.001). The small increase in immobility (15.7% increase) in the no shock group compared to pretraining (14±2%; p<0.05) may be due to habituation to the light cue.

For contextual freezing there was a significant group effect (F(1,26) = 100.867, p<0.001), time effect (F(1,26) = 254.042, p =0.007), and interaction (F(1,26) = 87.495, p<0.001). Freezing in the conditioned group (84±2%) was significantly higher than in the no shock group (30±3%; P<0.001).

nNOS KO mice

nNOS KO mice failed to acquire visually cued fear conditioning (Figure 4.2C) but did acquire contextual fear conditioning (Figure 4.2D) after multiple trainings. For visually cued freezing there was a significant time effect (F(1,20) = 110.814, p<0.001), but no significant group effect (p>0.05), and a significant interaction between group and time
Post hoc analysis revealed slightly increased freezing in the conditioned group (17±1%) and the no shock group (22±1%) compared to pretraining levels (6±1% and 5±2%, respectively) which may be due to habituation to the light cue. The finding that no differences were observed between the conditioned and no shock groups after 24 h suggests that the impairments for visually cued fear conditioning persisted after the multiple trainings.

For contextual freezing there was a significant group effect \((F_{(1,20)} = 56.361, p<0.001)\), time effect \((F_{(1,20)} = 160.902, p<0.001)\), and interaction \((F_{(1,20)} = 55.730, p<0.001)\). Freezing was significantly higher in the conditioned group (68±3%) compared to the no shock group (23±3%; \(p<0.001)\), suggesting that the multiple spaced trainings facilitated LTM for contextual fear conditioning. These results are in agreement with our previous studies in nNOS KO mice, showing improvement in contextual fear conditioning following multiple trainings (Kelley et al., 2009).

**Plasma corticosterone response correlates with fear learning**

**Single training**

Plasma corticosterone levels were measured on day 1, both 15 min pretraining and 15 min posttraining. Subsequently, 24 h later, we investigated whether re-exposure to the visual CS elicits an increase in the stress hormone. Figure 4.3A depicts results from WT and nNOS KO mice using a single training. There was a significant group effect \((F_{(1,63)} = 4.422, p<0.05)\), time effect \((F_{(2,63)} = 15.774; p<0.001)\), and interaction between genotype and group \((F_{(1,63)} = 4.811; p<0.05)\). Post hoc tests showed no significant differences between pretraining corticosterone levels across all groups. In the WT shock group, there were significant increases from pretraining to posttraining and Day 2 post
visual CS exposure (p<0.001). In nNOS KO mice, neither the exposure to the shock nor the visual CS increased plasma corticosterone levels (Figure 4.3A). Thus, the results in nNOS KO mice showed that the absence of corticosterone responses was associated with impaired LTM formation (Fig. 4.1), while in WT mice increases in corticosterone levels were associated with successful LTM formation (Fig. 4.1). Together, these results suggested a positive correlation between corticosterone responses and fear learning.

Multiple trainings

Figure 4.3B depicts results of corticosterone levels following multiple (4) fear conditioning trainings. There were significant effects of group (F(1,72) = 9.681; p=0.003) and time (F(2,72) = 16.512; p<0.001). The WT mice exhibited posttraining increases in corticosterone following multiple (4) US (p<0.001) and pseudotrainings (p<0.05) on Day 1. Increased corticosterone in the pseudotrained control mice suggests that the repeated handling and transfers were stressful. Importantly, re-exposure to the visual CS on Day 2 only elicited corticosterone response in the WT shock group (p<0.001). The nNOS KO shock group exhibited an increase in corticosterone levels from pretraining to posttraining (p<0.001) but not on Day 2 after visual CS re-exposure. The hormonal response of nNOS KO mice 15 min following four trainings appears to correlate with acquisition of contextual fear conditioning as we have previously reported (Kelley et al., 2009). The lack of hormonal response to the visual cue in nNOS KO mice on Day 2 correlated with the impaired acquisition of cued fear conditioning.

Correlation analysis

Correlations between corticosterone levels 15 min following re-exposure to the visual CS (Day 2) and behavioral freezing in the LTM test are shown in Figure 4.4.
Significant positive correlations were obtained for corticosterone and freezing responses after a single training (Figure 4.4A; \( r=+0.554, \ p=0.007 \)) and after multiple trainings (Figure 4.4B; \( r=+0.545, \ p=0.005 \)). These results suggested that the magnitudes of physiological and behavioral responses to conditioned fearful stimuli were correlated for both intensities of training.

**Western blotting studies**

**Expression of ERK1/2 and CREB in WT and nNOS KO mice**

We performed western blot analysis of tissue from naïve WT and nNOS KO mice to determine if there were genotypic differences in the expression of ERK1/2, CREB, and the respective phosphorylated isoforms, pERK1/2 and pCREB in the amygdala (Figure 4.5) and hippocampus (Figure 4.9). The levels of pERK1/2 and pCREB were normalized to total ERK1/2 and CREB levels, respectively, and expressed as arbitrary relative density units (Figures 4.5 and 4.9 panels B and E). The levels of total protein were normalized to beta-tubulin loading control (Figures 4.5 and 4.9 panels C and F). Student’s t-tests (unpaired, two-tail) revealed elevated pCREB in nNOS KO mice amygdala (2-fold; \( p=0.005 \); Figure 4.5E) and hippocampus (1.54-fold; \( p=0.005 \); Figure 4.9E) compared to WT mice. The levels of pERK1/2, ERK1/2, and total CREB were not different. The results suggest that elevated CREB phosphorylation in the amygdala and hippocampus of nNOS KO mice is independent of total CREB expression and ERK1/2 activity.
Effects of the pharmacological NO signaling modulators on ERK1/2 in the amygdala of WT and nNOS KO mice

Results showed that the pharmacological NO signaling modulators had no effects on ERK1/2 phosphorylation or total ERK1/2 expression in the amygdala of WT and nNOS KO mice 1 hour after drug administration (Figure 4.6).

Effects of SMTC and molsidomine on CREB in the amygdala of WT mice

We investigated the effects of the behaviorally effective doses of NO signaling modulators on CREB in the amygdala 30 min following drug administration in WT mice. Results are summarized in Figure 4.7. The nNOS inhibitor SMTC reduced pCREB expression by 75% of control vehicle (Figure 4.7B; p<0.001) and total CREB expression by 44% of control vehicle (Figure 4.7C; p<0.05). Conversely, the NO donor molsidomine increased pCREB expression by 51% of control vehicle (Figure 4.7E; p=0.007) but had no effect on total CREB expression (Figure 4.7F). The findings suggest that inhibition and activation of NO signaling pathway influenced CREB activation and expression in WT mice, which is consistent with the NO-cGMP transduction pathway.

Effects of NO donor molsidomine on pCREB expression in the amygdala of nNOS KO mice

Unexpectedly, molsidomine (20 mg/kg) reduced pCREB expression in the amygdala of nNOS KO mice by 48% of control vehicle (Figure 4.8B; p<0.05). The drug had no effect on total CREB expression (Figure 4.8C).
Effects of the pharmacological NO signaling modulators on ERK1/2 and CREB in the hippocampus of WT and nNOS KO mice

Results showed that the pharmacological NO signaling modulators had no effects on hippocampal expressions of phosphorylated or total ERK1/2 (Figure 4.10) and phosphorylated or total CREB (Figure 4.11). These findings suggest that hippocampal phosphorylation and expression of these proteins are less susceptible to acute pharmacological modulations of NO signaling than for the amygdala.

Discussion

Genetic deletion and pharmacological inhibition of nNOS impair cued and contextual fear conditioning

Previous studies have implicated NO signaling in contextual (Kelley et al., 2010; Kelley et al., 2009; Resstel et al., 2008) and auditory cued (Ota et al., 2008; Overeem et al., 2010; Schafe et al., 2005) fear conditioning. Our previous studies showed that nNOS KO mice had short- and long-term memory deficits for auditory cued (minor) and contextual (major) fear conditioning (Kelley et al., 2009) that were partially rescued by pretraining, but not posttraining, administration of NO donor (Kelley et al., 2010). Therefore, our previous studies implicated NO in the acquisition of fear learning. The present study investigated the effects of genetic deletion and acute pharmacological inhibition of nNOS on LTM for visually cued and contextual fear conditioning. The results of acute pharmacological inhibition of nNOS by SMTC corroborated the results of the genetic deletion of nNOS, suggesting a requirement for NO signaling in the acquisition and subsequent consolidation of LTM for visually cued and contextual fear conditioning. Yet, the finding that the NO donor molsidomine did not improve visually
cued fear conditioning in nNOS KO mice suggests that exogenously administered NO donor may be insufficient to reverse this particular impairment. Previous studies from our laboratory showed that lower doses of molsidomine (4 – 10 mg/kg) improved a) drug-induced conditioned place preference learning (Itzhak and Anderson, 2007), and b) contextual fear conditioning (Kelley et al., 2010) in nNOS KO mice. The present study showed a dose-dependent improvement in contextual fear conditioning in nNOS KO mice following administration of molsidomine (Figure 4.1D). Molsidomine (10 mg/kg) caused a 5.7-fold increase in cGMP in the hippocampus of nNOS KO mice (Kelley et al., 2010) which may be related to the improvements in contextual LTM.

It has been suggested that using a discrete visual CS for fear conditioning requires multiple CS-US pairings to elicit fear conditioned response (Heldt et al., 2000; Newton et al., 2004). Our results show, however, that WT mice acquired visually cued fear conditioning after a single training and that cued LTM was only moderately improved (19% increase in freezing) by multiple (4) spaced trainings. Further, the multiple spaced trainings failed to improve visually cued LTM in nNOS KO mice. These outcomes are similar to our previous studies with auditory cued fear conditioning in which multiple (4) trainings moderately improved auditory cued LTM in WT mice but not in nNOS KO mice (Kelley et al., 2009). However, the magnitude of auditory cued LTM deficits in nNOS KO mice was much smaller (10-15% less freezing than WT; Kelley et al., 2009) than the magnitude of visual cue LTM deficits (Figure 4.1A, 4.1C). Differential dependency of NO signaling in the auditory and visual CS processing pathways, such as within the basolateral amygdala, may underlie the differences between auditory and visually cued fear conditioning.
The current findings demonstrate that the acquisition of contextual LTM is facilitated by multiple (4) spaced trainings in WT and nNOS KO. Several studies suggest that single and multiple spaced trainings recruit different mechanisms and substrates for contextual LTM. For instance, spaced trainings improved LTM for contextual but not auditory cued fear conditioning through enhancement of hippocampal LTP (Scharf et al., 2002). Also, multiple trainings and the presence of an intertrial interval (spacing) can supersede the requirements of CREB (Kogan et al., 1997) and the hippocampus (Wiltgen et al., 2006) for contextual fear conditioning. Mutant CREB-deficient mice acquired LTM for contextual fear conditioning after spaced, but not massed, trainings (Kogan et al., 1997). Additionally, rats with hippocampal lesions acquired contextual LTM when multiple shocks, but not a single shock, were used (Wiltgen et al., 2006).

Our results suggest that LTM formation following a single fear conditioning training is NO-dependent, while LTM formation following multiple (4) spaced trainings may be NO-independent. First, nNOS KO mice had deficits in both cued and contextual fear memory following a single training (Figure 4.1). Second, administration of the nNOS inhibitor SMTC prior to a single training suppressed both cued and contextual fear memory in WT mice (Figures 4.1A, 4.1B). Third, administration of the NO donor molsidomine prior to a single training dose-dependently improved contextual fear conditioning in nNOS KO (Figure 4.1D). Fourth, nNOS KO mice acquired optimal contextual fear response following multiple (4) spaced trainings (Figure 4.2D), suggesting that the formation of LTM following multiple trainings no longer depends on the NO signaling pathway.
nNOS and the visual system

The findings that NO donor and multiple trainings did not improve LTM for visually cued fear conditioning in nNOS KO mice raises the possibility that neural adaptations in the KO mice may impede visual processing. Several studies have examined roles for nNOS and NO signaling in visual processing pathways (Cudeiro and Rivadulla, 1999). For example, in the mouse retina NO production is stimulated by light and can modulate light-evoked responses (Pang et al., 2010; Wang et al., 2007). Also, in the avian retina NO has been shown to stimulate ERK and CREB phosphorylation (Socodato et al., 2009). In the lateral geniculate nucleus, NO may play a role in neuronal light/dark responses (NuCCI et al., 2003). Finally, in the mouse visual cortex NO can contribute to LTP through modulation of cGMP production (Haghikia et al., 2007). The visual cortex may be relevant because indirect thalamo-cortico-amgydala pathways play a role in the development and maintenance of visually cued fear response (Shi and Davis, 2001). However, it should be noted that acute (Knepper and Kurylo, 1998) and chronic (Tobin et al., 1995) pharmacological inhibitions of NO signaling by the NOS inhibitor L-nitroarginine methyl-ester (L-NAME; IP) in rats did not impede visual discrimination task performance when a light cue was used. This suggests that nNOS inhibition does not prevent discrimination of the visual CS. We hypothesize that deficient NO signaling in the amygdala, rather than disturbances of visual processing, impairs visual cue-dependent learning. Inhibition of NO signaling in the amygdala should be sufficient to impair fear learning across modalities because the amygdala is the convergence point of CS, US, and hippocampal processing pathways in fear conditioning (LeDoux, 2000).
**Plasma corticosterone and fear conditioning**

The stress hormone corticosterone has been implicated in LTM formation for cued and contextual fear conditioning (Rodrigues et al., 2009). The current study investigated a relationship between elevations of plasma corticosterone and the magnitude of conditioned freezing elicited by re-exposure to the visual CS. Results showed that there is a positive correlation between the magnitudes of visually cued freezing and corticosterone responses 15 min following re-exposure to the visual CS 24 h posttraining (Figure 4. 4). Overall, the results show a relationship between the physiological response to stress (increase in corticosterone levels) and the behavioral fear response (freezing) to a visual cue that had been associated with aversive US. These findings suggest that measurements of neuroendocrine responses to cues associated with traumatic events may facilitate the monitoring of clinical treatments of PTSD.

**ERK1/2 and CREB expression in WT and nNOS KO mice**

Regulation of CREB activity in the basolateral and central nuclei of the amygdala is essential for the acquisition and consolidation of LTM of fear conditioning. Specifically, amygdalar activations of ERK1/2 (Schafe et al., 2000) and CREB (Han et al., 2007; Kida et al., 2002; Viosca et al., 2009a) are required for the development of cued fear conditioned response. We investigated the expression of ERK1/2 (upstream of CREB) and CREB and their phosphorylated isoforms in the amygdala of naïve WT and nNOS KO mice because they are a) required for fear conditioning, and b) downstream of NO-cGMP signal transduction.

In the amygdala of naive nNOS KO mice, pCREB levels were 2-fold higher than in WT mice while total CREB expression was the same (Figure 4.5). Also, in the
hippocampus of naïve nNOS KO mice pCREB levels were 1.54-fold higher than WT mice and total CREB expression were the same between the genotypes (Figure 4.9). These findings suggest a dysregulation of the mechanisms of CREB phosphorylation or dephosphorylation, rather than of overall CREB expression, in the absence of the nNOS gene. The reason for enhanced pCREB expression is not clear, however several studies have showed that genetic and chronic pharmacological inhibition of NO signaling led to increased CREB phosphorylation (Ser-133) in the dentate gyrus of the hippocampus and the subventricular zone of the olfactory bulb (Moreno-Lopez et al., 2004; Packer et al., 2003; Zhu et al., 2006). These studies demonstrated that NO can act as a negative regulator of neurogenesis and CREB phosphorylation; the mechanism of this interaction is unknown (Contestabile, 2008). The findings that pERK1/2 and total ERK1/2 were similar in both genotypes suggest that aberrant pCREB expression is independent of ERK1/2. It is possible that enhanced pCREB expression is the consequence of a compensatory mechanism in the nNOS KO mice, whereby the absence of NO signaling stimulates other transduction pathways that modulate the phosphorylation state of CREB.

Several studies have demonstrated that viral vector-mediated overexpression of CREB in rodent amygdala facilitated auditory cued LTM of fear conditioning (Han et al., 2007; Josselyn et al., 2001; Viosca et al., 2009a; Wallace et al., 2004). It has been suggested that overexpression of active CREB enhances learning by 1) reducing the threshold for late-phase LTP induction and 2) overexpression of plasticity-related proteins enabling rapid consolidation of LTM (Viosca et al. 2009a). Interestingly however, nNOS KO mice have increased amygdalar pCREB levels concurrent with significant deficits in cued LTM (Figures 2.1, 4.1, and 4.2). Although these findings are
difficult to reconcile, the consequences of enhanced pCREB expression in nNOS KO mice may be different than that in WT mice. First, the absence of nNOS leads to a deficit in late-phase LTP in nNOS KO mice (Hopper and Garthwaite, 2006). Second, a consequence of increased CREB transcription would normally be overexpression of nNOS because these form a positive feedback loop (Sasaki et al., 2000). Enhancement of nNOS and NO signaling in the CREB overexpression studies would be expected to facilitate LTM (Chien et al., 2005; Ota et al., 2008). Conversely, other studies have shown that overexpression of CREB impairs learning. For example, overexpression of CREB in the olfactory bulb reduced odor preference learning in rats (Yuan et al., 2003), and overexpression of an active isoform of CREB in the hippocampus impaired acquisition and retrieval of spatial LTM in Morris Water Maze task (Viosca et al., 2009b). Also, it has been shown that overexpression of CREB in the nucleus accumbens was shown to decrease natural and drug reward behavior, while reduction in CREB expression enhanced natural and drug reward behavior (Nestler, 2004). These studies demonstrated that the effects of increased CREB are not always associated with facilitation of learning and memory. The results of genetic and pharmacological studies support the conclusion that nNOS inhibition precludes potential gains in learning and memory associated with elevated pCREB.

Unlike chronic inhibition of nNOS, either pharmacologic or genetic, we found that acute inhibition of nNOS by SMTC reduced amygdalar expression of pCREB and total CREB. Our previous studies demonstrated that the same treatment decreased cGMP by 56% in the amygdala of WT mice (Kelley et al., 2010). This implies that the reduced production of amygdalar cyclic nucleotides also inhibits downstream CREB.
phosphorylation (Lu et al., 1999; Puzzo et al.; 2006). In agreement with this is the finding that the NO donor molsidomine (20mg/kg) increased CREB phosphorylation in the amygdala WT mice (Figure 4.6E). The finding that SMTC reduced total amygdalar CREB expression supports evidence that nNOS and CREB form a positive feedback loop (Sasaki et al., 2000). Under typical conditions, CREB phosphorylation in the basolateral and central nuclei of the amygdala is required for fear-related LTM formation. We posit that inhibition of CREB phosphorylation and transcription by acute administration SMTC during fear conditioning underlies the behavioral impairments observed in fear conditioning (Figure 4.1).

Interestingly, the NO donor had opposing effects on amygdalar pCREB expression in WT and nNOS KO mice. Molsidomine enhanced and decreased pCREB expression in WT and nNOS KO mice, respectively. The possibility of NO acting as a negative regulator of pCREB in nNOS KO mice is at odds with the previously described NO-cGMP transduction pathway; however, it may be consistent with the hypothesis that NO negatively regulates pCREB expression (Packer et al., 2003; Zhu et al., 2006). The differences of the two functions of NO may be reconciled by considering the roles and characteristics of the pathways. For learning and memory, NO signaling positively regulates CREB in a phasic manner that is dependent on neuronal activity and the N-methyl-D-aspartate (NMDA) receptor. For neurogenesis, the tone of neural proliferation and CREB activity is negatively regulated by NO through an alternate and currently unknown mechanism. The 47% reduction of pCREB in the amygdala of molsidomine-treated nNOS KO mice suggests that the net effect of the NO donor was inhibition of pCREB (Figure 4.8B). The reason for the opposite effects of molsidomine in WT and
nNOS KO mice is not known, however the results may be related to the baseline genotypic differences in pCREB expression (Figure 4.5E). Regardless of the mechanism, exogenous administration of NO to nNOS KO mice may function to restore (i.e. reduce) aberrant CREB phosphorylation and consequently to aid recovery of optimal contextual fear conditioning.

**Hippocampal ERK1/2 and CREB expressions in WT and nNOS KO mice**

As shown in Figure 4.9, pCREB levels were 1.54-fold higher in naïve nNOS KO hippocampus compared to WT while pERK1/2, total ERK1/2, and total CREB expressions remained the same. The elevated hippocampal pCREB findings were similar to the findings for the amygdala, and are consistent with studies showing pCREB overexpression following chronic NO signaling inhibition (Packer et al., 2003; Park et al., 2004; Zhang et al., 2010; Zhou et al., 2007; Zhu et al., 2006). In the Zhang et al. (2010) study, it was shown that nNOS KO mice had an elevated expression of pCREB in the hippocampus of nNOS KO mice.

**Conclusions**

The major findings of the present study are: 1) Results of both genetic and pharmacological modulations of NO signaling suggest that the development of visually cued fear conditioning is nNOS-dependent. 2) The physiological (plasma corticosterone) and behavioral (freezing) responses to visual aversive CS correlated with fear learning ability. 3) Modulation of pCREB expression in the amygdala by the NO signaling pathway is implicated in the consolidation of visual cue-dependent LTM.
Figure 4.1. Long-term memory (LTM) of visually cued and contextual fear conditioning in WT and nNOS KO mice. Mice (n=6-10/group) underwent a single training and LTM was determined 24 h and 7 d posttraining. Results are expressed as percent of total time spent freezing. “No shock” controls underwent the same training but did not receive the footshock. WT mice received the nNOS inhibitor SMTC (100 mg/kg) or vehicle and nNOS KO mice received the NO donor molsidomine (10 and 20 mg/kg) or vehicle 30 min before fear conditioning. Pretraining measurements reflect immobility before the footshock; no differences in % freezing between the groups were observed in baseline activity during the pretraining period. A. WT mice acquired visual cued LTM, and the nNOS inhibitor impaired visual cued LTM (*p<0.05). B. WT mice acquired contextual LTM, and the nNOS inhibitor impaired contextual LTM (*p<0.05). C. nNOS KO mice failed to acquire visual cued LTM, and the NO donor did not facilitate visually cued LTM. D. nNOS KO mice failed to acquire contextual LTM, but the NO donor dose-dependently improved contextual LTM (*p<0.05).
Figure 4.2. LTM for visually cued and contextual fear conditioning after multiple (4) trainings in WT and nNOS KO mice. Mice (n=5-8/group) underwent four trainings (10-12 min intertrial interval); “no shock” controls underwent the same trainings but did not receive the footshocks. Cued and contextual freezing were measured pretraining and LTM was measured 24 h posttraining. A. The multiple trainings improved visual cued LTM in WT mice (67±5% freezing vs. 49±4% freezing following a single training; Figure 4.1A) (*p<0.001 conditioned vs. no shock). B. WT mice acquired contextual LTM after multiple trainings (*p<0.001). C. nNOS KO mice failed to acquire visual cued LTM after the multiple trainings. D. The multiple trainings restored the contextual learning deficit observed after a single training (Figure 4.1D) in nNOS KO mice (*p<0.001).
Figure 4.3. Corticosterone levels before and after single and multiple trainings (n=5-8/group). A. For single training experiments, WT shock mice exhibited a significant increase in corticosterone levels from pretraining to 15 min posttraining, on Day 1, and 15 min post-exposure to the visual CS on Day 2 (*p<0.001). WT no shock control mice did not show significant increases in corticosterone levels. nNOS KO mice did not show significant increases in corticosterone levels after the administration of a single footshock training. B. For multiple training experiments, WT mice showed a significant increase in corticosterone levels from pretraining to 15 min posttraining, on Day 1, and 15 min post-exposure to the visual CS on Day 2 (*p<0.001). Control no shock WT mice showed an increase in corticosterone 15 min post pseudo-training (*p<0.01), but not after visual CS exposure on Day 2. nNOS KO shock group exhibited a significant increase in corticosterone levels from pretraining to 15 min posttraining on Day 1 (*p<0.001). However, exposure to the visual CS on Day 2 did not cause a significant increase in corticosterone levels.
Figure 4.4. Correlations between the expression of visually cued LTM (% Freezing) and plasma corticosterone (ng/ml) 15 min after the LTM tests. A. For a single training, the magnitude of freezing during the visual cue test correlated with the magnitude of corticosterone responses in WT and KO mice: WT shock (●, n=6); WT no shock (○, n=5); KO shock (▼, n=6); KO no shock (▽, n=5). B. For multiple trainings, the magnitude of freezing during the visual cue test correlated with the magnitude of corticosterone responses in WT and nNOS KO mice: WT shock (●, n=8); WT no shock (○, n=3); KO shock (▼, n=8); KO no shock (▽, n=5).
Figure 4.5. Genotypic comparisons of ERK1/2 and CREB expressions in the amygdala of naïve WT and nNOS KO mice (n=9-14/group). No differences in ERK1/2 phosphorylation or expression were observed, however pCREB was significantly elevated in nNOS KO mice compared to WT mice. A. Representative immunoblots for ERK1/2 experiments. B. No genotypic differences were observed for pERK1/2. C. No genotypic differences were observed for ERK1/2 expression. D. Representative immunoblots for CREB experiments. E. pCREB levels were 2-fold higher in nNOS KO mice than in WT mice (*p=0.005). F. No genotypic differences were observed for total CREB expression.
Figure 4.6. ERK1/2 phosphorylation and expression in the amygdala of WT (left) and nNOS KO mice (right) following pharmacological NO modulator administrations (n = 5/group). Mice received SMTC (100 mg/kg; WT), molsidomine (20 mg/kg; nNOS KO), or vehicle 30 min before sacrifice. Representative immunobLOTS from amygdala tissue are shown for WT mice (A) and nNOS KO mice (D). Quantification revealed no drug effects on ERK1/2 phosphorylation (B and E) or on total ERK1/2 expression (C and F) in the amygdala.
Figure 4.7. CREB phosphorylation and expression in the amygdala of WT mice following pharmacological NO modulator administrations (n=5/group). Mice received vehicle, SMTC (100 mg/kg) or molsidomine (20 mg/kg) in the home cage 30 min before sacrifice. The nNOS inhibitor and NO donor affected CREB phosphorylation in a manner that is consistent with the NO signal transduction pathway. A. Representative immunoblots following vehicle and SMTC administrations. B. SMTC reduced CREB phosphorylation by 75% of control vehicle (**p<0.001). C. SMTC reduced total CREB expression by 45% of control vehicle (*p<0.05). D. Representative immunoblots following vehicle and molsidomine administrations. E. Molsidomine increased CREB phosphorylation by 34% of control vehicle (*p<0.05). F. Total CREB expression was not affected by molsidomine administration.
Figure 4.8. CREB phosphorylation and expression in the amygdala of nNOS KO mice following pharmacological NO modulator administration (n=5/group). nNOS KO mice received vehicle or molsidomine (20 mg/kg) in home cage 30 min before sacrifice. A. Representative immunoblots following vehicle and molsidomine administrations. B. Molsidomine reduced CREB phosphorylation by 47% of control vehicle (*p<0.05) C. Total CREB expression was not affected by molsidomine administration.
Figure 4.9. Genotypic comparisons of ERK1/2 and CREB in the hippocampus of naïve WT and nNOS KO mice (n=9-14/group). No differences in ERK1/2 phosphorylation or expression were observed, however pCREB was significantly elevated in nNOS KO mice compared to WT mice. A. Representative immunoblots for ERK1/2 experiments. B. No genotypic differences were observed for pERK1/2. C. No genotypic differences were observed for ERK1/2 expression. D. Representative immunoblots for CREB experiments. E. pCREB levels were 1.54-fold higher in nNOS KO mice than in WT mice (*p=0.005). F. No genotypic differences were observed for total CREB expression.
Figure 4.10. ERK1/2 phosphorylation and expression in the hippocampus of WT (left) and nNOS KO (right) following pharmacological NO modulator administrations (n = 5/group). Mice received SMTC (100 mg/kg; WT), molsidomine (20 mg/kg; nNOS KO), or vehicle 30 min before sacrifice. Representative immunoblots from hippocampus tissue are shown for WT (A) and nNOS KO (D) mice. Quantification revealed no drug effects on ERK1/2 phosphorylation (B and E) or on total ERK1/2 expression (C and F) in the hippocampus.
Figure 4.11. CREB phosphorylation and expression in the hippocampus of WT (left) and nNOS KO (right) following pharmacological NO modulator administrations (n=5/group). Mice received SMTC (100 mg/kg; WT), molsidomine (20 mg/kg; nNOS KO), or vehicle 30 min before sacrifice. Representative immunoblots from hippocampus tissue are shown for WT (A) and nNOS KO (D) mice. Quantification revealed no drug effects on CREB phosphorylation (B and E) or on total CREB expression (C and F) in the hippocampus.
Chapter 5

Discussion

Summary

My studies have been instrumental in identifying novel roles of NO signaling in Pavlovian fear conditioning. First, it was determined that in the absence of the nNOS gene (nNOS KO mice) there were complete impairments in contextual and visually cued fear conditioning and a partial impairment in auditory cued fear conditioning (Kelley et al., 2011; Kelley et al., 2009). Second, it was shown that acute facilitation of NO signaling in nNOS KO mice by pharmacological NO donor improved the deficits in contextual fear conditioning, and that acute pharmacological inhibition of nNOS in WT mice impaired contextual and visually cued fear conditioning (Kelley et al., 2011; Kelley et al., 2010). Third, a positive correlation was shown between the magnitudes of the physiological (stress hormone) and behavioral (freezing) responses to conditioned fearful stimuli (Kelley et al., 2011). Fourth, it was determined that the behaviorally effective doses of the pharmacological NO signaling modulators had direct effects on amygdalar and hippocampal cGMP and CREB levels which implicated the NO signaling pathway for fear conditioning (Kelley et al., 2011). In summary, these studies have discovered novel roles of the NO signaling pathway in the acquisition of STM and subsequent consolidation of LTM for various forms of fear conditioning.

Given the relevancy of fear conditioning and associative memory formation for PTSD, the results suggest that inhibition of the NO signaling pathway impedes the long-term storage of trauma-associated memories. Importantly, the current discoveries provide a solid rationale for future investigations on the roles of NO signaling in other
forms of amygdala- and hippocampus-mediated learning. For example, the processes of LTM reconsolidation and extinction learning involve many of the same molecular pathways as for initial memory acquisition (Izquierdo et al., 2004) and they may reduce the persistence of intrusive trauma-associated memories. Also, the current studies implicated the hormonal stress response and molecular substrates of the NO signaling pathway in fear memory formation, thus providing specific molecular targets for pharmacotherapy development. These studies have significantly expanded the fields of NO signaling, fear conditioning, and memory research. Further, the findings have opened up several lines of research that are discussed at the end of this chapter.

**Contextual fear conditioning**

**Role of nNOS gene in contextual fear conditioning**

In Chapter 2 it was shown that male and female nNOS KO mice have complete impairments in short-term memory (STM) and long-term memory (LTM) of contextual fear conditioning compared to WT counterparts (Kelley et al., 2009). Only one other study has reported investigating a role of NO signaling in contextual fear conditioning and in that study, the nNOS inhibitor 7-NI did not have an effect on contextual fear conditioning in rats (Maren, 1998). However, as the author of the study concluded, the vehicle (DMSO) caused immobility which may have confounded the measurements of behavioral freezing. In our lab similar effects on locomotor behavior were observed following DMSO administration to mice (data not shown). The current investigations showed deficient STM for contextual fear conditioning in nNOS KO mice, which suggests impaired acquisition of hippocampus-mediated memory (Figure 2.2A).
Subsequently, LTM of contextual fear conditioning was also impaired in nNOS KO mice (Figure 2.2A).

The finding that nNOS KO mice have impaired contextual fear conditioning is in agreement with several lines of research which have implicated NO signaling and the nNOS gene in other forms of hippocampus-mediated processes. First, early- and late-phase hippocampal LTP are dependent on NO signaling (Bon and Garthwaite, 2003; Lu et al., 1999; Zhuo et al., 1998). Not surprisingly, hippocampal slices from nNOS KO mice exhibited deficient LTP which was restored by bath application of a NO donor (Hopper and Garthwaite, 2006). Second, the acquisition of certain hippocampus-mediated learning tasks, such as inhibitory avoidance and Morris Water Maze, is impaired by pharmacological inhibition of NO in rats (Bernabeu et al., 1995; Chapman et al., 1992). Third, a few studies have implicated NO signaling specifically for the process of LTM consolidation following hippocampus-mediated learning tasks (Furini et al., 2009; Rickard et al., 1994; Telegdy and Kokavszky, 1997). Fourth, behavioral studies in nNOS KO mice showed impaired performance in spatial learning for the Morris Water Maze (spatial reference memory) and the eight-arm radial maze (spatial working memory) (Kirchner et al., 2004; Tanda et al., 2009; Weitzdoerfer et al., 2004). Kirchner et al. (2004) also showed that nNOS KO mice have aberrant protein expression in the hippocampus. Most notably for learning, the expression of the vesicular-fusion protein NSF was downregulated, and the CREB-mediated gene products GRP78 (molecular chaperone) and guanine nucleotide-binding protein (G-protein mediated signaling) were upregulated in nNOS KO hippocampi (Kirchner et al., 2004). My studies showed in naïve nNOS KO mice hippocampus tissue significantly a) decreased cGMP levels
(Figure 3.4B) and b) elevated pCREB expression (Figure 4.9E). I believe that the dysregulations of cGMP and pCREB in the hippocampus of nNOS KO mice are related to the complete STM and LTM deficits for contextual fear learning.

**Pharmacological NO signaling modulators and contextual fear conditioning**

In Chapters 3 and 4 it was investigated whether pharmacological modulations of NO signaling have similar outcomes for contextual fear conditioning as was observed in WT and nNOS KO mice (Kelley et al., 2009). For this aim, it was tested whether a) pharmacological inhibition of nNOS impairs contextual fear conditioning, and b) whether pharmacological NO donor administration reverses the deficits in contextual fear conditioning in nNOS KO mice. These experiments utilized the selective nNOS inhibitor SMTC in WT mice and the NO donor molsidomine in nNOS KO mice; the drugs were delivered in a vehicle of water or saline, respectively. Importantly, there were no differences in baseline activity measures between drug- and vehicle- treated WT and nNOS KO mice indicating that the drugs had no acute or long-term behavioral effects on motor behavior (Table 3.1 and Figure 3.1). Pretraining administrations of the nNOS inhibitor to WT mice dose-dependently impaired the acquisition (STM) and LTM of contextual fear conditioning (Figure 3.2A). The nNOS inhibitor caused a U-shaped dose response curve in which the highest dose (200 mg/kg) had no effect but the mid-range doses of 50 – 100 mg/kg had the greatest effects. Administrations of SMTC immediately posttraining had no effects on contextual fear conditioning (Figure 3.2C), suggesting that posttraining administration alone did not impair LTM consolidation. In nNOS KO mice, pretraining administrations of the NO donor dose-dependently improved contextual fear conditioning (Figures 3.3A and 4.1D), and posttraining administration had no effect
(Figure 3.3C). Thus, the effects of pretraining pharmacological inhibition of nNOS in WT mice were similar to the effects of nNOS gene deletion for contextual fear conditioning (Figure 2.2A). Also, pretraining administration of the NO donor to nNOS KO mice partially restored the impairments in STM and LTM for contextual fear conditioning similar to WT levels (Figure 2.2A). Together, the pharmacological studies in WT and nNOS KO mice (Kelley et al., 2011; Kelley et al., 2010) corroborated with genetic studies (Kelley et al., 2009) and confirmed the role of NO signaling in the acquisition of contextual fear conditioning.

An important outcome of the pharmacological studies was that only pretraining, and not posttraining, administrations of the NO modulators affected contextual fear conditioning (Figures 3.2 and 3.3). Typically in memory studies, posttraining drug administration is used to investigate consolidation-specific effects; an effect is determined to be specific for the consolidation process when LTM, but not STM, is affected. The results of the posttraining studies suggest that NO signaling has a role in contextual fear acquisition, rather than the consolidation process alone. The findings that STM was affected by a) pretraining drug administrations (Figures 3.2A and 3.3A), and b) nNOS gene deletion (Figure 2.2A) supports a role of NO in memory acquisition. However, it is not clear at this time whether the pretraining administrations had direct effects on LTM consolidation. For instance, it is possible that the pretraining administrations (30 minutes prior) permitted drug effects during the initial stages of consolidation, which subsequently influenced the LTM results. It was shown in Chapter 4 that SMTC and molsidomine had effects on CREB expression, which suggests that the NO modulators likely influenced LTM. Overall, the data show that the NO modulators
had direct effects on memory acquisition (STM) which were subsequently consolidated into LTM.

**Multiple trainings and contextual fear conditioning**

Given the deficits observed in nNOS KO mice, it was investigated whether a more intense training strategy (i.e. more footshocks) will improve contextual fear conditioning (Chapter 2; Kelley et al., 2009). Multiple (4) spaced trainings (10-12 ITI) were used because this protocol is superior to “massed” training for improving contextual fear conditioning (Josselyn et al., 2001; Scharf et al., 2002). In massed training, the animal receives the same number of shocks but in a shorter time period and usually does not leave the training cage. The current findings were in agreement that multiple trainings facilitated contextual fear learning because, first, nNOS KO mice exhibited step-wise improvements in contextual STM during each successive training (Figure 2.4). Second, contextual LTM in nNOS KO mice was improved following the multiple trainings (Figure 2.5B and 2.5D) compared to a single training (Figure 2.2A). Thus, it was demonstrated that multiple trainings resulted in improvements in STM acquisition and LTM consolidation for contextual fear conditioning in nNOS KO mice (Kelley et al., 2009).

In the LTM tests following multiple trainings, a sex-dependent effect was observed (Figure 2.5). Female nNOS KO mice maintained remote contextual LTM after 7 days while male nNOS KO mice showed decreased contextual LTM after 7 days compared to 24 hours (Figure 2.5B and 2.5D). The finding that contextual fear LTM was more persistent in female than male mice is interesting considering the fact that women are nearly twice as likely to develop PTSD as men (Kessler et al., 2005). Nevertheless,
the sex-dependent effect appeared to be specific to the multiple trainings and to contextual LTM. This specificity is due to the finding that in the pharmacological studies in Chapter 4, the improved contextual LTM in male nNOS KO mice following NO donor administration persisted in the 7 day LTM test (Figure 4.1D). The differences in 7 day LTM tests in the two studies have implications for the stability and maintenance of remote contextual fear memory. The findings suggest that the mechanisms of improved contextual fear learning in male nNOS KO mice following multiple trainings and pharmacological NO donor are different, with NO donor resulting in more persistent contextual fear memory.

**NO dependency of contextual fear conditioning**

A major conclusion of this thesis is that the acquisition of contextual fear conditioning following a single training is NO-dependent, however memory formation following multiple trainings is NO-independent (Table 5.1). This is supported by the findings that 1) nNOS KO mice had impaired contextual fear conditioning following a single training compared to WT mice; 2) the pharmacological modulations of NO signaling affected single training fear conditioning in WT and nNOS KO mice; and 3) nNOS KO mice acquired near optimal contextual LTM following multiple trainings. Additional support comes from a preliminary study (data not shown) in which it was observed that pretraining molsidomine administration prior to the multiple trainings did not facilitate contextual LTM in nNOS KO mice compared to vehicle administration. Table 5.1 shows the NO dependencies of contextual fear conditioning following single and multiple trainings.
Previous investigations have shown that the mechanisms of contextual fear memory formation following single and multiple trainings are different, which may be in agreement with the current conclusions. For instance, hippocampaelectomized rats can acquire contextual fear conditioning following three spaced trainings, but not a single training (Wiltgen et al., 2006). This suggested that in the absence of the hippocampus, increased training intensity facilitated weaker associations between contextual elements and the footshock in an amygdala-dependent form of LTM (see Figure 1.1B “weak”). In another study, it was shown that CREB-deficient mice acquired contextual fear conditioning when trainings were spaced 60 minutes apart, but not following a single or massed training (Kogan et al., 1997). It is postulated that during multiple spaced trainings, accumulations of second messenger molecules and memory activating kinases have an additive effect on memory acquisition and LTM consolidation which is independent of CREB (Josselyn et al., 2001). My studies contribute to this line of research by demonstrating different dependencies of NO signaling for contextual fear conditioning following a single and multiple trainings (Table 5.1).

**Summary**

Given the prominent role of NO signaling in hippocampal LTP, the current studies have forged a solid link between the fields of NO signaling and LTP-mediated contextual fear conditioning. Specifically, the data showed that NO is required for the acquisition (STM) and subsequent consolidation (LTM) of contextual fear conditioning. It was also shown that contextual fear conditioning following multiple trainings no longer requires NO signaling. Since trauma-associated contextual memories in humans are
known to require the hippocampus and CREB (Grillon et al., 1996), the current findings contribute that NO signaling is also critical for that process.

**Auditory cued fear conditioning**

**Role of the nNOS gene in auditory cued fear conditioning**

In Chapter 2 it was shown that male and female nNOS KO mice had minor deficits in auditory fear conditioning compared to WT counterparts (Kelley et al., 2009). The deficits were observed for both STM and LTM of auditory cued fear conditioning (Figure 2.2B). Similar to contextual fear conditioning, these outcomes suggest impaired memory acquisition, rather than a consolidation-specific impairment. Importantly, the magnitudes of the deficits in auditory cued freezing in the nNOS KO mice were relatively small compared to the complete deficits for contextual and visually cued freezing (Figure 2.2A and 4.1C). This suggests a partial acquisition of auditory cued fear conditioning in nNOS KO mice. This may indicate that a NO-independent process in the amygdala facilitated partial acquisition of this type of learning. Based on the current studies in WT and nNOS KO mice (Kelley et al., 2009) and previous findings in rats (Schafe et al., 2005), it is concluded that NO signaling has a partial role in the acquisition of auditory cued fear conditioning following a single training (Table 5.1).

**Pharmacological NO signaling modulators and auditory cued fear conditioning**

In the pharmacological experiments in Chapter 3, it was observed that administrations of the nNOS inhibitor to WT mice and the NO donor to nNOS KO mice had no significant effects on auditory cued fear conditioning (Figures 3.2 and 3.3; Kelley et al., 2010). Unlike the results of contextual fear conditioning, the results of the genetic and pharmacological studies in auditory cued fear conditioning did not corroborate. This
suggests that the effects of global nNOS gene deletion and acute manipulations of NO signaling do not have the same effect on amygdala-mediated auditory cued fear conditioning. It is possible that WT mice acquired and consolidated auditory cued fear conditioning primarily through an NO-independent mechanism, and therefore the nNOS inhibitor had little effect. Similarly, it is possible that the nNOS KO mice partially acquired auditory cued fear conditioning by an NO-independent mechanism and therefore the NO donor had little effect. It should be noted however, that there was a non-significant trend of improved auditory cued fear conditioning in the nNOS KO following molsidomine administration (Figure 3.2B). The NO donor may have had a facilitating effect on auditory cued memory, however due to the small magnitude of the deficit in nNOS KO mice the size of the improvement was not statistically significant.

The first study to implicate NO signaling in auditory cued fear conditioning showed that direct intra-amygdalar injections of the nNOS inhibitor 7-NI prevented auditory cued LTM consolidation in rats (Schafe et al., 2005). Later studies from the Schafe group showed that downstream components of NO signaling, namely cGMP, PKG, and ERK, had roles in presynaptic and postsynaptic mechanisms required for amygdalar LTP in vitro and auditory cued fear learning in vivo (Ota et al., 2010; Ota et al., 2008; Overeem et al., 2010; Schafe et al., 2005). Clearly, the NO signaling pathway has a role in auditory cued fear conditioning. However, two major differences exist between the current findings described in Chapters 2 and 3 and the findings in Schafe et al., (2005). The first major difference is that STM was affected by nNOS gene deletion (Figure 2.2B); however STM was intact in the Schafe et al. (2005) study. The second major difference is that the systemic administrations of NO signaling modulators did not
affect auditory cued fear conditioning (Figures 3.2 and 3.3), however Schafe et al., (2005) showed that site-specific pharmacological inhibition of nNOS impaired auditory cued LTM. Several experimental differences exist which may in part explain the discrepancies. First, species differences may render differential dependency of NO signaling, presumably with mice having less requirement of NO for auditory fear conditioning than rats. Second, it is possible that neural adaptations in the amygdala of nNOS KO mice partially compensate for lack of NO signaling during auditory cued learning. Third, differences in drug pharmacokinetics between local 7-NI infusion and i.p. SMTC administration may have different outcomes for STM and LTM consolidation. The physiological relevance of systemic administrations of SMTC and molsidomine was confirmed as the drugs modulated the downstream NO signaling molecules cGMP and CREB in the amygdala at the time of fear conditioning (30 minutes later) (Kelley et al., 2011; Kelley et al., 2010). Overall, it appears that systemic and intra-amygdalar modulations of NO signaling have different effects on auditory cued fear conditioning.

**Multiple trainings and auditory cued fear conditioning**

No differences were observed between male and female WT and nNOS KO mice for auditory cued fear conditioning following multiple trainings (Figure 2.5; Kelley et al., 2009). The similar magnitudes of auditory cued freezing in WT and nNOS KO mice suggests that auditory cued fear conditioning following multiple trainings is NO-independent (Table 5.1). In addition, WT mice exhibited similar magnitudes of auditory cued freezing following a single and multiple trainings (Figures 2.2B and 2.5), suggesting that a ceiling effect was reached by a single training.
Summary

The major findings from the auditory cued fear conditioning studies are the following. First, the results of the genetic studies in Chapter 2 are in agreement with other studies that have shown that auditory cued fear conditioning is NO-dependent (Apergis-Schoute et al., 2005; Ota et al., 2010; Overeem et al., 2010; Schafe et al., 2005). Interestingly, the auditory cued fear conditioning deficits in nNOS KO mice were relatively small compared to other fear conditioning impairments. The finding that nNOS KO mice exhibited auditory cued freezing, albeit slightly reduced from WT, suggests a partial acquisition of auditory cued fear memory. Second, the systemic pharmacological modulators of NO did not significantly influence auditory cued fear conditioning. Third, auditory cued freezing following multiple trainings was similar in WT and nNOS KO mice. Taken together, these findings lead to the conclusions that auditory cued fear conditioning following a single training is partially NO-dependent, while auditory cued fear conditioning following multiple trainings is NO-independent (Table 5.1).

Visually cued fear conditioning

Role of the nNOS gene in visually cued fear conditioning

In Chapter 4, evidence is provided that the nitrergic system is critically involved in visually cued fear conditioning (Kelley et al., 2011). First, WT mice acquired visually cued fear conditioning following a single training, yet nNOS KO mice exhibited a complete impairment for visually cued fear conditioning (Figure 4.1). Second, pretraining administration of nNOS inhibitor to WT mice impaired visually cued fear conditioning (Figure 4.1A), however NO donor to nNOS KO failed to improve visually
cued fear conditioning (Figure 4.1C). Third, multiple trainings improved visually cued LTM in WT mice but did not improve visually cued LTM in nNOS KO mice (Figure 4.2A and 4.2C). The results demonstrate that, unlike contextual and auditory cued LTM, the formation of visually cued LTM is nNOS-dependent following both single and multiple trainings.

**Pharmacological NO signaling modulators and visually cued fear conditioning**

Only one other study has reported investigating NO signaling and visually cued fear conditioning. In that study, the non-selective NOS inhibitor L-NAME (40 mg/kg) resulted in partial (non-significant) inhibition of the acquisition of visually cued fear conditioning in rats (Johnson et al., 2000). The authors concluded that NO may be one of several Ca\(^{2+}\)-mediated signaling cascades that act in parallel to facilitate amygdalar synaptic plasticity. In the current studies, significant decreases in visually cued LTM were observed in WT mice following nNOS inhibitor administration (Figure 4.1A). The pharmacological results in WT mice corroborated the results in nNOS KO mice that exhibited a total impairment in visually cued LTM (Figure 4.1C). Taken together, these findings implicate nNOS in visually cued fear conditioning. Surprisingly, pretraining administration of the NO donor failed to improve visually cued fear conditioning in nNOS KO mice (Figure 4.1C) despite the improvements in contextual fear conditioning in the same animals (Figure 4.1D). This outcome suggests that exogenously administered NO is insufficient to reverse the particular impairment in visually cued LTM formation. It is possible that deletion of the nNOS gene results in disruptions of visual system that are not reversed by an acute administration of NO donor. Importantly, nNOS KO mice do not have any major CNS structural abnormalities (Huang et al., 1993). Also, visual
discrimination learning was intact in rodents following pharmacological inhibition of NO signaling (Knepper and Kurylo, 1998; Tobin et al., 1995). These findings suggest that genetic and acute pharmacological inhibition of NO signaling do not produce major disruptions of the visual processing.

**Multiple trainings and visually cued fear conditioning**

Increased training intensity by multiple trainings significantly improved visually cued LTM in WT mice; however it was not sufficient to improve visually cued fear conditioning in nNOS KO mice (Figure 4.2). These outcomes suggest that visually cued fear conditioning following multiple trainings is NO-dependent (Table 5.1). This conclusion is different than for contextual and auditory cued fear conditioning that are NO-independent following multiple trainings (Table 5.1). The dissimilarity may be due to differences in the modality-specific fear conditioning pathways. For instance, NO signaling is known to have numerous roles in brain substrates that are part of the visual CS processing pathways (Cudeiro and Rivadulla, 1999) and auditory CS processing pathways (Overeem et al., 2010). Deficient NO signaling in the visual CS processing pathway could have specific effects on visually cued learning. Another study showed that nNOS KO mice failed to acquire visually cued LTM of appetitive conditioning using a blinking light CS (Itzhak et al., 2010). Also, in a preliminary study it was determined that pretraining administration of NO donor to nNOS KO mice prior to multiple trainings did not improve visually cued LTM (data not shown). The findings that nNOS KO mice failed to acquire visually cued LTM following multiple trainings with and without the pharmacological NO donor supports the conclusion that visually cued LTM requires the nNOS gene.
Summary of fear conditioning studies

My findings on the dependency of NO signaling for contextual, auditory and visually cued fear conditioning are described in Table 5.1. The data suggest that memory formation following a single training of contextual, auditory, and visually cued fear conditioning is NO-dependent. This is based on the findings that a) WT mice acquired contextual, auditory and visually cued fear conditioning while b) nNOS KO mice had deficits in all of these forms of fear conditioning. Also, pharmacological experiments with inhibitors and facilitators of NO signaling, in general, corroborated the results of the genetic comparisons. The only exception was the lack of improvement in nNOS KO mice for visually cued fear conditioning following the NO donor (Figure 4.1C). However, the nNOS inhibitor impeded visually cued fear conditioning in WT mice (Figure 4.1A), supporting the conclusion that that form of learning is NO-dependent. Following multiple trainings, nNOS KO mice successfully consolidated LTM for contextual and auditory cued fear conditioning, suggesting that memory formation after multiple trainings is NO-independent. However, visually cued fear conditioning following multiple trainings remained NO-dependent, as nNOS KO mice failed to acquire visually cued STM and LTM under those conditions. This line of investigations has been on the forefront of research in the fields of fear conditioning and nitric oxide. The findings shed light on the development of contextual and cued fear memories under different intensities of trauma exposure, which has implications for the formation of trauma-associated memories in PTSD and specific phobia.
Role of the nNOS gene in corticosterone response to the conditioned and unconditioned stimuli

The primary goal of the corticosterone studies was to investigate the relationship between physiological and behavioral responses following re-exposure to the context and sensory cues (CS) that were associated with fear conditioning. To this end, plasma corticosterone (stress hormone) levels were measured 15 minutes after the LTM tests for contextual and cued fear conditioning (Kelley et al., 2011; Kelley et al., 2009). The results showed that WT mice had elevated corticosterone levels following re-exposures to the conditioned context and discreet CS following the single and multiple trainings (Figures 2.3 and 4.3). In contrast, nNOS KO mice did not exhibit elevated corticosterone levels after re-exposures to the context or the sensory CS following a single training (Figures 2.3 and 4.3A). However, after multiple trainings, the nNOS KO mice exhibited elevated corticosterone following context (females only) and auditory CS (male and female) but not visual CS re-exposures (Figures 2.6 and 4.3B). These data in WT and nNOS KO mice showed that when a context or sensory CS was deemed fearful, it was associated with both corticosterone release and behavioral freezing. Positive correlations confirmed the relationship between the corticosterone levels and the magnitude of behavioral freezing in response to the visual CS in WT and nNOS KO mice (Figure 4.4). The findings suggest that monitoring the stress response in humans may facilitate clinical management of anxiety related symptoms.

Another goal of the corticosterone studies was to investigate the relationship between physiological responses to the footshock-US 15 minutes after training and subsequent acquisition and consolidation of LTM for fear conditioning. For these
investigations, posttraining measurements of corticosterone after single and multiple trainings were analyzed and are discussed in relation to the LTM results. First, WT mice exhibited elevated corticosterone levels 15 minutes after the single and multiple trainings (Figures 2.3 and 4.3). The posttraining elevations in corticosterone levels in WT mice were followed by contextual, auditory, and visually cued freezing in the LTM tests (Kelley et al. 2011; Kelley et al., 2009). Second, nNOS KO mice did not exhibit elevated corticosterone levels after a single training (Figures 2.3 and 4.3A). A single training was also insufficient to support contextual, auditory (partial), and visually cued freezing in nNOS KO mice (Kelley et al. 2011; Kelley et al., 2009). Third, nNOS KO mice exhibited elevated corticosterone following multiple trainings (Figure 2.6 and 4.3B) and improved LTM for contextual and auditory cued freezing, but not visually cued freezing (Kelley et al., 2011; Kelley et al., 2009). Taken together, the investigations suggest a relationship between posttraining corticosterone response and subsequent fear memory formation. This raises the question of whether insufficient posttraining corticosterone response in the nNOS KO mice is related to the fear conditioning deficits. Accordingly, previous reports have shown that posttraining corticosterone facilitates contextual (Pugh et al., 1997; Thompson et al., 2004) and auditory cued (Marchand et al., 2007) fear conditioning. The mechanism of this improvement is believed to be a feed-forward facilitation of neurons involved in LTM consolidation mediated by circulating corticosterone reaching the brain (Rodrigues et al., 2009). It was shown that WT and nNOS KO mice have similar nociception and vocalization responses to footshock (Table 2.1), suggesting that the pain threshold is similar for both the genotypes. Future studies on the effects of glucocorticoid signaling facilitation in nNOS KO mice and inhibition in
WT mice during fear conditioning may help elucidate the role of posttraining corticosterone responses in fear-related memory formation.

**Molecular studies**

**Summary**

The expression of memory-related NO signaling molecules in the brain of WT and nNOS KO mice were investigated to elucidate a) the molecular consequences of genetic and pharmacological modulations of the NO signaling pathway and b) a potential relationship to the formation of fear-related memory. The investigations focused on cGMP, ERK1/2, and CREB (downstream of NO, Figure 1.6), which have been previously implicated in fear conditioning. I hypothesized that modulations of those molecules will correlate with the outcomes of fear conditioning.

Investigations of baseline expressions of cGMP, ERK1/2, and CREB in naïve WT and nNOS KO mice were performed. The results of the genotypic comparison studies showed that there were major differences in basal cGMP and phosphorylated CREB expressions and no differences in ERK expression (Kelley et al., 2011; Kelley et al., 2010). Next, investigations of the effects of the behaviorally effective doses of the pharmacological NO modulators were performed in mice 30 minutes after drug administrations. The results of the pharmacological studies were that the drugs significantly modulated cGMP production, CREB phosphorylation, and CREB expression (Kelley et al., 2011; Kelley et al., 2010). It is concluded that in relation to fear-related memory formation, first, aberrant expressions of cGMP and pCREB in the nNOS KO mice are related to the fear conditioning deficits. This is supported by the findings that the behaviorally effective doses of the NO donor partially restored the
aberrant expressions in nNOS KO mice. Second, the drug-mediated changes in cGMP and CREB expressions were consistent with the observed outcomes of STM and LTM formation in WT and nNOS KO mice.

**cGMP**

**Summary**

The investigations of basal cGMP levels in the hippocampus and amygdala of naïve mice revealed a) significant reductions in cGMP in nNOS KO mice compared to WT mice and b) dose-dependent modulations of cGMP following nNOS inhibition and NO donor administration to WT and nNOS KO mice (Figure 3.4). The findings of reduced cGMP levels in the hippocampus and amygdala of naïve nNOS KO mice are consistent with inhibition of the NO→sGC→cGMP signaling pathway (Figure 1.6). The findings that nNOS inhibitor in WT mice caused dose-dependent decreases in cGMP levels is also consistent with inhibition of that pathway. Elevated levels of cGMP in WT mice and nNOS KO mice with NO donor administration are consistent with facilitation of the NO→sGC→cGMP signaling pathway. The effects of the NO modulators on cGMP levels showed that the systemic administrations had direct effects on brain cGMP production. Importantly, the differences in cGMP levels shown in Figure 3.4 were present during fear conditioning (i.e. 30 minutes after drug administration). Therefore, the differences in the genotypic and drug-mediated changes in cGMP levels are relevant for the behavioral outcomes of fear conditioning as is described below.

**Hippocampal cGMP and contextual fear conditioning**

Several recent studies have implicated hippocampal cGMP-mediated signaling in LTP (Hopper and Garthwaite, 2006) and various hippocampus-mediated learning tasks
(Boess et al., 2004; Furini et al., 2009; Prickaerts et al., 2002; Taqatqeh et al., 2009). The studies in Chapter 3 are the first to demonstrate a relationship between hippocampal cGMP and contextual fear conditioning (Kelley et al., 2010). Specifically, it was shown that in WT mice dose-dependent decreases in cGMP production (Figure 3.4A) coincided with dose-dependent decreases in contextual freezing (Figure 3.2A). Similarly, in nNOS KO mice the NO donor resulted in increased cGMP production (Figure 3.4B) and also improved contextual freezing (Figures 3.3A and Figure 4.1D).

I believe that impaired hippocampal cGMP production in the absence of NO is a major determinant of the impairments of contextual fear conditioning in the current studies. This is supported by the findings that administrations of NO donor have the following effects in nNOS KO mice: 1) partial restoration of cGMP production (Figure 3.4B), 2) improved hippocampal LTP (Hopper and Garthwaite, 2006), and 3) dose-dependent improvements STM and LTM of contextual fear conditioning (Figures 4.1D). As described in the proposed cellular models in Figures 5.1 and 5.2, deficient cGMP levels in presynaptic terminals renders deleterious effects on early-phase LTP and STM (Arancio et al., 1995). Also, deficient intracellular cGMP-mediated signaling in nNOS KO mice reduces presynaptic and postsynaptic gene expression, which is required for late-phase LTP and LTM consolidation (Lu et al., 1999). Thus, the current findings implicate hippocampal cGMP production with the a) STM acquisition and b) LTM consolidation of hippocampus-mediated contextual fear conditioning.

**Amygdalar cGMP and cued fear conditioning**

The relationship between amygdalar cGMP and amygdala-mediated auditory cued fear conditioning is not entirely clear. Recent studies have implicated cGMP-mediated
signaling in the amygdala for auditory cued fear conditioning (Ota et al., 2008; Paul et al., 2008; Paul et al., 2010). The current findings may be at odds with those studies for the following reasons. First, nNOS KO mice partially acquired auditory cued fear conditioning despite decreased basal amygdalar cGMP levels (Figures 2.2B and 3.4B). Second, the pharmacological NO signaling modulators had significant effects on amygdalar cGMP levels (Figure 3.4) but they did not significantly affect auditory cued fear conditioning in WT and nNOS KO mice (Figures 3.2 and 3.3). These outcomes suggest that neural adaptations in the nNOS KO mice may enable the acquisition of auditory cued fear conditioning independently of cGMP-mediated signaling.

The role of amygdalar cGMP-mediated signaling in visually cued fear conditioning has not been reported. The current studies in WT mice showed that nNOS inhibitor administrations dose-dependently reduced amygdalar cGMP and also reduced visually cued LTM (Figure 4.1A). This suggests a relationship between amygdalar cGMP and visually cued fear conditioning in WT mice. However, this relationship was not apparent in nNOS KO mice. In nNOS KO mice, the NO donor increased amygdalar cGMP yet it did not improve visually cued fear conditioning (Figure 4.1C). Therefore, similar to auditory cued fear conditioning, in nNOS KO mice the levels of amygdalar cGMP did not correlate with visually cued fear conditioning.

**ERK1/2**

ERK1/2 serves to coordinate signals from cell surface receptors and second messenger systems to nuclear transduction mechanisms. During learning and memory formation, ERK1/2 is activated downstream of NMDAR-mediated NO→cGMP→PKG signaling (Figure 1.6). ERK1/2 phosphorylation (pERK1/2) has a role in LTP induction
through activation of nuclear CREB (Giovannini, 2006). Pharmacological inhibition of ERK1/2, and most recently partial genetic knockdown of ERK2, has confirmed the requirement of ERK2 phosphorylation for contextual and auditory fear conditioning (Satoh et al., 2007). Recently, a role for pERK2 in STM of hippocampus-mediated inhibitory avoidance task was shown (Igaz et al., 2006). I hypothesized that deficient NO→cGMP→PKG signaling in nNOS KO mice and in WT mice with administration of nNOS inhibitor will reduce pERK1/2 expression.

**nNOS gene deletion and ERK1/2**

The investigations of basal ERK1/2 levels in the amygdala and hippocampus of naïve WT and nNOS KO mice revealed no genotypic differences in ERK1/2 expression or activation (Figures 4.5 and 4.9). This suggests that ERK1/2 regulation is normal in nNOS KO mice compared to WT counterparts under basal conditions. This may be important considering ERK has several known regulatory roles in developmental and survival processes including cell division, proliferation, and apoptosis (Sweatt, 2001). The findings may in part explain why nNOS KO mice do not show major congenital or developmental abnormalities. For instance, homozygous ERK2 KO are embryonic lethal and heterozygous ERK2 KO have major anatomical abnormalities; ERK1 KO mice do not have major impairments (Selcher et al., 2001; Yao et al., 2003). Regardless, it is unclear at this time why basal ERK expression and phosphorylation are similar in nNOS KO and WT mice considering the basal dysregulations of cGMP (Figure 3.4) and pCREB (Figure 4.5E and 4.9E) that were observed in naive nNOS KO mice. It appears that the normal regulation of ERK1/2 in nNOS KO mice is independent of cGMP.
Pharmacological NO signaling modulators and ERK1/2

The investigations revealed that the pharmacological NO modulators had no effect on ERK1/2 expression or phosphorylation in the amygdala (Figure 4.6) or hippocampus (Figure 4.10) of WT and nNOS KO mice. This outcome was surprising considering the significant drug-mediated modulations of upstream cGMP (Figure 3.4) and downstream CREB (Figures 4.7 and 4.8). However, these findings are in agreement with the previous findings that no differences in ERK1/2 were observed due to deletion of the nNOS gene (Figures 4.5 and 4.9).

CREB

The expression and phosphorylation of CREB was investigated because CREB phosphorylation is implicated in LTM consolidation following fear conditioning (Bourtchuladze et al., 1994). Unlike cGMP and ERK1/2 which have roles in STM, CREB is exclusively associated with the protein-synthesis dependent phase of LTM consolidation. I hypothesized that genetic and pharmacological inhibitions of the NO signaling pathway will result in reduced CREB phosphorylation (pCREB) according to the NO→cGMP→pERK1/2→pCREB signaling pathway (Figure 1.6). Likewise, I hypothesized that facilitation of NO signaling by pharmacological NO donor will result in increased pCREB according to the NO signaling pathway (Figure 1.6). It was anticipated that enhancement of pCREB will correlate with improved LTM, and that reduced pCREB will correlate with impaired LTM consolidation.

Summary

The initial investigations of basal CREB expression in naïve WT and nNOS KO surprisingly revealed elevated expression of pCREB in the amygdala (Figure 4.5) and
hippocampus (Figure 4.10) of nNOS KO mice compared to WT mice. This effect was not observed in the prefrontal cortex (data not shown), which suggests region-specific overexpression. The investigations of the effects of the NO modulators on CREB also revealed the following interesting and unanticipated results. First, the NO modulators had specific effects in the amygdala (Figures 4.7 and 4.8) and not in the hippocampus (Figure 4.11). Second, NO donor administration had different effects on pCREB in WT amygdala (Figure 4.7) and nNOS KO amygdala (Figure 4.8). Specifically, in WT mice the NO donor increased pCREB, which was consistent with facilitation of the NO→cGMP→pCREB signaling pathway (Figure 1.6). In addition, the nNOS inhibitor reduced pCREB and CREB expression, which was consistent with inhibition of that pathway (Figure 5.1). However, in nNOS KO mice NO donor administration reduced pCREB. This finding may be consistent with the role of NO as a negative regulator of pCREB by an unknown mechanism (Figure 5.2).

**Genotypic differences in pCREB expression**

The magnitudes of the genotypic differences in basal pCREB expression in the amygdala and hippocampus of naïve WT and nNOS KO mice were striking (Figures 4.5E and 4.9E). It was confirmed that total CREB expression was the same between the genotypes (Figures 4.5F and 4.9F), therefore the elevations in pCREB are due to dysregulation of phosphorylation or dephosphorylation processes. Also, given that basal activation of ERK1/2 was similar in both genotypes, it is likely that the mechanism of elevated pCREB in nNOS KO mice is independent of ERK1/2. Candidate mechanisms in which CREB may be phosphorylated independent of ERK1/2 include the Ca^{2+}/calmodulin-dependent protein kinases II & IV and PKA; these kinases can
translocate to the nucleus where they directly lead to CREB phosphorylation (Selcher et al., 2002). Also, NO-mediated activation of cAMP→PKA→CREB signaling has been observed to act in parallel to cGMP-mediated signaling in hippocampal neurons thus providing an alternative pathway leading to CREB phosphorylation that is independent of ERK1/2 (Figures 1.6 and 5.1; Lu et al., 1999).

The findings of elevated pCREB in the amygdala and hippocampus are likely in agreement with several studies which have shown that genetic and chronic pharmacological inhibitions of NO signaling results in pCREB overexpression via an unknown mechanism (Moreno-Lopez et al., 2004; Packer et al., 2003; Zhu et al., 2006). It is suggested that tonic NO production has the role of inhibiting CREB phosphorylation and the expression of neural proliferation markers, suggesting that NO negatively regulates neurogenesis (Figure 5.2) (Park et al., 2004).

As it was previously described in the Introduction, the phasic production of NO linked with NMDAR activation is followed by LTP induction and CREB phosphorylation (Figure 1.6) (Hopper and Garthwaite, 2006; Lu et al., 1999; O'Dell et al., 1991). Accordingly, through the NO→cGMP→pCREB signaling pathway phasic NO production has the role of positive regulation of pCREB (Figure 1.6). However, tonic NO production has the role of negative regulation of pCREB (Contestabile, 2008). I suggest that in nNOS KO mice, the absence of negative regulation of pCREB resulted in overall elevated pCREB expression (Figure 5.2).

**Pharmacological NO signaling modulators and CREB**

The investigations of CREB expression and phosphorylation in the amygdala and hippocampus following the pharmacological administrations revealed specific effects on
CREB in the amygdala, but not the hippocampus (Figures 4.7, 4.8, and 4.11). This finding suggests that amygdalar CREB is more susceptible to the modulations of NO signaling and cGMP (Figure 3.4) than in the hippocampus. Importantly, contextual and cued fear conditioning are both dependent on the amygdala (Phillips and LeDoux, 1992), therefore LTM for contextual and cued fear conditioning may both be influenced by changes in amygdalar CREB. I believe that the modulations of CREB following administrations of the NO signaling modulators, which are shown in the proposed model figures (Figure 5.1 for WT mice and Figure 5.2 for nNOS KO mice), underlies LTM formation for NO-dependent forms of fear conditioning (Table 5.1).

In WT mice, administration of the nNOS inhibitor resulted in decreased CREB phosphorylation and expression; conversely, administration of the NO donor to WT mice resulted in increased pCREB (Figure 4.7). The changes in pCREB were consistent with inhibition and facilitation of the NO→cGMP→pCREB signaling pathway, respectively (Lu et al., 1999). The nNOS inhibitor also reduced total CREB expression (Figure 4.7C). The magnitude of the downregulation was surprising given the timeframe of 30 minutes; nevertheless the finding is consistent with the fact that nNOS and CREB form a positive feedback loop (Sasaki et al., 2000). Accordingly, acute inhibition of nNOS is expected to inhibit both CREB phosphorylation and expression. The finding that administration of the NO donor to WT mice resulted in increased amygdalar pCREB suggests that activation of the NO→cGMP→pCREB signaling pathway resulted in a positive regulation of pCREB.

In contrast to the effects in WT mice, administration of the NO donor to nNOS KO mice resulted in decreased pCREB (Figure 4.8). This finding may be in agreement
with the hypothesis that NO has a negative regulation of CREB phosphorylation. The data suggest that the net effects of exogenous NO donor to nNOS KO mice was to inhibit pCREB. The different outcomes of the NO donor in WT and nNOS KO mice for pCREB demonstrates that NO has a dual role in CREB phosphorylation. Under typical conditions in naïve WT mice, the NO donor modulated CREB via the canonical cGMP transduction pathway. However, under the conditions of elevated basal pCREB in the nNOS KO mice, the NO donor inhibited pCREB. It is possible that neural adaptations in the nNOS KO mice render the effects of exogenous NO donor differently than in WT mice. For instance, in the case of a dysregulation of pCREB expression the exogenous NO donor appeared to function by restoring the dysregulation.

In summary, pharmacological NO signaling modulators had the expected effects on pCREB in WT mice based on the NO→cGMP→pCREB signaling pathway. However, in the nNOS KO mice where basal expression of pCREB was dysregulated, the NO donor had the opposite effect which was consistent with a negative regulation of pCREB by NO. The biochemical effects of the nNOS inhibitor in WT mice are shown in Figure 5.1 (right panel). The biochemical effects of the NO donor in nNOS KO mice are shown in Figure 5.2 (right panel).

**Elevated pCREB expression and learning**

Despite the elevated pCREB expression in the amygdala and hippocampus, nNOS KO mice have deficits in amygdala- and hippocampus-mediated learning. This is surprising considering several studies have shown that viral and genetic facilitation of CREB activity in the amygdala and hippocampus improves contextual and cued fear conditioning (Han et al., 2008; Josselyn et al., 2001; Viosca et al., 2009a; Wallace et al.,
2004). It is believed that enhanced CREB activity facilitates LTM consolidation by two primary mechanisms. The first mechanism is a reduction in the threshold for LTP induction; this effect has been observed in CREB-overexpressing neurons (Viosca et al., 2009a). The second mechanism is by reducing the requirement for posttraining protein synthesis and translation during LTM consolidation. This is because CREB-overexpressing neurons already overexpress memory related proteins at baseline, therefore LTM consolidation is facilitated and occurs more rapidly (Han et al., 2008).

However, in the nNOS KO mice these two mechanisms likely do not occur despite the elevated pCREB expression (Figures 4.5E and 4.9E). For instance, the threshold for LTP induction in nNOS KO hippocampal slices is not reduced (Hopper and Garthwaite, 2006). Also, LTM consolidation is not facilitated in nNOS KO mice as has been determined using behavioral learning tasks (Kelley et al., 2011; Kelley et al., 2009; Kirchner et al., 2004; Tanda et al., 2009; Weitzdorfer et al., 2004). Rather, the findings in nNOS KO mice show that ultimately, inhibition of NO signaling precludes any potential gains in learning and memory afforded by overexpression of pCREB.

In contrast to the studies which have shown that pCREB overexpression facilitates memory, two studies have shown that pCREB overexpression impaired the acquisition and retrieval of memory in rats (Viosca et al., 2009b; Yuan et al., 2003). The results of these investigations suggested that the relationship between CREB and learning and memory may be an inverted U-function, and suggest that an optimal window of CREB phosphorylation may be required for learning. Interestingly, the current studies showed that the NO donor reduced amygdalar pCREB expression in nNOS KO mice, which coincided with significant improvements in contextual fear conditioning (Figures
4.1D and 4.8B). This suggests that restoration of pCREB to near WT levels is supportive of fear learning.

Given the importance of CREB for LTM consolidation, the molecular findings for CREB suggest that the drugs influenced LTM consolidation. However, this conclusion appears to contrast the results from the posttraining drug administration studies where it appeared that the drug administrations had no effects on LTM consolidation (Figures 3.2 and 3.3 panels B and D). In light of the current molecular results, it now appears that the drugs likely did have an influence on the consolidation of LTM through modulations of CREB. This conclusion is in agreement with previous studies that showed that pharmacological modulations of NO signaling directly influenced LTM consolidation (Furini et al., 2009; Kemenes et al., 2002; Schafe et al., 2005).

**Implications of the molecular findings for behavior**

In conclusion of the molecular studies, my findings have contributed significantly to our understanding of the effects of global modulations of the NO signaling pathway on brain levels of cGMP, ERK1/2, and CREB. The results showed that genetic and acute pharmacological inhibitions of the NO signaling pathway had the following effects: 1) decreased cGMP levels in the hippocampus and amygdala, 2) no effects on ERK1/2, and 3) opposing effects on amygdalar pCREB in WT and nNOS KO mice (Kelley et al., 2011; Kelley et al., 2010). In relation to the behavioral studies, the changes in cGMP and CREB in general corroborated the outcomes of fear conditioning. First, decreased cGMP levels in nNOS KO and WT mice with nNOS inhibitor administration was associated with impairments in STM and LTM for contextual, auditory, and visually cued fear conditioning. Second, increased cGMP levels following NO donor administration to
nNOS KO mice coincided with improvements in contextual fear conditioning. These outcomes implicated novel roles of cGMP in the acquisition of these types of fear conditioning. Third, the CREB studies showed that inhibition of pCREB and total CREB expression in WT mice following nNOS inhibitor administration coincided with impaired LTM for contextual and visually cued fear conditioning. Fourth, decreased pCREB in nNOS KO mice following NO donor administration was associated with improved LTM for contextual fear conditioning. These novel results indicate that clinical treatments which target the NO→cGMP→CREB signaling pathway may hold promise for modulating maladaptive memory formation and stress responses related to PTSD and specific phobia.

**Proposed models of NO signaling and fear conditioning**

Figures 5.1 and 5.2 summarize my findings on the molecular changes in NO signaling in WT and nNOS KO mice, and propose models for how the genetic and pharmacological modulations of the NO signaling pathways influenced the behavior for fear conditioning. In WT mice (Figure 5.1, left panel), NO acts as a retrograde signal (1) and intracellular messenger to activate soluble guanylyl cyclase (sGC) which produces cGMP. In the presynaptic terminal, cGMP-mediated signaling (2) is responsible for facilitating vesicle release which is important for early-phase LTP and STM (Arancio et al., 1995). Also in the presynaptic neuron, cGMP-mediated signaling activates ERK1/2 (3) and presynaptic gene transcription which is involved in synaptic plasticity and LTP (4) (Overeem et al., 2010). In the postsynaptic neuron, cGMP-mediated signaling (2) leads to pCREB activation (3) via two major signaling pathways (Lu et al., 1999). The first is the PKG→ERK1/2→pCREB signaling pathway and the second is the
cAMP→PKA→pCREB signaling pathway. Because there were no changes in ERK1/2 by the NO signaling modulators, I believe that activations of the latter pathway affected amygdalar pCREB expression. The phosphorylation of CREB is required for gene transcription related to late-phase LTP and LTM consolidation (4) (Kida et al., 2002).

Administration of the nNOS inhibitor to WT mice had the following effects (Figure 5.1, right panel). First, cGMP production was significantly reduced (2) (Kelley et al, 2010). Reduced cGMP production is expected to impair early-phase LTP and STM acquisition, which was observed for contextual and visually cued fear conditioning (Kelley et al., 2011; Kelley et al., 2010). Second, it was shown that the nNOS inhibitor significantly reduced pCREB expression in WT mice (3) (Kelley et al., 2010). Reduced pCREB expression likely impaired LTP and LTM consolidation, and LTM deficits in contextual and visually cued fear LTM were observed (Kelley et al., 2011; Kelley et al., 2010). Essentially, reduced cGMP and CREB activity following nNOS inhibitor administration impaired STM and LTM for NO-dependent forms of fear conditioning (Table 5.1).

In nNOS KO mice (Figure 5.2, left panel), deficient NO signaling results in a lack of retrograde signaling (1) and activation of cGMP-mediated signaling (2). It was shown that basal cGMP levels were reduced in the nNOS KO mice (Kelley et al., 2010). In the presynaptic neuron, deficient cGMP production likely impaired early-phase LTP and STM. The behavioral studies confirmed STM deficits for contextual and auditory cued fear conditioning (Kelley et al., 2010; Kelley et al; 2009). Acute inhibition of cGMP-mediated signaling was expected to lead to reduced activation of CREB (3). However, studies have shown that tonic NO production has a negative regulation of pCREB (Contestabile, 2008), therefore chronic inhibition of NO in the absence of the nNOS gene
resulted in elevated pCREB by elimination of the inhibitory pathway (4). The results showed that pCREB was elevated in the nNOS KO at baseline (Figures 4.5E and 4.9E), suggesting that the elimination of the inhibitory pathway outweighed the consequences of reduced cGMP-mediated signaling on pCREB expression. Administration of the NO donor to nNOS KO (Figure 5.2, right panel) increased cGMP levels (2) (Kelley et al., 2010), which improves early-phase LTP and STM (Arancio et al., 1995). Activation of cGMP-mediated signaling was expected to result in activation of pCREB via the cGMP→PKG→pCREB signaling pathway (3). However, the NO donor reduced pCREB in nNOS KO mice, suggesting that the exogenous NO had the effect of inhibiting pCREB consistent with the role of NO in the neurogenesis literature (4). Given the aberrant basal expression of pCREB in the nNOS KO, the NO donor appeared to homeostatically restore pCREB levels. In summary, increased NO and cGMP at the time of training (i.e. memory acquisition) and restoration of aberrant pCREB resulted in improved STM and subsequent consolidation of LTM of fear conditioning in nNOS KO mice.

**Future Directions**

My findings have opened several avenues of additional research. First, site-specific inhibitions of nNOS in the amygdala and hippocampus will help to further define the roles of the NO signaling pathway for fear conditioning. Second, since my studies implicated NO signaling in fear memory formation, future investigations on the role of NO signaling in fear extinction learning have a solid foundation because fear extinction shares similar mechanisms with initial fear learning. Third, differences in the hormonal response to the footshock US in WT and nNOS KO mice were intriguing and warrant further studies on how nNOS gene deletion impaired the hormonal response to stress.
Fourth, given that the pharmacological modulators of NO signaling significantly altered the expressions of cGMP, pCREB, and CREB in naïve mice, future studies should investigate how these molecules are influenced by fear conditioning with and without the drug administrations.

**Region-specific inhibition of nNOS**

The current studies showed that congenital and global deletion of the nNOS gene and acute systemic modulations of NO signaling had similar effects on contextual and cued fear conditioning. Future studies using region-specific and conditional nNOS KO mice will be of great value in specifying the roles of the anatomical substrates for fear conditioning. For instance, region-specific inhibitions of nNOS in the hippocampus and amygdala will elucidate the precise roles nNOS in each of these for contextual and cued fear conditioning. Conditional and inducible repression of the nNOS gene will minimize the potential of compensatory mechanisms that could influence fear learning. Along similar lines, pharmacological investigations utilizing direct injections of the NO modulators into the hippocampus and amygdala, for example, would complement the previously mentioned genetic studies. It has already been shown that intra-amygdalar infusions of the nNOS inhibitor 7-NI and the NO donor SNAP impaired and facilitated auditory cued fear conditioning, respectively (Schafe et al., 2005). Yet, the effects of intra-amygdalar and intra-hippocampal injections of NO modulators on visually cued and contextual fear conditioning have not been reported.

**Role of NO signaling in fear extinction learning**

Discovering a role for NO signaling in fear extinction learning would be of immense clinical value. The clinical analog of extinction learning is exposure based
therapy, which is the frontline of cognitive treatment strategies for PTSD and specific phobia (Blanco et al., 2003). In animals and humans alike, the hippocampus, amygdala, and prefrontal cortex have key roles in fear extinction learning (Barad et al., 2006; Knight et al., 2004). It has been suggested that fear extinction learning involves many of the same molecular mechanisms as the initial fear learning (Izquierdo et al., 2004; Lin et al., 2003). Presently, only one study has reporting investigating NO signaling and fear extinction learning (Johnson et al., 2000). In that study, the systemic administration of the nonspecific NOS inhibitor L-NAME failed to block the extinction of contextual fear conditioning. However, pretraining L-NAME administration also did not affect contextual fear conditioning which was in contrast to the current results with SMTC. Therefore, I believe that these investigations should be revisited using a more specific nNOS inhibitor, such as SMTC, and using nNOS KO mice. Recent studies have shown success for D-cycloserine, a partial NMDAR agonist, which facilitated the consolidation of extinction learning in humans (Garakani et al., 2006). Further exploration of the role of NMDAR-related signals in extinction learning, such as the NO signaling cascade, could have a significant impact on anxiolytic pharmacotherapy development.

**Differences in stress response**

The corticosterone studies demonstrated differences in the stress responses to the footshock US between WT and nNOS KO mice. Intriguingly, nNOS KO mice did not exhibit hormonal stress response following single training fear conditioning; single training fear conditioning was also insufficient to elicit fear-related STM and LTM in the nNOS KO mice. This finding raises the question of the role of nNOS in corticosterone response, and also the role of posttraining corticosterone for learning and memory in the
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nNOS KO mice. Studies investigating the impacts of glucocorticoid signaling modulations in WT and nNOS KO mice will help elucidate the relationship between posttraining corticosterone and LTM. Specifically, future investigations could utilize pretraining and posttraining administrations of corticosterone, glucocorticoid receptor agonists/antagonists, or adrenalectomy surgery to address this question. I hypothesize, based on the existing literature, that exogenous corticosterone administration will improve fear-related LTM in nNOS KO mice, and that inhibition of glucocorticoid signaling in WT will reduce fear-related LTM. These studies will significantly improve our understanding of a) the roles of stress hormones in memory formation and b) the role of nNOS in regulating hormonal stress response. Ultimately, these studies could have implications for pharmacotherapies that modulate glucocorticoid signaling in individuals that are prone to trauma exposure.

**Effects of fear conditioning on cGMP, ERK, and CREB expressions**

It was shown that modulations of cGMP and CREB in naïve mice were accompanied by significant and corresponding modulations of STM and LTM of fear conditioning. These studies implicated baseline expression of cGMP and CREB for the outcomes of fear conditioning. Future molecular studies should focus on investigating the expressions of cGMP, ERK1/2, and CREB after fear conditioning with and without the pharmacological modulators of NO. The future experiments will investigate how pretraining administrations of the NO modulators influence the molecular expressions after acquisition of STM and during LTM. As previously discussed, cGMP, ERK1/2, and CREB have been shown to play a role in fear conditioning, and the current studies
significantly contributed to the current knowledge. The outcomes of these future studies will most likely further implicate the NO signaling pathway in fear conditioning.

Several potential outcomes of those experiments would require careful interpretation. For instance, one potential outcome is that elevated pCREB levels in nNOS KO mice will increase even further as a result of activations of NO-independent pathways involved in fear conditioning, including the Ca$^{2+}$/calmodulin and PKA pathways. Alternatively, it could be determined that pCREB in nNOS KO mice does not increase following fear conditioning; this outcome may be due to a ‘ceiling effect’ of pCREB expression. Another potential outcome is that pCREB may decrease in nNOS KO mice following fear conditioning. While this outcome may be the least likely of the three, it would be similar to the results of NO donor administration to nNOS KO and would suggest that the restoration of aberrant CREB phosphorylation is paramount for optimal fear conditioning.
Table 5.1. Dependency of NO signaling in fear conditioning to contextual, auditory, and visual conditioned stimuli (CS).

Acquisition and consolidation of memory for contextual fear conditioning are NO-dependent following a single training session. nNOS gene deletion and pharmacological inhibition of nNOS resulted in impaired contextual fear response following a single training. Also, nNOS KO mice given the NO donor acquired contextual fear response after a single training. Following multiple (4) trainings, STM and LTM of contextual conditioning were NO-independent. This was demonstrated by similar magnitudes of contextual freezing in WT and nNOS KO mice following multiple trainings. Memory for auditory CS was partially NO-dependent. A single training of nNOS KO mice resulted in only a partial deficit in freezing response to the auditory CS. Following multiple trainings however, the development of auditory cued fear conditioning was NO-independent because auditory cued freezing was similar in WT and nNOS KO mice. Memory for visual CS was NO-dependent following single and multiple trainings. A complete impairment in visually cued freezing in nNOS KO mice was observed after both a single training and multiple trainings. Further, nNOS inhibition in WT mice impaired visually cued LTM. The current novel investigations showed differential dependencies of NO signaling for contextual and auditory cued fear conditioning depending on whether a single or multiple (4) trainings were used, and that memory for visually cued fear conditioning was NO-dependent for both types of training.

<table>
<thead>
<tr>
<th>CONDITIONED STIMULUS (CS)</th>
<th>NO-DEPENDENT FEAR CONDITIONING</th>
<th>NO-INDEPENDENT FEAR CONDITIONING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TRAINING</td>
<td></td>
</tr>
<tr>
<td>CONTEXT CS</td>
<td>Single</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>Multiple</td>
<td></td>
</tr>
<tr>
<td>AUDITORY CS</td>
<td>Single</td>
<td>✓*</td>
</tr>
<tr>
<td></td>
<td>Multiple</td>
<td></td>
</tr>
<tr>
<td>VISUAL CS</td>
<td>Single</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>Multiple</td>
<td>✓</td>
</tr>
</tbody>
</table>

* The results suggested partial.
Table 5.2. The effects of genotype and pharmacological modulators of NO signaling on CREB phosphorylation in the amygdala and hippocampus.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Genotype</th>
<th>Amygdala</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>WT</td>
<td>(baseline)</td>
<td>(baseline)</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>↑ ↑pCREB</td>
<td>↑ ↑pCREB</td>
</tr>
</tbody>
</table>

Pharmacological modulators of NO signaling

| nNOS inhibitor | WT      | KO       | =          |
|                | ↓ pCREB |          |            |

| NO donor       | WT      | KO       | =          |
|                | ↑ pCREB |          |            |

Abbreviations: WT, wild-type; KO, knock-out; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; pCREB, phosphorylated (Ser-133) cAMP response element binding protein
↑, increase  
↓, decrease  
=, no change  
–, not tested

Genotypic comparisons of pCREB expression in naïve vehicle-treated mice revealed significantly elevated (↑↑) pCREB in the amygdala and hippocampus of nNOS KO mice compared to WT mice (baseline). Thirty minutes after the systemic administrations of the pharmacological modulators of NO (nNOS inhibitor and NO donor) the expression of pCREB was determined in the amygdala and hippocampus. In WT mice, the nNOS inhibitor administration decreased pCREB in the amygdala (↓) but pCREB remained the same in the hippocampus (=) compared to vehicle-treated WT controls. The nNOS inhibitor was not tested in nNOS KO mice (–). In WT mice administered the NO donor, pCREB was increased in the amygdala (↑) and remained the same in the hippocampus (=) compared to vehicle-treated WT controls. In nNOS KO mice, the NO donor decreased pCREB expression in the amygdala (↓) and had no effects in the hippocampus (=). The studies with CREB showed: 1) basal pCREB expression is elevated in nNOS KO amygdala and hippocampus. 2) The systemic drug administrations had specific effects on amygdalar pCREB expression but not hippocampal pCREB. 3) In WT mice, the inhibition of nNOS decreased amygdalar pCREB expression and NO donor increased amygdalar pCREB expression; these findings were consistent with expected modulations of the NO→cGMP→pCREB pathway. 4) In nNOS KO mice, facilitation of NO signaling had the opposite effect as in WT mice; exogenous NO reduced pCREB which was consistent with the purported role of NO as a negative regulator of pCREB. The findings suggest that the levels of pCREB in the amygdala correlate with the behavioral outcomes of single training, NO-dependent fear conditioning.
Figure 5.1. Proposed model of NO signaling pathways in WT mice that are involved in fear conditioning.

In WT mice (left panel), nNOS activation produces NO which can act as a retrograde signal (1) or intracellular messenger to stimulate sGC to produce cGMP (2). In the presynaptic terminal, cGMP facilitates synaptic vesicle release and PKG-mediated signaling which have roles in promoting early-phase LTP (E-LTP) and short-term memory (STM) acquisition of fear conditioning. In the postsynaptic neuron, cGMP-mediated signaling leads to CREB phosphorylation (3) via PKG → pERK1/2 → pCREB and also via cAMP → PKA → pCREB. These signaling pathways are implicated in late phase LTP (L-LTP) and LTM consolidation of fear conditioning. Also, retrograde NO signaling can promote presynaptic gene expression via similar cGMP-mediated signaling pathways in the presynaptic neuron (4). Following nNOS inhibitor administration to WT mice (right panel), the production of NO is reduced and has the following downstream effects. Retrograde signaling is reduced (1) and therefore reduced cGMP is produced in the presynaptic terminal (2). This has the effect of impaired E-LTP and STM acquisition of fear conditioning. In the postsynaptic cell, reduced cGMP production (2) inhibits cGMP-mediated signaling cascades which lead to CREB phosphorylation (3). Reduced pCREB impairs LTM consolidation of fear conditioning.
Figure 5.2. Proposed model of NO signaling pathways in nNOS KO mice that are involved in fear conditioning.

In nNOS KO mice (left panel), deletion of the nNOS gene results in deficient NO production. This results in a lack of retrograde signaling (1) and the production of cGMP in the presynaptic terminal (2). Reduced presynaptic cGMP production impairs E-LTP and STM acquisition of fear conditioning. In the postsynaptic cell, reduced cGMP production inhibits cGMP-mediated signaling which inhibits the phasic activation of pCREB (3). However, NO also negatively regulates pCREB via an unknown mechanism (4). The lack of NO-mediated inhibition of pCREB results in overall high elevations in pCREB expression (↑↑pCREB). Following NO donor administration to nNOS KO mice (right panel), exogenous NO has the following effects. First, NO stimulates cGMP production in the presynaptic terminal which helps to restore the deficits in STM acquisition of fear conditioning. Second, NO in the postsynaptic cell restores cGMP-mediated signaling (3). Third, exogenous NO inhibits pCREB via its negative regulatory function (4). Overall, the facilitation of NO-mediated signaling in nNOS KO following NO donor administration had the effect of reduced pCREB expression by over-activation of the inhibitory pathway.
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