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The Contribution Of Nmda Receptors Within The Central Nucleus Of The Amygdala To The Suppression Of Pain Affect

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THE CONTRIBUTION OF NMDA RECEPTORS WITHIN THE CENTRAL NUCLEUS OF THE AMYGDALA TO THE SUPPRESSION OF PAIN AFFECT

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DEDICATION

This work is dedicated to those of my family who have contributed to my education either financially or emotionally. Your support has allowed me to attain my goals and fulfill my aspirations, and your selfless and empathetic contributions are unrivaled. From the bottom of my heart, I am truly grateful to: my Dad, James D. Spuz; my Mom, Camille A. Spuz; my Gramma, Wanda Piecuch; my “Bachi”, Bernice Spuz; my Grampa, Walter Spuz; my brother, Brian R. Spuz; and my cousin, Shannon M. McNulty.

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CHAPTER 1

Introduction

The human pain experience, both experimental and clinical, is composed of three dimensions (Gracely, McGrath, & Dubner, 1978; Price, Harkins, & Baker, 1987). The sensory-discriminative dimension of pain permits the individual to localize the painful stimulus, and to assess its intensity and physical properties. The affective-motivational dimension encourages avoidance and recuperation via the perception of negative affect associated with noxious stimulation. The cognitive-evaluative dimension induces an appraisal of the meanings and consequences associated with painful sensations and injury. These latter two dimensions interact to generate emotional disturbances such as fear, anxiety, frustration and depression that contribute to the suffering and physical disabilities of patients in chronic pain (Crombez, Vlaeyen, Heuts, & Lysens, 1999; McCracken, Zayfert, & Gross, 1992; Sullivan, Reesor, Mikail, & Fisher, 1992; Waddell, Newton, Henderson, Somerville, & Main, 1993; Wade, Dougherty, Hart, Rafii, & Price, 1992). Therefore, understanding the neurobiology that underlies the generation and suppression of pain affect is of clinical importance and warrants intensive study.

The amygdala is a forebrain structure critical for providing affective salience to sensory information for animals and humans (LeDoux, 2000). Functional magnetic resonance imaging studies in humans report that increased amygdala activation is associated with fear and anxiety (Adolphs, Tranel, & Damasio, 1998; Critchley, Mathias, & Dolan, 2002; Furmark, Fischer, Wik, Larsson, & Fredrikson, 1997; Tillfors, et al., 2001) or exposure to threatening stimuli (Carlsson, et al., 2004; Carretie, Hinojosa, Mercado, & Tapia, 2005; Isenberg, et al., 1999; Phelps, et al., 2001). Similarly,

amygdala activation is observed in fearful and anxious rats, or in rats exposed to aversive and threatening stimuli (Adell, Casanovas, & Artigas, 1997; Duncan, Knapp, & Breese, 1996; Figueiredo, Bodie, Tauchi, Dolgas, & Herman, 2003; Lehner, et al., 2006). On the other hand, damage to the amygdala suppresses responding to aversive and threatening stimuli in both humans and animals (Blanchard & Blanchard, 1972; Borszcz & Leaton, 2003; Hebben, Corkin, Eichenbaum, & Shedlack, 1985; Jelasic, 1966). These findings indicate that the amygdala acts as the "threat detector" of the brain. The amygdala processes stimuli that threaten the individual and contributes to the execution of affective behaviors that permit the individual to cope with the threat (Bernard & Bandler, 1998; LeDoux, 2000).

The prototypical threat to an individual is exposure to a noxious stimulus Within the brain, the amygdaloid central nucleus (CeA) and basolateral complex (BLC; includes lateral and basolateral subnuclei) receive nociceptive afferents via spinoamygdaloid (Giesler, Katter, & Dado, 1994; Newman, Stevens, & Apkarian, 1996) and spino-parabrachio-amygdaloid pathways (Bernard & Besson, 1990; Ma & Peschanski, 1988), directly from collaterals of the spinothalamic tract (Burstein & Potrebic, 1993), and indirectly from spinoreticulothalamic and spinopontothalamic tracts via inputs relayed by medial and intralaminar thalamic nuclei (Bourgeais, Gauriau, & Bernard, 2001; Krout & Loewy, 2000; Petrovicky, 1990; Su & Bentivoglio, 1990; Volz, et al., 1990), and the insular cortex (Shi & Davis, 1999). Noxious stimulation evokes neural activity in both CeA and BLC (Bernard, Huang, & Besson, 1990; Romanski, Clugnet, Bordi, & LeDoux, 1993).

In addition to nociceptive input, the BLC receives highly processed multimodal sensory information from several cortical regions (visual, auditory, somatosensory and olfactory cortices), as well as more direct but less processed sensory information from the corresponding thalamic nuclei. The BLC is proposed to process these inputs and allocate emotional salience to those that represent environmental threats. The outputs of the BLC engage the defense circuit that enables the individual to respond to the threat (McDonald, 1998; Pitkanen, 2000). The CeA is the major output sub-nucleus of the amygdala and the CeA receives afferents from the BLC. Efferents from CeA to the hypothalamus, periaqueductal gray, and medulla coordinate execution of defensive behaviors designed to cope with threats (LeDoux, Iwata, Cicchetti, & Reis, 1988; Petrovich, Canteras, & Swanson, 2001; Pitkanen, 2000). Therefore, the CeA was evaluated for its contribution to the production of pain affect in the present study.

1.1 Evidence for a Role of the Amygdala in Pain Processing

Case studies of patients that received ablations within the limbic forebrain implicate the amygdala as an essential structure involved in the affective experience of pain. The classic neurological patient H.M. received a bilateral resection of the medial temporal lobe for the treatment of epilepsy, resulting in the ablation of several limbic structures, including most of the amygdaloid complex (Corkin, Amaral, Gonzalez, Johnson, & Hyman, 1997). H.M. subsequently suffered from a range of neurological deficits, including anterograde amnesia, the inability to perceive odor quality, and most relevant to the current investigation, the failure to identify painful stimuli and to withdraw from such stimuli (Corkin, 1984; Hebben, et al., 1985). Hebben and colleagues (1985) demonstrated that thermal stimulation of H.M.'s hand or chest, as compared to normal

control patients and control patients with global amnesia, resulted in a failure to perceive the stimulation as noxious as measured by withdrawal latency (Hebben, et al., 1985). Although these results implicate the ablated region (i.e., amygdala) in pain sensation, H.M.'s inability to report internal states such as hunger and thirst and his inability to feel discomfort from the applied noxious thermal stimulation suggests a generalized deficit in affective responding.

In patients with trigeminal neuralgia, bilateral amygdalotomy reduces intractable pain (Jelasic, 1966). Patients reported moderate to intense pain during electrode implantation and during injection of the lesioning material into the left or right hemispheric amygdala. Following the procedure, patients reported decreased pain sensation and decreased pain affect to the neuralgia syndrome. Brown (1977) successfully treated intractable pain patients who experienced associated psychological consequences (e.g., drug dependence and psychogenic fixation) with lesions of the limbic system (Brown, 1977). Follow-up assessments of up to twenty years revealed that the majority of patients (90.5%) experienced improvement as measured by pain relief, requirement of medication, and ability to function at a job or in the home. These case studies demonstrate that the amygdala and other forebrain structures process nociceptive information, and most notably, mediate the affective dimension of the pain experience.

In accordance with the findings from lesion studies, human neuroimaging studies consistently reveal activation of the amygdala during noxious stimulation (Bingel, et al., 2002; Kulkarni, et al., 2005). Bingel and colleagues (2002) reported that application of noxious radiant stimuli via a thulium (Tm)-yttrium-aluminum-granate (YAG) laser to

either the left or right hand of healthy participants evoked bilateral increases in the blood-oxygen-level-dependence (BOLD) measure within the amygdala. Bilateral activation of the amygdala to a unilateral stimulus precludes amygdalar involvement in the sensory-discriminative aspect of pain processing and rather supports a role of the amygdala in the affective-motivational aspect of pain processing. Further, healthy participants instructed to attend to the unpleasantness associated with the application of a noxious CO_2 laser to the left dorsal forearm exhibited increases in regional cerebral blood flow (rCBF) within the amygdala (Kulkarni, et al., 2005). Participants instructed to attend to the location of the stimulus did exhibit a change in rCBF levels within the amygdala. These results support the notion that the amygdala is involved in affective-motivational processing of nociceptive information. It should be noted that the resolution of current imaging technology does not permit identification of the individual amygdaloid nuclei activated in these studies. Recently, however, pain-evoked neural activity was recorded in humans through electrodes implanted bilaterally in the medial temporal lobe during the investigation of intractable epilepsy (Liu, et al., 2010). Stimulation of the hand with a laser that selectively activated cutaneous nociceptors produced evoked responses from CeA. Consistent with the results of neuroimaging studies, pain-evoked responses in CeA were recorded bilaterally following stimulation of either hand.

Fos is the protein product encoded by the c-*Fos* gene that is expressed following neurotransmitter binding and membrane depolarization (Sheng & Greenberg, 1990), and provides an indirect measure of neuronal activation. *Fos* expression is increased in the amygdaloid complex following acute intra-plantar (i.pl.) formalin injection (Lei,

Zhang, & Zhao, 2004) and in response to noxious peripheral stimulation of the tail via hot water bath (Dai, Zhu, Li, Huang, & Xu, 1993). These studies do not discuss which subnuclei of the amygdala exhibited increased *Fos* expression, however, examination of the histological figures suggest primary labeling in the medial, lateral, basal, and central nuclei. Nakagawa and colleagues (2003) reported that i.pl. formalin injections significantly increased *Fos* expression in the lateral and basolateral amygdala, but not CeA, contralateral to the side of stimulus presentation. However, these investigators observed that intraperitoneal (i.p.) administration of acetic acid elevated *Fos* expression in CeA, and to a lesser degree in LA and BLA (Nakagawa, et al., 2003). Fear conditioning studies that utilize noxious foot-shock as an unconditional stimulus (US) revealed that foot-shock administered within a context during training results in increased CeA-*Fos* expression when compared to rats that did not receive foot-shock within that same context (Milanovic, et al., 1998; Radulovic, Kammermeier, & Spiess, 1998). Deep somatic pain induced by formalin injection into the rat multifidus muscle (i.e., low back muscle) produces significant *Fos* expression in the BLA (Ohtori, et al., 2000). The pharmacological activation of protein kinase C (PKC), via intrathecal phorbol 12,13-dibutyrate administration, within the spinal dorsal horn produces behavioral pain states (i.e., scratching, licking, biting, severe tail shaking, and vocalizations), and these nociceptive behaviors correlate with *Fos* expression in the cingulate cortex, parafascicular nucleus, and basolateral amygdala (Narita, et al., 2004). That cellular activation of the amygdala arises from such a diverse typology of noxious stimulation implicates the amygdala as a structure strongly involved in the experience of pain.

Excitotoxic lesions of CeA resulted in the abolition of conditioned place aversion (CPA) supported by noxious chemical stimulation (i.p. administered acetic acid) or formalin injection into the hind-paw, but failed to reduce associated nociceptive behaviors (Tanimoto, Nakagawa, Yamauchi, Minami, & Satoh, 2003). Acetic acid-induced pain behaviors included typical writhing behavior (i.e., contraction of abdominal muscles followed by extension of the hind limbs) and formalin-induced pain behaviors included the elevation and the biting, licking, or shaking of the injected paw. These results suggest a role of CeA in the generation of pain affect as measured by CPA, but not pain sensation as measured by the aforementioned writhing and formalin-induced pain behaviors. Because the forebrain does not integrate writhing behavior or formalin behavior (Hammond, 1989; Matthies & Franklin, 1992), amygdalar lesions would not affect these behaviors. Additionally, electrolytic lesions of the CeA resulted in the elevation of the threshold for tail-shock to elicit the vocalization after-discharge (VAD) response (Borszcz & Leaton, 2003). Research in this laboratory validated VADs as a model of pain affect (Borszcz, 1993, 1995a, 1995b; Borszcz, Johnson, & Fahey, 1994). These vocalizations occur following a brief noxious tail-shock and are spectrographically distinct from vocalizations that occur during tail-shock (VDS; Borszcz, 1995b, 2006). This evidence that amygdala lesions suppress affective pain responses strongly implicates the amygdala in the processing of the affective-motivational dimension of the pain experience.

1.2 Nociceptive Processing within CeA: Contribution of Glutamate Receptors

As described earlier, the CeA receives nociceptive afferents via a variety of pathways. In the rat, nociceptively responsive neurons in CeA are localized within the

lateral capsular sub-division (CeALC). CeALC neurons have either wide dynamic range (WDR) or nociceptive specific (NS) characteristics (Bird, et al., 2005; Han & Neugebauer, 2005; Li & Neugebauer, 2004a, 2004b). WDR neurons respond to both innocuous and noxious stimulation of the periphery with the rate of neural activity related to intensity of stimulation, and NS neurons respond only to noxious stimulation. Activation of both WDR and NS neurons occurs following stimulation of broad areas of the body, indicating large receptive fields for both types of neuron (Bernard & Besson, 1990; Bernard, Huang, & Besson, 1992; Neugebauer & Li, 2002).

Nociceptive input to NS neurons in CeALC appears to be provided by the spino-parabrachio-amygdaloid pathway (Bernard & Besson, 1990; Ma & Peschanski, 1988). Nociceptively responsive neurons in the external lateral pontine parabrachial (pPBel) and external medial pontine parabrachial (pPBem) nuclei that project to CeALC are innervated by projections from nociceptively responsive laminae I neurons of the spinal dorsal horn (Todd, et al., 2002). These PB neurons lack WDR characteristics, and only respond to noxious input from the periphery (i.e., possess NS characteristics; Bernard & Besson, 1990).

Noxious-evoked activity in WDR neurons in CeALC is mediated by glutamate receptors. Li and Neugebauer (2004a) recorded extracellular single-unit activity from WDR neurons in CeALC in anesthetized rats. The neurons' responses to graded brief (15 s) mechanical stimuli (noxious pinch) applied to the knee were challenged by administration of NMDA (APV) and non-NMDA (DNQX) receptor antagonists into the CeA via reverse microdialysis. Both receptor antagonists suppressed noxious-evoked neural activity in CeALC. APV failed to alter spontaneous background activity or the

response of CeALC neurons to innocuous mechanical stimulation. Alternately, DNQX also suppressed background activity and the response of CeALC neurons to innocuous stimulation.

Given the contribution of the CeA to the production of affective behaviors in response to threats, and the contribution of CeA-glutamate receptors to nociceptive processing within the CeA, we evaluated the effects of CeA-administration of NMDA and non-NMDA receptor antagonists on the generation of pain affect. We observed that administration of APV (NMDA antagonist) or CNQX (non-NMDA antagonist) into the CeA preferentially elevated the current intensity of tail-shock to elicit VADs (Spuz & Borszcz, in preparation; see Figure 1 for APV data). The current intensity to elicit VDS was elevated to a lesser degree; whereas, the current to elicit spinal motor reflexes (SMRs = tail-flicks) was not altered by injection of glutamate receptor antagonists into the CeA. These findings indicate that glutamate-mediated neuronal activation in CeA contributes preferentially to the production of the affective response to pain.

1.3 Evidence for the Involvement of NMDA Receptors within the Amygdaloid Central Nucleus in Antinociception

Whereas the aforementioned results indicate that NMDA (and non-NMDA) receptor-mediated neuronal excitation within CeA contributes to production of pain affect, a variety of evidence indicates that activation of the CeA suppresses responses to noxious stimulation. Electrical stimulation of the CeA results in antinociception measured as an increase in the tail-flick latency to noxious heating of the tail (Mena, Mathur, & Nayar, 1995; Oliveira & Prado, 1998), a reduction of the tonic phase in the formalin test and suppression of VDSs and VADs to noxious tail-shock (Mena, et al.,

1995). Additionally, stimulation of the CeA suppresses shock-induced vocalizations in guinea pigs (Leite-Panissi, Coimbra, & Menescal-de-Oliveira, 2003).

As systemic administration of NMDA agonists produces neuronal excitation within CeA (Inada, Farrington, Moy, Koller, & Duncan, 2007; Radulovic, Blank, Nijholt, Kammermeier, & Spiess, 2000) the possibility that NMDA receptor agonism within CeA (like NMDA receptor antagonism) suppresses pain affect was evaluated. Preliminary findings demonstrated that administration of NMDA into the CeA produced antinociceptive effects similar to that observed following injection of APV into the CeA (see Figure 2). That is, both treatments preferentially elevated VAD threshold. The present study was designed to evaluate the mechanisms through which NMDA receptor activation and antagonism within CeA produce suppression of pain affect.

Hypothesis

The central hypothesis of this study is that NMDA receptor antagonism within CeA blocks nociceptive transmission to efferent sites of CeA that coordinate affective responding to noxious stimulation; whereas, NMDA receptor activation within CeA engages efferent projections of CeA that activate endogenous antinociceptive mechanisms that suppress nociceptive transmission that contributes to production of pain affect. Support for the former mechanism is provided by the aforementioned findings that administration of an NMDA receptor antagonist into the CeA inhibits noxious evoked neuronal activity within CeA (Li & Neugebauer, 2004a), and our finding that administration of APV into the CeA suppresses pain-induced vocalizations The latter mechanism is supported by reports that antinociception elicited by stimulation of CeA is blocked by inactivation of the ventrolateral periaqueductal gray (vlPAG; Leite-

Panissi, et al., 2003; Oliveira & Prado, 1998). The vlPAG is a core mesencephalic structure that contributes to opiate-induced antinociception (Bodnar, Williams, Lee, & Pasternak, 1988; Borszcz, 1995a; Borszcz, Johnson, & Thorp, 1996; Jensen & Yaksh, 1986), and is reciprocally interconnected with CeA (Mantyh, 1982, 1983a, 1983b). Electrical stimulation of the CeA produces antinociception that is blocked by pretreatment of the vlPAG with the mu-opioid receptor antagonist beta-funaltrexamine (Oliveira & Prado, 2001). Moreover, glutamatergic stimulation of CeA activates projection neurons in vlPAG through enkephalin-mediated disinhibition (Behbehani, Jiang, & Chandler, 1990; da Costa Gomez & Behbehani, 1995; Sandkuhler, Willmann, & Fu, 1989). Projections of these vlPAG neurons to limbic, thalamic and brainstem sites contribute to the suppression of pain affect elicited by morphine injected into vlPAG (Borszcz, 1995a, 1999).

Specific Aims

Based on preliminary findings, the specific aims of this study were as follows:

Aim #1: *Complete the evaluation of the suppression of pain affect produced by administration of NMDA into the CeA.*

- Anatomical specificity: It was hypothesized that administration of NMDA into sites surrounding CeA will be less effective in suppressing VADs compared to administration into the CeA.
- Neurotoxicity: It was hypothesized that histological evaluation of CeA will reveal that NMDA-mediated suppression of VADs is not related to NMDA-induced neurotoxicity.

Aim #2: *Evaluate the functional interaction between the CeA and vlPAG in the suppression of pain affect.*

- It was hypothesized that if intra-CeA NMDA administration generates antinociception through activation of enkephalinergic interneurons in vlPAG, then suppression of VADs observed with intra-CeA NMDA administration will be attenuated or abolished following the intra-vlPAG administration of an enkephalin antagonist.

Aim #3: *Evaluate the cellular response of the vlPAG to NMDA and NMDA antagonist administered into the CeA.*

- It was hypothesized that if NMDA receptor antagonism blocks the through-put of nociceptive transmission at the level of the CeA, and if NMDA receptor agonism activates neural projections involved in endogenous antinociception, then the expression of the neural activity marker *Fos* within the endogenous antinociceptive circuit will differ following intra-CeA NMDA receptor antagonist vs. agonist.

CHAPTER 2

Methods

2.1 General Methods

Animals

A total of ninety Long-Evans rats (Charles River, Raleigh, NC) ranging from 100-200 days old were used in these experiments. Pairs of rats were housed in plastic cages in a climate-controlled vivarium (lights on 7 A.M. to 7 P.M.), and given ad libitum access to food and water. Testing occurred during the light portion of the cycle. Rats were handled two to three times over one week before surgery and before testing to minimize the effects of stress from human contact. All procedures were approved by the Institutional Animal Care and Use Committee of Wayne State University.

Stereotaxic Surgery

All surgeries were performed under aseptic conditions. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) following pretreatment with atropine sulfate (1 mg/kg, i.p.). For implants aimed at CeA, ventral to CeA, and dorsal to CeA, two stainless steel 26-gauge single-cannulae (Plastics One, Roanoke, VA) were stereotaxically, bilaterally implanted above CeA according to coordinates extrapolated from the rat brain atlas of Paxinos and Watson (Paxinos & Watson, 1998). The coordinates (in mm) relative to the bregma suture and the top of the flat skull are as follows: right implant (AP = -2.0, L = +4.0, DV= -6.0), left implant (AP = -2.0, L = +4.4, DV= -6.0). For implants aimed lateral to CeA, two single 26-gauge cannulae were bilaterally implanted above positions lateral to CeA using the following stereotaxic coordinates (in mm): right implant (AP = -2.0, L = +5.2, DV = -6.0), left implant (AP =

-2.0, L = +5.6, DV = -6.0). For implants aimed medial to CeA, two single 26-gauge cannulae were bilaterally implanted above positions medial to CeA using the following stereotaxic coordinates (in mm): right implant (AP = -2.0, L = +2.8, DV = -6.0), left implant (AP = -2.0, L = +3.2, DV = -6.0).

For implants aimed toward the vlPAG, one stainless steel 26-gauge single-cannula (Plastics One, Roanoke, VA) was implanted unilaterally above the vlPAG at a twenty-degree angle according to coordinates extrapolated from the rat brain atlas of Paxinos and Watson (Paxinos & Watson, 1998). Rats received vlPAG implants on either the left or right side based upon random assignment. The coordinates (in mm) relative to the bregma suture and the top of the flat skull were as follows: AP = -7.8, L = +2.6, DV= -3.6.

All cannulae were affixed to the skull with four stainless steel bone screws (3/16 in) and cranioplastic cement. Each guide cannula was fitted with a 33-gauge dummy cannula that extends the length of the guide to maintain its patency. Rats were given 7-10 days to recover before the initiation of testing.

Drug Injections

Intracerebral CeA injections were administered in a constant volume 0.25µl via 33-guage injectors. Injectors targeted at CeA extended 3mm beyond the end of the cannula. Injectors targeted at sites dorsal to CeA extended 1.8mm beyond the end of the cannula, and injectors targeted at sites ventral to CeA extended 4.2mm beyond the end of the cannula. Intracerebral vlPAG injections were administered in a constant volume 0.5µl via a 33-guage injector. Injectors targeted at vlPAG extended 3mm beyond the end of the cannula. All injections were made at a constant rate over 1 min

via an infusion pump (Harvard Model PHD 2000) and injectors were left in place for 2 min after the completion of injections to aid the diffusion of drugs into tissue. N-methyl-D-aspartate (NMDA; Tocris, Ellisville, MO), D-(-)-2-Amino-5-phosphopentanoic acid (APV; Tocris, Ellisville, MO), and H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP; Sigma-Aldrich, St. Louis, MO) were dissolved in normal sterile saline.

Histological Analysis

All rats with the exception of those that underwent *Fos* expression analysis were sacrificed by carbon dioxide asphyxiation at the completion of their testing sequence. Injection sites were marked by safrin-O dye (0.25µl) and brains were extracted and placed in a 20% (w/v) sucrose formalin solution for 48-72 hours. Brains were sectioned at 45µm on a freezing microtome, and injection sites were localized with the aid of the Paxinos and Watson (1998) brain atlas by a researcher unaware of the results from behavioral testing.

Rats from the NMDA dose response study that received the highest NMDA dose (1µg/.25µl per side) were stained using cresyl violet in order to assess the potential neurotoxic effects of NMDA qualitatively. Stained tissue was histologically examined at 10X magnification and compared to stained tissue from rats that received saline injections. Neuronal cell loss or proliferation of glial cells surrounding the NMDA injection site was considered evidence for neurotoxic damage.

2.2 Experimental Design

Dose Response Analysis

To quantify a dose-response relationship between CeA-administered NMDA agonist and SMR, VDS, and VAD thresholds, rats received bilateral injections of one dose of

NMDA (0.1µg, 0.25µg, 0.5µg, and 1µg/side) and saline into the CeA prior to two separate test sessions. Injections of drug were given over 2 min, and the injector remained in place for 1 min to aid in the diffusion of drug into tissue. Doses of NMDA were determined by preliminary results. Saline injections were maintained as the first test in order to ascertain baseline levels of responding. Test sessions were separated by 4 – 6 days, each drug group contained 6 or 7 rats, and the saline group contained 21 rats (summation of saline treatments from all rats in all drug treatment groups).

Anatomical Specificity

The anatomical specificity of NMDA mediated antinociception within the CeA was evaluated by administration of NMDA (1µg/side) into sites surrounding the CeA. Rats (n = 7) received a saline test followed by a test with NMDA agonist on separate test sessions at each of three separate anatomical sites by means of injectors that extend 1.8mm, 3.0mm, and 4.2mm beyond the length of the guide cannulae (see Figure 3 for a schematic of injector placement). Test sessions were separated by 5 – 7 days. NMDA administration within the CeA in this study permitted the replication of NMDA (1µg/side) on response thresholds, and these data were used to compare the effects of NMDA administered into sites surrounding the CeA.

CeA NMDA – vlPAG CTAP Interaction

Rats (n = 8) first received three testing sessions (sessions separated by 5 – 7 days) with different pairs of injections separated by 15 min in the following order: CeA saline + vlPAG saline; CeA saline + vlPAG CTAP (0.25µg/0.5µl); CeA NMDA (0.025µg/0.25µl per side) + vlPAG saline. These tests allowed for the assessment of baseline

responding, CTAP effects on baseline responding, and the replication of NMDA-induced antinociception, respectively.

The capacity for vlPAG mu-opiate receptors to mediate CeA-NMDA induced antinociception was evaluated by a final test using the following pair of injections: CeA NMDA + vlPAG CTAP.

The dose of NMDA was determined following preliminary experimentation to determine the dose of NMDA that consistently produced elevations in threshold that were below ceiling. This permitted the measurable capacity for CTAP to attenuate the effects of NMDA. The dose of CTAP used was determined following preliminary experiments.

2.3 Pain Testing

Testing Apparatus

Rats were placed into custom made Velcro body suits and restrained on a Plexiglas pedestal using Velcro strapping that passes through loops located on the underside of the suits. This design maintained the rat in a crouched posture throughout testing and permitted unobstructed access to the head for intracerebral injections. Testing was conducted within a sound attenuating, lighted, and ventilated chamber equipped with a small window that enabled visual monitoring of the animal during testing.

Tail-shock (20ms pulses at 25Hz for 1,000ms) was delivered by a computer controlled constant current shocker (STIMTEK, Arlington, MA) through electrodes (0-gauge stainless steel insect pins) placed intracutaneously on opposite sides of the tail, 7.0cm (cathode) and 8.5cm (anode) from the base. The utility of this form of tail-

shock as a noxious stimulus has been extensively discussed (Borszcz, 1993, 1995b; Borszcz, et al., 1994; Bromm & Meier, 1984).

Spinal motor reflexes (SMRs) were measured with a semi-isotonic displacement transducer (Lafayette Instruments Model 76614, Lafayette, IN) attached to the rat's tail with cotton thread. The output voltage of the transducer was amplified (x50) and then digitized (500Hz sampling rate) by an analog-to-digital converter of the microcomputer. SMR was defined as movement of the transducer arm by at least 1mm following shock onset.

Vocalizations were recorded by a pressure-zone microphone (Realistic model 33-1090, Tandy, Ft. Worth, TX) located on the wall of the testing chamber 15cm from the rat's head. The microphone was connected to an audio amplifier (Technics model SA-160, Tandy, Ft. Worth, TX) and a 10-band frequency equalizer adjusted to selectively amplify frequencies above 1500Hz. The filtering of low frequencies prevented extraneous noise (i.e., rats' respiration and movement artifacts) from contaminating vocalization records. The output of the amplifier was integrated by a Coulbourn Instruments (Allentown, PA) contour following integrator (2ms time base) and digitized (500Hz sampling rate) by a separate analog-to-digital converter of the microcomputer.

Performance Measurement

Performance variables for each animal were recorded by the microcomputer during every test. SMR performance consists of the latency (ms), peak amplitude (mm), and magnitude (cm x ms) of tail movement on each trial. Vocalization performance includes the peak intensity (in decibels: SPL, B scale), latency (ms), and duration (ms) of

vocalizations during the shock epoch (VDS = vocalization during shock) and for the 2,000 ms interval following shock termination (VAD = vocalization afterdischarges). Previous studies revealed that changes in these performance variables reflect the confounding influence of motor impairments on increases in response thresholds (Borszcz, 1993; Borszcz, et al., 1994).

Testing Protocol

For two consecutive days prior to testing, rats were be adapted to the testing apparatus for a period of 20 min each day to minimize the effects of restraint. For all studies, testing began 6-10 min following completion of intra-CeA injections. Test sessions consisted of 20 randomly presented trials. On 16 trials, tail-shocks between 0.02 mA and 2.50 mA were delivered, and on four trials no current was delivered so as to assess false alarm rates. Trials were presented with a minimum intertrial interval of 30 sec and each test session concluded within 20 min. These procedures cause no observable damage to the tail. Following each test session, the testing apparatus was cleaned with 5% ammonia hydroxide to eliminate stress odors (Fanselow, 1985).

Data Analysis

Threshold data was reorganized in ascending order according to tail-shock intensity. SMR, VDS, and VAD thresholds for each rat were calculated as the minimum current intensity from a string of at least two consecutive intensities that generated the response. All analyses used alpha = .05.

Response thresholds for the NMDA dose response experiment were directly compared using repeated-measures MANOVA. A significant omnibus MANOVA was followed by within-subjects contrasts of response thresholds. The effects of dose on

individual responses were analyzed by one-way ANOVA. The doses of NMDA that elevated response thresholds above baseline levels (i.e., saline thresholds) were determined by comparing thresholds following saline and NMDA treatments using Dunnett's test. The capacity of NMDA to elevate response thresholds following its injection into sites surrounding the CeA was analyzed for each response by one-way ANOVA. Post-hoc comparisons using independent samples t-tests were used to assess response thresholds generated following the administration of NMDA (1µg/.25µl per side) or saline into the CeA and into sites surrounding the CeA. The capacity of vlPAG-CTAP to reduce NMDA-CeA induced elevations of response thresholds was analyzed across treatment groups for each response by a one-way ANOVA followed by post-hoc independent samples t-test.

Testing sessions that included a threshold greater than 2.0 standard deviations from the mean threshold was considered an extreme outlier and was excluded from analysis (n = 2). Rats with incorrectly placed cannulae were also excluded from analysis (n = 2). Rats were also excluded from analysis due to death during surgery (n = 1), illness (n = 2) or complications related to their surgical cannulae implants (n = 3).

2.4 Immunohistochemistry

Experimental Design - CONTEXT

Male Long Evans rats were first randomly assigned to one of three context groups: Home Cage, Chamber Only, and Shock. Home Cage rats resided in the laboratory holding room for three consecutive days (at least 3h/day). Chamber Only rats were exposed to the afore-described tail-shock vocalization response test chamber, but did not receive shock, for three consecutive days (20 min/day) in addition to residing in the

laboratory holding room. Shock rats followed protocol identical to the Chamber Only group except that on Day 3 a tail-shock vocalization-response test (see above) was given to the rat in the chamber.

Rats within each Context group were further randomly assigned to one of three drug treatment groups: saline, APV, or NMDA. Rats were given intra-CeA treatments bilaterally. The doses used in these groups were as follows: saline (0.25µl/side), APV (4µg in 0.25µl/side), and NMDA (1µg in 0.25µl/side).

Free-Floating Immunohistochemistry Protocol

Two hours after the intracerebral injection, the animals were deeply anaesthetized with an overdose of sodium pentobarbital into the liver (120 mg/kg) and transcardially perfused with 0.1M phosphate-buffered saline (pH = 7.4) followed by 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4; 4° C). The brains were removed and post-fixed at 4° C for 1.5 hours in 4% PFA and then stored for at least 48 hours in 30% sucrose in 0.1M PBS at 4° C for cryoprotection or in long-term cryoprotectant (0.1M PBS + ethylene glycol + sucrose) at -20° C. Brains were sliced transversely at 45µm and vlPAG sections with the AP coordinates -7.64 through -8.72 mm posterior to Bregma were collected.

Tissue sections were collected in 0.1M PBS and subsequently processed free-floating according to the avidin–biotin-peroxidase complex (ABC) method. Primary antibody concentrations were determined (1:5000) following a titration protocol that varied the concentration of primary while leaving the secondary at a constant (1:200 per manufacturer). All reactions were carried out under agitation at room temperature.

On the first day, the sections were washed six times (10 min each) in 0.1M PBS and then incubated with 0.3% H_2O_2 for 30 min. Sections were then washed three times with 0.1M PBS (5 min each) and then incubated with blocking buffer (0.3% Triton X-100, 1% normal goat serum, 1% bovine serum albumin in 0.01M PBS, pH = 7.4) for 60 min to reduce non-specific antibody staining. Sections were then incubated overnight with the primary *Fos* rabbit polyclonal IgG (Santa Cruz, USA) at a concentration of 1:5000 in blocking buffer.

On the second day, sections were washed three times (10 min each) with PBST (0.1M PBS with 0.02% Triton X-100) and then incubated for 2 hr with secondary biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories) at a concentration of 1:200 in blocking buffer. The sections were then incubated for 1 hr with the avidin and biotin complex (A and B solution of the ABC kit, Vector Laboratories) at a concentration of 1:500 in PBST, and then again washed three times in PBST (5 min per wash) followed by two washes in 0.05M Tris-HCl (pH = 7.6; 5 min each). Finally, chromagen was visualized with 0.005% 3,3'V-diaminobenzidine (Sigma), 0.6% nickel ammonium sulfate, and 0.005% H_2O_2 in 0.05M Tris-HCl. Tissue sections were washed twice with 0.05 M Tris-HCl (5 min each) and then washed in distilled water for 10 min.

Quantification of Fos-*Positive Cells*

Tissue sections were mounted on gelatin-coated slides, dehydrated, coverslipped, and photographed at with a Nikon Eclipse 80i microscope with a digital camera attached to it (Cool Snap Photometrics EZ). The vlPAG was photographed at 40x and compared to the rat brain atlas (Paxinos & Watson, 1998) as an orientation aid for tracing the vlPAG. Images were magnified to 200x, and a 400µm x 300µm box was placed within

the confines of the vlPAG. *Fos* immunoreactivity was visualized as a dark reaction product inside neuronal nuclei. The number of *Fos*-positive nuclei was counted in the box by hand with the aid of a computerized cell counting system (Nikon Elements Software, 3.1). The vlPAG was bilaterally counted in each rat and counted on three to four separate sections collected from each rat. The results are expressed as the number of *Fos*-positive nuclei.

Data Analysis

The number of *Fos*-positive nuclei is expressed as mean ± S.E.M. Results were analyzed using a two-way between-subjects ANOVA with drug treatment (saline, APV, NMDA) and context (Home Cage, Chamber, Shock Test) as between-group factors followed by one-way ANOVAs with Dunnett's post-hoc tests. The alpha level was .05 for all analyses.

CHAPTER 3

RESULTS

3.1 NMDA Dose Response Analysis

The effects of the bilateral administration of NMDA into the CeA on SMR, VDS, and VAD thresholds are depicted in Figure 2, and the distribution of the bilateral administration sites are depicted as black triangles in Figure 4. The repeated-measures MANOVA (Wilk's λ) comparison of response thresholds revealed significant main effects of response ($F(2,41) = 122.06$, $p < 0.001$) and dose ($F(4,42) = 21.94$, $p < 0.001$), and a significant Response x Dose interaction, ($F(8,82) = 11.30$, $p < 0.001$). One-way ANOVA revealed that VDS and VAD thresholds were elevated in a dose-dependent manner by NMDA administration [VDS: $F(4,46) = 9.75$, $p < 0.001$; VAD: $F(4,46) = 32.28$, $p < 0.001$], but NMDA treatments did not affect SMR thresholds, $F(4,46) = 2.44$, $p > 0.05$. Post-hoc analysis using Dunnett's test revealed that 0.25µg NMDA was the minimum dose required to significantly elevate VDS thresholds ($M = 1.00$, $SEM = .31$) above those observed with saline treatment ($M = .06$, $SEM = .01$). Dunnett's test revealed that 0.1µg NMDA was the minimum dose required to significantly raise VAD thresholds ($M = .66$, $SEM = .14$) above those observed with saline treatment ($M = .10$, $SEM = .01$). Dunnett's test revealed that bilateral 0.1µg NMDA administration preferentially elevated the VAD response over the VDS response, (VAD: $M = .66$, $SEM = .14$; VDS: $M = .22$, $SEM = .05$, respectively).

3.2 NMDA Anatomical Specificity

Bilateral administration of saline into the CeA and sites surrounding CeA did not produce a significant difference in response thresholds, $t(17) = 0.20$, $p > .05$; thus, these

data were combined. The effects of bilateral administration of saline and 1µg NMDA into the CeA and sites surrounding CeA are depicted in Figure 5.

One-way ANOVAs revealed that vocalization thresholds differed with respect to treatment location (Fs(2,37) > 24.76, p < .05), but SMRs did not differ (F(2,37) = .88, p > .05). Independent samples t-tests revealed that 1µg NMDA administration into the CeA significantly elevated VDS and VAD thresholds above those observed with saline administration (ts(28) > 6.29, p < .05). Independent samples t-tests further revealed that 1µg NMDA administration into sites surrounding the CeA produced significantly weaker elevations on VDS and VAD thresholds compared to the administration of 1µg NMDA into the CeA (ts(17) > 3.27, p < .05). Figure 4 depicts administration sites where NMDA effectively elevated (black squares, NMDA-CeA) or failed to elevate (black circles, NMDA-other) VAD thresholds.

3.3 Neurotoxicity Analysis

Cresyl violet stained CeA sections from rats that received bilateral microinjections of 1µg NMDA did not produce any pattern of neuronal cell loss or proliferation of glial cells. See Figure 11 for representative sections from an animal treated with bilateral CeA-saline and bilateral CeA-1µg NMDA. As depicted, there is no evidence of neurotoxicity within the NMDA CeA section compared to the saline treated section.

3.4 CeA NMDA – vlPAG CTAP Interaction

The effects of the bilateral administration of 0.025µg NMDA into the CeA challenged by the unilateral administration of 0.25µg CTAP into the vlPAG on SMR, VDS, and VAD thresholds are depicted in Figure 6, and the distribution of the administration sites are depicted as black circles in Figure 7.

One-way ANOVA revealed a significant interaction between CeA treatment and vlPAG treatment for SMR and VAD thresholds (Fs(1,32) > 6.00, ps < .05), but not VDS threshold (F(1,32) = 2.64, p < .05). Two points can explain the SMR interaction. First, the variability of the NMDA-CeA + CTAP-vlPAG group is extremely small. Second, mean data suggests that sal-CeA + CTAP-vlPAG treatment tends to elevate SMR threshold, but NMDA-CeA + CTAP-vlPAG treatment tends to lower SMR threshold. Independent samples t-test (one-tail) revealed that, as expected given the dose-response analysis, NMDA-CeA + sal-vlPAG treatment resulted in a significant elevation of VAD threshold above that observed with sal-CeA + sal-vlPAG treatment (t(14) = 2.29, p < .05). Independent samples t-test (one-tail) revealed that, as hypothesized, NMDA-CeA + CTAP-vlPAG treatment significantly attenuated VAD threshold compared to NMDA-CeA + sal-vlPAG treatment (t(14) = 1.87, p < .05).

3.5 Fos Expression within vlPAG

The effects of bilateral intra-CeA treatment and context on the number of *Fos*-positive nuclei within the vlPAG are depicted in Figure 8. Representative sections with *Fos* expression are shown in Figure 9. The two-way ANOVA (treatment x context) revealed a significant main effect of treatment on the number of *Fos*-positive nuclei within the vlPAG (F(2,36) = 59.86, p < .05) and a significant treatment x context interaction (F(4,36) = 3.53, p < .05), but did not reveal a main effect of context (F(2,36) = .03, p > .05). One-way ANOVA revealed significant simple effects within the Home Cage, Chamber Only, and Shock contexts (Fs(2,11) > 4.46, ps < .05). Post-hoc analysis via Dunnett's revealed that within the Home Cage context, the number of intra-vlPAG *Fos*-positive nuclei was greater in animals that received bilateral intra-CeA 1µg

NMDA (*M* = 29.22 ± SEM = 4.23) compared to animals that received intra-CeA saline (*M* = 5.58 ± SEM = 0.89) and intra-CeA 4µg APV (*M* = 7.78 ± SEM = 0.85). Within the Chamber Only context, the number of intra-vlPAG *Fos*-positive nuclei was greater in animals that received bilateral intra-CeA 1µg NMDA (*M* = 21.07 ± SEM = 3.63) compared to animals that received intra-CeA 4µg APV (*M* = 8.73 ± SEM = 0.82). Within the Shock Test context, the number of intra-vlPAG *Fos*-positive nuclei was greater in animals that received bilateral intra-CeA 1µg NMDA (*M* = 32.05 ± SEM = 3.32) compared to animals that received intra-CeA saline (*M* = 4.35 ± SEM = 0.90) and intra-CeA 4µg APV (*M* = 5.67 ± SEM = 1.29).

One-way ANOVA revealed that SMR, VDS, and VAD thresholds in the Shock Test Context differed with respect to CeA treatment (*F*s(2,61) > 5.24, *p*s < .05). Independent samples t-tests revealed that compared to saline, SMR, VDS and VAD thresholds were elevated to a greater extent following 4µg APV (*t*s(45) > 2.73, *p*s < .05) and 1µg NMDA (*t*s(48) > 3.01, *p*s < .05). SMR and VAD threshold were not significantly different between 4µg APV and 1µg NMDA (*t*s25) < 1.83, *p*s > .05), but VDS threshold was significant (*t*(25) = 2.28, *p* <.05).

Figure 10 depicts mean threshold data for saline, APV, and NMDA from animals in the *Fos*, APV dose response, NMDA dose response, and CeA-vlPAG Interaction studies. Independent samples t-tests (one-tailed) revealed that compared to intra-CeA saline treatment, bilateral intra-CeA APV (*t*(45) = 7.86, *p* < .05) and NMDA (*t*(48) = 14.08, *p* < .05) significantly elevated VAD thresholds. Threshold elevations following APV treatment did not significantly differ from those following NMDA treatment (*t*(25) = 1.83, *p* < .05).

3.6 Performance Analysis

3.6.1 Response Profiles

Of the four experiments that composed this study, 388 test trials were sham trials (i.e., no shock given). False alarm rates were low (SMR = 1.80%, VDS = 0.00%, VAD = 0.00%) and indicate that behaviors did not occur spontaneously or as a result of drug treatment, but instead were generated via tail-shock. SMR, VDS, and VAD reflect nociceptive processing at progressively higher levels of the neuraxis. Analysis of rats that received transections of the neuraxis revealed that SMR responses are organized at the spinal level, VDS within the medulla below the pontomedullary border, and VAD within the forebrain (Borszcz, Johnson, Anderson, & Young, 1992; Carroll & Lim, 1960). On the remaining 1,552 trials where tail-shocks were administered, responses organized rostrally within the CNS were rarely generated without those integrated more caudally within the CNS. VAD generation, without concomitant elicitation of VDS and SMR, occurred on 0.58% of all trials. VDSs were elicited without SMR on 0.32% of the trials in which VDS was the most rostrally elicited response.

3.6.2 Response Characteristics

SMR reaction time (SMR.RT), amplitude (SMR.AMP), and magnitude (SMR.MAG); VDS reaction time (VDS.RT), amplitude (VDS.AMP), and duration (VDS.DUR); and VAD reaction time (VAD.RT), amplitude (VAD.AMP) and duration (VAD.DUR) were recorded at threshold for vehicle and each drug treatment condition (see Table 1, mean ± standard error of the mean), and compared to saline treatment threshold (i.e., baseline) using a one-way ANOVA with Dunnett's post-hoc tests (see Table 2).

NMDA Dose Response and Anatomical Control Studies

Comparison of SMR performance variables across saline and NMDA drug treatments revealed that SMR performance at threshold was not affected by NMDA treatments, *F*s(4,83) < 0.81, *p*s > .05. Comparison of VAD performance variables across saline and NMDA drug treatments revealed that VAD performance at threshold was not affected by NMDA treatments, *F*s(4,74) < 2.26, *p*s > .05. Comparisons of VDS performance variables demonstrated that reaction time of VDSs at threshold were not altered by NMDA treatment (*F*(4,79) = 1.21, *p* > .05), but the amplitude and duration of VDSs were significantly lower following NMDA treatments, *F*s(4,79) > 3.18, *p*s < .05. Post-hoc analysis revealed that the amplitude of VDS was decreased compared to baseline following bilateral administration of 1µg NMDA. Post-hoc analysis revealed that the duration of VDS was decreased compared to baseline following bilateral administration of 0.25µg NMDA, 0.5µg NMDA and 1µg NMDA. The effect on VDS amplitude is small ($M_{1\mu g\ NMDA}$ = 85.52 ± *SEM* = 1.22 vs. M_{saline} = 90.06 ± *SEM* = 0.94) and did not occur in other experiments. The effects on VDS duration also did not occur in the 1µg NMDA group in the *Fos* Expression Study – Shock Group.

CeA – vlPAG Interaction Study

Comparison of SMR, VDS, and VAD performance variables across baseline (saline CeA + saline vlPAG) and drug treatments revealed that all response characteristics were not significantly affected by treatment, *F*s(3,30) < 1.40, *p*s > .05.

Fos Expression Study – Shock Group

Comparison of performance variables across bilateral intra-CeA saline, 4µg APV, and 1µg NMDA revealed that all response characteristics were not significantly affected

by treatment (SMR: *F*s(2,11) < 1.54, *p*s > .05; VDS: *F*s(2,8) < 2.38, *p*s > .05; VAD: *F*s(1,6) < 2.32, *p*s > .05). Three animals in the 1µg NMDA group did not respond to any shock intensity with a VDS, and thus only one animal's threshold data is reported for VDS.RT, VDS.AMP, and VDS.DUR. Likewise, all four animals in the 1µg NMDA group did not respond to any shock intensity with a VAD, and thus no threshold data could be reported for VAD.RT, VAD.AMP, and VAD.DUR.

CHAPTER 4

DISCUSSION

The present study provides the first demonstration of behavioral antinociception generated by administration of an NMDA receptor agonist into the CeA. Administration of NMDA into the CeA produced dose-dependent increases in VAD and VDS thresholds but failed to elevate SMR threshold. Direct comparisons of response thresholds revealed that VAD threshold was preferentially elevated compared to VDS threshold by the intra-CeA injection of NMDA, and the minimum effective dose of NMDA to elevate VAD threshold was lower than the dose that raised VDS threshold. The increases in VAD threshold cannot be attributed to drug-induced motor deficits as increases in VAD threshold were not accompanied by performance decrements. Increases in VDS threshold may reflect the effects of drugs on performance. NMDA produced decreases in VDS duration at threshold and increases in VDS latency at threshold. Decrements in these performance variables following systemic drug treatments (i.e., morphine, diazepam) were shown to elevate response thresholds independent of the drugs' effect on sensory processing (Borszcz, et al., 1994). However, these decrements were relatively small and were not observed in the CeA-NMDA + vlPAG-CTAP interaction study.

Similar to the present results, administration of carbachol, serotonin (5-HT), the 5-$HT_{1A/7}$ agonist 8-hydroxy-dipropylaminotetralin (8-OH-DPAT), or morphine into either the basolateral amygdala (BLA), thalamic parafascicular nucleus (nPF), or ventral tegmental area (VTA) produced selective increases in VAD and VDS thresholds without an accompanying increase in SMR threshold (Harte, Hoot, & Borszcz, 2004; Harte,

Kender, & Borszcz, 2005; Harte, Lagman, & Borszcz, 2000; Kender, Harte, Munn, & Borszcz, 2008; Nandigama & Borszcz, 2003). The failure to observe increases in SMR threshold does not reflect the resistance of this response to antinociceptive treatments. In previous studies, administration of morphine into the rostral ventromedial medulla (RVM) or vlPAG produced significant increases in SMR, VDS, and VAD thresholds (Borszcz, 1995a; Borszcz, Johnson, & Thorp, 1996; Borszcz & Streltsov, 2000), and the intrathecal administration of morphine, 5-HT, or norepinephrine was equally effective in raising SMR, VDS, and VAD thresholds (Borszcz, Johnson, & Williams, 1996). The capacity of these central treatments to elevate SMR threshold also demonstrates that SMRs are not generated by direct stimulation of the tail musculature by the tail-shock. These findings indicate that the capacity to elevate SMR threshold depends on the site within the CNS at which antinociceptive treatments are administered.

The capacity of CeA-administered NMDA to elevate vocalization thresholds is likely limited to its action within CeA. Bilateral administration of the highest dose of NMDA (1µg/side) into sites surrounding the CeA produced greatly attenuated increases in VAD and VDS thresholds. Thus, it is unlikely that the effects of NMDA observed in the dose response study are the result of drug spread into these surrounding sites. Further, the effect of NMDA on vocalization thresholds cannot be the result of an excitotoxic lesion. NMDA is a known neurotoxin at high doses, but the doses used in the present study are well below those shown to produce cell loss (8 µg; Maisonnette, Kawasaki, Coimbra, & Brandao, 1996). Further, qualitative analysis of cresyl-violet stained tissue revealed that bilateral treatment with 1µg NMDA failed to produce cell loss. Additional evidence against elevations of vocalization thresholds being the result

of a lesion comes from the results of the CeA-NMDA + vlPAG-CTAP study, as CeA-NMDA induced vocalization threshold elevations were attenuated via vlPAG-CTAP administration. If the threshold elevations were due to a lesion of CeA, CTAP would be unable to reverse these elevations. This result also argues against the observed effects on vocalization thresholds being the consequence of NMDA induced elliptic-type neural activity producing a functional lesion in CeA (Frenk & Yitzhaky, 1981). Therefore, the capacity of NMDA administered into the CeA to suppress pain-induced vocalizations is the result of activation of NMDA receptors within CeA.

The preferential increase in VAD threshold after intra-CeA APV or NMDA administration reflects suppression of the affective reaction to noxious stimulation. Previous research in this laboratory validated VADs as a rodent model of pain affect. VADs have distinct spectrographic characteristics compared to VDSs (Borszcz, 1995b, 2006; Borszcz & Leaton, 2003), and are preferentially suppressed by systemically administered drug treatments that preferentially suppress the affective response of humans to pain (Borszcz, et al., 1994; Gracely, et al., 1978; Price, von der Gruen, Miller, Rafii, & Price, 1985). Generation of VADs is also suppressed by damage of or drug treatments into forebrain sites known to contribute to production of the affective response of humans to clinical and experimental pain (Borszcz, 1999; Borszcz & Leaton, 2003; Greer, 2007; Harte, et al., 2005; Harte, et al., 2000; Hoffmeister, 1968; Mark, Ervin, & Yakovlev, 1961; Nandigama & Borszcz, 2003; Sweet, 1980; Zubieta, et al., 2001). Additionally, the capacity of noxious tail-shock to support fear conditioning is directly related to its production of VADs (Borszcz, 1993, 1995b; Borszcz & Leaton, 2003), and fear conditioning supported by electrical stimulation of the ventromedial

hypothalamus is correlated with generation of VAD-like vocalizations (i.e., vocalizations with the same spectrographic characteristics as VADs; (Borszcz, 2006).

It is of interest that both the NMDA receptor antagonist APV (Spuz & Borszcz, in preparation) and the agonist NMDA produce similar behavioral effects when administered within the CeA. NMDA receptors have been identified in the medial (CeM) and capsular (CeC) subnuclei of the CeA in rat (Farb, Aoki, & Ledoux, 1995; Gracy & Pickel, 1995). It is proposed that manipulation of NMDA receptors within CeA produces affective analgesia, measured as elevations in VAD thresholds, via the action of APV and NMDA at separate neural populations within CeC and CeM, respectively.

4.1 Model of Intra-CeA APV-Induced Affective Analgesia

The CeA receives nociceptive-specific information from the spinal cord dorsal horn via the indirect spino-parabrachio-amygdaloid (Bernard & Besson, 1990; Ma & Peschanski, 1988; Todd, et al., 2002) pathway and the direct spino-amygdaloid pathway (Burstein & Potrebic, 1993; Cliffer, Burstein, & Giesler, 1991; Newman, et al., 1996). Electrophysiology studies indicate that the lateral region of CeC (CeALC) contains the greatest proportion of neurons activated by noxious peripheral stimulation of the rat body (Bernard, et al., 1992). Stimulation of these CeALC neurons antidromically activates pontine parabrachial (PB) neurons that respond exclusively to noxious cutaneous stimulation (Bernard & Besson, 1990). In-vitro whole-cell voltage-clamp recordings of rat CeALC neurons revealed that following PB electrical stimulation, CeALC neurons exhibit monosynaptic excitatory post-synaptic currents (EPSCs; Bird, et al., 2005). Bath application of APV to the slices does not alter evoked EPSCs, suggesting that glutamatergic pain transmission via the PB-CeALC pathway does not

interact with CeALC-NMDA receptors. However, in vivo investigations showed that administration of APV into the CeALC of rats does attenuate noxious, but not innocuous, evoked neural activity in the CeALC (Li & Neugebauer, 2004a). Thus, NMDA dependent nociceptive neural transmission interacting with the CeALC must project from regions other than the PB. An alternative pathway for NMDA dependent glutamate neurotransmission to interact with CeA is via the direct spino-amygdaloid pathway. To the best of our knowledge, no study has investigated whether glutamate is the major neurotransmitter involved in this direct spinoamygdaloid projection. Also as described earlier, the CeA receives nociceptive input relayed from the spinal dorsal horn via the intralaminar thalamus, yet no study has investigated the neurochemistry underlying these thalamic afferents or their contribution to noxious-evoked activity in the CeA.

Our previous demonstration that intra-CeA administration of APV produces dose-dependent increases in vocalization thresholds, with a preferential influence on VAD thresholds (Siegel, 2005; Spuz & Borszcz, in preparation) likely reflects the suppression of nociceptive processing at the level of the CeALC NMDA receptors. This blockade inhibits the further transmission of nociception to efferent sites of the CeA that coordinate affective responding to noxious stimulation. As noted earlier, CeA is the major output nucleus of the amygdaloid complex, and its projections to the ventromedial hypothalamus (VMH) and dorsolateral PAG (dlPAG) govern the execution of innate affective reactions to environmental threats, including pain (Borszcz & Spuz, 2009). The dorsomedial division of the VMH (dmVMH) and dlPAG are the core mesolimbic structures that control execution of affective responses to environmental threats (Siegel,

2005). Both the dmVMH and dlPAG exhibit *Fos* activation following exposure to either noxious or non-noxious threatening stimuli (Beckett, Duxon, Aspley, & Marsden, 1997a; Bullitt, 1990; Canteras, Chiavegatto, Ribeiro do Valle, & Swanson, 1997; Dielenberg, Hunt, & McGregor, 2001; Liu, Qiang, & Qiao, 1998; Parry, Semenenko, Conley, & Lumb, 2002; Rodella, Rezzani, Gioia, Tredici, & Bianchi, 1998; Sandner, et al., 1993), and inactivation or damage of these sites block naturally occurring affective responses to threats (Canteras, et al., 1997; Cheu & Siegel, 1998; Markham, Blanchard, Canteras, Cuyno, & Blanchard, 2004). Stimulation of the dmVMH and dlPAG elicits affective responses in rats, cats and monkeys (Fernandez De Molina & Hunsperger, 1962; Lipp & Hunsperger, 1978; Milani & Graeff, 1987), and in humans generates reports of fear, anxiety, and horror (Ervin, Mark, & Stevens, 1969; Heath, 1975; Iacono & Nashold, 1982; Tasker, 1982). For all these species, vocalizations are part of their affective reaction to imminent threat and are produced as part of their affective reaction to dmVMH or dlPAG stimulation (Blanchard, Hynd, Minke, Minemoto, & Blanchard, 2001; Blanchard, Flannelly, & Blanchard, 1986; Fernandez De Molina & Hunsperger, 1962; Jurgens & Pratt, 1979).

Previous research in this laboratory (Borszcz, 2006) demonstrated that electrical or chemical stimulation of dmVMH generates VAD-like vocalizations. Manipulation of $GABA_A$ neurochemistry within the dmVMH altered the threshold for elicitation of VADs by dmVMH electrical stimulation or tail-shock. Administration of the $GABA_A$ antagonist bicuculline or the $GABA_A$ agonist muscimol into the dmVMH lowered and elevated VAD threshold, respectively. These treatments did not alter thresholds of VDS or SMR elicited by tail-shock. Bicuculline and muscimol administered into the dmVMH also

elevated and lowered the asymptotic level of fear conditioning supported by dmVMH stimulation or tail-shock.

The dlPAG serves as the interface between limbic forebrain sites that process stimuli that threaten the individual and execution of innate affective responses that enable the individual to cope with the threat (Bernard & Bandler, 1998; Jurgens & Pratt, 1979; Keay & Bandler, 2001). Inputs from the dmVMH to the dlPAG activate descending dlPAG projections to the brainstem that coordinate the execution of the behavioral and autonomic responses that constitute affective responding. These projections are activated by nociceptive input to the dmVMH. Neurons within dmVMH that exhibit *Fos* expression following presentation of a noxious cutaneous stimulus are double-labeled by administration of a retrograde tracer into the dlPAG (Parry, et al., 2002). Projections from the dlPAG to the rostral ventrolateral medulla initiate the autonomic reactions associated with affective responses to threats (Lovick, 1992; Wang & Wessendorf, 2002). Projections from the dlPAG to the nucleus retroambiguus initiate activity in the laryngeal, articulatory, and respiratory motor neurons that generate affective vocalizations (Jurgens, 2002).

As depicted in Figure 13, nociceptive input to the CeA activates the dmVMH → dlPAG → brainstem circuit for generation of VADs. Suppression of VADs that follows administration of APV into the CeA is posited to reflect inhibition of nociceptive throughput to the dmVMH and related interconnected limbic sites. Consequently, nociceptive input is unable to gain access to the mesolimbic circuit responsible for generating affective behaviors to threats. As vocalizations are a common affective response to imminent threats and exposure to a noxious stimulus is the prototypical

imminent threat, the interruption of pain input to this circuit suppresses the rat's affective vocalizations to pain (i.e., VADs). As Charles Darwin noted concerning the emission of pain-induced vocalizations (Darwin, 1898):

- "When animals suffer from an agony of pain, they generally writhe about with frightful contortions; and those which habitually use their voices utter piercing cries or groans."
- "Great pain urges all animals, and has urged them during endless generations, to make the most violent and diversified efforts to escape from the cause of suffering…. As the muscles of the chest and vocal organs are habitually used, these will be particularly liable to be acted on, and loud, harsh screams or cries will be uttered."

4.2 Model of Intra-CeA NMDA-Induced Affective Analgesia

Alternately, elevations in vocalization thresholds following intra-CeA NMDA administration may reflect the action of this drug at NMDA receptors within the medial subdivision of the CeA (CeM). Figure 13 depicts excitatory projections from CeM that activate vlPAG inhibitory projections to the CeALC and dmVMH. NMDA receptor activation within CeM is postulated to engage efferent projections of CeM that activate endogenous antinociceptive mechanisms within the vlPAG. The CeM projects directly to the vlPAG (Rizvi, Ennis, Behbehani, & Shipley, 1991), a midbrain structure critically involved in endogenous antinociception. The neurochemistry of the CeM – vlPAG projection as involved in antinociception has yet to be elucidated, however, evidence suggests that substance P may play a role. Substance-P neurons in CeM project to the vlPAG (Gray & Magnuson, 1992), neurokinin (NK) receptors are localized on vlPAG enkephalin interneurons (Commons & Valentino, 2002), substance P binding to intra-vlPAG NK receptors evokes the local release of enkephalin (Drew, Mitchell, & Vaughan, 2005), and activation of vlPAG neurokinin receptors leads to antinociception (Rosen, Zhang, Lund, Lundeberg, & Yu, 2004).

Thus, CeM-NMDA receptor activation may activate substance P projections that interact with the antinociceptive neural circuitry of the vlPAG to produce elevations in vocalization thresholds. Specifically, the aforementioned substance P induced release of enkephalin in vlPAG may be the mechanism whereby NMDA administered into the CeA produced elevations of vocalization thresholds in the present study. The internal neurochemistry of the vlPAG that contributes to antinociception is well characterized and is depicted in Figure 12 (Reichling, 1991; Reichling, Kwiat, & Basbaum, 1988). The vlPAG contains tonically active GABA interneurons that suppress serotonergic output neurons. Enkephalin release within the vlPAG inhibits the GABA interneurons via binding with mu-opiate receptors on the GABA interneurons, thereby disinhibiting serotonin projection neurons in vlPAG. The serotonergic projection neurons activate antinociceptive processes at the level of the limbic system, thalamus, and brainstem. Congruent with this circuitry, morphine administration into the vlPAG and acting at mu-opiate receptors presumably located on the GABA interneuron, produces elevations in vocalization thresholds that are blocked via administration of a serotonin receptor antagonist (methysergide) into the amygdala, medial thalamus, or rostral ventral medulla (Borszcz, 1995a, 1999).

As noted earlier, behavioral antinociception generated by CeA activation is blocked by inactivation of the vlPAG via local lidocaine injection or administration of a mu-opiate receptor antagonist into vlPAG (Leite-Panissi et al., 2003; Oliveira & Prado, 2001). Further, electrophysiology experiments demonstrated that glutamatergic stimulation of the CeA alters vlPAG neural activity through intra-vlPAG opiate receptors (da Costa Gomez & Behbehani, 1995). Administration of D,L-homocysteic acid, a

glutamate agonist, into the CeA results in an approximate 1:1 ratio of vlPAG neural excitation to inhibition. These vlPAG neural responses were suppressed following the microiontophoretic application of the opiate antagonist naloxone to the vlPAG. The excitatory and inhibitory responses recorded from the vlPAG may reflect the recording of separate neural populations in line with the internal neurochemistry of the vlPAG. Excitatory responses following D,L-homocysteic acid likely reflects excitation of the vlPAG enkephalin interneurons and/or the serotonergic projection neurons. Alternately, inhibitory responses likely reflect inhibition of GABA interneurons.

Results of the present study are consistent with the hypothesis that NMDA receptor activation in CeA suppresses pain affect through the release of enkephalin in vlPAG, which engages antinociceptive projections from the vlPAG. Compared to following injection of saline into the CeA, the administration of NMDA into the CeA produced increased *Fos* expression in the vlPAG. This difference in *Fos* expression was observed in all three experimental contexts, and therefore is a reliable effect of NMDA receptor activation within the CeA. Presumably, *Fos* was expressed by enkephalinergic interneurons and serotonergic projection neurons of the vlPAG. This assumption is supported by the finding that elevations in VAD threshold induced via bilateral intra-CeA administration of NMDA were reduced following the unilateral intra-vlPAG administration of the mu-opiate specific antagonist CTAP.

That the vlPAG is involved in producing elevations in vocalization thresholds following intra-CeA NMDA, but not intra-CeA APV, is also supported by the present results investigating the expression of *Fos*-positive nuclei in the vlPAG. Although rats who received intra-CeA NMDA exhibited greater numbers of *Fos*-positive nuclei in the

vlPAG compared to rats administered saline into the CeA, this was not the case with rats who received APV into the CeA. *Fos* expression in vlPAG following administration of APV into the CeA did not differ from that observed following administration of saline into the CeA. Importantly, the doses of APV and NMDA administered into the CeA produced significant elevations in vocalization thresholds. These data suggest that administration of NMDA into the CeA activates the vlPAG, and the vlPAG contributes to the elevation of vocalization thresholds produced by intra-CeA NMDA. Conversely, administration of APV into the CeA does not activate the vlPAG, and thus the vlPAG is likely not involved in the elevation of vocalization thresholds produced by intra-CeA APV.

4.3 Interactions of Sub-populations of NMDA receptors in CeA

The results of the present study suggest the NMDA receptors within the CeA are segregated both anatomically and functionally with regard to the production of behavioral antinociception. Administration of the NMDA receptor antagonist APV or agonist NMDA produced a dose-dependent preferential elevation in the threshold of the VAD response, which is a validated measure of pain affect in the rat. Overall, these findings indicate that NMDA receptors within the CeA contribute to the processing of pain affect. Administration of APV into the CeA likely produces its antinociceptive effects via the inhibition of nociceptive transmission at the level of the CeALC. Conversely, the antinociceptive effects elicited via NMDA into the CeA likely are a result of the activation of CeM projection neurons that engage antinociceptive mechanisms within the vlPAG.

The present finding that NMDA receptor agonism and antagonism within a particular structure can produce similar effects on nociceptive processing is consistent with earlier findings of this laboratory. Previously, we reported that administration of NMDA into the rostral anterior cingulate cortex (rACC) and APV into the caudal ACC (cACC) both generated dose-dependent increases in vocalization thresholds similar to that observed in the present study (Greer, 2007). Because the rACC and cACC are sufficiently separated anatomically, it was possible in that study to separately administer drugs into either site using our microinjection technique. The CeA, however, is a much smaller structure and the microinjection technique does not permit the spatial resolution required to inject APV or NMDA within the boundaries of CeALC or CeM, respectively. Thus, administration of either drug likely activates both subpopulations of NMDA receptors.

It is possible to explain the similar behavioral effects of APV and NMDA using a model that describes the neurochemical and anatomical connections of CeALC and CeM with structures responsible for the generation of VADs (see Figure 13). Administration of NMDA into the CeA activates NMDA receptors within the CeALC. It would be expected that this effect would elicit vocalizations from the rat, given that activation of the CeALC would engage the neural circuitry (dmVMH and dlPAG) involved in the generation of VADs (Figure 13, CeALC → dmVMH → dlPAG).

That VADs are not elicited, but rather suppressed, by intra-CeA administration of NMDA is posited to be the result of concomitant NMDA receptor activation of the CeM. NMDA receptor activation of CeM is proposed to engage, via mu-opiate mediated disinhibition, antinociceptive projection neurons from vlPAG that suppress nociceptive

processing within the circuit that contributes to production of VADs (Figure 13, CeM → vlPAG → CeALC and dmVMH). Activation of serotonergic neurons of the vlPAG that project to the CeALC and dmVMH are hypothesized to suppress production of VADs. Immunohistochemical retrograde transport double labeling studies revealed that serotonergic neurons in vlPAG project to CeA and VMH (Li, Jia, Rao, & Shi, 1990; Li, Zeng, Dong, Rao, & Shi, 1991; Smith & Flynn, 1980). Mu-opiate mediated activation of serotonergic projections to CeA contributes to suppression of VADs. Administration of the serotonin receptor antagonist methysergide into the CeA reverses the increase in VAD threshold generated by injection of the mu-opiate receptor agonist morphine into vlPAG (Borszcz, 1999). This result is consistent with findings that stimulation of vlPAG or systemic administration of morphine increases the efflux and metabolism of serotonin in CeA (Spampinato, Esposito, Romandini, & Samanin, 1985; Viana, Graeff, & Loschmann, 1997). The contribution of serotonergic projections from vlPAG to dmVMH to the suppression of pain affect has not been evaluated; however, injection of the 5-HT_{1A} receptor agonist 8-OH-DPAT into the VMH suppressed defensive vocalizations in the cat (Hassanain, Bhatt, & Siegel, 2003).

Findings of electrophysiological studies of the amygdala provide additional support for the hypothesis that serotonergic projections from vlPAG to CeALC inhibit NMDA receptor mediated nociceptive processing within CeALC. Although no study to date has investigated the ability of serotonin agonists to suppress NMDA-evoked neural activity within CeA, several studies suggest such a mechanism. For example, microiontophoretic administration of serotonin onto neurons within the lateral amygdala decreased the number of action potentials elicited via microiontophoretic administration

of glutamate (Stutzmann & LeDoux, 1999; Stutzmann, McEwen, & LeDoux, 1998). It is likely that serotonin acts directly within the CeA to inhibit NMDA-induced excitation. First, 5-HT_{1A} is the primary receptor subtype that mediates post-synaptic serotonin induced inhibition (see Saxena, 1995). Second, 5-HT_{1A} is the predominate serotonin receptor found in the CeA (Radja, et al., 1991). In accordance, iontophoretic application of the 5-HT_{1A} receptor agonist 8-OH-DPAT produced a preferential inhibition of spontaneous neural activity within the CeA (Stein, Davidowa, & Albrecht, 2000).

Although the effect of serotonin on noxious-evoked unit activity in the amygdala has not been evaluated, serotonin modulates noxious-evoked activity in the parafascicular thalamic nucleus (nPF) that is also innervated by serotonergic projections of the vlPAG (Chen, Zeng, Rao, & Shi, 1992), and contains 5-HT_{1A} and 5-HT_7 receptors (Neumaier, Sexton, Yracheta, Diaz, & Brownfield, 2001; Pazos & Palacios, 1985). Iontophoretic application of serotonin in the nPF and administration of morphine into the vlPAG inhibits noxious evoked neural activity in the nPF. These effects were blocked by local iontophoretic application of the broad-spectrum 5-HT antagonist methysergide (Dafny, Reyes-Vazquez, & Qiao, 1990; Reyes-Vazquez, Qiao, & Dafny, 1989). Behavioral antinociception produced by vlPAG-administered morphine was also shown to be mediated by the activation of serotonin projections to the nPF (Borszcz, 1999; Borszcz & Streltsov, 2000). Increases in VAD and VDS thresholds generated by the injection of morphine into vlPAG were reversed in a dose-dependent manner by the injection of methysergide into nPF. Furthermore, systemic administration of morphine in a dose that selectively elevates vocalization thresholds increased the release and metabolism of serotonin in the nPF (Munn & Borszcz, 2002). Direct administration of 8-

OH-DPAT into the nPF selectively elevated VAD and VDS thresholds, which were blocked by concurrent administration of the 5-HT_{1A} antagonist WAY 100,635 (Harte, et al., 2005). These findings support the contention that serotonin acting in the CeALC may also generate antinociception through inhibition of noxious-evoked activity.

Previous findings from this laboratory demonstrated that increasing mu-opiate receptor activation in the vlPAG (via injection of increasing doses of morphine) generates progressive recruitment of serotonergic antinociceptive projections of the vlPAG that inhibit nociceptive processing at forebrain, medullary and spinal levels of the neuraxis (Borszcz, 1995a, 1999; Borszcz, Johnson, & Thorp, 1996; Borszcz & Streltsov, 2000). In the current study, administration of low doses of NMDA into the CeA presumably only engage antinociceptive projections of vlPAG that inhibit nociceptive processing at forebrain sites responsible for generating VADs (Borszcz, 2006; Borszcz & Leaton, 2003; Carroll & Lim, 1960; Hoffmeister, 1968). As the dose of NMDA administered into the CeA was increased, VDS threshold was also elevated. VDSs are organized within the medulla below the pontomedullary border (Carroll & Lim, 1960; Hoffmeister, 1968). The effect of NMDA on VDS threshold is likely due to the recruitment of descending serotonergic projections from the CeA that inhibit nociceptive processing by medullary neurons responsible for the generation of the VDS response. Following administration of a dose of morphine into vlPAG that selectively elevated VAD and VDS thresholds, the subsequent injection of methysergide into the RVM restored VDS threshold to baseline levels (Borszcz, 1999).

It is well established that mu-opiate receptor activation of vlPAG inhibits nociceptive processing within the spinal dorsal horn via engaging descending spinopetal

projections (Basbaum & Fields, 1984; Jensen, 1986; Yaksh & Malmberg, 1994), and thereby inhibit withdrawal reflexes (tail-flick, paw withdrawal) elicited by noxious stimuli (Carstens, Hartung, Stelzer, & Zimmermann, 1990; Jensen & Yaksh, 1986; Levy & Proudfit, 1979; Ossipov, Goldstein, & Malseed, 1984; Yaksh, Yeung, & Rudy, 1976). Previous studies in this laboratory revealed that vlPAG administration of morphine at high doses is capable of elevating SMR threshold along with VAD and VDS thresholds. This increase in SMR threshold is mediated via recruitment of spinopetal projections from the rostral ventomedial medulla (Borszcz, 1999). It is conceivable that administration of a dose of NMDA into the CeA larger than that used in the present study may indeed elevate SMR threshold. However, there are limitations to the dose of NMDA that can be administered into the CeA without producing a neurotoxic lesion of CeA (Maisonnette, et al., 1996).

4.4 Ethological Relevance

The studies presented here provide support for the involvement of NMDA receptors within subdivisions of the CeA in the generation of affective analgesia. The perceptual-defensive-recuperative (PDR) theory of fear and pain (Bolles & Fanselow, 1980) provides insight into the ethological relevance of the present findings. PDR theory contends that fear will inhibit pain because pain-related behaviors will interfere with defensive behaviors that occur in response to an imminent predator. For example, an animal engaged in a physical encounter with a predator and that has sustained an injury must prevent the emergence of pain-related behavior in order to maintain execution of defensive behaviors. If the animal were to tend to the injury, the animal would be rendered defenseless and would provide the predator with an advantage.

Suppression of pain signaling would allow the animal to devote attention to the predator rather than to the pain.

Ethological evaluation of the behavior of rodents, other mammals, and humans revealed that individuals produce a series of defensive behaviors when confronted with a threat (Blanchard & Blanchard, 1969, 1987; Blanchard, et al., 1986; Fanselow & Lester, 1988). Proximity of the individual to the threat (i.e., predatory imminence) and various environmental constraints (availability of escape routes) governs expression of particular behavior patterns within the series of defensive reactions. For the rat, the presence of a distant predator results in the cessation of movement (freezing) in order to make the individual less conspicuous. As a predator approaches and escape routes are available then rats engage in escape behaviors. When contact with the predator is imminent and an escape route is not available, rodents engage in defensive aggression as exemplified by threat-attack behaviors. The rat rears-up to face the predator, displays its teeth, and emits sonic vocalizations (see Figure 14). Continued approach by the predator elicits jump-attacks of the rat upon the predator. Resultant physical contact with the predator involves continued sonic vocalizations, struggling, biting, and escape attempts. Defensive aggression serves to thwart the attack of a predator, or to escape the predator's grasp or the source of noxious stimulation. Post-encounter defensive reactions include ultrasonic vocalizations, hyperalgesia, and finally recuperative behaviors.

Evaluation of defensive responding by humans revealed a similar pattern of defensive behaviors. The proximity of the subject to the source of threat and the availability of escape routes determined whether humans engaged in freezing, flight, or

defensive aggression (Blanchard, et al., 2001). Human imaging studies that utilized a virtual maze and virtual predators revealed activation of brain areas consistent with PDR theory (Mobbs, et al., 2009; Mobbs, et al., 2007). In these studies, participants are instructed to navigate a virtual maze and evade an approaching virtual predator. If the predator catches the participant in the maze, the participant will receive noxious shock to the hand. Functional MRI revealed that as the predator approached an inescapable distance from the participant, activation of the PAG and CeA occurred that correlated with post-imaging subjective results of dread (i.e., fear). Furthermore, humans that received stimulation of sites within the defensive aggression circuit (dorsolateral periaqueductal gray, medial hypothalamus, amygdala) reported feelings of dread, anxiety, anger, fear, and impending death (Ervin, et al., 1969; Jenck, Moreau, & Martin, 1995; Nashold, Wilson, & Slaughter, 1974; Tasker, 1982). The forms of defensive responding, the environmental variables that determine the pattern of defensive behaviors, and the underlying neural circuit that generate defensive responding is highly conserved across mammalian species.

As described above, the neural circuit that governs the execution of defensive responding to environmental threats is well characterized. The medial hypothalamus and dlPAG are the core structures of the defense circuit and they are modulated by inputs from the amygdala (Siegel, 2005). Environmental stimulus information (for example, sensory stimuli from a predator, such as a cat) enters the lateral amygdala of the rat via projections from the sensory thalamus and sensory cortex (LeDoux, 2007). The thalamic pathway to the amygdala is shorter and thus faster, but its capacity to represent a potentially threatening stimulus is limited (Bordi & LeDoux, 1994a, 1994b).

The thalamo-cortico-amygdala pathway, which involves several cortico-cortical links before reaching the amygdala (Romanski & LeDoux, 1993a, 1993b), is longer and slower, but provides detailed stimulus information about the threat. The thalamic inputs thus may be useful for producing rapid defensive responses on the basis of limited stimulus information. Rapid response to threats has obvious survival value (Ekman, 1992; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; LeDoux, Iwata, Pearl, & Reis, 1986; Ohman, 1986). Cortical inputs permit subsequent detailed appraisal of the stimulus to determine whether in fact it is a threat and the need for continued defensive responding. Sensory information from the lateral amygdala projects to the basolateral amygdala (BLA), and the BLA processes the affective salience of stimuli, including fear and other negative affective states associated with threatening stimuli (Davis & Whalen, 2001; Rosen & Schulkin, 1998). Threat stimuli-induced activation of the BLA activates projections to CeA, and CeA activation mediates the generation of defensive behaviors through its projections to the medial hypothalamus and the dlPAG. These defensive behaviors have a phylogenetic history of enabling the individual to cope with threatening stimuli (Davis & Whalen, 2001). Because exposure to a noxious stimulus represents an immediate and intimate threat, defensive behaviors activated under conditions of high predatory imminence are engaged. The preeminence of noxious stimuli in engaging the defense circuit is indicated by the fact that noxious stimuli bypass the thalamus and cortex, and project directly to BLA and CeALC (Bernard & Besson, 1990; Burstein & Potrebic, 1993; Cliffer, et al., 1991; Gauriau & Bernard, 2002; Li & Neugebauer, 2004a; Newman, et al., 1996).

I propose that noxious and non-noxious threats are processed by a common neural substrate within CeALC to produce defensive responding, and that activation of CeM modulates this processing so to optimize the animal's defense against environmental threats. It is speculated that pain information from the spinal cord and threat stimulus information from the BLA converge upon the same neural population within CeALC, and this neural population is responsible for the activation of the dmVMH and dlPAG, and thereby promotes the execution of defensive responding to noxious and non-noxious threatening stimuli (see Figure 15). As described earlier, the CeALC of the rat contains neurons that respond to noxious peripheral stimulation. The CeA of the rat (along with the LA, BLA, dmVMH and dlPAG) also exhibits neuronal activation in response to non-noxious threatening stimuli. Beckett and colleagues (1997) reported that exposure of naïve rats to 20 kHz ultrasonic tone increased *Fos* expression in all these sites (Beckett, Duxon, Aspley, & Marsden, 1997b). That is, all components of the defense circuit are activated. It is well documented that rats emit 22 kHz ultrasonic vocalizations (USV) when exposed to a predator (Blanchard, Blanchard, Rodgers, & Weiss, 1990; Knutson, Burgdorf, & Panksepp, 2002). As the production of these calls is enhanced by the presence of familiar conspecifics (Blanchard, Blanchard, Agullana, & Weiss, 1991; Brudzynski & Ociepa, 1992), it has been suggested that they provide a warning signal about an imminent environmental threat. Accordingly, these vocalizations (or 22 kHz pure tones) generate defensive responding in rats not directly exposed to a predator (Brudzynski & Chiu, 1995; Burgdorf, et al., 2008). The defense circuit is particularly attuned to these vocalizations as a high proportion of neurons in LA and BLA respond preferentially to acoustic stimuli in the 18 – 27 kHz range (Bordi &

LeDoux, 1992). Similarly, CeA, dlPAG and dmVMH exhibit enhanced *Fos* expression in rats following their exposure to cat odor (Dielenberg, et al., 2001). The medial amygdaloid nucleus (MeA), but not LA and BLA, also exhibited increased *Fos* expression. This latter finding is consistent with the fact that transmission of odor to CeA is via the MeA rather than LA and BLA. MeA is considered the 'vomeronasal amygdala', the principal limbic projection area of the accessory olfactory bulb (Kevetter & Winans, 1981; Luiten, Koolhaas, de Boer, & Koopmans, 1985; Martinez-Marcos & Halpern, 1999; Scalia & Winans, 1975). Therefore, non-noxious threatening stimuli (auditory or olfactory), like noxious stimuli, appear to gain access to the core structures of the defense circuit via CeA. The subdivisions of CeA that are activated by non-noxious stimuli has not been evaluated but it is speculated that CeALC is the principal target of these stimuli.

The notion that noxious and non-noxious threats are processed by a common neural substrate within CeALC to produce defensive responding, and that activation of CeM modulates this processing so to optimize the animal's defense against environmental threats, may be explained by the Yerkes-Dodson theory of arousal and performance (Yerkes & Dodson, 1908). This theory is illustrated with an inverted-U curve (see Figure 16), which indicates that as arousal increases, efficiency of performance increases until an optimum level of performance is achieved. With increasing arousal past this optimum level, efficiency of performance decreases. In the case of the rat and the predator cat, predator-stimulus specific information reaches the MeA (olfaction) and BLA (auditory and visual), which in turn sends excitatory projections to the CeALC. In turn, the CeALC → dmVMH → dlPAG pathway is engaged and

defensive responses aimed at thwarting the predator are generated (see Figure 15, green pathway). If the rat is injured during this confrontation, nociceptive information will project and add to the cellular activation within CeALC. In terms of Yerkes-Dodson theory, this extreme compounded cellular activation would drive the rat's defensive responding past optimum levels of performance and toward a decreased efficiency in performance, resulting in the inability for the rat to contend with the predator at optimum levels. The rat's behavior would be observed as a compound of defensive strategies to thwart the predator and strategies to tend to the injury. Such a compound of behaviors would render the animal unable to devote attention solely to the predator. This would serve in the predator's favor because the animal would be unable to contend with the predator efficiently.

In order to avoid this decreased efficiency in defensive responding to the predator, it is proposed that the CeALC engages a system that attenuates extreme levels of cellular arousal within CeALC. As shown in Figure 15, when nociceptive projections add to the predator-induced cellular excitation of the CeALC, an intra-amygdaloid projection from CeALC → CeM is recruited. The CeM then engages the endogenous antinociceptive vlPAG serotonergic inhibitory projections that synapse upon CeALC and dmVMH neurons. This inhibition attenuates the cellular excitation within the CeALC and dmVMH, and in terms of the Yerkes-Dodson theory, reverses the performance curve back toward optimum levels. Thus, the rat is able to contend effectively with the predator via predator-specific defensive behaviors without concomitant generation of pain-related behaviors that would interfere with optimum performance. Lastly, it is proposed that once the rat successfully fights off the predator,

or the rat is able to escape, the BLA-induced cellular activation of CeALC diminishes, the vlPAG serotonergic inhibition upon CeALC neurons releases, but the injury-induced cellular activation of CeALC remains and the rat will engage in pain-related defensive behaviors designed cope with the injury.

Evidence in support of this model includes studies that have examined the role of serotonin within the amygdala and medial hypothalamus on defensive aggression and pain behavior. Systemic administration of the serotonin agonists buspirone and gepirone to wild rats significantly reduced defensive aggression (e.g., jump attacks, sonic vocalizations, biting; Blanchard, Rodgers, Hendrie, & Hori, 1988). Studies on the anole lizard and mouse, bred for low or high levels of aggression, revealed that high aggression animals exhibit lower levels of serotonin within the amygdala (medial nucleus) and the medial hypothalamus (Serri & Ely, 1984; Summers, et al., 2005) and low aggression animals exhibit higher levels of serotonin within the amygdala (Young, et al., 2008). Administration of serotonin into the rat cortical amygdala decreased defensive aggression measured as a decrease in the number of aggressive postures/attacks in the shock-induced fighting test (Pucilowski, Plaznik, & Kostowski, 1985). Lastly, injection of the serotonin agonist 8-OH-DPAT into the VMH suppressed defensive vocalizations in the cat elicited via electrical stimulation of the PAG (Hassanain, et al., 2003). Studies have investigated the effect of intra-amygdaloid serotonin administration on pain responses, although no study to date has investigated this effect within the CeA. Administration of serotonin into the BLA elevates tail-shock induced vocalization thresholds (Nandigama, 2005), and intra-basomedial amygdala serotonin administration increases the pressure required to elicit a vocalization in the tail

compression pain test (Plaznik, Danysz, & Kostowski, 1985). Depletion of forebrain serotonin via severance of the medial forebrain bundle resulted in analgesia as measured via flinch-jump, stabilimetric, or hot-plate pain tests, which indicates forebrain serotonin attenuates pain-related behaviors (Harvey, Schlosberg, & Yunger, 1975). These studies suggest that increased levels of serotonin within the defensive circuit suppress defensive aggression and pain behaviors.

4.5 Future Directions

The present study focused on the contribution of intra-CeA NMDA receptors to the suppression of pain affect. This manuscript put forth the notion that activation of CeM-NMDA receptors activate endogenous antinociceptive mechanisms via vlPAG mu-opiate receptors. Intra-vlPAG administration of CTAP effectively suppressed the elevations in VAD threshold generated by intra-CeA NMDA administration. The present study did not assess the capacity of CTAP to alter intra-CeA APV elevations on VAD thresholds. Given the proposition that APV inhibits nociceptive transmission at the level of CeALC-NMDA receptors, it is expected that intra-CeA APV-induced elevations in VADs would not be suppressed by intra-vlPAG CTAP administration. This hypothesis is supported by the results from the present *Fos* study, which revealed that *Fos* levels within vlPAG are unchanged following intra-CeA APV treatment and indicates that the vlPAG is not involved in intra-CeA APV-induced elevations in vocalization thresholds.

The neurochemistry underlying nociceptive afferents to CeALC directly via the spinal cord and indirectly via the pontine parabrachial nucleus and intralaminar thalamus have yet to be investigated. The present study and others (Li & Neugebauer, 2004a, 2004b) provide evidence that intra-CeA NMDA receptors are implicated in the

transmission of nociception at the level of the CeALC, but it is not known whether glutamate is the primary neurotransmitter involved in these ascending projections. In order to assess whether glutamate is the primary neurotransmitter involved in ascending nociception to the CeALC, immunocytochemistry studies could be performed. A potential study may involve labeling of glutamate-containing nociceptive fibers to the CeA (e.g., spinoamygdaloid, spinoparabrachioamygdaloid, or spinothalamoamygdaloid fibers that respond to noxious stimulation) and receptor staining of glutamate receptors within CeALC. Contacts of labeled fibers upon stained glutamate receptors would provide evidence that glutamate is the primary neurotransmitter involved in ascending nociception to CeALC. It would also be of interest to assess whether BLA → CeALC projections that convey non-noxious threat information also utilize glutamate as the primary neurotransmitter.

In order to bolster the notion that pain information from the spinal cord and non-noxious threat information from the BLA converge upon the same neural population within CeALC in order to produce affective responding via the CeA → dmVMH → dlPAG pathway, single unit recording of CeALC neurons may be evaluated. One such study may investigate the evoked responses of CeALC neurons to noxious stimuli presented to the periphery and to non-noxious predator stimuli presented in the environment. Should a significant number of CeALC neurons respond to both noxious and non-noxious stimuli, it may be concluded that these forms of threat information converge upon the same population of neurons within CeALC.

In addition, microdialysis may be used to measure levels of serotonin within CeALC and dmVMH following administration of NMDA into the CeA. It is predicted that

following intra-CeA NMDA treatment, levels of serotonin within the CeALC and dmVMH would increase, reflecting engagement of the CeM → vlPAG circuit that provides serotonergic inhibition upon the CeALC and dmVMH. Further, it is predicted that APV treatment of the CeA would not produce increases in serotonin levels in these structures because APV induced increases in vocalization thresholds is postulated to not rely upon a vlPAG serotonergic inhibition mechanism.

In terms of the ethological relevance of the present study, it is critical that the capacity for CeA-administered serotonin to alter pain behaviors and defensive aggression be evaluated. As previously discussed, serotonin administration into several amygdaloid structures has been shown to suppress defensive responding and to suppress pain behaviors, yet these effects have not been demonstrated following serotonin administration into the CeA. It would be possible to utilize the tail-shock vocalization response test and the microinjection technique to assess the capacity of intra-CeA serotonin to inhibit tail-shock induced vocalizations. Additionally, it would be possible to use the microinjection technique to administer serotonin into the CeA and present the rat with a cat in an environment with limited escape routes so as to increase predatory imminence. It is hypothesized that intra-CeA serotonin would inhibit pain affect in the form of VADs and inhibit defensive responding in response to the imminent predator cat.

The ability of intra-CeA serotonin to modulate NMDA-induced cellular excitation has yet to be evaluated. Electrophysiology experiments may be used to record neural activity within the CeALC following application of NMDA to the CeALC. It is predicted that administration of a serotonergic antagonist onto CeALC neurons would suppress

NMDA-induced neural activity. It would follow that blockade of serotonergic inhibition of the CeALC and dmVMH would lead to the capacity of intra-CeA NMDA treatment to elicit defensive behaviors. Thus, administration of a serotonin receptor antagonist to either CeALC, dmVMH, or both structures followed by intra-CeA NMDA administration should elicit VAD-like vocalizations and defensive aggression behaviors (e.g., rearing, biting, jump attacks). With serotonergic receptors blocked, it is predicted that NMDA would be able to activate the dmVMH → dlPAG neural circuit responsible for the generation of defensive behaviors.

In summary, exploration of these future directions would provide strong support for the model presented here regarding the contribution of CeA-NMDA receptors to the generation of defensive behaviors in response to noxious and non-noxious stimuli.

APPENDIX A

FIGURES

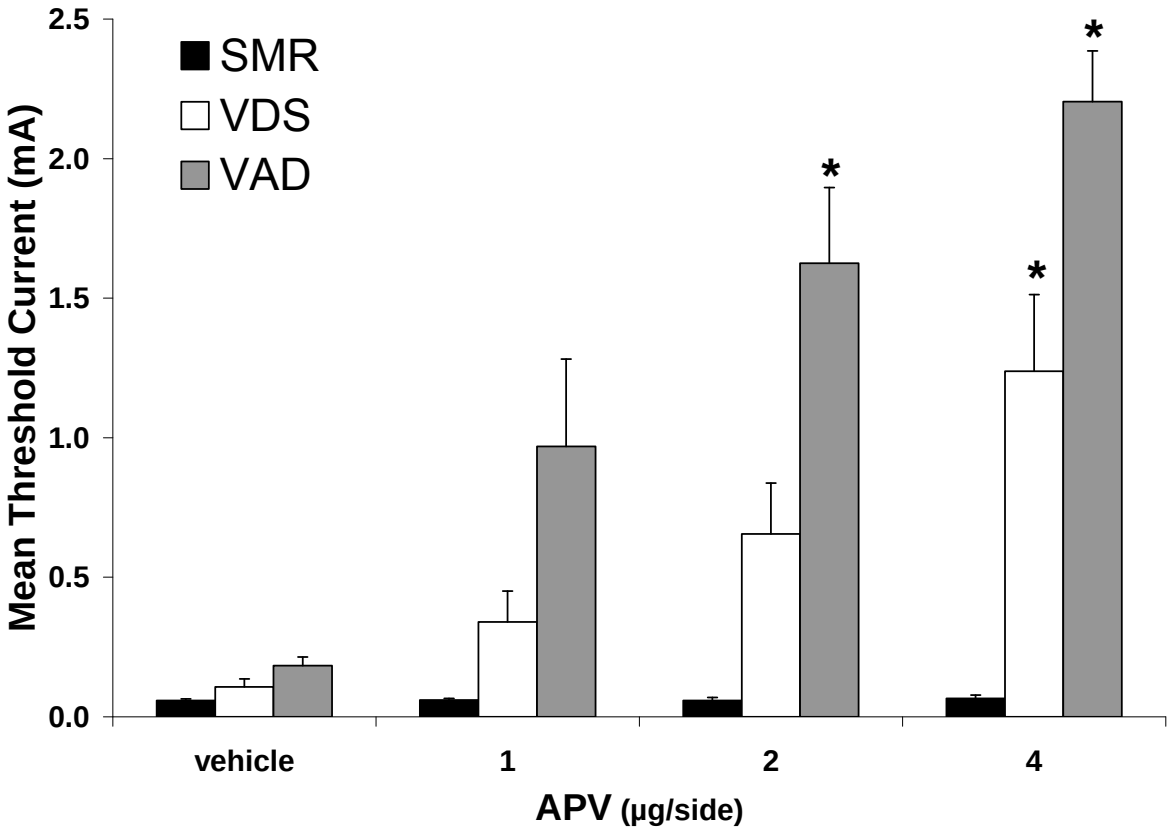

Figure 1. Mean (± S.E.M.) threshold current (mA) of spinal motor reflex, (SMR), vocalization during shock (VDS), and vocalization after-discharge (VAD) of rats who received bilateral vehicle (saline) and APV microinjections into the central nucleus of the amygdala (CeA).

* = significantly elevated over vehicle (saline) treatment, $p < .05$

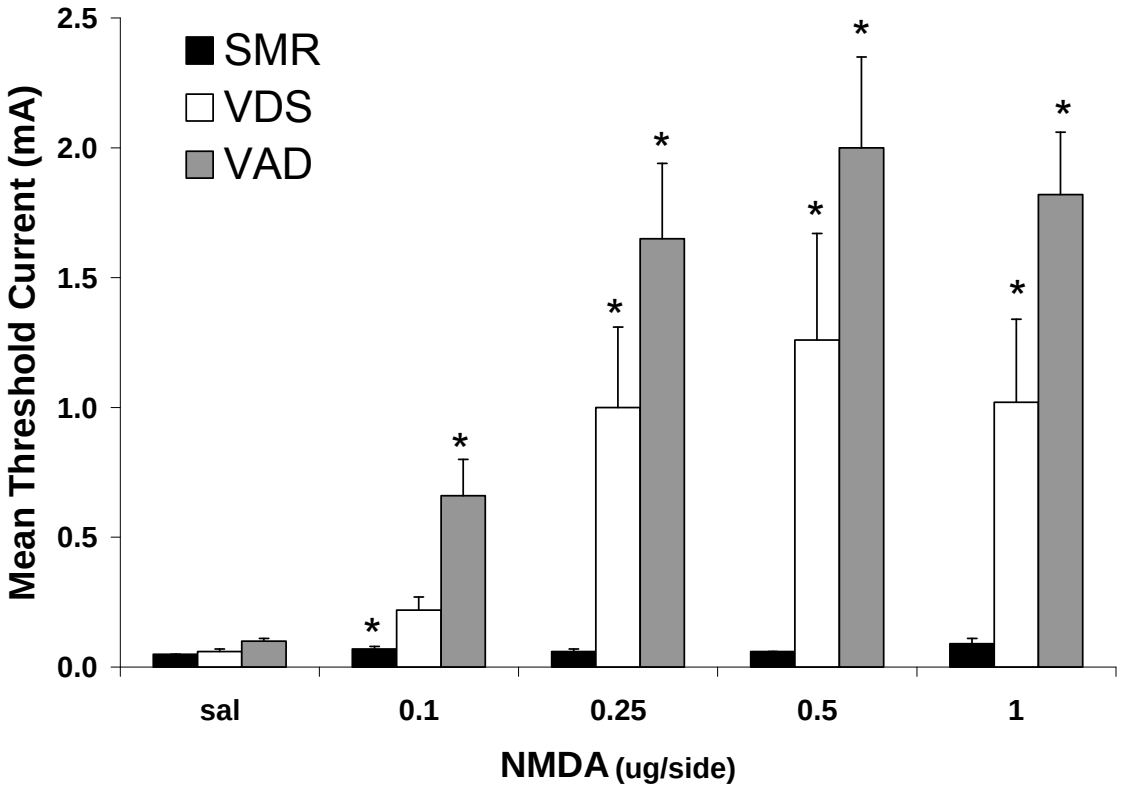

Figure 2. Mean (± S.E.M.) threshold current (mA) of spinal motor reflex, (SMR), vocalization during shock (VDS), and vocalization after-discharge (VAD) of rats who received bilateral saline and NMDA microinjections into the central nucleus of the amygdala (CeA).

* = significantly elevated over saline treatment, $p < .05$

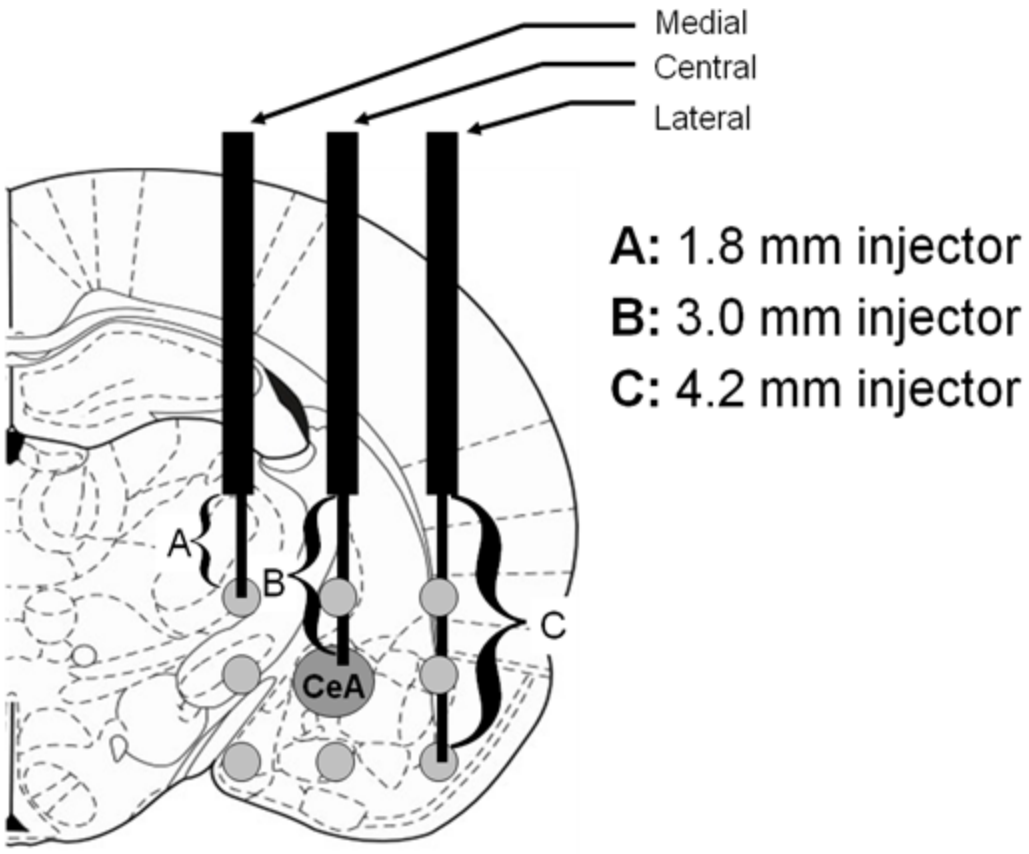

Figure 3. Schematic depicting the placement of cannulae for the NMDA anatomical control study. Medial = 1.8mm medial to the central nucleus of the amygdala (CeA). Central = directly above the CeA. Lateral = 1.8mm lateral to the CeA. Each animal with a medial, central, or lateral cannula placement receives injections with (A) a 1.8mm injector, (B) a 3.0mm injector, and (C) a 4.2mm injector.

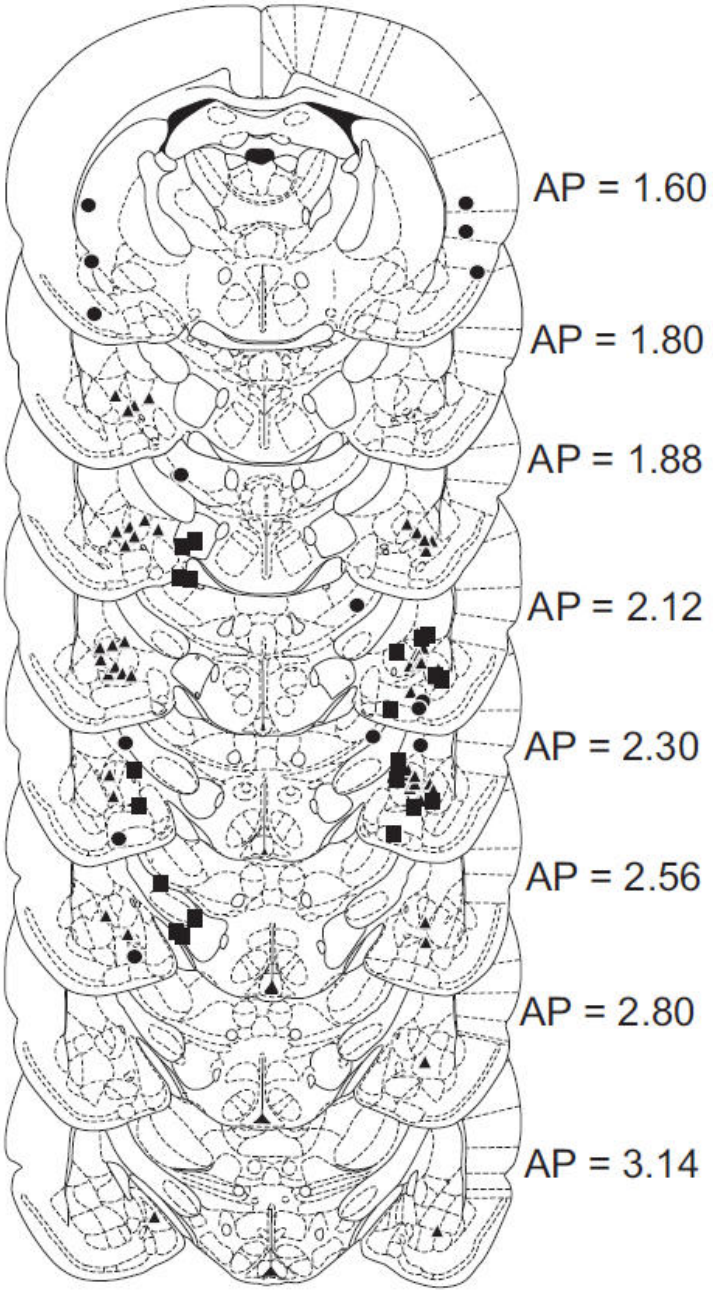

Figure 4. Distribution of injection sites within and around the central nucleus of the amygdala (CeA) that received bilateral injections of NMDA and saline. Black triangles indicate injection sites from the dose response study where bilateral 1µg NMDA produced vocalization threshold increases. Black circles indicate injection sites from the anatomical control study where bilateral 1µg NMDA failed to produce vocalization

threshold increases (Other-NMDA). Black squares indicate injection sites from the anatomical control study where bilateral 1µg NMDA produced vocalization threshold increases (CeA-NMDA). Coordinates are millimeters posterior to bregma. Figures are adapted from The Rat Brain in Stereotaxic Coordinates by Paxinos and Watson (1998).

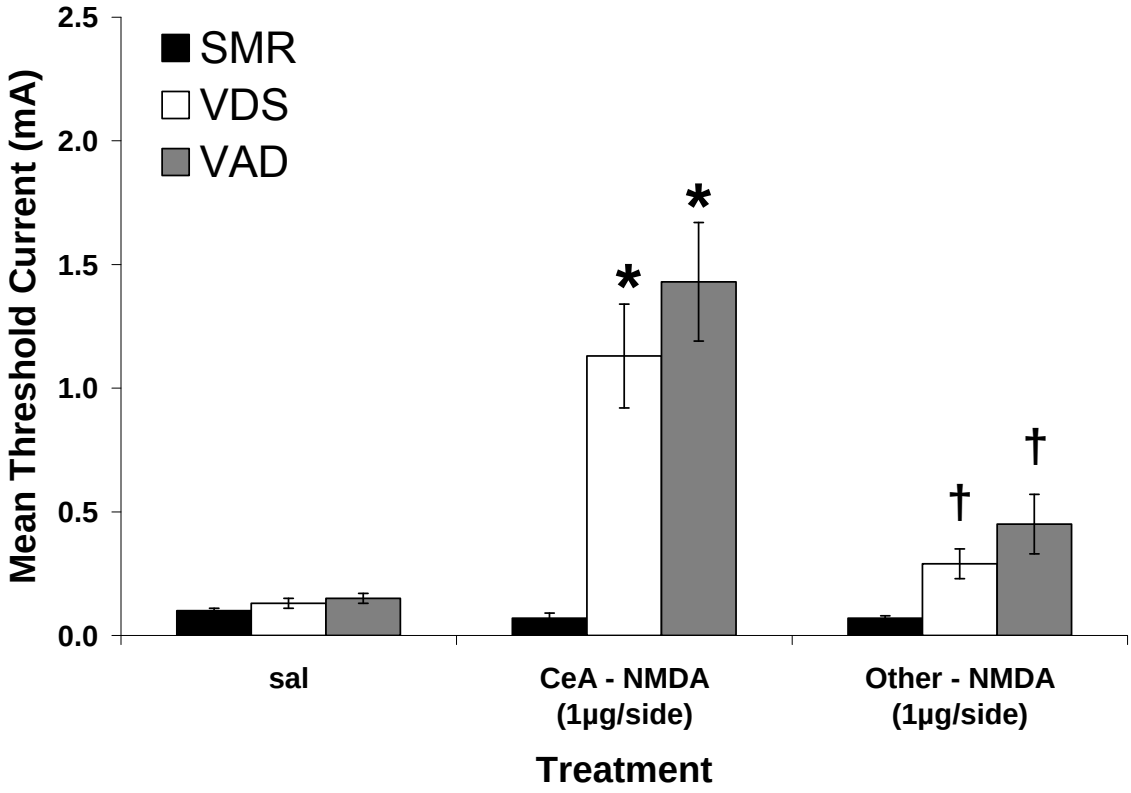

Figure 5. Comparison of the mean (± S.E.M.) threshold current (mA) of the spinal motor reflex, (SMR), vocalization during shock (VDS), and vocalization after-discharge (VAD) of rats produced by the bilateral administration of saline and 1µg NMDA into the CeA (CeA-NMDA) and sites surrounding the CeA (Other-NMDA).

* = significantly elevated compared to saline treatment, $p < .05$

† = significantly decreased compared to CeA – NMDA treatment, $p < .05$

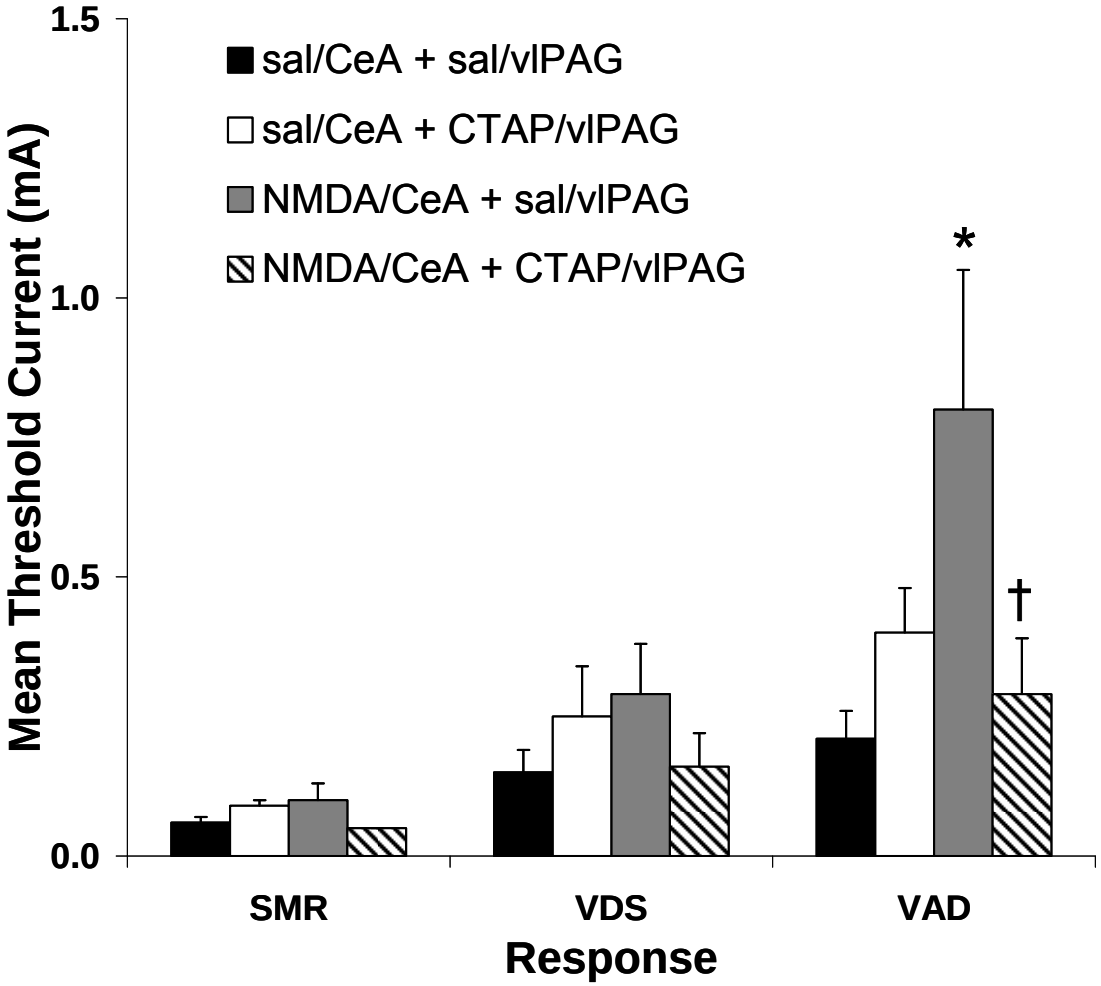

Figure 6. Mean (± S.E.M.) threshold current (mA) of spinal motor reflex, (SMR), vocalization during shock (VDS), and vocalization after-discharge (VAD) of rats who received microinjections of saline or 0.025µg NMDA into the central nucleus of the amygdala (CeA) and saline or 0.25µg CTAP into the ventrolateral periaqueductal gray (vlPAG).

* = significantly elevated over sal/CeA + sal/vlPAG, $p < .05$

† = significantly decreased to NMDA/CeA + sal/vlPAG, $p < .05$.

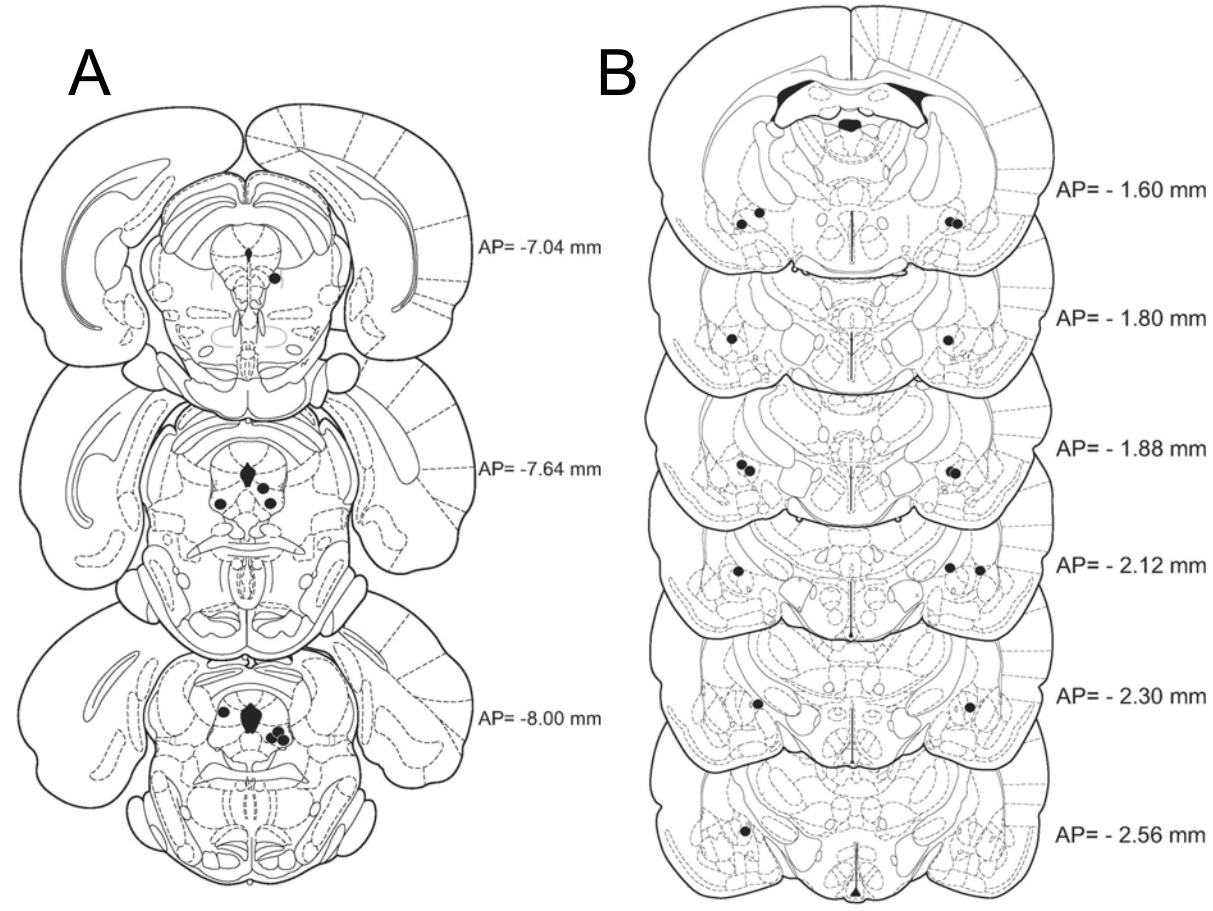

Figure 7. Distribution of injection sites within (A) the ventrolateral periaqueductal gray (vlPAG) that received unilateral injections of 0.25µg CTAP and saline and (B) the central nucleus of the amygdala (CeA) that received bilateral injections of 0.025µg NMDA and saline. Coordinates are millimeters posterior to bregma. Figures are adapted from The Rat Brain in Stereotaxic Coordinates by Paxinos and Watson (1998).

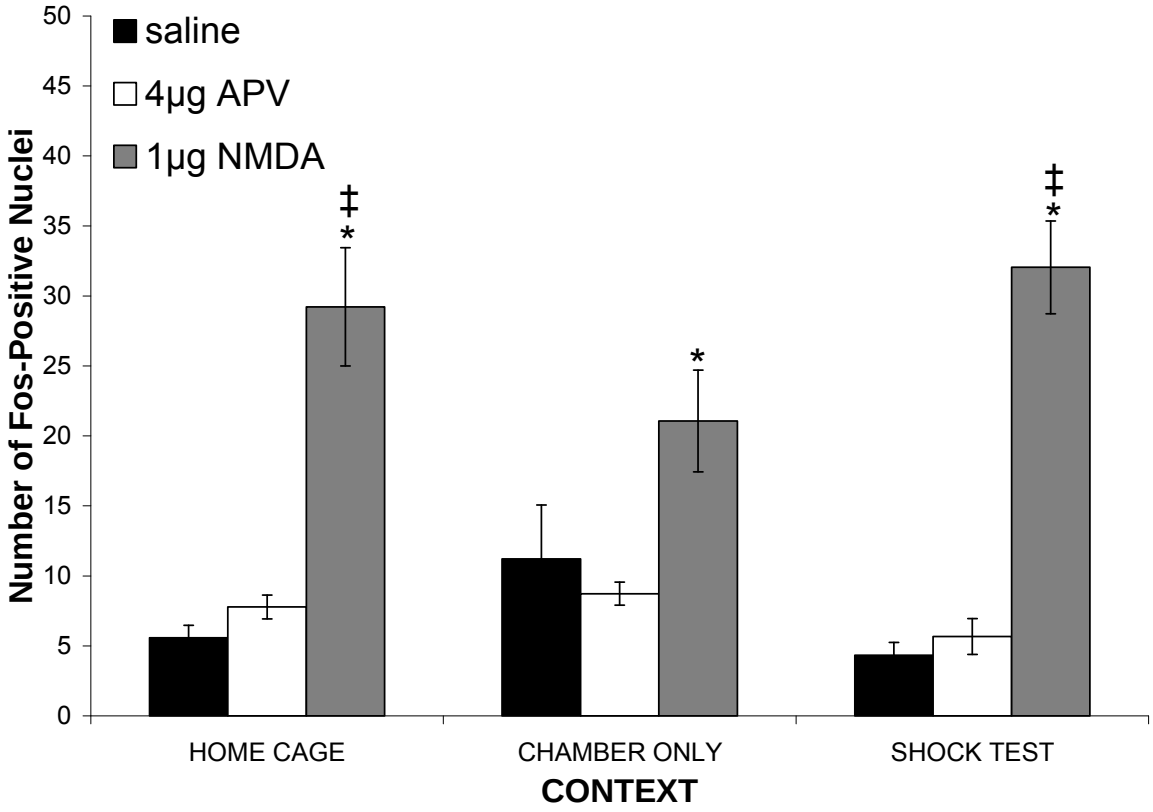

Figure 8. Number of *Fos*-immunoreactive cells in the vlPAG following bilateral microinjection of saline, APV, or NMDA into the central nucleus of the amygdala (CeA). Data are expressed as mean±S.E.M. of *Fos*-positive neurons in a 400µm x 300µm area of tissue within the vlPAG.

* = significantly elevated over saline, $p < .05$

‡ = significantly elevated over 4µg APV, $p < .05$

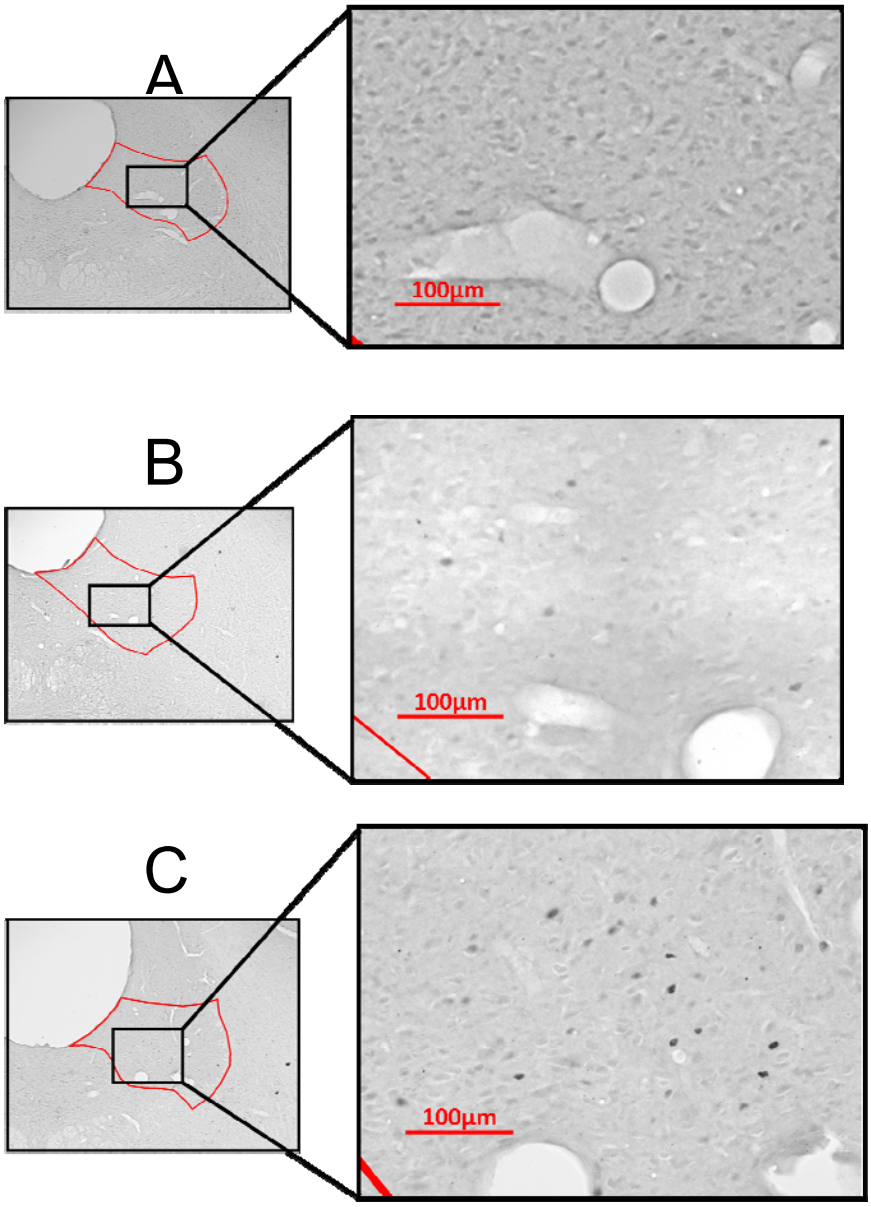

Figure 9. Representative vlPAG photomicrographs of *Fos*-immunoreactive cells (dark dots) from rats in the *Fos* expression Home Cage group and treated with bilateral microinjection of (A) saline, (B) 4µg APV, or (C) 1µg NMDA into the central nucleus of the amygdala (CeA). Note the greater number of nuclei with dark stain in (C), but not in (A) or (B). Sections on the left were photographed at 40x, and sections on the right were photographed at 200x.

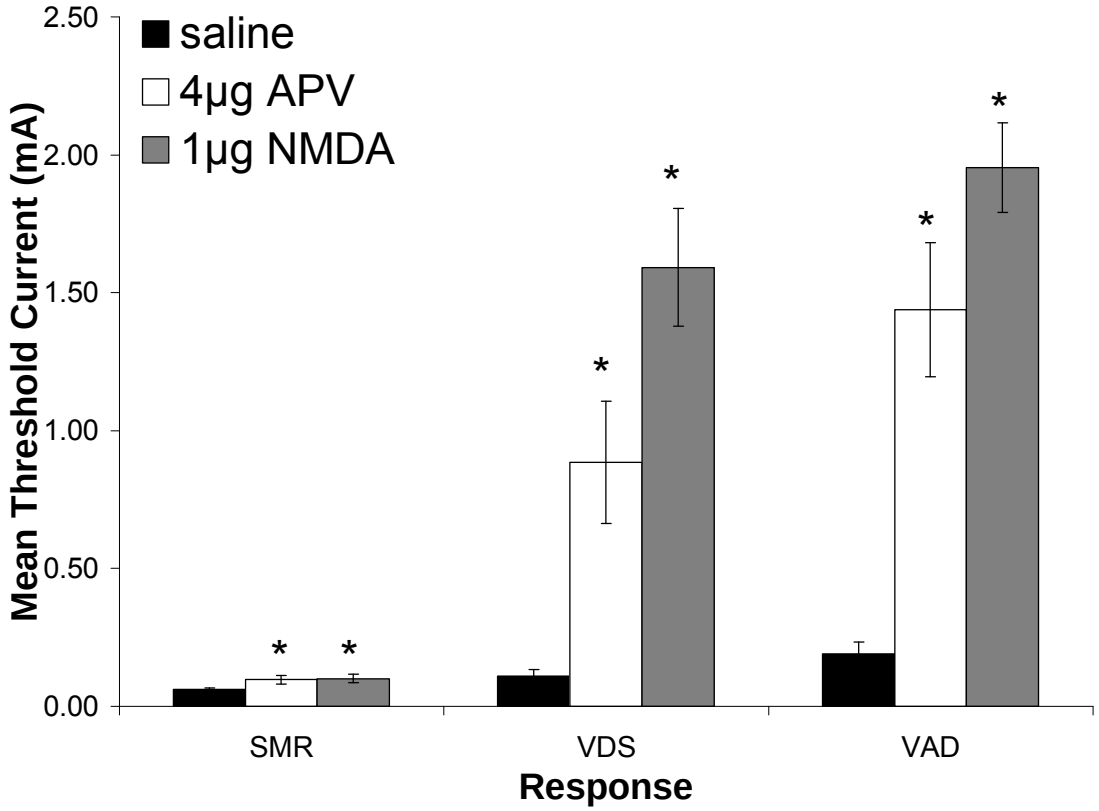

Figure 10. Comparison of the mean (± S.E.M.) threshold current (mA) of the spinal motor reflex, (SMR), vocalization during shock (VDS), and vocalization after-discharge (VAD) of rats produced by the bilateral administration of saline, 4µg APV, and 1µg NMDA into the CeA.

* = significantly elevated compared to saline treatment, $p < .05$

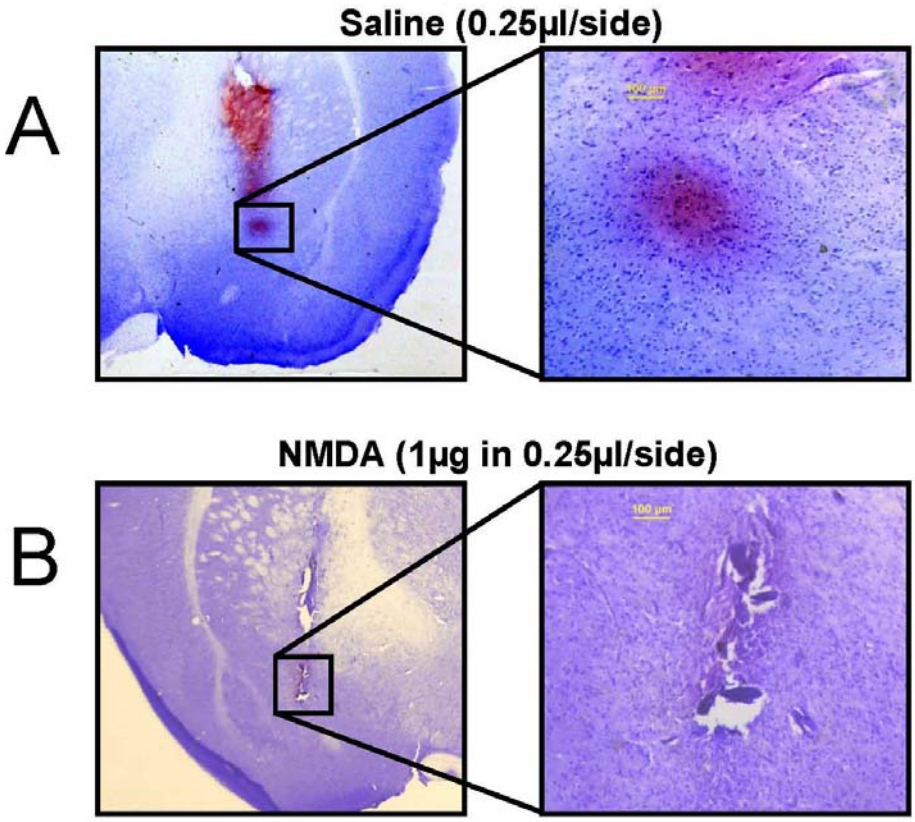

Figure 11. Representative slices for qualitative analysis of the potential neurotoxic effects of NMDA administration into the central nucleus of the amygdala (CeA). (A) slice from a rat that received saline microinjections and (B) slice from a rat that received 1µg NMDA. Note the lack of tissue blanching surrounding the CeA injection site in (B), indicating a lack of excitotoxicity. Slices at -1.88mm posterior to bregma.

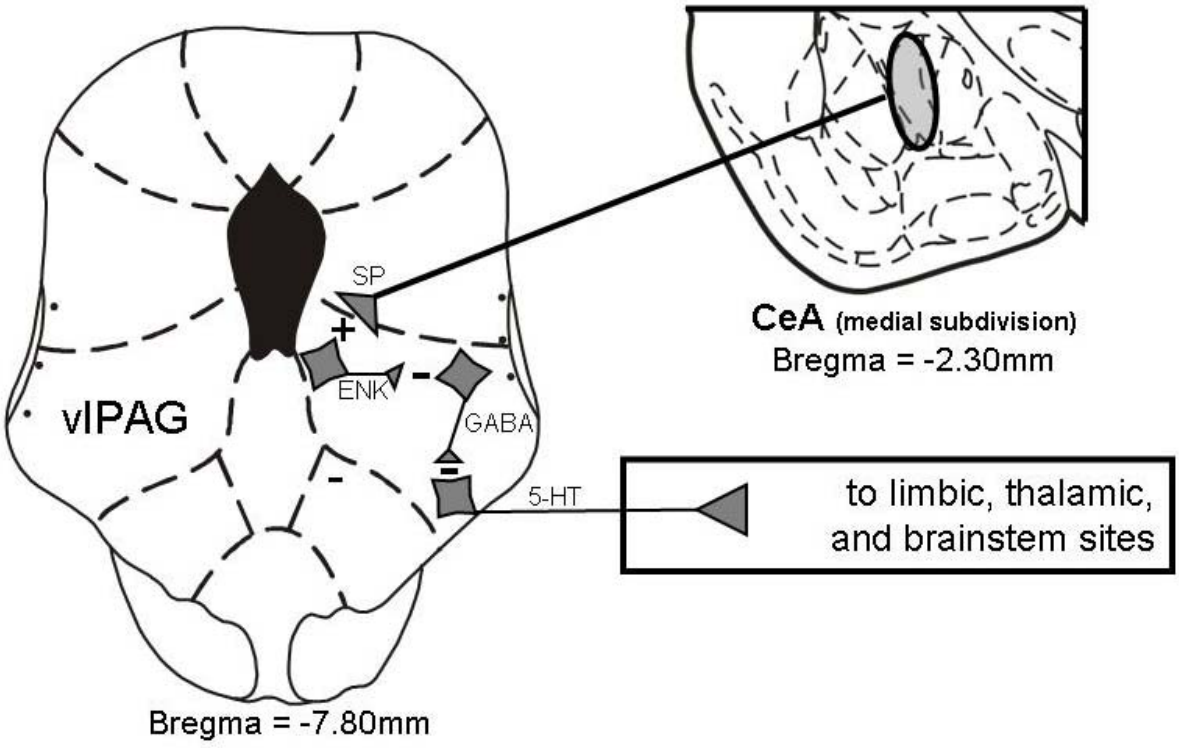

Figure 12. Schematic representation of the internal circuitry of the ventrolateral periaqueductal gray (vlPAG). The vlPAG receives substance P (SP) efferents from the CeA that likely interact with enkephalin (ENK) neurons. Enkephalin release onto GABAergic interneurons releases tonic GABA inhibition on the serotonergic (5HT) projection neurons, resulting in disinhibition of serotonergic (5HT) projection neurons to limbic, thalamic, and brainstem sites that contribute to the suppression of pain affect elicited by morphine injected into vlPAG.

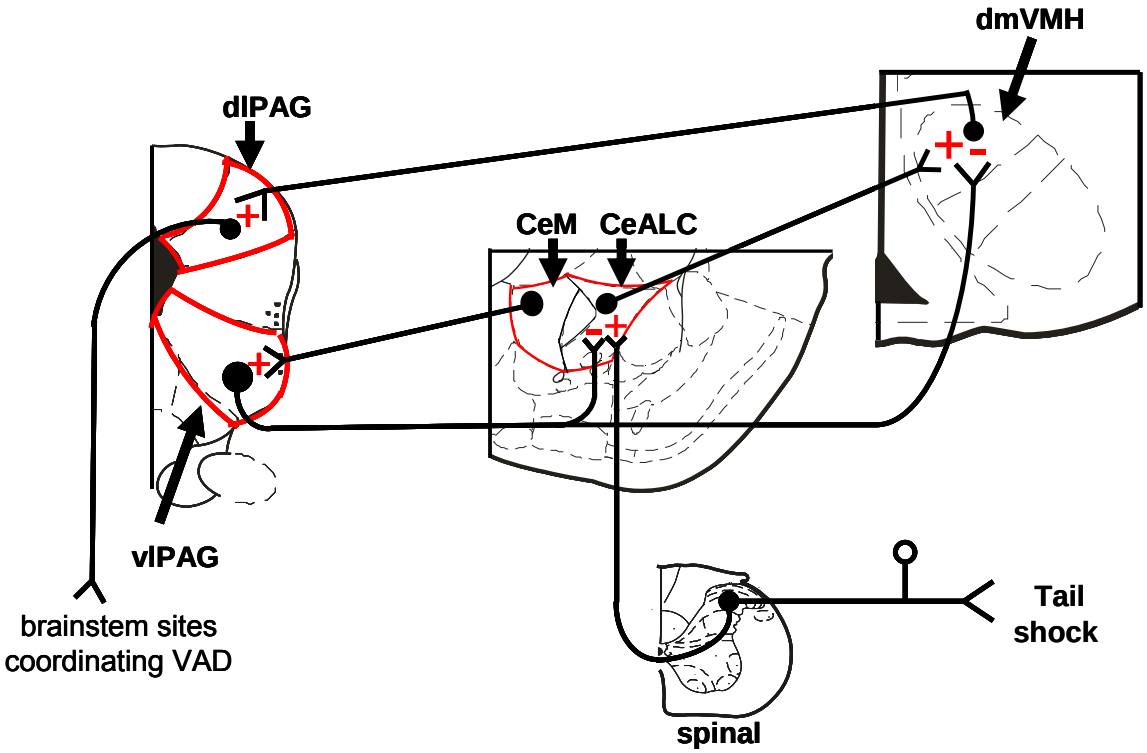

Figure 13. Schematic representation of the neurochemistry leading to inhibition of VAD responding in APV and NMDA treated rats. APV likely blocks NMDA receptors within the CeALC, which directly blocks nociceptive transmission from the spinal cord at this level. NMDA likely activates NMDA receptors within CeM. CeM projections (likely utilizing substance P as a transmitter), activate vlPAG serotonergic projection neurons that synapse within CeALC to inhibit excitatory responses within this structure. This mechanism likely prevents any action of NMDA on CeALC NMDA receptors. Additionally, vlPAG-serotonergic projection neurons synapse within dmVMH. DmVMH and dlPAG together form the core structures responsible for the generation of VADs. Presumably, serotonin within dmVMH inhibits excitatory transmission to dlPAG, and thus VADs are not generated.

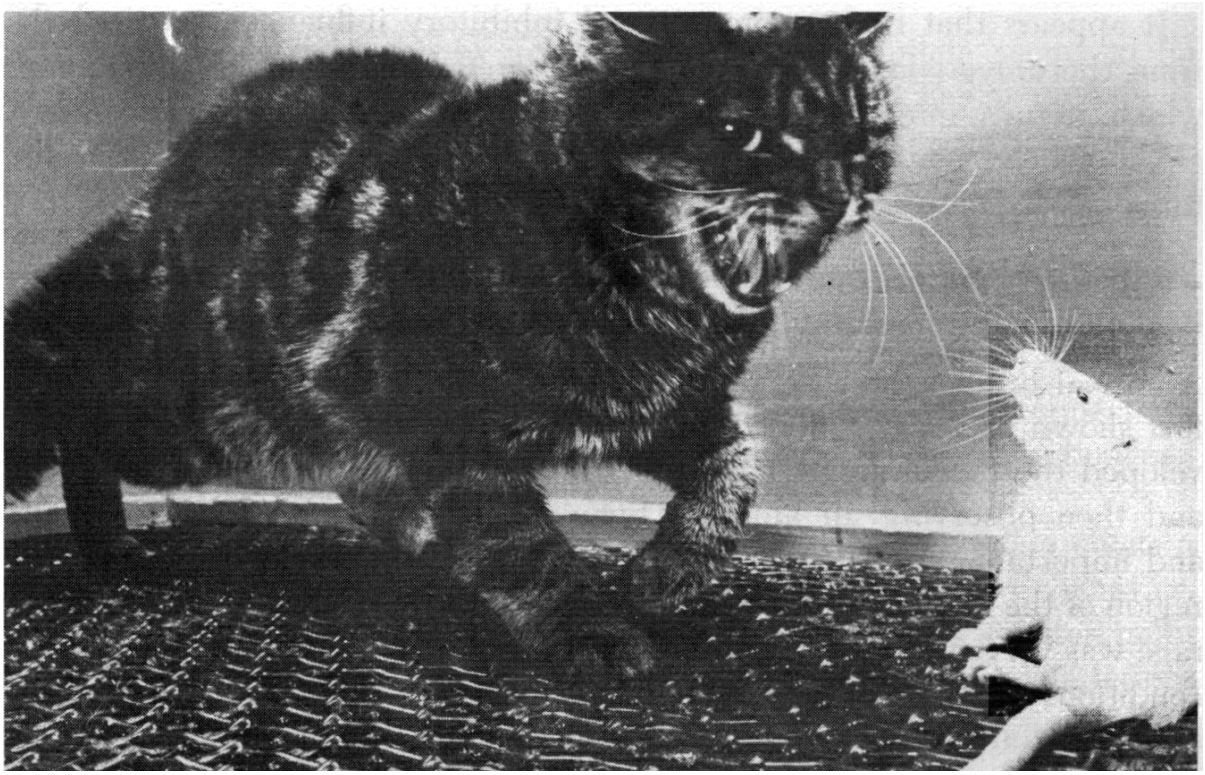

Figure 14. Photograph of a rat engaged in defensive aggression behaviors in response to an imminent threat, a predator cat. Note that the rat is cornered with limited escape routes, and the cat is proximal to the rat. The rat is reared-up on its hind limbs and has its mouth open to bear its teeth and emit sonic vocalizations. From Flynn, 1967.

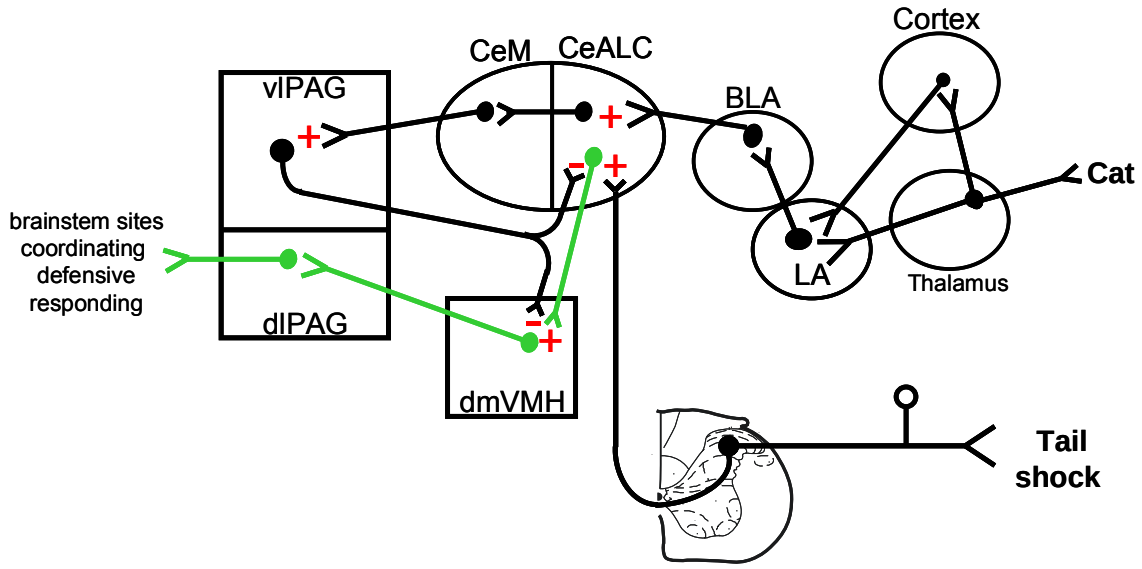

Figure 15. Model of noxious and non-noxious input convergence within CeALC on the generation of defensive aggression. Pain information from the spinal cord and threat stimulus information from the BLA converge upon the same neural population within CeALC. This neural population is responsible for the activation of the dmVMH and dlPAG, which thereby promotes the execution of defensive responding to noxious and non-noxious threatening stimuli (circuit depicted in green). Compound activation of the CeALC by noxious (e.g., tail-shock) and non-noxious (e.g., cat) stimuli results in the recruitment of an intra-CeM excitatory projection to vlPAG. The vlPAG sends inhibitory serotonergic projections to the CeALC (and dmVMH) that attenuate the intra-CeALC cellular activation and allows the individual to perform defensive behaviors at an optimum level of performance.

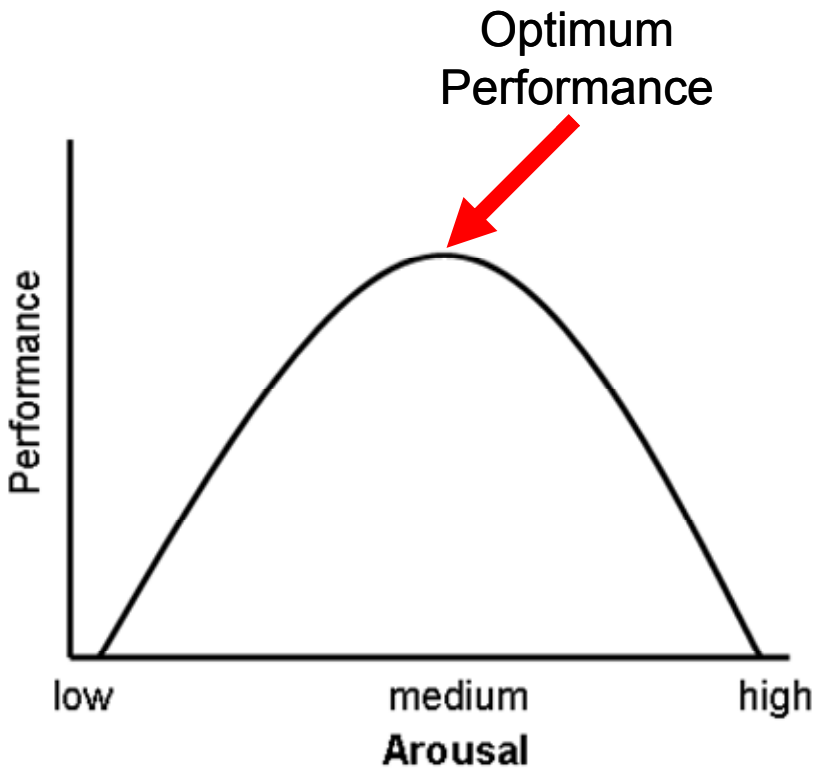

Figure 16. Yerkes-Dodson Curve. As arousal increases, efficiency in performance increases until an optimum level of performance is achieved. With increasing arousal past this optimum level, efficiency in performance decreases. Based on Yerkes and Dodson, 1908.

APPENDIX B

TABLES

Table 1

Descriptive Statistics (Mean ± SEM) of SMR, VDS, and VAD Response Characteristics at Drug Treatment Threshold vs. Saline Threshold

	SMR.RT Latency (ms)	**SMR.AMP** Amplitude (mm)	**SMR.MAG** Magnitude (cm x ms)	**VDS.RT** Latency (ms)	**VDS.AMP** Amplitude (dB)	**VDS.DUR** Duration (ms)	**VAD.RT** Latency (ms)	**VAD.AMP** Amplitude (dB)	**VAD.DUR** Duration (ms)
NMDA Dose Response & Anatomical Control Studies									
saline	309.20 ± 39.79	16.03 ± 2.38	115.02 ± 16.42	295.05 ± 20.58	90.06 ± 0.94	558.20 ± 22.89	1211.50 ± 32.34	86.99 ± 0.79	657.20 ± 55.46
.1µg NMDA	248.86 ± 96.09	27.14 ± 4.59	168.40 ± 51.75	286.29 ± 84.89	87.26 ± 1.97	392.29 ± 81.53	1114.86 ± 12.86	92.41 ± 2.24	747.71 ± 138.11
.25µg NMDA	286.57 ± 85.71	17.87 ± 5.98	97.07 ± 38.98	206.86 ± 53.39	84.70 ± 1.06	310.29 ± 44.09	1523.60 ± 294.04	89.96 ± 2.74	541.20 ± 176.14
.5µg NMDA	400.80 ± 93.88	18.80 ± 8.32	111.54 ± 36.91	255.00 ± 120.21	86.53 ± 1.71	334.50 ± 57.42	1102.00 ± 0.00	87.30 ± 3.20	312.00 ± 20.00
1µg NMDA	303.44 ± 50.95	19.90 ± 3.54	113.37 ± 19.06	223.18 ± 26.39	85.52 ± 1.22	408.45 ± 43.66	1287.14 ± 59.99	88.42 ± 1.39	606.67 ± 74.88
CeA - vlPAG Interaction Study									
saline CeA + saline vlPAG	369.71 ± 108.48	14.59 ± 5.06	111.46 ± 39.07	265.43 ± 26.19	89.81 ± 2.80	510.00 ± 69.80	1102.00 ± 0.00	87.30 ± 2.59	541.43 ± 106.30
0.025µg NMDA CeA + saline vlPAG	239.75 ± 77.98	26.30 ± 4.61	223.73 ± 56.76	229.25 ± 52.38	90.53 ± 2.62	438.50 ± 72.63	1351.75 ± 94.85	89.76 ± 1.69	685.00 ± 77.62
saline CeA + 0.25µg CTAP vlPAG	179.00 ± 32.75	18.36 ± 5.71	131.49 ± 43.22	256.75 ± 52.37	92.89 ± 2.02	593.25 ± 57.10	1239.00 ± 102.45	87.45 ± 2.41	726.38 ± 169.70
0.025µg NMDA CeA + 0.25µg CTAP vlPAG	201.00 ± 47.85	19.01 ± 7.61	141.05 ± 53.42	266.75 ± 38.53	89.34 ± 2.51	432.00 ± 76.39	1379.50 ± 148.50	87.20 ± 1.02	630.00 ± 111.99
Fos Expression Study - Shock Group									
saline	306.00 ± 119.41	25.25 ± 7.83	202.85 ± 69.59	247.00 ± 63.43	89.98 ± 0.54	476.50 ± 99.49	1104.00 ± 2.00	89.23 ± 4.67	727.50 ± 231.00
4µg APV	254.50 ± 98.13	38.68 ± 13.20	231.75 ± 95.61	118.50 ± 14.57	90.90 ± 3.70	425.00 ± 65.59	1123.33 ± 21.33	97.90 ± 1.67	347.33 ± 97.24
1µg NMDA	292.00 ± 132.93	22.15 ± 12.06	70.35 ± 22.19	92.00*	92.70*	388.00*	---**	---**	---**

* = mean is based on n = 1, see text for details
** = n is equal to zero, see text for details

Table 2

SMR, VDS, and VAD Response Characteristics at Drug Treatment Thershold vs. Saline Threshold: ANOVA and Tukey's HSD

Characteristic	*F*	*df*	*p*	Treatment for which *Dunnet's* is significant
NMDA Dose Response and Anatomical Control Studies				
SMR.RT	0.29	4, 83	0.89	--
SMR.AMP	0.81	4, 83	0.53	--
SMR.MAG	0.51	4, 83	0.73	--
VDS.RT	1.21	4, 79	0.31	--
VDS.AMP	3.18	4, 79	0.02*	1µg NMDA/side
VDS.DUR	6.16	4, 79	0.00*	0.25µg NMDA/side 0.5µg NMDA/side 1µg NMDA/side
VAD.RT	2.26	4, 74	0.07	--
VAD.AMP	1.62	4, 74	0.18	--
VAD.DUR	0.77	4, 74	0.55	--
CeA - vlPAG Interaction Study				
SMR.RT	1.40	3, 30	0.26	--
SMR.AMP	0.67	3, 30	0.58	--
SMR.MAG	1.01	3, 30	0.41	--
VDS.RT	0.15	3, 30	0.93	--
VDS.AMP	0.41	3, 30	0.75	--
VDS.DUR	1.21	3, 30	0.32	--
VAD.RT	1.37	3, 30	0.27	--
VAD.AMP	0.39	3, 30	0.76	--
VAD.DUR	0.41	3, 30	0.75	--
FOS Expression Study - Shock Group				
SMR.RT	0.05	2, 11	0.95	--
SMR.AMP	0.61	2, 11	0.57	--
SMR.MAG	1.54	2, 11	0.27	--
VDS.RT	2.38	2, 8	0.18	--
VDS.AMP	0.11	2, 8	0.9	--
VDS.DUR	0.16	2, 8	0.86	--
VAD.RT	1.15	1,6	0.33	--
VAD.AMP	2.32	1,6	0.19	--
VAD.DUR	1.78	1,6	0.24	--

** p < .05*

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ABSTRACT

THE CONTRIBUTION OF NMDA RECEPTORS WITHIN THE CENTRAL NUCLEUS OF THE AMYGDALA TO THE SUPPRESSION OF PAIN AFFECT

by

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The amygdala processes stimuli that threaten an individual and organizes the execution of affective behaviors designed to cope with the threat. The prototypical threat to an individual is exposure to a noxious stimulus. The central nucleus of the amygdala (CeA) receives nociceptive afferents and exhibits neuronal activation in response to noxious peripheral stimulation. NMDA receptors within CeA mediate this noxious-evoked neural excitation, and previous studies in the laboratory have shown that blockade of CeA NMDA receptors via the antagonist APV elevates the threshold for noxious tail-shock-induced vocalization afterdischarges (VADs), a validated measure of pain affect in the rat. The present study further evaluated the contribution of NMDA receptors to the suppression of pain affect.

Intra-CeA NMDA receptor activation via the agonist NMDA elevated VAD thresholds in a dose dependent manner. That the NMDA receptor agonist and antagonist produce similar behavioral effects is hypothesized as the result of targeting separate neural populations within the CeA. Whereas the antagonist likely inhibits nociception at the level of the lateral capsular division of the CeA, the agonist likely activates

antinociceptive efferents at the level of the vlPAG. In support of this hypothesis, the present study revealed that *Fos* expression within vlPAG is greater in rats that received intra-CeA agonist NMDA treatment compared to those that received the antagonist APV or saline. Lastly, intra-CeA NMDA agonist-induced elevations in VAD thresholds were blocked via the pre-treatment of the vlPAG with the mu-opiate antagonist CTAP. These studies provide the first demonstration of the contribution of CeA NMDA receptors to the generation of pain affect in the rat.

AUTOBIOGRAPHICAL STATEMENT

Upon graduation from Lake Shore High School (St. Clair Shores, MI) in 1999, I enrolled at Wayne State University. In 2003, I entered the laboratory of Dr. George S. Borszcz as an undergraduate research assistant investigating the neuronanatomical and neurochemical mechanisms within the medial thalamus that underlie the emotional dimension of the pain experience. I received a Cum Laude Bachelor of Science degree in Psychology in 2004 from Wayne State. I enrolled in the Psychology graduate program, Behavioral and Cognitive Neuroscience area, at Wayne State University in 2005. In May of 2008, I graduated with my Master of Arts degree in Psychology with the thesis *NMDA or Non-NMDA Receptor Antagonism within the Amygdaloid Central Nucleus of the Rat Produces Antinociception.* I intend to continue my scientific training following graduation with a post-doctoral position at the University of Michigan with Dr. Martin Sarter beginning January 2011.