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Increase in Conditioned Place Preference and Feeding After Mu-opioid Activation in the Bed Nucleus of the Stria Terminalis

by

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Abstract

The bed nucleus of the stria terminalis (BNST), part of the extended amygdala, has been shown to have an important role in anxiety, stress, and drug withdrawal. Recent research hints that the BNST also modulates appetitive motivation, including drug reinforcement and feeding. However, it remains unclear whether increased chow consumption following intra-BNST opioid microinjection is simply the result of stress-induced feeding. In the present study, rats were classically conditioned to associate intra-BNST μ-opioid microinjections with a distinct environmental context using a conditioned place preference paradigm. Results showed that rats preferred an environment paired with BNST opioid activation, suggesting that opioids in the BNST preferentially activate positive affective circuitry. This suggests that appetitive states, such as feeding, induced by intra-BNST opioids are not simply a response to increased stress, and hints at a more active role for the BNST in mediating the motivational effects of drugs and natural rewards.

Increase in Conditioned Place Preference and Feeding After Mu-opioid Activation in the Bed Nucleus of the Stria Terminalis

The term "extended amygdala" refers to a system of parallel pathways which form a caudo-rostral continuum from the central and medial nuclei of the amygdala into the bed nucleus of the stria terminalis (BNST) (de Olmos & Heimer, 1999). The extended amygdala forms a ring-like structure around the internal capsule (see Figure 1) and receives input from areas of the cortex involved in emotional processing (i.e. infralimbic cortex, etc.) as well as from the basolateral amygdaloid complex, while sending important projections to the hypothalamus, hippocampus, and brainstem (Heimer, 2003). Previous research has implicated the extended amygdala in such varying pathologies as mood disorders, schizophrenia, Alzheimer's disease, obsessive compulsive disorders, and anxiety and stress disorders (de Olmos & Heimer, 1999), and is viewed by some as forming a parallel corticostriatopallidal loop developed to coordinate autonomic and somatomotor responses (Dong & Swanson, 2006). Increased attention has been given to this macrosystem due to its role in drug addiction (specifically its role in withdrawal and relapse).

The BNST, in particular, has an emerging role as a key integrative structure involved in the brain mechanisms of stress, anxiety, and drug abuse. Phelix, Liposits, and Paull (1994) refer to the region as "the major telencephalic relay center for the modulatory control of the hypothalamic and brain stem centers responsible for the behavioral, endocrine, and physiological portions of the peripheral stress response"(p. 115). In addition to massive inputs from the medial and central amygdaloid nuclei the BNST receives afferents from such varied regions as the prefrontal cortex, hypothalamus, hippocampus, and ventral tegmental area (VTA) while sending strong to moderate efferents to VTA, nucleus accumbens, paraventricular nucleus of the

hypothalamus, lateral hypothalamus, and medial preoptic area in addition to other extended amygdala structures (Dong & Swanson, 2004, 2006; Phelix et al., 1994).

To fully understand the BNST's connection to stress and relapse, it is important to be familiar with the body's allostasis-maintaining hormonal system, the hypothalamic pituitary adrenal axis (HPA axis). The HPA axis is responsible for maintaining a stable level of circulating glucocorticoids during normal conditions (Whitnall, 1993). Physiological or psychological stressors result in a release of corticotrophin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (PVN), which triggers the secretion of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH in the blood reaches the adrenal gland, which stimulates the release of the stress hormones corticosterone and cortisol into the bloodstream. As they act to initiate the stress response throughout the body they also feed back into the brain to shut down further HPA axis activation, protecting the body from excessive cortisol exposure (Adam & Epel, 2007). Prominent projections to the parvocellular division of the PVN implicate the BNST as the key integrative relay structure for limbic-HPA axis interactions (Alheid, 2003), due to the fact that the structure receives projections from a large number of limbic-related areas, including the basolateral amygdala (BLA), medial amygdala, medial prefrontal cortex, and ventral subiculum (Herman, Ostrander, Mueller, & Figueiredo, 2005; Walker, Toufexis, & Davis, 2003).

As mentioned earlier, the CeA and BNST are very similar anatomically and neurochemically. The CeA is known to be highly involved in autonomic responses to conditioned fear (Kapp, Frysinger, Gallagher, & Haselton, 1979), which has caused researchers to try to differentiate the role of the BNST in fear responses from that of the CeA. Walker and Davis (2008) suggested that this difference lies in short-term versus long-term fear responses based on studies showing that glutamate receptor blockade in the BNST decreases late fear

responses, whereas in the CeA glutamate blockade had no effect (Davis, Schlesenger, & Sorenson, 1989). Waddell, Morris, and Bouton (2006) found that BNST lesions attenuated conditioned fear to long-duration stimuli but had no effect on responses to short-duration stimuli, while Resstel, Alves, Reis, Crestani, Correa, and Guimaraes (2008) showed that reversible lesions of the BNST decreased freezing behavior and suppression of punished responses in rats. This suggests that the role of the BNST in fear seems to be akin to anxiety, defined by Walker et al. (2003) as "slower-onset, longer-lasting responses that frequently accompany sustained threats, and that may persist even after threat termination", while the CeA seems to play more of a role in stimulus-specific fear (Davis & Shi, 1999).

Large efferents from the locus coeruleus and ventral noradrenergic bundle (VNAB; the collection of noradrenergic projections originating from the A1 and A2 cell groups in the brainstem) project to the BNST and give it the highest concentration of norepinephrine (NE) in the entire brain (Koob, 1999; Forray & Gysling, 2004). The abundant NE, especially in the ventral BNST, has been shown to have a key role mediating the aversive symptoms of withdrawal, as ß-NE antagonists in the BNST have been shown to greatly reduce opiate withdrawal symptoms (Aston-Jones, Delfs, Druhan, & Zhu, 1999; Egli, Kash, Choo, Savchenko, Matthews, Blakely, et al., 2005). BNST-projecting cells in the VNAB are activated during opiate withdrawal, and lesions of the VNAB but not the locus coeruleus drastically decrease opiate withdrawal-induced conditioned place aversion, suggesting that VNAB NE plays a critical role in mediating the aversive symptoms of withdrawal in the BNST (Delfs, Zhu, Druhan, & Aston-Jones, 2000). In addition, NE in the BNST likely has a role in stress-induced relapse to drug seeking, as NE antagonists block the reinstatement of drug seeking behaviors after footshock (Leri, Flores, Rodaros, & Stewart, 2002). Wang, Cen, and Lu (2001) showed that rats receiving

footshocks on a regular basis maintained a morphine-induced conditioned place preference while rats that received sham footshocks did not, and this effect was significantly attenuated by injection of a α_2-noradrenergic receptor antagonist (clonidine) into the BNST. Additionally, they showed a single footshock could reinstate a conditioned place preference that had been extinguished 21 days before, with clonidine also blocking this effect. This suggests that the BNST has a major role in stress-induced reinstatement of drug-seeking behavior.

The BNST receives CRH projections from the CeA and contains a high concentration of CRH receptors (Koob & Heinrichs, 1999). During ethanol withdrawal, extracellular CRH levels in the BNST are elevated, likely due to NE terminals synapsing onto CRH neurons in the BNST (Phelix et al., 1994), and are decreased by subsequent ethanol uptake (Olive, Koenig, Nannini, & Hodge, 2002). These heightened levels do not play a role in the aversive symptoms of the withdrawal syndrome (McNally & Akil, 2002), but instead intra-BNST CRH is more closely associated with the reinstatement of drug-seeking behavior. Direct injection of CRH into to the BNST induces reinstatement in absence of a stressor, and injection of the CRH antagonist D-Phe CRF_{12-41} into the BNST blocks footshock-induced reinstatement of cocaine seeking behavior (Erb & Stewart, 1999) and reinstatement of morphine conditioned place preference (Wang, Fang, Liu, & Lu, 2006).

The role of the BNST in stress responses coupled with projections from the hippocampus (via the ventral subiculum and CA1 regions) suggests a role for the BNST in learning during stressful contexts (McDonald, Shammah-Lagnado, Shi, & Davis, 1999). The hippocampus has the highest levels of glucocorticoid receptivity of any brain structure, which it uses to regulate glucocorticoid levels in the body by inhibiting HPA axis activity via inhibitory cholinergic projections to PVN-projecting structures, mainly the BNST (Herman & Cullinan, 1997; Zhu,

Umegaki, Suzuki, Miura, & Iguchi, 2001). These projections synapse in the BNST (Zhu et al., 2001), which can inhibit hippocampal HPA axis regulation when activated by the basolateral amygdala (Forray & Gysling, 2004). Shors and Mathew (1998) showed that the BLA is necessary for enhanced learning during exposure to a stressor, but this enhanced learning was prevented by excitotoxic BNST lesions. After being exposed to a tail shock stressor, temporary inactivation of the BNST during eye-blink conditioning prevented stress-enhanced learning whereas inactivation during the stressor itself did not (Bangasser, Santollo, & Shors, 2005), suggesting that the BNST maintains the stress response in anticipation of new learning situations by inhibiting hippocampal mediation of HPA axis activity (Shors, 2006).

The BNST has assumed an important role in drug addiction studies due to the moderate to high densities of mu-, delta-, and kappa opioid receptors found within it (Mansour, Fox, Akil, & Watson, 1995). Microiontophoretically-applied morphine showed a bidirectional effect on the firing rate of neurons in the BNST, though cells that increased their firing rate upon application of acetylcholine tended to also increase their firing rate upon application of morphine, and were disproportionately located in the ventral BNST (Casada & Dafny, 1993). The similarity of ventral BNST neuronal responses between morphine and acetylcholine suggests that opioids in the BNST may activate neuronal circuits that inhibit the hippocampal HPA axis regulation system, leading to an increased stress response. However, Choi, Furay, Evanson, Ostrander, Ulrich-Lai, and Herman (2007) found differential effects of BNST lesions on HPA axis activity, with posterior lesions inhibiting and anterior lesions exciting the stress response. This makes it difficult to predict with confidence what effect opioid activation in the ventral BNST will have on HPA axis activity, as HPA response may differ based on the focus and spread of the activation.

Previous experiments in our lab have shown that opioid microinjection into the BNST stimulates an increase in chow consumption (Jackson & Berridge, 2007), but the mechanism driving this increase is unknown. It is possible that opioids in the BNST trigger an activation of the HPA axis and therefore an increase in subjective feelings of psychological stress, meaning that the subsequent increase in food intake may be a method of coping. If increased chow consumption is instead due to stimulation of the brain's motivational pathways, it may be that intra-BNST opioids increase feeding via enhancements of hedonic pleasure, reward desire, or other measures.

The purpose of this experiment was to explore the effect of μ-opioid activation in the BNST on place conditioning in order to determine whether this activation was rewarding or stressful. The conditioned place preference apparatus has been shown to be a popular and consistent protocol to measure reward in laboratory animals (Bardo & Bevins, 2000; Tzschentke, 2007), and μ-opioids were chosen having already been shown to increase feeding upon application in the BNST (Jackson & Berridge, 2007). One group of rats was classically conditioned to associate μ-opioid-induced activation of the BNST with a distinct environmental context using a conditioned place preference paradigm, and then tested to determine whether this environmental association was appetitive or aversive. In addition, we also tested the same rats for the effect of μ-opioid microinjection on feeding. A second group of rats underwent Fos-like protein immunohistochemical testing to determine the spread and intensity of drug activation in neural tissue surrounding the microinjection target.

Method

Subjects

Eleven experimentally naive female Sprague Dawley rats obtained from in-house breeding colonies were housed in group cages in a climate-controlled colony room (~21ºC; 12 hr reverse light/dark cycle; lights on at 9:00 PM). Seven of the rats participated in behavioral testing (experiment 1), while the remaining four were used for Fos-like protein immunohistochemistry (experiment 2). Rats were given ad libitum access to food pellets (Labdiet® 5001 Rodent Diet) and water (tap water) when not in testing, and all animals weighed 230-340 g at the time of surgery. Testing was conducted during the dark phase of the light-dark cycle. All experimental procedures fell within guidelines approved by the University of Michigan's University Committee on the Use and Care of Animals.

Microinjection Cannula Surgery.

All animals were handled for a total of 15 minutes spread over two days before surgery. Rats were pretreated with 0.2 mL atropine sulfate (i.p) and anesthetized with a mixture of ketamine HCl (Ketaset®; 80 mg/kg, i.p.) and xylazine (AnaSed®; 5 mg/kg, i.p.) before being placed in a Stoelting Co. (Wood Dale, IL) stereotaxic apparatus with the incisor bar set 3.3 mm below interaural zero (flat skull). Rats were implanted with bilateral 14.0 mm stainless-steel guide cannulae (23 gauge) 2.0 mm above the intended target. Targets in the BNST differed only in their anteroposterior (AP) values (two rats were targeted at -.15 mm while the remaining five were targeted at -.45 mm posterior to bregma), while the mediolateral (ML) coordinates (±1.60 mm) and dorsoventral (DV) coordinates (-4.8 mm below skull surface) stayed constant for all animals. Microinjection guide cannulae were anchored to the skull with screws and acrylic cement (Bosworth Company, Skokie, IL). A stainless-steel stylet was placed into the cannulae to

prevent occlusion. After surgery each rat received injections of the analgesic Buprenex (0.3 mg/mL diluted with 9.0 mL sterile water; 0.25mL s.q. per rat) and antibiotic chloramphenicol sodium succinate (0.1 mL i.p. per rat). Five days were allowed for recovery before behavioral testing, with 15 minutes of handling spread over the final two days of recovery.

Drugs and intracerebral injections

To prepare the drug, DAMGO (Sigma Aldrich), a selective μ-opioid agonist, was dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus). aCSF alone was used for vehicle control microinjections. Microinjections were given with stainless-steel injector cannulae (28 gauge) extending 2.0 mm beyond the ventral tip of the guide cannulae, attached to a syringe pump via PE-20 surgical tubing. During microinjections, animals were gently hand-held and each side infused with a volume of 0.2 μL (0.1 mg DAMGO) at a rate of 0.2 μL/min for a total bilateral dose of 0.4 μL (0.2 mg DAMGO). Immediately after infusion the injectors remained in place for an additional 60 sec to allow the drug to diffuse away from the injector tips before the stylet was replaced and the animal placed in the appropriate testing apparatus. DAMGO and vehicle microinjections were spaced at least 24 hr apart, in a counterbalanced order across rats.

Experiment 1: Conditioned place preference and food intake testing

Place preference testing procedure

Apparatus description. Conditioned place preference (CPP) testing was performed using a three-compartment apparatus. Two large conditioning compartments (28 x 21 x 21 cm) were separated from each other by a smaller central "starting" compartment (12 x 21 x 21 cm). One of the large chambers had black walls with a wire grid floor and was brightly lit (intensity 1,000-1,300 lux) using a Fiber-Lite® MI-150 fiber optic surgical light (Dolan-Jenner Industries,

Boxborough, MA) on a medium setting. The other large chamber had white walls with a wire mesh floor and received lighting only from overhead room lights (intensity 550-650 lux). The center compartment had gray walls and a solid gray floor and was inaccessible to rats on training days. Each compartment of the apparatus had a clear Plexiglas lid, and compartments were separated from each other using removable dividers that were appropriately colored to match the chambers they divided. Prior to the experiment, the effectiveness of the place conditioning procedure was validated using a separate group of rats conditioned to have a place preference for a compartment paired with diazepam (1 mg/kg, i.p.) (Spyraki, Kazandjian, & Varonos, 1985).

Natural preference testing. Rats were habituated to the place preference apparatus for three days after the post-surgical handling session. During habituation sessions, the rats were placed in the central starting chamber and allowed to explore the apparatus in its entirety for 30 minutes under normal testing conditions, after which they were returned to their home cages. The final habituation day was videotaped in order to measure any existing natural preferences. This videotaping was performed using cameras mounted above the chambers and pointed downward to capture the important transition area of the apparatus (e.g. the central starting compartment and half of each conditioning compartment). Bilateral mock injections of 0.2 µL aCSF were given after the natural preference test was completed in order to accustom the animals to the microinjection procedure.

Place conditioning training procedure. After natural preference testing was completed, each group of rats was assigned in a counterbalanced manner to have one conditioning compartment paired with DAMGO microinjection and the other side paired with vehicle. Rats then progressed through four consecutive daily conditioning trials containing two DAMGO microinjections paired with their assigned conditioning compartment on days 2 and 4 and two

vehicle microinjections paired with the opposite conditioning compartment on days 1 and 3. Throughout the entire experiment chambers were thoroughly cleaned with a 70% ethanol solution after each session in order to remove visible excrement and smells from the wire floors, walls, and plexiglass lids of the chamber. On each conditioning day, rats received bilateral microinjections (0.2 µL per side) before being placed in the appropriate conditioning compartment, where they were confined for 30 minutes before being returned to their home cage. While the microinjections were being given the rat's stylet was cleaned using a sterile alcohol pad and replaced after the microinjection cannulae were removed.

Conditioned place preference test. On the day following the final place conditioning session, rats were not given microinjections. Instead their stylets were cleaned normally before they were placed into the central starting compartment and allowed to freely explore the entire apparatus for 30 minutes. During the conditioned place preference test, the rats were videotaped using the same method that was used during the natural preference test.

Place preference videotape analysis. Each rat's natural preference and conditioned place preference videotape was scored offline by an observer blind to the experimental conditions. A rat was considered to be in a particular compartment whenever its head and both forelimbs were inside the compartment and on the ground for more than two seconds. A rat that traveled directly from one conditioning compartment to the other and spent less than two seconds in the central starting compartment was considered to have gone directly from one conditioning compartment to the other, with no time scored for the central compartment. After exactly 30 minutes had passed the test was considered complete and the total time spent (seconds) in each compartment was tabulated.

Food intake testing procedure

Apparatus description. Food intake testing was conducted using transparent test chambers (23 x 20 x 45 cm) with corncob bedding spread evenly across the chamber floor. A pre-measured amount of food pellets (30 ± .5 g; Labdiet® 5001 Rodent Diet) was placed in the right front corner of the chamber floor, and tap water was available during each 60 minute test session. Each rat was assigned to its own test chamber for the entirety of the food intake stage of the experiment.

Food intake chamber habituation. Rats were habituated to their assigned habituation chamber for three consecutive days preceding food intake testing, starting the day after the conditioned place preference testing was complete. During habituation days, each rat had its stylet cleaned and replaced before being placed into their assigned test chamber for 60 minutes. Conditions for each habituation day were identical with test day conditions with the exception of microinjections.

Food intake testing. Prior to food intake testing, rats were assigned in a counterbalanced order to receive microinjections of DAMGO and vehicle (both 0.2 µL per side), with the two microinjections spaced 24 hours apart (identical to conditioned place preference/aversion testing microinjection procedure). During microinjections, stylets were cleaned and replaced after microinjection cannulae were removed. The rat was then placed in the food intake test chamber for 60 minutes. After the session, the rat was returned to its home cage and the remaining food in the chamber was measured. Each food intake test session was videotaped for detailed off-line analysis.

Food intake videotape analysis. The food intake test videotapes were analyzed for 1) total time spent eating (defined by the total amount of time the animal's mouth was either

nibbling/biting a food pellet or chewing), 2) total time spent drinking (amount of time that the animal's tongue was touching the water spout), 3) cage crosses (number of times the animal completely crossed the center of the cage; incompletely crossing the center before returning to the initial side of the cage was counted as a single cross rather than a cross over and a cross back), 4) paw treading (a natural defensive behavior defined as a rapidly repeated forward-and-backward movement of forepaws in order to spray bedding forward), 5) food sniffing (defined as orienting the nose in a pointed manner towards a food pellet while in close proximity to it), 6) grooming bouts (defined as systematic paw strokes over the face or licking of the body), 7) total time spent asleep (the animal was considered asleep if it remained immobile in a resting position for more than a minute; the sleeping bout was terminated by locomotion lasting more than 30 seconds or one minute if the locomotion happened as a result of the experimenter entering the room), 8) total number of rears (defined as the animal standing on its hind limbs with both forepaws approximately one inch off the ground; if the animal touched ground for less than a second between rears, then only one rear was counted), 9) total amount of time carrying food (defined time spent locomoting while being in the possession of a food pellet; food carry duration was equal to the total amount of time between when the animal gripped the food in its jaw and when either the food left its jaw or the it ceased locomoting). These criteria were adopted from Reynolds and Berridge (2001).

Histology

After completion of the final food intake test day, rats were deeply anesthetized with sodium pentobarbital (0.5 mL/rat, i.p.; Fatal-Plus®) and decapitated. Brains were extracted and immediately placed in a 10% paraformaldehyde solution for approximately four days until the brains sank, then transferred to a 30% sucrose/70% sodium phosphate buffer solution for

approximately four days. They were then sectioned (60 μm) using a sliding microtome, mounted on slides, and stained with cresyl violet. Cannula placements were mapped onto a stereotaxic atlas (Paxinos & Watson, 2007) and confirmed to be in the bed nucleus of the stria terminalis.

Statistical Analysis

Conditioned place preference. A rat was considered to have formed a conditioned place preference if the time it spent in the drug-paired compartment during the conditioned place preference test (CPP-Drug) was greater than the time it had spent in the same color compartment during the natural preference test (NP-Drug), whereas a conditioned place avoidance/aversion was considered to have been formed if NP-Drug was greater than CPP-Drug. Diagnostic histograms showed a non-normal distribution of values in both NP-Drug and CPP-Drug, so nonparametric testing using the Wilcoxon signed-rank test was performed. A measure of the observed effect size was calculated using Cohen's *d*. One rat from the experiment was excluded from the analysis because it displayed highly abnormal behavior after recovering from illness during its post-surgical recovery period. Analyses were completed using SSPS 16.0 and Cohen's *d* was calculated using R 2.7.2.

Food intake. Diagnostic histograms showed non-normal distributions of values for both food intake and behavioral measures in both DAMGO and vehicle conditions, so nonparametric testing using the Wilcoxon signed-rank test was performed for the food intake total between conditions as well as for each behavioral measure between conditions. For measures that were significant ($p < .05$) or marginally significant ($.05 < p < .10$), Cohen's *d* was calculated to measure the observed effect size. All rats that were included in the conditioned place preference statistical analysis were also included in the food intake analysis. Analyses were completed using SSPS 16.0 and Cohen's *d* was calculated using R 2.7.2.

Experiment 2: Fos plume analysis of local impact

Fos-like protein immunohistochemistry. Rats assigned to the Fos plume analysis group underwent identical microinjection guide cannula surgery procedures to those that participated in behavioral testing. A "sham" surgery was performed on one of the rats, with no microinjection cannulae implanted. Seven days after surgery animals were perfused transcardially 90 minutes after bilateral microinjection of either DAMGO or vehicle. After perfusion, brains were removed, placed in 4% paraformaldehyde overnight, and then transferred to 30% sucrose. Local Fos activation was performed using immunohistochemistry and immunofluorescence techniques described in Faure, Reynolds, Richard, and Berridge (2008). Briefly, the brain was sliced into two adjacent series of 40 µm coronal sections using a sliding microtome and stored in 0.1 M sodium phosphate buffer (pH 7.4). Alternate series of sections were processed for immunofluorescence by successive immersion (with gentle agitation and intervening rinses in 0.1 M sodium phosphate buffer) in solutions containing 0.1 M sodium phosphate buffer plus 0.2% Triton and (1) 5% normal donkey serum (NDS) for 30 minutes, (2) 5% NDS and goat anti-c-Fos (1:10) overnight at 4ºC, (3) 5% NDS plus 2 drops signal enhancer for 30 minutes, and (4) 5% NDS, donkey anti-goat Alexa Fluor 488 (excitation, 488 nm; emission, 519 nm; Invitrogen), and 2 drops signal enhancer for 2 h. After incubation of the final immersion sequence, the tissue was rinsed twice for 10 minutes in 0.1 M sodium phosphate buffer plus 0.2% Triton and once more for ten minutes in 0.1 M sodium phosphate buffer. Sections were then mounted, air dried, and coversliped with ProLong Gold antifade reagent (Invitrogen).

Fos plume identification. Imaging of the processed sections was completed using a Leica microscope equipped for both brightfield and fluorescence microscopy. Visualization of fluorescence utilized a filter with an excitation band at 480~505 and an emission band at 505-

545. Immunofluorescence-processed sections were used to identify Fos plumes normally located at the ventral limit of microinjector tip damage. Using Adobe Illustrator CS3, a grid of 68 x 68 µm sample squares was placed on top of the image, with the central square of the grid lined up with the center of the microinjection damage (10x magnification). Eight radial arms emanated from the center of the grid (0º, 45º, 90º, 135º, 180º, 225º, 270º, 315º), and a count of Fos positive cells was produced for each grid box in each radial arm.

Fos plume assessment and mapping procedure. We used previously described methods to measure drug-induced Fos plumes surrounding a microinjection site (Mahler, Smith, & Berridge, 2007; Faure et al. 2008; Reynolds & Berridge, 2008). DAMGO-induced Fos plumes were assessed against baseline sham and vehicle Fos expression densities (see Figure 2). Areas of 2x and 3x Fos expression over sham and vehicle controls were mapped and the spread of each level of expression was determined for each radial arm; spread was defined as the distance from the injection site to the last box in each radial arm that expressed Fos greater than or equal to each designated level of expression (2x or 3x). Maps were created showing the 2x and 3x Fos elevation zones (plumes) for each site, and the radii of these plumes were averaged for each of the expression levels. Circular symbols with a size based on the average radius of each Fos plume were nested to create a representation of the intensity and spread of activation around the microinjection site. Behavioral effects for each microinjection site were represented by colors of these symbols, and their placement on anatomical maps displays both the location of microinjection placement for each rat and the size of expected neuronal activation around each microinjection site. Symbols used in the mapping of behavioral effects are based on DAMGO-induced activation over baseline vehicle activation. Both cannula microinjection sites for each rat were plotted on coronal slices, and these bilateral placement sites were collapsed onto unilateral

planes for placement on sagittal, and horizontal slices (two sites for each rat) in order to create a three-dimensional depiction of the position of Fos plumes in the brain and the location of behavioral effects (Figures 4 and 7).

Results

Statistical analyses were run using data collected from six animals that successfully made it through the entire testing regimen. One of the original seven rats was removed due to illness. Marginally significant data ($0.05 < p < 0.10$) and notable trends ($0.10 < p < 0.20$) are listed along with significant results because the final sample size was very small ($n = 6$). Based on the large effect sizes calculated using Cohen's *d*, it is expected that most if not all of these data points would become significant if the experiment were to be replicated with a sufficiently large number of participants.

Conditioned Place Preference

A Wilcoxon signed-rank test showed that rats spent more time (seconds) in the conditioning compartment paired with drug microinjections ($M = 1026.83$, $SD = 556.16$) during the CPP test than they did during the natural preferences test ($M = 460.00$, $SD = 285.15$), $Z = 1.99$, $d = 1.28$, $p = 0.05$ (see Figures 3 and 4). A second Wilcoxon signed-rank test was run to test whether the time spent in the middle compartment differed between the natural preferences and CPP test. This test showed a decreasing trend in the total amount of time spent (seconds) in the middle compartment between the natural preferences test ($M = 226.14$, $SD = 206.51$) and the CPP test ($M = 109.14$, $SD = 46.86$), $Z = -1.57$, $d = 0.80$, $p > 0.1$. For a visual depiction of the amount of time spent in each chamber for each rat between the natural preferences test and conditioned place preference test, see Figure 5.

Food Intake

A Wilcoxon signed-rank test showed a marginally significant increase in total chow consumption (g) after DAMGO microinjection (M_{DAM} = 1.59, SD_{DAM} = 1.64) as compared to vehicle microinjection (M_{VEH} = 0.23, SD_{VEH} = 0.32). Tests for behavioral measures during food intake testing showed that rats spent significantly more time eating (seconds) after DAMGO microinjection (M_{DAM} = 274.67, SD_{DAM} = 297.64) than after vehicle microinjection (M = 36.50, SD = 68.63), Z = 2.02, d = 1.10, p = 0.04 (see Figures 6 and 7). Results also showed a marginally significant increase in the number of food sniffs for DAMGO-injected rats than vehicle-injected rats (M_{DAM} = 14.50, SD_{DAM} = 14.43; M_{VEH} = 6.00, SD_{VEH} = 2.76), Z = -1.75, d = 0.82, p = 0.08. Notable trends in the data included increases for DAMGO-injected animals in time carrying food (seconds) (M_{DAM} = 4.33, SD_{DAM} = 6.98; M_{VEH} = 0.33, SD_{VEH} = 0.82), Z = -1.34, d = 0.81, p = 0.18, and drinking (M_{DAM} = 27.67, SD_{DAM} = 38.32; M_{VEH} = 4.50, SD_{VEH} = 8.22), Z = -1.46, d = 0.84, p = 0.14, relative to vehicle-injected animals.

Fos plume mapping

Fos plume mapping helps determine the extent and intensity of microinjection activation on surrounding neural tissue. Our plumes showed characteristics that were consistent with plumes measured in previous studies (Mahler et al., 2007; Faure et al., 2008; Reynolds & Berridge, 2008). We assumed roughly equal diffusion of drug outward from the microinjection site in each direction, so each plume represents a spherical area of neural activation and indicates the functional zone likely to be responsible for the behavioral effects of DAMGO microinjection. It is likely however that functionally inert levels of the drug drifted outside of the defined levels of the plume.

Fos plume analysis normally employs group sizes of 3-5 drug-injected animals, 4-6 vehicle-injected animals, and 3-5 normal (sham) control animals (Mahler et al., 2007; Reynolds

& Berridge, 2008). Due to time constraints, both of the normal and vehicle groups for this study contained only one animal each, whereas the DAMGO microinjection group contained only two animals, one of which was found to have a unilateral brain infection that had heavily damaged the microinjection area.

Binding of antibodies to non-Fos entities (non-specific binding) is a normal occurrence during Fos immunohistochemical procedures, usually in small, negligible amounts. However, in some cases non-specific binding can become elevated, increasing the difficulty in differentiating between Fos positive cells and other fluorescent binding. Unfortunately for our study, non-specific binding occurred at a higher level than usual, and as a result our Fos counts may not be as accurate as normal. Still, because elevated zones of activation did occur, we are confident that our Fos counting technique captured the major trends in the data.

Discussion

Our results indicate that opioid activation of the BNST produces a conditioned place preference, which is consistent with our hypothesis that increased food intake after intra-BNST opioid activation is the result of rewarding processes rather than stress-inducing processes. BNST opioids also increased chow consumption and related behaviors (e.g. food sniff and total time spent eating), which is consistent with earlier lab findings. Our findings suggest a larger role for the BNST in appetitive aspects of reward rather than coping mechanisms of stress.

BNST opioid-induced place preference may be due to modulation of ventral tegmental area (VTA) mesolimbic dopamine circuitry that has been implicated in reward and positive motivation. The VTA provides dopamine (DA) to structures in the ventral striatum and prefrontal cortex, areas known to be involved in evaluating the positively-valenced effects of drugs and other rewards (White, 1996; Berridge, 2007). The ventral BNST sends a strong

projection to the VTA which upon glutamatergic stimulation increases VTA DA discharge (at low doses) nearly 125% (Georges & Aston-Jones, 2001) and upon electrical stimulation activates nearly 85% of VTA cells (Georges & Aston-Jones, 2002). This strongly implicates the ventral BNST in potently modulating the excitability of VTA DA cells and by extension mesolimbic mechanisms of reward and motivation.

Functionally, this BNST-VTA connection may be part of a larger pathway for cortical modification of reward or motivational processing. Massi, Elezgarai, Puente, Reguero, Grandes, Manzoni, et al. (2008) showed that excitatory inputs exclusively from the infralimbic cortex converge on the ventral BNST, which relays cortical input to the VTA using the previously described connection, and that application of a cannabinoid agonist within the ventral BNST inhibited this excitatory drive and its associated increase in VTA DA discharge. Dumont, Mark, Mader, and Williams (2005) showed that self-administered cocaine and palatable food enhanced excitatory glutamatergic transmission in the ventrolateral BNST whereas passively administered cocaine or food had no effect. It is possible that opioids in the BNST could interact with the connection between the VTA and BNST and cause a substantial increase of dopamine discharge and related reward-seeking behavior.

We originally considered stress-induced eating to be a possible explanation of the food intake increase seen upon opioid application in the BNST. The effect of stress on eating differs based on the palatability of the offered food. Stress-induced eating is greatest when the available foods are highly palatable “comfort foods” (Dallman, Pecoraro, & la Fleur, 2005), leading Adam and Epel (2007) to propose a “Reward Based Stress Eating” model with cortisol and reward circuitry mediating the effect. Using palatable foods, Boggiano, Chandler, Viana, Oswald, Maldonado, & Wauford (2005) were able to double food intake by subjecting rats to both caloric

restriction and footshock stress. The effect of stress on intake of less palatable foods shows the opposite trend.

Our findings indicate that a stress-induced eating explanation for the observed μ-opioid increase in food intake is unlikely. Intracerebroventricular CRH as well as BNST- and PVN-localized CRH decrease food intake (Krahn, Gosnell, Grace, & Levine 1986; Krahn, Gosnell, Levine, & Morley, 1988; Ciccocioppo, Fedeli, Economidou, Policani, Weiss, & Massi, 2003). Interestingly, nociceptin/orphanin-opioid receptor agonist microinjection into the BNST blocks the anorectic effect of later CRH BNST microinjection (Ciccocioppo et al., 2003). The BNST has elevated levels of CRH expression during stress (Kim, Park, Choi, Moon, Lee, Kang, et al., 2006), and BNST CRH has been shown to form a conditioned place aversion in rats which can be prevented with the application of CRH antagonists (Sahuque, Kullberg, Mcgeehan, Kinder, Hicks, Blanton, et al., 2006). The fact that BNST CRH causes both an anorectic effect and a conditioned place aversion suggests that opioids are initiating their effects through a mechanism other than stress.

Our results implicate intra-BNST opioids in aspects of reward, but we do not know exactly which specific aspect of reward was most effectively manipulated. Berridge and Robinson (2003) parsed the concept of "reward" into three separate components based on neurobiological manipulations: 'Liking' or the hedonic impact of rewards, 'wanting' or incentive motivation to pursue rewards, and learning of the predictive-value of reward-paired cues (for a brief explanation of these components along with recent findings, see Berridge, Robinson, & Aldridge (2009)). The conditioned place preference paradigm as a classical or Pavlovian conditioning tool measures aspects of reward learning – pairing the appetitive aspects of the drug (unconditioned stimulus) with a particular context (conditioned stimulus) (Bardo & Bevins,

2000) – while the food intake test measures changes in food consumption that could be a result of manipulating either the hedonic feelings felt upon food ingestion ('liking') or the motivation to increase the total amount eaten ('wanting') (Berridge & Robinson, 2003).

Positive explanations for increased chow consumption after intra-BNST opioid microinjections could be the result of a change in either the perceived hedonic aspects of the food or the overall motivation to consume the food. These predictions are based on the effects of opioids on food intake when injected into the CeA and PVN, structures with very strong connections to the BNST. Stanley et al. (1989) found that injection of opioids into the CeA elicited a very strong increase in feeding, and Giraudo, Grace, Welch, Billington, & Levine (1993) noted that the opioid antagonist naloxone's ability to decrease food intake depended on the palatability of the food offered, suggesting that opioid activation of the CeA increases food consumption by increasing the perceptual affective or "emotional" qualities of the food ingested (Glass, Billington, & Levine, 1999). In contrast, intra-PVN opioid activation also increases food intake (Gosnell, Morley, & Levine, 1985; Stanley, Lanthier, & Leibowitz, 1989), but this is independent of the palatability of the food offered (Koch, Glass, Cooper, & Bodnar, 1995). Therefore the PVN is implicated in controlling the total energy consumption independent of the palatability of food ingested. Our findings suggest that increases in chow consumption after intra-BNST opioid microinjection were the result alterations to brain mechanisms of reward, primarily due to the fact that intra-BNST opioids formed a conditioned place preference, but as stated earlier, we are not sure which aspect of reward was altered.

The primary limitation in the current study is the small number of rats that completed the testing regimen, though the effect we displayed was very robust for the conditioned place preference test. Figure 5 shows how great of an impact opioid activation in the BNST had on

forming a conditioned place preference for many of the rats in the study. For some rats, the difference in time spent in the drug-paired side between the natural preferences test and the conditioned place preference test exceeded ten minutes (600 sec) and two of the tested rats actually flipped their preference, which is indicative of how salient the alterations in behavior were after opioid manipulation. For food intake testing, the time spent eating (in seconds) was significantly different between the groups even though the actual amount consumed was not, though a larger amount of rats would have likely led to a significant result.

It is possible that our strong effect was influenced by the short time between microinjections for each animal. Normally, microinjections are spaced 48 hr apart (Reynolds & Berridge, 2001, 2002, 2008) to allow drug to diffuse completely from brain tissue, but due to time constraints the current study necessitated decreasing this to 24 hr. It is assumed that most of the drug is cleared from targeted synapses in this time; however, as discussed earlier the BNST is highly involved in both withdrawal and anxiety, so the magnitude of our effect could potentially be a result of a conditioned preference for the drug-paired side combined with a conditioned aversion for the vehicle paired side, caused by an activation of either withdrawal- or anxiety-driven responses. However, it is unclear whether the very small doses of active drug given are sufficient to elicit a drug dependence syndrome in the animal, which is necessary for heightened levels of NE and CRH associated with withdrawal and fear/anxiety.

As discussed earlier, much of the existing research concerning the BNST focuses on the structure's involvement in negative situations (stress, anxiety, withdrawal, relapse, etc.), but the current experiment suggests that the BNST may be more of a bi-valent structure than previously thought. Further exploration of extended amygdala circuitry should be conducted to discover the exact mechanism(s) of action through which opioids in the BNST exhibit their behavioral

effects. Future experiments should also explore the BNST's connection to the components of reward by using procedures that can more thoroughly measure the structure's contributions to each component, such as taste reactivity, autoshaping, and Pavlovian-to-instrumental transfer (PIT).

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Figure Captions

Figure 1. Illustration showing the various components of the extended amygdala and major surrounding structures. Figure taken from Koob, 2003.

Figure 2. Summary of the Fos scoring procedure. The top row contains images taken at 10x magnification (white lines have been added to show the approximate boundaries of the bed nucleus of the stria terminalis; ac, anterior comissure; BNST, bed nucleus of the stria terminalis; ic, internal capsule, LV, lateral ventricle). On each image, a grid was placed with the center grid box located over the estimated center of microinjection (marked with a 'C'; second row). The number of Fos-activated cells in each box was counted for each image, and average counts from DAMGO-injected animals were compared to both normal and vehicle controls. Areas of 2x and 3x activation relative to both normal and vehicle controls were noted, and plumes were drawn for each image (row 3).

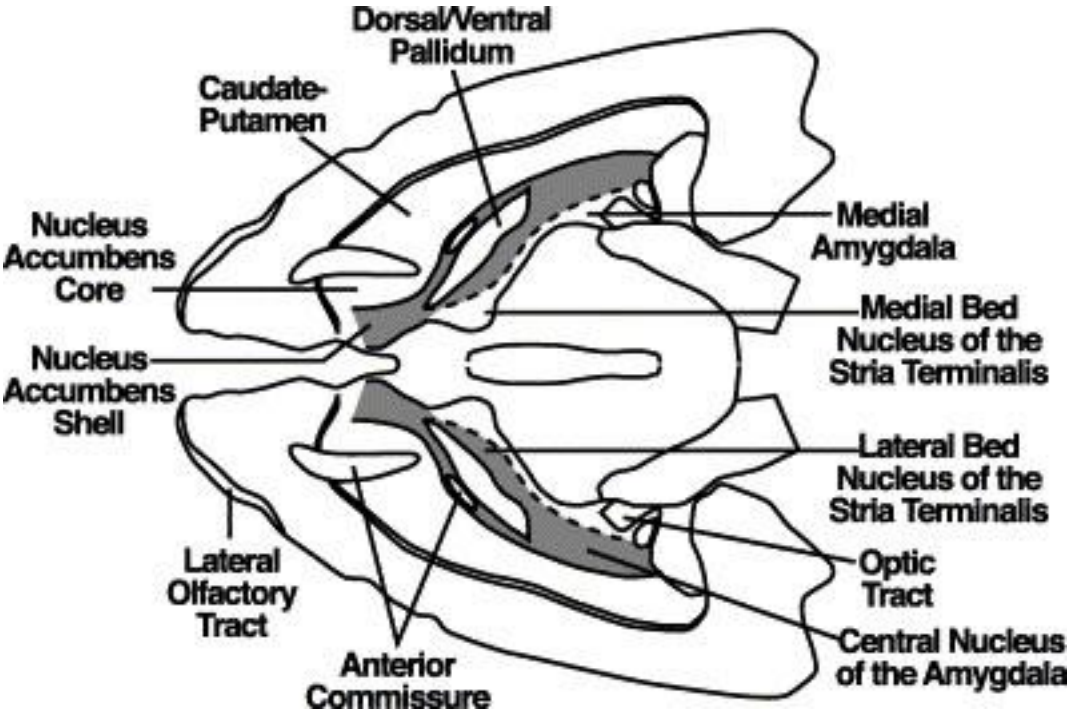
Figure 3. Anatomical map showing the difference in time spent in the drug-paired side between the natural preference test and conditioned place preference test. Symbol placements are based on based on position of microinjection cannula in the BNST, shown in horizontal and sagittal views. Rats formed a conditioned place preference for a chamber paired with DAMGO. Values shown are the time spent in the drug-paired side during the conditioned place preference test minus the natural preferences test, with higher numerical values indicating stronger place preferences. The inner circle represents the area around the microinjection site with 3x activation and the semi-transparent halo surrounding it represents the area around the microinjection site with 2x activation relative to vehicle controls. The thick black line shows the maximal extent of the BNST in each view.

Figure 4. Bar graph showing the average time spent in the drug-paired side during the natural preferences test compared to the conditioned place preferences test. Rats spent more time in the drug-paired side during the conditioned place preference test than during the natural preferences test.

Figure 5. Stacked bar graph showing the distribution of time spent in each chamber for each rat during both the conditioned place preference and natural preference test. The average for both natural preference and conditioned place preference tests is displayed as the final pair of bars. Five of the six rats tested displayed a conditioned place preference, and for some the difference in time from natural preference to conditioned place preference tests was more than ten minutes.

Figure 6. Anatomical map showing the difference in total time spent eating (seconds) between testing after DAMGO microinjection and vehicle microinjection. Symbol placements are based on based on position of microinjection cannula in the BNST, shown in horizontal and sagittal views. Rats microinjected with DAMGO spent more time eating (seconds) than they did on days that they were injected with vehicle. Values shown are the total time spent eating after DAMGO microinjection test minus the total time spent eating after vehicle microinjection. The inner circle represents the area around the microinjection site with 3x activation and the semi-transparent halo surrounding it represents the area around the microinjection site with 2x activation relative to vehicle controls. The thick black line shows the maximal extent of the BNST in each view.

Figure 7. Bar graph showing the difference in total time spent eating (seconds) on DAMGO-injected days versus vehicle-injected days. Rats spent more total time eating after microinjections of DAMGO than they did after microinjections of vehicle.


Dorsal/Ventral
Pallidum
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Nucleus
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Medial
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Medial Bed
Nucleus of the
Stria Terminalis
Lateral Bed
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Stria Terminalis
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Central Nucleus
of the Amygdala
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Tract
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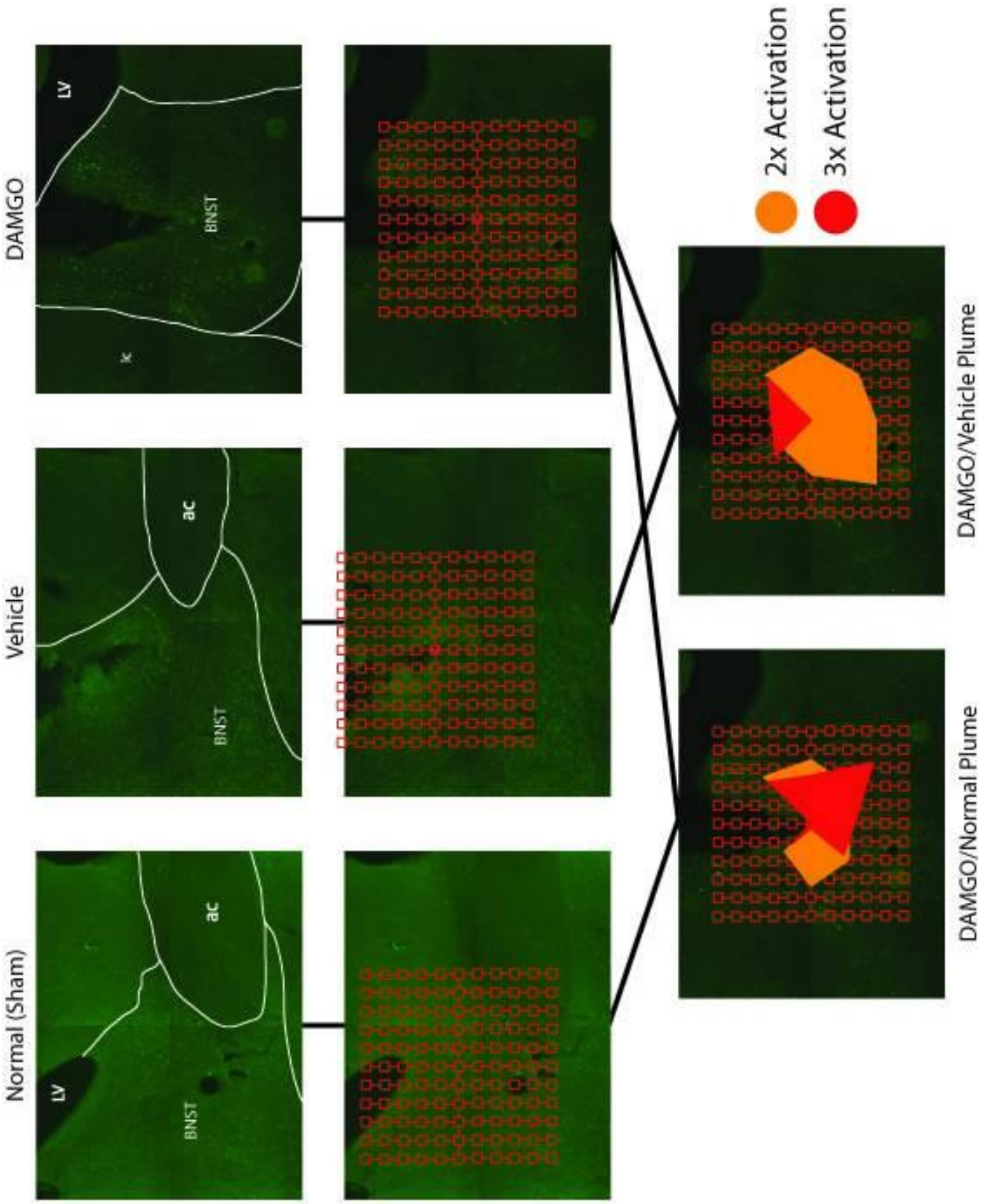
DAMGO
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3x Activation
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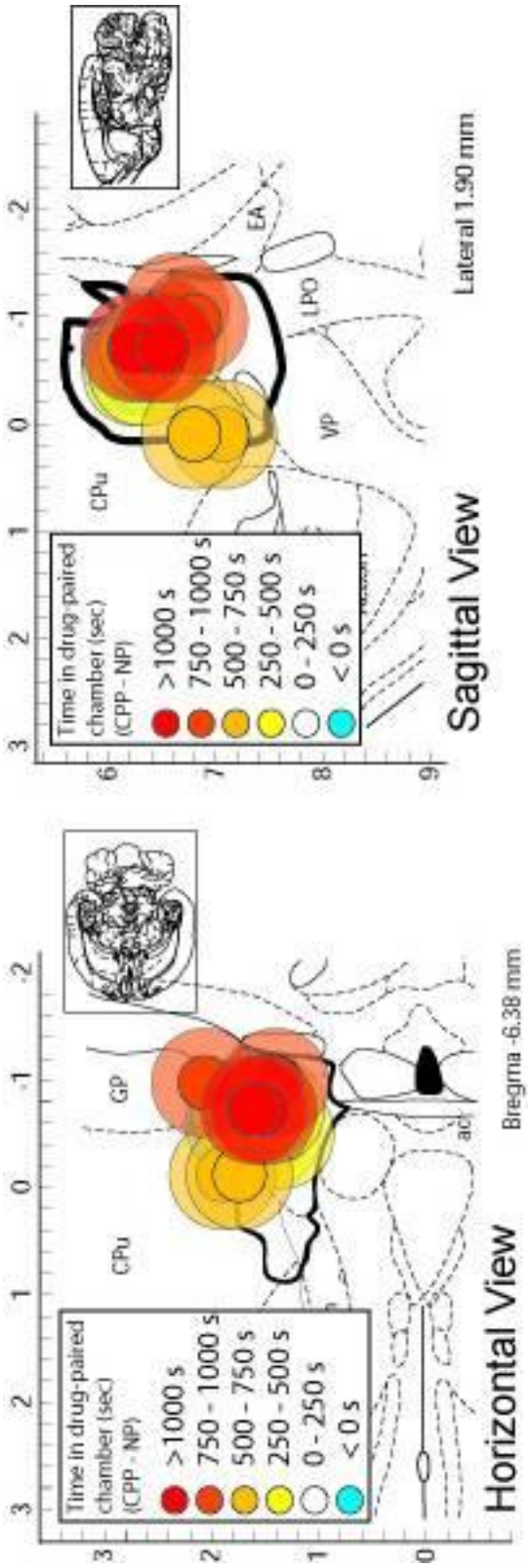
DAMGO/Normal Plume


Time in drug-paired chamber (sec) (CPP - NP)
>1000 s
750 - 1000 s
500 - 750 s
250 - 500 s
0 - 250 s
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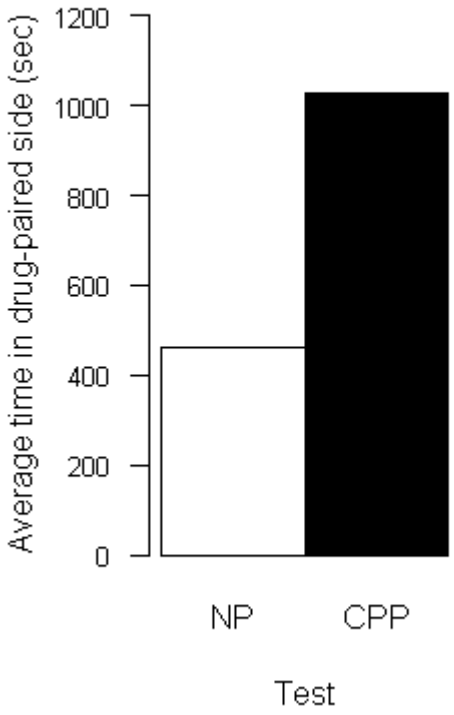
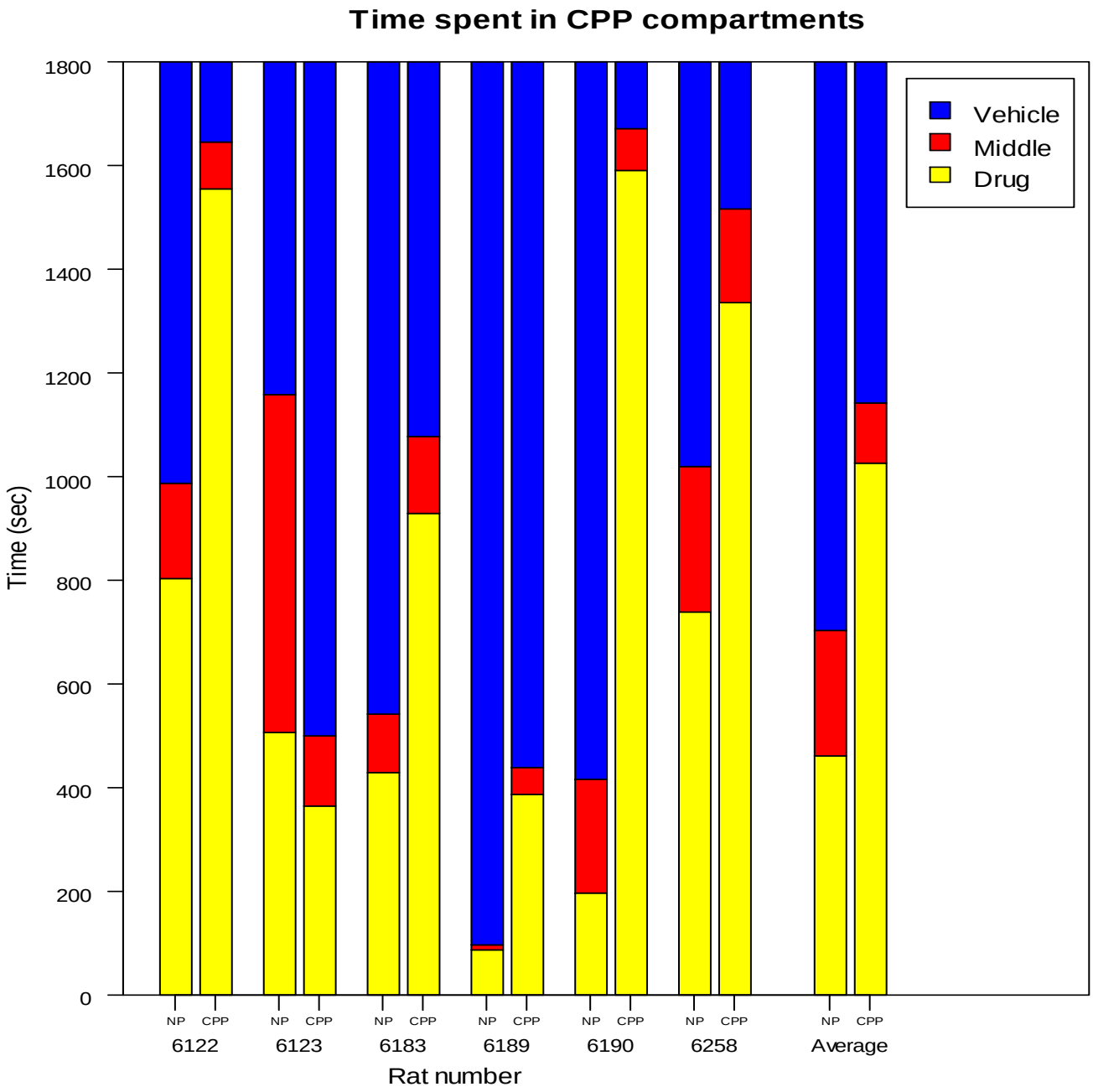
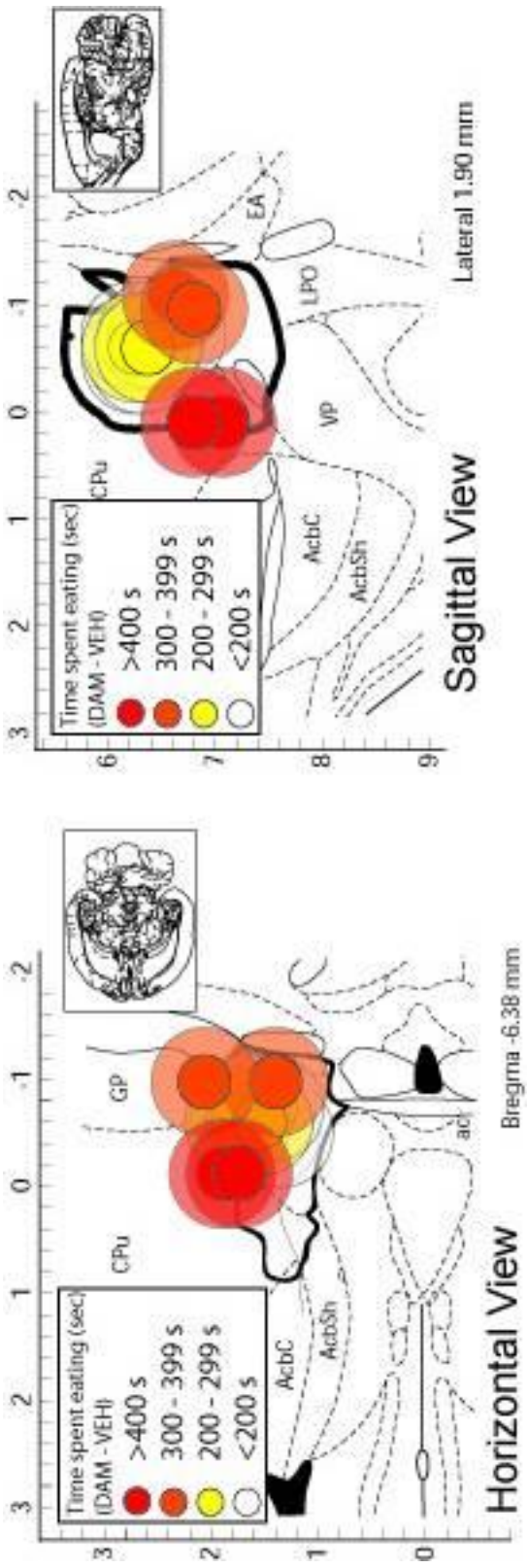
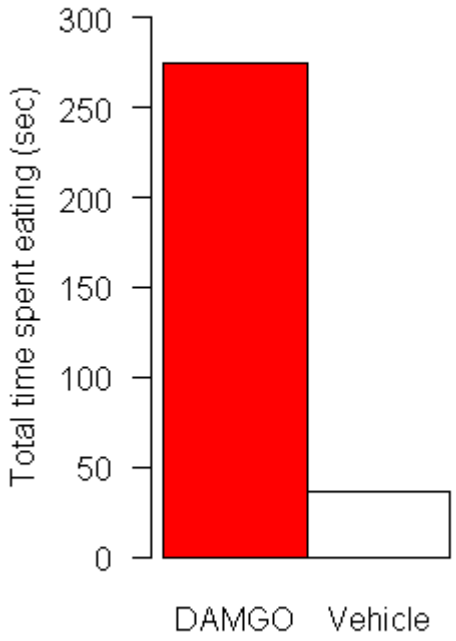
CPu
GP
ac
Horizontal View
Bregma -6.38 mm
Time in drug paired chamber (sec) (CPP - NP)
>1000 s
750 - 1000 s
500 - 750 s
250 - 500 s
0 - 250 s
< 0 s
CPu
EA
LPO
VP
Sagittal View
Lateral 1.90 mm


Average time in drug-paired side (sec)
1200
1000
800
600
400
200
0
NP
CPP
Test


Time spent in CPP compartments
Vehicle
Middle
Drug
Time (sec)
1800
1600
1400
1200
1000
800
600
400
200
0
NP
CPP
6122
6123
6183
6189
6190
6258
Average
Rat number


Time spent eating (sec)
(DAM - VEH)
>400 s
300 - 399 s
200 - 299 s
<200 s
CPu
GP
AcbC
AcbSh
ac
Horizontal View
Bregma -6.38 mm
Time spent eating (sec)
(DAM - VEH)
>400 s
300 - 399 s
200 - 299 s
<200 s
CPu
EA
LPO
VP
AcbC
AcbSh
Sagittal View
Lateral 1.90 mm


300
250
200
150
100
50
0
Total time spent eating (sec)
DAMGO
Vehicle