Nitric oxide signalling in the basolateral complex of the amygdala: an extension of NMDA receptor activation during Pavlovian fear conditioning and expression

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By

Kathie Overeem

University of Canterbury

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Abbreviations

AMPA	L-α-amino-3-hydroxy-5-mrthyl-4-isoxazole propionate
ANOVA	Analysis of variance
AP5	Amino-5-phosponovaleric acid
BSC	Basolateral complex of the amygdala
BL	Basolateral nucleus of the basolateral complex
BM	Basomedial nucleus of the basolateral complex
CE	Central Nucleus of the amygdala
CREB	Cyclic adenosine monophosphate response element-binding protein
CS	Conditioned stimulus
cGMP	Cyclic guanosine monophosphate
DA	Dopamine
ERK	Extracellular regulated protein kinase
GABA	γ-Aminobutyric acid
ISI	Inter stimulus interval
L-NAME	N-nitro-l-arginine methyl ester
LA	Lateral Nucleus
LC	Locus coeruleus
mRNA	Messenger ribonucleic acid
NMDA	N-Methyl-D-Aspartate
NA	Noradrenalin
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PKG	cGMP-dependent protein kinase
PSD-95	Post synaptic density protein-95
sGC	Soluble guanylate cyclase
SIN-1	3-morpholino-sydnonimine
US	Unconditioned stimulus
VTA	Ventral tegmental area
7-Ni	7-nitroindazole

Abstract

N-methyl-D-asparate (NMDA) receptors located within the basolateral complex of the amygdala are required for the consolidation and expression of Pavlovian conditioned fear. The events downstream of receptor activation that mediate these processes are not well defined. An intermediate step that may be of significance is the synthesis of the gas nitric oxide (NO). Nitric oxide is synthesised as a result of NMDA receptor activation and acts as an unconventional neurotransmitter freely diffusing across cell membranes interacting with its targets in a non-synaptic manner. The targets of NO include cellular components that play significant roles during the consolidation of conditioned fear and the neurotransmission associated with its expression. This implies that NO may be an important intermediary of NMDA receptor activation and both these processes. The current study sought to examine this possibility using fear potentiated startle to examine the expression of learned fear. Three experiments were conducted, fifty rats received intra-BSC microinfusions of the global nitric oxide synthase inhibitor L-NAME either prior to fear conditioning, fear testing, or examination of the shock sensitization of the acoustic startle affect. The results indicated that NO was indeed required for both the consolidation and expression of learned fear, whereas it was not required for shock enhanced startle responding. This study provides new information about the sub-cellular basis of conditioned fear, and highlights the pivotal role played by NO in processes associated with conditioned fear.

1. Introduction

1.1 General introduction

Fear is one of the most intense emotions that an individual can experience. From an evolutionary perspective, fear is extremely important as it can deter an individual from situations and stimuli that could be dangerous. The strength of the emotion, specifically, its ability to strongly regulate behaviour, makes it adaptive. However, fear can be a double-edged sword. It can also be irrepressible, playing roles in psychological disturbances such as post-traumatic-stress disorder; anxiety related disorders; and many phobias. A detailed understanding of the molecular and cellular underpinnings of fear would, in turn, provide insight into the possible neuroanatomical basis of such emotional psychopathology.

The amygdala is a temporal lobe structure intimately involved in fear processing. More specifically, it links the perception of aversive stimuli with the neuroanatomy that regulates the behavioural and autonomical processes associated with fear responding. Furthermore, it plays the most predominate role in neuroanatomical underpinnings of fear memory formation, specifically Pavlovian conditioned fear. The fact that the amygdala plays a role in fear conditioning is well established. Research concerning fear memory formation is currently focussing on the cellular and sub-cellular events in the amygdala that mediate processing associated with learned fear.

Research has shown that amygdaloid N-methyl-D-asparate (NMDA) receptors play a crucial role in both the consolidation and expression of learned fear (Bauer, 2000; Campeau, 1992; Fendt, 2001; Goosens, 2004; Lee, 2001; Maren, 1996; Miserendino, 1990; Rodrigues, 2004; Walker, 2000, 2002). However, a detailed understanding of the events downstream of receptor activation that mediate these processes is lacking. A significant event could be the synthesis of the gas nitric oxide (NO). NO is second messenger that is ultimately synthesised

by calcium that passes through the activated NMDA receptor channel. It freely diffuses across cell membranes where it can directly interact with a large number of targets, and set in motion numerous signal transduction pathways. Many of the targets of NO, or the consequences of its signalling, are involved in both the consolidation and expression of learned fear. Thus, NO signalling may be a significant mediatory of NMDA receptor activation and these processes.

Thus, the main aim of this study was to determine whether NO signalling is required for processes association with conditioned fear. More specifically, the significance of NO synthesis during fear memory consolidation, the expression of learned fear, and the expression of unconditioned fear was examined. No previous study has analysed whether NO could play a role during these processes, specifically when a light is used as a conditioned stimulus and fear potentiated startle is used to examine the expression of learned fear.

Below, to assist in understanding how NO could modulate process associated with learned fear, the literature concerning the structure and function of the amygdala are discussed in section 1.4, followed by an overview of the neurochemical and cellular processes associated with fear memory consolidation and expression in sections 1.5 through to 1.7. This includes an overview of the role of the NMDA receptor in conditioned fear in section 1.7.2. Section 1.8 concerns how NO could affect the expression and consolidation of learned fear. Finally sections 1.9 and 1.10 detail the importance of this research and the experimental procedures employed in this current study. Firstly, however the historical background concerning the role of the amygdala in emotional possessing is presented.

1.2 Historical background

Research suggesting that the amygdala may play a role in emotion originated some 60 year ago. Kluver and Bucy (1939) reported that temporal lobe lesions in primates caused extreme changes in emotional behaviour (cited in Weiskrantz, 1956). They reported, amongst other deficits, that visual stimuli and events appeared to lose their emotional value. For example, the animals tried to eat uneatable objects; copulate with animals of the same sex; and lost their fear of snakes and humans. Kluver and Bucy (1939) referred to this as psychic blindness. Today psychopathology in humans resulting from temporal lobe damage of a comparable magnitude is often referred to as the Kluver Bucy syndrome. Most importantly, this innovative research revealed that the temporal lobe is a key brain area involved in processing emotion. However, the lesions inflicted in the primates were large and encompassed many anatomically distinct structures including the amygdala, hippocampus, and surrounding cortical areas. The precise details regarding the neuroanatomical underpinnings of the behavioural deficits were consequently lacking.

Weiskrantz (1956) subsequently discovered that damage to the amygdala was the primary cause of the Kluver Bucy syndrome. He reported that primates with bilateral amygdalectomies exhibited behaviour abnormalities consistent with those reported by Kluver and Bucy (1939) after more sizeable lesions of the temporal lobe. Weiskrantz (1956) also went a step further and experimentally analysed the behavioural change associated with amygdaloid damage. He demonstrated that the amygdala is crucial for avoidance conditioning, a process in which an animal learns to avoid a fearful stimulus, such as a shock, by performing a particular response. Based on this, Weiskrantz proposed that the amygdala was necessary for making the association between objects or events and their emotional consequence. Research concerning exactly how the amygdala underpins this and other

behavioural processes proliferated during the years that followed. It appears that the amygdala also plays a role in events including attention, object reward associations, and social behaviour (Alexander, 2000; Bachevalier, 2000; Compton, 2003). Notwithstanding the complexity of amygdaloid functioning and consistent with Weiskrantz's proposal, most of the research regarding the amygdala has concerned how it imbues an event or stimulus with emotion, especially fear. One of the most predominant and reliable methods used to examine this is Pavlovian fear conditioning.

1.2.1 Pavlovian fear conditioning

During the fear conditioning procedure an animal, usually a rat, is presented with contiguous pairings of an emotionally neutral conditioned stimulus (CS), such as a light or a tone, and an innately feared unconditioned stimulus (US), most often a footshock. The animal consequently learns to fear the CS because it associates it with the US. That is, the CS acquires the emotional value of the US. Subsequent presentation of the CS produces a fear response equivalent to that formerly only provoked by the US. The most common laboratory measures used to ascertain whether the animal has indeed learnt the association are CS-induced freezing and fear potentiated startle (FPS) (LeDoux, 2000). Defensive freezing is an adaptive response of small animals characterised by complete immobilization of all movement except for that required for normal respiration. FPS, on the other hand, is characterised by a fast sequential muscle contraction in response to a sudden stimulus that is augmented in the presence of the CS. Generally, freezing is used to assess fear when the CS is auditory, while increased reflex potential is used when the CS is visual, most commonly a light. Both light and tone are discrete cues and have been used extensively in fear conditioning research. It is noteworthy, however, that the context in which the US is presented can also serve as a CS. In this case the CS is not discrete; it is instead comprised of

the features that make up the environment including the smell, size, illumination, and other salient cues observable by the animal. This current study concerns conditioning involving discrete cues, consequently such research where freezing and FPS have been employed to measure fear will be discussed below. Research involving discrete cues, and complementary *in vitro* studies, have provided a wealth of information concerning how the amygdala mediates fear processing. The discussion below will begin with the amygdala's place at a systems level within the fear circuitry, its neuroanatomy, and its neurophysiology.

1.3. The Neuroanatomy of the amygdala

The general composition of the amygdala is now quite well characterised. It is an almond shaped multinuclear structure located within the medial edge of the temporal lobe. It is comprised of 13 diverse nuclei and cortical regions, which are often further divided into two or more subdivisions (McDonald, 1998; Pitkanen, 2000; Sah, 2003). These richly interconnected structures are grouped according to their electrophysiological properties, cytoarchitecture, histochemistry, and their extrinsic and intrinsic nuclear connections. Research from lesion, pharmacological, stimulation, and recording studies have indicated that both the basolateral complex (BSC) and the central nucleus (CE) of the amygdala play crucial roles in the neuroanatomical and neurophysiological underpinnings of conditioned fear (for a review see Kim, 2005). Essentially, together they form the interface between stimulus processing and the psychological and physiological characteristics of a fear response.

1.3.1 The Basolateral complex

Tract tracing studies have revealed that the BSC is the preponderate area of sensory input, receiving extensive excitatory afferents from sensory and higher order processing areas (Pitkanen, 1997). Essentially, sensory information concerning conditioned fear travels via the thalamus before reaching the BSC. From this point it can either journey directly to the BSC, or it can travel indirectly to the complex via higher order cortical areas (Davis, 1994; Doron, 1999; Lanuza, 2004; Shi, 1999a, 2001b). The BSC is composed of the lateral (LA), basal lateral (BL), and the basal medial (BM) nuclei. Incoming sensory information enters at different nuclei depending on its origin. The LA predominantly receives CS and US sensory information directly from the thalamus, while the BL and BM, primarily receive information from internal or higher order processing areas such as the hippocampus, hypothalamus,

perirhinal cortex, and other secondary and polymodal cortical areas (Davis, 1994; Doron, 1999; Lanuza, 2004; Pitkanen, 1997; Shi, 1999a, 2001b). All forms of excitatory neurotransmission, however, is modulated, integrated, and processed within the complex by each nucleus (Pitkanen, 1997). Thus, the BSC acts as the primary interface of the amygdala by processing incoming sensory information. When sensory information is deemed to be fearful the neurotransmission that it provokes in the BSC ultimately excites cells in the CE (Pitkanen, 1997).

1.3.2 Afferents of the Central Nucleus

The CE, which is subdivided into capsular, lateral, intermediate, and medial divisions, is the major output component of the amygdala. During neuronal activity associated with learned fear it is excited by the neurotransmission that originates from the BSC. It must be noted, however, that the CE also receives excitatory efferents from visual, auditory, and somatosensory areas via structures such as the posterior thalamic nucleus and parabrachial pathways (Lanuza, 2004). Nonetheless, projections from the BSC play an important role in conditioned fear, especially when the CS is discrete rather than contextual (Koo, 2004). Tract tracing studies have revealed that projections from the BL and BM predominantly target the medial portion of the CE. The only other component that receives BSC projections is the lateral CE, however its afferents are light (Maren, 1999; Pare, 1995). The LA, on the other hand, does not project directly to the CE. Output projections from the LA synapse onto a net of interconnected GABAergic cell clusters situated in between the BSC and CE, which are referred to as the intercalated cell masses. These cells, in turn, project to the medial portion of the CE (Millhouse, 1986; Pare, 1993; Royer, 1999). Research by Royer and colleagues (1999) has revealed that the intercalated cells tonically inhibit excitatory activity in the medial portion of the CE, and consequently gate information flow between the LA and CE.

During the initiation of a fear response excitation in the LA alleviates the inhibition imposed by the intercalated cells; this as well as direct projections from the BL and BM increases cellular excitation in the medial portion of the CE (Royer, 1999).

1.3.3 Efferents of the Central Nucleus

The medial portion of the CE is dense with spiny excitatory projection neurons, these synapses onto a number of brain stem and hypothalamic regions that mediate the autonomic and behavioural characteristics of a fear response (for a review see Davis, 2000a). For example, projections to the lateral hypothalamus mediate the autonomic characteristics of fear, such as increased blood pressure and pupil dilation (LeDoux, 1988). Projections to the paraventricular nucleus of the hypothalamus regulate the activation of the hypothalamo-pituitary-adrenocortical axis, which is intimately involved in mediating a general stress response (Herman, 2004). Efferents to the midbrain central grey and the reticularis pontis caudalis regulate freezing and FPS respectively, both of which, as mentioned, are predominantly used to assess fear learning. Finally, efferents to mid brain areas such as the ventral tegmental area (VTA) and the locus coeruleus (LC) initiate the release of excitatory catecholamines. These neurotransmitters act as neuronal modulators, enhancing excitation in various brain areas including the amygdala. Accordingly, their influence on conditioned fear is discussed in more detail below. Thus, the CE is the output component of the amygdala, activating brain regions that regulate the autonomic and behavioural characteristics of a fear response. The fear response is, in turn, characterised by a general increase in arousal in both the central and peripheral nervous system.

Thus, the CE mediates a fear response, while the BSC is the sensory interface of the amygdala. Because the BSC, especially the lateral component of the complex, processes CS and US associated sensory information it is the neuroanatomical location that plays the most

predominant role in the varying aspects of fear conditioning. Processes that occur within the BSC associated with fear memory will consequently dominate the rest of the introduction. Manipulation of the neurochemistry within this complex during either fear conditioning or expression has shed light on the chemical modulation of the complex associated with learned fear.

1.4 Neurochemistry of conditioned fear

Many neurochemical influences of BSC functioning have been described. However, only those applicable to this current study, specifically γ-aminobutyric acid (GABA), glutamate, and the catecholamines, will be discussed below.

1.4.1 GABA

In the resting BSC GABA is found at a high concentration in the extracellular fluid; as a result it imposes a global blanket of inhibition on the structure. This is predominantly mediated by the ionotropic $GABA_A$ receptor (Millan, 2003). Activation of the receptor results in an influx of negative chloride ions into the cell, hyperpolarizing the cell membrane and impeding cellular excitation. Accordingly, stimulation of amygdaloid $GABA_A$ receptors obstructs fear processing. For example, infusing the $GABA_A$ receptor agonist muscimole into the amygdala immediately prior to either fear conditioning or testing significantly attenuates both processes (Muller, 1997). Thus, for any aspect of conditioned fear processing to take place the global blanket of inhibition imposed by GABA must subside. Indeed, the extracellular concentration of GABA declines upon presentation of the CS is conditioned animals (Stork, 2002). Thus, GABA inhibits excitatory neurotransmission in the BSC. During conditioned fear associated processes this inhibitory influence is alleviated, consequently permitting excitatory neurotransmission.

1.4.2 Glutamate

Glutamatergic neurotransmission is the principle means of excitatory communication both within the BSC as well as from sensory areas to the complex (Walker, 2002). This is, in turn, mediated by a number of different glutamate receptors. However, only the activity of the L-α-amino-3-hydroxy-5-mrthyl-4-isoxazole propionate (AMPA) and NMDA receptors

relates to this current study and will be discussed below. AMPA receptors are ubiquitously located throughout the BSC and are the primary means by which glutamatergic neurotransmission is mediated (Carlson, 2001b). Accordingly, administration of AMPA receptor antagonists or agonists into the BSC impairs or facilitates the expression of conditioned fear respectively (Maren, 1999). NMDA receptors, on the other hand, appear to play a more global role in fear processing. Their activation is crucial for the consolidation of conditioned fear. However, *in vitro* examination of excitatory neurotramission in the BSC has also revealed that NMDA receptors work in conjunction with AMPA receptors during excitatory synaptic communication, especially in the spiny projection neurons of the complex (Sah, 2003). Thus, NMDA receptors may play a role in the neural dynamics of conditioned fear expression. This assumption, however, is the topic of considerable debate (see below). Nonetheless, excitatory neurotransmission associated with conditioned fear is predominantly mediated by glutamate.

1.4.3 The Catecholamines

The catecholamines, dopamine (DA) and noradrenalin (NA), modulate excitatory activity in the BSC during processing associated with conditioned fear. The dopaminergic and noradrenergic cell bodies are located in the VTA and LC respectively. The arborisations from these cells project throughout the brain including a dense projection to the amygdala (Chow, 2000). Here these neurotransmitters function as neuronal modulators, since they do not directly partake in excitatory neurotransmission. Instead, they notably modulate neuronal activity that, in turn, influences cellular excitation (Carlson, 2001b). For example, antagonism of D1 and D2 DA receptors in the BSC prevents fear conditioning (for a review see Pezze, 2004), while inhibiting cellular excitation in the VTA impedes fear expression (Borowski, 1996; Munro, 1997). In a similar manner, infusing cloidine, the agonist for the α_2 NA

presynaptic autoreceptor, into the BSC prevents both fear conditioning and expression (Davies, 2004; Schulz, 2002). As a result, it has been hypothesized that NA and DA significantly facilitate cellular excitation in the BSC, essentially gating information flow (Borowski, 1996; McGaugh, 2004; Munro, 1997). Consequently, they act in concert with glutamate to induce cellular excitation in the BSC. This increase in excitation is crucial for neurotransmission associated with the consolidation and expression of conditioned fear.

1.5. Electrophysiology of fear conditioning

Examination of the electrophysiological consequences of fear conditioning has revealed that it potentiates CS-evoked excitatory post synaptic potentials (EPSP) in the BSC (Collins, 2000; Quirk, 1997; Quirk, 1995). For example, fear conditioning increases the magnitude of CS-evoked firing and shortens the latency of firing onset after stimulus presentation (Quirk, 1995). This increase in cellular excitation presumably enables the CS to induce a fear response via activation of the CE. Research has suggested that this CS modification is dependent on the abovementioned convergence of CS- and US-associated neurotransmission within the LA (Blair, 2001; Romanski, 1993; Sah, 2003; Schafe, 2005; Shi, 1999b, 2001a). Indeed, neurotransmission concerning both stimuli converge on the same cells in the LA (Romanski, 1993). Presumably this results in an associative form of synaptic potentiation. Whereby, CS associated synapses are potentiated in cells that are receiving CS and US associated neurotransmission. Thus, this is thought to be the principal neuroanatomical area where the CS fear association is represented. It is noteworthy, however, that areas outside of the LA, such as the CE, thalamus, and geniculate nuclei, are also implicated in the formation of a fear memory (Apergis-Schoute, 2005; Cahill, 1999; Pare, 2004; Samson, 2005; Schroeder, 2005). Nonetheless, cellular modifications in the LA appear to be especially crucial (Blair, 2005; Maren, 2003; Rodrigues, 2004; Schafe, 2005). Research concerning the sub-cellular events within the BSC that could lead to this increase in CS-evoked activity has revealed that *de novo* protein synthesis is a key requirement.

1.6 Sub-cellular basis of fear memory consolidation

1.6.1 Fear memory consolidation

Fear memory consolidation is synonymous with long-term memory formation and is characterised by *de novo* protein synthesis in the BSC (Bailey, 1999; Schafe, 2000). Presumably these new protein components change the synaptic structure of the cells involved, potentiating CS-associated neurotransmission. The synthesis of new cellular components requires the activation of a number of protein kinases. Protein kinases act as cellular switches; they phosphorylate and functionally change target proteins. These enzymes are vital components of signalling cascades that set in motion cellular events required for protein synthesis.

A protein kinase signalling cascade that plays a pivotal role in memory consolidation is the extracellular regulated kinase (ERK) signalling cascade. The ERK cascade is a member of the mitogen activated protein kinases (MAPK) family of intracellular kinase signalling cascades. The primary way in which ERK pathway is activated is by Ras, a small GTPase that is localized at the presynaptic and postsynaptic membrane (Kennedy, 2005; Seeger, 2004). Furthermore, signal transduction pathways initiated by many of the other kinases implicated in fear conditioning are known to activate the ERK pathway (Ohtsuka, 1996; Selcher, 2002). These include calcium dependent protein kinases (PKC); cyclic-AMP dependent protein kinase (PKA); phosphatidylinositol 3-kinsase (PI3-kinase) (Goosens, 2000; Lin, 2001; Schafe, 2000). Thus, the ERK transduction cascade appears to be a pivotal pathway that integrates a number of upstream cellular signals into a cohesive message. Activation of the ERK pathway results in gene transcription and subsequent protein synthesis. This involves the transcription factor cyclic-adenosine-monophosphate response element binding protein (CREB) (Adams, 2000; Sweatt, 2001, 2004). More specifically,

CREB promotes the transcription of genes resulting in the production of messenger ribonucleic acid (mRNA), which is later transcribed into cellular proteins. Thus, activation of the ERK signalling cascade ultimately results in *de novo* protein synthesis via CREB.

ERK signalling in both the pre and postsynaptic cell is required for fear memory consolidation. Pre-conditioning and intra-LA infusions of the ERK antagonist U0126 prevents fear memory consolidation while leaving short-term memory, or the acquisition of conditioned fear, unaffected. Similar results were obtained when U0126 was infused into thalamus, where cell bodies for presynaptic sensory afferents reside (Schafe, 2000, 1999). Thus, the ERK signalling transduction pathway plays a pivotal role during fear conditioning in both pre and postsynaptic cells. Research concerning the events that lead to ERK activation has revealed that calcium influx through the activated NMDA receptor is a key prerequisite (English, 1997; Xia, 1996).

1.6.2 The NMDA receptor

Over the years many researchers have reported that antagonism of the NMDA receptor in the BSC impairs fear conditioning (Bauer, 2000; Rodrigues, 2004; Walker, 2000, 2002). Its characteristic method of activation has provided insight into how it could mediate the process. The NMDA receptor is unique as it requires three prerequisites for its activation. Firstly, an obligatory glycine and / or d-serine co-agonist must be bound on the extracellular side of the receptor. Secondly, the cellular membrane in which the receptor is situated must be strongly depolarized. This induces a change in the conformation of the receptor, dislodging a magnesium ion that acts as a plug in the receptor channel. Finally, glutamate must bind to the receptor free of the magnesium plug. Thus for the receptor to activate during synaptic transmission the presynaptic cell must release glutamate while the postsynaptic cell is depolarized (Carlson, 2001a). It has been suggested that this occurs during fear

conditioning when incoming CS and US neurotransmission converge on the same cells within the LA. The US would provide the first prerequisite of receptor activation. Simultaneous weak glutamatergic inputs from CS-associated sensory areas would fulfil the second requirement (Blair, 2001). This associative input result in NMDA receptor activation, specifically at synapses receiving excitatory-CS information (Blair, 2004). Thus, without the US, CS-associated excitatory neurotransmission would not activate the receptor. Thus, the fact that the NMDA receptor acts as a coincidence detector makes it a valid possibility for initiating the cellular events that lead to the CS modifications that underpin fear conditioning. However, as mentioned above, in some areas of the BSC, NMDA receptors also appear to subserve routine synaptic transmission. However, behavioural examination of unconditioned fear has revealed that NMDA receptor activation in the BSC is not required (Van Nobelen, 2006). Therefore, it is likely that CS-associated neurotransmission in conditioned animals' results in NMDA receptor activation. Whether this actually occurs has been disputed in the fear conditioning literature.

It has been debated whether NMDA receptors in the BSC specifically underpin learning, or whether they also play a role in CS associated neurotransmission in fear conditioned animals. More specifically, a number of researchers examining the expression of conditioned fear have reported that the NMDA receptor is not required (Campeau, 1992; Goosens, 2004; Miserendino, 1990). Conversely, others have reported that antagonism of the receptor induces a deficit in the retrieval of conditioned fear, indicating that NMDA receptors are involved in CS associated neurotransmission in a manner that may account for their involvement in the consolidation of learned fear (Fendt, 2001; Lee, 2001; Lindquist, 2004; Maren, 1996). One possibility concerning the discrepancy is that NMDA receptors with different subcomponents could underlie each process. The NMDA receptor is composed of a

principal NR1 subunit and one or more of the modulatory NR2 subunits NR2A-D (Stephenson, 2001). NMDA receptors with NR2A and NR2B subunits are highly concentrated within the LA (Sah, 2003). The abovementioned researchers, however, have used global NMDA receptor antagonists to assess the involvement of NMDA receptors in fear conditioning. Selective antagonism of receptors that contain NR2B subunits has revealed that these receptors are predominantly involved in fear consolidation (Rodrigues, 2002). Receptors that contain the NR2A subunit, on the other hand, are thought largely to play a role in neurotransmission (Blair, 2001). Both receptor types are able to bind the scaffold protein post synaptic density protein-95 (PSD-95) via their NR2 subunit (Kornau, 1995). The partnership between NMDA receptors and this protein serves to assemble multi-protein complexes at the postsynaptic membrane necessary for NMDA receptor mediated synaptic responses (Wyneken, 2004). Thus, NMDA receptors with NR2A and NR2B subunits are able to associate with the same intracellular signalling proteins. One example is the enzyme neuronal-nitric oxide synthase (nNOS), which binds directly to PSD-95. Furthermore, nNOS is highly concentrated in the LA where, as mentioned, both types of NMDA receptors predominate (McDonald, 1993; Unger, 1992). This places the enzyme in an applicable position to mediate NMDA associated processes. Neuronal-NOS is activated by calcium that passes through the activated receptor via calcium-calmodulin, resulting in the production of the small gaseous molecule nitric oxide (NO). Therefore, this gas could be a significant intermediary of NMDA receptor activation and fear consolidation and expression.

1.7. Nitric oxide

NO is predominantly known as a retrograde neurotransmitter that plays a crucial role in the molecular underpinnings of various forms of learning and memory including inhibitory avoidance (Calixto, 2001; Meyer, 1998), spatial memory (Holscher, 1996; Zou, 1998), habituation (Yamada, 1995), working memory (Cobb, 1995), and olfactory memory (Bohme, 1993; Dawson, 1994; Samama, 1999). NO is quite a unique signalling molecule for a number of reasons: firstly, it is not stored in synaptic vesicles and released via exocytosis, instead it is synthesised on demand. Secondly, it is synthesised and released at the postsynaptic membrane, rather than at the presynaptic membrane. Thirdly, it can freely diffuse across cell membranes. Finally, it has a half life of up to thirty seconds, after which it spontaneously decays into nitrate (Lowenstein 1994). In that time, however, it can diffuse in a radius of ~300 to 350μm and interact with its targets in a non-synaptic manner (Lancaster Jr, 1997; Vizi, 2000). Given that the size of a synaptic cleft is approximately 20nm and the size of a cell body is a few micrometers (Kiss, 2000), NO has the ability to diffuse across a large portion of the cells in the BSC, intercalated masses, and part of the CE, significantly modulating activity in its radius.

The effects of NO can be broadly divided into two types: either soluble guanylate cyclase (sGC)-dependent, or sGC-independent (Krumenacker, 2004). In both types of signalling NO sets in motion complex signal transduction pathways with numerous subdivisions. For example, during sGC dependent process NO binds to sGC, activating the enzyme. This results in the synthesis of the second messenger cyclic-guanosine-monophosphate (cGMP) which, in turn, activates three principal targets: cGMP dependent protein kinase (PKG); cyclic-nucleotide gated channels; and cyclic nucleotide

phosphodiesterases (Ahen, 2002). Activation of these proteins broadly modifies cellular activity; some examples of their actions are mentioned in detail below. NO signalling independent of sGC predominantly involves radox reactions. For example, NO can directly interact with a target causing its nitrosylation, or NO can interact with an oxygen species forming peroxynitrite. Peroxynitrite can subsequently decompose into a number of other reactive species, peroxynitrite as well as its products can, in turn, influence cellular activity (Ohkuma, 2001). Thus, NO signalling is extremely complex and widespread.

Overall, NO is an unconventional neurotransmitter and neuronal modulator that acts as an extension of NMDA-receptor-mediated glutamatergic activity (Dawson, 1994). It sets in motion extensive parallel cellular signal transduction pathways that influence cellular activity in a broad manner. Furthermore, because NO is capable of diffusing in such a large radius, the synthesis of the gas in the LA could result in NO's effects reverberating throughout a large portion of the amygdala. Consistent with the fact that nNOS could be associated with NMDA receptors with either the NR2A or NR2B subunits, many of the sGC-dependent and independent targets of NO are involved in cellular processes required for amygdaloid synaptic transmission or fear memory consolidation.

1.7.1 Nitric oxide as a modulator of cellular excitation

As discussed above GABA, glutamate, and the catecholamines all play significant roles in modulating neurotransmission in the BSC. Research has indicated that NO can modulate the activity of these neurotransmitters. Given that the NMDA receptor is required for glutamatergic synaptic transmission in the BSC associated with the recall of conditioned fear, NO synthesis may be a significant intermediate step in between NMDA receptor activation and excitatory synaptic transmission in the BSC.

1.7.1.1 The $GABA_A$ receptor

NO is able to significantly reduce GABAergic activity, thus facilitating cellular excitation. As discussed above, GABA plays an essential role in inhibiting cellular excitation in the BSC via the $GABA_A$ receptor, and gates information flow between the BSC and CE via the intercalated cells (Lang, 1998; Li, 1996; Royer, 1999; Sanders, 1995; Szinyei, 2000; Yamanda, 1999). A number of researchers have reported that NO significantly reduces the inhibitory chloride currents that pass through the $GABA_A$ receptor in a sGC-dependent manner (Robello, 1996; Wall, 2003; Wexler, 1998; Zarri, 1994). For example, Robello and colleagues (1996) used whole cell patch clamp recordings to examine $GABA_A$ receptor functioning in cerebral cells. Bath application of L-arginine, the precursor for NO, significantly reduced the chloride current induced by GABA application. This effect was diminished by a NOS antagonist and a PKG inhibitor. This indicates that NO mediates its effect on the $GABA_A$ receptor via activation of this cGMP-dependent kinase (Robello, 1996). If NO works in a similar manner in amygdaloid cells it could reduce the inhibitory tone imposed by GABA, consequently facilitating excitatory neurotransmission both within the BSC and between the BSC and CE.

1.7.1.2 Glutamate

Research has indicated that NO can increase the extracellular concentration of glutamate, thus directly facilitating excitatory neurotransmission (Ohkuma, 2001). For example, *in vivo* microdialysis research in the conscious rat has revealed that the NO donor 3-morpholino-sydnonimine (SIN-1) increases the extracellular concentration of glutamate in the hippocampus and striatum up to 197% above baseline levels (Segovia, 1994). It appears that NO can induce this release by directly interacting with vesicle docking proteins at the presynaptic cell membrane, consequently facilitating glutamate liberation into the synaptic

cleft (Meffert, 1996, 1994). If NO acts this way in the LA it could directly facilitate excitatory neurotransmission by increasing the extracellular concentration of glutamate.

1.7.1.3 The Catecholamines

NO can also modulate excitatory neurotransmission by increasing extracellular catecholamine concentrations (Guevara-Guzman, 1994; Kiss, 2000; Prast, 2001). NO does this by functionally altering the catecholamine reuptake systems. Firstly, NO is able to inhibit their reuptake transporters, preventing the catecholamines that are liberated during neurotransmission from being taken back up into the presynaptic cell (Kiss, 2001, 2004). For both the catecholamines, NO modulates the reuptake systems in a sGC-independent manner. More specifically, NO directly interacts with the NA transporter preventing it from functioning; conversely it inhibits the DA transporter via peroxynitrite (Fleckenstein, 1997; Kaye, 2000). Additionally, Lonart and colleges have reported that NO can reverse each transporter, consequently increasing the extracellular concentration of both neurotransmitters (Lonart, 1993, 1992). Thus, NO is able to enhance the extracellular concentration of both DA and NA by altering their reuptake systems. Because the catecholamines significantly facilitate cellular excitation in the BSC NOs ability to modulate their activity could have considerable effects on neurotransmission associated with conditioned fear.

1.7.1.4 Summary

NO could, therefore, significantly increase the excitatory tone of the BSC, and thus play an important role during the expression of learned fear. More specifically, CS associated neurotransmission in the amygdala could activate NMDA receptors, especially those containing the NR2A subunit, resulting in the synthesis of NO. NO would diffuse in a spherical manner increasing cellular excitation in its radius. Thus, NMDA receptor activation

could influence the activity of many cells in the vicinity, thus having a more global effect on the BSC structures rather than only affecting the cell in which the NMDA receptor resides. However, a general increase in cellular excitation could also facilitate the initial consolidation of learned fear. Thus, NO acting in the above mentioned manner could influence both fear memory recall and its consolidation. However, NO can also modulate some of the cellular machinery specifically required for long-term fear memory formation.

1.7.2 Nitric oxide in memory consolidation

As discussed above *de novo* protein synthesis is a fundamental requirement of fear memory consolidation. Moreover, the NMDA receptor has been implicated in this activity. As discussed next, NO can directly modulate the cellular machinery required for protein synthesis. Thus, it could be the intermediate step between NMDA receptor activation and fear memory consolidation.

1.7.2.1 Extracellular regulated kinase

NO can link NMDA receptor activation with ERK signalling. NO is able to activate Ras, a signalling protein that is required for fear conditioning (Brambilla, 1997, Merino, 2006) and is a principle activator of the ERK signalling pathway (Lander, 1996, 1995; Yun, 1999, 1998). NO activates Ras by directly interacting with the protein (Lander, 1995;. Yun, 1998). Given that ERK signalling in both the pre and postsynaptic cell is required for fear memory consolidation and that Ras is located at both the presynaptic and postsynaptic membrane (see above), it seems plausible that NO could activate this signalling pathway in both cells. Thus, NO may link NMDA receptor activation with ERK signalling. However, NO's possible contribution to protein synthesis does not end there. As mentioned, ERK signalling leads to gene transcription via CREB; NO can also activate CREB.

1.7.2.2 CREB

NO can promote the activation of CREB, consequently playing a more direct role in protein synthesis by facilitating the transcription of mRNA. NO can activate CREB in a sGC-dependent manner. Specifically, researchers examining late phase long-term potentiation (L-LTP) in hippocampal cells, an *in vitro* phenomenon that is analogous to memory consolidation (Blair, 2001; Dityatev, 2005; Laroche, 2000; Maren, 2005; Silva, 2003), reported that inhibiting PKG activity blocked L-LTP (Lu, 1999). Examination of how PKG mediates its effects revealed that inhibiting the kinase reduced CREB phosphorylation (Lu, 1999). Thus, the NO-sGC-cGMP-PKG pathway contributes to CREB phosphorylation in the hippocampus. As a result, this pathway can modulate the transcription of genes from the cell nucleus. NO could also play a similar role in the BSC during fear memory consolidation, thereby contributing to NMDA-receptor-mediated protein synthesis.

1.7.2.3 Summary

Overall, NO is able to modulate ERK and CREB signalling, contributing to protein synthesis in both a sGC-independent and sGC-dependent manner respectively. Thus, NO could link NMDA receptor activation, specifically those that contain the NR2B subunit, with cellular events required for the long-term cellular modifications that underpin fear memory consolidation. Indeed, this seems feasible when considering that fear memory consolidation requires the post and pre synaptic cell, indicating that a retrograde messenger may be involved.

1.8 Reasons for this current study

NO is an extremely promiscuous molecule, it essentially acts as an extension of glutamatergic synaptic transmission via activation of the NMDA receptor. The fact that NO can modulate neurotransmission as well as cellular processes required for fear memory consolidation is fitting when considering that NMDA receptors, especially those with NR2A and NR2B subunits respectively, have been implicated in both processes. Thus, NO synthesis could significantly subserve NMDA receptor activation and both processes. It must be mentioned, however, that the above overview of NO's actions is in no way exhaustive and predominantly mentions research where brain areas outside of the amygdala were examined. Nonetheless, there is no reason to believe that NO would not act in a similar manner in the BSC.

The first step in this research area is to determine whether NO plays a significant role in the BSC during processing associated with conditioned fear. Recently there has been an increase in research concerning this very endeavour. For example, examination of LTP in amygdaloid cells has revealed that an increase in NO activity significantly augments the process (Chien, 2003). This indicates that NO could play a role in amygdaloid mediated memory formation. Moreover, infusion of the NO donor SIN-1 into the amygdala results in activation of the hypothalamic-pituitary-adrenal axis, a process that is known to occur during the initiation of a fear response (Herman, 2004; Seo, 2001), thereby, showing that NO activity pays a role in fear associated amygdaloid functioning. Direct examination of NO's role in fear conditioning, however, has revealed that it is exclusively required for fear memory consolidation. More specifically, Schafe and colleagues (2005) reported that intra-LA microinfusions of the selective nNOS inhibitor 7-nitroindazole (7-Ni) did not effect short-term memory formation, fear expression, and the re-consolidation of learned fear. However,

the CS used in this series of experiments was auditory and fear learning was assessed by examining CS induced defensive freezing. Thus, it is important to ascertain whether these findings can extend to fear conditioning when the CS is visual and FPS is used to examine fear expression.

1.9 Experimental procedures employed in this study

1.9.1 Fear potentiated startle

A FPS paradigm was employed in the current study to determine whether rats can learn a CS-US fear association, and express conditioned fear when NO synthesis is inhibited in the BSC during each process. During the conditioning procedure the animals were conditioned to fear a light. During the subsequent testing session, baseline acoustic startle reflex amplitudes of the fear conditioned animals were obtained and compared with those acquired when the acoustic stimulus and the CS were presented simultaneously. Because of the arousing characteristic of fear if the animals had learnt the fear association their acoustic startle responses were potentiated in the presence of the CS. One of the strengths of the FPS paradigm is that it is not species typical and can be reliably observed in humans by using the eye-blink component of FPS. Researchers using this method have reported that FPS in humans shares many characteristics to those observed in animal populations. For example, FPS is enhanced when human subjects are exposed to a CS previously paired with a US (Grillon, 1997; Jovanovis, 2005), when they anticipate shock (Grillon, 1995; Morgan, 1995), and when they have been exposed to an aversive stimulus (Bradley, 2001). Furthermore, FPS in humans also requires an intact temporal lobe (Buchanan, 2004). Thus, because of these similarities the FPS paradigm has a great deal of face validity in experimental analyses of the neural systems and molecular events involved in fear and anxiety. Thus, FPS was employed in the current study to assess whether rats learnt a CS-US fear association, and whether they could express conditioned fear.

1.9.2 Shock sensitization of the acoustic startle

The shock sensitization of the acoustic startle paradigm was used to determine whether animals can express fear when NO signalling is inhibited in the BSC. The shock sensitization effect is characterised by an enhancement of acoustic startle amplitudes immediately following several un-signalled shock presentations. It has been suggested that this is a manifestation of an unconditioned fear response (Davis, 1989). An alternative view is that it is an expression of conditioned fear. More specifically, it has been suggested that the shock increased acoustic startle results from rapid conditioning to the context in which the shock was delivered (Richardson, 1999; 1998). Research from our laboratory supports the prior hypothesis. For example, infusion of the global NMDA receptor agonist D(-)-2-Amino-5-phosphonopentanoic acid (AP-5), into the BSC did not influence the shock sensitization effect (Van Nobelen, 2006). Contextual conditioning, on the other hand, does require NMDA receptor activation (Maren, 1996), thus suggesting that shock increased acoustic startle is indeed an expression of unconditioned fear. Thus, a shock sensitization paradigm was used in this current study to examine unconditioned fear.

1.10 The aims and hypotheses of the current study

The first aim of this study was to determine whether NO signalling in the BSC is required for fear memory consolidation. To ascertain whether this is indeed the case, the effect of two doses of the global NOS inhibitor N-nitro-l-arginine methyl ester (L-NAME) on the consolidation of conditioned fear were examined. No previous study has examined whether NO is required for fear conditioning when the CS is visual and FPS is used to examine fear responding. This is surprising for a number of reasons: firstly, the enzyme responsible of NO synthesis, nNOS, is highly concentrated in the LA, which is the neuroanatomical location of the amygdala where the crucial component of CS-US engram is thought to reside. Secondly, with this paradigm NMDA receptor activation is the prerequisite for memory consolidation. Thirdly, NO is able to modulate cellular processes that underpin fear memory consolidation. Fourthly, research is emerging that suggests NO signalling plays a role in amygdaloidal functioning. Finally, research over the last decade has revealed that NO signalling plays a crucial role in the formation of many forms of memory. Therefore, it was hypothesised that inhibiting NO signalling in the BSC will prevent fear memory consolidation.

The second aim of the current study was to determine whether NO is required for the expression of learned fear. No previous study has examined whether NO could be involved in the expression of visual conditioned fear using a FPS paradigm. Again this is unexpected when considering that NMDA receptor activation is required for the expression of fear using this testing procedure and that NO is able to modulate processes required for neurotransmission in the amygdala. Therefore, it was hypothesised that inhibiting NO synthesis would prevent the expression of learned fear.

The third aim of this study was to determine whether NO signalling in the BSC is required for the enhanced acoustic startle observed in the shock sensitization of the acoustic startle paradigm. Once again, no previous researchers have examined whether NO could play such a role. Because the NMDA receptor is not required for this effect it was hypothesised that NO signalling in the amygdala is not required for this effect (Van Nobelen, 2006). Nonetheless, it was important to determine whether the animals can experience and express unconditioned fear so that the results from the fear consolidation and fear expression experiments can be attributed specifically to memory deficits rather than deficits in emotional responding.

The final aim of this study was to eliminate two other possible confounds. Specifically, whether inhibiting NO synthesis modifies normal baseline acoustic startle responding or the perception of footshock. The first of which could effect the interpretations drawn from the results of the fear expression experiment. The second could effect how one interprets the fear conditioning experiment. It was hypothesised that inhibiting NO synthesis would not modify these behaviours.

Overall, it was hypothesised that intra-BSC NO signalling is a significant extension of NMDA receptor activation associated with the consolidation of learned fear and its subsequent expression. Inhibiting NO's synthesis in the BSC should accordingly interfere with both processes. It was, conversely, anticipated that NO is not required for the expression of unconditioned fear, baseline acoustic startle responding, or the perception of footshock. Therefore, inhibiting the synthesis of NO in the BSC should not modify shock enhanced acoustic startle responding, or alter these routine behaviours.

2. Method

2.1 Subjects

Fifty male albino rats of the Wistar strain bred in the Psychology Department at the University of Canterbury served as subjects. They were group-housed 4 per cage in a colony room maintained at 22ºC and 48% rH. The colony was kept on a 12-h light dark cycle, lights on at 8.00 A.M, behavioural testing was conducted in the light portion of the cycle. At the beginning of the experiment the animals were approximately 3 months of age and weighed between 300 and 350 g. Throughout the study the animals had free access to food and water. All experimental procedures conformed to national and international guidelines for the ethical use of laboratory animals and followed protocols approved by the University of Canterbury Animal Ethics Committee.

2.2 Apparatus

The acoustic startle reflex amplitudes of the animals were measured in four identical startle cages (16.5 X 8.0 X 9.0 cm) (Med Associates, Fairfield, VT), located in sound attenuating melamine chambers (60 X 34 X 56 cm). A 2.8-W lamp and a 6.0-cm speaker were positioned 10 cm from each startle cage. The lid and walls of each cage consisted of horizontal stainless steel rods 0.25 cm in diameter and spaced 1.5 cm apart. The chamber floors were also comprised of stainless steel rods 0.45 cm in diameter and spaced 1.10 cm apart. The cages were located on top of a startle platform (25 x 11.5 x 4.5 cm) that contained a load-based cell that measured startle amplitudes. The analogue amplitudes were filtered to control for electrical noise through a Med associates startle transducer, amplified, and converted to a digital signal (0.5 V peak voltage amplitude equals 100 units). Med associates

startle reflex software version 5.6 recorded the startle amplitudes, and controlled the presentation of light, shock, and white noise stimuli. The acoustic stimulus was produced by a programmable audio generator and was a 100 ms white noise burst with a rise and fall time of 5 ms. The ambient noise level in the chambers was 36 dB, measured with a Bruel and Kjaer (Model 2235; Denmark) sound level meter (A-scale). The 600-μA footshock was presented through the grid floor of the startle chambers by a constant current scrambled shock generator.

2.3 Drug:

The global NOS inhibiter N(G)-nitro-l-arginine methyl ester (L-NAME), was purchased from Sigma-Aldrich (Auckland, New Zealand), dissolved in physiological saline (pH = 7.4) and infused in a volume of 0.5 μl per side. The dosages used (1.0 and 0.1 μg) were within the range used by previous researchers who have infused L-NAME into the central nucleus of the amygdala, and the dorsal raphe nucleus before conducting behavioural tests (Grahn, 2000; Zarrindast, 2002). The most effective dose ascertained from the fear consolidation experiment was subsequently used in both the fear expression and shock sensitization experiments.

2.4 Surgical procedures

Stereotaxic surgery was preformed under aseptic conditions. Thirty min before anaesthetic was administered each animal received an intraperitoneal (IP) injection of atropine (0.2 mg/kg) in order to prevent mucus build-up in the respiratory system and to facilitate respiration. They were subsequently anesthetized with an IP injection of sodium pentobarbitone (92 mg/kg). After the induction of anaesthesia 0.2 ml of the local anaesthetic mepivicane was subcutaneously administered at the top of the animal's skull at the

approximate location where an incision would be subsequently made. This was followed by subcutaneous administration of 0.2 mg/kg of the analgesic ketophen between the neck and shoulders. Four min later the animals were mounted in a stereotaxic instrument (Wood Dale, IL, USA). The incisor bar was adjusted so that the horizontal plane was level at Lambda and Bregma. An incision was then made to expose the bone at the top of the animal's skull. All rats received bilateral 22-gauge (0.71 mm) guide cannula (C313G; Plastics One, Roanoke, VA) implants aimed 1.0 mm above the lateral portion of the BSC in both hemispheres (AP: -2.8mm posterior from bregma; ML: ±4.8mm from the sagittal suture; DV: - 8.2mm from the skull surface). Both implants were perpendicular to the horizontal plane. Coordinates were obtained from the Paxinos and Watson (1997) stereotaxic brain atlas for the rat. The cannulae were fixed to the skull with stainless steel screws and dental cement, and 28-guage (0.36 mm) dummy stylets (C313DC, Plastic one) were placed within the shaft of each cannula to prevent blockage.

2.5 Base line assessment of acoustic startle amplitudes

For each animal, the dB noise level required to produce average acoustic startle amplitudes approximating 200 units was determined 5 to 7 days after surgery. Rats were placed in the startle chambers and allowed 4 min to adapt to the environment. Next they were presented with 60 noise bursts (95db). The following day, the noise level for each animal was adjusted so that acoustic startle amplitudes would approximate the desired level (generally an increase or decrease of 3 dB was required). The acoustic startle of each subject was then reassessed using the same procedures as the previous day. For each animal, the dB noise level that provoked average startle amplitudes approximating 200 units was used for all subsequent experiments; noise levels for all animals were between (87-98 dB).

2.6 Microinfusion process

During the infusion process the animals were hand held, and the dummy stylets removed. Two 28-guage (0.36 mm) infusion needles (C3131, Plastic One) preloaded with the appropriate drug concentration were inserted into each guide cannulae. Both extended 1.0 mm below the tip of the guide cannula and were attached to drug-filled polyethylene tubes (PE20, Plastics One) connected to 2.0 µl Hamilton syringes (Hamilton Co., Reno, NEV). The syringes were located in a Stoelting infusion pump (model 310; Stoelting Co., Wood Dale, IL). The bilateral infusions were made over 1 min, and the infusion needles were kept in place for a further 2 min to allow the drug to diffuse from the tip. Following the removal of the needles the dummy stylets were promptly reinserted.

2.7 The Fear conditioning experiment

Twenty-four hours after baseline assessment 21 rats were randomly assigned to 3 groups (N = 7), and received intra-BSC microinfusions of either saline or one of two doses of L-NAME (1.0 or 0.1µg) using the methods outlined in section 2.6.

2.7.1 The fear conditioning procedure

Immediately after drug infusion the rats were placed in the startle chambers and allowed 10 min to adapt to the apparatus and for the drug to diffuse throughout the target area. This time frame is consistent with that used by Grahn et al. (2000) who waited 10 min before behavioural testing after micro-infusing L-NAME into the dorsal raphe nucleus. The rats were then presented with 10 light + footshock pairings, at an inter-stimulus-interval (ISI) of 2 min. The light was presented for 3500 ms, and co-terminated with a 250-ms footshock.

2.7.2 Assessment of fear potentiated startle

For each rat, fear potentiation of acoustic startle was assessed 24 h after conditioning. The rats were placed in the startle chambers and allowed 4 min to adapt. Firstly, they were exposed to a block of 30 noise bursts. This first block was presented so that the animals would habituate to the stimuli, as animals exhibit abnormally high startle responses occur over the first few noise presentations. Consequently, startle responses from this block were not included in the subsequent analysis. Next, the animals were presented with a block of 5 noise-alone trials, followed by a block of 5 light + noise presentations. The light was presented for 3.5 s and co-terminated with a 100-ms noise burst. Across all blocks the ISI was 30 s (see Figure 1).

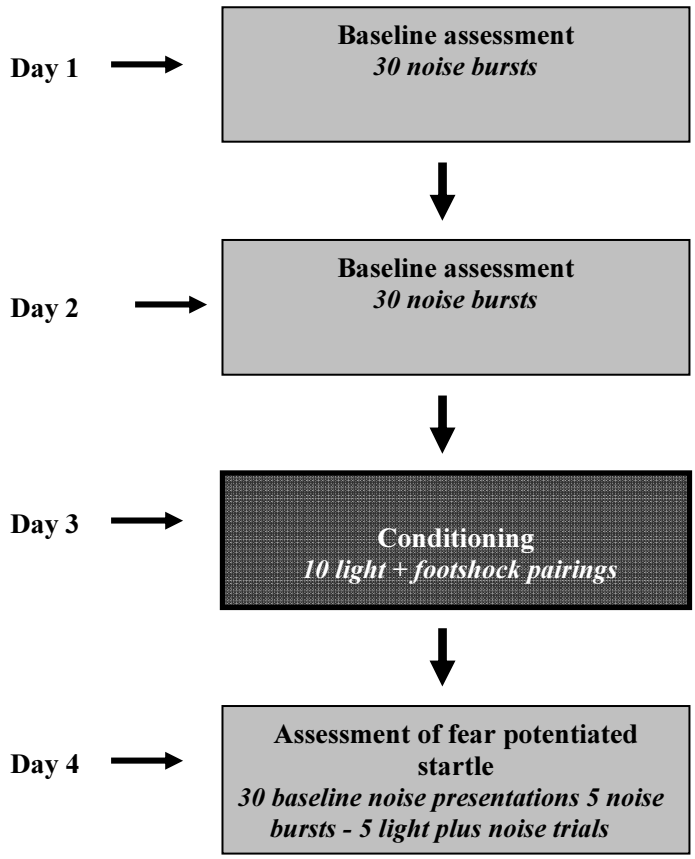

Figure 1 Schematic of the procedure used to determine whether intra-BSC microinfusions of L-NAME (1.0 and 0.1μg) prevented fear memory consolidation. Animals were tested at approximately the same time each day. Darkened box denotes that the procedure occurred immediately after drug infusion.

2.7.3 Data analysis

For each rat, acoustic startle amplitudes were average within the second block of five noise-alone trials and the third block of five light + noise trials. The average acoustic startle amplitude from the light + noise trails was subtracted from the average acoustic startle score from the noise-alone trails to give an average difference score. Results were analysed using a 2 (Block) X3 (Group) repeated-measures ANOVA design with the block factor as the repeated measure. Simple effects analyses were conducted to examine differences in group means. Furthermore, mean difference scores from the three groups were compared with each

other using a one-way ANOVA. Simple effects analyses were conducted to determine which means differed from each other.

2.8 The expression of learned fear experiment

Twenty-four hours after baseline assessment (see 2.5) 13 animals were fear conditioned using the same methods as described above (see 2.7.1). However, the animals received 20 light + shock pairings, and the shock duration was 500 ms. Approximately 24 h later the animals were randomly assigned to two groups and received bilateral microinfusions of either saline (N = 7) or 0.10 µg of L-NAME (N = 6) see section 2.6. After infusion the rats were promptly placed in the startle chambers and allowed 4 min to adapt. Next, they were presented with 10 noise bursts. Consistent with above, this first block of noise bursts was presented in order to allow the animals to habituate to the presentation of stimuli, and results were not included in the subsequent analysis. The number of noise presentations in this first block was substantially less than the FPS assessment in the previous fear conditioning experiment, as it had been determined from the findings of the previous study that the drug is effective 10 min after infusion. Thus, the procedure for this experiment was adjusted so that the animals were presented with the final two blocks approximately 10 min after infusion. Next the animals were presented with a second block of 5 noise-alone trails, and a final block of 5 noise + light trials. As with the previous experiment, the light was presented for 3.5 s and co-terminated with a 100-ms noise burst. The ISI across all blocks was 30 s (see Figure 2).

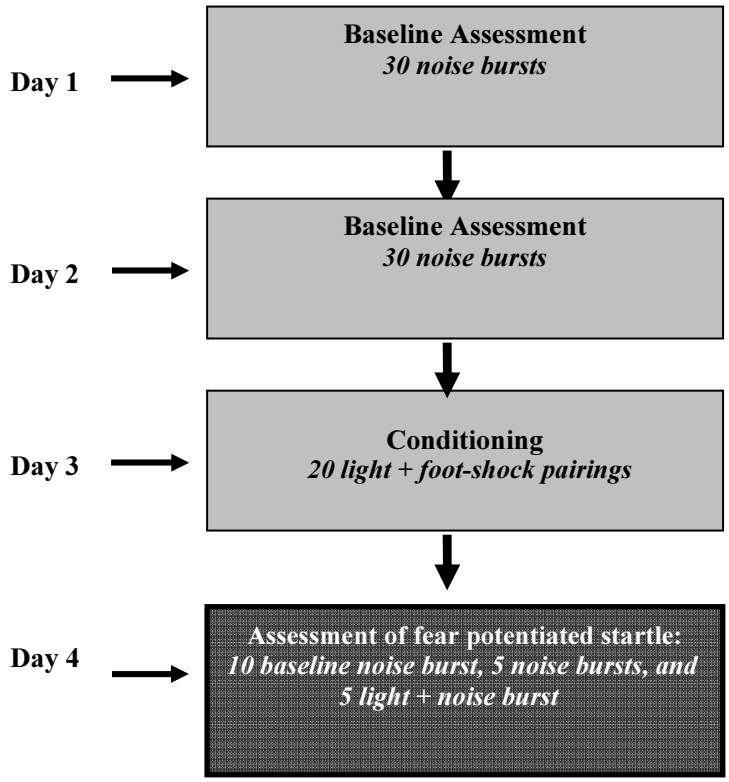

Figure 2. Schematic of the procedure used to determine whether 0.1µg L-NAME microinfused into the BSC significantly attenuated the expression of conditioned fear. Animals were tested at approximately the same time each day. Darken box denotes that the procedure occurred immediately post infusion.

2.8.1 Data analysis

Group means were calculated in the same manner as the fear conditioning experiment (2.7.3). Data was analysed using a 2 (Block) X2 (Group) repeated measures ANOVA design with the block factor as the repeated measure. Simple effects analyses and a *t*-test were conducted to determine whether differences between group means were significant.

2.9. The shock sensitization experiment

Twenty-four hours after baseline assessment (see 2.4) 16 animals were randomly divided into two groups: a saline-control group (N = 8) and an L-NAME group (N = 8). The

rats were placed in the startle chambers and allowed 4 min to adapt. They were then presented with a pre-infusion block of 20 noise bursts. The animals were promptly removed from the chambers and received bilateral microinfusions of either 0.1 μg of L-NAME, or saline (see 2.6). Immediately following infusion the animals were returned to their original startle chambers, and allowed 1 min to re-adapt. The rats were then presented with a second post-infusion / pre-shock block of 20 noise bursts. Followed by 10 500-ms foot shocks at an ISI of 10 s, finally they were presented with a post-shock block of 20 noise bursts. All noise burst were presented at an ISI of 30 s (see Figure 3).

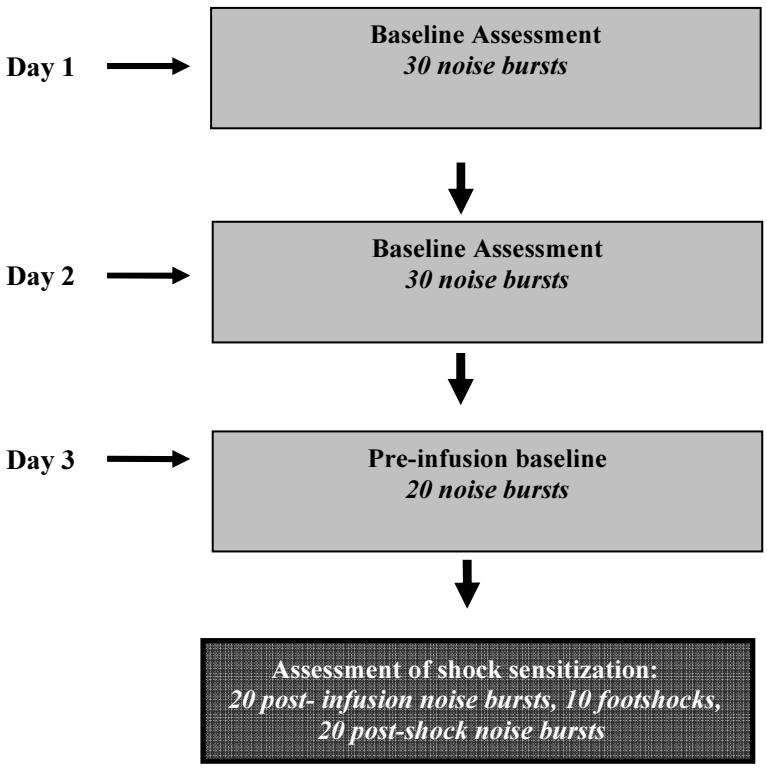

Figure 3. Schematic of the procedure used to determine whether 0.1 μg of L-NAME microinfused into the BSC significantly affected baseline acoustic startle amplitudes, shock reactivity, or shock sensitization of the acoustic startle. Animals were tested at approximately the same time each day. Darken box indicates that the procedure occurred immediately after drug infusion.

2.9.1 Data analysis

2.9.1.1 Effect of L-NAME on baseline acoustic startle reactivity

For each rat, the average acoustic startle scores in the pre-drug infusion block and the post-drug infusion block were determined. The average from the post-drug infusion block was subtracted from the average from the pre-infusion block to give an average difference in drug induced acoustic startle responding. Results were analysed in the same manner as described in section 2.8.1.

2.9.1.2 Effect of L-NAME on shock reactivity

For each rat, the average movement amplitudes 250-ms pre-shock onset and 250-ms post-shock onset were calculated. Average movement amplitudes from the post-shock were subtracted from average movement amplitudes pre-shock to give an average increase in movement amplitude induced by footshock. Inferential analyses were conduct in the same manner as described in section 2.8.1.

2.9.1.3 Effect of L-NAME of shock sensitization of acoustic startle

For each rat, average acoustic startle responses in the post-shock block were calculated. The average acoustic startle scores from the post-drug block (see 2.7.1.1) were subtracted from the average startle in the post-shock block to give an average difference score indicative of shock enhanced arousal. Again, inferential analyses were conducted in the same manner as described in section 2.8.1.

2.10. Histology

The rats were euthanazed with an overdose of sodium pentobarbitone and perfused intracardially, first with 200 ml of saline followed by 200 ml of 10% formaldehyde. The brains were promptly removed and stored in ten percent formaldehyde for approximately 2

days, and then transferred to a long-term 70% sucrose solution for a minimum of a week. Coronal sections of 50 μm were cut using a cryostat, mounted on gelatine coated slides, and stained with Cresyl Violet. Cannulae placements were subsequently verified using a light microscope. The placements were confirmed and reconstructed on representative coronal sections taken from the Paxinos and Watson rat brain stereotaxic atlas (Paxinos, 1997). Data from animals with placements that indicated that the infusion stylets were not within 0.5 mm of the basolateral complex, or where the guide cannulae had damaged the amygdala, were not included in the subsequent analysis.

3. Results

3.1 Histology:

Figure 4 depicts the intra-BSC injections sites of L-NAME for groups from the fear conditioning, fear expression, and shock sensitization of the acoustic startle experiments. All placements are superimposed on coronal sections taken from Paxinos and Watson (1997). As illustrated, injection sites were predominantly in the medial-lateral portion of the BSC.

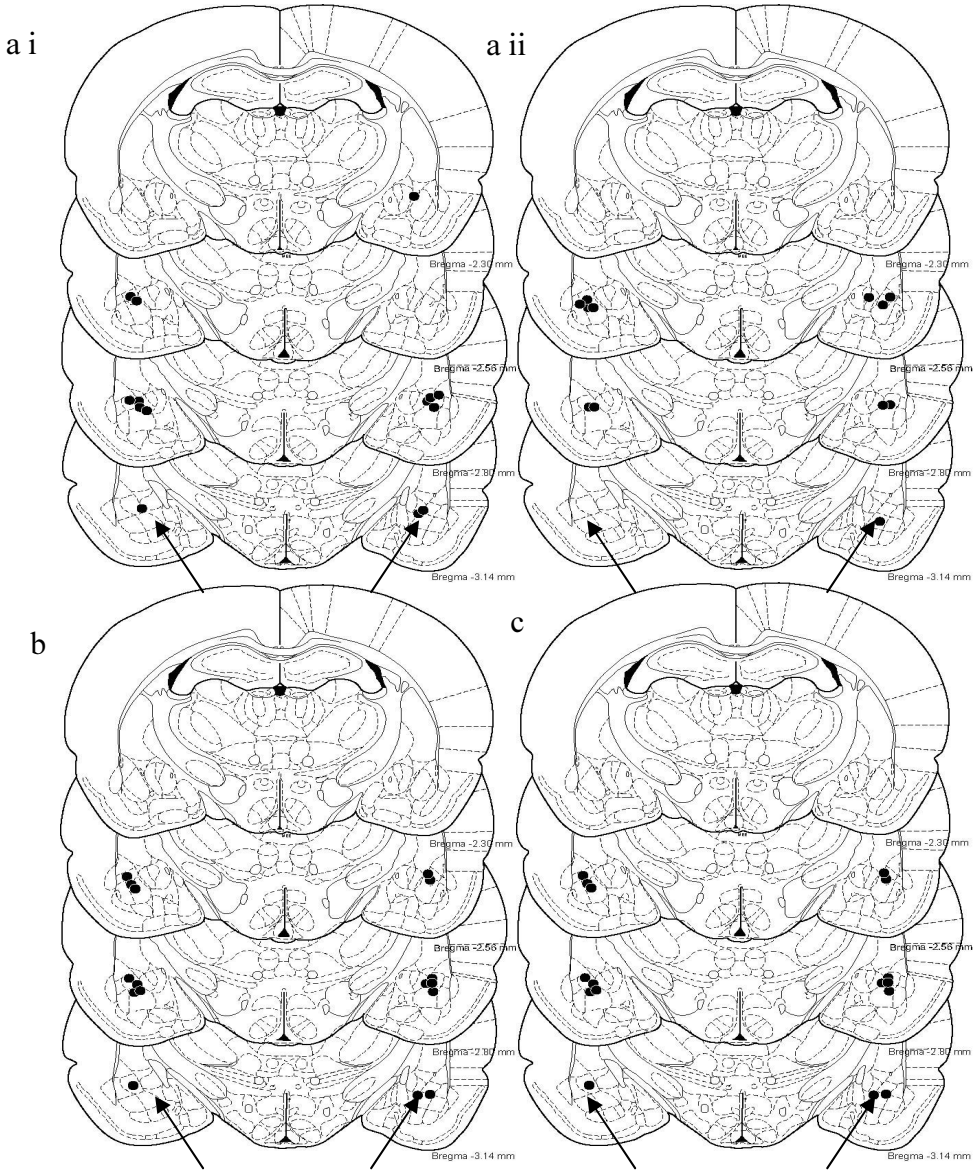

Figure 4. Schematic coronal sections through the rat brain showing the intra-BSC injection sites of L-NAME. The 1.0 and 0.1 µg fear conditioning groups are depicted in a i and ii respectively. The 0.1 µg fear expression group is depicted in b. The 0.1 µg shock sensitization group is shown in c. Arrows indicate the site of the BSC. Schematics are adapted from Paxinos and Watson (1997). Numbers are distances from Bregma

3.2 Fear conditioning experiment

The aim of the fear conditioning experiment was to determine whether NO plays a significant role in the molecular underpinnings of fear memory consolidation. It was also necessary to establish at what concentration L-NAME is most effective at inhibiting this phenomenon, in order to determine the most desirable drug concentration that could be used for the subsequent experiments. During the conditioning procedure the animals were required to learn that the presentation of a light-CS predicts the occurrence of a footshock-US. The consolidation of this association was assessed using a FPS paradigm with the dependent measure being the animal's acoustic startle response during the noise-alone trials and the noise + light trials. For each rat, average acoustic startle responses in the noise + light trials were subtracted from the average acoustic startle responses from the noise-alone trials to produce an average difference score (Appendix A). Thus, high difference scores were indicative of fear induced potentiation of the acoustic startle and thus fear memory consolidation.

Figure 5 shows the average noise-alone and noise + light startle amplitudes, along with and the average difference between these two blocks for the three groups. The figure shows that the acoustic startle levels for each group were equivalent in the noise-alone block. However, in the noise + light block the acoustic startle responses of the saline group increased substantially compared with the two drug groups. Thus, the difference score for the control group was substantially larger than those for two drug groups.

A 2(Block: noise-alone and noise + light) X3 (Group: saline, 1.0, and 0.1µg of L-NAME) repeated-measures ANOVA with Blocks as the repeated factor yielded a significant effect of Blocks, $F(1,18)=9.51, p<0.007$, and a significant Group X Block interaction, $F(2,18)=3.90, p<0.04$, without a Group main effect (Appendix B). Simple effect analyses

confirmed that the acoustic startle amplitudes of the control group increased significantly on the noise + light as compared with the noise-alone, F=16.45,p<0.0008. Conversely, the two drug groups exhibited similar startle amplitudes on the baseline and CS + Noise stimulus blocks, 1.0µg F=0.57,p>0.4; and 0.1µg F=0.28,p>0.6. The difference scores for the three experimental groups differed significantly when compared with each other: F(2,18)=3.90,p<.04 (Appendix C). Simple effects analysis revealed that the difference score of the control group was significantly larger than those of the two drug groups, 1.0µg, F=5.46,p<0.04; 0.1µg, F=6.21,p<0.03, while the two drug groups did not differ significantly from each another, F=0.024, p>0.8. Thus, both dosages of L-NAME impaired the capacity of animals to condition to a light CS.

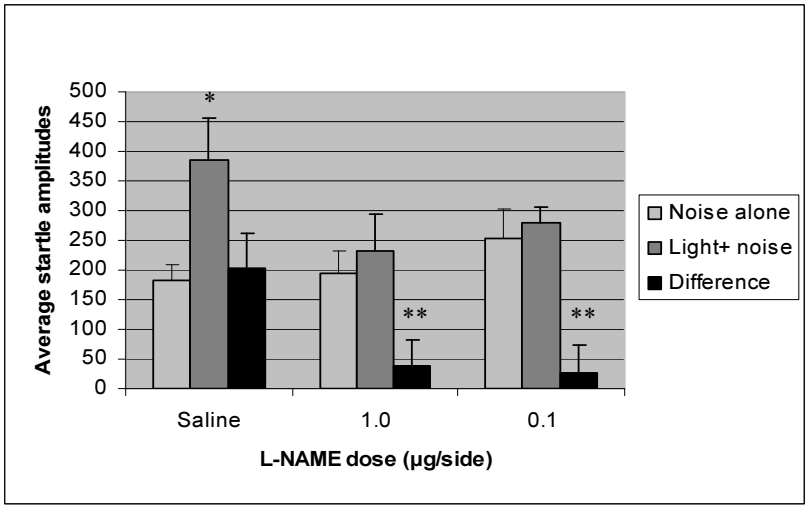

Figure 5. Average acoustic startle scores (+SEM) for groups that received intra-BSC microinfusions of L-NAME (1.0, 0.1µg) or saline per side prior to conditioning 24 hours previously. N=7 in each group. * $p < 0.05$ relative to noise-alone trials, ** $p < 0.05$ relative to saline group.

***3.3 Fear expression experiment**:*

The aim of the fear expression experiment was to determine whether NO is required for the expression of conditioned fear. Specifically, it was conducted to determine whether the lowest dose of L-NAME the significantly impaired memory consolidation would also prevent fear memory recall. As with the previous experiment, the animals were required to learn that the CS predicts the US, and a FPS paradigm was used to assess whether the animals had indeed learnt this association. Accordingly, the dependent measures were the animal's acoustic startle response during the noise-alone trails and the noise + light trials. For each animal, average acoustic startle responses in the noise + light trials were subtracted from the average acoustic startle responses from the noise-alone trials to produce an average difference score (Appendix D). Thus, high difference scores were indicative of fear potentiation of acoustic startle and thus the expression of learned fear.

Figure 6 shows the average acoustic startle response during the noise-alone trials, the noise + light trails, and the average difference between these blocks for both the saline-control and L-NAME groups. The figure illustrates that all groups exhibited similar acoustic startle amplitudes in the noise-alone trails. In the noise + light block the acoustic startle amplitude of the saline-control group increase substantially compared with that of the L-NAME group. Thus, the control group appeared to have a significantly large difference score.

A 2(Blocks: noise-alone and noise + light) X2 (Group: saline and L-NAME) repeated-measures ANOVA with the Blocks factor as the repeated measure yielded a significant main effect of Blocks, $F(1,11)=5.73, p<0.04$, and a significant Block x Group interaction $F(1,11)=4.87, p<0.05$, in the absence of a Group main effect (Appendix E). A simple effects analysis confirmed that the startle responses of the control group were significantly higher on the noise + light presentations compared with the noise-alone trials,

F=11.47, p<0.007, while the acoustic startle amplitudes of the L-NAME group did not differ significantly across these two stimulus blocks, *F*=0.02,p>0.9. Further comparison of the difference scores using a *t*-test for dependent means revealed that the score for the control group was significantly larger than that of the L-NAME group *t*(11)=2.21,p<0.05. Thus, L-NAME microinfusions into the BSC impaired the capacity of animals to recall a light-CS fear association.

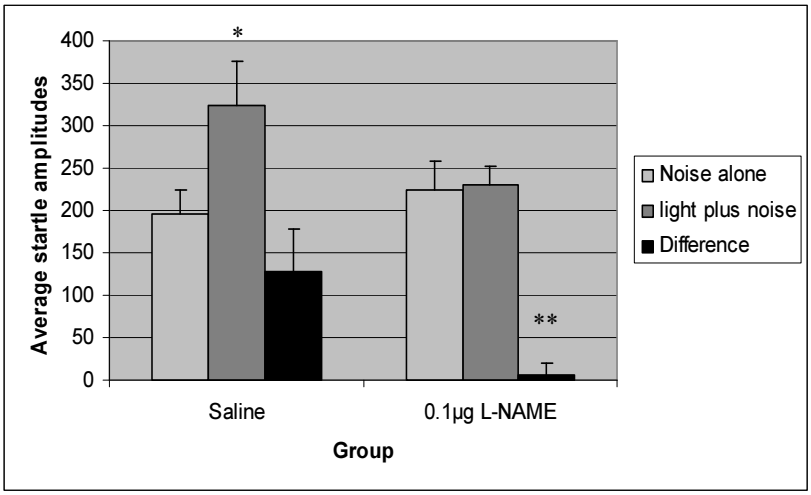

Figure 6. Mean acoustic startle amplitudes (+SEM) after intra-BSC bilateral microinfusions of saline or 0.1µg of L-NAME prior to fear testing. L-NAME group (N = 7), Saline group (N = 6). Animals were fear conditioned 24 hours previously. * p < 0.05 relative to the noise-alone trails, ** p < 0.05 relative to the saline group.

3.4 Shock sensitization experiment:

The first aim of the shock-sensitization experiment was to assess whether NO is required for the shock sensitization of the acoustic startle effect. That is, this experiment was conducted to ascertain whether the animals can express fear when L-NAME is infused into the BSC, thus ensuring that the results from the fear expression experiment can be attributed to a deficit in memory retrieval rather than emotional responding. Furthermore, it would ensure that the results of the fear conditioning experiment can be attributed to a deficit in fear learning rather than the animals not perceiving fear in the presence of the US. More over the procedure employed in this experiment served to address to possible confounds, specifically whether (1) L-NAME microinfused into the BSC modified the animal's baseline acoustic startle responding; or (2) perception of footshock.

3.4.1 Effect of L-NAME infusion on baseline acoustic startle reactivity

The dependent measure in this experiment was the animals' acoustic startle responses during the pre-drug infusion and post-drug infusion blocks of noise bursts. For each animal, the average acoustic startle responses in the pre-drug infusion block were subtracted from the average acoustic startles from the post-drug infusion block to give a difference score (Appendix F). If L-NAME did affect baseline acoustic startle amplitudes then amplitudes in the pre-infusion block would have been substantially different from those observed in the post-infusion block, resulting in either a significantly large or small difference score.

Figure 7 shows the average pre-infusion and post-infusion acoustic startle scores and the average difference between these two blocks for both the control and L-NAME groups. It illustrates that the pre-infusion and post-infusion scores for the L-NAME group were equivalent, resulting in small difference score. On the other hand, the acoustic startle

responses of the control group increased somewhat in the post-infusion block compared with the pre-infusion block, resulting in a moderate difference score.

However, a 2(Blocks: pre-infusion and post-infusion) X2 (Group: saline and L-NAME) repeated-measures ANOVA with the Blocks factor as the repeated measure revealed no significant main effects or interaction effects (Appendix G). Thus, simple effect analysis indicated that the difference between the pre-infusion and post-infusion blocks was not significant for either the control, F=2.46,p>0.1, or L-NAME, F=0.03,p>0.8 group. Also, a t-test for independent means revealed that the control and L-NAME groups differences scores were equivalent, $t(14)$=0.98,p>0.3. Thus, we can conclude that the L-NAME infusions into the amygdala does not alter baseline startle responding.

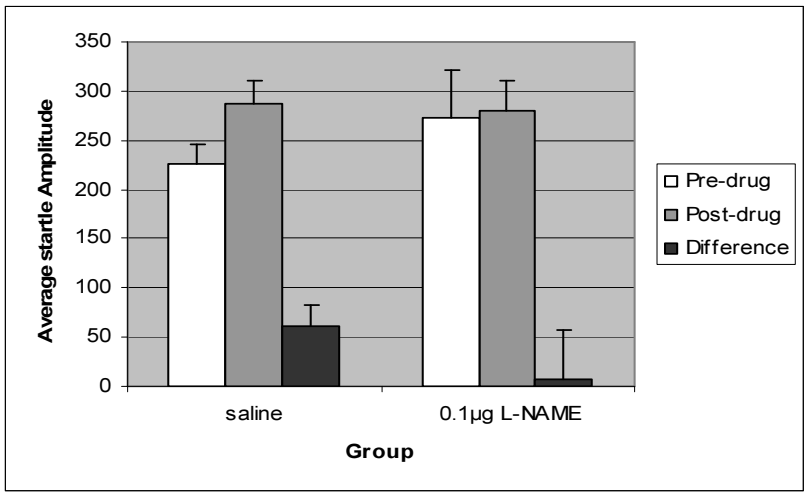

Figure 7. Mean acoustic startle amplitudes (+SEM) as a function of intra-BSC microinfusions of saline (N = 8) or 0.1µg of L-NAME (N = 8) into the BSC.

3.4.2 Effect of L-NAME infusion on shock reactivity

The dependent measure in this experiment was the animals' average movement amplitudes 250-ms pre-shock onset and 250-ms post-shock onset. Average movement amplitudes post-shock onset were subtracted from the average movement amplitudes pre-shock onset to give an average difference in movement reactivity that was indicative of shock perception (Appendix H). Thus, if the animals perceived footshock in a normal manner then the difference score were substantially large.

Figure 8 shows the average movement amplitudes 250-ms pre-shock onset and 250-ms post-shock onset for both the L-NAME and saline groups. The figure shows that both groups exhibited a substantial and equivalent increase in acoustic startle amplitude in the 250-ms post-shock block compared with the 250-ms pre-shock block, resulting in equally large difference scores.

A 2 (Shocks: 250-ms pre-onset and 250-ms post-onset) X2 (Group: saline and L-NAME) repeated-measures ANOVA with Shocks as the repeated factor did, indeed, yield a highly significant main effect of Shocks $F(1,14)= 394.70, p<0.000000$, without any other significant effects (Appendix I). A simple effect analysis indicated that both the saline and L-NAME groups showed a highly significant increase in acoustic startle responses in the 250-ms post-shock-onset block compared with the 250-ms pre-shock-onset block: saline, $F=206.47, p<0.000000$; L-NAME, $F=188.43, p<0.000000$. Further analysis of the difference scores using a *t*-test for independent means indicated that the two groups were responding in a similar manner, $t(14)=0.45, p>0.6$. Thus, both the control and L-NAME group exhibited a substantial and equivalent increase in movement amplitude as a result of shock presentation.

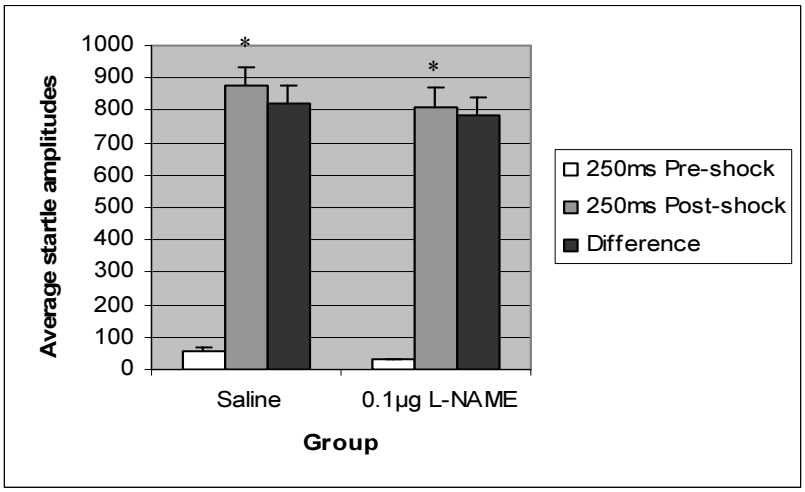

Figure 8. Average movement amplitudes (+ SEM) 250 ms pre- and 250 ms post-shock onset as a function of shock reactivity after intra-BSC microinfusion of saline (N = 8) or 0.1µg of L-NAME (N = 8). * $p < 0.001$ relative to baseline movement amplitudes.

3.4.3 Effect of L-NAME on the shock sensitization of the acoustic startle affect

The dependent measure in this experiment was the animal's average acoustic startle amplitudes in the post-drug / pre-shock block and the post-shock blocks of noise bursts. Average acoustic startle responses from the post-shock block were subtracted from acoustic startle amplitudes from the post-drug block to give a difference score that was indicative of shock enhanced startle responding (Appendix F). If the animals were exhibiting the shock sensitization effect then their difference scores should be substantial.

Figure 9 shows the average post-infusion and post-shock acoustic startle amplitudes and the difference between these two blocks for both the Saline and L-NAME groups. The figure illustrates that the acoustic responses of both the saline and L-NAME groups increased substantially in the post-shock block compared with the post-infusion block. Thus, both groups had considerably large and similar difference scores.

A 2(Blocks: post-infusion, and post-shock) X2 (Group: saline and L-NAME) repeated measures ANOVA with Blocks as the repeated factor confirmed that there was a significant

main effect of Blocks, $F(1,14)=58.97, p<0.000002$, in the absence of any other significant effects (Appendix J). A simple effect analyses revealed that the increase in acoustic startle responding in the post-shock block compared with the post-infusion block was highly significant for both the control group $F=18.02$, $p<0.0009$, and L-NAME group, $F=43.76$, $p<0.00002$. Moreover, a *t*-test for independent means showed that the difference scores for the saline and L-NAME groups were equivalent, $t(14)=-0.09, p>0.9$.

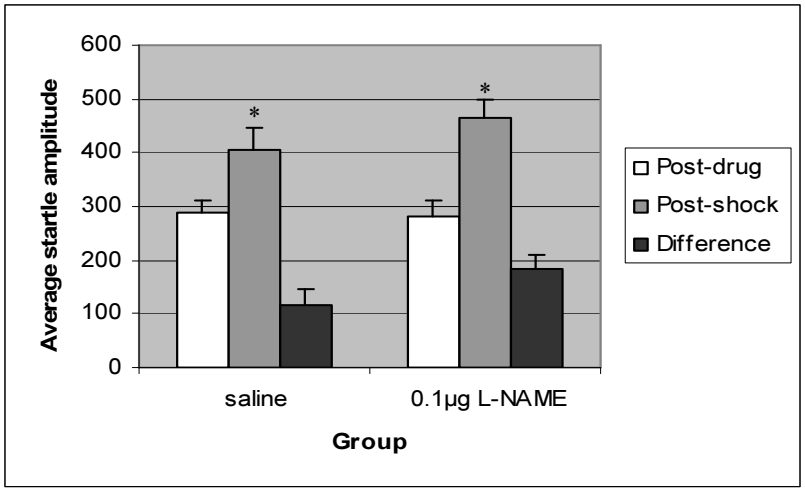

Figure 9. Mean acoustic startle amplitudes (+SEM) post intra-BSC microinfusions of saline (N = 8) or 0.1µg of L-NAME (N = 8), and post-shock. * p <0.001 relative to post-drug startle amplitudes.

4. Discussion

4.1 Main findings

Activation of NMDA receptors in the BSC is required for both the consolidation and expression of conditioned fear. Additionally, these two processes are thought to be mediated by NMDA receptors with different subunits. Those with NR2A subunits are thought to play a more predominant role in neurotransmission, and thus the expression of fear; while those with NR2B subunits are thought to chiefly underpin fear memory consolidation. However, the events downstream of activation of these receptors that subserve either event are not well characterised. Signalling via the gas NO could be a significant mediatory event. The enzyme responsible for the synthesis of NO, nNOS, can associate with either receptor type, and is ultimately activated by the calcium influx through activated NMDA receptors. Most importantly, nNOS is highly concentrated in the lateral component of the BSC, the neuroanatomical area that plays the most crucial role in the molecular underpinnings of conditioned fear. It was, therefore, hypothesised that NO could significantly subserve NMDA receptor associated processes in the BSC.

This current study sought to determine whether NO signalling in the BSC plays a significant role in during the consolidation and expression of conditioned fear, specifically when the CS is visual and FPS is used to examine learned fear expression. The results indicated that firstly, intra-BSC microinfusions of 1.0 or 0.1μg of L-NAME prior to fear conditioning severely impaired the capacity of the animals to condition to a light-CS. Secondly, intra-BSC microinfusions of 0.1 μg of L-NAME, the smallest and most effective dosage used in the fear conditioning experiment, impaired the expression of learned fear. Conversely, intra-BSC microinfusions of 0.1 μg did not effect shock enhanced acoustic

startle responding. Further examination of some possible confounds revealed that L-NAME microinfused into the BSC did not affect baseline acoustic startle responding. Thus, the results from the fear expression experiment cannot be attributed to a sensiomotor deficit induced by the drug. Finally, intra-BSC infusions of L-NAME did not modify the animal's perception of footshock. Therefore, the results from the fear conditioning experiment cannot be attributed to the animals not perceiving shock in a normal manner during the conditioning procedure. Overall, this current study is the first of its kind to illustrate that NO signalling is required for processes associated with conditioned fear, specifically when the CS is visual and fear is assessed using FPS. Below, the significance of these findings with regards to the relevant literature is discussed.

4.2 The fear conditioning study

The results of this study indicated that NO signalling in the BSC is required for the animals to condition to a light CS. Moreover, because the animals were capable of perceiving unconditioned fear, as illustrated by the results of the shock sensitization experiment, the results of this study illustrate that NO signalling in the BSC is required for animals to associate the light-CS with a fear provoking US, rather than a deficit in emotional responding during the conditioning procedure. This finding is consistent with the fact that NO plays a significant role during the acquisition and consolidation of other forms of learning (Bohme, 1993; Calixto, 2001; Cobb, 1995; Holscher, 1996; Meyer, 1998; Samama, 1999; Yamada, 1995; Zou, 1998). In regards to fear conditioning, one other study using a tone-CS and defensive freezing has reported a similar finding (Schafe, 2005). Taken together the results of these studies indicate that NO signalling in the BSC mediates the ability of a CS to acquire the fear-provoking properties of the US.

4.3 The expression of learned fear study

Unlike defensive freezing (Schafe 2005) inhibiting the synthesis of NO impaired the expression of learned fear when examined using FPS. Given that the animals were able to express unconditioned fear when L-NAME was microinfused into the BSC these results can be attributed to a deficit in memory recall rather than emotional responding. Thus, it is reasonable to conclude that in addition to its important role in fear conditioning, NO synthesis is required for the recall of a conditioned fear memory when the CS is visual.

4.4 The shock sensitization study

This experiment revealed that NO signalling is not required for the expression of unconditioned fear. Because NO is synthesised as a result of NMDA receptor activation, this study supports the recent finding that the NMDA receptor antagonist AP5 does not effect the shock sensitization of the acoustic startle effect (Van Nobelen 2006). Furthermore, as discussed above, the results of this study indicate that the animals were able to express unconditioned fear when NO synthesis was inhibited in the BSC, thus the results of the fear conditioning and fear expression experiments can be attributed to a deficit in memory associated processes rather than emotional responding. Taken together these studies indicate that NO synthesis is required for processes specifically associated with conditioned fear rather than unconditioned fear.

4.5 Limitations

This research would have been improved if it had demonstrated the animals from the fear conditioning experiment were in fact capable of learning. In the same manner, it would have been advantageous to show that the animals from the fear expression experiment were able to express conditioned fear. This would illustrate that the mechanical damage caused by

cannulae implantation did not result in the behavioural deficits. One way to address these issues is by counteracting the effect of L-NAME by microinfusing the nNOS agonist L-arginine into the BSA simultaneously. This is a common manipulation used by past researchers to ascertain whether the NO signalling pathway was indeed manipulated during their experimental procedures. Thus, this precautionary measure would ensure that the animals had the capacity to learn or express fear, but would also demonstrate that the NO signalling pathway was, undoubtedly, manipulated in this experiment. However, given that the control animals were capable of conditioning or expressing conditioned fear, and that L-NAME microinfusion into the BSC only affected behaviours that are known to require NMDA receptor activation, specifically fear conditioning and expression; it seems plausible that the NO signalling pathway was examined in this current study and it was, indeed, its manipulation that resulted in the memory impairments. Nonetheless, to be certain that this is in fact the case, this additional measure could be included in subsequent experiments.

4.6 Future research and perspectives

Future studies will need to examine precisely how NO signalling contributes to the formation of a fear memory. More specifically, pre-conditioning drug infusions, as was employed in this study, effect both the acquisition and consolidation of learned fear. Post conditioning drug infusions, on the other hand, specifically no more than six hours after the procedure, target processes that occur during the consolidation phase of memory formation. More specifically, over the hours post fear conditioned the cell signalling cascades that eventuate in memory consolidation are in progress (Rodrigues 2004). Thus, it may be possible to determine whether NO signalling is indeed required for the consolidation of learned fear by infusing a nNOS antagonist into the BSC at successive time points after the conditioning procedure. Thus, if nNOS activation over the minutes or hours after

conditioning significantly contributes to memory consolidation this manipulation will reveal its contribution and the time period of its involvement. However, if the synthesis of NO is transient specifically occurring during the conditioning procedure, whereby NO works upstream of many of the cell signalling cascades that lead to memory consolidation by initiating such events, post-conditioning infusions of a nNOS antagonist may not have an effect. Nonetheless, future research of this manner would undoubtedly contribute to understanding precisely how NO subserves memory formation.

The results of the fear expression experiment, however, do allude to the possibility that NO signalling simply contributes to routine neurotransmission in a manner that could account for the impaired fear conditioning. Indeed, in other brain areas, NO is able to increase cellular excitation by attenuating GABAergic inhibition, and augmenting the extracellular concentration of the catecholamines and glutamate (Fleckenstein, 1997; Guevara-Guzman, 1994; Kaye, 2000; Kiss, 2000; Lander, 1995; Lonart, 1993, 1992; Lu, 1999; Meffert, 1996; Meffert, 1994; Ohkuma, 2001; Prast, 2001; Robello, 1996; Segovia, 1994; Wall, 2003; Wexler, 1998; Yun, 1998; Zarri, 1994). NO's ability to modulate these neurochemicals may contribute to fear memory retrieval. Therefore, perhaps NO signalling simply subserves a general increase in excitatory tone in the amygdala that is crucial for the formation, or more specifically the acquisition, of a fear memory, without affecting the cellular machinery specifically required for memory consolidation. Thus, in addition to the post-conditioning drug infusions discussed above, future research could include examining whether NO was able to activate the cellular components required for fear memory consolidation.

Fear memory consolidation requires *de novo* protein synthesis, an event that requires the activation of the ERK signalling pathway and CREB (Bailey, 1999; Josselyn, 2001;

Schafe, 2000, 1999). Research concerning brain areas outside of the amygdala has revealed that NO signalling plays a key role activating both these cellular components (Lander, 1995; Lu, 1999; Yun, 1998). More specifically, NO is able to activate Ras, which, in turn, activates the ERK transduction pathway. Interestingly, Ras in located at both the pre and postsynaptic membrane, and ERK signalling in both the pre and postsynaptic cell is required for memory consolidation. Thus, given NOS ability to act as a retrograde transmitter it seems probable that NO can initiate ERK signalling in both cells as a result of fear conditioning. Furthermore, NO is known to facilitate CREB activation in hippocampal cells via the NO-sGC-cGMP-PKG pathway. Perhaps NO worked in a similar manner in the BSC. Therefore, future research will need to examine whether NO did, in fact, activate, or at least contribute to the activation, of ERK and CREB. Such research findings will also help to determine the precise role played by nitric oxide in the formation of a fear memory, and would consequently contribute to understanding the role played by the NMDA receptor in conditioned fear.

Given that NO signalling is a consequence of NMDA receptor activation, the findings of this study support research that has demonstrated that NMDA receptor is required for both fear conditioning and the expression of conditioned fear, probably mediated by receptors with NR2B and NR2A subunits respectively (Blair, 2001; Fendt, 2001; Lee, 2001; Lindquist, 2004; Maren, 1996; Rodrigues, 2002) Therefore, the results of his study indicate that NMDA receptor activation is a crucial requirement of fear memory expression. However, NMDA receptor activation during the expression of conditioned fear does not appear to simply subserve routine neurotramission; it also required for the animal to learn new information about the CS. For example, activation of the NMDA receptor in the BSC is required for fear memory extinction and second order fear conditioning (Davis, 2002; Gewirtz, 1997; Lee,

1998). Fear extinction occurs when the animal is repetitively exposed to the CS free of the US. As a result, the animal relearns not to fear the CS. Second order fear conditioning, on the other hand, occurs when a fear conditioned animal is presented with the CS paired with another CS (CS2). As a result the animal learns to fear CS2 because it associates it with the CS previously paired with the US. The interesting point here is that CS2 essentially becomes associated with the US even though it was never explicitly paired with the noxious stimulus. Future research concerning the role of NO signalling in the BSC could examine whether the gas is an important intermediate step in the formation of these additional NMDA dependent memories. However, findings from such research would still raise the question of whether NO mediates its effects by subserving routine neurotransmission or memory consolidation. Given, the manner in which NO acts in brain areas outside of the amygdala, and the fact that it can associate with NMDA receptors with either NR2A or NR2B subunits, it is possible that it mediates both processes concurrently.

Nitric oxide signalling in the amygdala could link fear memory recall and memory consolidation together. More specifically, during the initial formation of a fear memory, NO synthesis could significantly, and simultaneously, contribute to fear conditioning in two ways. Firstly, it could enhance cellular excitation facilitating excitatory neurotransmission, ensuring that the BSC is adequately excited to a level required for memory consolidation. Secondly, it could activate, or at least significantly prime, the cellular machinery required for memory consolation. Later, NO synthesis during the expression of conditioned fear could also serve two simultaneous mechanisms. Firstly, it could significantly facilitate cellular excitation thus insuring that the fear memory is easily recalled. Secondly, it could modulate processes required for memory consolidation thus facilitating new learning. Consequently, additional or new information about the recalled memory could be easily acquired, as is seen

during fear extinction and second order fear conditioning. Thus, because memory about an event or stimulus is subject to change, with new information being added to the existing memory with experience, it would seem reasonable to conclude that recall of a previously consolidated fear memory would concurrently prime new learning about the fearful event. Both process could be subserve by NO signalling, thus eliminating unnecessary signalling redundancy during two processes that are so closely related. The above proposition could still be consistent with the suggestion that NMDA receptors with NR2A and NR2B subunits predominantly mediate neurotransmission associated with conditioned fear and fear memory consolidation respectively. Other events downstream of activation of either receptor, or the characteristics of receptor activation, could subserve this processing distinction. Though, if NO did act in this manner there would be some degree in overlap in the functioning of each receptor. This, however, could help to explain why NMDA receptor activation, a process that is so intimately tied with memory formation, is also required for the recall of a fear memory. As mentioned above, however, future research concerning the precise consequences of NO signalling in the BSC is required in order to ascertain whether this is indeed how NO acts in this complex.

4.7 General conclusions

The BSC of the amygdala is a key structure involved in fear conditioning. The NMDA receptor within this complex has been implicated in both the consolidation and expression of conditioned fear. This study has demonstrated that the gas NO is a crucial intermediate step in between glutamatergic mediated activation of the receptor and both these processes. That is, this study is the first of its kind to illustrate the importance of NO signalling during processes associated with conditioned fear. It therefore makes a new and important contribution to the research area concerning the molecular underpinnings of

learned fear in the amygdala. Although this study did not demonstrate how NO mediates its effects it nonetheless highlights a number of areas for future research.

4.7.1 Clinical implications

The finding that NO signalling contributes significantly to both the consolidation and expression of conditioned fear may help to clarify the molecular underpinning of psychological disorders associated with exaggerated fear and anxiety. Indeed, research concerning the fear related disorders schizophrenia and chronic stress has reported that NO signalling may be involved (Krukoff, 1997; Salum, 2006; Yao, 2004). In the case of schizophrenia, this is appropriate when considering that the NMDA receptor has been implicated in this disorder (Coyle, 2004). Thus, this research may illustrate a component of how NO may play a role in such fear associated disturbances. Overall, the current study provides new details concerning the molecular bases of fear processing and illustrates the significant role played by NO in mediating amygdaloid activity associated with conditioned fear.

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Appendix A

Individual fear potentiated acoustic startle scores for animals in the fear conditioning experiment. The table shows individual average acoustic startle scores, for the noise-alone trials, noise + light trials, and the difference between these two blocks for all three groups. Group means are depicted in bold.

Saline group		
5 noise-alone	**5 light-plus-noise**	**Difference**
118.4	422.4	304
227.2	250.8	23.6
267.2	610.8	343.6
95.4	242.2	146.8
143.8	384.6	240.8
255.8	632.2	376.4
170	156.6	-13.4
182.54	**385.66**	**251.66**

1.0 µg of L-NAME group		
5 noise-alone	**5 light-plus-noise**	**Difference**
398	537.2	139.2
234	117.8	-116.2
118.4	193.2	74.8
167.2	41.4	-125.8
163.6	314.4	150.8
156.2	275.6	119.4
120.4	142	21.6
193.97	**231.66**	**37.68**

Appendix A continued

5 noise-alone	0.1 µg of L-NAME group 5 light-plus-noise	Difference
127.8	210.2	82.4
226.2	274.6	48.4
137.4	225.6	88.2
234.2	359	124.8
437.4	219	-218.4
454.6	403.2	-51.4
251.66	**278.31**	**26.66**

Appendix B

2X3 repeated-measures Analysis of variance for the fear conditioning experiment
Blocks (average noise alone and noise + light acoustic startle amplitudes) X Group (saline, 1.0, 0.1µg of L-NAME).

Source of Variation	Sum of Squares	DF	Mean Square	F	PROB
Drug	38121	2	19061	0.7756	0.475204
Error	4423	18	24574		
Blocks	83456	1	83456	9.5079	0.006407
Blocks X Drug	68396	2	384198	3.8961	0.039270
Error	157995	18	8778		

Appendix C

One-way Analysis of Variance for the fear conditioning experiment
Difference scores (average noise-alone acoustic startle amplitudes subtracted from the average noise + light acoustic startle amplitudes) against Group (saline, 1.0, 0.1µg of L-NAME)

Source of Variation	Sum of Squares	DF	Mean Square	F	PROB
Group	136.792.5	2	68396.3	3.896101	0.039270
Error	3159909.9	18	17555.1		

Appendix D

Individual FPS scores for animals in the fear expression experiment. The table shows individual average acoustic startle scores, for the noise-alone trials, noise + light trials, and the difference between these two blocks for both groups. Group means are depicted in bold.

Saline group		
5 noise-alone	**5 light-plus-noise**	**Difference**
259	414.8	155.8
116.4	228.6	112.2
230.8	501.6	270.8
261.2	178.4	-82.8
77.2	207.2	130
241.6	262.2	20.6
190.6	480.6	290
196.68	**324.77**	**128.09**

L-NAME group		
5 noise-alone	**5 light-plus-noise**	**Difference**
215.4	227.2	11.8
299.8	273.8	-26
108.2	130.2	22
249.6	267.4	17.8
321	273.4	-47.6
153.4	206.6	53.2
224.57	**229.77**	**5.2**

Appendix E

2X2 repeated-measures analysis of variance for the fear expression experiment
Blocks (average noise-alone and noise + light acoustic startle amplitudes) X Group (Saline and L-NAME)

Source of Variation	Sum of Squares	DF	Mean Square	F	PROB
Group	7278	1	7278	0.5762	0.463773
Error	138954	11	12632		
Blocks	28697	1	28697	5.7299	0.035626
Blocks X Drug	24394	1	24394	4.8706	0.049482
Error	55092	11	5008		

Appendix F

Individual acoustic startle amplitudes for animals in the shock sensitization of the acoustic startle experiment. The table shows individual average acoustic startle scores, pre-drug, post-drug/pre-shock, post shock, and the difference between the pre-drug and post-drug blocks (difference), and the difference between post-drug/pre-shock and post-shock blocks (sensitization) for both groups. Group means are depicted in bold.

Saline group				
Pre-drug	**Post-drug**	**Difference**	**Post-shock**	**Sensitization**
218.6	223.6	5	285.9	62.3
206.8	322.2	115.4	486.6	164.4
218.7	298.5	79.8	367	68.5
238.2	400	161.8	559.2	159.2
213.1	295.6	82.5	460.8	165.2
224.5	261.9	37.4	210.1	-51.8
152.9	196.6	43.7	402.2	205.6
341.6	305.8	-35.8	472.2	166.4
226.8	**288.03**	**62.25**	**405.5**	**117.48**

L-NAME group				
Pre-drug	**Post-drug**	**Difference**	**Post-shock**	**Sensitization**
277.2	290.5	13.3	503.8	213.3
141.9	319	177.1	425.3	106.3
165.4	123.8	-41.6	320.3	196.5
234.6	235.7	1.1	394.8	158.8
156.3	360.8	204.5	581.1	220.3
276.5	251.6	-24.9	570.9	319.3
529.9	269.6	-260.3	371.4	101.8
406.8	393.8	-13	542.3	148.5
273.58	**280.6**	**7.03**	**405.5**	**183.1**

Appendix G

2X2 repeated-measures Analysis of variance for the shock sensitization experiment. Blocks (average acoustic startle amplitudes pre-drug and post-drug) X Group (saline and L-NAME)

Source of Variation	Sum of Squares	DF	Mean Square	F	PROB
Drug	3097	1	3097	0.3147	0.583687
Error	137775	14	9841		
Blocks	9316	1	9316	1.5295	0.236526
Blocks X Drug	5875	1	5875	0.9646	0.342695
Error	85271	14	6091		

Appendix H

Individual movement amplitudes during assessment of shock reactivity for animals from the shock sensitization experiment. The table shows individual average movement amplitude 250ms pre shock onset, 250ms post shock onset, and the difference between these two blocks (shock reactivity) for both groups. Group means are depicted in bold.

Saline group		
250ms pre shock onset	**250ms post shock onset**	**Shock reactivity**
25.1	784.4	759.3
55.4	860.9	805.5
25.6	951.6	926
30.7	973.6	942.9
120.7	628.7	508
95.1	1141.8	1046.7
40.6	769.1	755.5
59.6	865.7	806.1
56.6	**875.35**	**818.75**

L-NAME group		
250ms pre shock onset	**250ms post shock onset**	**Shock reactivity**
26.1	916	889.9
32.7	827.5	794.8
22.4	571.5	549.1
19.6	1067.1	1047.5
39.2	683.4	644.2
36.2	932	895.8
34.2	794.9	760.7
22.1	697.4	675.3
29.06	**811.23**	**782.17**

Appendix I

2X2 repeated-measures analysis of variance for the shock sensitization experiment. Blocks (movement amplitude 250ms pre shock-onset and 250ms post shock onset) X Group (Saline and L-NAME)

Source of Variation	Sum of Squares	DF	Mean Square	F	PROB
Drug	16804	1	16804	1.4042	0.255745
Error	167536	14	11967		
Blocks	5125842	1	5125842	394.6972	0.000000
Blocks X Drug	2677	1	2677	0.2062	0.656757
Error	181815	14	12987		

Appendix J

2X2 repeated-measures analysis of variance for the shock sensitization experiment. Blocks (average movement amplitudes post-drug/pre-shock and post-shock) X Group (Saline and L-NAME)

Source of Variation	Sum of Squares	DF	Mean Square	F	PROB
Drug	5156	1	5156	0.3741	0.550578
Error	192958	14	13783		
Blocks	180691	1	180691	58.9676	0.000002
Blocks X Group	8613	1	8613	2.8109	0.115803
Error	42899	14	3064		