The Nucleus Accumbens: Involvement in the modulation of memory for arousing events.

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Abstract

Some of the key factors that contribute to many memory disorders involve either a reduced or exaggerated capacity to experience arousal. These outcomes are related to impaired neurotransmitter release in brain areas that process memory such as the amygdala and hippocampus. Although there are multiple pathways that emotionally arousing information may be consolidated into memory, very little attention has been devoted to identifying how norepinephrine release in the nucleus accumbens contributes to this process. Of particular interest to this project is the shell region of the accumbens because it receives noradrenergic terminals exclusively from neurons in the nucleus of the solitary tract (NTS) that convey information regarding increased peripheral autonomic and neuroendocrine activity in response to emotionally arousing events. Noradrenergic input from the NTS may contribute to enhanced memory for emotional events by increasing responsiveness of accumbens neurons to the constellation of inputs transmitted to this area from the amygdala and the hippocampus. A major theme of the proposed studies is that the multitude of inputs that converge upon the accumbens during learning, places this structure in an ideal position to integrate and bind information regarding the individual features of learned events into memory storage. Thus, studies discussed in the dissertation were developed to examine specific elements of this central hypothesis.

The dissertation consists of five main chapters. The first presents background literature representing the foundation and rationale for conducting

the proposed studies. **Chapter 2** examines whether a functional relationship exists between noradrenergic A2 neurons and the nucleus accumbens shell in processing memory for emotional events. Findings from this chapter are the first to identify the specific noradrenergic receptor subtype in the nucleus accumbens shell that mediates the beneficial actions on memory produced by chemical stimulation of NTS neurons.

Experiments in **Chapter 3** were integral in demonstrating that accumbens shell neurons play a fundamental role in consolidating converging information initially processed by the amygdala and hippocampus. These experiments also reveal that separate limbic structures provide a unique contribution in creating different representations in memory of an emotionally arousing event. **Chapter 4** further investigates the integrative role of the accumbens in processing limbic information. These studies addressed a possible mechanism by which glutamate released from amygdala or hippocampal inputs may facilitate noradrenergic neurotransmission in the accumbens shell to modulate memory storage.

Collectively, these findings indicate that, along with the amygdala and hippocampus, the shell division of the nucleus accumbens provides a critical contribution in integrating and consolidating information following learning. More importantly, the data presents a mechanism by which information emanating from these key limbic structures interacts with noradrenergic signals from the NTS. Implications and significance of these studies are discussed in **Chapter 5**.

Chapter 1: Introduction

Emotionally arousing experiences have a unique feature of creating vivid and detailed memories for several facets of events that have transpired. The indelible representations created by emotional events are attributed to the changes these experiences initiate on peripheral hormonal secretion and the central release of specific neurotransmitters in limbic brain regions. Traditionally, these areas have included the amygdala and hippocampus, both of which have well established roles in encoding and processing memory for emotional events (Diamond, et al., 2005; Hernandez-Rabaza et al., 2008; Joels, et al., 2004; Laurent & Westbrook, 2008; McGaugh 2004; Richter-Levin & Akirav, 2003; Vianna, et al., 2004). Recent findings suggest that the shell division of the nucleus accumbens may play an equally important role in integrating and consolidating representations of new experiences following emotionally arousing events (Kerfoot, Chattillion & Williams, 2008). This view derives from anatomical findings demonstrating that the accumbens shell receives neural input from the basolateral nucleus of the amygdala concerning the affective components of experiences (French & Totterdell, 2003; Mogenson, et al., 1980; Petrovich, et al., 1996), projections from the ventral subiculum region of the hippocampus regarding contextual features from the environment (French & Totterdell, 2003; Groenewegen, et al., 1987; Meredith, et al., 1990) and reward related

components of learning experiences from the ventral tegmental area (Ikemoto, 2007; Nauta, et al., 1978).

Although a diverse number of anatomical inputs convey separate aspects of emotional experiences directly to the shell division of the accumbens, very little attention has been devoted to understanding which neuromodulators regulate accumbens activity during the memory encoding and storage process. Since the interactions between separate neurotransmitter systems in this region of the accumbens has not been fully investigated, the contribution of the accumbens in integrating affective, contextual and emotional features of new events into memory storage remains to be discovered. The following sections provide a brief overview of the known involvement of the nucleus accumbens in behavior and memory, the anatomical organization of the accumbens and evidence suggesting that anatomical inputs from the brainstem to the shell play a significant role in modulating neuronal activity in the accumbens during memory formation.

Anatomical Organization of the Nucleus Accumbens

The accumbens is a recipient of highly processed information regarding decision-making (prefrontal cortex; French & Totterdell, 2002; Sesack & Pickel, 1992), affective components of experiences (basolateral nucleus of the amygdala; French & Totterdell 2003; Mogenson, et al., 1980; Petrovich, et al., 1996), contextual features from the environment (ventral subiculum; French & Totterdell, 2003; Groenewegen, et al., 1987; Meredith, et al., 1990) and hedonics or reward (ventral tegmental area; Ikemoto, 2007; Nauta, et al., 1978). In addition

to receiving vital limbic input, the shell region of the accumbens also receives a dense supply of noradrenergic terminals exclusively from brainstem neurons in the A2 region of the NTS (Delfs, et al., 1998; Figure 1). These cells play a pivotal role in memory formation by receiving input from the periphery regarding elevations in secretion of the stress related hormone epinephrine and heightened physiological states in response to emotionally arousing events. The A2 neurons also convey this information to brainstem and limbic regions that are involved in the memory storage process (Ricardo & Koh, 1978; van Bockstaele, Bajic, Proudfit & Valentino, 2001).

It is interesting to note that projections from the basolateral amygdala and hippocampus converge monosynaptically on projection neurons within the caudomedial region of the accumbens shell (French & Totterdell, 2003). Of equal importance is the finding that the caudomedial division of the shell is also heavily innervated by noradrenergic terminals from the NTS (Delfs, et al. 1998). *This arrangement of inputs provides the foundation by which neural input from the NTS regarding physiological arousal may amplify or modulate the encoding of emotional and contextual information into memory in the accumbens shell.*

Efferent fibers in the accumbens shell contact several structures. These structures include areas in the basal forebrain, diencephalon, brainstem, ventral pallidal areas, hypothalamus and the ventral tegmental area (Groenewegen & Russchen., 1984; Groenewegen, Wright & Beijer, 1996; Heimer, Zahm, Churchill, Kalivas & Wohltmann, 1991; Mogenson, Swanson & Wu, 1983; Nauta, et al., 1978). Most of the target structures of accumbens shell efferents also send

reciprocal projections to the accumbens. Of particular interest is the projection from the extended amygdala/accumbens shell complex to the NTS as well as the dorsal motor nucleus of the vagus (Zahm, 2000). The reciprocal connection between the NTS and accumbens may serve an important feedback loop to transmit continually updated information regarding heightened levels of peripheral arousal during the extended process of memory consolidation. In addition, the accumbens is involved in a thalamocortical-basal ganglia loop via projections through the ventral pallidum and mediodorsal thalamus that innervate the prefrontal cortex and return to the accumbens (Groenewegen, Wright & Beijer, 1996).

Electrophysiological Characteristics of Accumbens Neurons

The afferent organization of inputs into the accumbens and the membrane potential properties of its neurons provide some insight into the integrative role this structure may play in memory. Because the accumbens receives converging inputs from multiple areas, it is important to understand how the separate inputs may regulate neuronal firing in this structure. Output neurons in the accumbens that project primarily to the ventromedial aspect of the ventral pallidum have very low resting membrane potentials (approximately -81mv). In addition, the membrane potential of these neurons are considered bistable suggesting that they fluctuate between a very negative resting potential (“down state”) and a slightly depolarized, less negative potential (“up state”; approximately -63mv; O’Donnell & Grace, 1995; 1998; O’Donnell, Greene, Pabello, Lewis & Grace,

1999; Yim & Mogenson, 1989). Whereas the very negative resting membrane potential is maintained by an inward rectifier potassium conductance displayed in accumbens output neurons (Wilson & Kawaguchi, 1996), the less negative “up state”, requires synaptic activation to depolarize the membrane potential enough to allow additional synaptic inputs to fire action potentials. In fact, several studies report that accumbens neurons require activation from more than one source to reach firing threshold (Callaway Hakan, & Henriksen, 1991; DeFrance, Marchand, Sikes, Chronister & Hubbard, 1985). This constraint on activity may explain why accumbens neurons receive inputs from several memory related areas in addition to the NTS.

An emerging idea in the literature dealing with the functionality of the nucleus accumbens is the hypothesis of neural networks. The traditional idea of one or multiple inputs influencing a single accumbens neuron to have either an excitatory or inhibitory outcome may not be the most accurate description of how accumbens neurons function. Pennartz and colleagues (1994) posit that the accumbens is comprised of neuronal ensembles. These ensembles are best understood in terms of their collective influence on target areas rather than the outcome of a single neuron. This is a critical point when trying to decode the consequences of activating multiple afferent inputs to accumbens neurons. For example, paired-pulse stimulation of the basolateral amygdala and hippocampus results in very different response outcomes in the accumbens depending on the sequence in which these areas are stimulated. Studies show that under some conditions, stimulation of the basolateral amygdala followed 25-50ms by

fornix/fimbria (hippocampus) stimulation increases the probability that an accumbens neuron fires an action potential. However, stimulation of the fimbria/fornix followed 25-75ms by basolateral amygdala stimulation results in a decreased probability of firing an action potential (Mulder, Hodenpijl & Lopes da Silva, 1998). It could be that the differential outcome in accumbens firing reflects how target areas specify which inputs from the accumbens are conveying affective or contextual information.

Since more than one source of input is required for neurons in the accumbens to reach threshold and fire an action potential, the type and intensity of signals conveyed by the NTS may also play a role in the propagation of information through this structure. Findings from electrophysiological studies reveal that neurons in the accumbens shell exhibit excitation following peripheral stimulation of ascending fibers of the vagus nerve (Mehendale, Xie, Aung, Guan & Yuan, 2004) or the infusion of glutamate onto neurons in the NTS that synapse within the accumbens shell (Kirouac & Ciriello, 1997). However, no studies have directly examined the significance of this input in biasing the responsiveness of the accumbens during the processing of affective and contextual features of new experiences conveyed by inputs originating in the hippocampus and amygdala. Thus, activation of NTS neurons following peripheral arousal might allow both the basolateral amygdala and hippocampus to have strong synaptic influences that would then lead to robust encoding within the accumbens shell during emotionally arousing events.

Involvement of the Nucleus Accumbens in Spatial Memory

The accumbens receives direct projections from the hippocampus (French & Totterdell, 2003; Groenewegen, et al., 1987; Meredith, et al., 1990) and these inputs may contribute to the capacity of the accumbens to process declarative forms of information into memory. Early studies showed that posttraining functional inactivation of the nucleus accumbens impairs spatial memory as evidenced by more entries into arms without a food reward in a spatially cued radial arm maze task. However, accumbens inactivation is ineffective in determining the outcome on tasks that do not require the hippocampus such as a radial arm maze task that utilizes conditioned visual cues to signal food baited arms (Seamans & Phillips, 1994).

Interestingly, hippocampal innervation is differentially segregated within the accumbens in that the shell region receives direct projections from the ventral subiculum and the core region receives projections from the dorsal hippocampus (Groenewgen, et al., 1987). This becomes an important distinction given that dorsal and ventral hippocampal areas are differentially involved in memory processing. Previous studies demonstrated that lesions of the ventral hippocampus impair contextual memory for fear conditioning involving a heightened state of emotion, whereas dorsal lesions impair memory for spatial learning in the Morris water maze task (Burhans & Gabriel, 2007; Richmond, et al., 1999). Given that the ventral hippocampus projects to the accumbens shell, it is not surprising that animals with lesions to the accumbens shell showed reduced freezing to the context in which an aversive footshock was administered

(Jongen-Relo, Kaufmann & Feldon, 2003). Together these studies provide evidence for a functional relationship between the ventral hippocampus and the accumbens shell in processing contextual information. Disruption of either the ventral hippocampus (which innervates the shell) or the accumbens shell, results in impaired contextual encoding.

Glutamate plays an important role in modulating accumbens neurons during learning in spatial environments. Findings show that posttraining intra-accumbens injection of NMDA or AMPA receptor antagonists impair reference memory performance following radial maze training, whereas only AMPA antagonists disrupt working memory during this form of spatial learning (Klein, Hadamitky, Koch & Schwabe, 2004). These experiments, along with more recent findings showing an involvement of the accumbens in allocentric (in relation to context/space) and egocentric (in relation to self) spatial memory, implicate the accumbens as a central site for spatial/contextual memory consolidation (De Leonibus, Oliverio & Mele, 2005).

Relationship between the Nucleus Accumbens and Amygdala

Information conveyed from the basolateral amygdala to the accumbens shell plays a crucial role in learning and storing into memory the motivational valence of stimuli. Rats with a unilateral lesion in the basolateral nucleus and a contralateral accumbens lesion, fail to acquire second-order conditioned responses. Results showed that although animals learn that a light signals the availability of food, they fail to learn that presenting a tone before the

presentation of the light also signals the availability of food (Setlow, et al., 2002). This means that after several second-order conditioning trials (tone preceding the light), animals with interrupted connections between the basolateral amygdala and accumbens fail to approach the food cup during presentation of the tone. These findings suggest that both structures are important for an organism to flexibly learn that multiple cues may predict the availability of a food reward.

Other findings show an involvement of the accumbens in processing information regarding aversive outcomes. Rats with accumbens lesions are capable of responding to cues previously paired with positive consequences very rapidly, but unlike sham lesioned animals, fail to modify response latencies or reaction time to cues that lead to aversive outcomes, such as the delivery of quinine in the place of an anticipated liquid sucrose reward (Schoenbaum & Setlow, 2003). The consolidation of memory for these and other types of emotionally arousing events is a time dependent process that does not happen instantly, but rather occurs over hours. Studies employing functional inactivation techniques to produce reversible lesions demonstrate that neuronal activity in the accumbens is crucial for consolidation for at least 90 minutes following learning. For example, retention of a footshock given during inhibitory avoidance learning is significantly disrupted even when infusions of tetrodotoxin into the accumbens are delayed until 1.5 hours following training (Lorenzini, Baldi, Bucherelli & Tassoni, 1995).

Evidence from these studies suggests the accumbens may integrate information from both the amygdala and hippocampus. The importance of the

accumbens as a site of convergence of inputs that are initially processed in the amygdala and hippocampus is revealed by findings from behavioral studies. For example, pretraining inactivation of the nucleus accumbens blocks acquisition of contextual fear conditioning, a task which requires both spatial and affective input (Haralambous & Westbrook, 1999). This finding indicates that the accumbens could be a site of integration of information and suggests an involvement of the accumbens in consolidating affective, spatial and contextual aspects of the environment during new learning.

Significance of Brainstem Input for Memory Processing

A complete understanding of the mechanisms that modulate accumbens activity during memory processing is far from complete. This shortcoming is due to the lack of available evidence demonstrating how information conveyed by brainstem neurons representing changes associated with heightened states of arousal in the periphery may influence accumbens functioning during memory formation. It is well established that the improvement in memory for emotionally arousing experiences is mediated in part by the initial secretion of epinephrine from the adrenals and the subsequent impact this hormone has in potentiating norepinephrine output in the hippocampus and amygdala (Miyashita & Williams, 2004; Williams, Men, Clayton & Gold, 1998). Epinephrine does not traverse the blood brain barrier to directly affect brain limbic structures, but activates receptors along the vagus nerve (Lawrence, Watkins & Jarrott, 1995) that in turn release glutamate on A2 neurons in the NTS (Allchin, Batten, McWlliams &

Vaughn, 1994; Sumal, Blessing, Joh, Reis & Pickel, 1983). The A2 norepinephrine containing neurons of the NTS project a dense supply of noradrenergic terminals to neurons in the shell division of the accumbens (Delfs, et al., 1998). More recently, findings revealed that increasing discharge along ascending fibers of the vagus nerve with electrical stimulation potentiates neuronal firing in the accumbens shell (Mehendale, et al., 2004). In addition, similar changes in the firing properties of accumbens neurons are observed following either electrical or glutamatergic activation of neurons in the NTS (Kirouac & Ciriello, 1997). Glutamatergic activation of A2 neurons has recently been shown to enhance memory for an arousing footshock experience (Kerfoot, Chattillion & Williams, 2008). This effect, however, is contingent upon accumbens cell functioning.

Relationship between Accumbens Norepinephrine and Brainstem Input

A number of early immunofluorescence and immunohistochemical experiments reported the presence of norepinephrine in the accumbens. However, these studies were conducted before an anatomical distinction was developed between the shell and core regions (Gaspar, Berger, Alvarez, Vigny & Henry, 1985; Lindvall & Stenevi, 1978; Swanson & Hartman, 1975). Recent investigations that delineated between core and shell found dopamine-β-hydroxylase immunoreactive fibers in the caudal shell region and very few, if any, in the core region (Berridge, Stratford, Foote & Kelley, 1997). Although the locus coeruleus and NTS project to the accumbens shell (Brog Salypongse, Deutch &

Zahm, 1993), only projections from NTS neurons contain norepinephrine (Delfs, et al., 1998). Additional studies suggest that noradrenergic innervation of the accumbens shell derives from the NTS and not the locus coeruleus (LC). For example, a recent study by McKittrick and Abercrombie (2007) demonstrated a dose of d-amphetamine, in a range that influences cognitive processing (i.e. 2.0 mg/kg ip), potentiates norepinephrine release in the accumbens for a period exceeding 3 hours. This is comparable to the time period norepinephrine release has been measured in either the amygdala or hippocampus following NTS activation or systemic administration of compounds that are released by amphetamine administration (Hassert, Miyashita & Williams, 2004; Miyashita & Williams, 2003; 2004). Furthermore, Holdefer and Jensen (1987) demonstrated that systemic injection of amphetamine does not increase discharge of LC neurons, but actually suppresses LC activity. Given findings showing d-amphetamine potentiates norepinephrine output in the accumbens combined with the finding showing that d-amphetamine suppresses LC activity (Holdefer & Jensen, 1987), it is apparent that treatments that affect physiological arousal, must therefore increase norepinephrine output in the accumbens shell through the only other noradrenergic pathway innervating this structure, the NTS.

Recent evidence demonstrates that glutamatergic activation of NTS neurons enhance memory for emotionally arousing events and also increase extracellular release of norepinephrine in the amygdala (Miyashita & Williams, 2002). Noradrenergic projections from the NTS send peripheral information to limbic structures regarding autonomic changes in the periphery in response to

emotional stimuli. Thus, it is possible that the beneficial effects on memory produced by activating NTS neurons following emotional learning experiences may be mediated not only by activation of noradrenergic receptors in the amygdala, but by producing similar excitatory actions in the accumbens shell as well. Therefore, it could be that peripheral inputs conveyed by noradrenergic brainstem neurons to the accumbens shell may be a critical step in signaling the significance of information encoded during an emotional event.

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Figures

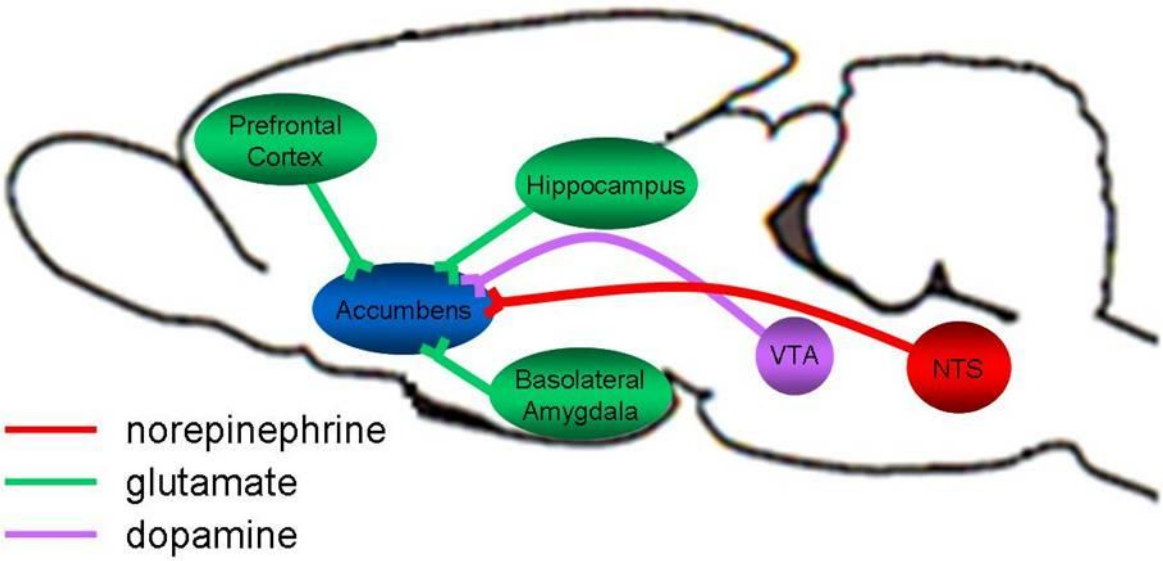

Figure 1. Sagittal diagram of the rat brain depicting selected areas that send afferent projections to the accumbens shell. Limbic areas such as the prefrontal cortex, amygdala and hippocampus send glutamatergic contacts whereas dopamine and norepinephrine are supplied by the ventral tegmental area (VTA) and nucleus of the solitary tract (NTS), respectively.

Chapter 2: Noradrenergic Mechanisms Involved in the Enhancement of Memory Following Activation of NTS Neurons

Introduction

During learning, the shell division of the nucleus accumbens receives highly processed information regarding the affective and contextual features of new experiences from the amygdala and hippocampus respectively (Brog, Salyapongse, Deutch & Zahm, 1993; French & Totterdell, 2003; Groenewegen, et al., 1987; Meredith, et al., 1990; Mogenson, et al., 1980; Petrovich, et al., 1996; Wang, Rao & Shi, 1992). These limbic inputs are complemented by norepinephrine releasing axons that originate from A2 noradrenergic neurons in the brainstem region of the nucleus of the solitary tract (NTS; Delfs et al., 1998). Norepinephrine release from A2 NTS neurons play an important role in conveying information regarding experience induced changes in the physiological state of the organism. The A2 neurons are activated during times of heightened arousal by the release of glutamate from peripheral vagal fibers (Allchin, Batten, McWlliams & Vaughn, 1994). Highly arousing events lead to an increase in the secretion of epinephrine that binds to beta-adrenergic receptors along the vagus nerve. The ascending branches of the vagus nerve, in turn, increase impulse flow to the brainstem (Lawrence, Watkins & Jarrott, 1995; Miyashita & Williams, 2006). Epinephrine administration, stimulation of the vagus nerve or direct

infusion of glutamate onto A2 NTS neurons are all known to significantly potentiate norepinephrine release within the amygdala and hippocampus (Hassert, Miyashita & Williams, 2004; Izumi & Zorumski, 1999; Liang, Chen & Huang, 1995; Roosevelt, Smith, Clough, Jensen & Browning, 2006; Segal, Markram & Richter-Levin, 1991; Williams, Men, Clayton & Gold, 1998; Miyashita & Williams, 2004). Elevated concentrations of norepinephrine in these limbic areas play a key role in facilitating memory for responses acquired under a wide range of emotionally arousing learning conditions (Bevilaqua, et al., 1997; Ferry, Roozendaal & McGaugh, 1999; Hatfield & McGaugh, 1999; Liang, Chen & Huang, 1995).

Memory enhancement produced by activating the NTS after emotional learning experiences is attributed in part to influences on noradrenergic receptors in the amygdala or hippocampus (Clayton & Williams, 2000; Miyashita & Williams, 2002; 2003; 2004; Williams, Men, Clayton & Gold 1998). However, recent findings question whether these mnemonic changes may be mediated through similar actions of NTS axons on neurons restricted to the shell division of the nucleus accumbens (Kerfoot, Chatillion & Williams, 2008). This subdivision of the accumbens receives innervations from both the locus coeruleus and NTS, although only axons originating from A2 cells contain the norepinephrine precursor, dopamine-beta-hydroxylase (Delfs, et al., 1998). Electrophysiological findings also indicate a strong relationship between the viscera, NTS and the accumbens since increasing discharge along ascending vagal fibers that terminate within the NTS potentiates neuronal firing in the accumbens shell

(Mehendale, Xie, Aung, Guan & Yuan, 2004). Similar excitatory changes in the firing properties of accumbens neurons are observed in response to activating NTS neurons with microinfusions of glutamate or current stimulation (Kirouac & Ciriello, 1997). Despite these anatomical connections, evidence demonstrating a functional relationship between A2 noradrenergic neurons and their capacity to activate the accumbens through norepinephrine release is currently not available.

Findings from a recent behavioral study revealed that chemical inactivation of the accumbens shell with the long acting anesthetic bupivacaine, completely reverses the improvement in memory produced by exciting NTS neurons with glutamate (Kerfoot, et al., 2008). These findings suggests that synaptic plasticity within the accumbens shell plays a key role in mediating the improvement in cognitive functioning produced by exciting noradrenergeric neurons in the NTS following emotional learning. They are limited however, in identifying the mechanism involved in mediating these changes in cognitive processing. Since the presence of dopamine-beta-hydroxylase in the shell originates exclusively from NTS axons innervating this region (Delfs, et al., 1998), it may be that adrenoceptors in the accumbens shell play a pivotal role in improving memory for new learning experiences following NTS activation.

Both alpha and beta-adrenergic receptors are embedded in the nucleus accumbens (Rainbow, Parsons & Wolfe, 1984; Unnerstall, Kopajtic & Kuhar, 1984), but mounting evidence suggests differences in the efficacy of these receptor subtypes in mediating the influence of norepinephrine on accumbens activity. For example, the GABA antagonist picrotoxin increases excitatory

postsynaptic potentials in slice preparations of the accumbens. These EPSP's are reduced by the addition of norepinephrine (Nicola & Malenka, 1998). This reduction in synaptic responding is only reversed by blocking alpha-adrenergic receptors with phentolamine while no changes in synaptic transmission are observed by antagonizing beta-adrenergic receptors with propranolol (Kombian, Ananthalakshmi & Edafiogho, 2006; Nicola, Kombian & Malenka, 1996; Nicola & Malenka, 1998). These findings indicate that noradrenergic influences within the shell are mediated via alpha adrenergic mechanisms rather than beta. The reduction in excitatory responses seen following norepinephrine exposure could underlie how neural input from the NTS regarding physiological arousal may amplify signal to noise encoding of information into memory.

In addition to electrophysiological evidence supporting a role of alpha-adrenergic receptors in mediating noradrenergic actions within the shell, other studies demonstrate that beta-adrenergic receptors may play a more important role in regulating dopamine release (Nurse, Russell & Taljaard, 1984; Reisine, Chesselet, Lubetzki, Cheramy & Glowinski, 1982). Specifically, beta rather than alpha receptors activate a-methyl-p-tyrosine (MpT)-sensitive, reserpine-resistant pools in the nucleus accumbens (Tuinstra & Cools, 2000). These pools are where dopamine is continuously synthesized. Therefore, stimulation or blockade of beta noradrenergic receptors in the shell directly influences dopamine release. This makes it quite difficult to determine the selective role of norepinephrine in this area via beta-adrenergic mechanisms. Studies addressing the role of beta noradrenergic receptors suggest that they are not involved in modulating memory

during associative learning. For example, drugs of abuse are easily associated with environmental contexts and specific external stimuli that are present during the time of ingestion. Injection of the beta-adrenergic receptor antagonist propranolol does not disrupt this form of drug-place associative memory for conditioned place preference tasks (Fricks-Gleason & Marshall, 2008). Although beta noradrenergic receptors do not influence learning under these behavioral conditions, the role of alpha-adrenergic receptors was not investigated. In a more recent study, direct intra-accumbens infusion of the alpha-adrenergic agonist, phenylephrine significantly improved long-term retention in a reward reduction task (Kerfoot et al., 2008). These findings illustrate the significance of alpha-noradrenergic activation in influencing synaptic changes within the accumbens shell that are necessary at the time of encoding to facilitate long-term memory.

It is still not clear however, whether the enhancement in mnemonic processes following NTS activation is influenced by noradrenergic transmission in the accumbens shell. Therefore, the current study employed *in vivo* microdialysis with HPLC to assess whether glutamate activation of NTS neurons alters norepinephrine concentrations in the accumbens shell of behaving animals. A second objective of this study was to determine whether brainstem activation of NTS neurons modulates activity in the accumbens shell by activating alpha-adrenergic receptors within this nucleus. If the enhancement in memory following glutamatergic activation of the NTS is influenced by noradrenergic transmission in the accumbens, then blockade of alpha-noradrenergic receptors in the shell should attenuate this memory enhancement.

General Methods

Subjects

Fifty-one male Sprague-Dawley rats (275-300 g) obtained from Charles River Laboratories (Wilmington, MA) were used in Experiment 1 (n=14) and Experiment 2 (n=37). The rats were individually housed in polypropylene cages with corncob bedding and maintained on a standard 12:12 hour light-dark cycle with lights on at 7:00 A.M. Food and water were available ad libitum during the 7 day adaptation period to the vivarium.

Surgery

Each rat received an injection of atropine sulfate (0.1 mg/kg i.p., American Pharmaceutical Partners, Inc., Schaumburg, IL) and was then anesthetized with sodium pentobarbital (50 mg/kg, i.p., Abbot Laboratories, North Chicago, IL). For Experiment 1, a midline scalp incision was made and a unilateral microdialysis cannula was implanted above the nucleus accumbens shell (AP +0.7, ML + 1.0 from bregma, DV -5.4 from skull surface) and bilateral 15 mm long extra thin wall stainless steel guide cannula (25 gauge, Small Parts, Miami Lakes, FL) were secured above the NTS (AP -13.3, ML +1.0 from bregma, DV -5.6 from skull surface). In Experiment 2, bilateral drug infusion cannulae were implanted 2 mm above the nucleus accumbens shell and NTS. All coordinates were adapted from the atlas of Paxinos and Watson (1986). The microdialysis cannula, drug infusion cannulae and jeweler's screws were affixed to the skull with dental cement and the scalp was closed with sutures. Stylets (15 mm, 00 insect dissection pins)

were then inserted into the injection cannulae to prevent occlusion. Penicillin (0.1 ml i.m., Fort Dodge Animal Health, Fort Dodge, IA) was administered immediately after surgery along with the analgesic, buprenex (0.05 ml s.c., Hospira, Inc., Lake Forrest, IL). The rats remained in a temperature-controlled chamber for at least one-hour following surgery and were given seven days to recover before initiating food or water deprivation procedures and behavioral training.

Microinjection Procedure

Each rat was restrained by hand in the experimenter's lap, the stylets were removed and 17 mm, 30 gauge injection needles were inserted bilaterally into the NTS guide cannula in Experiment 1. In the second study, injection needles were lowered into the accumbens guide cannulae first and these infusions were followed approximately two minutes later by bilateral injections into the NTS. The tip of the injection needles extended 2 mm beyond the base of the guide cannulae. The needles were connected to 10µl Hamilton syringes by PE-20 (polyethylene) tubing. An automated syringe pump (Sage-Orion, Boston MA) delivered the respective drugs for a total volume of 0.5 µl over 60 seconds. The injection needles were left in place for an additional 60 sec following infusions to ensure complete delivery of the drugs and the stylets were then reinserted into the cannulae.

Methods for Experiment 1

Microdialysis Procedure

Probes. CMA/12 (Carnegie/Medecin, Stockholm, Sweden) dialysis probes with a 2-mm membrane tip were used to collect norepinephrine from the shell region of the nucleus accumbens. The inlet arm was connected to a 1 ml Hamilton syringe by FEP tubing, and a CMA-1000 microinfusion pump (Carnegie/Medecin) was used to drive the syringes. The outlet arm of the probe was connected by FEP tubing to 350 µl collection vials containing 15 µl of dihydroxybenzylamine (1.0 pg/µl) that serves as an internal standard for HPLC analysis. The probes were perfused continuously with artificial cerebral spinal fluid (aCSF; pH 7.4; 145.0 mM NaCl, 4.0 mM KCl, 1.2 mM CaCl2 and 2.0 mM Na2HPO4) at a flow rate of 1.0 µl/min. Dialysate samples of norepinephrine were collected every 20 minutes and stored on ice until assayed with high performance liquid chromatography (HPLC).

Microdialysis Chamber. Samples of dialysate were collected in a CMA/120 system round-bottomed transparent bowl with a diameter of 400 mm at the top designed for microdialysis experiments in conscious, freely moving animals. The system enables long term combined studies of animal behavior and concurrent microdialysis experiments.

Microdialysis Sample Collection. The microdialysis experiment consisted of five phases: Habituation, Baseline 1 collection, Control injection, Baseline 2 collection, and Experimental treatment. Subjects were first transported to the laboratory and left undisturbed for 20 minutes. Each rat was habituated to the

chamber for 1 hour after probe implant and no samples were collected during this time. The concentrations of norepinephrine in the first three samples collected after the habituation period were averaged to yield the Baseline 1 value. Each subject was then administered an intra-NTS infusion of PBS as a control for injection, and three additional samples were collected over the next 60 min. Afterward, two additional samples were collected, and the mean concentration of norepinephrine contained in these samples represented the Baseline 2 value. Rats then received an infusion of either 50.0 ng/0.5 µl (n=4) or 100.0 ng/0.5 µl (n=5) of l-glutamic acid into the NTS. Seven more samples were collected before the probe was removed from the guide cannula and the rat was returned to the home cage. The vials containing each sample were sealed with parafilm (Fisher Scientific, Pittsburgh, PA) and stored on ice until assayed with HPLC.

A third group of rats was habituated to a Coulbourn behavioral chamber (12”W x 10”D x 12”H, Model #: H13-16) for 17 minutes on the day prior to microdialysis collection. The front and back walls of the chamber were made of clear plastic with stainless steel sides and a removable stainless steel grid floor. On the day of microdialysis collection, animals experienced the same procedures described above. However, these animals received the lower dose of glutamate in the NTS immediately after the type of footshock that was used during training in Experiment 2 (n=5; 0.35 mA footshock for 2 seconds). Animals were removed from the collection bowls and placed in the Coulbourn chamber for one minute before a 2 second, 0.35 mA footshock was delivered. Animals remained in the chamber for an additional 60 seconds before being removed and administered

NTS infusions. Following injections, animals were placed back into the CMA bowls and collection proceeded for 2 hours and 20 minutes.

It is important to note that all experimental manipulations were initiated in the final 10 minutes of the collection period that preceded the experimental treatment. This 10 minute period reflects the amount of time required for the dialysate samples to be transported from the membrane of the microdialysis probe through the FEP tubing to the sample collection vials.

Norepinephrine Assay. Norepinephrine concentrations in the dialysate sample were assayed by HPLC electrochemical detection (ESA, Chelmsford, MA). At the end of the microdialysis experiment, 35 µl of each dialysate sample was loaded into a Waters 717 autosampler, automatically injected with a flow rate of 1.0 ml/min. The mobile phase consisted of 50 mg disodium EDTA, 13.8 mg monobasic sodium phosphate and 58 mg octane sulfonate adjusted to pH 3.2 by adding 85% phosphoric acid. Norepinephrine concentrations and peak heights were measured in comparison with those of a known norepinephrine standard (32 pg/35 µl). The concentration, peak height, and retention time for dialysate samples of norepinephrine were analyzed with the Millennium software package (Waters).

Statistical Analysis. The levels (pg/ml) of norepinephrine from the 3 baseline samples were averaged to yield a standard baseline value. Comparisons between norepinephrine levels at baseline and each 20 minute time point was analyzed with repeated measures ANOVA. Fischer's post hoc test will be used to analyze specific comparisons between treatment groups.

Histology

Rats were deeply anesthetized with a euthanasia solution and perfused intracardially with 0.9% saline followed by 10% formalin to verify microinjection cannulae placement. The brains were stored in a 10% formalin and 12% sucrose solution until sectioned on a vibratome. Sections were cut 60 µm thick, mounted on glass slides, subbed with chromium-aluminum and stained with cresyl violet. The location of the cannulae and injection needle tips were verified by examining enlarged projections of the slides (Figure 1a and 1b).

Methods for Experiment 2

Water-Motivated Inhibitory Avoidance Task

Apparatus. A trough-shaped, two compartment rectangular apparatus (91cm long, 21cm wide at the top and 6.4cm wide at the bottom) with a hinged lid was used to train the rats in a water-motivated inhibitory avoidance task. A sliding metal door (14.5 cm) separated a neutral and dark compartment. The neutral compartment was constructed of white opaque Plexiglas (31cm long) and brightly illuminated by a 60 watt light located directly above the compartment. The dark compartment was constructed of stainless steel plates (60 cm long). A curved stainless steel water spout connected to a 30-cc plastic syringe containing water was placed 1 cm above the floor at the end of the dark compartment.

Pre-Training Manipulations. One-week after surgery, rats were placed on a water maintenance schedule with daily access to water during behavioral training

and for twenty minutes in their home cage. Body weights were monitored daily to insure that they remained at 10% of their ad-lib feeding weights throughout the experiment.

Training. Animals were habituated during a 5 min period of exploration with the opportunity to cross between the white and dark compartments of the inhibitory avoidance apparatus. During training, each rat was placed in the dark compartment facing the retractable door that separated the dark from the illuminated compartment. The metal door was lowered to 2/3 of its length (i.e. creating a 4 cm hurdle), a timer was started and the a) latency to begin drinking, b) total amount of time spent drinking, c) total amount of time spent in the dark compartment and d) total amount of time spent in the white illuminated compartment was recorded. Each rat received one training trial lasting 120 seconds on each of six consecutive days.

On Day 7 (i.e. experimental day), each rat was placed in the dark compartment as before however, a 0.35 mA electrical footshock was administered as soon as the rat initiated the first lick towards the water spout. The shock remained on until the animal escaped from the dark compartment by crossing over the 4 cm high hurdle into the illuminated neutral compartment. Each animal was retained in the neutral compartment for 30 sec with the door two-thirds open, the door was then raised and the animals remained in the neutral compartment for an additional 30 sec. During this time, they were given the opportunity to cross between the white and dark compartment (shown in Figure 2a and 2b) and to explore the drinking spout. Hence, the animals were

allowed 60 sec to learn that the white illuminated compartment was safe relative to the dark compartment where footshock was just experienced. Each animal was then removed from the apparatus and given an intra-accumbens infusion of phosphate buffered saline (PBS) or the α-adrenoceptor antagonist, phentolamine (0.5 µg/0. 5 µl). The accumbens injections were then followed by bilateral intra-NTS infusion of either PBS or the dose of l-glutamic acid (50 ng/0.5 µl) previously shown to improve retention under these behavioral conditions (Kerfoot et al., 2008; Miyashita & Williams, 2002). The dose of phentolamine was selected from those demonstrated to be low enough not to affect memory when given alone, but sufficient to block the actions of noradrenergic agonists in previous studies (Cools, Ellenbroek, van den Bos & Gelissen, 1987; Roozendaal & Cools, 1994).

Microinjection Procedure. Rats were randomly assigned to one of four groups that received PBS into both the accumbens shell and NTS (PBS/PBS: n=8), phentolamine in the accumbens and PBS in the NTS (phentolamine/PBS: n=10), PBS into the accumbens and glutamate into the NTS (PBS/glutamate: n=9) or microinjections of phentolamine in the accumbens and glutamate in the NTS (phentolamine/glutamate: n=7).

Retention Test. Memory for the surprising footshock in the dark compartment was assessed 24 hours later and consisted of two phases. During Phase 1, the rats were placed in the dark compartment facing the partially lowered metal door and given 60 sec to enter the neutral compartment or alternatively, to initiate the first lick from the water spout. If the rat entered the neutral compartment, the metal door was raised and the rat remained in the

neutral compartment for 30 sec. Phase 2 of retention began after this 30 sec period. Those that did not enter the dark compartment after 60 sec were removed and placed in the neutral compartment with the metal door raised for 30 sec. Measures recorded during Phase 1 included percentage of animals to drink from the spout, latency to drink, amount time spent drinking and latency to escape into the neutral compartment. During Phase 2, the metal door was lowered and the time spent avoiding the dark compartment, latency to drink from the spout, total time spent drinking and the total amount of time spent in the neutral compartment was recorded over a period of 300 sec. Figures 2a and 2b depict the separate measures recorded for Phase 1and Phase 2 of the retention test, respectively.

Statistical Analysis. The behavioral measures from the water-motivated inhibitory avoidance task are expressed as mean ± standard errors (SE). Between-group comparisons for the behaviors measured during retention testing were made with a two-way analysis of variance (ANOVA; NTS x nucleus accumbens shell injections) followed by post hoc tests. Comparisons between the last day of training before footshock and Phase 2 of the retention test for the latency to first lick the spout from the beginning of Phase 2 and the total amount of time spent in the neutral compartment on were made with a two-way repeated measures ANOVA followed by a post-hoc test.

Histology

Histological procedures were the same as those described in the previous histology section for Experiment 1. The location of injection needle tips in this study is displayed in Figure 3a and 3b.

Results for Experiment 1

As shown in Figure 4, samples of norepinephrine were collected with in vivo microdialysis from the nucleus accumbens shell at 20 minute intervals following microinjections of phosphate buffered saline (PBS) into the NTS followed 2 hours later with either 50 or 100 ng/0.5 µl of l-glutamic acid. The larger dose of glutamate was selected to approximate the level of glutamate release that results from the combined treatment of footshock (0.35 mA, 2 sec), and glutamate (50 ng) that has been shown to improve memory in an emotionally arousing learning task (Kerfoot, Chattillion & Williams, 2008; Miyashita & Williams, 2002). As an additional measure, a third group received the memory enhancing treatment (50 ng of glutamate in the NTS along with a 0.35 mA footshock). A one-way repeated measures ANOVA revealed a significant overall change in accumbens norepinephrine concentrations relative to baseline values across the 160 minute period of collection, $F (2, 12) = 13.0$, $p < 0.01$. There was also a significant interaction for the change in norepinephrine over time and the drug treatments, $F (2, 24) = 6.1$, $p < 0.01$.

Between-group comparisons made with factorial ANOVAS revealed that norepinephrine levels did not differ between any of the three treatment groups following intra-NTS infusion of PBS. These levels remained constant for an hour

following PBS injections. Following Baseline 2 (time point 180 minutes), infusion of the low dose of glutamate (50 ng) into the NTS also did not significantly potentiate norepinephrine release in the accumbens when given alone (open squares). However, the same dose produced a significant increase in norepinephrine output when combined with the low intensity (0.35 mA), 2 sec footshock (167% increase from Baseline 2; $p < 0.01$, open diamonds). Intra-NTS infusion of a higher, 100ng, dose of glutamate alone produced a less dramatic, albeit significant increase in accumbens noradrenergic output (40% increase from Baseline 2; $p < 0.05$, closed squares). Norepinephrine levels for these two groups remained significantly elevated above Baseline 2 values throughout the next eight periods of sampling. These findings are consistent with those reported by McKittrick and Abercrombie (2007) showing that norepinephrine in the accumbens remains elevated for as long as 240 minutes in response to peripheral administration of d-amphetamine. Extracellular levels of norepinephrine remained unchanged in the group given the low (50 ng) dose of glutamate in the NTS.

Results for Experiment 2

Phase 1 of Retention Testing

Latency to Emit an Active Avoidance Response or Drink from the Spout. During Phase 1 of the retention test each animal was placed in the dark compartment facing the lowered metal door. The latency to lick the water spout (measured from the very beginning of the retention test until the end of the 60

sec test) or alternatively to enter the safe/neutral compartment was measured. A two-way ANOVA on the mean seconds to exit the dark and enter the neutral-illuminated compartment revealed no significant interaction between the treatment groups, $F(1, 30) = 0.12$, $p = ns$ (PBS/PBS 46.5 ± 4.2, phentolamine/PBS 42.0 ± 5.5, PBS/glutamate 45.0 ± 7.6, phentolamine/glutamate 45.0 ± 8.3). Although animals did not emit an active avoidance response and escape from the dark compartment, they did not initiate drinking from the spout either, $F(1, 30) = 3.52$, $p = ns$ (PBS/PBS 53.1 ± 7.0, phentolamine/PBS 56.7 ± 2.6, PBS/glutamate 60.0 ± 0.0, phentolamine/glutamate 46.7 ± 6.6). The Phase 1 test is essential in establishing that the surprise shock given during training is effective in promoting adequate learning and retention of this arousing experience in all control and experimental groups. The long latencies displayed across all groups reveal that the intensity of the footshock given 24 hours previously was sufficient for this purpose. Phase 2 testing evaluated treatment induced differences in the strength of this memory and the potential contribution of accumbens α-receptors in mediating these effects.

Phase 2 of Retention Testing

Contextual Memory: Time Spent Avoiding the Shock / Dark Compartment. Each animal began Phase 2 of the retention test in the neutral compartment. After 30 sec, the metal door separating the neutral-illuminated and shock (dark) compartment was lowered and the latency to enter the dark compartment was

recorded. If memory of the context where footshock was experienced 24 hrs earlier was retained, then latencies to exit the neutral area and reenter the section of the maze where the footshock occurred on Day 7 of training should be extended. A two-way ANOVA indicated a significant interaction between NTS and accumbens treatments for the latency to enter the shock compartment, $F(1, 30) = 9.14$, $p < 0.01$. As shown in Figure 5, animals given intra-accumbens PBS followed by glutamate in the NTS spent significantly more time in the neutral compartment before entering the context where footshock was administered 24 hours previously ($p < .01$ compared to all other treatment groups). This dose of glutamate was not effective in the group given glutamate after accumbens receptors were blocked with phentolamine. The time spent in the neutral compartment for this group was not different from that of PBS controls but was significantly lower than that of the PBS-Glutamate group ($p < 0.01$).

During Phase 2 of the retention test, the door separating the neutral and shock compartment remained open. Consequently, animals could freely move between both compartments. As such, time spent in the illuminated compartment provides an indirect measurement of the total time animals spent avoiding the shock compartment. A repeated measures ANOVA revealed a significant Day x NTS x Accumbens interaction between the groups on this measure, $F(3, 60) = 5.44$, $p < 0.01$. As depicted in Figure 6, all subjects spent the majority of time in the dark compartment containing the water-spout during the last day of training (D6). Although all animals spent significantly more time in the neutral compartment during Phase 2 of the retention test compared to the last day of

training, animals given PBS in the accumbens and glutamate in the NTS spent even more time in the neutral compartment compared to all other treatment groups (PBS/PBS $p < 0.01$; phentolamine/PBS $p < 0.01$; phentolamine/glutamate $p < 0.05$). More importantly, the extended period of time displayed by the PBS/glutamate group in the neutral compartment was not observed in animals given an identical dose of glutamate into the NTS after the α-adrenergic receptors were functionally inactivated by phentolamine injection (phentolamine/glutamate).

Response Specific Memory: Latency to First Lick the Spout. Given the possibility of both contextual and response-based memory formation in this task, two additional measures were recorded to assess response-specific memory. If the last behavior emitted before initiation of the arousing footshock (approaching the spout to drink) was retained in memory, then subjects should take significantly longer to initiate contact with the spout and spend less time drinking from the spout. To address this view, the time required to initially drink from the spout after entering the shock compartment was recorded for Phase 2 of retention testing. A two-way ANOVA revealed a significant interaction between NTS and accumbens injections, $F(1, 30) = 6.32$, $p < 0.05$ (Figure 7). The group given PBS/glutamate required a significantly longer period of time to initiate drinking from the spout relative to PBS/PBS controls ($p < 0.01$) or animals given phentolamine in the accumbens and PBS in the NTS ($p < 0.01$). However, this effect was prevented by blocking alpha adrenoceptors in the accumbens shell. Unlike the PBS/glutamate animals, subjects given phentolamine into the shell

before intra-NTS infusion of glutamate (phentolamine/glutamate) exhibited significantly shorter latencies to drink from the water spout ($p < .01$) during Phase 2 of retention testing.

The amount of time each animal spent drinking after initial contact was made with the water spout served as an additional measure of retention for the response emitted immediately before the delivery of footshock. As shown in Figure 8, a two-way ANOVA revealed significant differences between treatment groups in the mean time spent drinking from the spout where footshock occurred 24 hours earlier, $F (1, 30) = 1.95$, $p < 0.05$. Animals given PBS/glutamate spent significantly less time drinking from the spout relative to PBS/PBS controls ($p < 0.01$) or the phentolamine/PBS group ($p < 0.01$). Without α-adrenoceptor functioning, animals in the phentolamine/glutamate group did not display the high level of avoidance behavior observed in the PBS/glutamate ($p < 0.01$).

Discussion

Traditionally, a great deal of attention has been devoted to understanding the contribution of the nucleus accumbens in forming associations between rewarding stimuli and increases in motivated behaviors. The functional significance of the accumbens is now being broadened to also include a role in processing memory for responses acquired in fear conditioning and contextual discrimination learning tasks. Current research demonstrates that memory enhancement produced by activating A2 neurons with glutamate can be completely abolished by reversible inactivation of the accumbens shell (Kerfoot,

Chattillion & Williams, 2008). These findings can be interpreted to suggest that information conveyed by NTS neurons in the form of visceral and hormonal changes indicative of heightened states of arousal, play a crucial role in modulating activity in the shell division of the accumbens during the storage of new events into memory. However, because a reversible anesthetic was used to silence the accumbens before exciting noradrenergic A2 NTS neurons with glutamate, it was unclear as to whether the attenuation in memory was due to inhibition of accumbens output neurons that respond to norepinephrine released from A2 neurons. The aim of the first study was to assess whether or not excitation of NTS neurons with glutamate facilitates norepinephrine output within the accumbens. The second study demonstrated that noradrenergic release from NTS terminals affects mnemonic processing in the accumbens by selective actions on postsynaptic alpha-noradrenergic receptors.

Experiment 1 addressed the first objective with the use of in vivo microdialysis. This approach revealed that control (PBS) or low dose (50 ng) infusions of glutamate into the NTS produced no appreciable fluctuations in norepinephrine levels in the accumbens shell. This finding demonstrates that the smaller concentration of glutamate alone may not be sufficient to activate A2 NTS neurons and impact the release of norepinephrine from these terminals within the accumbens. However, infusion of the low dose of glutamate in combination with an arousing footshock (similar to that used during training in Experiment 2) was sufficient to potentiate norepinephrine release in the accumbens. This is consistent with findings from Miyashita and Williams (2002)

showing that footshock and 50 ng of glutamate in the NTS potentiates norepinephrine release in the basolateral amygdala. The current data extends these findings to demonstrate that peripheral activation via footshock is required along with the low dose of glutamate into the NTS to increase extracellular levels of norepinephrine in the accumbens (Experiment 1). This treatment also leads to a significant enhancement in memory for the arousing experience (results from Experiment 2; see below). The increase in norepinephrine seen in the SHOCK/GLUT 50ng group can also be simulated by infusing a higher dose of glutamate (100 ng) in the absence of the emotionally arousing footshock. The magnitude of the effect of activating NTS neurons is revealed by the finding that norepinephrine concentrations in the accumbens remained elevated for at least 2 hours and 20 minutes in groups given the high dose of glutamate or the low dose with a footshock. Norepinephrine concentrations escalated to 229% above Baseline 2 for the PBS / GLUT 100ng group and 119% for the SHOCK / GLUT 50ng group. Norepinephrine levels may not have remained as high compared to the initial collection following footshock and injection because animals are removed from the context where the footshock occurred.

Findings from Experiment 1 are consistent with those reported by Kirouac and Ciriello (1997) that showed potentiation in neuronal firing in the accumbens following either electrical or glutamatergic activation of neurons in the NTS. The current results extend these data and suggest that the increase in accumbens neuronal firing in response to NTS activation may be due to a long lasting release of norepinephrine from NTS terminals. In addition, it has been shown that

peripheral administration of d-amphetamine leads to a significant release of norepinephrine in the accumbens that persist for up to four hours (McKittrick & Abercrombie, 2007) and that increasing discharge along ascending fibers of the vagus nerve with electrical stimulation potentiates neuronal firing in the accumbens shell (Mehendale, et al., 2004). Taken together, the traditional pathway that includes the hippocampus and amygdala as the major recipients of noradrenergic input crucial for processing peripheral information, can be updated to include the accumbens.

Experiment 2 demonstrated that intra-NTS infusion of glutamate improves memory for emotionally arousing learning experiences, consistent with previous findings (Kerfoot, Chattillion & Williams, 2008; Miyashita & Williams, 2002). The beneficial actions of activating the NTS was evident by the number of separate measures that animals in the PBS/glutamate group displayed enhanced retention performance. For example, animals in this group were the only subjects to require an extended amount of time to enter the shock compartment. The extended delay to enter this compartment provides some validation that the PBS/glutamate group remembered the context in which the emotionally arousing footshock was given 24 hours earlier. Findings from Experiment 2 were also instrumental in revealing that norepinephrine output from NTS terminals influences accumbens neurons via alpha-adrenergic receptors. Of particular interest are the results demonstrating that blocking alpha-noradrenergic receptors with phentolamine in the accumbens attenuates memory improvement produced by exciting the NTS (PBS/glutamate). Therefore, our understanding of

how the NTS influences processing in the accumbens can be extended to include facilitation of contextual memory (Figure 5 and 6) as well as response specific memory (Figure 7 and 8).

A number of early immunofluorescence and immunohistochemical experiments reported the presence of norepinephrine in the accumbens. Recent investigations that delineated between core and shell found dopamine-β-hydroxylase immunoreactive fibers in the caudal shell region and very few, if any, in the core region (Berridge, Stratford, Foote & Kelley, 1997). Although the locus coeruleus and NTS are known to project to the accumbens shell (Brog Salypongse, Deutch & Zahm, 1993), only projections from NTS neurons contain norepinephrine (Delfs, et al., 1998). Additional studies suggest that noradrenergic innervation of the accumbens shell derives from the NTS and not the LC. For example, Holdefer and Jensen (1987) demonstrated that systemic injection of amphetamine does not increase discharge of LC neurons, but actually suppresses LC activity. Given findings showing d-amphetamine potentiates norepinephrine output in the accumbens combined with the finding showing that d-amphetamine suppresses LC activity (Holdefer & Jensen, 1987), it is apparent that treatments that affect physiological arousal, must therefore increase norepinephrine output in the accumbens shell through the only other noradrenergic pathway innervating this structure, the NTS. This is important to establish given findings that norepinephrine released from A1, A2 and A5 neurons act on alpha receptors whereas A4 and A6 cell groups act on beta receptors (Cools, et al., 1991). Results from the current study add support to

those that suggest noradrenergic influences within the shell are mediated via alpha-adrenergic mechanisms rather than beta (Kombian, Ananthalakshmi & Edafiogho, 2006; Nicola, Kombian & Malenka, 1996; Nicola & Malenka, 1998).

Peripheral inputs conveyed by noradrenergic brainstem neurons to the accumbens shell play a critical role in signaling the significance of information encoded during an emotional event. The current study shows that activation of A2 noradrenergic neurons in the NTS facilitates norepinephrine release in the accumbens shell as assessed by *in vivo* microdialysis measures. Norepinephrine released from NTS terminals acts on alpa-adrenergic receptors to facilitate memory consolidation following arousing events. Interestingly, the same region of the shell that receives noradrenergic input from the NTS also receives projections from the basolateral amygdala and hippocampus (Delfs, et al., 1998; French & Totterdell, 2003). This arrangement provides the foundation for studies that suggest information from either the amygdala or hippocampus is dependent upon an intact amygdala/accumbens or hippocampus/accumbens pathway (Roozendaal, de Quervain, Ferry, Setlow & McGaugh, 2001). The accumbens shell therefore may be in a position to modulate information not only from the NTS but also information emanating from the amygdala and hippocampus. Future studies are required to assess whether the accumbens can modulate information initially processed in the amygdala or hippocampus.

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Figures

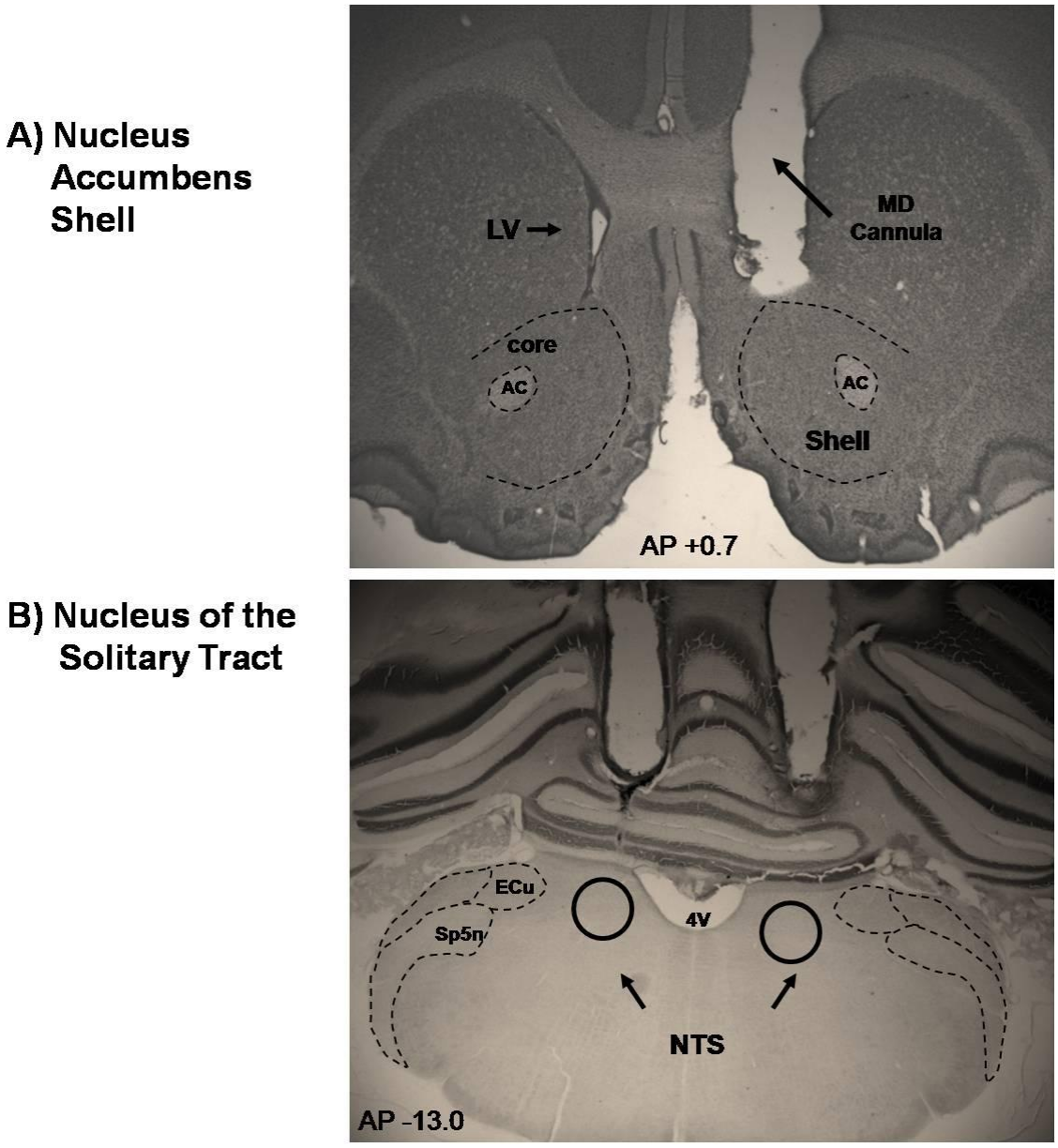

Figure 1. Location of A) microdialysis cannula placements in the nucleus accumbens shell and B) needle tip placements in the nucleus of the solitary tract from animals trained and tested in Experiment 1 overlaid onto representative photomicrographs. Abbreviations: 4V = fourth ventrical, AC = anterior commissure, core = nucleus accumbens core, ECu = ext cuneate nucleus, LV = lateral ventricle, MD cannula = unilateral microdialysis cannula (counterbalanced for side), Shell = nucleus accumbens shell and Sp5n = spinal trigeminal nucleus.

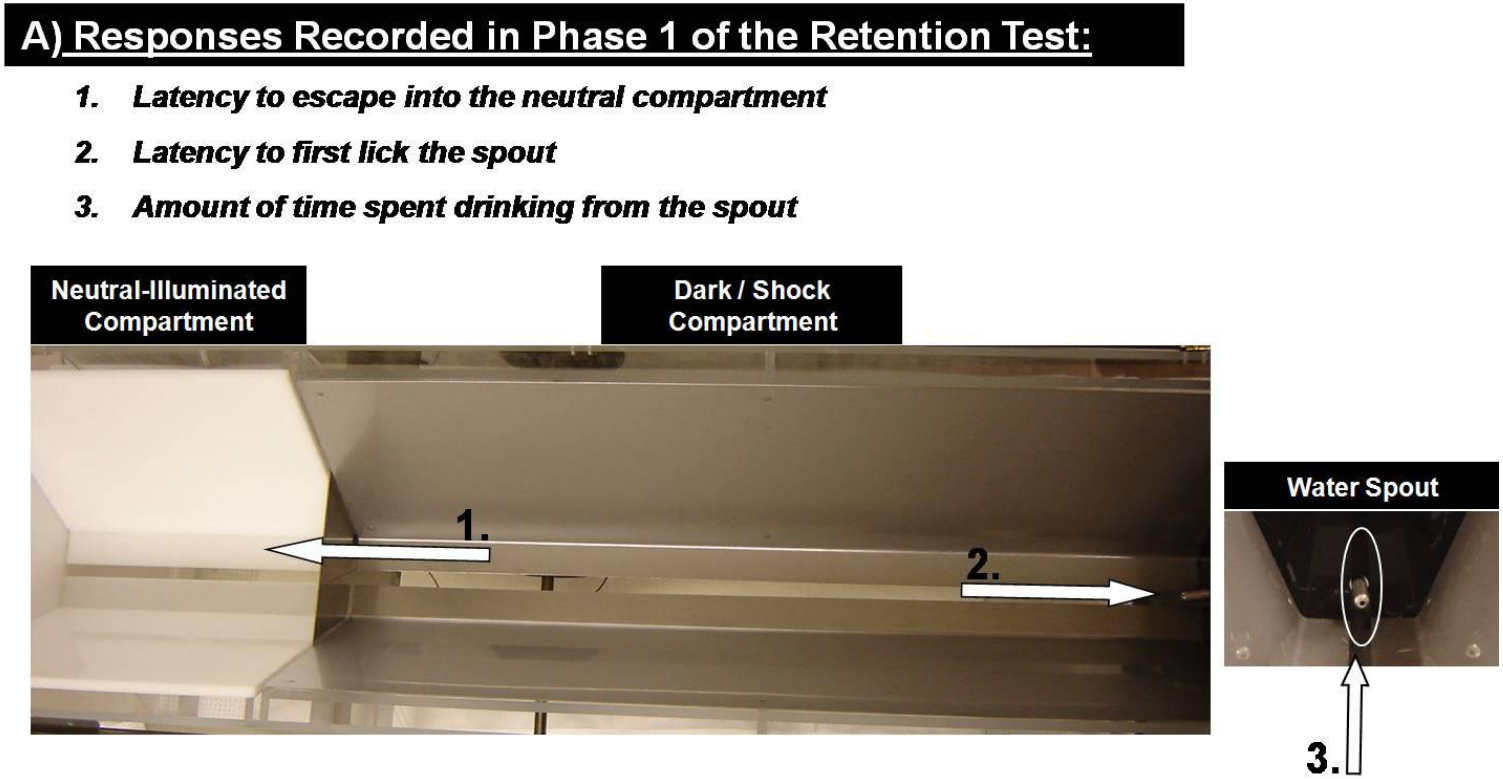

Figure 2a. Overhead view of the inhibitory avoidance apparatus along with the measures recorded during the first Phase of retention testing. Subjects were placed in the dark compartment facing the sliding door and the (1) latency to either escape into the neutral compartment or alternatively (2) lick the spout at the end of the dark compartment was recorded. The total amount of time each animal spent drinking during the 60 sec test in Phase 1 was also assessed (3).

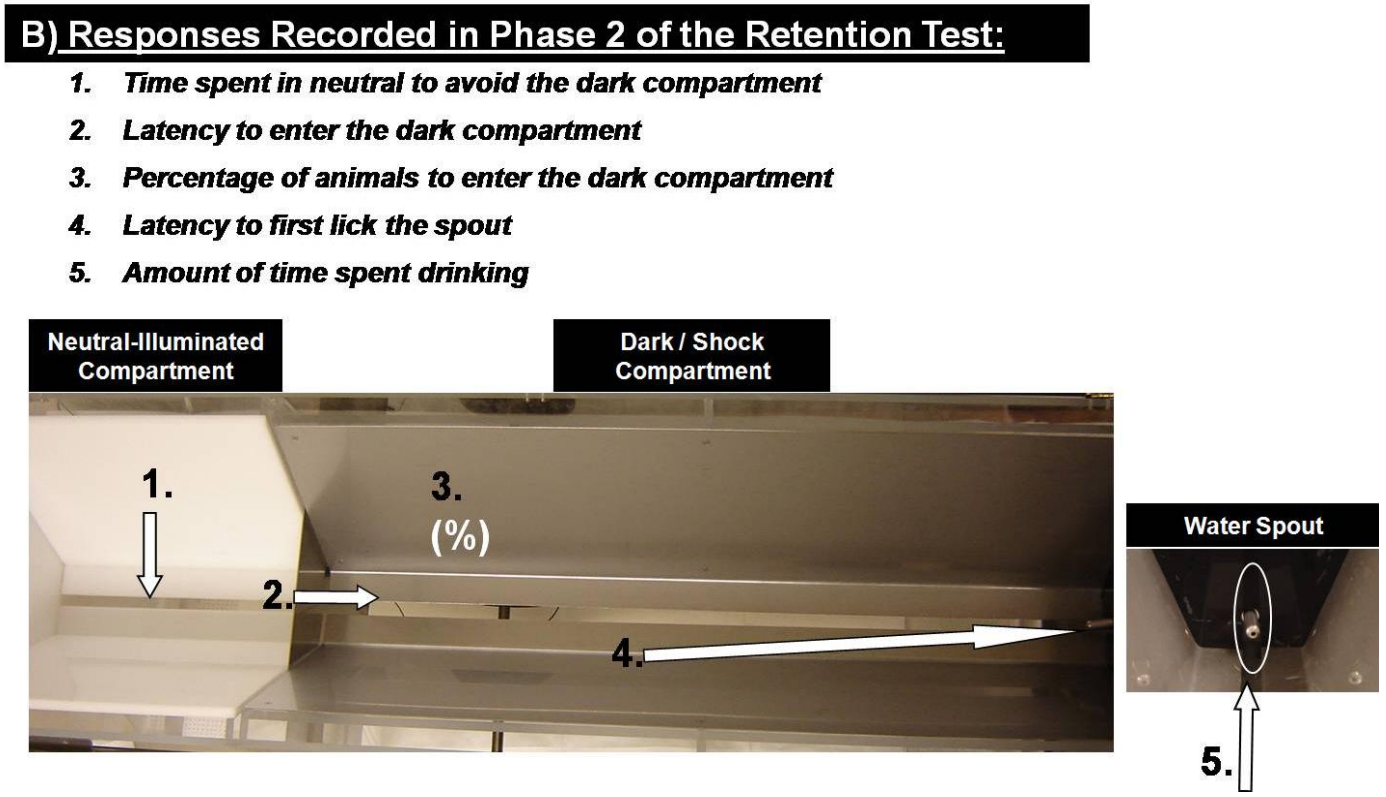

Figure 2b. Overhead view of the inhibitory avoidance apparatus and a description of the sequence of responses recorded to assess memory during Phase 2 of retention testing. The subjects were placed in the neutral compartment and the following behaviors were recorded: (1) time spent avoiding the dark compartment, (2) latency to enter the shock compartment, (3) percentage of animals to enter the dark compartment, (4) time required to initiate contact and lick from the waterspout and (5) total time spent drinking from the spout once initial contact was made.

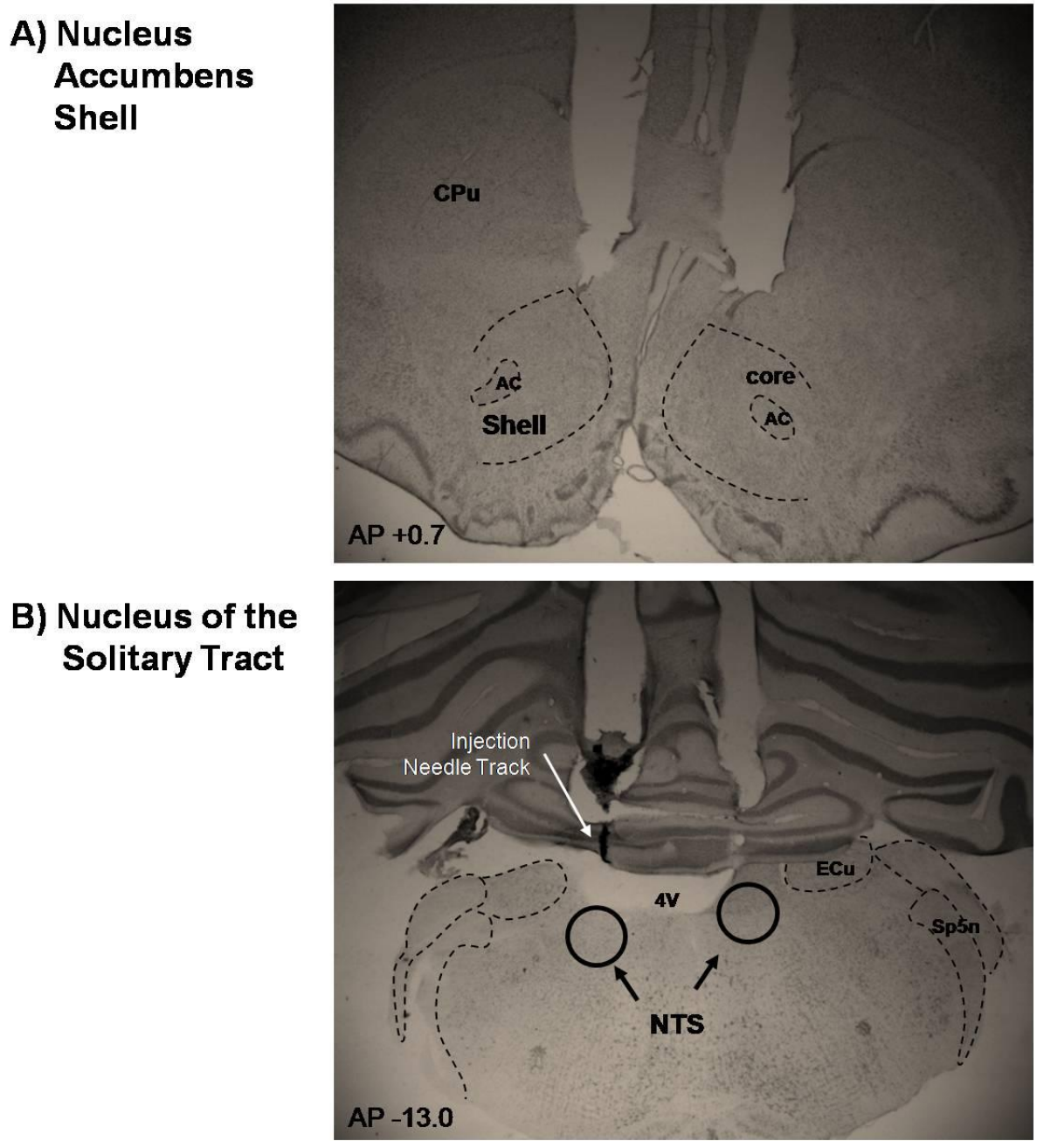

Figure 3. Location of injection needle tip placements in A) the nucleus accumbens shell and B) the nucleus of the solitary tract overlaid onto a representative photomicrograph from animals trained and tested in Experiment 2. Abbreviations: 4V = fourth ventrical, AC = anterior commissure, core = nucleus accumbens core, CPu = caudate putamen, ECu = ext cuneate nucleus, Shell = nucleus accumbens shell and Sp5n = spinal trigeminal nucleus.

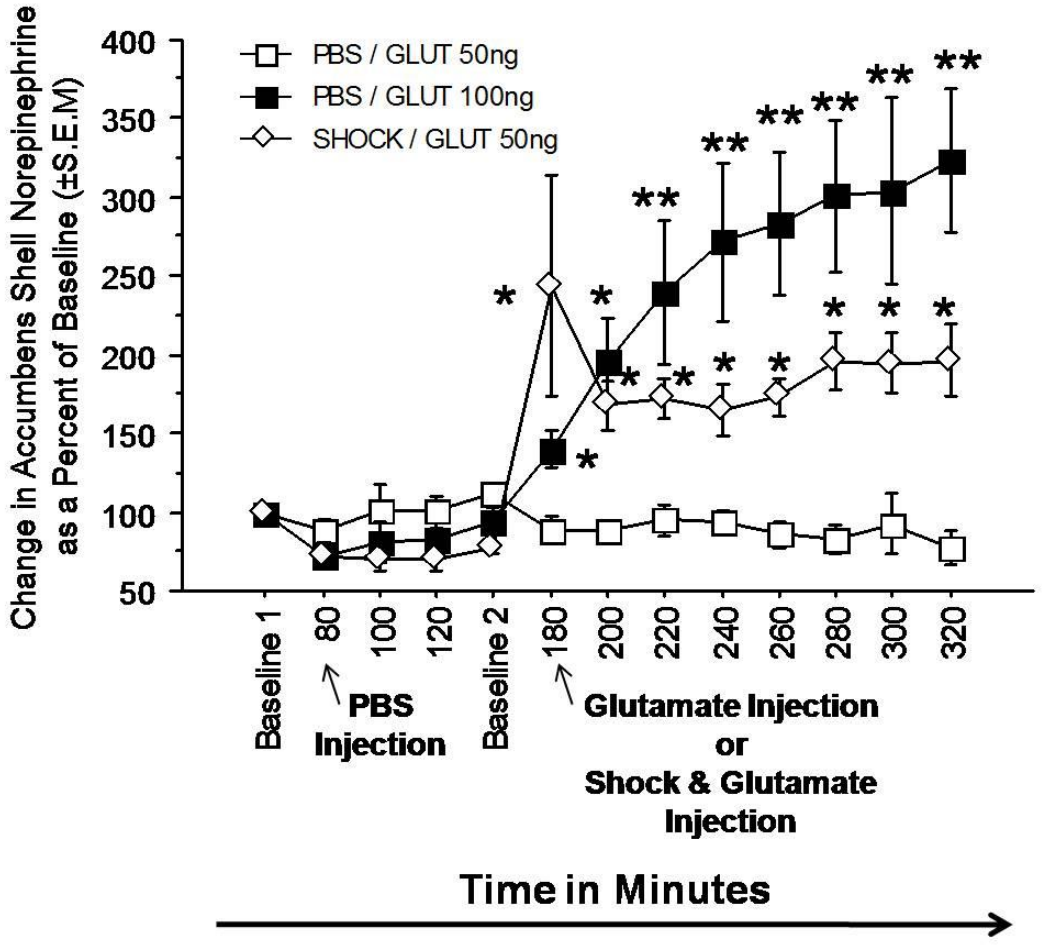

Figure 4. Mean (± SE) change in accumbens norepinephrine levels as a percent of baseline. NTS infusions of PBS or a low dose of glutamate (50ng) immediately following 60 minutes of baseline measurements produced no appreciable fluctuations in norepinephrine levels in the accumbens shell. However, similar infusions of 50ng glutamate into the NTS in combination with a mild footshock 120 minutes following baseline caused a 167% increase in extracellular concentrations of norepinephrine collected from the accumbens. This effect could be simulated in animals given intra-NTS infusions of a higher, 100ng, dose of glutamate alone. * denotes $p<0.05$, ** denotes $p<0.01$.

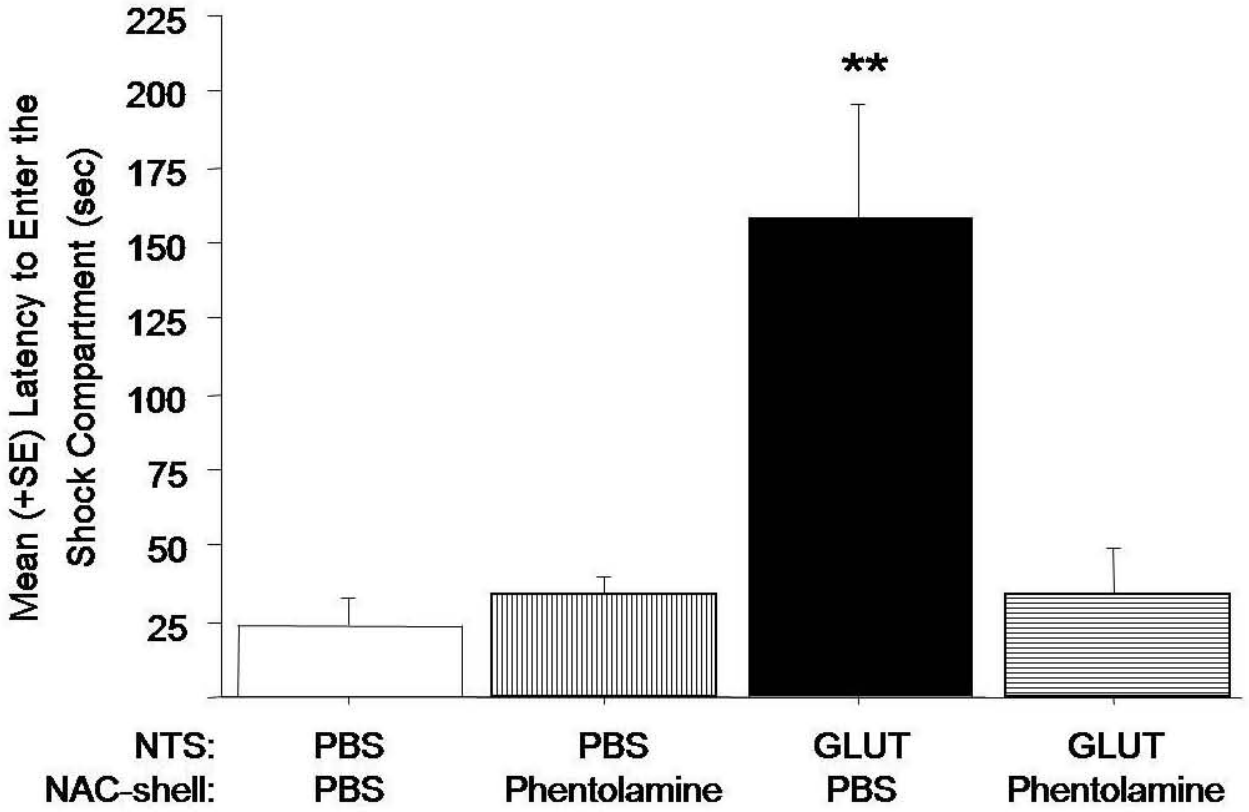

Figure 5. Mean (+ SE) latency to enter the dark compartment in Phase 2 retention testing. Animals in the PBS/GLUT group took significantly longer than all other treatment groups ($p<0.01$) to enter the dark compartment where footshock had been administered 24 hours previously. ** denotes $p<0.01$.

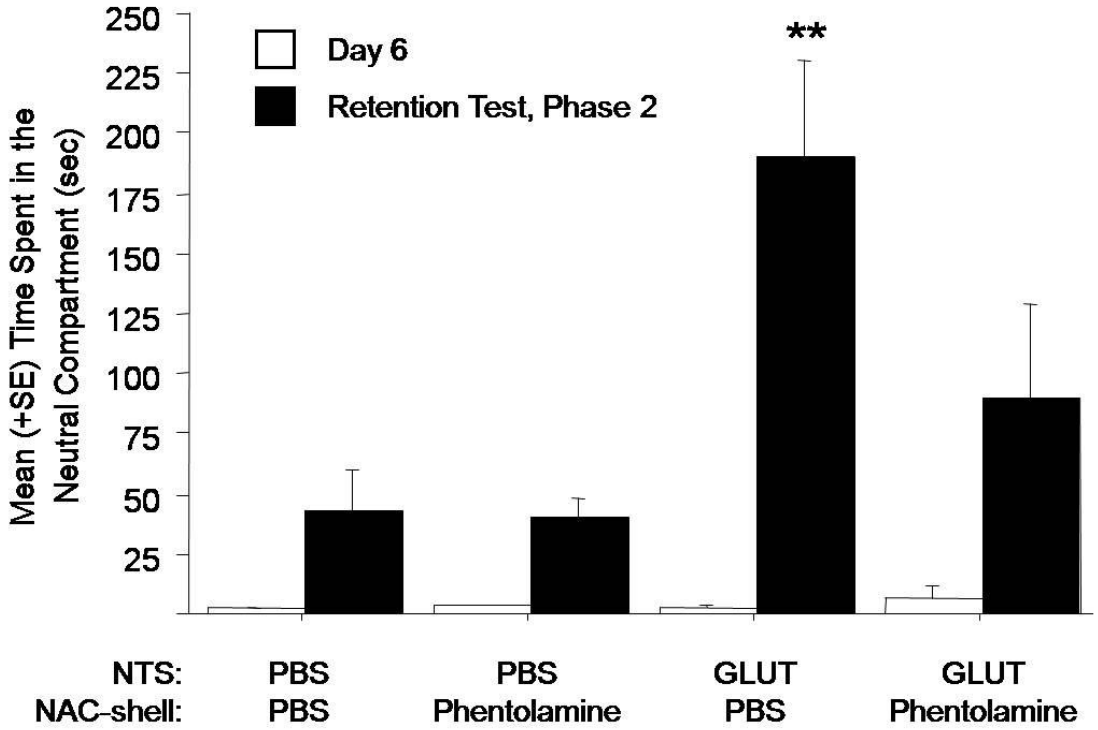

Figure 6. Mean (+ SE) time spent in the neutral compartment avoiding entry into the dark compartment on the last day of training (day 6) and Phase 2 of the retention test. On day 6, all groups spent approximately the same amount of time in the neutral compartment. Twenty-four hours later following shock and injections, all treatment groups spent more time in the white compartment compared to day 6. However, only animals in the PBS/GLUT group spent significantly more time avoiding the dark compartment ($p<0.01$). Blockade of α-noradrenergic receptors in the shell prior to NTS activation attenuated this enhancement as evidenced by the reduction in time spent in the neutral compartment. ** denotes $p<0.01$.

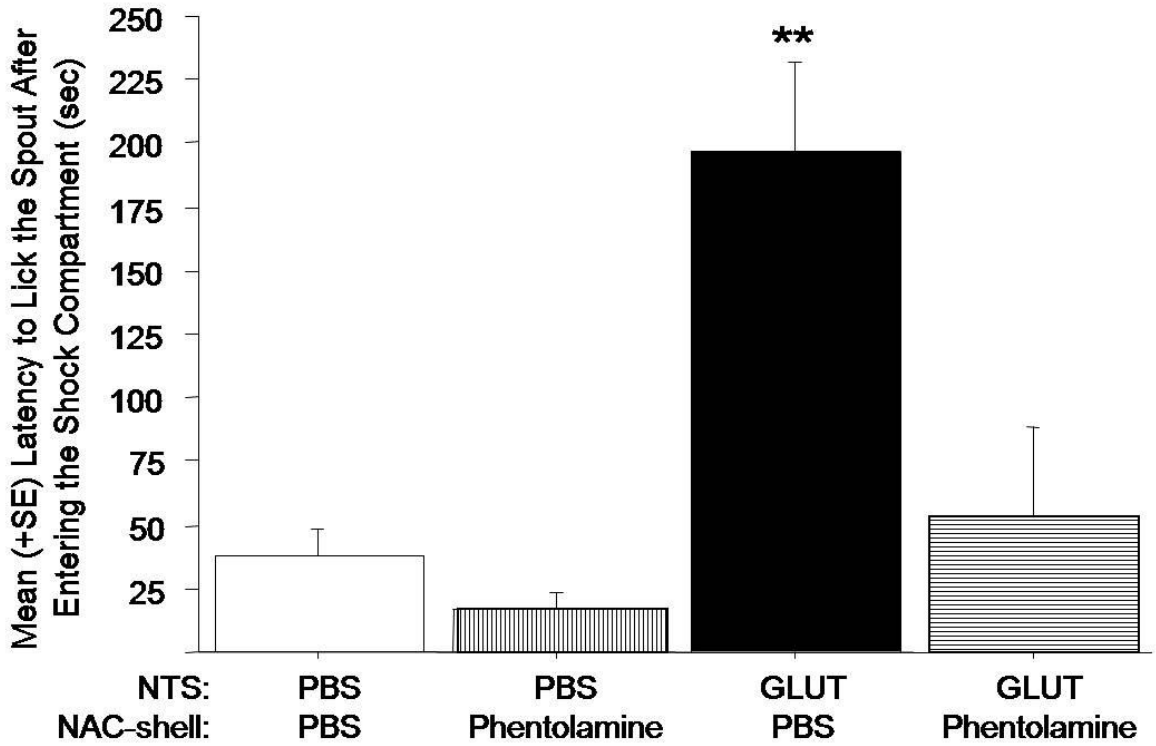

Figure 7. Mean (+ SE) latency to lick the spout after entering the shock compartment in Phase 2 retention testing. This measure represents the memory for the last action emitted prior to delivery of the arousing footshock. Activation of NTS neurons (PBS/GLUT) produces an enhancement in memory given that only animals in this group took significantly longer ($p<0.01$) to lick the water spout. Accumbens α-noradrenergic receptors are needed for this enhancement to occur as evidenced of how soon animals in the Phentolamine/GLUT group licked from the spout. ** denotes $p<0.01$.

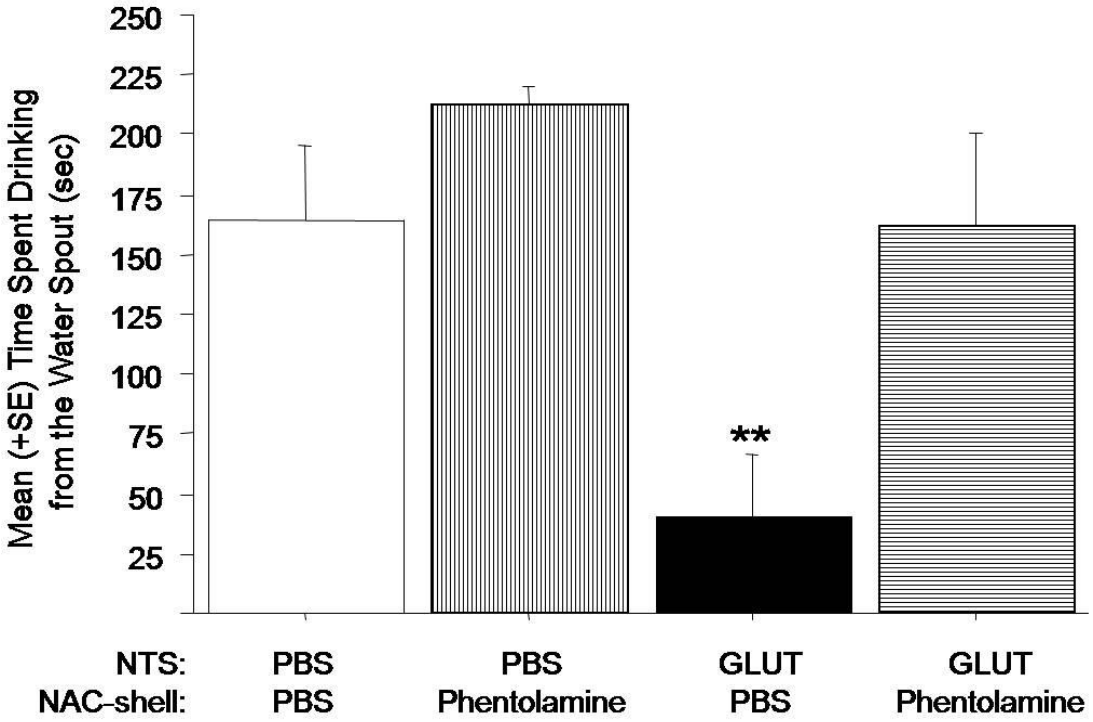

Figure 8. Mean (+ SE) amount of time spent drinking from the water spout during the retention test (Phase 2). Even though all animals are on water restriction, only animals in the PBS/GLUT group fail to drink for a majority of the time during Phase 2 of the retention test ($p<0.01$). Again animals treated with the same dose of glutamate in the NTS, but phentolamine in the accumbens, look similar to controls and drink for approximately 150 seconds of a 300 second test. ** denotes $p<0.01$.

Chapter 3: Consequences of Activating Noradrenergic Inputs to Either the Amygdala or Hippocampus are Mediated within the Nucleus Accumbens Shell

Introduction

The nucleus accumbens shell receives a constellation of inputs representing peripheral physiological arousal from brainstem nuclei, affective appraisal of stimuli from the amygdala, and contextual and temporal relationships of stimuli from the ventral hippocampus (Al'bertin, 2003; Brog, Salyapongse, Deutch & Zahm, 1993; Delfs, et al., 1998; French & Totterdell, 2003; Groenewegen, et al., 1987; Jongen-Relo, Kaufmann & Feldon, 2003; McGinty & Grace, 2009; Meredith, et al., 1990; Mogenson, et al., 1980; Petrovich, et al., 1996; Wang, Rao & Shi, 1992). Interestingly, projections from the basolateral amygdala (BLA) and hippocampus (HIPP) converge monosynaptically on projection neurons within the accumbens shell (French & Totterdell, 2003). Based on this anatomical arrangement, it is suggested that information transmitted from either the amygdala or hippocampus in response to new learning may require critical processing within the nucleus accumbens (Roozendaal, et al., 2001). The accumbens shell therefore, may be in a position to modulate information emanating from limbic structures that encode separate features of newly experienced events.

Information conveyed from the basolateral amygdala to the accumbens shell plays a crucial role in encoding the motivational value of stimuli as well as the affective components of novel learning conditions. Evidence supporting the former idea shows that disruption of inputs between the basolateral amygdala and accumbens impairs the capacity to acquire second-order conditioned responses (Setlow, et al., 2002). This study revealed that although animals may learn that a light stimulus signals food availability, rats with unilateral basolateral and contralateral accumbens lesions fail to learn that a tone presented before the light also signals food availability. Additionally, the accumbens plays a role in integrating information from the amygdala regarding the saliency of an event. A study by Haralambous and Westbrook (1999) revealed that pre-training inactivation of the nucleus accumbens blocks acquisition of contextual fear conditioning. Findings from these two studies suggest that both the amygdala and accumbens are important for organisms to flexibly learn that environmental cues predict upcoming rewards or impending emotionally arousing events such as footshock. Because accumbens inactivation also disrupts contextual learning during fear conditioning, the accumbens shell may be in a position to modulate representations of new experiences that are initially processed not only within the amygdala, but the hippocampus as well.

Similar to the amygdala, hippocampal innervation is differentially segregated within the accumbens. Specifically, the shell region receives direct projections from the ventral subiculum whereas the core region receives projections from the dorsal hippocampus (Groenewgen, et al., 1987). This is an important distinction

given that dorsal and ventral hippocampal areas are attributed different roles in memory processing. Previous studies reveal that ventral hippocampus lesions impair memory for contextual fear conditioning, whereas dorsal lesions disrupt spatial learning in the Morris water maze task (Burhans & Gabriel, 2007; Richmond, et al., 1999). Given that the ventral hippocampus projects to the accumbens shell, it is not surprising that animals with accumbens shell lesions show reduced freezing when returned to the training context where an aversive footshock was administered (Jongen-Relo, Kaufmann & Feldon, 2003). Together these studies provide evidence of a functional relationship between the ventral hippocampus and accumbens shell in processing contextual information. They are also instrumental in showing that disruption of either the ventral hippocampus or the accumbens shell leads to deficits in encoding contextual representations of a learning environment.

Results from electron microscopy studies have confirmed that both the amygdala and hippocampus converge on single output neurons in the accumbens shell (French & Totterdell, 2003). Based on this anatomical arrangement, accumbens neurons are in an ideal position to integrate representations of new learning experiences that are initially processed by the amygdala and hippocampus. In support of this view, Roozendaal and colleagues (2001) found that posttraining infusions of compounds that facilitate later retention when given in either the amygdala or hippocampus after inhibitory avoidance training, are ineffective in influencing memory storage in animals with pretraining chronic accumbens lesions. Other manipulations that interrupt normal

accumbens synaptic activity such as microinfusions of tetrodotoxin also impair retention of a footshock given in a similar inhibitory avoidance task even when the injections are delayed beyond 90 minutes following training (Lorenzini, Baldi, Bucherelli & Tassoni, 1995). Together, these results suggest that processing in the hippocampus or amygdala alone may not be sufficient to influence memory, but may require additional integration within the accumbens.

Additional evidence indicating that the accumbens plays an important role in integrating information conveyed by limbic afferents is provided by findings emerging from neurochemical studies. For example, the accumbens receives excitatory glutamatergic innervations from limbic areas including the amygdala and hippocampus (Blaha et al., 1997; Callaway, Hakan & Henriksen, 1991; Cano-Cebrian et al., 2003; Finch, 1996; Floresco et al., 2001; Floresco et al., 1998; Howland et al., 2002; Legault et al., 2000; Legault et al., 1999). Activation of either limbic structure induces excitatory responses in accumbens shell neurons (Charara & Grace, 2003). During arousing experiences, these cues are especially important for appraisal of the context in which emotionally laden events have transpired. It is well known that processing of arousing experiences requires initial secretion of epinephrine from the adrenals and the subsequent impact this hormone has in potentiating norepinephrine output in the hippocampus and amygdala (Liang, Chen & Huang, 1995; Miyashita & Williams 2004; Wallace, Magnuson & Gray, 1989; Williams, Men, Clayton & Gold, 1998). The hippocampus and amygdala receive noradrenergic innervation via indirect projections from the nucleus of the solitary tract (NTS) to the locus coeruleus

(LC; Haring & Davis, 1985; Loughlin, Foote & Grzanna, 1986; Loy, Koziell, Lindsey & Moore, 1980; Petrov, Krukoff & Jhamandas, 1993). Previous evidence demonstrates that infusions of norepinephrine into the amygdala (Miranda et al., 2003; Roozendaal et al., 2008; Roozendaal et al., 2006; Tully, Li, Tsvetkov & Bolshakov, 2007; van Stegeren et al., 2005; van Stegeren, Wolf, Everaerd & Rombouts, 2008) or hippocampus (Bevilaqua et al., 1997; Birthelmer, Stemmelin, Jackisch & Cassel, 2003; Dommett, Henderson, Westwell & Greenfield, 2008; Izumi & Zorumski, 1999;) improve memory in a wide range of learning conditions. Other studies reveal the source of norepinephrine release within these areas is mediated in part by excitation of NTS neurons in the brainstem. For example, infusion of glutamate into the NTS increases extracellular release of norepinephrine in the amygdala (Miyashita & Williams, 2002). Moreover, in the absence of a functioning NTS, norepinephrine levels in the hippocampus fail to increase in response to peripheral arousal induced by systemic epinephrine injection (Miyashita & Williams, 2004). Recent findings show that glutamate activation of the NTS requires accumbens shell processing (Kerfoot, Chattillion & Williams, 2008). The collective evidence suggests that the mnemonic consequences of activating noradrenergic systems within the amygdala or hippocampus require additional processing within the nucleus accumbens shell.

Given the behavioral, neurochemical and anatomical evidence, Experiment 1 of the present paper addresses whether neuronal processing within the accumbens shell contributes to the enhancement in memory produced by activating the basolateral amygdala or hippocampus with norepinephrine. If the

accumbens is necessary during this consolidation process, it is important to identify the timeframe in which information that is initially encoded by the amygdala and hippocampus is further modified and processed within the accumbens shell. To address these questions, subjects were given posttraining intra-amygdala or hippocampal infusions of norepinephrine at a dose previously shown to enhance memory (Hatfield & McGaugh, 1999; LaLumiere, Buen & McGaugh, 2003). Later, all subjects were given intra-accumbens infusion of muscimol to functionally inactivate the shell. Muscimol inactivation of the accumbens shell was delayed to allow sufficient time for norepinephrine to activate intracellular cascades that lead to long-term synaptic modifications involved in forming new memories. If the accumbens mediates the consequences of limbic activation, then inactivation of the shell should attenuate memory for the aversive experience despite noradrenergic activation of either the hippocampus or amygdala. Experiment 2 examined the strength of the memories formed following footshock and brain infusions. If memories are well formed by activating these limbic structures, then certain aspects of the memory trace should be manifested when the same animals are given a discrimination test in a new apparatus constructed with similar and different contextual features as the original training environment.

General Methods

Subjects

Seventy-eight male Sprague-Dawley rats (275-300 g) obtained from Charles River Laboratories (Wilmington, MA) were used in the following experiments. The rats were individually housed in polypropylene cages with corncob bedding and maintained on a standard 12:12 hour light-dark cycle with lights on at 7:00 A.M. Food and water were available ad libitum during the seven day adaptation period to the vivarium.

Surgery

Each rat received an injection of atropine sulfate (0.1 mg/kg i.p., American Pharmaceutical Partners, Inc., Schaumburg, IL) and was then anesthetized with sodium pentobarbital (50 mg/kg, i.p., Abbot Laboratories, North Chicago, IL). A midline scalp incision was made and bilateral 15 mm long extra thin wall stainless steel guide cannula (25 gauge, Small Parts, Miami Lakes, FL) were implanted 2 mm above the nucleus accumbens shell (AP +0.7, ML ±1.0 from bregma, DV -5.4 from skull surface) and either the basolateral amygdala (AP -3.0, ML ±5.0 from bregma, DV -6.7 from skull surface) or the ventral subiculum of the hippocampus (AP -5.3, ML ±4.5, DV -8.6 from skull surface). All coordinates were adapted from the atlas of Paxinos and Watson (1986). Guide cannulae and two skull screws for anchoring were affixed to the skull with dental cement. The scalp was then closed with sutures and stylets (15 mm, 00 insect dissection pins) were inserted into the injection cannulae to prevent occlusion. Penicillin (0.1 ml i.m., Fort Dodge Animal Health, Fort Dodge, IA) was administered immediately after surgery along with the analgesic, buprenex (0.05 ml s.c., Hospira, Inc., Lake Forrest, IL). The rats remained in a temperature-controlled chamber for at least

one-hour following surgery and were given seven days to recover before initiating food or water deprivation procedures and behavioral training.

Histology

Rats were deeply anesthetized with a euthanasia solution and perfused intracardially with 0.9% saline followed by 10% formalin to verify microinjection cannulae placement. The brains were stored in a 10% formalin and 12% sucrose solution until sectioned on a vibratome. Sections were cut 60 µm thick, mounted on glass slides, subbed with chromium-aluminum and stained with cresyl violet. The location of the cannulae and injection needle tips were verified by examining enlarged projections of the slides (Figure 1a, 1b and 1c).

Methods for Experiment 1

Behavioral Paradigm: Water-Motivated Inhibitory Avoidance Task

Apparatus. A trough-shaped, two compartment rectangular apparatus (91 cm long, 21 cm wide at the top and 6.4 cm wide at the bottom) with a hinged lid was used to train the rats in a water-motivated inhibitory avoidance task. A sliding metal door (14.5 cm) separated a neutral and dark compartment. The neutral compartment was constructed of white opaque Plexiglas (31 cm long) and brightly illuminated by a 60 watt light located directly above the compartment. The dark compartment was constructed of stainless steel plates (60 cm long). A curved stainless steel water spout connected to a 30-cc plastic syringe containing water was placed 1 cm above the floor at the end of the dark compartment.

Training. One-week after surgery, rats were placed on a water restriction schedule with access to water for twenty minutes a day in addition to water consumed during behavioral training. Body weights were monitored daily to insure that weights did not deviate below 10% of their ad-lib feeding weights throughout the experiment. Animals were habituated by placing each in the inhibitory avoidance apparatus for 300 sec. During this time, they were given the opportunity to cross between the white and dark compartment and to explore the drinking spout.

For the next six days of training each rat was placed in the dark compartment facing the retractable door that separated the dark from the illuminated compartment. The metal door was lowered to 2/3 of its length (i.e. creating a 4 cm hurdle). A timer was started and the following measures were recorded until the completion of the trial: a) latency to begin drinking, b) total amount of time spent drinking, c) total amount of time spent in the dark compartment and d) total amount of time spent in the white illuminated compartment. Each training day consisted of one trial lasting 120 seconds.

On Day 7 (i.e. experimental day), each rat was placed in the dark compartment as before however, a 0.35 mA electrical footshock was administered once the rat initiated a lick toward the water spout. The shock remained on until the animal escaped from the dark compartment by crossing over the 4 cm high hurdle into the illuminated neutral compartment. Each animal was retained in the neutral compartment for 30 sec with the door two-thirds open, the door was then raised and the animals remained in the neutral compartment

for an additional 30 sec. Hence, the animals were allowed 60 sec to learn that the white illuminated compartment was safe relative to the dark compartment where footshock was just experienced. Each animal was then removed from the apparatus and given intra-amygdala or hippocampal infusions of PBS or a dose of norepinephrine (0.2 µg) previously shown to enhance memory (Hatfield & McGaugh, 1999; LaLumiere, Buen & McGaugh, 2003). At specific time points after the shock and amygdala or hippocampus injection, all subjects were removed from their home cages and given an intra-accumbens infusion of either muscimol (100 ng) or PBS. The dose of muscimol was based upon those that have been shown to impair memory in the accumbens shell (Reynolds & Berridge, 2001; 2002).

As a basis of comparison, 5 animals in the basolateral group and 5 animals in the hippocampal group were never shocked or injected on this experimental day. These animals were placed into the neutral compartment after reaching the end of the dark compartment before initiating drinking from the spout. They remained in the neutral compartment for 60 sec with the metal door raised. Animals were then removed and placed back in their homecages.

Microinjection Procedure. Each experimental rat was restrained by hand in the experimenter's lap, the stylets were removed and 17 mm, 30 gauge injection needles were inserted bilaterally into either the basolateral amygdala or ventral hippocampus followed an hour later by bilateral injections into the accumbens shell. The tip of the injection needles extended 2 mm beyond the base of the guide cannulae. The needles were connected to 10 µl Hamilton syringes by PE-

20 (polyethylene) tubing. An automated syringe pump (Sage-Orion, Boston MA) delivered 0.5 µl PBS, norepinephrine (0.2µg; Sigma Aldrich, St. Louis, MO) or muscimol (100ng; Sigma Aldrich, St. Louis, MO) over 60 sec. Rats were randomly assigned injection groups. An outline of the injection groups is provided in Table 1. The injection needles were left in place for an additional 60 sec following infusions to ensure complete delivery of the drugs and the stylets were then reinserted into the cannulae.

Retention Test. Retention of the footshock experience in the dark compartment was assessed 24 hr later and consisted of two phases. During Phase 1, the rats were placed in the dark compartment facing the partially lowered metal door and given 60 sec to enter the neutral compartment or alternatively, to initiate the first lick from the water spout. If the rat entered the neutral compartment, the metal door was raised and the rat remained in the neutral compartment for 30 sec. Phase 2 of retention began after this 30 sec period. Those that did not enter the dark compartment after 60 sec were removed and placed in the neutral compartment with the metal door raised for 30 sec. During Phase 2, the metal door was lowered and the time spent avoiding the dark compartment, latency to drink from the spout, total time spent drinking and the total amount of time spent in the neutral compartment was recorded over a period of 300 sec.

Statistical Analysis. The behavioral measures from the water-motivated inhibitory avoidance task are expressed as mean ± standard errors (SE). Between-group comparisons for the behaviors measured during retention testing

were made with a two-way analysis of variance (ANOVA) followed by post hoc tests. Comparisons between the non-shock and non-injection animals and the shock and injection groups were made with paired t-tests.

Methods for Experiment 2

Behavioral Paradigm: Y-Maze Task

Apparatus. In Experiment 2, a trough-shaped Y-maze constructed of stainless steel was used to examine the strength of the memory for the footshock experienced in Experiment 1 (Figure 2). The three alleys of the maze were each 49 cm long x 18.5 cm high. The floor and ceiling were 4 and 19 cm wide, respectively. The floor of the stem arm was covered by a removable cardboard panel with beads attached. This served as a neutral environment, one in which the animals had never been exposed. The left and right alleys each were constructed of two stainless steel plates that were separated lengthwise by a 0.5 cm gap, similar to the shock context in Experiment 1. However, an additional cardboard panel with bedding used in the homecages, was inserted into either the left or right arm in a counterbalanced fashion. This created two distinct arms; one that resembled a safe environment (bedding from the homecage) and one that resembled the context in which the animals had been previously shocked (steel plates). In addition, each of the two alley ways also contained a water spout located at the end of the arm.

Testing. Forty-eight hours following footshock in the water-motivated inhibitory avoidance task of Experiment 1, animals that received either PBS or

norepinephrine injections in the basolateral amygdala or hippocampus and PBS or musicmol 1 hour later in the accumbens shell were placed in the neutral arm of the Y-maze. Each animal was allowed 300 seconds to explore the maze. The location of the “safe” and “shock” arm were counterbalanced so as to control for left or right biases. Measurements included 1) latency to enter the “shock” arm, 2) latency to enter the “safe” arm, 3) latency to lick from either spout located at the end of each arm, 4) cumulative time spent drinking from the water spout and 5) cumulative time spent in each of the three arms.

Statistical Analysis. The behavioral measures from the Y-maze task are expressed as mean ± standard errors (SE). Between-group comparisons for the behaviors measured during retention testing were made with analysis of variance (ANOVA) followed by post hoc tests.

Results for Experiment 1

Basolateral Amygdala Injections

Phase 1 Retention Testing:

Latency to Escape the Shock Compartment. A two-way ANOVA on the mean latency to exit the shock context and enter the neutral compartment revealed no significant interaction between the treatment groups, $F(2, 26) = 1.81$, p = ns. Although all animals remained in the shock compartment for a similar amount of time, separate t-tests revealed that the latency to first lick and initiate drinking from the spout was significantly longer in all of the experimental groups given footshock 24 hours relative to the non-shock control group ($p < 0.05$

compared to each shocked group). A comparison of latencies between shocked and non-shocked controls groups on this measure establishes that all experimental groups learned something about the footshock experienced 24 hours earlier as compared to animals that never got shocked.

Phase 2 Retention Testing:

Contextual Memory. Animals begin the second phase of retention testing in the white neutral compartment of the apparatus. After a 30 sec delay, access to the dark (shock) compartment is made possible by lowering a guillotine door that separates the two different contextual sections of the apparatus. The latency to enter the dark compartment is then recorded and serves as an index of memory for the shock experienced in this context 24 hours earlier. A two-way ANOVA indicated no significant interaction between basolateral amygdala (BLA) and accumbens treatments for the latency to enter the darker shock compartment, $F(2, 26) = 0.49$, $p = ns$. As shown in Figure 3, all experimental groups spent a similar length of time in the neutral compartment before entering the context where footshock was administered 24 hours previously.

Response Specific Memory. Since the footshock that is given on day 7 of training is not initiated until each subject approaches the spout to begin drinking, there are two possible representations of this event that may be encoded into memory. These include, 1) the context in which the footshock occurred and 2) the instrumental action emitted before delivery of the footshock (approaching the spout to drink). To assess memory for the second possible representation, the time required to drink from the spout after entering the shock context as well as

the duration of time spent drinking was recorded. As shown in Figure 4, a two-way ANOVA revealed a significant interaction between BLA and accumbens treatments, $F(2, 26) = 3.95$, $p < 0.05$. Although all animals in the shock and injection groups readily entered the shock context, only animals that received norepinephrine in the BLA and PBS in the shell took significantly more time to initiate drinking from the water spout ($p < 0.01$, compared to all other treatment groups). Muscimol in the accumbens given 1 hour or 7 hours later attenuated memory in animals given the same dose of norepinephrine in the BLA as evidenced by how quickly these animals initiated licking. Individual t-tests revealed no significant difference in latency to begin licking the spout between non-shock controls and all other treatment groups, excluding NE/1hr PBS animals.

As an additional measure, the amount of time each animal spent drinking after initial contact was made with the water spout was recorded. A two-way ANOVA revealed significant differences between treatment groups in the mean time spent drinking from the spout where footshock occurred 24 hours earlier, $F(2, 26) = 4.29$, $p < 0.05$ (Figure 5). Animals in the NE/1hr PBS group spent significantly less time drinking from the spout relative to all other treatment groups ($p < 0.01$). Again, regardless of the time delay between BLA and accumbens injections (1hr vs. 7hr), muscimol in the accumbens blocked the influence of activating noradrenergic receptors in the BLA.

Hippocampus Injections

Phase 1 Retention Testing:

Latency to Escape the Shock Compartment. Similar to the findings obtained with the basolateral amygdala groups, the mean latency to exit the shock context and enter the neutral compartment was not statistically different between the separate hippocampal treatment groups ,two-way ANOVA, $F(2, 26) = 1.42$ p = ns. Although all groups remained in the shock compartment for a similar amount of time, a t-test revealed that only animals in the non-shock control group initiated drinking from the spout faster than shocked animals ($p < 0.05$ compared to all other treatment groups).

Phase 2 Retention Testing:

Contextual Memory. To measure memory for the context where footshock was delivered, the latency to enter the shock compartment was measured from the beginning of Phase 2 (animals start in the neutral compartment). As shown in Figure 6, a two-way ANOVA revealed a significant interaction between hippocampal (HIPP) and accumbens infusions, $F(2, 32) = 10.56$, $p < 0.01$. All treatment groups preformed in a similar fashion as those animals in the non-shock control group, except the NE/1hr PBS group. Animals given norepinephrine in the hippocampus and PBS in the accumbens an hour later, took significantly more time to enter the context where footshock was delivered ($p < 0.01$ compared to all treatment groups). Infusion of muscimol in the accumbens either 1 hour or 7 hours later attenuated the memory enhancing

effect of activating the hippocampus with norepinephrine ($p < 0.01$ for NE/1hr MUSC and NE/7hr MUSC groups compared to NE/PBS group).

Response Specific Memory. A two-way ANOVA also revealed significant differences between treatment groups on the latency to lick the spout after entering the dark compartment, $F(2, 32) = 50.84$, $p < 0.01$. The NE/1hr PBS group took significantly longer to enter the shock context than any other group and also required a significantly longer period of time to lick the spout ($p < 0.01$, compared to all treatment groups; Figure 7). The long latencies are reflective of the fact that 4 of 5 animals in this group never left the neutral compartment during this phase of testing (300 seconds). Blocking neuronal transmission in the accumbens with muscimol attenuated the extended avoidance response to both enter the dark or to drink from the spout displayed by the group given posttraining intra-hippocampal infusion of norepinephrine. Individual t-tests revealed that animals in the NE/1hr MUSC and NE/7hr MUSC groups performed similarly to animals that never experienced a shock. Moreover, there was a significant interaction between treatment groups on the cumulative time spent drinking from the spout as revealed by a two-way ANOVA, $F(2, 32) = 16.32$, $p < 0.01$ (Figure 8). All of the groups experienced long bouts of licking from the spout, similar to non-shock controls, except those animals given norepinephrine in the hippocampus and PBS an hour later in the shell.

Comparison Between BLA and HIPP Treatments

Since all groups in Experiment 1 experienced identical training and footshock procedures, it is possible to determine whether posttraining treatments rendered within the basolateral amygdala versus the hippocampus, differentially affects memory for the separate responses that are measured during retention testing. The water-motivated inhibitory avoidance task has been used to assess the contribution of amygdala processing during arousing situations (Miyashita & Williams, 2002). The task design also has strong contextual features that require hippocampal processing. Thus, an assessment of performance in groups given PBS or NE within these structures should determine which aspects of this task are more sensitive to amygdala versus hippocampal processing.

Table 2 shows the means, standard error, mean difference, and significance between groups for three critical measures, as assessed by individual t-tests. Animals given PBS in either the basolateral amygdala or ventral hippocampus perform in a similar fashion across measures of latency to enter the shock compartment, latency to initiate licking from the spout after entering the dark compartment and cumulative time spent drinking. However, there are significant differences in basolateral amygdala norepinephrine infusions compared with hippocampal norepinephrine infusions. Animals given norepinephrine in the hippocampus took significantly longer to enter the shock compartment ($p < 0.01$) and drink from the water spout ($p < 0.01$) relative to the basolateral groups given the same treatment. The hippocampus group also spent significantly less time

drinking compared to animals given norepinephrine in the basolateral amygdala ($p < 0.01$).

Results for Experiment 2

A second experiment was conducted to assess the strength of the representation of footshock training retained in memory for the separate norepinephrine and muscimol treatment groups used in Experiment 1. For this purpose, subjects were given a discrimination test in a Y-maze apparatus. This apparatus was modified such that only one of the maze alleys contained the same contextual attributes (i.e. metal walls and footshock plates) that were present during footshock delivery in Experiment 1 whereas the remaining two alleys were constructed with completely different contextual features.

Two-way ANOVAs were used to compare performance between groups that received PBS or norepinephrine in the amygdala or hippocampus that were followed one hour later by PBS or muscimol infusions into the accumbens shell during Experiment 1. Results showed a significant difference between treatments on the latency to enter the arm that resembled the shock compartment, $F (1, 33) = 4.58$, $p < 0.05$ as well as the latency to first lick from the spout in the arm that resembled the shock compartment, $F (1, 33) = 4.22$, $p < 0.05$. As shown in Figure 9, only animals with hippocampal infusions of norepinephrine and accumbens infusions of PBS took significantly longer to enter the shock arm ($p < 0.01$ compared to all treatment groups). These animals also took significantly more time to initiate licking from the spout located in the “shock” arm ($p < 0.01$

compared to all treatment groups; Figure 10). Disruption of accumbens processing 48 hours previously with muscimol attenuated these two effects. Infusions of norepinephrine in the basolateral amygdala did not produce any appreciable differences in performance as compared to PBS/PBS controls. Figure 11 shows there were no group differences on the cumulative time spent drinking from the spout, $F(1, 33) = 3.77$, p = ns. This means that although animals with hippocampal infusions of norepinephrine and accumbens infusions of PBS took longer to enter the shock context and drink from the shock context, they drank from the shock arm spout for the a similar amount of time as all other treatment groups.

Discussion

Although findings from anatomical and physiological studies revealed that amygdala and hippocampal inputs converge on single neurons in the accumbens shell (French & Totterdell, 2003), evidence suggesting that accumbens processing is necessary to integrate new learning experiences from these limbic areas into memory is scarce. Moreover, experimental findings implicating the actual time frame in which the accumbens contributes to encoding novel events into memory storage has not been successfully documented. Results emerging from the present experiments are instrumental in addressing both of these shortcomings in the literature.

Findings from Experiment 1 confirm previous studies demonstrating that posttraining activation of noradrenergic receptors within the basolateral amygdala

or hippocampus facilitate subsequent retention performance (Bevilaqua et al., 1997; Birthelmer et al., 2003; Dommett et al., 2008; Izumi & Zorumski, 1999; Miranda et al., 2003; Roozendaal et al., 2008; Roozendaal et al., 2006; Tully et al., 2007; van Stegeren et al., 2005; van Stegeren et al., 2008). The present results also extend these findings by revealing that noradrenergic activation of the amygdala or hippocampus differentially facilitates the category of representations formed after emotionally arousing events involving unexpected footshock. For example, noradrenergic activation of the amygdala facilitates memory for response specific representations directly associated with footshock delivery (Figure 4), whereas activation of the hippocampus enhances memory for the context in which the emotionally arousing footshock is delivered (Figure 6).

Second, animals given intra-hippocampal infusions of norepinephrine (HIPP-NE) took significantly longer than all other treatment groups to enter the Y-maze alley in Experiment 2 that was contextually similar to the dark compartment where footshock was delivered in the first study. This finding indicates that noradrenergic activation of the hippocampus not only leads to more stable representations of the footshock event over time, but this memory also generalizes to new learning conditions involving similar contextual stimuli. In contrast, the response specific memory associated with licking the spout that was evident in subjects given intra-amygdala infusions of norepinephrine was not as robust when this group was placed in the Y-maze although it contained similar contextual features. These animals readily entered the context of the Y-maze

containing the metal footshock plates and did not hesitate before drinking from the water spout (Figure 10).

The most intriguing findings of the current study reveal that the consequences of activating noradrenergic receptors in the amygdala or hippocampus are mediated in part by actions initiated within the accumbens shell. The data show that inactivation of the shell with the GABAergic agonist muscimol, attenuates memory enhancement produced by activating either the amygdala or hippocampus. This attenuation in memory was evident when neuronal activity in the accumbens shell as interrupted either 1 or 7 hours after the limbic drug infusions. These results extend what is currently known regarding the time frame in which the accumbens contributes to mnemonic processing (Lorenzi et al., 1995) and demonstrates that this activity is critical during the initial and late stages of consolidation.

Differences in Amygdalar and Hippocampal Processing

Results from the current study are in concordance with previous findings (Bevilaqua et al., 1997) that noradrenergic activation of the amygdala or hippocampus facilitates memory for an arousing footshock experience. Using a one-trial step down inhibitory avoidance task, Bevilaqua and colleagues (1997) found that basolateral activation enhances memory only when norepinephrine is administered immediately posttraining. Noradrenergic activation of the hippocampus, however, enhances memory for the footshock experience when administered 0, 3 or 6 hours posttraining. These data suggest that memory

modulation in the hippocampus occurs for up to 6 hours compared to amygdala modulation. However, measures of step-down latency used in the previous study fail to discern the specific contributions each limbic structure provides to the memory representation of the footshock experience. For example, information conveyed from the basolateral amygdala to the accumbens shell plays a crucial role in the learning and storing into memory the motivational value of stimuli. In contrast, hippocampal afferents to the accumbens provide information regarding contextual features of the environment. Results from the present work not only show that noradrenergic activation of limbic structures enhances memory, but reveal key differences in activating the amygdala or hippocampus (Table 2).

The behavioral paradigm used in Experiment 1, was developed to dissociate representations in memory for the contextual versus the response specific aspects of learning that occur following unexpected footshock delivery. Therefore, it was possible to evaluate the differential contributions each limbic structure provides following activation. Results showed that activation of the basolateral had no effect on the latency to enter the context where footshock had been administered 24 hours previously (Figure 3). However, this treatment was shown to facilitate memory on measures relating to response specific aspects of the task such as latency to lick the spout and the cumulative time spent drinking (Figure 4 and 5). In contrast to amygdala activation, infusions of norepinephrine in the ventral hippocampus facilitate memory for the context in which the footshock transpired (Figure 6). Because these animals took significantly longer to enter the shock context, they also have longer latencies to initiate drinking

from the water spout. These behavioral findings provide functional evidence that supports electrophysiological data showing that hippocampal activation generates longer durations of accumbens activity as compared to amygdala stimulation (Grace, 2000). Together with the current behavioral data, it can be suggested that the longer periods of neuronal activity in the accumbens in response to hippocampal activation reflect the attention required to process contextual cues. During initial training in the water-motivated inhibitory avoidance task, animals learn that a context that was once pleasant (provided the availability of water) is now aversive (footshock). On the other hand, it can be suggested that the brief period of accumbens neuronal activity following amygdala stimulation reflects event-related processing. This is supported by findings that, although animals infused with norepinephrine in the amygdala readily enter the shock compartment, they still require a significantly longer period of time to begin drinking from the spout (last response emitted before the footshock was delivered). The difference in the magnitude of memory enhancement between basolateral and hippocampal animals shown in the current study provide behavioral support for the view that during emotionally salient events, hippocampal input may dominate with contextual processing compared to basolateral amygdala input, which may tag the affective value of the situation (Grace, 2000).

In contract to the current results, another study found the basolateral amygdala to be involved in contextual learning and that processing in the hippocampus is not required 6 hours posttraining (Sacchetti et al., 1999). Several

procedural dissimilarities underlie the discrepancy reported between these data and the current findings. First, it should be noted that Sacchetti and colleagues (1999) used Pavlovian fear conditioning procedures such that a tone preceded a footshock. The pairing of tone with a shock occurred seven times, giving the animal ample time to associate the tone and shock with the context. In the current study, animals were given only a single footshock that did not persists beyond 2 seconds before they escaped into the neutral compartment. An additional difference between the two behavioral paradigms is that the current study required an instrumental response of approaching the spout before the footshock was delivered. This allows for a more precise association between action and stimulus (shock). The second discrepancy that should be noted is the target area of the hippocampus. Sacchetti and colleagues (1999) found that processing in the dorsal hippocampus is not required 6 hours after the footshock experience. This means that 6 hours posttraining, blockade of dorsal hippocampal neurons has no influence on memory. But the current study investigated the contribution of **ventral** hippocampal processing to memory consolidation. If the ventral hippocampus were similar to the dorsal, then only animals given norepinephrine in the hippocampus and muscimol in the accumbens1 hour later would show attenuation in contextual memory. However, the current results showed that an intact pathway from ventral hippocampus to accumbens is required 7 hours posttraining in order to facilitate memory for where the footshock occurred. Taken together, these findings suggest that dorsal and ventral hippocampal areas not only differ in the pattern of innervation to the

accumbens, but also in the length of time that neuronal processing is required to facilitate contextual and spatial memories.

Gating of Limbic Information within the Nucleus Accumbens Shell

Several studies indicate that accumbens neurons require activation from more than one source to reach threshold (Callaway Hakan, & Henriksen, 1991; DeFrance, Marchand, Sikes, Chronister & Hubbard, 1985). This constraint on activity may explain why accumbens neurons receive inputs from several memory related areas. In particular, projections from the basolateral amygdala and ventral hippocampus converge monosynaptically on projection neurons within the caudomedial region of the accumbens shell (French & Totterdell, 2003). Because the accumbens receives converging inputs from multiple areas, it is important to understand how separate inputs may regulate neuronal firing in this structure. An emerging idea in the literature dealing with the functionality of the nucleus accumbens is the hypothesis of neural networks. Pennartz and colleagues (1994) propose that the accumbens is comprised of neuronal ensembles. These ensembles are best understood in terms of their collective activation.

In a recent study conducted by McGinty and Grace (2009), neurons in the nucleus accumbens were shown to integrate limbic and cortical innervations depending on the intensity and timing of inputs. Specifically, weak stimulation of two inputs generates more excitation of accumbens neurons than activation of either structure alone. When these stimulations occur at the same time,

accumbens neurons become active. This electrophysiological characteristic of neurons in the accumbens establishes a coincidence detection system such that areas that fire together have direct influence over accumbens activity. However, an interesting finding by McGinty and Grace (2009) showed that strong activation of one input may disrupt processing of the second input. This electrophysiological feature may serve as the mechanism underlying behavioral differences in hippocampal and amygdala activation reported in the present study. Although activation of both limbic structures facilitates memory for certain aspects of the water-motivated inhibitory avoidance task, the magnitude of the facilitation was greater in hippocampal animals (Table 2). The reason may be due to the fact that delivery of the footshock in Experiment 1 continued the whole length of the dark compartment until animals escaped into the safe/neutral compartment. The animals remained in the neutral compartment for 30 seconds before the retractable door was raised. During this 30 second period of time, animals could see the dark compartment and form a distinct representation between the "dark" shock and "illuminated" safe compartments of the apparatus. This component of the training procedure may account for the stronger degree of activation in the hippocampus. This strong activity may have disrupted amygdala processing as proposed by McGinty and Grace (2009), leading to a facilitation in contextual measures in animals with norepinephrine infusions in the hippocampus as compared to the amygdala.

Significance of Accumbens Involvement in Long-term Consolidation

The accumbens not only plays a role in the integration of information emanating from the hippocampus or amygdala, but is also involved in the consolidation of these processes into memory. For example, rats with accumbens lesions fail to modify response latencies or reaction time to cues that lead to aversive outcomes, such as the delivery of quinine in the place of an anticipated liquid sucrose reward (Schoenbaum & Setlow, 2003). The consolidation of memory for these and other types of emotionally arousing events is a time dependent process that does not happen instantly, but rather occurs over hours. Studies employing functional inactivation techniques to produce reversible lesions demonstrate that neuronal activity in the accumbens is crucial for consolidation for at least 90 minutes following learning (Lorenzini, Baldi, Bucherelli & Tassoni, 1995).

While findings from the current study are in agreement with previous results (Lorenzini et al., 1995), they also extend what is currently known about the integrative nature of accumbens neurons and the timeframe in which accumbens processing is required. First, the present work demonstrates that activation of the amygdala or hippocampus is not sufficient to enhance memory when accumbens activity is disrupted with muscimol. Second, and most importantly, results from the present study determine the temporal window in which neurons from the accumbens are required to process the beneficial information emanating from the amygdala or hippocampus. Findings show that without accumbens processing 1 or 7 hours posttraining, memory for a footshock experience is attenuated despite limbic activation. This timeframe corresponds to synaptic changes identified in

other brain regions. For example, three phases of synaptic plasticity have been identified in the hippocampus posttraining: 1) synaptic loosening (2-6 hours), 2) synaptic reorganization (6-9 hours) and 3) synaptic selection (9-24 hours). Microarray analysis revealed two distinct waves of gene upregulation during these phases (O'Sullivan et al., 2007). The first wave occurs between 0 and 2 hours whereas the second wave occurs between 6 and 9 hours. Results from the current study reveal that the accumbens is necessary not only during the first wave, but second wave time point as well. Together these findings suggest that along with the hippocampus, the accumbens shell is also involved in the consolidation process during this second wave of gene upregulation.

Furthermore, studies have shown norepinephrine in the hippocampus increases cAMP and PKA activity as well as pCREB levels 6 hours later (Bevilaqua et al., 1997). Initial activation of these second messenger cascades immediately posttraining is thought to correspond to the phase in synaptic plasticity involved in synaptic loosening. However, the second wave of cAMP, PKA, CREB and subsequent gene regulation may be involved in the final stages of synaptic plasticity. According to O'Sullivan and colleagues (2007), genes that are upregulated 6 to 9 hours after learning were those involved in synaptic selection. In light of this finding, the current data can be interpreted to suggest that the accumbens shell is involved in the late phase of consolidation which gives rise to the selection of synapses that will represent the engram for the learning experience.

Conclusions

This study provides evidence that blocking accumbens functioning with muscimol an hour or even seven hours following amygdala or hippocampus activation attenuates the improvement in memory seen following noradrenergic activation of the amygdala or hippocampus alone. These findings suggest that the accumbens shell plays an integral role modulating information initially processed by limbic structures following exposure to emotionally arousing events. Additionally, results are integral in determining the involvement of the accumbens shell in long-term consolidation processes lasting over 6 hours. These processes may involve synapse selectivity during memory formation.

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Figures

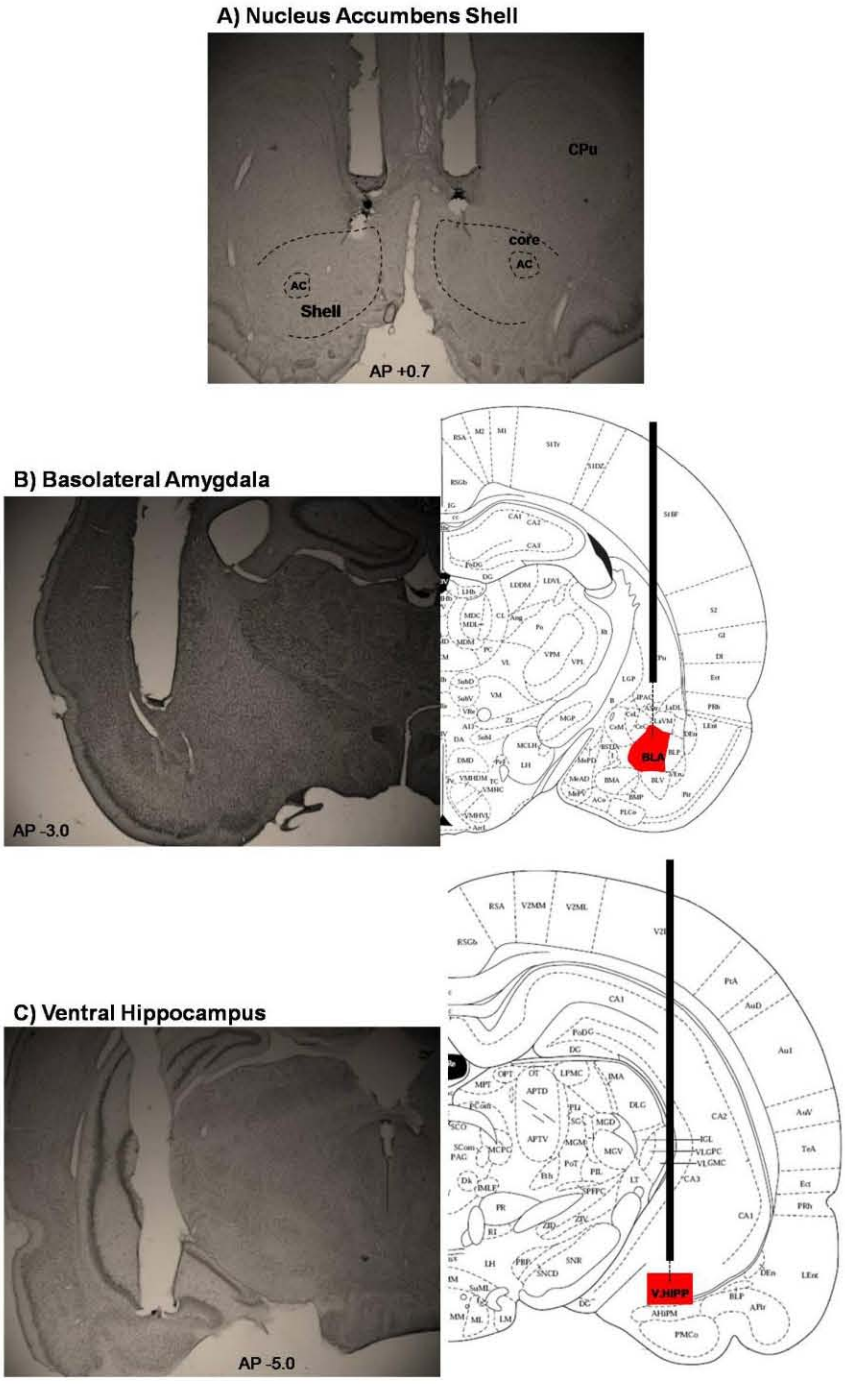

Figure 1a, 1b and 1c. Location of injection needle tip placements in the A) nucleus accumbens shell, B) basolateral amygdala and C) ventral hippocampus. Abreviations: AC = anterior commissure, core = nucleus accumbens shell, CPu = caudate putamen and Shell = nucleus accumbens shell. Brain diagram from “The Rat Brain in Stereotaxic Coordinates”; adapted from Paxinos and Watson.

Table 1: Injection Groups

Basolateral Amygdala **BLA Injection**	**Time Delay →**	**NAC-Shell Injection**	**Total N**
PBS	1hr	PBS	n = 5
PBS	1hr	MUSC	n = 5
PBS	7hr	MUSC	n = 7
NE	1hr	PBS	n = 5
NE	1hr	MUSC	n = 5
NE	7hr	MUSC	n = 5

Ventral Hippocampus **HIPP Injection**	**Time Delay →**	**NAC-Shell Injection**	**Total N**
PBS	1hr	PBS	n = 6
PBS	1hr	MUSC	n = 6
PBS	7hr	MUSC	n = 7
NE	1hr	PBS	n = 5
NE	1hr	MUSC	n = 5
NE	7hr	MUSC	n = 7

Table 1. This table depicts the injection treatments for the basolateral amygdala (BLA), ventral hippocampus injections (HIPP) and the accumbens shell (NAC). Also included is the time delay between limbic injections and accumbens infusions.

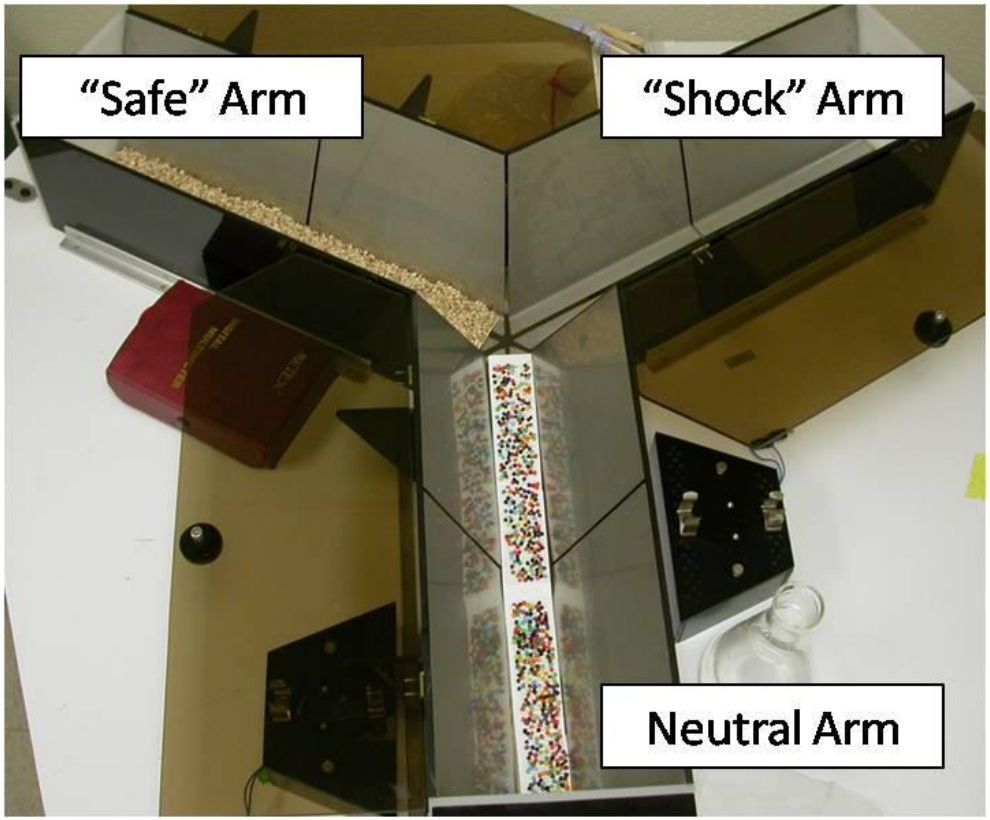

Figure 2. Picture of the Y-maze used in Experiment 2. The neutral arm served as the main stem of the maze. Flooring for the neutral arm consisted of a beaded cardboard insert; a novel environment and texture. The left and right arms of the maze were counterbalanced so there was an equally likely chance of the right arm resembling the "shock" or "safe" arm. The "safe" arm consisted of corncob bedding used in the animal's home cage. The "shock" arm had metal floors similar to those in the water-motivated inhibitory avoidance task in which animals were previously shocked.

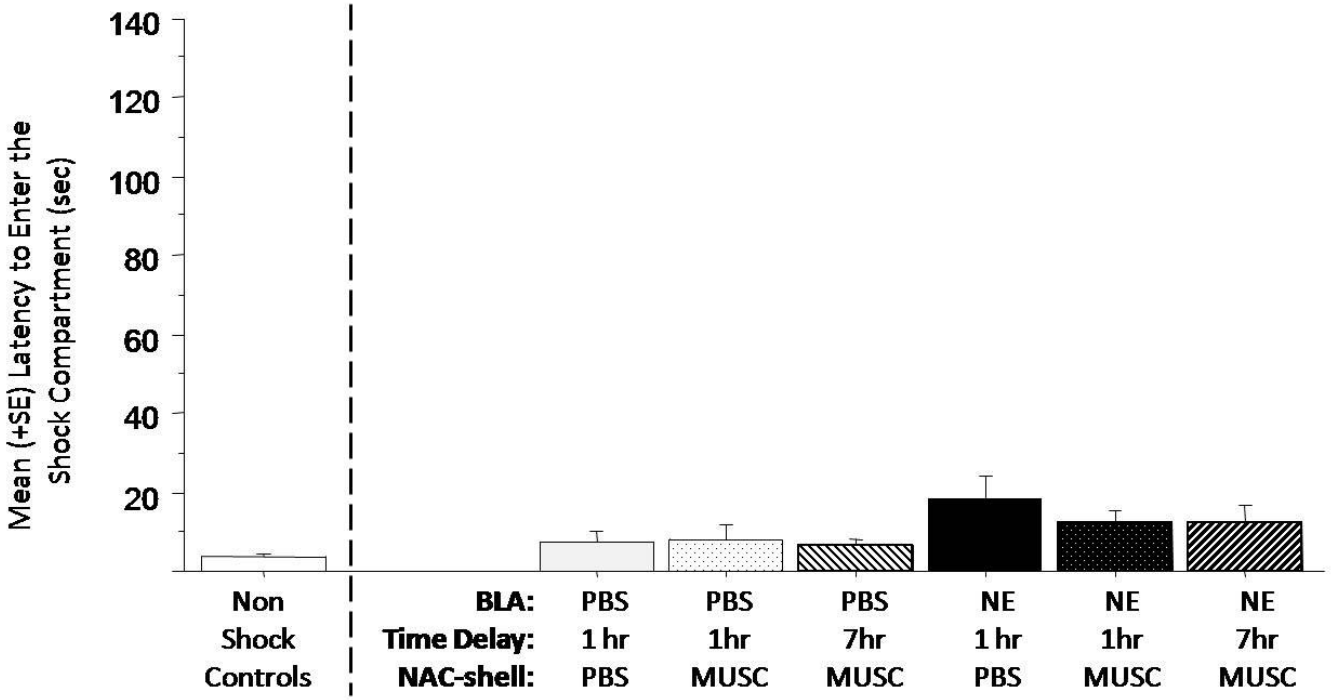

Figure 3. Mean (+ SE) latency for subjects with bilateral basolateral and accumbens shell cannulae implants to enter the compartment where shock was delivered 24 hours earlier. There were no group differences in the time it took animals to enter the shock context. Individual t-tests revealed that all groups performed in a similar fashion as animals that never experienced shock or injections.

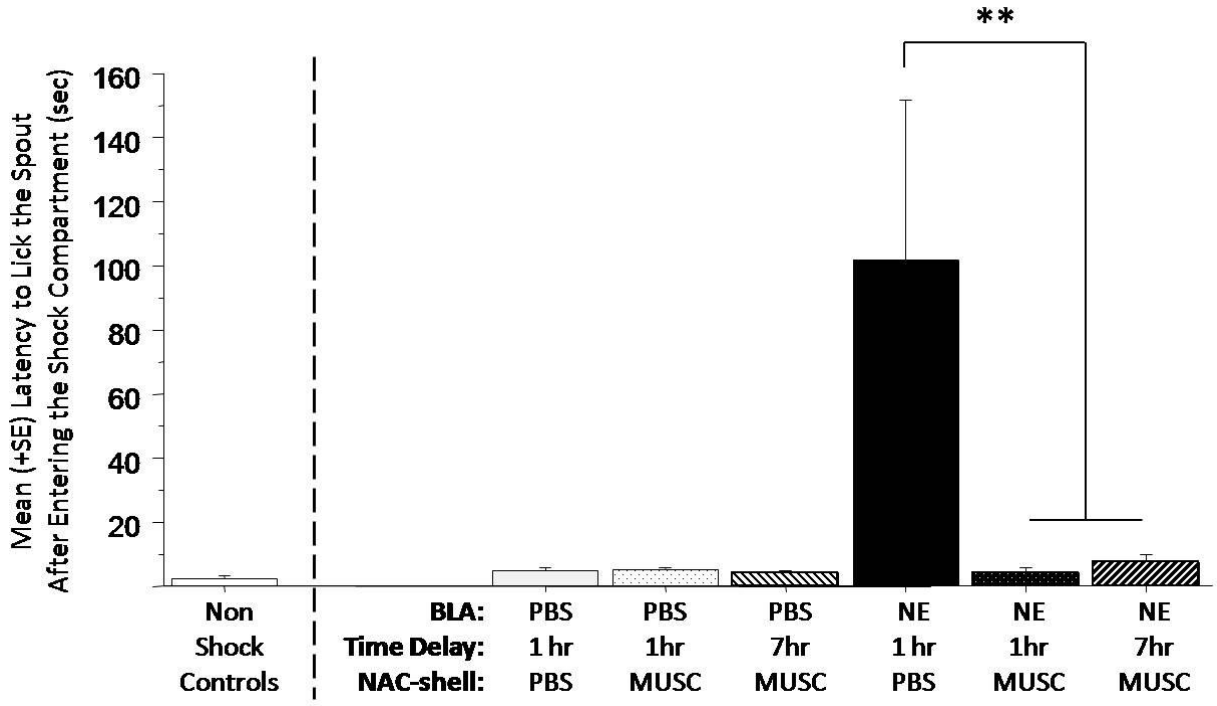

Figure 4. Mean (+ SE) latency for animals with basolateral amygdala and accumbens shell cannulae implants to lick the spout after entering the shock compartment. Although all groups readily entered the dark compartment, only animals given norepinephrine in the basolateral amygdala (BLA) and PBS in the accumbens (NAC) took significantly longer to traverse the dark compartment and initiate licking from the spout ($p<0.01$). Infusions of musicmol in the accumbens 1 or 7 hours later were able to attenuate the enhancement in memory following noradrenergic activation of the BLA. ** denotes $p<0.01$.

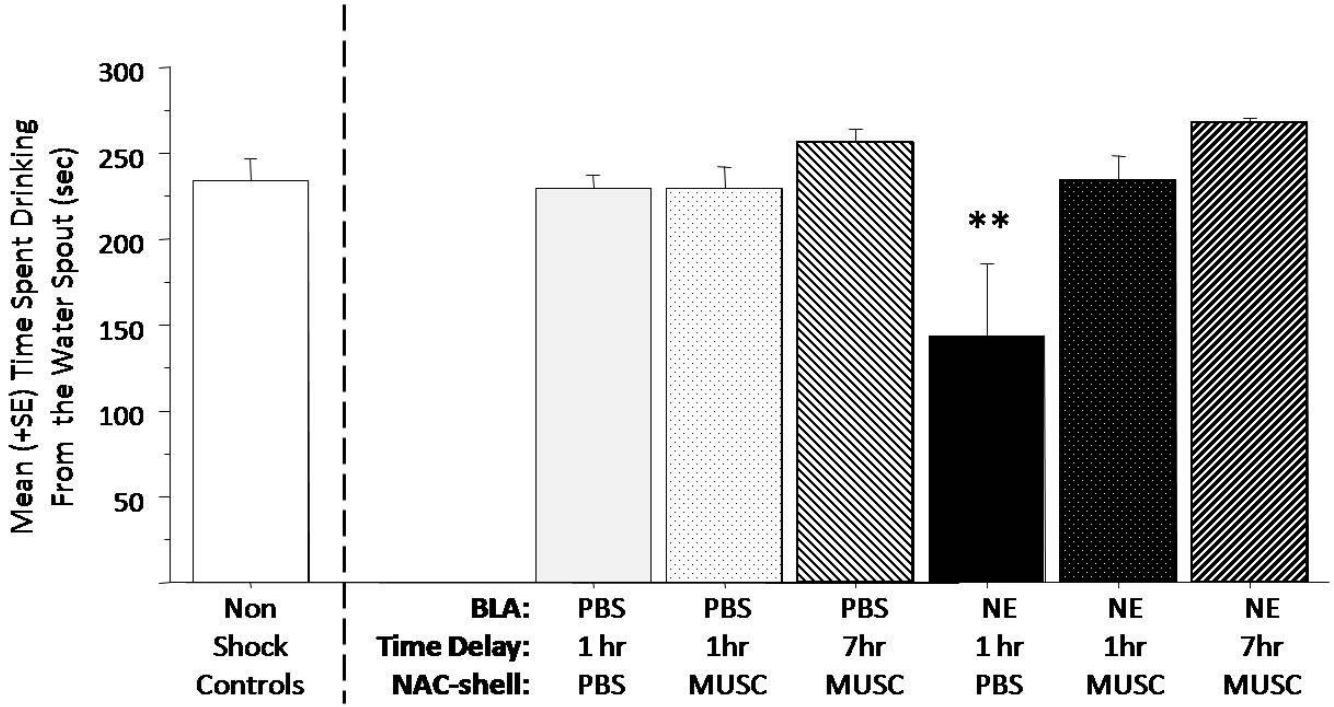

Figure 5. Mean (+ SE) time spent drinking from the water spout located in the dark compartment in animals with basolateral and accumbens shell cannulae implants. The cumulative time spent drinking was significantly reduced in animals given intra-basolateral infusion of norepinephrine and intra-accumbens PBS ($p < 0.01$). Again, inactivation of the accumbens shell with muscimol 1 or 7 hours later blocked this effect. Animals in the NE/1hr MUSC and NE/7hr MUSC groups drank for a similar amount of time as non-shock control animals. ** denotes $p<0.01$.

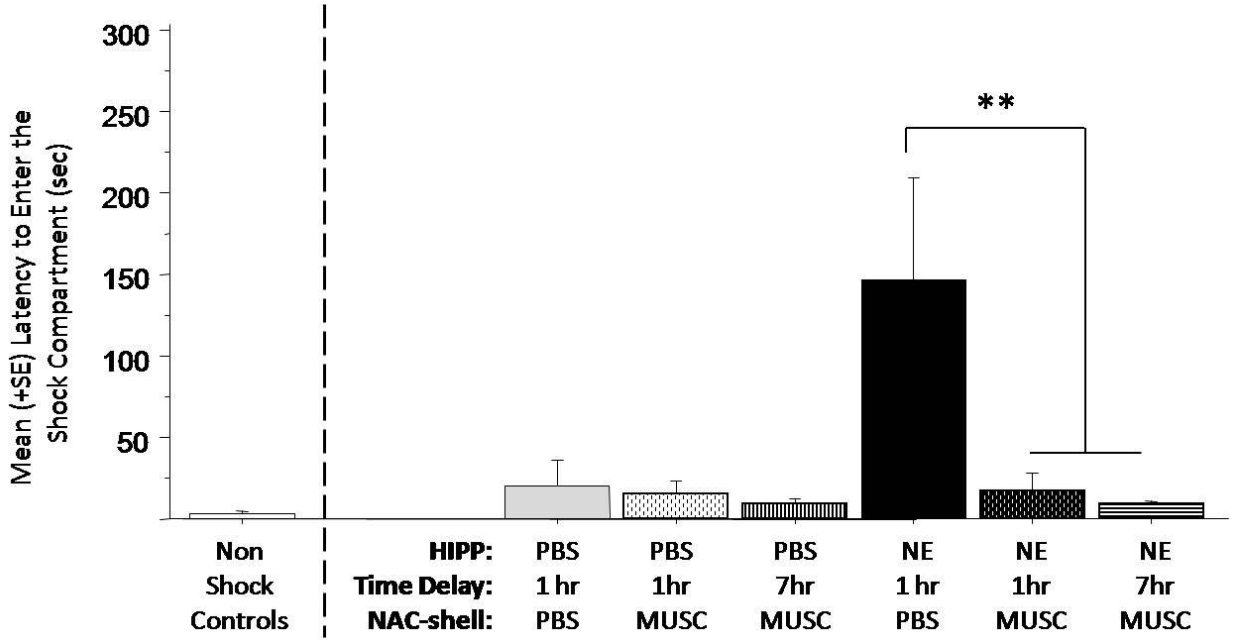

Figure 6. Mean (+ SE) latency to enter the compartment where shock was administered 24 hours previously in animals with ventral hippocampal and accumbens shell cannulae implants. Only animals given norepinephrine in the hippocampus took significantly longer to enter the context where footshock had been delivered 24 hours previously ($p < 0.01$). Infusion of muscimol in the accumbens either 1 hour or 7 hours later attenuated the memory enhancing effect ($p < 0.01$ for NE/1hr MUSC and NE/7hr MUSC groups compared to NE/PBS group).This is in direct contrast to animals given the same dose of norepinephrine in the basolateral amygdala that readily entered the shock compartment (Figure 3). ** denotes $p<0.01$.

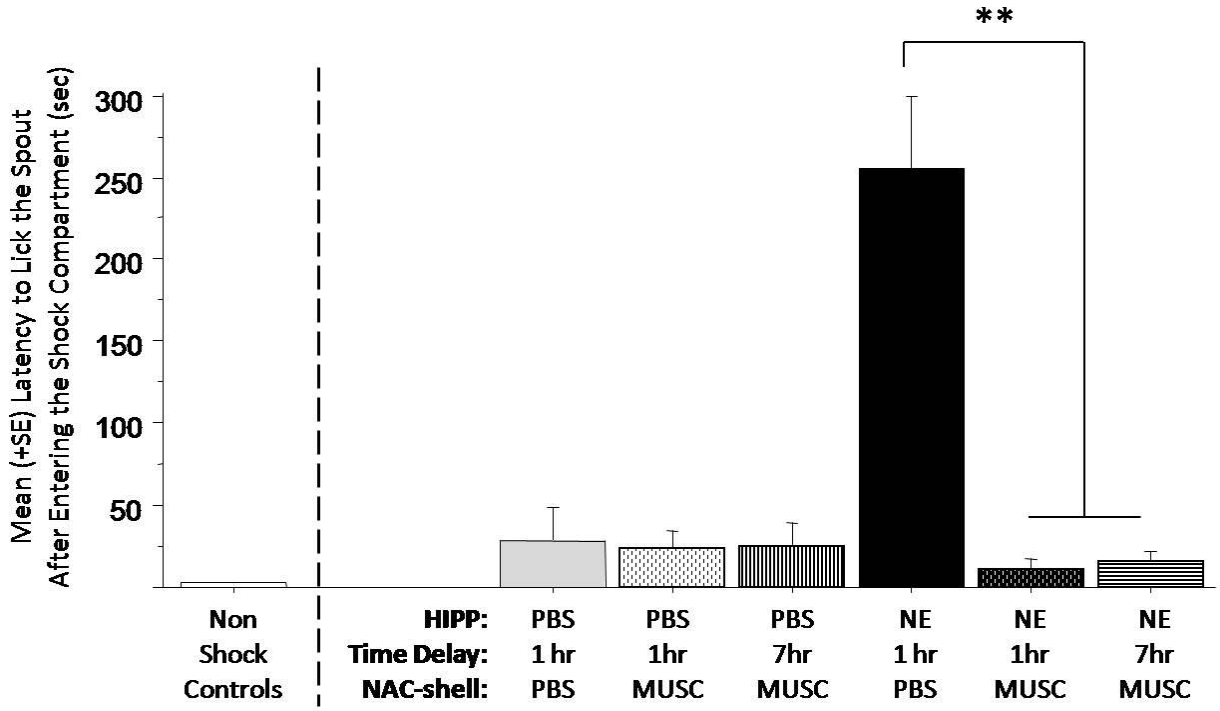

Figure 7. Mean (+ SE) latency for animals with ventral hippocampal and accumbens shell cannulae implants to initiate licking from the spout after entering the dark compartment. Not only did animals in the NE/1hr PBS group take longer to enter the shock context, but they also took significantly longer to initiate drinking from the spout compared to all other treatment groups ($p<0.01$).

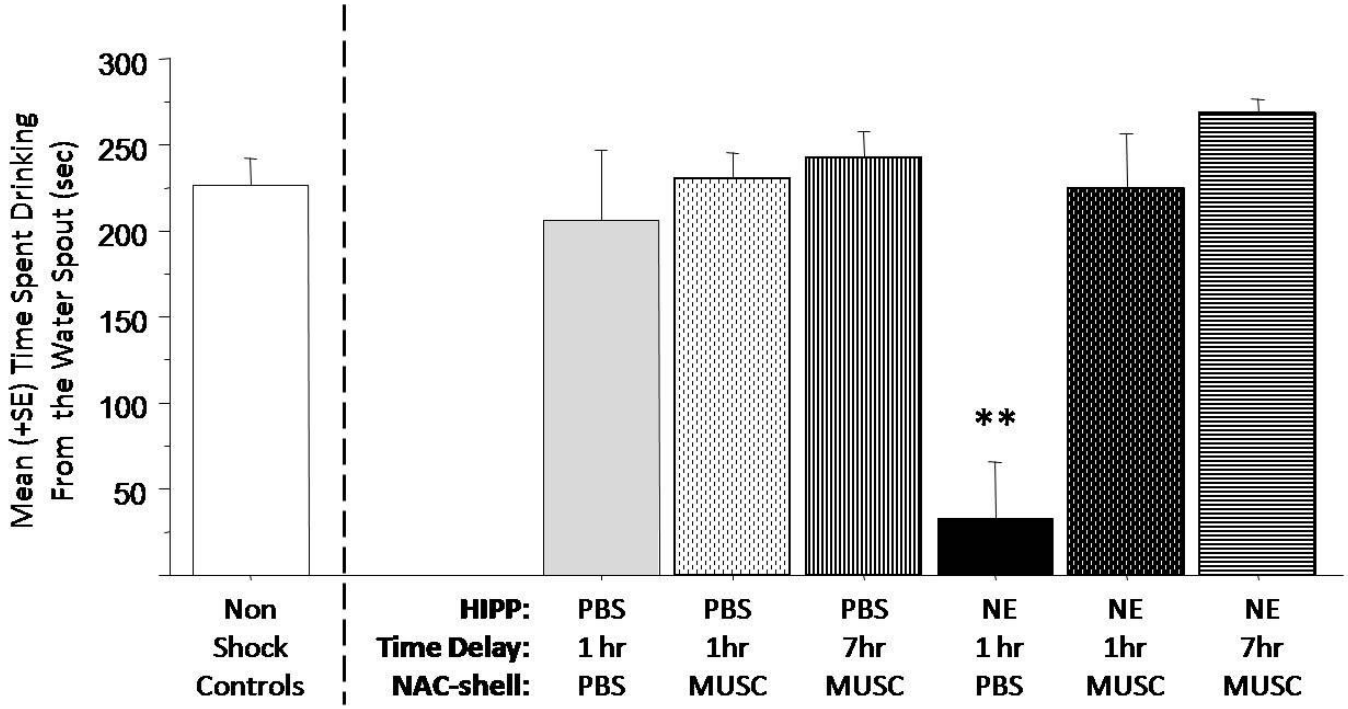

Figure 8. Mean (+ SE) time spent drinking from the water spout located in the dark compartment in subjects with ventral hippocampal and accumbens shell cannulae implants. Disruption of accumbens neuronal functioning via infusions of muscimol either 1 hour or 7 hours later blocks the memory enhancement of activating hippocampal neurons with norepinephrine. This is evidenced by how long animals in these groups spent drinking compared to animals treated with the same dose of norepinephrine in the hippocampus and PBS in the accumbens. Only NE/1hr PBS animals spent a significantly less amount of time drinking from the spout ($p<0.01$).

Table 2: Comparison of BLA and HIPP Treatments

**Only those groups in which the accumbens received PBS are shown*

Latency to Enter the Dark Compartment

BLA	Mean ± SE	HIPP	Mean ± SE	Mean Diff.	p Value
PBS	7.0 ± 3.1	PBS	21.1 ± 14.5	-14.05	0.75
NE	18.1 ± 6.1	NE	146.3 ± 63.0	-128.24	0.01

Latency to Lick the Spout After Entering the Dark Compartment

BLA	Mean ± SE	HIPP	Mean ± SE	Mean Diff.	p Value
PBS	4.9 ± 1.2	PBS	28.5 ± 20.2	-23.58	0.62
NE	101.5 ± 50.4	NE	256.1 ± 43.9	-154.54	0.006

Total Time Spent in the Neutral Compartment

BLA	Mean ± SE	HIPP	Mean ± SE	Mean Diff.	p Value
PBS	7.4 ± 3.0	PBS	24.1 ± 17.5	-16.64	0.37
NE	46.4 ± 23.6	NE	193.4 ± 52.3	-147.00	0.003

Table 2. Only animals from Experiment 1 that received PBS in the accumbens shell are shown as a way to make comparisons between basolateral amygdala (BLA) and hippocampus (HIPP) treatments. Comparisons were made between BLA-PBS and HIPP-PBS as well as BLA-NE and HIPP-NE for selected measures during Phase 2 of retention testing. All comparisons were made with unpaired t-tests.

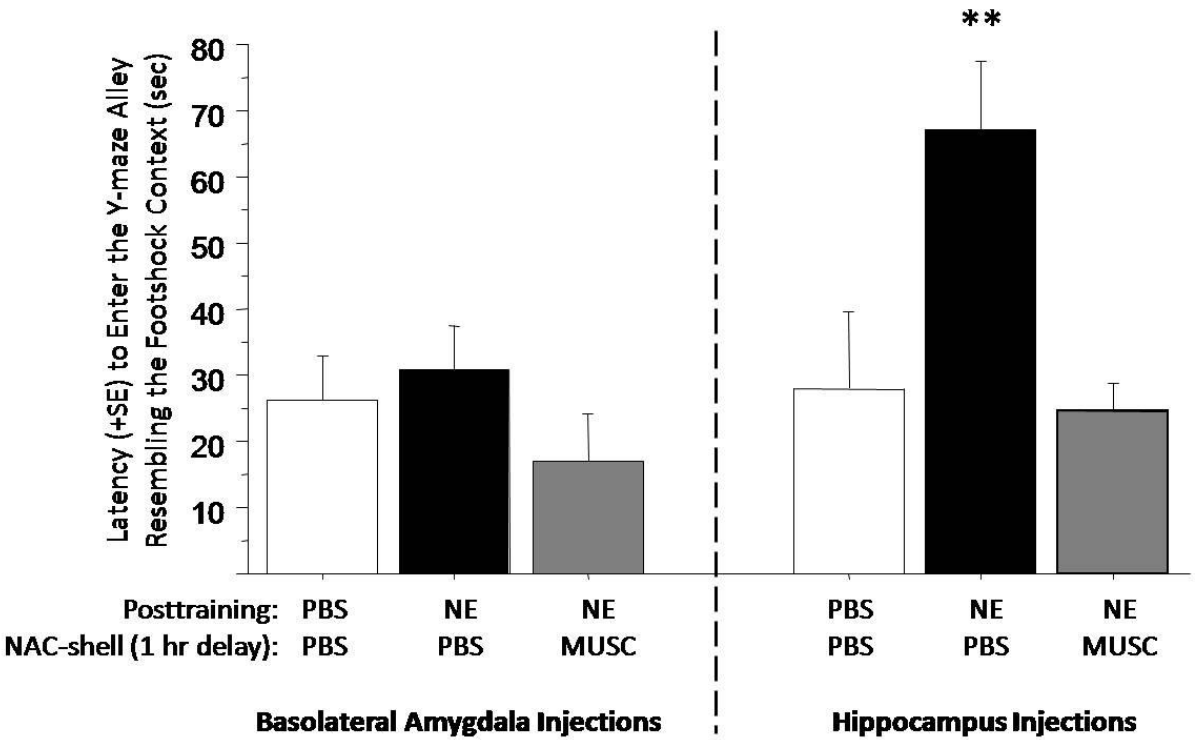

Figure 9. Mean (+ SE) latency to enter the Y-maze alley resembling the footshock context in animals with accumbens shell cannulae implants and implants in either the basolateral amygdala or ventral hippocampus. Only animals given norepinephrine (NE) in the hippocampus took significantly longer to enter the arm of the Y-maze that resembled the footshock context ($p<0.01$). Although the shock and injection was given 48 hours previously, these animals maintain high contextual memory for the shock compartment. This effect is blocked in animals given muscimol in the accumbens. ** denotes $p<0.01$.

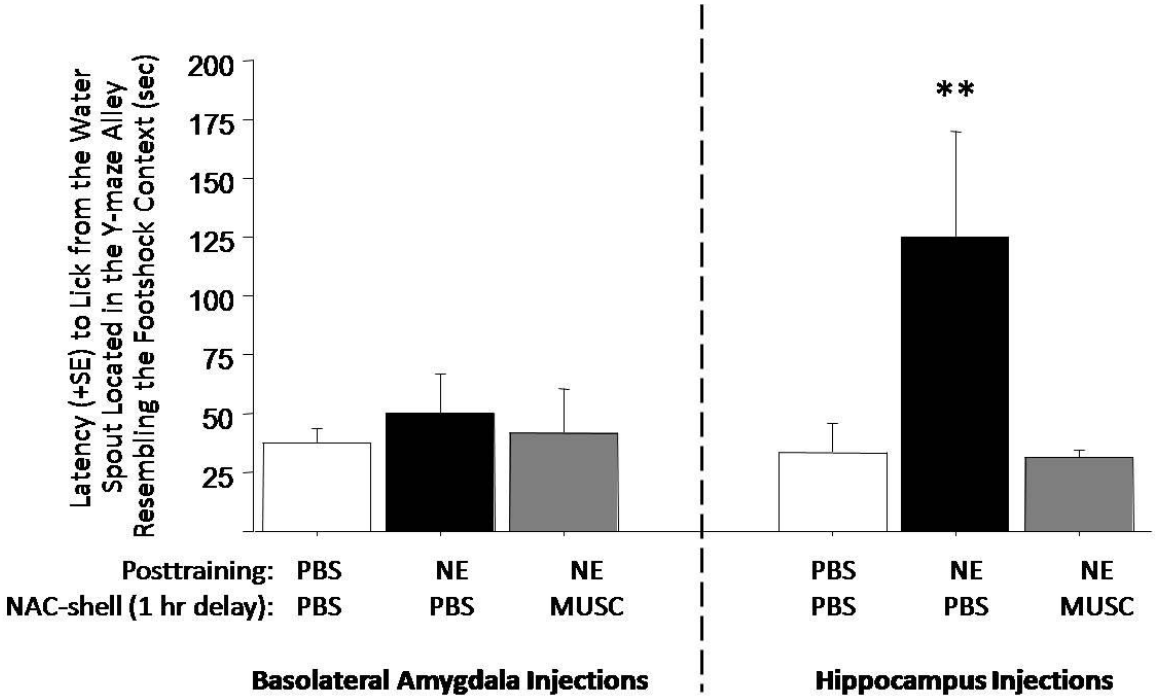

Figure 10. Mean (+ SE) latency to lick from the water spout located in the Y-maze ally resembling the footshock context in animals with accumbens shell cannulae implants and implants in either the basolateral amygdala or ventral hippocampus. Animals in the BLA-NE/PBS not only readily entered the similar shock context, but they also readily drank from the spout located at the end of the arm. However, the same dose of norepinephrine in the hippocampus produced significantly longer latencies to lick from the spout compared to all other treatment groups ($p<0.01$).

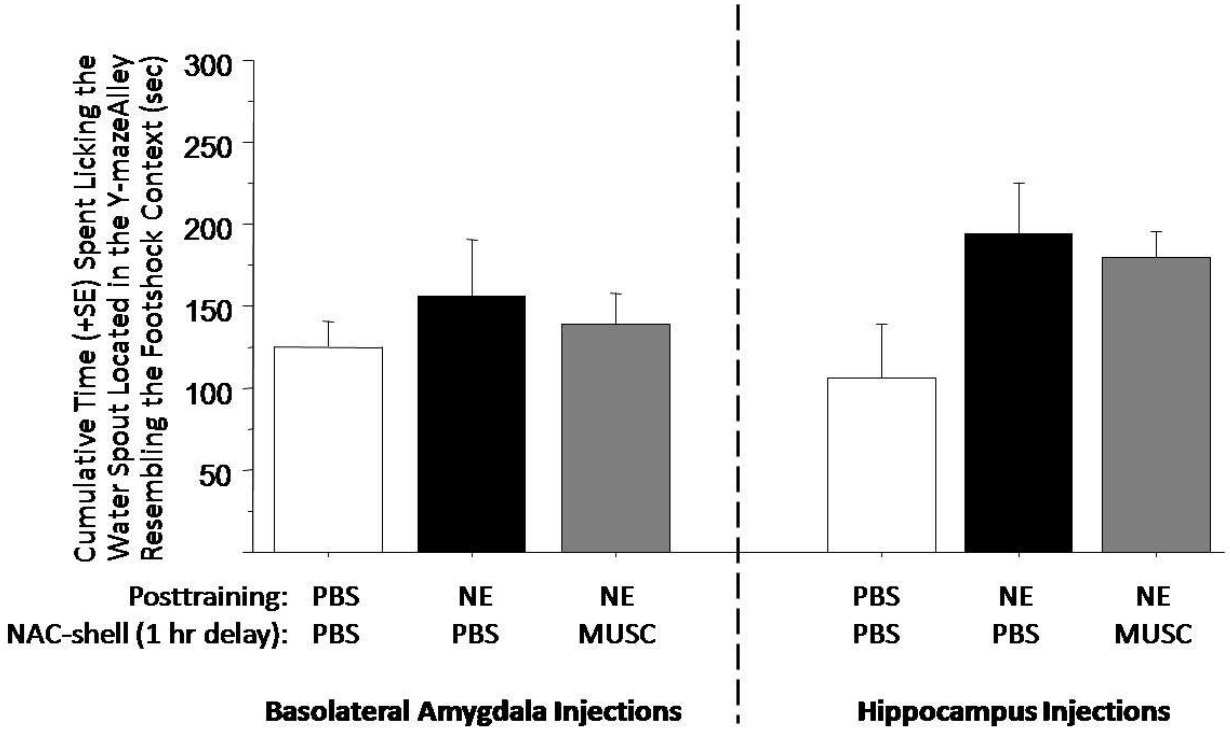

Figure 11. Mean (+ SE) time spent drinking from the spout located in the arm resembling the footshock context in animals with accumbens shell cannulae implants and implants in either the basolateral amygdala or ventral hippocampus. The cumulative time spent licking from the spout in the “shock” arm was the only measure that failed to reveal contextual transfer effects. All animals drank for a similar amount of time, suggesting that memory representation for this measure is not strong enough to transfer 48 hours following shock and norepinephrine treatment.

Chapter 4: Mechanism by which Glutamatergic Innervation from the Ventral Hippocampus Modulates Norepinephrine Release within the Nucleus Accumbens Shell

Introduction

The nucleus accumbens shell receives a constellation of inputs from brain regions involved in forming new memories following emotionally arousing events. The most critical inputs arise from structures that process affective and contextual components of new learning experiences such as the basolateral amygdala and ventral subiculum of the hippocampus (French & Totterdell, 2003; Groenewegen et al., 1987; Meredith, Wouterlood & Pattiselanno, 1990; Mogenson, Jones & Yim, 1980; Petrovich, Risold & Swanson, 1996). The accumbens also receives information regarding heightened states of peripheral visceral arousal from brainstem neurons in the nucleus of the solitary tract (NTS; Delfs et al., 1998). Although the shell is innervated heavily by both the locus coeruleus and NTS, only axons originating from A2 cells within the NTS proper contain the norepinephrine precursor, dopamine-beta-hydroxylase (Delfs, et al., 1998) indicating that these neurons provide the primary source of norepinephrine to the shell. Electrophysiological findings also indicate a strong relationship between information transmitted between the viscera and NTS. This information is then conveyed to the accumbens since increasing discharge along ascending

vagal fibers that terminate within the NTS, potentiates neuronal firing in the accumbens shell (Mehendale, Xie, Aung, Guan & Yuan, 2004). Of particular importance is the finding that the basolateral amygdala and hippocampus synapse in the caudomedial region of the accumbens shell (French & Totterdell, 2003). This is the same area that also receives noradrenergic projections exclusively from the NTS (Delfs et al., 1998). Taken together, the caudomedial region of the shell may represent a critical area that integrates limbic and visceral information following exposure to emotionally arousing learning conditions.

Behavioral evidence supports the idea that accumbens neurons are beneficial to the integration of limbic and visceral processes. For example, posttraining infusions of compounds that facilitate later retention when given in either the amygdala or hippocampus after inhibitory avoidance training are ineffective in influencing memory storage in animals with pretraining chronic accumbens lesions (Roozendaal et al., 2001). Additionally, memory enhancement for an aversive footshock experience following glutamatergic activation of NTS neurons is contingent upon accumbens cell functioning (Kerfoot, Chattillion & Williams, 2008). Anatomical and behavioral evidence suggests that the accumbens is in a position to process contextual features of an environment and affective components of an event as well as integrate physiological responses to a situation. However, the mechanism by which these three areas may interact in the accumbens shell is not fully understood.

Afferents from the basolateral amygdala and ventral hippocampus that terminate within the accumbens shell contain the excitatory amino acid,

glutamate as their primary transmitter (Blaha et al., 1997; Callaway, Hakan & Henriksen, 1991; Cano-Cebrian et al., 2003; Finch, 1996; Floresco et al., 2001; Floresco et al., 1998; Howland et al., 2002; Legault et al., 2000; Legault et al., 1999). This is an important distinction to make given that glutamate potentiates norepinephrine release in other brain structures. For example, early studies demonstrate that activation of presynaptic glutamate receptors in isolated nerve terminals in the hippocampus or prefrontal cortex increases norepinephrine release from vesicular transmitter stores (Wang, Andrews & Thukral, 1992). Furthermore, in the rat dorsal striatum, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor agonists induce norepineprhine release more efficiently than either N-methyl-D-aspartic acid (NMDA) or kinate receptor agonists (Ohta et al., 1994). These findings provide the impetus for examining whether or not glutamatergic afferents emanating from the amygdala or hippocampus, may regulate activity in the ventral striatum (accumbens) by influencing noradrenergic output from NTS axons.

Activation of these particular limbic structures can be achieved through noradrenergic influences. The hippocampus and amygdala receive noradrenergic innervation via the locus coeruleus (LC; Haring & Davis, 1985; Loughlin, Foote & Grzanna, 1986; Loy, Koziell, Lindsey & Moore, 1980; Petrov, Krukoff & Jhamandas, 1993). Following an arousing event, norepinephrine is released in widespread regions of both areas (Liang, Chen & Huang, 1995; Miyashita & Williams, 2002; 2004; Wallace, Magnuson & Gray, 1989; Williams, Men, Clayton & Gold, 1998) that in turn, send glutamatergic projections to the accumbens shell

(Christie, Summers, Stephenson, Cook & Beart, 1987; Roberts, Woodhams, Polak & Crow, 1982). Although anatomical connections between the accumbens shell and these limbic areas are firmly established, findings demonstrating that basolateral or hippocampal activation influences accumbens functioning by potentiating norepinephrine output are currently nonexistent.

Given this shortcoming in the literature and the documented finding that limbic and NTS axons converge together within the caudomedial shell (Delfs et al., 1998; French & Totterdell, 2003), the present study investigated whether glutamate release from activated amygdala or hippocampal inputs produce any changes in norepinephrine output measured within the accumbens shell. If the ventral striatum (accumbens) is similar to the dorsal striatum, this may occur via activation of AMPA receptors on noradrenergic terminals (Ohta et al., 1994). However, it has yet to be determined whether noradrenergic fibers in the accumbens shell contain glutamatergic receptors. Therefore, an immunofluorescent approach was adopted in Experiment 1 to examine whether AMPA receptors are distributed along noradrenergic fibers that innervate the accumbens shell. To further address this possible configuration of inputs, Experiment 2 used a neurochemical approach involving *in vivo* microdialysis with HPLC to determine if activation of limbic structures that send glutamatergic fibers to the accumbens, potentiates norepinephrine release in the accumbens shell. One limitation of our HPLC electrochemical detection equipment is that it is not possible to assess norepinephrine and glutamate release simultaneously. This limitation was circumvented by using an indirect measure of glutamate activity

that involved infusion of a glutamatergic antagonist in the shell with reverse phase microdialysis (i.e., drug infusion via the microdialysis cannula) 40 minutes following limbic activation. If glutamate activation of AMPA receptors distributed along NTS terminals modulates norepinephrine output in the accumbens, then these changes should be attenuated by infusing the AMPA receptor antagonist CNQX within the accumbens shell.

General Methods

Subjects

Twenty-nine male Sprague-Dawley rats (275-300 g) obtained from Charles River Laboratories (Wilmington, MA) were used in Experiment 1 (n=5) and Experiment 2 (n=24). The rats were individually housed in polypropylene cages with corncob bedding and maintained on a standard 12:12 hour light-dark cycle with lights on at 7:00 A.M. Food and water were available ad libitum during the seven day adaptation period to the vivarium.

Methods for Experiment 1

Immunofluorescent Procedure

Animals were perfused transcardially with a 0.9% saline solution followed by 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4. The brains were then removed and submerged in a 4% paraformaldehyde solution for 24 hours. The following day, the brains were dissected on a vibratome at a thickness of 50 μm and tissue sections were collected serially. To visualize both noradrenergic fibers

and AMPA receptors (n=4), the tissue was incubated in antibodies against mouse monoclonal to DBH (AB 31126, 1:500, Abcam Inc, Cambridge, MA) and rabbit polyclonal to GluR2/3 (AB 1506, 1:100, Chemicon, Temecula, CA) for two days. Primary antibodies were diluted in 1% sodium borohydride, 0.05% sodium azide and 0.1% triton. On day three, the tissue was thoroughly rinsed in PBS and then incubated in secondary antibodies against mouse (Tetramethylrhodamine, T-2762, 1:500, Invitrogen, Carlsbad, CA) and rabbit (Alexa Fluor 488, A-11008, 1:500, Invitrogen, Carlsbad, CA). As a control (n=1), sections were incubated with only secondary antibodies to ensure the absence of any non-specific secondary binding. In addition, other sections were incubated with mouse anti-DBH primary antibody and anti-rabbit secondary antibody to ensure there was no non-specific Alexa 488 binding. After Immunocytochemistry, tissue was mounted and coverslipped with Prolong Gold anti-fade in order to prevent photo-bleaching.

Data Analysis

Confocal Imaging. Tissue was imaged on an Olympus IX70 microscope equipped with the Fluoview 5.0 confocal laser scanning system (Olympus America, Melville, NY) using a 60x oil immersion objective (N.A. = 1.4) with an additional 1.5x optical zoom to give a final magnification of 90x. Tissue containing the caudomedial region of the accumbens shell was imaged with sequential passes of the blue (to detect GluR2/3 label, argon-ion laser) and green (to detect DBH label, helium-neon; 543 nm) lasers and collected as separate channels. Images were then deconvolved using Autoquant's (MediaCy)

theoretical point-spread-function algorithm and visualized with Volocity (Improvision).

Colocalization Analysis. Using Volocity software, the average pixel intensity for the selected area was generated for each label. Any pixel below three standard deviations of the mean intensity was considered background and not counted as label. Only those areas of tissue in which there were strong labeling (more than three standard deviations above the mean) for both GluR2/3 and DBH were considered areas of colocalization. In addition, it was possible to determine the percentage of DBH labeled fibers containing GluR2/3 receptors. These percentages are expressed as the mean ± standard errors (SE).

Methods for Experiment 2

Surgery

All animals received an injection of atropine sulfate (0.1 mg/kg i.p., American Pharmaceutical Partners, Inc., Schaumburg, IL) and was then anesthetized with sodium pentobarbital (50 mg/kg, i.p., Abbot Laboratories, North Chicago, IL). A midline scalp incision was made and a unilateral microdialysis cannula was implanted above the nucleus accumbens shell (AP +0.7, ML + 1.0 from bregma, DV -5.4 from skull surface) and bilateral 15 mm long extra thin wall stainless steel guide cannula (25 gauge, Small Parts, Miami Lakes, FL) were secured above either the basolateral amygdala (AP -3.0, ML ±5.0 from bregma, DV -6.7 from skull surface) or the ventral subiculum of the hippocampus (AP -5.3, ML ±4.5, DV -8.6 from skull surface). All coordinates were adapted from the atlas of Paxinos and Watson (1986). The microdialysis cannula, guide cannulae and

two skull screws for anchoring were affixed to the skull with dental cement and the scalp was closed with sutures. Stylets (15 mm, 00 insect dissection pins) were then inserted into the injection cannulae to prevent occlusion. Penicillin (0.1 ml i.m., Fort Dodge Animal Health, Fort Dodge, IA) was administered immediately after surgery along with the analgesic, buprenex (0.05 ml s.c., Hospira, Inc., Lake Forrest, IL). The rats remained in a temperature controlled chamber for at least one hour following surgery and were given seven days to recover before initiating food or water deprivation procedures and behavioral training.

Microdialysis Procedure

Probes. CMA/12 (Carnegie/Medecin, Stockholm, Sweden) dialysis probes with a 2-mm membrane tip were used to collect norepinephrine from the shell region of the nucleus accumbens. The inlet arm was connected to a 1 ml Hamilton syringe by FEP tubing, and a CMA-1000 microinfusion pump (Carnegie/Medecin) was used to drive the syringes. The outlet arm of the probe was connected by FEP tubing to 350 µl collection vials containing 15µl of dihydroxybenzylamine (1.0 pg/µl) that serves as an internal standard for HPLC analysis. The probes were perfused continuously with artificial cerebral spinal fluid (aCSF; pH 7.4; 145.0 mM NaCl, 4.0 mM KCl, 1.2 mM CaCl2 and 2.0 mM Na2HPO4) at a flow rate of 1.0 µl/min. Dialysate samples of norepinephrine were collected every 20 minutes and stored on ice until assayed with high performance liquid chromatography (HPLC).

Microdialysis Chamber. Samples of dialysate were collected in a CMA/120 system round-bottomed transparent bowl with a diameter of 400 mm at the top designed for microdialysis experiments in conscious, freely moving animals. The system enables long term combined studies of animal behavior and concurrent microdialysis experiments.

Microdialysis Sample Collection. The microdialysis experiment consisted of three main phases: habituation, Baseline collection and experimental treatment. Subjects were first transported to the laboratory and left undisturbed for 20 minutes. The rat was habituated to the chamber for 1 hour after probe implant and no samples were collected during this time. The concentrations of norepinephrine in the first three samples collected after the habituation period were averaged to yield the Baseline value. The rat was then administered an intra-basolateral amygdala or hippocampus infusion of PBS or norepinephrine (BLA-PBS n=5; BLA-NE n=5; HIPP-PBS n= 5; HIPP-NE n=5; HIPP-NE rp/CNQX n=4). The dose of norepinephrine was chosen to approximate the level of norepinephrine release that results from infusions of norepinephrine (0.2 µg) in the hippocampus and amygdala shown to improve memory (Hatfield & McGaugh, 1999; LaLumiere, Buen & McGaugh, 2003) combined with a mild footshock (0.35 mA, 2 sec). Five more samples were collected before the probe was removed from the guide cannula and the rat was returned to the home cage. A separate group of animals (n=4) received intra-accumbens infusion of the AMPA receptor antagonist CNQX (0.5 µg/0.5 µl) forty minutes following limbic activation via reverse phase microdialysis (i.e., drug infusion via the microdialysis

cannula). The vials containing each sample were sealed with parafilm (Fisher Scientific, Pittsburgh, PA) and stored on ice until assayed with HPLC. It is important to note that all experimental manipulations were initiated in the final 10 minutes of the collection period that preceded the experimental treatment. This 10 minute period reflects the amount of time required for the dialysate samples to be transported from the membrane of the microdialysis probe through the FEP tubing to the sample collection vials.

Norepinephrine Assay. Norepinephrine concentrations in the dialysate sample were assayed by HPLC electrochemical detection (ESA, Chelmsford, MA). At the end of the microdialysis experiment, 35 µl of each dialysate sample was loaded into a Waters 717 autosampler, automatically injected with a flow rate of 1.0 ml/min. The mobile phase consisted of 50 mg disodium EDTA, 13.8 mg monobasic sodium phosphate and 58 mg octane sulfonate adjusted to pH 3.2 by adding 85% phosphoric acid. Norepinephrine concentrations and peak heights were measured in comparison with those of a known norepinephrine standard (32 pg/35 µl). The concentration, peak height, and retention time for dialysate samples of norepinephrine were analyzed with the Millennium software package (Waters).

Statistical Analysis. The levels (pg/ml) of norepinephrine from the 3 baseline samples were averaged to yield a standard baseline value. Comparisons between norepinephrine levels at baseline and each 20 minute time point was analyzed with repeated measures ANOVA. Fischer's post hoc test will be used to analyze specific comparisons between treatment groups.

Histology

Rats were deeply anesthetized with a euthanasia solution and perfused intracardially with 0.9% saline followed by 10% formalin to verify microinjection cannulae placement. The brains were stored in a 10% formalin and 12% sucrose solution until sectioned on a vibratome. Sections were cut 50 µm thick, mounted on glass slides, subbed with chromium-aluminum and stained with cresyl violet. The location of the cannulae and injection needle tips were verified by examining enlarged projections of the slides. The location of microdialysis cannula tips in the accumbens shell and injection needle tips in either the basolateral amygdala or ventral hippocampus are displayed in Figures 1a, 1b and 1c.

Results for Experiment 1

Figure 2 shows the representative area of the accumbens shell (AP +0.7, from bregma) from which images were taken. Following control measures described previously, neither the presence of DBH fibers nor GluR2/3 receptors were discernable (Figure 3A-F) in the control sections. However, for those animals in which tissue was stained for both DBH and GluR2/3, there were obvious signs of fiber and receptor labeling (Figure 4A and 4B, respectively). Superimposition of DBH and GluR2/3 staining shows areas of overlap as discerned by white regions (Figure 4C). These regions of DBH and GluR2/3 colocalization were found to occur in approximately 20% of all DBH labeled fibers in the selected region of the accumbens shell (Figure 5).

Results for Experiment 2

Dialysate samples of norepinephrine were collected from the nucleus accumbens shell following microinjections of PBS or norepinephrine (NE) into the basolateral amygdala or ventral hippocampus. A one-way repeated measures ANOVA revealed no significant interaction between norepinephrine concentrations across the different time periods and basolateral amygdala drug treatment, $F(1, 6) = 0.28$, $p = 0.94$ (Figure 6). Because norepinephrine levels remained similar to those collected during baseline regardless of whether the PBS or NE was infused into the basolateral, reverse phase of CNQX infusions into the accumbens were not employed.

However, as shown in Figure 7, there was a significant interaction between hippocampal drug treatment (PBS vs. NE) and norepinephrine concentrations, $F(1, 6) = 5.25$, $p < 0.01$. Infusion of norepinephrine in the hippocampus significantly elevated extracellular norepinephrine levels in the accumbens shell. Between group comparisons revealed that norepinephrine remained significantly high in the HIPP-NE as compared to the HIPP-PBS group for two hours ($p < 0.05$). Because AMPA receptors were found to colocalize with DBH fibers in Experiment 1, it may be that the increase in accumbens norepinephrine concentrations following hippocampal activation is due to hippocampal glutamatergic activation of those receptors. To examine this possibility, a third group of hippocampal animals received the same dose of norepinephrine in the hippocampus as the HIPP-NE group. However, the third group also received the AMPA receptor antagonist CNQX in the accumbens 40 minutes following the

hippocampal infusion using reverse phase techniques (n=4). Between-group comparisons revealed that norepinephrine levels did not differ between the HIPP-NE and HIPP-NE/rpCNQX treatment groups for the two collection periods following hippocampal infusions ($p > 0.05$). However, immediately following CNQX infusions into the accumbens, the concentration of norepinephrine in the accumbens decreased to levels similar to that obtained in PBS treated animals and continued at this lower concentration for the remainder of the experiment. Only animals in the HIPP-NE group continued to display significantly high levels of accumbens norepinephrine.

Discussion

The present experiments employed immunocytochemistry and *in vivo* microdialysis with HPLC to address the hypothesis that norepinephrine output from NTS terminals innervating the accumbens (Delfs et al., 1998) is regulated in part, by amygdala and hippocampal projections that release glutamate (Blaha et al., 1997; Callaway, Hakan & Henriksen, 1991; Cano-Cebrian et al., 2003; Finch, 1996; Floresco et al., 2001; Floresco et al., 1998; Howland et al., 2002; Legault et al., 2000; Legault et al., 1999) to activate AMPA receptors distributed along NTS axons. As glutamate release is reported to augment norepinephrine output in other brain structures, the current study addressed whether glutamatergic receptors (AMPA) are in a position to modulate norepinephrine release within the accumbens. Results from the current study show that AMPA receptors are colocalized with norepinephrine containing fibers identified in the accumbens

shell (Figure 4). Within the sampling of accumbens sections, the prevalence of AMPA receptor and norepinephrine colocalization was approximately 20%. In addition to the anatomical evidence provided in the present work, the neurochemical findings that emerged from Experiment 2 revealed that activation of the hippocampus leads to a marked increase in accumbens norepinephrine levels. The continued release of norepinephrine following hippocampal activation is contingent upon AMPA receptors. This is evidenced by the immediate decrease in accumbens noradrenergic levels following infusions of an AMPA antagonist, CNQX (Figure 7). Unlike the hippocampus, activation of the basolateral amygdala did not lead to any significant changes in the concentration of norepinephrine sampled from the accumbens (Figure 6). This finding suggests that although the hippocampus, basolateral amygdala and NTS (noradrenergic innervation) synapse within the same region of the shell, only hippocampal inputs interact with NTS fibers to facilitate norepinephrine activity in the shell.

Previous findings have revealed that presynaptic glutamatergic receptors regulate the release of neurotransmitters in humans as well as rats (Cartmell & Schoepp, 2000; MacDermott, Role & Siegelbaum, 1999; Raiteri, 2006). More importantly, AMPA receptors induce noradrenergic release more efficiently than either NMDA or kinate receptor agonists in the dorsal striatum (Ohta et al., 1994). Results from the current study extend these findings by showing that AMPA receptors are not only located on noradrenergic fibers in the ventral striatum (accumbens), but are required to facilitate norepinephrine release following limbic activation. This new finding provides evidence that along with the hippocampus

and prefrontal cortex (Wang, Andrews & Thurkral, 1992), the accumbens shell is an additional region where activation of presynaptic glutamate receptors facilitates the release of norepinephrine.

An additional finding from the current study is that only activation of the hippocampus, as compared to the basolateral amygdala, facilitates norepinephrine release via AMPA receptor mechanisms within the accumbens shell. While these findings are among the first neurochemical results to support hippocampal modulation of noradrenergic release, there is anatomical support for this finding. Sesack and Pickel (1990) found that terminals from the ventral hippocampus make axo-axonal contact with terminals stained for tyrosine hydroxylase (TH). However, staining for tyrosine hydroxylase, the rate limiting enzyme for dopamine, does not preclude the possibility that TH-positive terminals could be noradrenergic. One of the most interesting measures from Sesack and Pickel's (1990) study revealed that approximately 17% of hippocampal inputs made axo-axonal connections with TH-labeled terminals. This is extremely interesting given the findings from the current study showing that AMPA receptor colocalization occurs in approximately 20% of DBH stained fibers in the accumbens and that activation of the ventral hippocampus potentiates norepinephrine release in the accumbens via AMPA receptor mechanisms. Further investigation is required to determine if other limbic areas that supply glutamatergic afferents to the accumbens shell, such as the prefrontal cortex, are also in a position to influence noradrenergic release.

The anatomical and neurochemical evidence supporting convergence and integration underlies a vital characteristic of accumbens neurons. Several studies show that accumbens neurons require activation from more than one source to reach threshold (Callaway Hakan, & Henriksen, 1991; DeFrance, Marchand, Sikes, Chronister & Hubbard, 1985). Therefore, during times of heightened arousal, inputs from the basolateral amygdala, hippocampus and NTS may be required in order to facilitate neuronal firing in the accumbens shell. It may also be the case that during this emotionally salient event, hippocampal processing dominates so as to encode the context in which the event has occurred. This is consistent with findings from McGinty and Grace (2009) that show strong activation of one input may disrupt processing of the second input. The current results show that activation of the hippocampus, but not the amygdala, facilitates norepinephrine release in the accumbens. This interaction between hippocampal and NTS noradrenergic fibers may provide the mechanism by which contextual memory is strengthened during emotionally arousing events.

Conclusions

This study provides evidence that AMPA receptors are located on noradrenergic fibers in the accumbens shell. Although both the basolateral amygdala and ventral hippocampus send glutamatergic innervations to the accumbens shell, only hippocampal activation facilitates accumbens norepinephrine release. Additionally, results show that the potentiation of norepinephrine release following hippocampal activation is dependent on AMPA

receptor functioning. Blockade of accumbens AMPA receptors decreases norepinephrine output to basal levels. These findings are among the first to reveal the mechanism by which the hippocampus interacts and modulates norepinephrine release from NTS terminals within the shell division of the nucleus accumbens.

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Figures

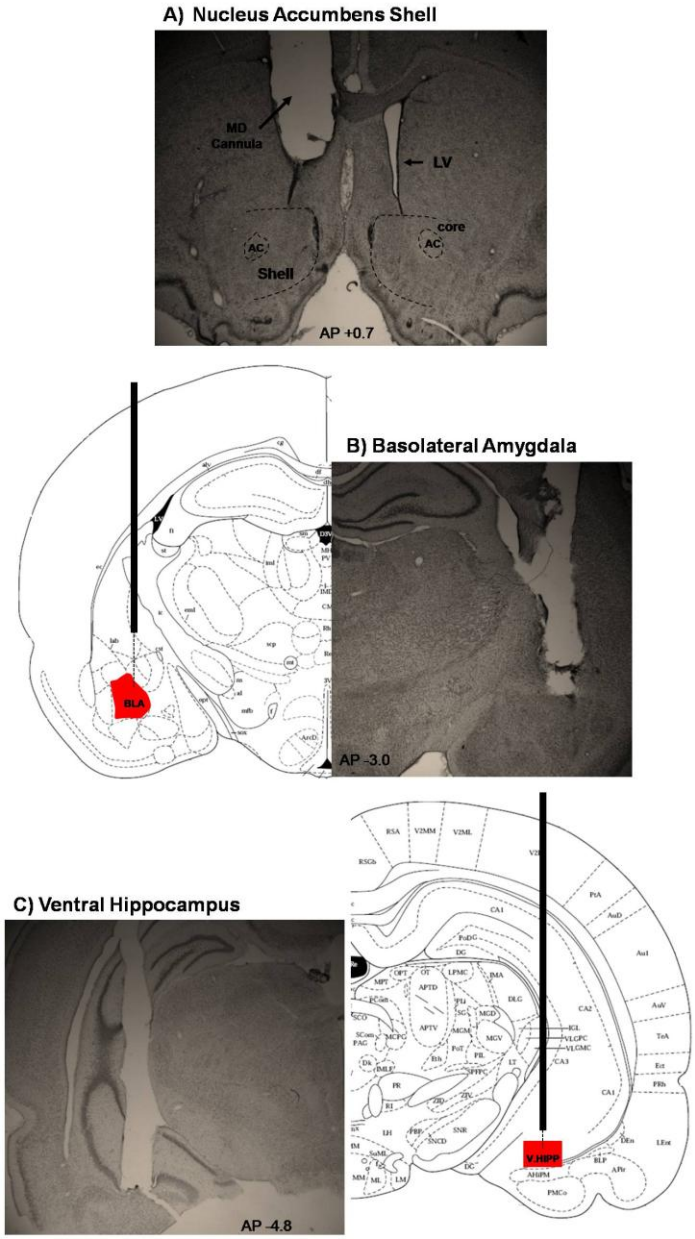

Figure 1a, 1b and 1c. Location of cannula tip placements in the A) nucleus accumbens shell as well as the injection needle tip placements for the B) basolateral amygdala and C) ventral hippocampus. Abreviations: AC = anterior commissure, core = nucleus accumbens core, LV = lateral ventricle, MD cannula = unilateral microdialysis cannula (counterbalanced for side) and Shell = nucleus accumbens shell. Brain diagram from “The Rat Brain in Stereotaxic Coordinates”; adapted from Paxinos and Watson.

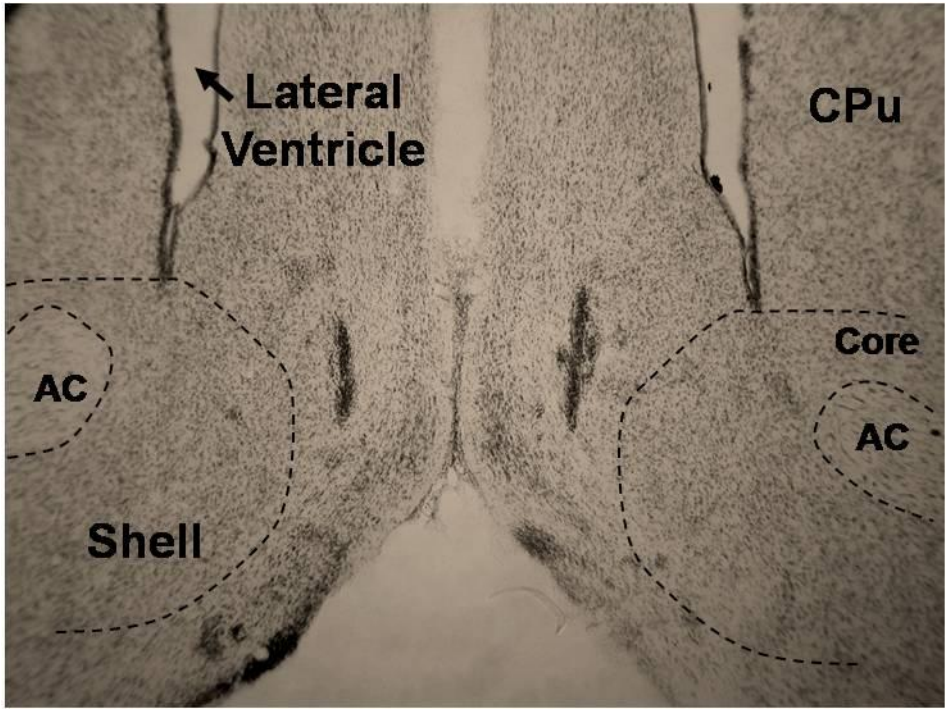

Figure 2. Photomicrograph depicting the area of the accumbens shell in which immunofluorescent images were taken (AP +0.7; adapted from the atlas of Paxinos & Watson, 1986). AC = anterior commisure, CPu = caudate putamen.

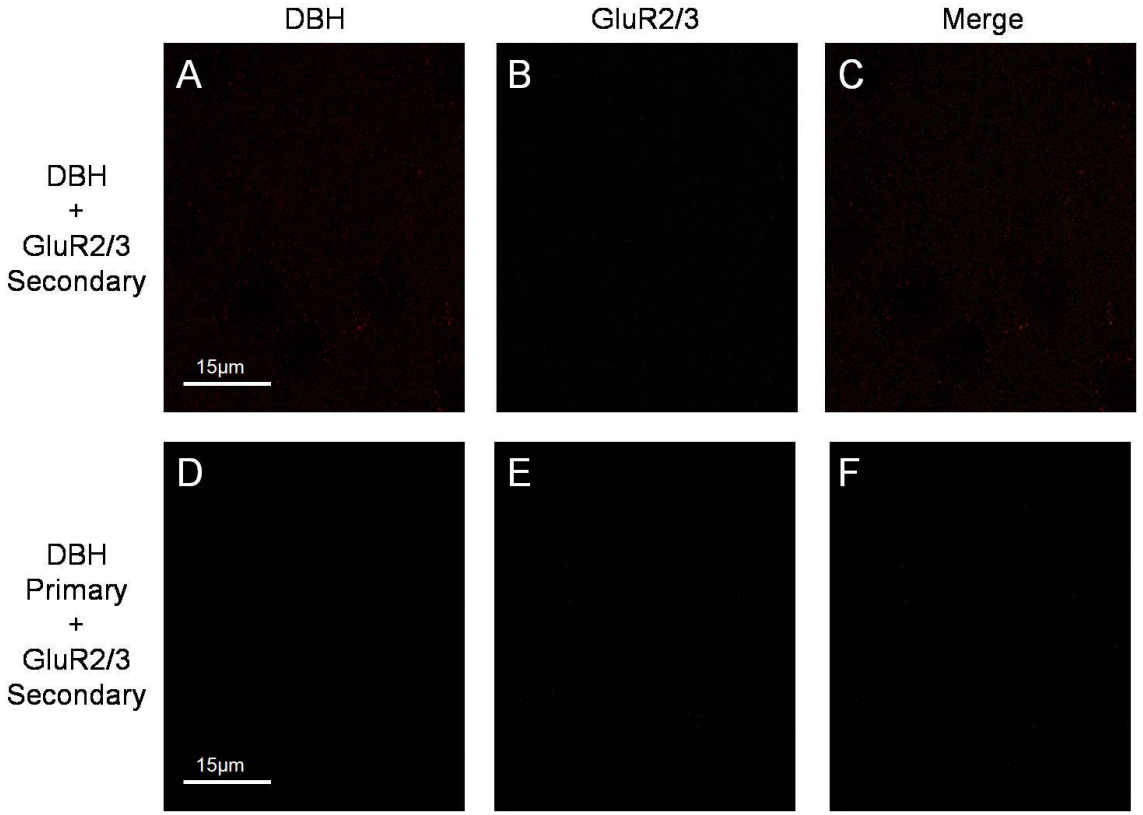

Figure 3. Control fluorescence confocal micrographs of the nucleus accumbens shell. These micrographs were included to control for the possibility of non-specific secondary binding. The top row was immunolabeled with no primary antibodies and then treated with secondary antibodies against DBH and GluR2/3. The bottom row was immunolabeled with primary antibodies against DBH and then treated with secondary antibodies against GluR2/3. (A and D) DBH, (B and E) GluR2/3, (C and F) Superimposition image of DBH and GluR2/3. Scale bar = 15µm.

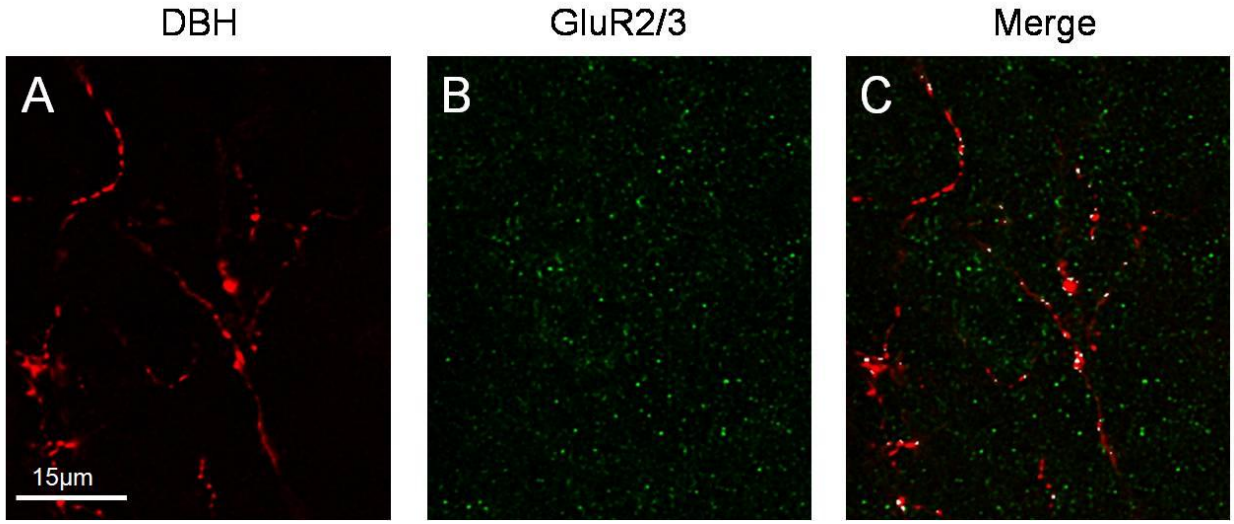

Figure 4. Fluorescence confocal micrographs of the nucleus accumbens shell immunolabeled with primary antibodies against DBH and GluR2/3 and treated with their corresponding secondaries. A) DBH fiber labeling. B) Receptor labeling for the GluR2/3 subunits of the AMPA receptor. C) Superimposition image of DBH and GluR2/3 with areas of colocalization in white. Scale bar = 15µm

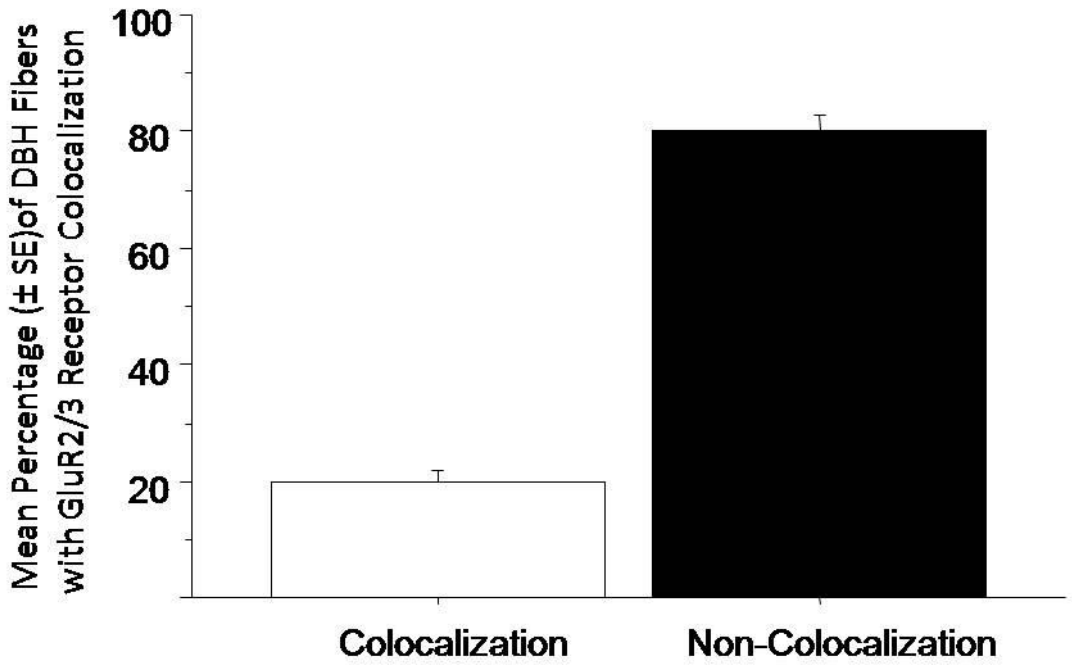

Figure 5. Mean (+ SE) level of DBH fibers that have positive labeling for the GluR2/3 subunit of the AMPA receptor. These areas were considered areas of colocalization whereas DBH fiber labeling that contained no discernable level of GluR2/3 labeling was considered to be non-colocalized.

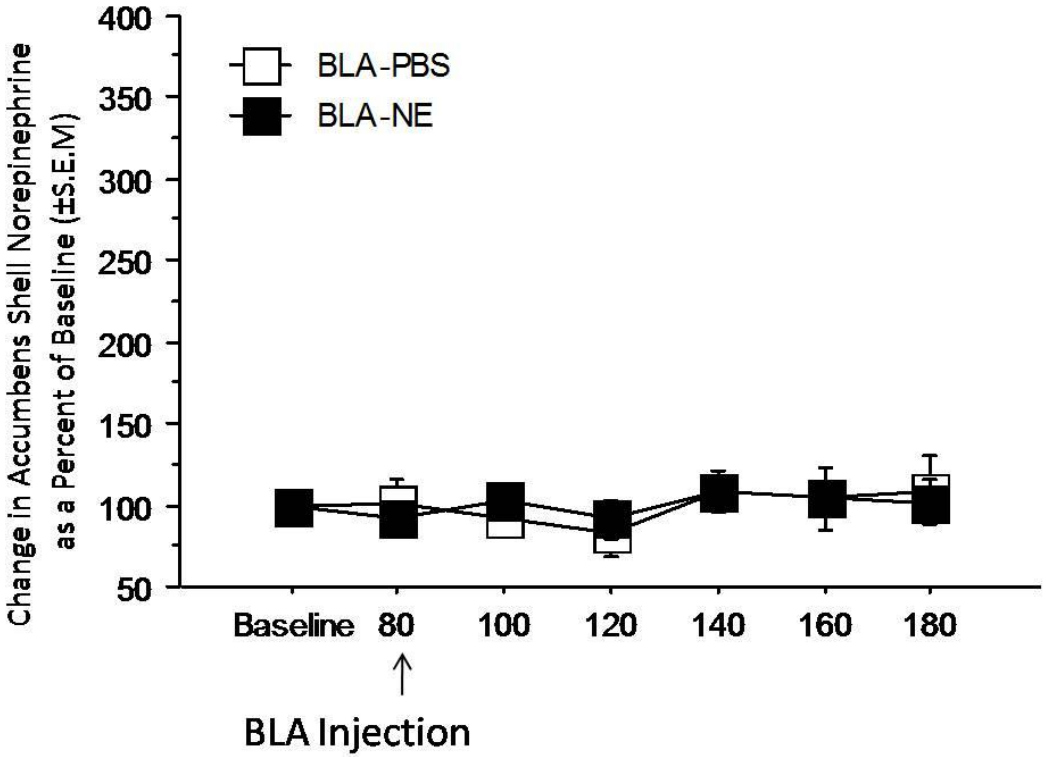

Figure 6. Mean (± SE) change in accumbens shell norepinephrine levels as a percent of baseline following basolateral amygdala injections. Infusions of norepinephrine or PBS in the basolateral amygdala produced no appreciable fluctuations in norepinephrine levels in the accumbens shell.

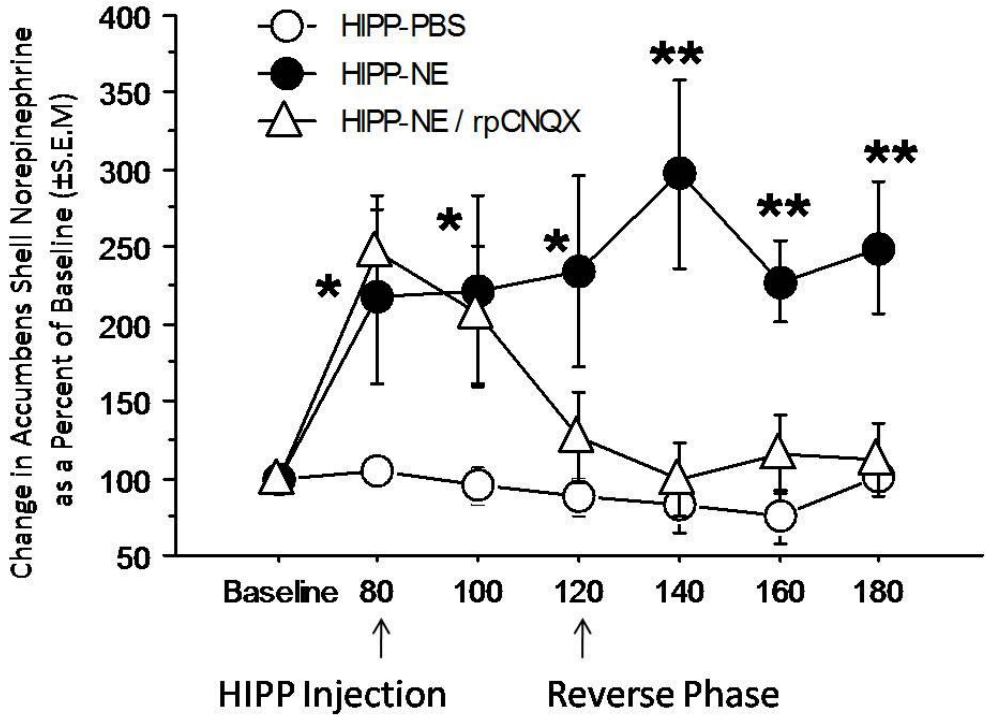

Figure 7. Mean (± SE) change in accumbens shell norepinephrine levels as a percent of baseline following ventral hippocampus injections. Unlike basolateral amygdala infusions, injections of norepinephrine in the hippocampus produced a marked increase in accumbens norepinephrine. The continued release of norepinephrine following hippocampal activation is contingent upon glutamatergic receptor activation. Animals in the HIPP-NE/rpCNQX group showed a significant decrease in accumbens norepinephrine levels following infusions of CNQX into the shell 40 minutes following hippocampal treatment. * denotes $p<0.05$, ** denotes $p<0.01$.

Chapter 5: Conclusions

This dissertation project contains a series of experiments that were developed to provide a better understanding of the integrative role of the accumbens shell in processing memory for emotional events and to address several shortcomings in the literature. The current dissertation determined 1) whether NTS activation modulates memory for arousing events by releasing norepinephrine within the accumbens shell, 2) if the shell integrates information regarding new learning experiences that is initially processed in either the amygdala or hippocampus, 3) whether the accumbens shell is involved in the later phases of memory consolidation and 4) if glutamatergic inputs from limbic terminals interact with or modulate norepinephrine release within the accumbens shell. An additional aim was to provide insight into a possible mechanism by which limbic structures and noradrenergic inputs interact within the accumbens shell.

Experiment 1 in **Chapter 2**, established whether neuromodulators that improve memory when infused into the NTS affect norepinephrine levels in the accumbens shell. Electrophysiological evidence supports a relationship between NTS and accumbens (Kirouac & Ciriello, 1997), however, the neurochemical consequences of NTS activation had not been established in the accumbens shell. Results from Experiment 1 in **Chapter 2** reveal that activation of NTS neurons with a high dose of glutamate (100ng) potentiates norepinephrine output

in the shell by 40% as compared to basal levels. Additionally, findings show that although the smaller concentration of glutamate (50ng) in the NTS alone may not be sufficient to impact the release of norepinephrine, infusion of the low dose of glutamate in combination with an arousing footshock is sufficient to potentiate accumbens norepinephrine levels.

After establishing that norepinephrine is released following NTS activation, Experiment 2 in **Chapter 2** identified the receptors involved in the enhancement of memory following activation of noradrenergic input to the nucleus accumbens. Although the accumbens has both alpha and beta-adrenergic receptors (Rainbow, Parsons & Wolfe, 1984; Unnerstall, Kopajtic & Kuhar, 1984), mounting evidence suggests differences in the efficacy between these receptor subtypes in mediating the influence of norepinephrine on accumbens activity. Therefore, Experiment 2 determined whether brainstem activation of NTS neurons modulates activity in the accumbens shell by activating alpha-adrenergic receptors within this nucleus. Results show that blocking alpha-noradrenergic receptors prior to activating NTS neurons attenuates the improvement in memory seen following NTS activation alone. Results from **Chapter 2** suggest that norepinephrine released from NTS terminals enhances the memory representation for the footshock experience by acting via alpha-adrenergic postsynaptic receptors within the accumbens shell.

Although findings from **Chapter 2** show the significance of the nucleus accumbens in integrating information from the periphery to modulate memory for arousing events, it is not known if the accumbens plays an equally important role

in consolidating information that is initially processed in the amygdala and hippocampus. Studies in **Chapter 3** assessed whether the convergence of inputs from these limbic regions within the accumbens shell contributes to successful encoding of emotional events into memory. Results from Experiment 1 confirm previous studies demonstrating that posttraining activation of noradrenergic receptors within the basolateral amygdala or hippocampus facilitate subsequent retention performance (Bevilaqua et al., 1997; Birthelmer et al., 2003; Dommett et al., 2008; Izumi & Zorumski, 1999; Miranda et al., 2003; Roozendaal et al., 2008; Roozendaal et al., 2006; Tully et al., 2007; van Stegeren et al., 2005; van Stegeren et al., 2008). However, the present results revealed that noradrenergic activation of the amygdala or hippocampus differentially facilitates the category of representations formed after emotionally arousing events. Specifically, noradrenergic activation of the basolateral amygdala facilitates response specific representations as compared to hippocampal activation that enhances memory for the context in which the event transpired. The most interesting and novel finding in **Chapter 3** is that the consequences of activating noradrenergic receptors in the amygdala or hippocampus are mediated in part by actions initiated within the accumbens shell for up to 7 hours posttraining. This timeframe corresponds to synaptic changes identified in other brain regions (O'Sullivan et al., 2007). Results from the current study suggest that the accumbens is necessary not only during the first wave of consolidation (0-2 hours), but second wave time point (6-9 hours) as well.

Findings thus far from **Chapters 2 and 3** demonstrate that accumbens norepinephrine is involved in memory enhancement following NTS activation and that the accumbens is involved in modulating information emanating from the hippocampus and basolateral amygdala. Given that these three areas (NTS, amygdala and hippocampus) innervate the same region of the accumbens (French & Totterdell, 2003), it was essential to examine how these areas interact to influence mnemonic processes. In other brain areas, glutamate was shown to potentiate norepinephrine release via AMPA receptor activation (Ohta et al., 1994). Therefore, Experiment 1 of **Chapter 4** determined whether AMPA receptors were located on noradrenergic fibers in the accumbens shell. The current findings revealed that AMPA receptors colocalized on fibers in the shell containing norepinephrine in approximately 20% of stained noradrenergic fibers. This is consistent with the idea that glutamatergic afferents make direct axo-axonal connections with catecholaminergic fibers (Seasack & Pickel, 1990).

Furthermore, the second Experiment in **Chapter 4** investigated the interactions between direct limbic activation of structures that send glutamatergic innervations to the accumbens and norepinephrine release within the shell. Using *in vivo* microdialysis and reverse phase techniques, results showed that activation of the basolateral amygdala did not lead to an increase in extracellular norepinephrine release. However, activation of the hippocampus leads to a marked increase in norepinephrine levels in the shell. The continued release of norepinephrine in the shell following hippocampal activation is contingent on AMPA receptor activation. This is evidenced by the finding demonstrating that

infusions of an AMPA receptor antagonist, CNQX, into the shell produced an immediate and drastic decrease in accumbens norepinephrine levels. Together these results from **Chapter 4** show that activation of the hippocampus facilitates norepinephrine release in the accumbens by activating AMPA receptors located on noradrenergic fibers.

In conclusion, the nucleus accumbens shell is a critical area involved in the integration of affective, contextual and physiological information. Processing in the shell is required for an extended period of time during the consolidation process. Glutamatergic limbic interactions with noradrenergic fibers from the NTS within the shell may be one mechanism through which mnemonic representations are formed following an aversive event. The current dissertation used behavioral, anatomical and neurochemical techniques to provide a better understanding of the integrative role the accumbens plays in consolidating memory. The experiments discussed within this dissertation describe a unique involvement of the accumbens in modulating information in the accumbens that lead to strong memory representations.

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