Published in final edited form as:

J Pain. 2014 December ; 15(12): 1305–1318. doi:10.1016/j.jpain.2014.09.007.

NMDA Receptor Agonism and Antagonism within the Amygdaloid Central Nucleus Suppresses Pain Affect: Differential Contribution of the Ventrolateral Periaqueductal Gray

Catherine A. Spuz, Michelle L. Tomaszynski, and George S. Borszcz

Department of Psychology, Behavioral & Cognitive Neuroscience Program, Wayne State University, Detroit, MI 48202

Abstract

The amygdala contributes to the generation of pain affect and the amygdaloid central nucleus (CeA) receives nociceptive input that is mediated by glutamatergic neurotransmission. The present study compared the contribution of N-methyl-D-aspartate (NMDA) receptor agonism and antagonism in CeA to generation of the affective response of rats to an acute noxious stimulus. Vocalizations that occur following a brief tail shock (vocalization afterdischarges) are a validated rodent model of pain affect, and were preferentially suppressed, in a dose dependent manner, by bilateral injection into CeA of NMDA (.1 µg, .25 µg, .5 µg, or 1 µg/side), or the NMDA receptor antagonist D-2-amino-5-phosphonovalerate (AP5, 1 µg, 2 µg, or 4 µg/side). Vocalizations that occur during tail shock were suppressed to a lesser degree, whereas, spinal motor reflexes (tail flick and hind limb movements) were unaffected by injection of NMDA or AP5 into CeA. Injection of NMDA, but not AP5, into CeA increased c-Fos immunoreactivity in the ventrolateral periaqueductal gray (vlPAG), and unilateral injection of the µ-opiate receptor antagonist H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP, 0.25 µg) into vlPAG prevented the antinociception generated by injection of NMDA into CeA. These findings demonstrate that although NMDA receptor agonism and antagonism in CeA produce similar suppression of pain behaviors they do so via different neurobiological mechanisms.

Perspective—The amygdala contributes to production of the emotional dimension of pain. NMDA receptor agonism and antagonism within the central nucleus of the amygdala suppressed rats' emotional response to acute painful stimulation. Understanding the neurobiology underlying emotional responses to pain will provide insights into new treatments for pain and its associated affective disorders.

© 2014 The American Pain Society. All rights reserved.

Corresponding Author: George S. Borszcz, Telephone: 313-577-2895, FAX: 313-577-7636, borszcz@wayne.edu, URL: <http://sun.science.wayne.edu/~gborszcz/>.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures – There were no conflicts of interest.

Keywords

nociception; emotion; c-Fos; amygdala; AP5; NMDA; vocalization; rat; periaqueductal gray; glutamate; vocalization

Introduction

The affective-motivational dimension of pain motivates individuals to seek medical attention, and underlies the suffering and disability associated with the pain state.⁴⁸ Despite this preeminent clinical significance, little is known regarding the neuronal mechanisms responsible for production or modulation of the affective component of the pain experience. The amygdaloid central nucleus (CeA) contributes to the execution of affective behaviors to environmental threats.⁴⁴ The prototypical threat to an individual is exposure to a noxious stimulus,^{9,12,22} and the CeA receives direct nociceptive inputs from the spinal cord dorsal horn^{18,56} and indirect nociceptive inputs relayed via the parabrachial nucleus (PB)^{2,41} and intralaminar thalamic nuclei.^{58,74}

Electrophysiological recordings within CeA demonstrate increases in evoked neural activity to acute noxious stimuli or during chronic pain.^{3,46} Acute nociceptive stimuli also increase immunoreactivity and expression of the immediate early gene, c-Fos, in the CeA that is reduced following antinociceptive treatments.^{35,53} Neuroimaging studies implicate a role of the amygdala in the processing of pain affect in humans.^{43,66,75} Although human neuroimaging has yet to attain the resolution necessary to identify individual amygdaloid nuclei, recent electrophysiological recordings from human brain revealed noxious-evoked activity in CeA.⁴⁷ Additionally, damage of the amygdala in humans^{17,36,42} and CeA in rats^{11,72} suppresses their affective responding to noxious peripheral stimulation.

The rat CeA contains NMDA and non-NMDA receptors that contribute to nociceptive processing.⁴⁶ In normal rats, nociceptive transmission to CeA involves glutamatergic afferents from PB that interact with non-NMDA receptors (but not NMDA receptors), and glutamatergic afferents from extra-PB sources that interact with NMDA receptors.^{4,46} The source of nociceptive glutamatergic afferents to CeA that interact with NMDA receptors is not known, but they may be provided directly via the spino-amygdaloid pathway or indirectly via relays from the intralaminar thalamus.^{58,74}

We previously reported that injection of NMDA (AP5) and non-NMDA (CNQX) receptor antagonists into CeA produced dose-dependent suppression of vocalization afterdischarges (VADs) in rats.⁶⁸ VADs occur immediately following application of a brief noxious tailshock and are a validated model of pain affect in rats (see Methods). In preliminary studies, we were surprised to observe that NMDA receptor agonism within CeA, via microinjection of NMDA into CeA, also preferentially suppressed production of VADs. The present study compared the suppression of pain affect elicited by NMDA receptor agonism and antagonism within CeA, and evaluated differences in the number of cells immunopositive for the immediate early gene c-Fos in the ventrolateral periaqueductal gray (vlPAG) following injection of NMDA or AP5 into CeA, because antinociception elicited from CeA is dependent on its interaction with vlPAG.⁵ Antinociception generated by the

administration of NMDA and AP5 into CeA was challenged by injection of the μ -opiate receptor antagonist CTAP into vIPAG as μ -opiate receptors within vIPAG mediate antinociception elicited from CeA.⁵⁹ Our goal was to assess whether the action of NMDA and an NMDA antagonist within CeA produces affective analgesia through different neurobiological mechanisms.

Methods

Animals

Eighty-six naïve male Long-Evans rats (Charles River, Raleigh, NC) were housed as pairs in polypropylene cages (52cm $\times$ 28cm $\times$ 22cm) with hardwood chip bedding and given *ad libidum* access to Rodent Lab Diet 5001 (PMI, Nutrition International, Inc., Brentwood, MO) and water. Housing was provided in a climate-controlled vivarium maintained on a 12:12-hr circadian cycle with lights on at 0700 hrs. All testing was conducted between 0800 and 1700 hrs. Upon arrival, rats were given 5–7 days of acclimatization prior to handling. Rats were handled 2–3 times during the week prior to surgery to minimize effects of stress from human contact. Following surgery, rats were handled once per day for at least one week before testing to check on their recovery and to further minimize the effects of stress from human contact. All experiments were performed following the guidelines of the United States National Institutes of Health *Guide for the Care and Use of Laboratory Animals* using protocols approved by the Wayne State University Institutional Animal Care and Use Committee.

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 bilaterally implanted above CeA according to coordinates extrapolated from the rat brain atlas of Paxinos and Watson.⁶⁰ The coordinates (in mm) relative to the bregma suture and the top of the flat skull are as follows: AP = -2.0 , L = ± 4.0 , 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): AP = -2.0 , L = ± 5.4 , 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): AP = -2.0 , L = ± 3.0 , DV = -6.0 .

For implants aimed toward the vIPAG, one stainless steel 26-gauge single-cannula (Plastics One, Roanoke, VA) was implanted unilaterally above the vIPAG at a twenty-degree angle according to coordinates extrapolated from the rat brain atlas of Paxinos and Watson⁶⁰. Rats received vIPAG 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 extended the length of the guide to maintain its patency. Rats were given 7–10 days to recover before the initiation of testing.

Histological Analysis

All rats, except those whose brains were processed for c-Fos immunoreactivity, 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 Watson⁶⁰ brain atlas by a researcher unaware of the results from behavioral testing.

A Nissl stain (cresyl violet) was used on the tissue of rats from the NMDA dose response study that received the highest NMDA dose (1µg/.25µl per side) to qualitatively assess the potential neurotoxic effects of NMDA treatment. 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.

c-Fos immunohistochemistry

Two hours after the intracerebral injection, the rats were rapidly and 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 in the coronal plane at 45µm and vIPAG sections with the AP coordinates –7.64 mm through –8.28 mm posterior to bregma were collected.

Free-floating tissue sections were processed as follows. All steps were preceded by washes in 0.1M PBS. All reactions were carried out on a shaker table at room temperature. First, tissue was incubated in 0.3% H₂O₂ for 30 min and blocked in 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. Sections were then incubated for 48 hours with a primary c-Fos rabbit polyclonal IgG (1:5000, #sc52, Santa Cruz Biotech, Santa Cruz, CA) at 4° C. This concentration was determined using multiple concentrations on sample tissue. The 1:5000 concentration yielded the highest specificity and only nuclear-staining

On the second day, sections were incubated for 2 hr with biotinylated goat anti-rabbit IgG secondary (1:200, #BA-1000, Vector Laboratories, Burlingame, CA). Visualization was accomplished with 1 hr incubation in an avidin and biotin complex (1:500, #PK-6100, ABC Elite Standard kit, Vector Laboratories, Burlingame, CA) followed by 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO) with 0.6% nickel ammonium sulfate until optimal color was achieved.

Quantification of c-Fos-positive cells

Tissue sections were mounted on gelatin-coated slides, dehydrated, cover slipped, and photographed with a Nikon Eclipse 80i microscope with Nikon Elements software (version 3.1). The vIPAG was photographed at 40x and compared to the rat brain atlas⁶⁰ as an orientation aid for tracing the vIPAG. Images were magnified to 200x, and a 400µm × 300µm box was centered within the vIPAG (see Fig 4). c-Fos immunoreactivity was visualized as a dark reaction product inside neuronal nuclei. The number of c-Fos-positive nuclei was counted in the box by hand with the aid of the Nikon Elements software. The vIPAG was counted in three to four separate sections representing both hemispheres in each rat. The results for each rat are expressed as the average number of c-Fos-positive nuclei across all sections and both hemispheres.

Assessment of pain affect

Research in this laboratory validated vocalization afterdischarges (VADs) as a rodent model of pain affect. These vocalizations occur immediately following application of noxious tailshock, are organized within the forebrain, and have distinct spectrographic characteristics compared to vocalizations that occur during shock (VDS).^{7,9,20,37} Systemically administered drug treatments that preferentially suppress the affective response of humans to pain also preferentially suppress production of VADs.^{13,28,61} Generation of VADs is 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.^{8,11,32–34,37,50,54,71} Additionally, the capacity of noxious tailshock to support fear conditioning is directly related to its production of VADs.^{6,7,9,11} In the present study, the effects of experimental treatments on VAD threshold were compared with their effects on the thresholds of other tail shock-elicited responses that are organized at medullary (vocalizations during shock, VDS) and spinal (spinal motor reflexes, SMR) levels of the neuraxis.^{15,20}

Apparatus

Testing was controlled by custom computer programs via a multifunction interface board (DT-2801, Data Translation, Marlboro, MA) installed in a PC. 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 maintains the rat in a crouching posture throughout testing, permits normal respiration and vocalizing, and allows unobstructed access to the head for intracerebral injections (see photograph⁷). Testing was conducted within a sound attenuating, lighted, and ventilated chamber equipped with a small window that enabled visual monitoring of rats during testing.

Tailshock (20 ms pulses at 25 Hz for 1,000 ms) 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, 7.0 cm (cathode) and 8.5 cm (anode) from the base. The intensity, duration, and timing of tailshocks were controlled by the computer. Current intensity was monitored by an analog-to-digital converter that digitized (500 Hz sampling rate) an output voltage of the shocker that was proportional to the current delivered.

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 arm of the transducer was positioned behind and perpendicular to the tail such that the thread extended in a straight line directly behind the rat. The output voltage of the transducer was amplified ($\times 50$) and then digitized (500 Hz sampling rate) by an analog-to-digital converter of the interface board. SMR was defined as movement of the transducer arm by at least 1.0 mm following shock onset. The computer recorded the latency (ms), peak amplitude (mm), and magnitude ($\text{cm} \times \text{ms}$) of tail movement on each trial. Displacements up to 100 mm can be detected, and latencies in 2 ms increments can be measured.

Vocalizations were measured by a pressure-zone microphone (Realistic model 33-1090, Tandy, Ft. Worth, TX) located on the wall of the testing chamber 15 cm 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 1500 Hz. 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 (2 ms time base) and digitized (500 Hz sampling rate) by a separate analog-to-digital converter of the interface board. The peak intensity (in decibels: SPL, B scale), latency (ms), and duration (ms) of vocalizations during the shock epoch (VDS) and for the 2,000 ms interval following shock termination (VAD), were recorded by the computer.

Pain Testing

For two consecutive days prior to testing, rats were adapted to the testing apparatus for a period of 20 min each day to minimize the effects of restraint stress. 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.²⁵

Drug Injections

Intracerebral CeA injections were administered in a constant volume 0.25 μl via 33-gauge injectors. Injectors targeted at CeA extended 3 mm beyond the end of the cannula. Injectors that targeted sites dorsal to CeA extended 1.8 mm beyond the end of the cannula, and injectors that targeted sites ventral to CeA extended 4.2 mm beyond the end of the cannula. Intracerebral vIPAG injections were administered in a constant volume 0.5 μl via a 33-gauge injector. Injectors that targeted vIPAG extended 3 mm 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 (AP5; Tocris, Ellisville, MO), and H-D-Phe-Cys-Tyr-

D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP; Sigma-Aldrich, St. Louis, MO) were dissolved in normal sterile saline.

Procedures

Experiment 1: AP5 and NMDA Dose-Response Analysis

We previously reported on the dose-response relationships between CeA administered AP5 and SMR, VDS, and VAD thresholds⁶⁸, and they are reproduced here (Fig 2A) for comparison with the effects of intra-CeA administered NMDA. The NMDA treatment groups received one bilateral intra-CeA microinjection of NMDA (0.1 µg, 0.25 µg, 0.5 µg, or 1 µg/side) and saline prior to two separate test sessions ($n = 5-7/\text{group} = 22$ total). Doses of NMDA were determined from the results of a preliminary study. Saline injections were maintained as the first test in order to ascertain baseline levels of responding. Test sessions were separated by 4 to 6 days.

Experiment 2: Anatomical Specificity

Anatomical specificity of the antinociceptive effects of AP5 treatments into CeA was established in our previous article⁶⁸, and those procedures were used here to establish anatomical specificity of NMDA treatments. Rats ($n = 8$) received bilateral microinjections of the high dose of NMDA (1 µg/side) from the dose-response experiment and saline into sites within and surrounding CeA. Only rats that completed all test sessions were included in the analysis. Rats were assigned to one of three groups: Central, Medial, and Lateral. Groups were named based on the medial-lateral plane of guide cannulae implants relative to CeA stereotaxic coordinates. The Central group ($n = 3$) received injections 1.2 mm dorsal to, 1.2 mm ventral to, and within CeA. The Medial group ($n = 3$) received injections 1.2 mm medial to CeA, and also 1.2 mm dorsal to and 1.2 mm ventral to this vicinity. The Lateral group ($n = 2$) received injections 1.2 mm lateral to CeA, and also 1.2 mm dorsal to and 1.2 mm ventral to this vicinity. Each rat in these groups received saline and NMDA treatments using an injector that extended 1.8 mm, 3.0 mm, and 4.2 mm past the end of the guide cannulae. Thus, each rat received a total of 6 treatments on 6 separate test sessions in 3 different brain regions (test sessions were separated by 5 to 10 days). Vehicle was administered on the first test, and NMDA was given on the second test at each site.

Experiment 3: Induction of c-Fos

Rats were randomly assigned to one of three groups: Home Cage, Chamber Only, and Pain Test. Each group was composed of three subgroups that were bilaterally administered saline (0.25 µl/side), NMDA (1 µg in 0.25 µl/side), or AP5 (4 µg in 0.25 µl/side) into CeA. All subgroups contained 4 rats (36 total rats). Home Cage rats were moved from the vivarium to the laboratory holding room for three consecutive days (3 hours/day). On the third day, following 1 hour in the holding room rats were administered saline or drugs into CeA and then brains were removed 2 hours later as described above. For three consecutive days, Chamber Only rats were brought to the laboratory holding room from the vivarium and remained in the holding room for 40 minutes prior to being restrained in the pain testing apparatus for 20 minutes. No tailshock was administered during these exposure sessions. Prior to chamber exposure on the third day rats received bilateral injections of saline or

drugs into CeA. Following exposure sessions rats were returned to the holding room for 2 hours, and on the third day brains were collected for c-Fos analysis. The Pain Test group followed the identical protocol of the Chamber Only group except on the third day pain testing was conducted.

Experiment 4: CeA to vIPAG interaction

The capacity of μ -opiate receptor antagonism within vIPAG to attenuate increases in response thresholds generated by intra-CeA administered NMDA ($n = 5$) or AP5 ($n = 4$) was evaluated. Rats received the following pairs of injections into CeA and vIPAG prior to four separate pain testing sessions: CeA saline + vIPAG saline, CeA saline + vIPAG CTAP (0.25 μ g), CeA NMDA (1 μ g per side) or AP5 (4 μ g per side) + vIPAG saline, and CeA NMDA (1 μ g per side) or AP5 (4 μ g per side) + vIPAG CTAP (0.25 μ g). Test sessions were separated by 5 – 7 days. Injections into vIPAG immediately preceded injections into CeA.

Data Analysis

Rats with incorrectly placed cannulae were excluded from analysis ($n = 4$). Rats were also excluded from analysis due to death during surgery ($n = 1$), illness ($n = 2$) or complications related to their cannulae implants ($n = 4$).

Pain Testing

After each test session, data were reorganized in ascending order according to tail shock intensity. SMR, VDS, and VAD thresholds for each rat were calculated as the lesser current intensity from a string of at least 2 consecutive intensities that generated the response. For Experiment 1, dose-dependent effects of NMDA on response thresholds were directly compared using repeated-measures multivariate analysis of variance (MANOVA). The effects of dose on individual responses were analyzed by one-way analysis of variance (ANOVA). The doses of NMDA that elevated response thresholds above baseline levels were determined by post hoc comparisons of thresholds after saline and NMDA treatments using Dunnett's test.

For Experiment 2, comparisons of response thresholds generated following the bilateral administration of saline and 1 μ g NMDA into the CeA and into sites surrounding the CeA were made using one-way ANOVA for each response. Post hoc pairwise comparisons of the effects of treatments on response thresholds were made using Tukey HSD test.

For Experiment 3, the capacity of bilateral injection of 1 μ g NMDA or 4 μ g AP5 into CeA to increase the number of c-Fos immunoreactive cells in vIPAG was analyzed using a two-way between-subjects ANOVA with drug treatment (saline, AP5, NMDA) and context (Home Cage, Chamber Only, Pain Test) as between-group factors followed by one-way ANOVAs with Dunnett's post-hoc tests.

For Experiment 4, the capacity of CTAP administered into vIPAG to reduce elevations of response thresholds generated by injections of NMDA or AP5 into CeA were analyzed across treatment groups for each response by a one-way ANOVA. Post-hoc pairwise treatment comparisons of thresholds was made using Tukey HSD test.

Results

Behavioral Profile

The SMR, VDS, and VAD responses reflect nociceptive processing at progressively higher levels of the neuraxis.^{11,21} Analysis of rats that received transections of the neuraxis revealed that SMRs are organized at the spinal level, VDSs within the medulla below the pontomedullary border, and VADs within the forebrain. Responses 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. VDS were elicited without SMRs on 0.32% of the trials in which only these responses were generated. False alarm rates were low (SMR = 1.8%, VDS = 0.0%, VAD = 0.0%), indicating that responses were not induced by drug administration, were not occurring spontaneously, and were not conditioned responses to the context, but instead were generated by tail shock.

Assessment of performance variables (see Table 1) demonstrated that latency, duration and amplitude of VADs at threshold were not influenced by administration of NMDA. In the dose-response experiment (Experiment 1), comparison of VAD performance variables at threshold across saline and NMDA drug treatments revealed no changes in the capacity to generate VADs, $F_s(4,74) < 2.26$, $ps > .05$. The latency of VDSs at threshold was also not altered by NMDA treatments ($F(4,79) = 1.21$, $p > .05$), but the amplitude and duration of VDSs were significantly lower following NMDA treatments, $F_s(4,79) > 3.18$, $ps < .05$. Post-hoc analysis revealed that the amplitude of VDS was decreased compared to baseline following bilateral administration of 1 μ g NMDA. Post-hoc analysis revealed that the duration of VDS was decreased compared to baseline following bilateral administration of 0.25 μ g NMDA, 0.5 μ g NMDA and 1 μ g NMDA. The effect on VDS amplitude is small ($M_{1\mu\text{g NMDA}} = 85.5 \text{ dB} \pm SEM = 1.22$ vs. $M_{\text{saline}} = 90.1 \text{ dB} \pm SEM = 0.94$) and did not occur in other experiments. The effects on VDS duration also did not occur in the 1 μ g NMDA group in the c-Fos Expression Study – Pain Test group (Experiment 3) or the CeA-vIPAG interaction study (Experiment 4). SMR performance (latency, amplitude, or magnitude) was not affected by NMDA treatments, $F_s(4,83) < 0.81$, $ps > .05$. These findings are similar to those we previously reported for AP5.⁶⁸ Across doses of AP5, VAD and SMR performance at threshold was not affected, but VDS duration was reduced following administration of the two highest doses of AP5 (2 μ g and 4 μ g). In the present study, 4 μ g AP5 did not alter any VDS performance variable in Experiments 3 and 4.

Experiment 1: Dose-Response Analysis

Injections were administered bilaterally into CeA in all rats. Fig 1A is a photomicrograph of a representative injection site for NMDA in CeA. It is unlikely that increases in response thresholds that followed NMDA administration were the result of excitotoxic lesions of CeA. Fig 1B is a photomicrograph of a Nissl stained section of CeA from a rat that was administered the highest dose (1 μ g/side) of NMDA prior to pain testing. CeA did not exhibit a reduction in the number of stained neurons in any rat that received the highest dose of NMDA. Additionally, a test session preceded by saline treatment was administered following (4 – 6 days) the test session that was preceded by NMDA (1 μ g/side) treatment. Response thresholds returned to baseline during this additional test session (data not shown).

Comparison of the effects of AP5 and NMDA into CeA on SMR, VDS, and VAD thresholds are depicted in Fig 2. Analysis of the effects of AP5 injections on response thresholds was previously reported.⁶⁸ Comparison of response thresholds across doses of NMDA (repeated measures MANOVA, Wilk's Lambda) revealed significant main effects of dose ($F(4,39) = 27.26, p < 0.001$), and response ($F(2,38) = 101.20, p < 0.001$), and a significant Dose $\times$ Response interaction, $F(8,76) = 11.30, p < 0.001$. This interaction reflects the finding that NMDA only elevated VAD and VDS thresholds, and VAD threshold was elevated to a greater degree than VDS threshold. One-way ANOVA revealed that VDS and VAD thresholds were elevated in a dose-dependent manner by NMDA administration (VDS: $F(4,43) = 13.32, p < 0.001$; VAD: $F(4,43) = 32.46, p < 0.001$), but NMDA treatments did not affect SMR threshold, $F(4,42) = 2.01, p > 0.05$. Lower doses of NMDA were able to elevate VAD versus VDS thresholds. Compared with vehicle treatment, VAD threshold was significantly elevated following bilateral administration of 0.1 μg NMDA, whereas VDS threshold was significantly elevated following bilateral administration of 0.25 μg NMDA, Dunnett, $ps < .05$. Direct comparison of VAD and VDS thresholds across doses of NMDA revealed a significant Dose $\times$ Response interaction, $F(4,39) = 10.16, p < 0.001$.

Experiment 2: Anatomical Specificity

Increases in VAD and VDS thresholds generated by injection of AP5 into CeA were previously shown to be limited to its action within CeA. Similarly, increases in VAD and VDS thresholds produced by injection of NMDA (1 $\mu\text{g}/\text{side}$) into CeA were found not to be due to its spread outside of the CeA. Fig 3A depicts the distribution of injection sites of NMDA within and surrounding the CeA. No systematic differences were observed on SMR, VDS, or VAD thresholds following bilateral administration of NMDA into sites surrounding the CeA, and thus these data were combined into a single group (Other Sites). Bilateral administration of saline into the CeA and sites surrounding CeA did not produce a significant difference in response thresholds, $ts(17) < .20, ps > .80$; thus, these data were combined for comparison of the effects on response thresholds of NMDA injected in CeA and Other Sites.

The effects on response thresholds of administering NMDA into sites surrounding CeA are depicted in Fig 3B. Comparison of response thresholds following administration of NMDA or saline into CeA or sites surrounding CeA revealed significant differences for VAD, $F(2,37) = 112.51, p < .001$, and VDS thresholds, $F(2,37) = 36.81, p < .001$, but not for SMR threshold, $F(2,37) = 0.11, p > .80$. Compared to following saline treatment, administration of NMDA into CeA produced significant elevations in VAD and VDS thresholds, Tukey HSD, $ps < .001$, but did not alter SMR threshold, Tukey HSD, $ps > .90$. Injections of NMDA into sites outside of the CeA were ineffective in elevating any response threshold, Tukey HSD, $p > .50$.

Experiment 3: c-Fos & Pain Thresholds

Fig 4 provides representative photomicrographs with c-Fos positive nuclei in vIPAG following bilateral administration of saline, AP5 (4 $\mu\text{g}/\text{side}$), and NMDA (1 $\mu\text{g}/\text{side}$) into CeA. Drug effects on c-Fos immunoreactivity were assessed in rats exposed to one of three contexts (Home Cage, Chamber Only, or Pain Test). The effect of drug treatments on the

number of c-Fos positive nuclei in vIPAG is shown in Fig 5A. Two-factor (drug and context) ANOVA revealed a significant main effect drug, $F(2,27) = 116.74, p < .001$. The main effect of context, $F(2,27) = .02, p > .90$, and the Drug $\times$ Context interaction, $F(4,27) = 1.07, p > .35$, were nonsignificant. These results reflect the finding that in each context, the expression of c-Fos in vIPAG was greater in rats administered NMDA into CeA. For each context, one-way ANOVA revealed significant group differences, all $F(2,11) > 26.35$, all $ps < .001$. Post-hoc analyses demonstrated that in each context the number of c-Fos positive nuclei in vIPAG was greater in groups that received bilateral injections of NMDA, as compared to either saline or AP5, into CeA, Tukey HSD, $ps < .001$. The number of c-Fos positive nuclei in vIPAG did not differ in groups administered saline or AP5 into CeA in any context, Tukey HSD, $ps > .75$.

Response thresholds of rats given pain testing are shown in Fig 5B. Consistent with the previous experiments, bilateral administration of NMDA (1 $\mu\text{g/side}$) or AP5 (4 $\mu\text{g/side}$) into CeA produced significant increases in VAD and VDS thresholds, but did not alter SMR threshold. Comparison of response thresholds across saline, AP5, and NMDA treatments (repeated measures MANOVA, Wilk's Lambda) revealed significant main effects of drug ($F(2,9) = 16.53, p < 0.001$), and response ($F(2,8) = 34.74, p < 0.001$), and a significant Drug $\times$ Response interaction, $F(4,16) = 4.97, p < 0.005$. This interaction reflects the finding that only VAD and VDS thresholds were affected by AP5 or NMDA treatments into CeA. One-way ANOVA across drug treatments revealed increases VDS and VAD thresholds (VDS: $F(2,11) = 11.99, p < 0.005$; VAD: $F(2,11) = 17.13, p < 0.001$), but not SMR threshold, $F(4,42) = 0.56, p > 0.55$. Compared to following saline treatment, VDS and VAD thresholds were significantly elevated following bilateral administration of AP5 or NMDA into CeA, Dunnett, $ps < .01$.

Experiment 4: CeA - vIPAG Interaction

Fig 6A depicts the distribution of unilateral injection sites within vIPAG that received CTAP (0.25 μg) injections following bilateral administration of AP5 (4 $\mu\text{g/side}$) or NMDA (1 $\mu\text{g/side}$) into CeA. A representative photomicrograph of an injection site within vIPAG is shown in Fig 6B.

The capacity of CTAP administered into vIPAG to reduce increases in response thresholds generated by injection of NMDA or AP5 into CeA is shown in Fig 7. For groups administered NMDA(1 $\mu\text{g/side}$) or AP5(4 $\mu\text{g/side}$) into CeA, one-way ANOVA across treatments revealed significant effects for VAD (NMDA, $F(3,19) = 43.82, p < .001$; AP5, $F(3,15) = 18.96, p < .001$) and VDS (NMDA, $F(3,19) = 37.82, p < .001$; AP5, $F(3,15) = 18.11, p < .001$) thresholds, but not SMR threshold (NMDA, $F(3,19) = .11, p > .90$; AP5, $F(3,15) = 1.61, p > .20$). Post-hoc threshold comparisons showed, consistent with the preceding experiments, that VAD and VDS thresholds following CeA-NMDA(1 $\mu\text{g/side}$) + vIPAG-saline or CeA-AP5(4 $\mu\text{g/side}$) + vIPAG-saline treatments were significantly elevated compared to thresholds following CeA(saline) + vIPAG(saline) treatments, Tukey HSD, $ps < .001$.

Administration of CTAP into vIPAG antagonized increases in vocalization thresholds produced by injection of NMDA into CeA. Compared to following CeA-NMDA(1 $\mu\text{g/side}$)

+ vIPAG (saline) treatment, VAD and VDS thresholds were significantly reduced following CeA-NMDA(1 µg/side) + vIPAG-CTAP(0.25 µg) treatment, Tukey HSD, $ps < .001$. Following injection of CTAP into vIPAG, NMDA-induced increases in VAD and VDS thresholds did not differ from baseline levels, Tukey HSD, $ps > .75$. Alternately, administration of CTAP into vIPAG had no effect on increases in vocalization thresholds generated by injection of AP5 into CeA. VAD and VDS thresholds did not differ following CeA-AP5(4 µg/side) + vIPAG-saline and CeA-AP5(4 µg/side) + vIPAG-CTAP(.25 µg) treatments, Tukey HSD, $ps > .90$.

Discussion

The present study demonstrated that administration of NMDA into 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. As VADs are a validated rodent model of pain affect, these findings indicate that NMDA receptor activation in CeA contributes to production of affective analgesia.

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 interference with the capacity of rats to fully perform the response. In Experiment 1, NMDA-induced increases in VDS thresholds were accompanied by decreases in VDS duration and increases in VDS latency. 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.^{6,13} However, these decrements were relatively small and were not observed in the CeA-vIPAG interaction study (Experiment 4).

The capacity of CeA-administered NMDA to elevate vocalization thresholds is limited to its action within CeA. Bilateral administration of the highest dose of NMDA (1 µg/side) into sites surrounding the CeA failed to increase vocalization thresholds. Furthermore, the effect of NMDA on vocalization thresholds cannot be the result of an excitotoxic lesion. NMDA is a known neurotoxin but the doses used in the present study are well below those shown to produce cell loss (8 µg),⁴⁹ and qualitative analysis of Nissl-stained tissue revealed that bilateral treatment with 1 µg NMDA failed to produce cell loss. Additional evidence is provided by the finding that elevations in vocalization thresholds produced by injection of NMDA into CeA were prevented by administration of CTAP into vIPAG. 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 epileptic-type neural activity producing a functional lesion in CeA.²⁷ Therefore, the capacity of NMDA administered into CeA to suppress pain-induced vocalizations is the result of activation of NMDA receptors within CeA.

Failure of NMDA to elevate SMR threshold indicates that glutamate acting at NMDA receptors in CeA does not activate descending projections that inhibit nociceptive processing at the spinal dorsal horn. This finding is consistent with our earlier report that administration

of morphine into the amygdala elevated vocalization thresholds but not SMR threshold.⁵⁴ However, a recent report demonstrated that injection of glutamate into CeA raised mechanical paw-withdrawal threshold that was blocked by pretreatment of CeA with the NMDA receptor antagonist MK-801.¹⁶ These differences may reflect the different pain submodalities employed in these studies.

Increases in vocalization thresholds generated by intra-CeA administered NMDA is consistent with findings that systemic administration of NMDA results in neuronal excitation within CeA^{40,62}, and that electrical stimulation of CeA suppresses pain-induced vocalizations in rats and guinea pigs.^{45,51} However, these findings appear to be inconsistent with reports that administration of a NMDA receptor antagonist into CeA suppresses both noxious-evoked neural activity within CeA and pain-induced vocalizations in rats.^{46,68} Indeed, the present study replicated our earlier finding that injection of AP5 into CeA also elevated VAD and VDS thresholds.

NMDA receptors have been identified in the medial (CeM) and latero-capsular (CeLC) subdivisions of the CeA in rat (see Fig 1A).^{26,29} We propose that manipulation of NMDA receptors within CeA produces affective analgesia via the action of NMDA and AP5 at separate neural populations within CeM and CeLC, respectively. These effects are mediated through different efferent projections of CeM and CeLC that respectively contribute to antinociception and nociception. For example, the number of c-Fos immunoreactive neurons increases in CeM, but not CeLC, following systemic morphine treatment that generates antinociception, and increases in CeLC, but not CeM, with hyperalgesia generated by precipitated withdrawal from morphine.³¹ This segregation within the CeA may account for the reported involvement of CeA in antinociception and nociception.⁵⁵ The differential selectivity of AP5 and NMDA for NMDA receptor subtypes (NR2A–D)^{57,63} may also contribute the antinociceptive actions these treatments. For example, differential expression of NMDA receptor subtypes on CeA neurons that are involved in the expression or suppression of pain affect may contribute to the different mechanisms whereby both AP5 and NMDA inhibit pain-induced vocalizations.

Nociceptive glutamatergic input to CeA is largely limited to CeLC, and NMDA receptors in CeLC contribute to noxious-evoked activity within CeLC.^{2,46} This glutamatergic input to CeLC activates projection neurons of CeLC that target limbic-forebrain sites that integrate execution of affective pain behaviors.^{9,12,55} Increases in vocalization thresholds following AP5 injection into CeA are presumably mediated by suppression of noxious evoked neural activity in CeLC.

Alternately, CeM contains projection neurons that target the vIPAG.²¹ The vIPAG is a nodal structure in the endogenous antinociceptive circuit and its projections to limbic-forebrain and medullary sites, and its activation of spinothalamic projections contributes to suppression of pain-induced vocalizations.^{8,10,14} Glutamatergic stimulation of CeA activates antinociceptive projection neurons in vIPAG through μ -opiate-mediated disinhibition.^{1,24,65} Increases in vocalization thresholds following NMDA injection into CeA are presumably mediated by activation of CeM projections to vIPAG that activate antinociceptive efferents. In the present study, we found an increase in c-Fos immunoreactivity in vIPAG following

NMDA administration into CeA, but no effect following AP5 administration. This increase in c-Fos immunoreactivity presumably reflects activation of enkephalinergic interneurons and antinociceptive projection neurons. That NMDA-induced increases in vocalization thresholds are mediated by μ -opiate disinhibition of vIPAG antinociceptive efferents is supported by the results in the present study that unilateral injection of CTAP into vIPAG blocked increases in vocalization thresholds that followed bilateral administration of NMDA into CeA. This suppression of NMDA-induced antinociception is consistent with the bilateral projection of CeA to vIPAG^{30,64}, and reports that antinociception elicited by bilateral administration of μ -opiates into the amygdala or medial thalamus is blocked by unilateral administration of μ -opiate or neurotensin antagonists into vIPAG⁷³, or inactivation of the vIPAG by unilateral administration of muscimol.⁵²

Glutamatergic projections that interact with NMDA receptors in CeM to produce antinociception may originate in the medial thalamus (nucleus parafascicularis, PF). We previously reported that increases in vocalization thresholds generated by injection of morphine into PF were blocked by pretreatment of AP5 into the rostral anterior cingulate cortex (rACC), and injection of NMDA into rACC elevated vocalization thresholds.³⁴

Given the limitations of our microinjection technique (see dye spread in Fig 1A) we assume that injection of AP5 or NMDA into CeA interacts with NMDA receptors in both CeLC and CeM. Therefore, administration of AP5 into CeA may inhibit activation of CeM projections that contribute to antinociception, but also blocks nociceptive processing by CeLC and therefore prevents activation of CeLC efferents that contribute to generation of affective responses to noxious stimulation. On the other hand, injection of NMDA into CeA is expected to activate CeLC neurons involved in nociceptive processing and its efferent projections that mediate affective responses to noxious stimulation. However, NMDA may also activate CeM projections to vIPAG that activate its antinociceptive efferents and thereby suppress affective responses generated via CeLC projections to the limbic forebrain. For example, serotonergic efferents from the vIPAG contribute to μ -opiate mediated antinociception elicited from vIPAG. We previously reported that increases in vocalization thresholds elicited by microinjection of morphine into vIPAG are blocked by pretreatment of CeA with the broad spectrum serotonin receptor antagonist methysergide into CeA.^{8,10} We propose that these serotonergic projections, engaged by NMDA receptor activation in CeM, target nociceptively responsive neurons in CeLC and thereby inhibit their processing of nociceptive inputs. Although no study has investigated the ability of serotonin agonists to suppress NMDA-evoked neural activity within CeA the microiontophoretic administration of serotonin onto neurons within the lateral amygdala decreased the number of action potentials elicited via microiontophoretic administration of glutamate.^{69,70}

CeLC projects to the dorsomedial subdivision of CeM that projects to the vIPAG²¹, and CeLC and CeM interact in generating autonomic stress responses.³⁹ We propose the CeLC and CeM may also interact during the course of nociceptive processing. Stress generates antinociception through μ -opiate mediated activation of vIPAG efferents and CeA contributes to this stress-induced analgesia.¹⁹ When nociceptive processing by CeLC reaches a level that elicits stress then CeLC projections to CeM may be engaged that in turn activate the vIPAG antinociceptive circuitry to limit further nociceptive processing by

CeLC. For example, application of a traumatic pain stimulus that elicited an intense emotional experience in humans evoked neural activity in the amygdala, hypothalamus and PAG.³⁸ The hypothalamus contributes to innate defensive responses⁶⁷ and to generation of pain-induced VADs.^{9,12} Processing of noxious input by CeLC that is sufficient to activate the hypothalamus may also engage the CeM projections to the vlPAG that moderate further nociceptive processing. A similar compensatory mechanism was proposed for the NMDA receptor activation in the rostral versus caudal anterior cingulate cortex that contributed to antinociception and nociception, respectively.^{23,34} As CeLC contributes to the processing of non-noxious stressful stimuli⁴⁴ that also suppresses responses to noxious stimulation¹⁹ the interaction between CeLC and CeM may provide a general mechanism underlying stress-induced analgesia.

Acknowledgments

We thank S. Kuk, T. Brandenstein, R.M. Dick, R. Timbuc, A. Amine, Y. Habib, and A. Nemeth for their excellent technical assistance.

This work was funded by National Institute of Neurological Disorders and Stroke grant (R01 NS-045720) and a faculty research development grant from Wayne State University. This research was conducted in partial fulfillment of the requirements for a doctorate of philosophy in Psychology from Wayne State University by C.A.S.

References

- Behbehani MM, Jiang M, Chandler SD. The effect of [Met]enkephalin on the periaqueductal gray neurons of the rat: an in vitro study. *Neuroscience*. 1990; 38:373–380. [PubMed: 2263320]
- Bernard JF, Besson JM. The spino(trigemino)pontoamygdaloid pathway: Electrophysiological evidence for an involvement in pain processes. *J Neurophysiol*. 1990; 63:473–490. [PubMed: 2329357]
- Bernard JF, Huang GF, Besson JM. Nucleus centralis of the amygdala and the globus pallidus ventralis: electrophysiological evidence for an involvement in pain processes. *J Neurophysiol*. 1992; 68:551–569. [PubMed: 1527575]
- Bird GC, Lash LL, Han JS, Zou X, Willis WD, Neugebauer V. Protein kinase A-dependent enhanced NMDA receptor function in pain-related synaptic plasticity in rat amygdala neurones. *J Physiol*. 2005; 564:907–921. [PubMed: 15760935]
- Bodnar RJ. Supraspinal circuitry mediating opioid antinociception: antagonist and synergy studies in multiple sites. *J Biomed Sci*. 2000; 7:181–194. [PubMed: 10810236]
- Borszcz GS. The capacity of motor reflex and vocalization thresholds to support avoidance conditioning in the rat. *Behav Neurosci*. 1993; 107:678–693. [PubMed: 8397872]
- Borszcz GS. Pavlovian conditional vocalizations of the rat: a model system for analyzing the fear of pain. *Behav Neurosci*. 1995; 109:648–662. [PubMed: 7576209]
- Borszcz GS. Differential contributions of medullary, thalamic, and amygdaloid serotonin to the antinociceptive action of morphine administered into the periaqueductal gray: A model of morphine analgesia. *Behav Neurosci*. 1999; 113:612–631. [PubMed: 10443787]
- Borszcz GS. Contribution of the ventromedial hypothalamus to generation of the affective dimension of pain. *Pain*. 2006; 123:155–168. [PubMed: 16564622]
- Borszcz GS, Streltsov NG. Amygdaloid-thalamic interactions mediate the antinociceptive action of morphine microinjected into the periaqueductal gray. *Behav Neurosci*. 2000; 114:574–584. [PubMed: 10883807]
- Borszcz GS, Leaton RN. The effect of amygdala lesions on conditional and unconditional vocalizations in rats. *Neurobiol Learn Mem*. 2003; 79:212–225. [PubMed: 12676520]

12. Borszcz, GS.; Spuz, CA. Hypothalamic control of pain vocalization and affective dimension of pain signaling. In: Brudzynski, SM., editor. *Handbook of Mammalian Vocalization*. Oxford: Academic Press; 2009. p. 281-292.
13. Borszcz GS, Johnson CP, Fahey KA. Comparison of motor reflex and vocalization thresholds following systemically administered morphine, fentanyl, and diazepam in the rat: assessment of sensory and performance variables. *Pharmacol Biochem Behav*. 1994; 49:827–834. [PubMed: 7886094]
14. Borszcz GS, Johnson CP, Thorp MV. The differential contribution of spinopetal projections to increases in vocalization and motor reflex thresholds generated by the microinjection of morphine into the periaqueductal gray. *Behav Neurosci*. 1996; 110:368–388. [PubMed: 8731064]
15. Borszcz GS, Johnson CP, Anderson ME, Young BJ. Characterization of tailshock elicited withdrawal reflexes in intact and spinal rats. *Physiol Behav*. 1992; 52:1055–1062. [PubMed: 1336601]
16. Bourbia N, Sagalajev B, Pertovaara A. Descending effect on spinal nociception by amygdaloid glutamate varies with the submodality of noxious test stimulation. *Neurosci Lett*. 2014; 570:26–31. [PubMed: 24747685]
17. Brown, MH. Limbic target surgery in the treatment of intractable pain with drug addiction. In: Sweet, WH.; Obrador, S.; Martin-Rodrequez, JG., editors. *Neurological treatment in psychiatry, pain and epilepsy*. Baltimore: University Park Press; 1977. p. 661-672.
18. Burstein R, Potrebic S. Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. *J Comp Neurol*. 1993; 335:469–485. [PubMed: 8227531]
19. Butler RK, Finn DP. Stress-induced analgesia. *Progress in Neurobiology*. 2009; 88:184–202. [PubMed: 19393288]
20. Carroll MN, Lim KS. Observations on the neuropharmacology of morphine and morphinelike analgesia. *Arch Int Pharmacodyn Ther*. 1960; 125:383–403. [PubMed: 13849328]
21. Cassell MD, Freedman LJ, Shi C. The intrinsic organization of the central extended amygdala. *Ann N Y Acad Sci*. 1999; 877:217–241. [PubMed: 10415652]
22. Chapman CR, Tuckett RP, Song CW. Pain and stress in a systems perspective: reciprocal neural, endocrine, and immune interactions. *J Pain*. 2008; 9:122–145. [PubMed: 18088561]
23. Cho ZH, Son YD, Kang CK, Han JY, Wong EK, Bai SJ. Pain dynamics observed by functional magnetic resonance imaging: differential regression analysis technique. *J Magn Reson Imaging*. 2003; 18:273–283. [PubMed: 12938121]
24. da Costa Gomez TM, Behbehani MM. An electrophysiological characterization of the projection from the central nucleus of the amygdala to the periaqueductal gray of the rat: the role of opioid receptors. *Brain Res*. 1995; 689:21–31. [PubMed: 8528703]
25. Fanselow MS. Odors released by stressed rats produce opioid analgesia in unstressed rats. *Behav Neurosci*. 1985; 99:589–592. [PubMed: 3843728]
26. Farb CR, Aoki C, Ledoux JE. Differential localization of NMDA and AMPA receptor subunits in the lateral and basal nuclei of the amygdala: a light and electron microscopic study. *J Comp Neurol*. 1995; 362:86–108. [PubMed: 8576430]
27. Frenk H, Yitzhaky J. Effects of amygdaloid kindling on the pain threshold of the rat. *Exp Neurol*. 1981; 71:487–496. [PubMed: 7461075]
28. Gracely RH, McGrath P, Dubner R. Validity and sensitivity of ratio scales of sensory and affective verbal pain descriptors: manipulation of affect by diazepam. *Pain*. 1978; 5:19–29. [PubMed: 673439]
29. Gracy KN, Pickel VM. Comparative ultrastructural localization of the NMDAR1 glutamate receptor in the rat basolateral amygdala and bed nucleus of the stria terminalis. *J Comp Neurol*. 1995; 362:71–85. [PubMed: 8576429]
30. Gray TS, Magnuson DJ. Peptide immunoreactive neurons in the amygdala and the bed nucleus of the stria terminalis project to the midbrain central gray in the rat. *Peptides*. 1992; 13:451–460. [PubMed: 1381826]
31. Hamlin AS, McNally GP, Osborne PB. Induction of c-Fos and zif268 in the nociceptive amygdala parallel abstinence hyperalgesia in rats briefly exposed to morphine. *Neuropharmacology*. 2007; 53:330–343. [PubMed: 17631915]

32. Harte SE, Lagman AL, Borszcz GS. Antinociceptive effects of morphine injected into the nucleus parafascicularis thalami of the rat. *Brain Res.* 2000; 874:78–86. [PubMed: 10936226]
33. Harte SE, Kender RG, Borszcz GS. Activation of 5-HT1A and 5-HT7 receptors in the parafascicular nucleus suppresses the affective reaction of rats to noxious stimulation. *Pain.* 2005; 113:405–415. [PubMed: 15661450]
34. Harte SE, Spuz CA, Borszcz GS. Functional interaction between medial thalamus and rostral anterior cingulate cortex in the suppression of pain affect. *Neuroscience.* 2011; 172:460–473. [PubMed: 21034797]
35. Hayashi T, Miyata M, Nagata T, Izawa Y, Kawakami Y. Intracerebroventricular fluvoxamine administration inhibited pain behavior but increased Fos expression in affective pain pathways. *Pharmacol Biochem Behav.* 2009; 91:441–46. [PubMed: 18817806]
36. Hebben N, Corkin S, Eichenbaum H, Shedlack K. Diminished ability to interpret and report internal states after bilateral medial temporal lobe resection. Case H.M. *Behav Neurosci.* 1985; 29:1031–1059. [PubMed: 3843537]
37. Hoffmeister, F. Effects of psychotropic drugs on pain. In: Soularic, A.; Cahn, J.; Charpentier, J., editors. *Pain.* New York: Academic Press; 1968. p. 309-319.
38. Hsieh JC, Stahle-Backdahl M, Hagermark O, Stone-Elander S, Rosenquist G, Ingvar M. Traumatic nociceptive pain activates the hypothalamus and the periaqueductal gray: a positron emission tomography study. *Pain.* 1996; 64:303–314. [PubMed: 8740608]
39. Huber D, Veinante P, Stoop R. Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. *Science.* 2005; 308:245–248. [PubMed: 15821089]
40. Inada K, Farrington JS, Moy SS, Koller BH, Duncan GE. Assessment of NMDA receptor activation in vivo by Fos induction after challenge with the direct NMDA agonist (tetrazol-5-yl)glycine: effects of clozapine and haloperidol. *J Neural Transm.* 2007; 114:899–908. [PubMed: 17318306]
41. Jasmin L, Burkey AR, Card JP, Basbaum AI. Transneuronal labeling of a nociceptive pathway, the spino-(trigemino-)parabrachio-amygdaloid, in the rat. *J Neurosci.* 1997; 17:3751–3765. [PubMed: 9133395]
42. Jelasic F. Relation of the lateral part of the amygdala to pain. *Confin Neurol.* 1966; 27:53–55. [PubMed: 5955974]
43. Kulkarni B, Bentley DE, Elliott R, Youell P, Watson A, Derbyshire SW, Frackowiak RS, Friston KJ, Jones AK. Attention to pain localization and unpleasantness discriminates the functions of the medial and lateral pain systems. *Eur J Neurosci.* 2005; 21:3133–3142. [PubMed: 15978022]
44. LeDoux JE. Emotion circuits in the brain. *Annu Rev Neurosci.* 2000; 23:155–184. [PubMed: 10845062]
45. Leite-Panissi CR, Coimbra NC, Menescal-de-Oliveira L. The cholinergic stimulation of the central amygdala modifying the tonic immobility response and antinociception in guinea pigs depends on the ventrolateral periaqueductal gray. *Brain Res Bull.* 2003; 60:167–178. [PubMed: 12725905]
46. Li W, Neugebauer V. Block of NMDA and non-NMDA receptor activation results in reduced background and evoked activity of central amygdala neurons in a model of arthritic pain. *Pain.* 2004; 110:112–122. [PubMed: 15275758]
47. Liu CC, Ohara S, Franaszczuk P, Zagzoog N, Gallagher M, Lenz FA. Painful stimuli evoke potentials recorded from the medial temporal lobe in humans. *Neuroscience.* 2010; 165:1402–1411. [PubMed: 19925853]
48. Loeser JD. Pain and suffering. *Clinical Journal of Pain.* 2000; 16:S2–S6. [PubMed: 10870733]
49. Maisonneuve SS, Kawasaki MC, Coimbra NC, Brandao ML. Effects of lesions of amygdaloid nuclei and substantia nigra on aversive responses induced by electrical stimulation of the inferior colliculus. *Brain Res Bull.* 1996; 40:93–98. [PubMed: 8724425]
50. Mark VH, Ervin FR, Yakovlev PI. Correlation of pain relief, sensory loss, and anatomical lesion sites in pain patients treated with stereotactic thalamotomy. *Trans Am Neurol Assoc.* 1961; 86:86–90. [PubMed: 14469949]
51. Mena NB, Mathur R, Nayar U. Amygdalar involvement in pain. *Indian J Physiol Pharmacol.* 1995; 39:339–346. [PubMed: 8582745]

52. Munn EM, Harte SE, Lagman A, Borszcz GS. Contribution of the periaqueductal gray to the suppression of pain affect produced by administration of morphine into the intralaminar thalamus of rat. *J Pain*. 2009; 10:426–435. [PubMed: 19231299]
53. Nakagawa T, Katsuya A, Tanimoto S, Yamamoto J, Yamauchi Y, Minami M, Satoh M. Differential patterns of c-fos mRNA expression in the amygdaloid nuclei induced by chemical somatic and visceral noxious stimuli in rats. *Neurosci Lett*. 2003; 344:197–200. [PubMed: 12812839]
54. Nandigama P, Borszcz GS. Affective analgesia following the administration of morphine into the amygdala of rats. *Brain Res*. 2003; 959:343–354. [PubMed: 12493624]
55. Neugebauer V, Li W, Bird GC, Han JS. The amygdala and persistent pain. *Neuroscientist*. 2004; 10:221–234. [PubMed: 15155061]
56. Newman HM, Stevens RT, Apkarian AV. Direct spinal projections to limbic and striatal areas: anterograde transport studies from the upper cervical spinal cord and the cervical enlargement in squirrel monkey and rat. *J Comp Neurol*. 1996; 365:640–658. [PubMed: 8742308]
57. Ogden KK, Traynelis SF. New advances in NMDA receptor pharmacology. *Trends Pharmacol Sci*. 2011; 32:726–733. [PubMed: 21996280]
58. Ottersen OP, Ben-Ari Y. Afferent connections to the amygdaloid complex of the rat and cat. *J Comp Neurol*. 1979; 187:401–424. [PubMed: 489786]
59. Pavlovic ZW, Cooper ML, Bodnar RJ. Opioid antagonists in the periaqueductal gray inhibit morphine and beta-endorphin analgesia elicited from the amygdala of rats. *Brain Res*. 1996; 741:13–26. [PubMed: 9001699]
60. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. 4 ed.. New York: Academic Press; 1998.
61. Price DD, Von der Gruen A, Miller J, Rafii A, Price C. A psychophysical analysis of morphine analgesia. *Pain*. 1985; 22:261–269. [PubMed: 2993984]
62. Radulovic J, Blank T, Nijholt I, Kammermeier J, Spiess J. In vivo NMDA/dopamine interaction resulting in Fos production in the limbic system and basal ganglia of the mouse brain. *Brain Res Mol Brain Res*. 2000; 75:271–280. [PubMed: 10686348]
63. Risgaard R, Hansen KB, Clausen RP. Partial agonists and subunit selectivity at NMDA receptors. *Chemistry*. 2010; 16:13910–13918. [PubMed: 20945316]
64. Rizvi TA, Ennis M, Behbehani MM, Shipley MT. Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: Topography and reciprocity. *J Comp Neurol*. 1991; 303:121–131. [PubMed: 1706363]
65. Sandkuhler J, Willmann E, Fu QG. Blockade of GABAA receptors in the midbrain periaqueductal gray abolishes nociceptive spinal dorsal horn neuronal activity. *Eur J Pharmacol*. 1989; 160:163–166. [PubMed: 2540988]
66. Schneider F, Habel U, Holthausen H, Kessler C, Posse S, Muller-Gartner HW, Arndt JO. Subjective ratings of pain correlate with subcortical-limbic blood flow: an fMRI study. *Neuropsychobiology*. 2001; 43:175–185. [PubMed: 11287797]
67. Siegel, A. *The Neurobiology of Aggression and Rage*. Boca Raton: CRC Press; 2005.
68. Spuz CA, Borszcz GS. NMDA or non-NMDA receptor antagonism within the amygdaloid central nucleus suppresses the affective dimension of pain in rats: evidence for hemispheric synergy. *J Pain*. 2012; 13:328–337. [PubMed: 22424916]
69. Stutzmann GE, LeDoux JE. GABAergic antagonists block the inhibitory effects of serotonin in the lateral amygdala: a mechanism for modulation of sensory inputs related to fear conditioning. *J Neurosci*. 1999; 19:RC8. [PubMed: 10341269]
70. Stutzmann GE, McEwen BS, LeDoux JE. Serotonin modulation of sensory inputs to the lateral amygdala: dependency on corticosterone. *J Neurosci*. 1998; 18:9529–9538. [PubMed: 9801389]
71. Sweet, WH. Bonica, JJ. *Pain*. New York: Raven Press; 1980. Central mechanisms of chronic pain (neuralgias and certain other neurogenic pain); p. 287–303.
72. Tanimoto S, Nakagawa T, Yamauchi Y, Minami M, Satoh M. Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. *Eur J Neurosci*. 2003; 18:2343–2350. [PubMed: 14622196]

73. Tershner SA, Helmstetter FJ. Antinociception produced by mu opioid receptor activation in the amygdala is partly dependent on activation of mu opioid and neurotensin receptors in the ventral periaqueductal gray. *Brain Res.* 2000; 865:17–26. [PubMed: 10814729]
74. Volz HP, Rehbein G, Triepel J, Knuepfer MM, Stumpf H, Stock G. Afferent connections of the nucleus centralis amygdalae A horseradish peroxidase study and literature survey. *Anat Embryol (Berl).* 1990; 181:177–194. [PubMed: 2327598]
75. Zubieta JK, Smith YR, Bueller JA, Xu Y, Kilbourn MR, Jewett DM, Meyer CR, Koeppe RA, Stohler CS. Regional mu opioid receptor regulation of sensory and affective dimensions of pain. *Science.* 2001; 293:311–315. [PubMed: 11452128]

Research Highlights

- NMDA and AP5 injected into the CeA suppressed pain-induced vocalizations in rats.
- NMDA, but not AP5, increases c-fos expression in the ventrolateral PAG (vlPAG).
- CTAP injected into vlPAG blocked the antinociception elicited by NMDA but not AP5.

Figure 1.

(A) Schematic and photomicrograph of a coronal section of the rat brain depicting a typical cannula tract and injection site with dye spread plume within the central nucleus of the amygdala (shaded in right side of schematic). Schematic identifies the three subdivisions of CeA: medial (CeM), lateral (CeL), and latero-capsular (CeLC). (B) Nissl stained coronal section of CeA following microinjection of 1µg NMDA. Schematic was modified from the rat brain atlas of Paxinos and Watson.⁶⁰

Figure 2.

The effects of bilateral administrations of (A) AP5 or (B) NMDA into the central nucleus of the amygdala on the mean ($\pm$ SEM) thresholds of spinal motor reflexes (SMRs), vocalizations during shock (VDSs), and vocalization afterdischarges (VAD). Asterisk (*) indicates thresholds significantly elevated above vehicle treatment ($\alpha = .05$). Data following AP5 injections were previously reported⁶⁸, and reproduced here for direct comparison with data following NMDA injections.

Figure 3.

(A) Histological reconstruction of sites that received injections of NMDA (1mg/side). The distribution of sites that received AP5 was nearly identical.⁶⁸ Coordinates are in millimeters posterior to bregma. Schematics were derived from the rat brain atlas of Paxinos and Watson.⁶⁰ (B) Comparison of the effects on response thresholds produced by NMDA (1mg/side) when administered into CeA (black circles in A) or sites surrounding CeA (gray circles in A). Data are plotted as the mean ($\pm$ SEM) thresholds of spinal motor reflexes (SMRs), vocalizations during shock (VDSs), and vocalization afterdischarges (VADs). Asterisk (*)

indicates thresholds significantly elevated above those observed following saline treatment. Pound sign (#) indicates thresholds significantly reduced compared to following NMDA treatment into CeA. For all comparisons, $\alpha = .05$.

Figure 4.

Representative photomicrographs of c-Fos-immunoreactive cells (dark dots) in the ventrolateral periaqueductal gray (as defined in the Paxinos and Watson⁶⁰ brain atlas – outlines in photographs on the left) from rats in the Home Cage group treated with bilateral microinjection of (A) saline, (B) 4µg AP5, or (C) 1µg NMDA into the central nucleus of the amygdala. Note the greater number of nuclei with dark stain in (C) when compared to (A) or (B). Sections on the left were photographed at 40x, and sections on the right were photographed at 200x.

Figure 5.

(A) Mean number ($\pm$ SEM) of c-Fos-immunoreactive (IR) cells in the ventrolateral periaqueductal gray (vlPAG) following bilateral microinjection of saline, 4μg AP5, or 1μg NMDA into the central nucleus of the amygdala. Groups were exposed to one of the following Contexts: Home cage, testing Chamber, or Pain Test. (B) Mean ($\pm$ SEM) thresholds of spinal motor reflexes (SMR), vocalizations during shock (VDS), and vocalization afterdischarges (VAD) in the groups in (A) that received pain testing (Pain

Test context). Asterisk (*) indicates cell counts or thresholds significantly elevated above those observed following saline treatment. For all comparisons, $\alpha = .05$.

Figure 6.

(A) Histological reconstruction of sites within the ventrolateral periaqueductal gray (vlPAG) that received unilateral injection of CTAP (0.25 μ g) prior to bilateral injection of NMDA (1 μ g, solid circles) or AP5 (4 μ g, gray circles) into the central nucleus of the amygdala (CeA). Coordinates are in millimeters posterior to bregma. Schematics were derived from the rat brain atlas of Paxinos and Watson.⁶⁰ (B) Photomicrograph of a coronal section of the rat brain depicting a typical cannula tract and injection site with dye spread plume within the vlPAG.

Figure 7.

Effects of unilateral pretreatment of CTAP (0.25 μ g) into the ventrolateral periaqueductal gray (vlPAG) on increases in response thresholds produced bilateral injection of (A) NMDA (1 μ g) or (B) AP5 (4 μ g) into the central nucleus of the amygdala (CeA). Data are plotted as the mean ($\pm$ SEM) threshold of spinal motor reflexes (SMRs), vocalizations during shock (VDSs), and vocalization afterdischarges (VADs). Asterisk (*) indicates thresholds significantly elevated above saline + saline treatments. Pound sign (#) indicates thresholds

significantly reduced compared to following NMDA + saline or AP5 + saline treatments.
For all comparisons, $\alpha = .05$.

Table 1

Mean($\pm$ SE) Performance at Threshold of Spinal Motor Reflex (SMR), Vocalization During Shock (VDS), and Vocalization Afterdischarge (VAD)

	Latency ¹ (ms)	SMR Amplitude (mm)	Magnitude (cm $\times$ ms)	Latency ¹ (ms)	VDS Amplitude (dB)	Duration (ms)	Latency ² (ms)	VAD Amplitude (dB)	Duration (ms)
<i>NMDA Dose Response Study</i>									
saline	309.20 $\pm$ 69.79	22.03 $\pm$ 2.38	115.02 $\pm$ 26.42	295.05 $\pm$ 20.58	90.06 $\pm$ 0.94	558.20 $\pm$ 22.89	211.50 $\pm$ 32.34	88.99 $\pm$ 0.79	657.20 $\pm$ 55.46
.1 μ g NMDA	312.86 $\pm$ 66.09	24.14 $\pm$ 4.59	116.40 $\pm$ 31.75	286.29 $\pm$ 84.89	87.26 $\pm$ 1.97	522.29 $\pm$ 41.53	214.86 $\pm$ 22.86	92.41 $\pm$ 2.24	677.71 $\pm$ 38.11
.25 μ g NMDA	286.57 $\pm$ 85.71	18.87 $\pm$ 5.98	107.07 $\pm$ 38.98	206.86 $\pm$ 53.39	87.70 $\pm$ 1.06	310.29 $\pm$ 44.09	223.60 $\pm$ 24.04	89.96 $\pm$ 2.74	641.20 $\pm$ 46.14
.5 μ g NMDA	300.80 $\pm$ 53.88	18.80 $\pm$ 8.32	111.54 $\pm$ 36.91	255.00 $\pm$ 62.21	86.53 $\pm$ 1.71	334.50 $\pm$ 57.42	212.00 $\pm$ 23.00	87.30 $\pm$ 3.20	612.00 $\pm$ 40.00
1 μ g NMDA	303.44 $\pm$ 50.95	19.90 $\pm$ 3.54	113.37 $\pm$ 29.06	223.18 $\pm$ 56.39	85.52 $\pm$ 1.22	408.45 $\pm$ 43.66	237.14 $\pm$ 29.99	88.42 $\pm$ 1.39	626.67 $\pm$ 44.88
<i>c-Fos Expression Study - Pain Group</i>									
saline	306.00 $\pm$ 69.41	25.25 $\pm$ 7.83	112.85 $\pm$ 29.59	247.00 $\pm$ 53.43	89.98 $\pm$ 0.54	516.50 $\pm$ 49.49	224.42 $\pm$ 22.06	89.23 $\pm$ 4.67	627.50 $\pm$ 43.00
4 μ g AP5	294.50 $\pm$ 48.13	25.68 $\pm$ 10.20	131.75 $\pm$ 30.61	268.50 $\pm$ 44.57	90.90 $\pm$ 3.70	515.00 $\pm$ 55.59	242.50 $\pm$ 21.24	88.43 $\pm$ 4.50	626.50 $\pm$ 31.76
1 μ g NMDA	292.00 $\pm$ 52.93	22.15 $\pm$ 8.06	117.35 $\pm$ 22.19	233.00 $\pm$ 43.00	88.18 $\pm$ 3.18	497.00 $\pm$ 57.00	224.31 $\pm$ 25.8	89.95 $\pm$ 3.89	635.51 $\pm$ 36.18
<i>CeA - vIPAG Interaction Study</i>									
saline CeA + saline vIPAG	299.21 $\pm$ 68.32	24.59 $\pm$ 5.06	111.46 $\pm$ 32.07	265.43 $\pm$ 46.19	89.81 $\pm$ 2.80	520.00 $\pm$ 49.80	222.00 $\pm$ 23.36	87.30 $\pm$ 2.59	641.43 $\pm$ 46.30
1 μ g NMDA CeA + saline vIPAG	289.75 $\pm$ 77.98	26.30 $\pm$ 4.61	123.73 $\pm$ 36.76	229.25 $\pm$ 52.38	90.53 $\pm$ 2.62	498.50 $\pm$ 52.63	261.75 $\pm$ 34.85	89.76 $\pm$ 1.69	685.00 $\pm$ 37.62
saline CeA + 0.25 μ g CTAP vIPAG	279.00 $\pm$ 52.75	20.36 $\pm$ 5.71	131.49 $\pm$ 43.22	256.75 $\pm$ 52.37	92.89 $\pm$ 2.02	553.25 $\pm$ 57.10	239.00 $\pm$ 32.45	87.45 $\pm$ 2.41	626.38 $\pm$ 69.70
1 μ g NMDA CeA + 0.25 μ g CTAP vIPAG	301.00 $\pm$ 67.85	19.01 $\pm$ 7.61	121.05 $\pm$ 43.42	266.75 $\pm$ 38.53	89.34 $\pm$ 2.51	532.00 $\pm$ 66.39	279.50 $\pm$ 48.50	87.20 $\pm$ 1.02	630.00 $\pm$ 51.99
saline CeA + saline vIPAG	309.71 $\pm$ 58.48	21.96 $\pm$ 4.89	121.17 $\pm$ 29.90	253.42 $\pm$ 63.91	91.16 $\pm$ 2.97	521.67 $\pm$ 49.07	212.00 $\pm$ 20.35	90.13 $\pm$ 2.88	660.21 $\pm$ 48.22
4 μ g AP5 CeA + saline vIPAG	302.11 $\pm$ 60.40	23.34 $\pm$ 6.26	124.32 $\pm$ 34.77	232.21 $\pm$ 55.95	92.21 $\pm$ 2.72	515.58 $\pm$ 49.75	218.00 $\pm$ 30.03	90.44 $\pm$ 2.59	656.60 $\pm$ 41.73
saline CeA + 0.25 μ g CTAP vIPAG	299.55 $\pm$ 50.19	19.02 $\pm$ 8.77	127.11 $\pm$ 38.53	259.79 $\pm$ 47.03	92.89 $\pm$ 2.02	523.10 $\pm$ 54.22	245.92 $\pm$ 27.20	88.23 $\pm$ 2.71	647.18 $\pm$ 50.25
4 μ g AP5 CeA + 0.25 μ g CTAP vIPAG	303.78 $\pm$ 61.51	21.04 $\pm$ 8.02	119.85 $\pm$ 44.26	238.36 $\pm$ 51.13	88.92 $\pm$ 2.74	518.67 $\pm$ 53.27	228.33 $\pm$ 26.41	91.38 $\pm$ 3.02	658.11 $\pm$ 54.84

¹ Latency from shock onset.

² Latency from shock offset.

Highlighted cells indicate performance significantly different from that observed following saline control treatments ($p < .05$).