# The Extended Amygdala in Appetitive Motivation for Reward: Role of the Bed Nucleus of the Stria Terminalis

**by**

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**To my wife, Jennifer**
**and my mother, Karen**

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## ABSTRACT

The Extended Amygdala in Appetitive Motivation for Reward:
Role of the Bed Nucleus of the Stria Terminalis

by

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The extended amygdala is an emerging neuroanatomical concept for a basal forebrain macrosystem, containing the bed nucleus of the stria terminalis (BNST) and several other highly interconnected nuclei. BNST has received increasing attention following the discovery that it connects to limbic brain regions involved in stress, homeostasis, and reward. Most of the literature on BNST function emphasizes its role in aversive motivational processes such as stress, anxiety and drug withdrawal. However, some circumstantial evidence suggests that BNST also plays a role in appetitive motivation (e.g., reward 'wanting'), although direct tests have not yet been made. Here, I present a series of experiments designed to provide the first direct evidence for a role of BNST in appetitive motivation for food reward. I found that stimulation of μ-opioid receptors in BNST potently increased eating behavior in non-deprived rats. By contrast, temporary suppression of BNST yielded increased aversive behaviors such as defensive treading and escape dashes. Was eating caused by BNST stimulation truly appetitive or

only instead due to aversive stress? I found that rats exhibited a conditioned place preference for an environment paired with μ-opioid stimulation in BNST, confirming that this stimulation produced primarily appetitive effects. I also found that opioid stimulation in BNST diffusely increased the motivational magnet qualities of a conditioned stimulus for a food reward in an autoshaping test. This stimulation spilled elevated motivation into inappropriate moments, enhancing responding even when the reward cue was absent. By contrast, stimulation of another limbic structure, nucleus accumbens, only increased motivational attractiveness in the presence of the cue. Accordingly, stimulation of nucleus accumbens, but not BNST, also elevated an animal's willingness to earn presentations of the autoshaping conditioned stimulus in conditioned instrumental reinforcement testing. Finally, I showed that increased feeding after μ-opioid stimulation in BNST occurs in spite of decreased hedonic 'liking' for sweet taste. Together, these experiments provide direct evidence that BNST mediates appetitive motivation, and further clarify the function of μ-opioid circuits in the extended amygdala.

# Chapter 1

## Introduction

The extended amygdala is an emerging neuroanatomical concept that lies in the basal forebrain and contains several independent but highly interconnected brain nuclei, including the bed nucleus of the stria terminalis (BNST). This macrosystem, and BNST in particular, have received a great deal of attention following the discovery that it receives a diverse array of input from limbic brain regions and sends output to a variety of midbrain and brainstem nuclei involved in stress, homeostasis, and reward. Although much of the current literature on BNST focuses on its role in aversive motivational processes such as stress, anxiety and drug withdrawal, there is at least circumstantial evidence suggesting that BNST also plays a role in appetitive motivation, possibly via dense concentrations of the reward-related μ-opioid receptor. In this dissertation, I present a series of experiments exploring the role of BNST in appetitive motivation. After first establishing a broad appetitive role for BNST using tests of voluntary feeding, I proceed to use targeted behavioral tests to examine discrete psychological processes that support appetitive behavior, including incentive motivation and hedonic impact. These experiments are designed to provide the first direct evidence that manipulation of BNST can result in the enhancement of appetitive motivation.

***Studying the neural substrates of appetitive motivation and reward***

Appetitive motivation for reward often feels like a single experience, a relatively seamless experience that flows across the desire for, consumption, and enjoyment of a pleasant drink or snack. But the single word *reward* belies a more complicated interplay between multiple distinct psychological mechanisms. Berridge, Robinson, and colleagues have argued for three reward processes that, though often experienced simultaneously, can be teased apart using the experimental techniques of affective neuroscience (Berridge & Robinson, 2003; Berridge, Robinson, & Aldridge, 2009; Robinson & Berridge, 1993). These processes are 1) incentive salience 'wanting,' which makes rewards or reward cues desired and elicits approach and consumption, 2) 'liking,' which conveys the hedonic pleasure of actually consuming the reward, and 3) learning, which helps to link experience with previously 'liked' rewards to future 'wanting' for that same reward or cues that have been associated with it.

Though at least a portion of the interest in the neuroscience of reward is surely the drive to understand and explain our own experiences, it is important to note that brain systems of reward, and especially their dysfunction or manipulation, likely underlie a number of serious clinical conditions. For example, the process of using and, in some cases, becoming addicted to drugs of abuse has been hypothesized to turn predominantly on structural or functional changes in brain reward systems, though a fierce debate continues about precisely which reward mechanism(s) and neurotransmitter system(s) are involved, and at what stage in the addictive process (Aston-Jones & Harris, 2004; Everitt et al., 1999; Koob & Le Moal, 2008; Le Moal & Koob, 2007; Redish, 2004; Robinson &

Berridge, 1993, 2000; Stewart, 2000). Though drugs of abuse are rather novel (evolutionarily speaking) and can essentially hijack brain mechanisms of reward (Nesse & Berridge, 1997), naturally rewarding stimuli, like food or sex, and behavioral addictions, such as gambling, likely tap into the same brain networks (Davis, Strachan, & Berkson, 2004; Goudriaan, Oosterlaan, de Beurs, & Van den Brink, 2004; Grant, Brewer, & Potenza, 2006; Volkow, Wang, Fowler, & Telang, 2008; Wang, Volkow, Thanos, & Fowler, 2004). Interestingly, but perhaps not surprisingly, each of these clinical conditions shows dramatic individual differences in both initial susceptibility and also the probability of recovery once the disease is acquired. This adds an additional layer of difficulty, requiring not only the uncovering of which brain regions and neurotransmitter systems mediate reward, but also how responding to reward may differ across individuals, and if possible how those differences in behavior relate back to differences in the brain (Cecchi, Capriles, Watson, & Akil, 2007; Flagel, Akil, & Robinson, 2009; Yacubian & Buchel, 2009).

Many different neurotransmitter systems, acting in a distributed network of brain regions, have been implicated in reward. Limbic brain regions, so named because they reside on the "rim" of the cerebrum adjacent to evolutionarily older structures in the midbrain, have received particular attention, including the nucleus accumbens (both core and shell) (Carlezon & Thomas, 2009; Pecina, Smith, & Berridge, 2006; Thorpe & Kotz, 2005; Zhang, Balmadrid, & Kelley, 2003), ventral pallidum (K. S. Smith, Tindell, Aldridge, & Berridge, 2009; Tindell, Berridge, Zhang, Pecina, & Aldridge, 2005), prefrontal cortical regions (de Araujo, Rolls, Kringelbach, McGlone, & Phillips, 2003; Knutson & Cooper, 2005; Rolls, 2006), and amygdala (El-Amamy & Holland, 2007;

Everitt et al., 1999; Holland, Han, & Winfield, 2002; Mahler & Berridge, 2009). The amygdala, in particular, has received recent attention in light of an emerging anatomical concept called the extended amygdala (reviewed in detail below). Briefly, this concept builds on the already prevalent distinction between so-called cortical regions of amygdala (basolateral nucleus) and sub-cortical regions (central and medial nuclei), highlighting the unique anatomical relationship between central/medial amygdala and a nearly unbroken continuum of cells extending rostrally (hence *extended* amygdala) all the way to the bed nucleus of the stria terminalis (Alheid & Heimer, 1988; Heimer, Trimble, Van Hoesen, & Zahm, 2007). Although much of the attention on the extended amygdala has focused on aversive aspects of addiction (Aston-Jones & Harris, 2004; Koob, 2003; Koob & Le Moal, 2008), emerging evidence suggests that the extended amygdala macrosystem may also play a critical role in mediating reward and appetitive motivation (Johnson, de Olmos, Pastuskovas, Zardetto-Smith, & Vivas, 1999; Newman, 1999; Waraczynski, 2006).

***The neurobiology of extended amgydala***

The basal forebrain contains a number of distinct brain nuclei, which can be usefully grouped into neuroanatomic 'macrosystems,' based on histological, architectural, and developmental evidence (Figure 1). Such macrosystems, though composed of individual functional units, often act together as large circuits in the generation of behavioral responses, much like relatively autonomous states combine to form a larger country. Macroystems of the basal forebrain include the ventral-striato-

pallidal system (part of the basal ganglia), the septal-diagonal band, and the extended amygdala.

The extended amygdala is a forebrain macrostructure, composed of two particular nuclei of the amygdala (central nucleus, CeA, and medial nucleus, MeA), the sublenticular extended amygdala (SLEA, sometimes referred to as caudal substantia inominata), the interstitial nucleus of the posterior limb of the anterior commisure (IPAC), and BNST (Figure 1). In volume, the BNST constitutes approximately 20-25% of the extended amydala.

More controversially, the caudal shell of the nucleus accumbens has sometimes been included as a member of extended amygdala, due primarily to the nearly seamless anatomical contiguity of rostal BNST and caudal accumbens shell. However, substantial differences in output patterns between accumbens shell and other extended amygdala nuclei have led recent investigators to argue against including accumbens shell as a standard extended amygdala component (de Olmos & Heimer, 1999; Zahm, 1998).

Proponents of the extended amygdala note the extensive intrinsic connections among these nuclei, parallel architecture, and common output pathways (Alheid, 2003; de Olmos & Heimer, 1999; Heimer et al., 2007). Though the nearly unbroken rostro-caudal continuum of cells extending from centro-medial amygdala through BNST was originally noted in the work of J.B. Johnston in the early 1920's (Johnston, 1923), widespread adoption of the extended amygdala concept did not arise until the late 1980's with the work of neuroanatomists George Alheid, Lennart Heimer and Jose de Olmos (Alheid & Heimer, 1988; Heimer & Van Hoesen, 2006).

Extended amygdala is now gaining acceptance as a valid and useful anatomical construct. There remains some debate, however, about the appropriateness of segregating the extended amygdala from the neighboring striatopallidal system. The most prominent advocate against a separate extended amygdala system is Larry Swanson, who has argued that the CeA – SLEA/IPAC – BNST continuum is best viewed as following the same anatomical rule as the more classic striatopallidal system, with CeA as primarily striatal and BNST as primarily pallidal (with some noted role reversals) (Swanson, 2000, 2003, 2005). Swanson's view is admirably parsimonious, integrating the extended amygdala seamlessly into a larger master plan of descending cortical (glutamate) --> striatal (GABA) --> pallidal (GABA) projections that may offer a useful sense of the typical flow of information through the extended amygdala system. But whether one accepts the extended amygdala as distinct from the basal ganglia or merely a slight variation from its standard plan does not detract from the close anatomical relationship between the extended amygdala nuclei: either they are their own macrosystem or an equally similar functional circuit/loop within the basal ganglia, and in both cases their deeply interconnected neuroanatomy is an equally useful springboard for behavioral investigation.

*Central vs Medial Divisions within the extended amygdala*

There are two parallel components of the broader extended amygdala, termed the central and medial divisions. The central division is comprised of the central amygdala, dorsal SLEA, IPAC, and lateral BNST. The medial division is composed of the medial amygdala, ventral SLEA, and medial BNST (Alheid, 2003; de Olmos & Heimer, 1999)

These parallels are further evident at the level of the amygdala and BNST; subnuclei present in the central amygdala are nearly always mirrored by similar subnuclei in the later BNST. A similarly parallel relationship holds for medial amygdala and medial BNST. Interestingly, the two divisions of extended amygdala note show robust intrinsic connectivity but relative sparse connections across the central/medial division (Alheid, 2003).

*Afferents and efferents*

The extended amygdala receives substantial input from a variety of limbic brain structures, including the basolateral amygdala complex (BLA, especially posterior regions), ventral hippocampus, and limbic cortex (including several medial prefrontal regions) (Alheid, 2003), as well as rich catecholamine innervation from brainstem and midbrain, including the densest population of norepinephrine terminals in forebrain (Aston-Jones, Delfs, Druhan, & Zhu, 1999; Forray & Gysling, 2004; R. J. Smith & Aston-Jones, 2008). The primary output targets include hypothalamus (medial and lateral divisions), midbrain dopaminergic cell population (ventral tegmental area and substantia nigra), a variety of brainstem nuclei including noradrenergic populations in medulla, and relatively weak projections to thalamic feedback loops (Alheid, 2003; de Olmos & Heimer, 1999). These output channels differ slightly across the two divisions of extended amgydala, with the central EA projecting to primarily *lateral* hypothalamus and the medial EA projecting to primarily *medial* hypothalamus.

Note an important deviation from the substantial output of basal ganglia output to thalamus; although extended amygdala does send modest output to thalamic nuclei

(including medial midline intralaminar and paraventricular regions) (Dong & Swanson, 2006a, 2006b), these projections are much less robust that the efferents from basal ganglia (de Olmos & Heimer, 1999; Dong, Petrovich, & Swanson, 2000; Dong & Swanson, 2004a). The robust projections to hypothalamus suggest a strong role for the extended amygdala in the modulation of the neuroendocrine access, which has been confirmed in behavioral studies outlining a key role for extended amygdala in processes of reward, stress, anxiety, and reproduction (reviewed below).

The extended amygdala is composed primarily of GABA-ergic cells, much like the neighboring striato-pallidum (Cassell, Freedman, & Shi, 1999). However, the extended amygdala also expresses a rich array of additional neuropeptides and neurotransmitters, including glutamate, acetycholine, enkephalins, substance P, corticotropic releasing factor (CRF), angiotensin II, vasopressin, and oxytocin (Alheid, 2003; Alheid, de Olmos, & Beltramino, 1995; de Olmos & Heimer, 1999). Although there is significant overlap between immunochemical staining in extended amygdala and the nearby striatum, there are also several notable cases of distinct macrosystem staining. For example, although the striatal and pallidal complexes both stain heavily for adenosine receptors, extended amygdala does not stain at all for these receptors (Rosin, Robeva, Woodard, Guyenet, & Linden, 1998). Notably, similar staining dissociations can be used to visualize the medial vs. central divisions of extended amygdala, such as oxytocin in the former and vasopressin in the latter (Alheid, 2003).

***Neurobiology of BNST***

BNST is a structure  at the far rostral end of the extended amygdala. It is located at the rostral tip of the stria terminalis, just caudal to the accumbens shell and surrounding the anterior commissure as it crosses the midline, and is roughly $3mm^3$ in volume in the rat (just slightly larger than accumbens shell). Though modest in size, BNST contains over a dozen distinct sub-nuclei, each with unique afferent and efferent patterns, as well as with occasionally divergent influences on behavior (see below for further review of the role of BNST in behavior). These sub-nuclei are often broadly assigned to larger divisions within BNST, most prominently the medial and lateral divisions. As noted above, the medial/lateral divisions of BNST are based primarily on interconnection with subcortical amygdala, with medial BNST interconnected most strongly to medial amygdala, and lateral BNST most strongly to central amygdala. However, further anatomical investigation has also revealed notable distinctions between anterior and posterior regions of BNST, especially in regards to regulation of hypothalamic-pituitary-adrenal (HPA) axis activity (Choi et al., 2007).

In an elegant and authoritative series of neuroanatomical tract-tracing experiments, Hong-Wei Dong, Larry Swanson and colleagues used small injections of the anterograde tracer Phaseolus vulgaris leucoagglutinin (PHAL) to target individual sub-nuclei of BNST, revealing in exquisite detail the pattern of efferent projections (Dong et al., 2000; Dong, Petrovich, & Swanson, 2001; Dong, Petrovich, Watts, & Swanson, 2001; Dong & Swanson, 2003, 2004a, 2004b, 2006a, 2006b, 2006c). These studies reveal an extremely complicated and diverse set of terminal fields reflecting

BNST's output to a wide range of brain regions, though the primary pattern mimics extended amygdala as a whole. The densest projections from BNST are to other areas of extended amygdala (SLEA, IPAC, and CeA/MeA), emphasizing the strong associative fibers that characterize the extended amygdala macrosystem. Similarly dense outputs target hypothalamus, and in particular the paraventricular region of hypothalamus (PVN) that is so critical in controlling neuroendocrine function, with medial and lateral BNST primarily targeting medial and lateral hypothalamus, respectively. BNST also sends significant outputs to midbrain (including VTA) and brainstem (in particular caudal medulla but also taste centers in the pontine parabrachial nucleus and the nucleus of the solitary tract) (Fudge & Haber, 2001; Kang & Lundy, 2009; C. S. Li & Cho, 2006).

As with efferents, afferent projections to BNST are densest from other regions of extended amygdala. The CeA and MeA appear to be the richest source of upstream information to BNST, sending dense projections to both medial and lateral divisions of BNST (Dong, Petrovich, & Swanson, 2001). BNST receives only weak direct input from BLA (Dong, Petrovich, & Swanson, 2001), with most of the link between BNST and BLA occurs via CeA, in support of Swanson's cortex—striatum—pallidum role for BNST, CeA, and BLA, respectively (Swanson, 2003). Other limbic projections to BNST include hippocampus and limbic cortex, in particular the infralimbic region of medial prefrontal cortex (Massi et al., 2008), and reciprocal, though relatively weak, connections with the shell of the nucleus accumbens. Notably, BNST receives significant projections from dopaminergic regions of midbrain (particularly VTA), noradrenergic regions of the brainstem (primarily in anterior-ventral BNST), and also direct input of taste information from the parabrachial nucleus (Alden, Besson, & Bernard, 1994; Norgren, 1976).

BNST, like the rest of extended amygdala, contains primarily GABA-ergic neurons, but also expresses a wide variety of additional receptors and neurotransmitters. Most notable are the dense expression of CRF (receptors and neurotransmitter) (Koob & Heinrichs, 1999), dopamine (receptors) (Carboni, Silvagni, Rolando, & Di Chiara, 2000), norepinephrine (receptors) (Aston-Jones et al., 1999; Aston-Jones & Harris, 2004; Delfs, Zhu, Druhan, & Aston-Jones, 2000; R. J. Smith & Aston-Jones, 2008), and opioids (receptors) (Casada & Dafny, 1993; Mansour, Fox, Akil, & Watson, 1995). The presence of this constellation of neurotransmitter systems would by itself be of interest, but it is their interaction that lends BNST some if its most interesting roles in behavior (McElligott & Winder, 2009). For example, dopamine in BNST modulates fast excitatory transmission of glutamate via a CRF dependent mechanism (Kash, Nobis, Matthews, & Winder, 2008), while norepinephrine in BNST modulates CRF release associated with drug withdrawal (Aston-Jones & Harris, 2004).

In general, dorsal regions of BNST appear to be less responsive than ventral regions to a range of neurochemical stimuli, including morphine, acetylcholine, and norepinephrine (Casada & Dafny, 1993), a finding paralleled by the subsequent report that the neurophysiological properties of neurons in BNST also show some differences along the dorso-ventral axis (Egli & Winder, 2003). Such findings have sparked particular interest in the characteristics of anterior-ventral BNST, which receives the bulk of BNST's dense noradrenergic input. Electrical and glutamatergic stimulation of neurons in this region have been shown to potently increase population firing of dopaminergic neurons in VTA (Georges & Aston-Jones, 2001). A recent study indicates that this excitatory input to BNST likely arises from infralimbic cortex, where stimulation

can potently drive firing in anterior-ventral BNST and, one synapse on, in VTA, and which can be directly inhibited by cannabinoid agonists in BNST (Massi et al., 2008). Notably, the study by Massi et al. (2008) sampled neurons from both ventral and dorsal regions of BNST, suggesting that perhaps dorsal regions may also contribute to this cortical-BNST-midbrain circuit, as well as other circuit interactions. Indeed, only dorso-lateral BNST neurons receiving VTA input were found to show morphine-dependent changes in neuroplasticity (Dumont, Rycroft, Maiz, & Williams, 2008).

### *Established behavioral roles of BNST*

Similar to its neurobiology, BNST is involved in a diverse array of behaviors, many of which are intimately intertwined. Here, I review the primary established roles for BNST in the modulation of aversive aspects of motivated behavior, derived mostly from studies in rodents.

#### *Stress*

BNST is perhaps best known for its role in the brain's stress network, and a large body of literature describes both the neuroanatomical and behavioral functions of BNST in both acute and chronic stress circuitry.

Though early lesion studies sometimes generated inconclusive results regarding the roles of BNST in altering activity of stress circuitry (Crane, Buller, & Day, 2003; Gray et al., 1993), possibly due to the large extent of the lesioned area, Dennis Choi, James Herman and colleagues (2007) utilized restricted lesions of anterior and posterior regions to clarify the role of BNST in modulating the HPA axis in response to acute

stress. They found that lesions of anterior BNST markedly reduced plasma corticosterone and decreased c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN), while lesions of posterior BNST had roughly the opposite effect (Choi et al., 2007). This suggests that anterior BNST typically acts to *excite*, while posterior BNST tonically *inhibits*, HPA axis activity. Further studies by this group have confirmed these opposing roles for anterior vs. posterior BNST, and additionally speculated that these roles especially relevant to acute stressors (Choi, Evanson et al., 2008; Choi, Furay et al., 2008), though see (Dallman et al., 2003) for indication of a role for BNST in chronic stress.

Behaviorally, the largest body of research linking BNST and stress involves the study of immobilization and restraint. Electrophysiological recording during acute immobilization stress revealed potent modulation of firing of BNST neurons (both excitation and inhibition), and a similar response was found when rats were presented with a auditory tone that was previously paired with immobilization (Henke, 1984). Subsequent research found that immobilization and electrical stimulation of BNST produced a similar constellation of behaviors, including increased locomotor and exploratory activities, though direct stimulation of BNST resulted in extended duration (3 hrs vs. 1 hr) of these stress-like behaviors (Casada & Dafny, 1991). BNST stimulation also reportedly caused more intense aversive behaviors than immobilization alone, causing vigorous escape attempts and even aggressive biting behavior. Finally, immobilization also caused dramatic increases in norepinephrine release in BNST (Pacak, McCarty, Palkovits, Kopin, & Goldstein, 1995).

Immobilization, though stressful, does not pose any immediate and life-threatening danger to rodents, and has been termed a 'processive' stressor. This is in contrast to 'systemic' stressors, like hemorrhage and cardiopulmonary depression, that are acutely dangerous and life-threatening (Herman & Cullinan, 1997). Systemic stressors usually mobilize HPA responses by utilizing direct connections from brainstem to PVN, whereas processive stressors often engage higher-order cognitive and emotional regions, including limbic brain regions. Yet most limbic system nuclei lack direct connections to PVN, indicating the need for a limbic-HPA axis relay. Indeed, BNST, with afferents from a variety of limbic brain regions and the ability to bi-directionally modulate HPA axis activity, appears to fill precisely this role (Herman, Ostrander, Mueller, & Figueiredo, 2005).

*Anxiety*

In addition to stress, BNST is also involved in fearful responses to environmental stimuli, a role that has been advanced most frequently by Michael Davis and colleagues. Rats will reliably display a startle response when presented with an aversive acoustic stimulus (such as a very loud noise), and this acoustic startle can be potentiated by either exposure to a cue that has previously predicted another aversive conditional stimulus (e.g. tone linked to footshock) or in the presence of an aversive unconditional stimulus such as extended exposure to a very bright light. Davis and colleagues, consistent with the work of others (Fendt & Fanselow, 1999; Goosens & Maren, 2001), showed that amygdala, and in particular CeA, was necessary for the expression of potentiated startle to a previously learned fear conditioned stimulus (CS) but not for the unconditioned startle to

the bright light. In contrast, BNST was found to be necessary for expression of the light-potentiated startle, but not the fear CS potentiated startle (Walker & Davis, 1997; Walker, Toufexis, & Davis, 2003). This distinction has caused Davis and colleagues to argue for a role of CeA in *fear*, which is expressed at a particularly dreaded discrete object or association, and a separate role for BNST in *anxiety*, a more diffuse response to a non-specific but still ominous unconditioned stimuli (M. Davis, 1998; M. Davis, Walker, & Lee, 1997b).

This perspective has been met with mixed support. Consistent with Davis' view, temporary inactivation of BNST has been shown to block the freezing normally elicited by exposure to trimethylthiazoline, an odor found in fox feces (Fendt, Endres, & Apfelbach, 2003). However, in two other tests of unconditioned fearful behaviors, the elevated plus maze and the shock-probe test, lesions of BNST were not found to affect typical measures of fear and anxiety (Treit, Aujla, & Menard, 1998). Additionally, and also inconsistent with the view that BNST does not mediate conditioned stimuli, lesions of BNST were found to disrupt behavioral and neuroendocrine responses following exposure to a context (though not a tone) previously paired with a fear conditioning context (Sullivan et al., 2004).

In summary, though it is clear that BNST does play a role in the response to fearful and aversive stimuli, it remains unclear whether BNST is chiefly involved in the diffuse, anxious response to long-duration unconditioned stimuli, or whether it also plays a role in the expression of conditioned fear.

*Drugs of Abuse and Addiction*

The extended amygdala as a whole has been implicated as a critical node in the development and maintenance of drug use (Everitt et al., 1999; Harris & Aston-Jones, 2007; Koob, 1999, 2003). Research has led investigators such as George Koob to suggest that BNST is particularly important in two processes that accompany extended drug use: withdrawal and dysphoria-triggered relapse.

*Withdrawal*

Repeated use of many drugs of abuse can result in an aversive motivational state that accompanies cessation of drug use, generally known as withdrawal. Withdrawal can involve both physical (such as trembling) as well as psychological (such as dysphoria) symptoms, and has been argued by some to constitute a significant component of the maintenance of drug-seeking that characterizes addiction (Aston-Jones & Harris, 2004; Koob, 2006; Koob & Le Moal, 2008; Le Moal & Koob, 2007).

Precipitated removal of ethanol caused an increase in the release of CRF in BNST, which quickly declined to basal levels following relatively swift return of the ethanol-containing solution (Olive, Koenig, Nannini, & Hodge, 2002). However, extending the duration of ethanol removal only served to enhance CRF concentration in BNST, which eventually surged to nearly double baseline levels. It has recently been reported that extended withdrawal from heroin, alcohol, and cocaine has a significant impact on the excitability of neurons in the juxtacapsular nucleus of BNST, a small sub-nuclei in the anterior-lateral region (Francesconi et al., 2009). This change in

neuroplasticity appeared to be dependent on CRF signaling, as the administration of a CRF-1 receptor antagonist normalized the intrinsic excitability of juxtacapsular neurons.

In addition to CRF, norepinephrine signaling in BNST is also critical to the expression of withdrawal symptoms, in particular to opiates. BNST neurons show increased activation, as measured by c-Fos expression, during withdrawal from opiates, an effect that can be attenuated by the ß-adrenergic antagonist propranolol (Aston-Jones et al., 1999). Further, animals made dependent on morphine will display robust avoidance of an environment that is paired with precipitated withdrawal, yet this avoidance (as well as somatic symptoms of withdrawal) can be abolished in rats by the disruption of noradrenergic signaling in BNST (Delfs et al., 2000).

*Relapse*

The above findings show a clear role for CRF and norepinephrine in BNST in the neural response to acute drug withdrawal. However, animals remain vulnerable to resumption of drug-seeking long after withdrawal symptoms subside, often resuming drug intake after weeks or months of remaining drug free. This process, known as relapse, can be triggered by a variety of factors, including exposure to drugs, drug cues, or stress (Robinson & Berridge, 2000; Shaham, Rodaros, & Stewart, 1994; Stewart, 2000).

As might be suspected given its known role in modulating the HPA axis, BNST appears to play a crucial role in relapse due to stress, demonstrated in a series of experiments conducted by Jane Stewart and colleagues. Administration of a stressful footshock will stimulate relapse of drug-seeking in a rat where this behavior was

previously extinguished. However, administration of the CRF receptor antagonist D-Phe directly into BNST, but not in CeA, prevented relapse following footshock; moreover, stimulation of CRF receptors by direct microinjection of CRF into BNST – without any accompanying footshock – was able to trigger relapse in drug-seeking equal to that observed following shock exposure (Erb & Stewart, 1999). Stewart and colleagues subsequently found that at least a portion of the CRF projections to BNST that contribute to stress-induced relapse do arise from CeA, despite the absence of any effect of CRF infusion in CeA on relapse (Erb, Salmaso, Rodaros, & Stewart, 2001). The stress-related relapse circuitry in BNST was later expanded to include a role for norepinephrine, as it was shown that disruption of noradrenergic signaling in BNST prevented stress-induced, but not drug-induced, relapse in rats trained to self-administer cocaine (Leri, Flores, Rodaros, & Stewart, 2002).

In summary, BNST plays a key role in a variety of aversive motivational processes. It is a key node in the brain stress system, acting as an interface between the limbic system and the HPA axis. BNST also plays a related role in anxiety, mediating behavioral responses to long-duration, unconditioned cues (and perhaps some conditioned cues) and mobilizing behaviors adaptive for dealing with diffuse, non-specific threats. Finally, CRF and noradrenergic transmission within BNST are critical to withdrawal and relapse to a variety of drugs of abuse, including ethanol, opiates, and stimulants.

***Potential role of BNST in appetitive motivation***

There is also reason to believe BNST might play a role in appetitive motivational processes, in addition to the variety of aversive and stressful motivational processes described earlier. The reason comes primarily from neuroanatomical and neurochemical considerations, with a few supporting functional observations from behavioral studies.

First, BNST shares strong connections with several reward-related brain regions. As noted earlier, BNST is able to potently modulate population firing in dopaminergic nuclei in the midbrain, including VTA (Georges & Aston-Jones, 2001; Kash et al., 2008; Massi et al., 2008). Ascending dopamine from midbrain has long been associated with reward processes, initially as a signal of the hedonic or euphoric qualities of rewarding stimuli (Wise & Bozarth, 1985; Wise & Rompre, 1989) and, more recently, as a signal of reward prediction (Schultz, Dayan, & Montague, 1997), a mediator of effort in obtaining rewarding stimuli (Salamone, 2007; Salamone, Correa, Farrar, & Mingote, 2007), and the primary signal of incentive salience 'wanting' (Berridge, 2007; Robinson & Berridge, 1993), among others. Within extended amygdala, lateral regions of BNST shares robust and bidirectional connections with CeA, which has been shown to potentiate food intake (Gosnell, 1988; Gosnell, Morley, & Levine, 1986) as well as incentive salience 'wanting' for cues that predict a food reward (Mahler & Berridge, 2007, 2009).

Second, and closely related to dopamine, several pieces of evidence suggest that BNST is linked to the rewarding properties of drugs of abuse. For example, acute, systemic administration of a variety of reinforcing drugs, including morphine, cocaine, nicotine, and ethanol, all stimulate dopamine release in BNST (Carboni et al., 2000).

Further, the disruption of GABA-ergic, dopaminergic, or opioid signaling within BNST disrupts the reinforcing properties of ethanol, cocaine, and heroin, respectively, in rats taught to self-administer those drugs (Epping-Jordan, Markou, & Koob, 1998; Hyytia & Koob, 1995; J. R. Walker, Ahmed, Gracy, & Koob, 2000). Interestingly, BNST may play a particular role in instrumental responding for reward, as self-administration of cocaine or food pellets, but not passive receipt of indentical patterns and amounts of cocaine or food, enhanced excitatory synaptic transmission in BNST (Dumont, Mark, Mader, & Williams, 2005).

Finally, BNST appears to mediate at least some aspects of appetitive sexual behavior, particularly in males, for both birds and mammals. In a study of male Japanese quail, it was found that presentation of a CS that predicted the availability of a receptive female (a common technique to increase reproductive success) led to increased c-Fos expression in medial BNST and medial preoptic area (Taziaux, Kahn, Moore, Balthazart, & Holloway, 2008). In contrast, when posterior BNST is lesioned in male rats, the normal preference for a female odor over a male odor is abolished even though normal olfaction remains intact (Been & Petrulis, 2008). Indeed, it has been suggested that the entire medial extended amygdala (including medial BNST and medial amygdala) constitutes an important circuit in the execution of male sexual behavior (Newman, 1999).

### *Opioids and reward*

The endogenous opioid system, and in particular the μ-opioid receptor, is strongly linked to reward processing throughout limbic brain regions, and as such affords a useful

initial point for investigating possible appetitive roles of BNST. Opioid receptors are distributed throughout extended amygdala in the rat, and BNST in particular shows robust expression of the μ-opioid receptor, in addition to ∂ and κ receptors (Mansour et al., 1995). In primates, BNST also displays dense labeling for the μ-opioid receptor, with medial regions exhibiting slightly denser staining than lateral regions (Daunais et al., 2001).

The μ-opioid receptor has often been implicated in the hedonic impact, or 'liking', as well as 'wanting', of rewards (Barbano & Cador, 2007; Berridge, 2000; Kelley et al., 2002; Pecina, Smith et al., 2006; K. S. Smith et al., 2009). Recently in the rat, a pair of μ–opioid hedonic hotspots have been identified in basal forebrain, one in rostro-dorsal accumbens shell and the other in caudal ventral pallidum (Pecina & Berridge, 2000, 2005; K. S. Smith & Berridge, 2005). In these small regions (each ~1mm$^3$), μ-opioid receptor stimulation results in potent increases in the hedonic impact of a sweet sucrose reward, elevating orofacial reactions characteristic of palatable rewards. Interestingly, these distinct hotspots in accumbens shell and ventral pallidum appear to interact in the generation of reward 'liking,' with disruption of opioid transmission in one hotspot effectively vetoing the enhancement of hedonic impact normally generated by opioid stimulation of the other (Smith & Berridge, 2007). In humans, systemic opioid blockade with naloxone attenuated the reported pleasure derived from large rewards in a gambling task (Petrovic et al., 2008) and reduced consumption of sweet, high-fat foods in binge eaters (but not non-binging controls) (Drewnowski, Krahn, Demitrack, Nairn, & Gosnell, 1995).

The latter finding, in particular, bridges the role of mediating hedonic 'liking' with μ-opioid's concomitant modulation of voluntary feeding, a measure of reward 'wanting.' Opioid stimulation throughout the basal forebrain, including ventral striatal and central amygdala regions, has been shown to dramatically increase food intake (Bakshi & Kelley, 1993a, 1993b; Gosnell, 1988; Gosnell et al., 1986). In particular, μ-opioid stimulation seems to favor the intake of energy dense, high-caloric foods that are high in fat and sugar content (Glass, Billington, & Levine, 1999; Zhang & Kelley, 2000). Additionally, μ-opioid stimulation has also been shown to increase 'wanting' for cues associated with rewards. Throughout nucleus accumbens, for example, the μ-opioid receptor agonist DAMGO increases both the conditioned reinforcement value of a reward cue (Phillips, Robbins, & Everitt, 1994) and also facilitates the transfer of the incentive motivational value of a Pavlovian reward CS to an available instrumental option linked to the same UCS reward in the Pavlovian-to-instrumental transfer paradigm (PIT) (Pecina & Berridge, In Preparation). Within extended amygdala, μ-opioid stimulation with DAMGO similarly enhances PIT (Mahler & Berridge, 2007), and also increases appetitive behaviors directed at a reward CS in an autoshaping (also known as sign-tracking) test (Mahler & Berridge, 2009).

***Rationale***

The goal of my dissertation is to further characterize the role of the BNST in reward and appetitive motivation. In particular, we chose to target primarily μ-opioid receptors as a substrate for motivation in BNST. As noted above, these receptors are densely expressed throughout BNST, and have been shown in many forebrain regions to

modulate appetitive and rewarding processes. Indeed, if extended amygdala follows the general cortex-striatum-pallidum plan of the brain, then BNST would be most functionally equivalent to a structure such as ventral pallidum, where μ-opioid stimulation has been shown to potently increase appetitive motivational processes, including feeding and hedonic 'liking' (K. S. Smith & Berridge, 2005, 2007; K. S. Smith et al., 2009).

We reasoned that food intake would serve as a direct and simple measure of appetitive motivation, and so in Experiment 1 tested the ability of direct microinjections in BNST to modulate feeding. Based on the evidence reviewed above that μ-opioids mediate enhanced feeding throughout the basal forebrain, including other regions of extended amygdala, we chose to test the ability of the μ-opioid agonist DAMGO in BNST to potentiate feeding. We also examined the ability of temporary lesions of BNST to modulate feeding, using the $GABA_A$ agonist muscimol.

Following our finding that DAMGO in BNST potently increased appetitive motivation for a food reward, we chose to investigate a series of possible discrete psychological mechanisms that could help explain increase in food intake. One possibility is that opioid stimulation in BNST increases incentive salience 'wanting' for rewards and rewards cues. In order to test this prediction, in Experiment 2 we examined the effect of microinjection on BNST in two different test of 'wanting': autoshaping and conditioned reinforcement. We also compared the effect of this manipulation to identical stimulation of μ-opioid receptors in the nearby accumbens shell. This allowed us to directly compare the roles of μ-opioid stimulation in extended amygdala and ventral striatum, and also

offered a novel test of the prediction that accumbens shell opioids may enhance 'wanting' for all available reward cues, rather than only an animal's preferred cue.

In addition to increased reward 'wanting,' enhanced food intake could also be the result of an increase in the hedonic impact, or 'liking,' of the food reward. In order to test the prediction that μ-opioid stimulation in BNST enhanced hedonic 'liking,' in Experiment 3 we utilized the taste reactivity paradigm to examine the hedonic impact of intra-oral injections of sweet sucrose and bitter quinine infusions following DAMGO microinjection in BNST. As with food intake, we also examined whether temporary inactivation of BNST with muscimol would impact taste reactivity. Additionally, we added a voluntary food intake session after taste reactivity where animals had the choice between palatable chocolate candies and standard lab chow to test the prediction that BNST μ-opioid stimulation specifically enhances consumption of palatable food rewards.

Finally, given the BNST's established role in stressful responding, it was important to test the prediction that the appetitive motivation we assigned to BNST due to increased feeding may instead be the result of increased stress. Indeed, stressful manipulations can frequently stimulate appetitive behavior, including feeding, so in Experiment 4 we examined whether DAMGO microinjection in BNST is inherently stressful. We utilized the conditioned place preference/avoidance paradigm, predicting that if DAMGO in BNST was indeed primarily stressful, then animals should avoid spending time in a chamber paired with DAMGO microinjection.

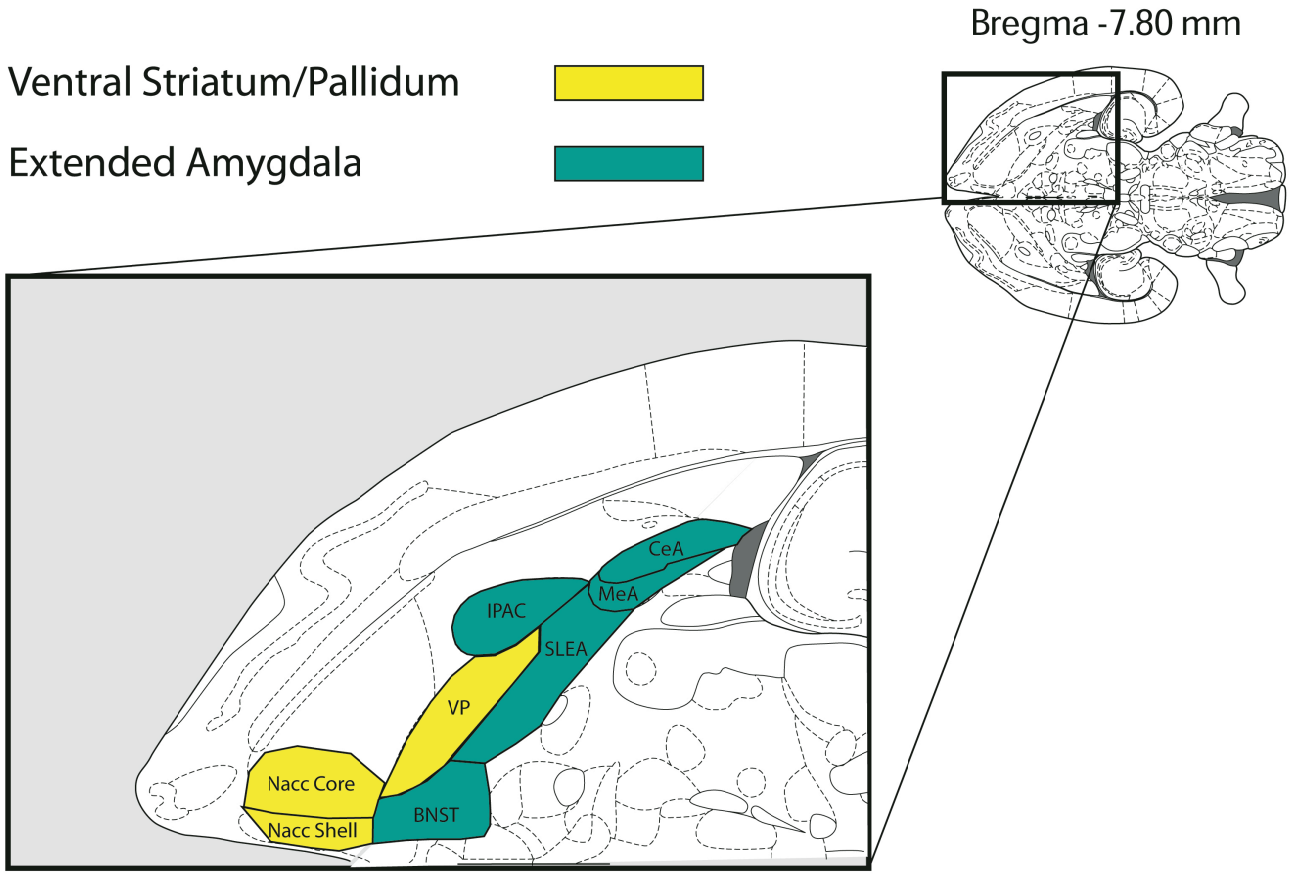



Figure 1.1 Overview of extended amygdala and ventral -striato-pallidal macrosystems in the forebrain of the rat. A horizontal slice through the basal forebrain of the rat, color coded to highlight the extended amygdala (blue-green) and ventral-striato-pallidal (yellow) macrosystems. BNST = bed nucleus of the stria terminalis; CeA= central nucelus of the amygdala; IPAC = interstitial nucleus of the posterior limb of the anterior commisure; MeA = medial nucleus of the amygdala; Nacc Core = core of the nucleus accumbens; Nacc Shell = shell of the nucleus accumbens; SLEA = sublenticular extended amygdala; VP = ventral pallidum. Adapted from Paxinos & Watson (2007).

## Chapter 2

## Modulation of Feeding by Opioid and GABA stimulation in the Bed Nucleus of the Stria Terminalis

### *Introduction*

The extended amygdala has been an increasing focus of researchers interested in the neural substrates of reward (Harris & Aston-Jones, 2007; Waraczynski, 2006) and addiction (Aston-Jones & Harris, 2004; Everitt et al., 1999; Koob, 1999; Koob & Le Moal, 2008). An important component of the extended amygdala is the bed nucleus of the stria terminalis (BNST), though this component has been relatively unstudied in experiments on reward. The BNST is a bilateral set of forebrain nuclei sitting just caudal to the shell of the nucleus accumbens and just below the lateral ventricles. It is a principle component of the extended amygdala, an emerging forebrain neuroanatomical macrostructure composed of the subcortical portions of the amygdala (central and medial nuclei), the sublenticular extended amygdala (SLEA), the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), and the BNST. Proponents of the extended amygdala note the extensive intrinsic connections among these nuclei, parallel architecture, and common output pathways (Alheid, 2003; de Olmos & Heimer, 1999; Heimer & Van Hoesen, 2006), though see (Swanson, 2003) for an alternative perspective. However, in order fully to understand how the extended amygdala regulates these and

other behaviors, it will be important to carefully explore the neurobiological and psychological properties of each of its component nuclei.

Most of the known behavioral roles of the BNST are related to aversive motivational states. BNST has been proposed as a major forebrain node for conveying stress-related information to the paraventricular nucleus of the hypothalamas (PVN) (Herman & Cullinan, 1997), and subsequent studies have shown that BNST lesions modulate Fos expression in PVN as well as plasma levels of corticosterone and ATCH (Choi et al., 2007). Michael Davis and colleagues have argued persuasively that BNST plays a particular role in diffuse anxiety (as opposed to more focused fear), based on experiments measuring alterations in fear-potentiated startle (M. Davis, 1998; M. Davis & Shi, 1999; M. Davis, Walker, & Lee, 1997a; M. Davis et al., 1997b; D. L. Walker & Davis, 1997; D. L. Walker et al., 2003). BNST has also been implicated in stressful facets of addiction to drugs of abuse, including maladaptive shifts in the allostatic response (Koob, 1999, 2003; Koob & Le Moal, 2008), expression of withdrawal from opiate drugs (Aston-Jones & Harris, 2004; Delfs et al., 2000), and relapse in drug taking after exposure to stress (Erb et al., 2001; Erb & Stewart, 1999). In sum, these findings paint BNST as a forebrain nucleus predominantly involved in aversive states and processes.

There are a few indications, however, that the BNST may also be involved in appetitive motivational processes. One indicator is anatomical: for example, mu-opioid activation in the central nucleus of the amygdala (CeA), another prominent extended amygdala structure with robust reciprocal projection to BNST (especially lateral BNST), is able to increase food intake (Giraudo, Billington, & Levine, 1998; Gosnell, 1988; Mahler & Berridge, In press), autoshaping for a conditional stimulus associated with a

food reward (Mahler & Berridge, In press), and Pavlovian to instrumental transfer (Mahler & Berridge, 2007). Another indicator is functional activation: BNST shows robust increases in Fos expression in response to several feeding manipulations, including ingestion of a small, highly palatable meal (Park & Carr, 1998) and ingestion of a sweet liquid sucrose solution (Mungarndee, Lundy, & Norgren, 2008). The BNST is also part of a network of ventral forebrain structures activated after a variety of orexigenic neurochemical manipulations, including central administration of neuropeptide Y (B. H. Li, Xu, Rowland, & Kalra, 1994), microinfusion of orexin A into lateral hypothalamus (Mullett, Billington, Levine, & Kotz, 2000), and microinjection of muscimol into the shell of the nucleus accumbens (Stratford, 2005). Finally, blockade of GABA, opioid, and dopamine neurotransmission in BNST result in significant disruptions of responding for alcohol, heroin, and cocaine, respectively (Epping-Jordan et al., 1998; Hyytia & Koob, 1995; J. R. Walker et al., 2000).

Combined, these findings provide indirect evidence for the possibility that BNST may play a role in appetitive motivation processes, in addition to its well established functions in the aversive motivational states of stress, anxiety, withdrawal, and relapse, yet to date there is no direct evidence of BNST's function in appetitive motivation. The purpose of this study was to evaluate the role of the BNST in a simple and direct measure of appetitive motivation, voluntary food intake. We assessed the effect of intra-BNST microinjections of a mu-opioid agonist, DAMGO, and a $GABA_A$ agonist, muscimol, and found that both manipulations were able to potently modulate feeding and related behaviors. These results indicate that BNST can powerfully influence appetitive

motivation, though further experiments will be necessary to pinpoint precisely which reward processes are driving this increase in feeding.

***Methods***

*Subjects*

A total of 28 Sprague-Dawley rats (females = 21, 250-500g at the time of surgery) were used for food intake testing. An additional 27 Sprague-Dawley rats (females, 250-400g at the time of surgery) were used for Fos plume analysis and mapping. All animals were housed in pairs (~21˚C; 12hr light/dark cyle, lights on at 9am) with *ad libitum* access to food (Purina 5001 chow; Purina Mills, St. Louis, MO) and tap water. All procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan in accordance with National Institute of Health guidelines.

*Surgery*

All animals were handled twice for a total of fifteen minutes prior to undergoing surgery. Rats were pretreated with atropine (0.04mg/kg) and then anesthetized with ketamine (80mg/kg) and xylazine (5mg/kg). Rats were then placed in a stereotaxic device and implanted with bilateral, chronic guide cannula (23 gauge, stainless steel), 14mm in length, aimed so that the ventral tip would rest 2mm above the BNST (AP: +0.24 to -0.84mm; ML: +/-1.2 to 1.7mm; DV: -4.5 to -4.95mm; incisor bar: -3.3mm [flat skull]). Guide cannula were secured to the skull using four stainless steel screws and dental acrylic, and fitted with stainless steel stylets to prevent occlusion. All rats were given post-operative analgesic (0.3 mg/kg buprenorphine) and prophylactic antibiotic (50

mg/kg chloramphenicol), and allowed to recover for at least 7 days before the onset of behavioral testing.

*Drugs and Microinjections*

All drugs were dissolved and diluted to dose in artificial cerebrospinal fluid (aCSF; Harvard Labs, Cambridge, MA). DAMGO was prepared at 0.05µg and 0.1µg doses (total bilateral dose of 0.1µg and 0.2µg, respectively), while muscimol was prepared at 75ng and 225ng doses (total bilateral dose of 150ng and 450ng, respectively). aCSF alone was used for vehicle microinjections. Microinjection schedules were counter-balanced across subjects using a Latin Square design.

On test days, animals were gently handled as the stylets were removed. Rats then received bilateral microinjections (0.2µL per side, total bilateral volume) via 16mm stainless steel microinjection tips (29 gauge), which extended 2mm beyond the ventral tip of the guide cannula. Microinjection tips were attached via PE-20 surgical tubing to a microinfusion pump, which delivered the infusion over the course of 60 seconds. Microinjection tips were left in place for an additional 60 seconds after the infusion ended to allow for drug diffusion, after which the stylets were replaced and the rat placed immediately in the food intake test chamber.

*Food Intake Testing*

During food intake testing, rats were placed in clear plastic cages containing a pre-measured pile of standard lab chow (~25g), a water spout, and corncob bedding. Prior

to food intake testing, rats were handled on two separate days for a total of 15 minutes and then habituated to the test environment for three additional days.

On test days, food intake was tested for 1 hour immediately following microinjection of vehicle, DAMGO, or muscimol. The entire session was videotaped for subsequent offline analysis. After the test was completed, remaining chow (including crumbs) was carefully removed from the cage and weighed. Test days were always separated by at least 48 hours.

*Food Intake Video Scoring*

Video recordings of food intake test sessions were scored offline by observers blind to the experimental condition. The following behaviors were recorded: eating time (in seconds), eating bouts (triggered by interruptions of eating of more than 5 seconds), food sniffing, food carrying, drinking time (in seconds), drinking bouts (same criteria as eating bouts), grooming, cage crosses, sleeping, rearing, and defensive treading. Treading is a natural defensive behavior emitted by rodents, and involves rapid forelimb strokes away from the body that push debris (e.g. dirt or bedding) in the direction of a threat.

*Histology*

After testing was completed, subjects used for behavioral testing were deeply anesthetized with sodium pentobarbital (0.2mg/kg; Fatal-Plus) and decapitated. Brains were extracted and placed in a 10% paraformaldehyde solution for 24-48 hours, and then placed in a 30% sucrose solution for 3-5 days, until the brains sank. The brains were then sliced on a freezing microtome (Leica) into 60μm coronal sections, mounted onto glass

slides, allowed to dry for at least 24 hours, and then stained with cresyl violet. Stained slices were viewed under light magnification and used to map the microinjection centers in each hemisphere on coronal sections taken from a rat brain atlas. (Paxinos & Watson, 2007) The majority of microinjection centers were bilateral hits of BNST (n= 25), and the remaining were unilateral hits (n=3).

*Fos-Like Protein Immunohistochemistry*

Rats utilized for Fos plume analysis underwent identical procedures for cannula implantation (except sham surgery control animals, who underwent surgery but did not receive cranial guide cannula) and pre- and post-surgical handling.

On the day of Fos plume testing, animals were given bilateral microinjections of vehicle (n=5), 0.05µg DAMGO (n=5), 0.1µg DAMGO (n=4), 75ng muscimol (n=4), and 225ng muscimol (n=4). Sham surgery animals (n=5) were handled gently for an amount of time equivalent to the animals receiving microinjections. Ninety minutes after microinjection, rats were transcardially perfused and their brains placed in 4% formaldehyde for 4-6 hours, then moved to a 30% sucrose solution for 3-4 days. Brains were then sliced on a freezing microtome in alternating 40µm coronal sections, with one series processed for Fos expression and the other retained for placement verification, if needed.

Fos activation following neurochemical manipulations of the BNST was measured using immunohistochemistry and immunofluorescence.(Faure, Reynolds, Richard, & Berridge, 2008; Reynolds & Berridge, 2008) Briefly, sections were immersed and gently agitated in successive baths of 0.1M sodium phosphate buffer (SPB) and 0.2%

Triton containing (1) 5% normal donkey serum (NDS) for 30 minutes, (2) 5% NDS and goat anti-c-Fos (1:10) overnight at 4˚C, (3) 5% NDS and signal enhancer for 30 minutes and, (4) 5% NDS and donkey anti-goat Alexa Fluor 488 (excitation: 488nm; emission: 519nm; Invitrogen) for 1 hour. Sections were then mounted, air dried for 2-4 hours, and then coverslipped with ProLong Gold antifade reagent (Invitrogen).

*Fos Plume Mapping of Neural Activation and Suppression*

Local Fos activation was visualized using a Leica microscope (DM 6000; Nussloch, Germany) equipped for both brightfield and fluourescent microscopy. A filter with an excitation band at 480-505nm and an emission band at 505-545 were used for fluorescent visualization, and images were captured at 10x magnification (2x2 tiled) using a Regita-SRV camera (Q-Imaging) and MCID Elite software. Fos-labeled cells were individually counted by an observer blind to treatment condition within ten adjacent sampling squares (68µm by 68µm) along each of seven radial arms extending from the center of the drug microinjection (45˚, 90˚, 135˚, 180˚, 225˚, 270˚, and 315˚).

Baseline levels of Fos expression were established by quantifying expression in two control conditions, (1) normal BNST tissue of sham surgery to assess expression in the absence of damage from guide cannula implantation and microinjector tip insertion and (2) following vehicle microinjection in BNST to assess expression following microinjection track and vehicle-induced Fos expression. These baseline values were compared to Fos densities in each of the four drug conditions to assess the functional spread of neural activation or inhibition following DAMGO or muscimol microinjection.

DAMGO and muscimol Fos plumes were mapped as >500%, >300%, >200%, 75% (-25%), and 50% (-50%) of Fos expression relative to vehicle microinjections and to normal tissue. In each case, the distance of each range of Fos expression was measured from the microinjection center along each radial arm. Spread was considered to extend to the furthest sampling square that contained greater than or equal to the particular level of activation or suppression being evaluated. Finally, the distance was averaged across all seven radial arms to produce an average radius of elevation or suppression. This procedure was repeated for every level of activation and suppression. The resulting drug-induced change in Fos expression relative to controls was then mapped to visualize the plume of activation and/or suppression for each drug and dose.

In the final stage of mapping, the Fos plume data identifying functional drug spread was merged with the behavioral data. DAMGO excitatory plumes symbols were created based on the radius of intense (>500%), moderate (>300%), and low (>200%) changes in Fos expression compared to vehicle controls. Muscimol plumes were constructed similarly, but had a slightly different structure comprising a small inner excitatory plume (>300% increase) surrounded by an inhibitory anti-plume with intense (-50%) and moderate (-25%) regions of Fos suppression. The verified bilateral microinjection centers of each rat are indicated by a pair of these symbols, and then color-coded to indicate specific behavioral effects. Microinjection centers for each rat were mapped in the coronal, sagittal, and horizontal planes; for the latter two, bilateral placements were collapsed onto a single unilateral map. Separate maps were constructed for each drug treatment and dependent variable. Thus, each symbol conveys information for each individual subject about (1) the location of drug microinjection, (2) the

functional spread of drug *in vivo* and (3) the behavioral effect of the drug treatment on the particular dependent variable being presented.

*Statistical Analyses*

The effect of each drug condition on all dependent variables was assessed using within-subjects ANOVA (drug), followed by paired-samples *post hoc* comparisons when appropriate. Between subjects ANOVA (site) was used for each drug treatment condition to test for regional differences in behavior within BNST, and also to compare the size of fos plumes and anti-plumes in the (dose,intensity). Between subjects ANOVA (injection day) was also used to assess the impact of injection order.

## ***Results***

*Summary*

In general, mu-opioid stimulation with DAMGO at sites throughout the BNST potently increased feeding behaviors. By contrast, BNST inhibition with muscimol decreased the number of feeding bouts. In addition, muscimol (but not DAMGO) increased fearful treading behavior and general locomotion, especially at the highest dose tested. The feeding suppression and fearful elicitation effects of muscimol, especially at the lower dose, were most prominent at more ventral sites in the BNST. No anatomical gradients were found for mu-opioid stimulation at either dose.

There was no effect of drug injection order on any of the dependent variables tested in this study, so treatment conditions were collapsed across days. A main effect of sex [$F(1,26)=7.71$, $p=0.01$] was found, with males eating more across all treatment

conditions than females. This effect is most likely the result of the larger size of the males (on average 150-200g heavier than the females), and not sex differences in response to drug condition [all Drug*Sex interactions = n.s.].

*Fos plume mapping*

Fos plumes indicating the functional spread of our neurochemical manipulations were constructed using histology of neural tissue from a separate group of rats. Analysis of Fos expression in each of the microinjection conditions used during food take testing indicated that for the drugs/doses/volumes used above, neuronal modulation was primarily limited to within BNST. (see Figure 1 and Table 1)

DAMGO microinjections were predominately excitatory. Fos expression increased by five times relative to control tissue in a small, intense excitatory plume near the microinjection center (0.05ug mean radius = 0.11mm; 0.1ug = 0.11mm). In a larger, intermediate zone of excitation, DAMGO tripled Fos expression (0.05ug = 0.29mm; 0.1ug = 0.25mm), and an even larger zone of low excitation displayed double the Fos expression of control tissue (0.05ug = 0.44mm; 0.1ug = 0.35mm). Assuming that these plumes are roughly spherical, the inner, intermediate, and outer plumes would have total volumes of approximately 0.005$mm^3$, 0.104$mm^3$, and 0.35 $mm^3$, respectively for 0.05ug dose. Plume volumes for the 0.1ug dose of DAMGO were slightly smaller, measuring 0.006$mm^3$, 0.062$mm^3$, and 0.175$mm^3$ for the inner, intermediate, and outer plumes, respectively. However, the plumes of the two DAMGO doses were not statistically distinct in size at any intensity level [Dose*Intensity, $F(2,45)=0.919$, p=n.s]. The estimated total volume of BNST is approximately 3$mm^3$ (~2mm rostro-caudal, 0.4 to

1.2mm medio-lateral, and 0.6 to 2mm dorso-ventral), meaning that the outer excitatory plume for the 0.05ug dose of DAMGO filled only about 15% of the BNST, while the outer plume for the 0.1ug dose filled only about 12%. The small, more intense inner plume filled only 4% of BNST for both DAMGO doses.

Interestingly, in every DAMGO microinjection at both doses of DAMGO we also saw outer skins of inhibition (-50% of control Fos expression). These were normally smaller than the regions of excitation in the same tissue, and had an average radius that fell between the intermediate and outer excitatory plume (data not shown). These mixed regions of excitation and inhibition are consistent with a prior report describing the reaction of BNST neurons to local microinfusion of morphine. (Casada & Dafny, 1993) Of the neurons sampled in that study which displayed responsiveness to morphine, roughly half responded with excitation while the other half responded with inhibition.

In contrast to DAMGO, both doses of muscimol produced very small inner zones of moderate excitation surrounded by large inhibitory anti-plumes. The inner zone of triple Fos expression relative to controls was roughly the size of the inner DAMGO plume (75ng = 0.1mm; 225ng = 0.11mm), though weaker than the five times expression seen with opioid stimulation in this zone. A larger intermediate anti-plume was found that displayed half the Fos expression of control tissue (75ng = 0.45mm; 225ng = 0.42mm), and an outer anti-plume with 25% less Fos expression than control tissue was found to extend nearly to the edge of our sampling grid (75ng = 0.59mm; 225ng = 0.53mm). As with DAMGO, the two doses of muscimol tested here generated identical plumes at all levels of intensity [Dose*Intensity, $F(2,42)=1.347$, p=n.s.] Although the muscimol plumes were larger in total volume (75ng = 0.84$mm^3$; 225ng = 0.61$mm^3$) than the

DAMGO plumes [F(4,120)=25.1, p=0.001], the maximum volume for the plumes of both muscimol doses were still much small than the total estimated volume of BNST (75ng = 28% of BNST volume; 225ng = 20%).

*DAMGO enhances food intake and muscimol decreases feeding bouts*

A within-subjects ANOVA resulted in a significant main effect of drug treatment on total food eaten in grams [F(2.96, 77.12)=27.80, p=0.001], total time eating [F(2.36,63.60)=24.41, p=0.001], and number of feeding bouts [F(2.47,66.86)=23.65, p=0.001]. Post hoc comparisons revealed that both the 0.05ug and 0.1ug doses of DAMGO significantly enhanced all three of these feeding measures relative to treatment with vehicle by nearly 300% (Figure 2,4). However, there was no difference in the effectiveness of the two DAMGO doses at stimulating feeding behavior.

Post hoc comparisons also revealed suppression of feeding bouts with the 225ng dose of muscimol, which were reduced to only 50% of vehicle levels (post hoc, $p<0.05$; Figure 4), as well as non-significant reductions in both food intake in grams and total time eating (Figure 3,4). The 75ng dose of muscimol did not significantly impact any feeding behaviors in this analysis.

In addition to feeding behavior, rats also had access to water during the test session. Drug treatment moderately reduced total time spent drinking [F(2.63,70.94)=2.93,p=0.046], and post hoc tests revealed a significant decrease for the 0.1ug dose of DAMGO (Figure 4). There was also a similar main effect of drug treatment on drinking bouts [F(3.5, 94.6)=3.61, p=0.012], which post hoc tests linked to significant suppression of drinking bouts by both doses of DAMGO (Figure 4).

*Muscimol increases defensive treading behavior and locomotion*

A significant main effect of drug treatment on defensive treading was found [$F(2.05,55.48)=11.20$, $p=0.001$], and post hoc comparisons revealed a significant increase in treading with the 225ng dose of muscimol (Figure 5,6). Treading was also increased with the 75ng dose, though not significantly. There was also a significant main effect of treatment on cage crosses [$F(2.13,57.43)=20.39$, $p=0.001$], and post hoc comparisons revealed significantly increased crosses with both doses the 75ng and 225ng doses of muscimol, with no significant dose effect (Figure 6). DAMGO had no effect on either treading or cage crosses (Figure 6). Rearing behavior was not affected by any drug treatment condition, indicating that muscimol does simply increase all locomotor behaviors (Figure 6).

The observed behavior of muscimol-treated animals helps to further clarify the enhancement of both treading and cage crosses. Muscimol-treated rats would typically stay close to the perimeter of the test chamber and make repeated circuits. Treading typically occurred upon an encounter with chamber corners and was generally directed towards the corner itself. This is consistent with previous reports of defensive treading in a test chamber devoid of a discrete threatening stimulus. (K. S. Smith & Berridge, 2005)

*Other behavioral effects*

There was a significant main effect of drug treatment on total time spent sleeping during the test session [$F(1.95, 52.72)=12.34$, $p=0.001$; Figure 4]. Post hoc tests revealed that all drug treatment conditions decreased sleeping relative to the vehicle condition,

though the above results indicate that DAMGO and muscimol treatments reduced sleep for different reasons (increase in time spend engaged in feeding vs. fearful and locomotor behaviors, respectively).

*Anatomical gradients*

Given the prominent anatomical, physiological, and neurochemical differences along the rostro-caudal(Choi et al., 2007; Dong & Swanson, 2004b), medio-lateral(de Olmos & Heimer, 1999), and dorso-ventral(Casada & Dafny, 1993) axes of the BNST, we also examined whether the behavioral effects we observed showed any distinct anatomical gradients.

Placements were classified as dorsal BNST (n=23) if they were above DV: -6.8mm from skull surface (roughly the level of the anterior commissure), and ventral BNST (n=5) if they were below this line (derived from (Casada & Dafny, 1993) and (Massi et al., 2008)). The 75ng dose of muscimol was the only drug treatment to generate significant dorso-ventral gradients, which encompassed both feeding and fearful behaviors (Figure 7). Ventral microinjection sites of 75ng muscimol significantly suppressed investigatory sniffs of the chow pellets [(F1,20)=4.33, p=0.05] and marginally decreased time spent eating [F(1,20)=3.05, p=0.096] and number of eating bouts [F(1,20)=3.05, p=0.096], relative to more dorsal sites. Additionally, ventral microinjections of 75ng muscimol increased defensive treading behavior [F(1,20)=4.45, p=0.048]. It is worth noting that these gradients existed only at our lower dose of muscimol; at our higher 225ng dose, there were no significant dorso-ventral gradients in

behavior. This indicates that sites in ventral BNST are particularly sensitive to the both the feeding and fearful effects of muscimol.

Along the rostro-caudal axis, placements were assigned to one of four categories: BNST/accumbens shell transition zone (n=5, AP: 0.84mm to 0.48mm), anterior BNST (n=11, AP: >0.48mm to 0.00mm), central BNST (n=9, AP: >0.00mm to -0.48mm), and posterior BNST (n=3, AP: >-0.48mm). No significant gradients were observed withing any drug condition along the rostro-caudal axis. Interestingly, anatomical mapping of muscimol feeding indicated possible differences on feeding time *between* doses in anterior regions of BNST (see Figure 3). In fact, when the effect of 75ng muscimol was compared to the effect of 225ng muscimol at regions anterior to -0.2mm behind bregma, it was found that the low dose of muscimol increased feeding by 234% relative to vehicle while the higher dose reduced feeding by 75% [t-test comparing percent change in feeding: $t(17)=2.2$, $p=0.043$].

Placements were categorized as either medial BNST (n=9) or lateral BNST (n=18) based on the classification scheme of (Paxinos & Watson, 2007). One rat was excluded from the medial-lateral analysis because both microinjection centers were precisely on the medial-lateral boundary. Medial placements in animals treated with 75ng of muscimol generated more incidents of carrying chow pellets than lateral placements [$F(1,20)=4.93$, $p=0.018$]. No other significant medial-lateral gradients were observed.

There were no significant gradients in any of the axes for either dose of DAMGO, indicating that the behavioral changes we observed following opioid manipulation are not linked to any particular sub-region of BNST, and consistent with a relative homogenous distribution of mu-opioid receptors thoughout BNST.

***Discussion***

We found that mu-opioid stimulation throughout BNST substantially increased intake of standard chow pellets in *ad libitum* fed rats. We also found that GABA-ergic disruption of neuronal activity within BNST suppressed feeding bouts at the higher of two doses tested and simultaneously increased locomotion and defensive treading behavior. Although the effect of opioid stimulation was homogenous throughout BNST at the doses tested here, a dorso-ventral gradient was detected for the effect of our lower dose of muscimol on both feeding and aversive behaviors.

*Mu-opioids stimulate feeding in BNST*

Previous research has shown that BNST is one of a series of nuclei that consistently showed enhanced activation (as measured by Fos expression) in response to either food intake or infusion (centrally or locally) of feeding related neuropeptides. (B. H. Li et al., 1994; Mullett et al., 2000; Mungarndee et al., 2008; Park & Carr, 1998) BSNT has also been identified as a substrate of corticotropin releasing factor (CRF) induced anorexia and its subsequent reversal by the opioid peptide Nociceptin/Orphanin FQ (N/OFQ). (Ciccocioppo et al., 2003) N/OFQ, however, does not act alone to stimulate feeding, but merely rescues the standard level of feeding found in vehicle-treated animals (Ciccocioppo, Cippitelli, Economidou, Fedeli, & Massi, 2004). To our knowledge, the present study is the first to demonstrate an increase in feeding following neurochemical manipulation of BNST.

Our finding of potently increased food intake following intra-BNST mu-opioid stimulation is consistent with numerous reports of opioid-induced feeding at locations in the basal forebrain, both within and outside the extended amygdala. Opioid stimulation in the nucleus accumbens, ventral and dorso-medial striatum, caudal ventral pallidum, ventro-medial hypothalamus and PVN have all been shown to enhance food intake (Bakshi & Kelley, 1993a, 1993b; Giraudo et al., 1998; Gosnell et al., 1986; Kelley et al., 2002; Pecina & Berridge, 2000, 2005; K. S. Smith & Berridge, 2005), in particular of highly palatable and energy dense foods (Naleid, Grace, Chimukangara, Billington, & Levine, 2007; Zhang & Kelley, 2000). In accumbens, opioids also to increase the breakpoint for responding for a food reward (Zhang et al., 2003). Within extended amygdala, similar feeding effects are found after opioid stimulation of the CeA (Giraudo et al., 1998; Gosnell, 1988), and possibly also at others sites in the extended amygdala including the SLEA and IPAC (Na, 2008). Together, these sites form a large network of opioid-sensitive feeding sites within the ventral forebrain, a virtually unbroken chain of nuclei extending from rostral accumbens to caudal CeA (a nearly 6mm rostro-caudal corridor).

We did not find any difference in effectiveness between the two doses of DAMGO (0.05ug and 0.1ug) used in this study, a finding mirrored in the highly similar size and intensity of the Fos plumes observed across both doses. Future studies will be necessary to evaluate lower DAMGO doses to establish the threshold for BNST sensitivity to mu-opioid induced feeding. Our doses of DAMGO are similar or indentical to those found to stimulate robust feeding in the shell of the nucleus accumbens (Pecina & Berridge, 2005), caudal ventral pallidum (K. S. Smith & Berridge, 2005), and CeA

(Gosnell, 1988; Mahler & Berridge, In press). Given the apparent homogeneity in the ability of mu-opioids to induce feeding in these sites, it would be of interest to explore whether there is similar homogeneity in the sensitivity of these regions to mu-opioid stimulation. Mu-opioid receptor binding and density does appear to be somewhat heterogeneous among these nuclei, with CeA displaying lower expression of both membrane-bound receptors and receptor mRNA than BNST, accumbens, or ventral pallidum (Mansour et al., 1995).

*Intra-BNST muscimol enhances defensive treading*

In contrast to opioid stimulation, GABA-ergic stimulation in BNST resulted in decreased feeding bouts and increased cage crosses and treading. Although the highest dose of muscimol tested here reduced several other feeding measures (including total intake in grams and total time eating), these reductions did not maintain statistical significance. One possible explanation is the low level of baseline feeding under vehicle treatment, likely the result of testing in non-deprived subjects using standard lab chow. Previous studies showing diminished food intake following intra-BNST CRF microinjection utilized food deprived subjects that demonstrated elevated baseline food intake, allowing relatively more room to observe a decline in feeding. (Ciccocioppo et al., 2004; Ciccocioppo et al., 2003) It would be of interest in future studies to either employ mild food restriction before testing with intra-BNST muscimol, or to use a more palatable food to encourage high baseline feeding.

Treading is a natural defensive behavior emitted by rodents as an adaptive response to an environmental threat, (Owings & Coss, 1977; Treit, Pinel, & Fibiger,

1981) and is also displayed in laboratory environments following neurochemical manipulations that generate fearful or aversive motivational states (Faure et al., 2008; Reynolds & Berridge, 2001, 2008). Although there was not a discrete aversive stimulus present at which the rodents might direct treading (such as a shock prod), there are several reasons to believe that the behavior we observed more closely resembles defensive treading rather than neutrally valenced locomotor stereotypy. First, we observed treading directed primarily at the corners of the testing chamber in conjunction with repeated circling of the chamber perimeter, instead of randomly oriented treading throughout the chamber. Second, we also observed distress vocalizations upon removal of many muscimol-treated animals from the testing chamber, as well as occasional escape attempts; similar vocalizations and escape attempts were never observed in DAMGO-treated animals. These aversive behaviors will be more carefully quantified in additional studies.

Treading has also been observed in several other forebrain sites following GABA agonism. Within extended amygdala, muscimol at a concentration similar to our highest dose generated robust treading in the CeA,(Mahler & Berridge, In press) indicating yet another homogeneous behavioral effect in extended amygdala. Muscimol in caudal accumbens shell results has also been shown to produce treading, which can be further enhanced by the addition of aversive environmental qualities such as bright lights and loud music (Reynolds & Berridge, 2001, 2008). This is in stark contrast to the effect of muscimol in rostral accumbens shell, where the manipulation greatly increases feeding and place preference (Reynolds & Berridge, 2002). The finding here of increased defensive treading after inactivation of BNST is consistent with previous reports of a role

for BNST in other aversive and stressful motivational states, including anxiety and drug withdrawal (M. Davis, 1998; Koob, 2003).

*Anatomical localization of behavioral effects*

Given the density of GABA- and opioid-sensitive feeding sites within the ventral forebrain, one could potentially argue that the feeding effects that we observed after microinjections into BNST were not, in fact, driven by BNST but instead by the diffusion of microinjections to other neighboring sites. However, data from our fos plume analyses of functional drug spread indicates that microinjection of the doses and volumes tested in this experiment do not spread substantially beyond the boundaries of BNST. This allows us to confidently conclude that BNST can now be added to the vast forebrain network of sites where opioids can influence feeding behavior and GABA agonism can generate aversive behavior, and provides strong evidence for the localization of these effects in BNST and not to neighboring sites such as the nucleus accumbens or ventral pallidum.

Our DAMGO doses were equally effective at stimulating food intake throughout BNST. However, the lack of anatomical specificity with feeding does not preclude localization for other behaviors, as evidenced by the restricted mu-opioid hedonic hotspot located in rostro-dorsal quadrant of accumbens shell, where DAMGO stimulates feeding equivalently at all locations (Pecina & Berridge, 2005). We did find a significant dorso-ventral gradient in both feeding behavior and treading for muscimol, though this gradient only emerged for our lower dose of muscimol. This may indicate that dorsal BNST is less sensitive to GABA-ergic inhibition than ventral BNST, which is supported by the lower

responsiveness of dorsal BNST neurons to acetylcholine, norepinephrine, and morphine (Casada & Dafny, 1993).

*What is the mechanism for BNST food intake?*

Now that BNST has been implicated in appetitive motivational processes, it will be important to identify precisely what psychological mechanisms are driving the observed increased in feeding. Although voluntary food intake is a general measure of appetitive motivation, there are a number of distinct (though not exclusive) possibilities that could result in increased feeding. Additional studies will be required that more precisely test individual explanations. Previous research suggests several explanations that merit particular attention: stress, general appetitive motivation, and incentive salience 'wanting.'

*Stress*: Stress, both acute and chronic, can have dramatic impacts on feeding in both humans and non-human animals (Dallman et al., 2003; Rutters, Nieuwenhuizen, Lemmens, Born, & Westerterp-Plantenga, 2009). BNST has been repeatedly implicated in the regulation of hypothalamic-pituitary-adrenal (HPA) axis activity, (Choi, Evanson et al., 2008; Choi et al., 2007; Choi, Furay et al., 2008) and also argued to comprise part of brain stress and anti-reward systems that are persistently dysregulated during drug addiction. (Aston-Jones & Harris, 2004; Koob, 2003; Koob & Le Moal, 2008) Behaviorally, BNST has been linked to the acute response to stressful events such as immobilization (Casada & Dafny, 1991; Henke, 1984), as well as relapses in drug seeking following exposure to acute stressors (Erb et al., 2001; Erb & Stewart, 1999),.

There are reasons to believe that our current mu-opioid feeding effect in BNST is not driven by stress. For one, our food intake testing was done using only standard rodent chow rather than a highly palatable food; studies of stress-induced feeding tend to show much more pronounced increases in highly palatable rather than standard food source. Additionally, infusion of a stress-related peptide, CRF, into BNST actually decreases feeding, rather than stimulating intake (Ciccocioppo et al., 2003). However, given the pronounced role of BNST in both acute and chronic brain stress systems, the possibility of stress-induced feeding must be evaluated. If, in fact, the feeding we observed was purely the result of stressful intake, this would be evidence against BNST's role in appetitive motivational processes.

*General appetitive motivation*: Enhanced feeding could also be the result of a broad increase in general appetitive motivation. For example, increased BNST activity, as measured by c-Fos expression, has been observed after a variety of different appetitive manipulations, including feeding and exposure to sexual stimuli (B. H. Li et al., 1994; Mungarndee et al., 2008; Taziaux et al., 2008). These suggest that BNST may broadly act to increase adaptive appetitive behaviors in situations where reward stimuli are present or where cues signal that a rewarding UCS may soon become available.

*'Wanting'*: More specifically, opioid stimulation in BNST could increase incentive salience 'wanting,' a discrete mechanism of appetitive motivation, causing the food pellets to be more desirable and eliciting greater approach and consumption. (Robinson & Berridge, 1993, 2000) There are several reasons to think that BNST could be involved in the generation and attribution of 'wanting.'

First, BNST sends strong, excitatory projections to midbrain dopaminergic nuclei, including the ventral tegmental area (VTA). (Georges & Aston-Jones, 2001; Massi et al., 2008) Dopamine is considered to be the primary neurotransmitter involved in the assignment of incentive salience 'wanting,' (Berridge, 2007; Berridge & Robinson, 1998; Robinson & Berridge, 1993) and BNST opioids could potentially drive increase feeding by enhancing the firing of dopaminergic cell populations in the VTA, and subsequently increasing dopamine release in limbic and cortical targets. Second, BNST is not only a source of excitatory projections to dopaminergic nuclei, but also a target of enhanced dopamine release following administration of reinforcing drugs. (Carboni et al., 2000) Enhanced dopaminergic transmission, either via prior sensitization or acute treatment with amphetamine, in both the shell of the nucleus accumbens and also the ventral pallidum enhance 'wanting' for reward cues. (Tindell et al., 2005; Wyvell & Berridge, 2000, 2001) Finally, mu-opioid stimulation in CeA, another extended amygdala nucleus, directly enhances 'wanting' for Pavlovian reward cues in autoshaping testing, in addition to increasing food intake. (Mahler & Berridge, In press) CeA and BNST, in particular the lateral divisions of BNST, share strong reciprocal connectivity and strikingly similar neural architecture (Alheid, 2003; de Olmos & Heimer, 1999). Based on these strong anatomical similarities and the identical opioid feeding effect in both structures, it is possible that BNST is also a substrate for mu-opioid 'wanting.'

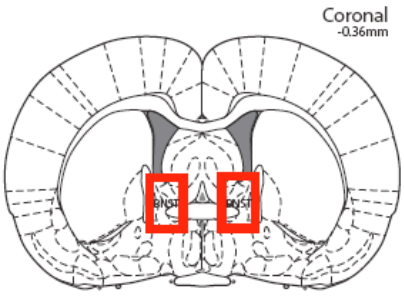
*Conclusion*

In conclusion, we have shown that mu-opioid stimulation of BNST can potently increase feeding, while GABA-ergic agonism in the same structure inhibits some

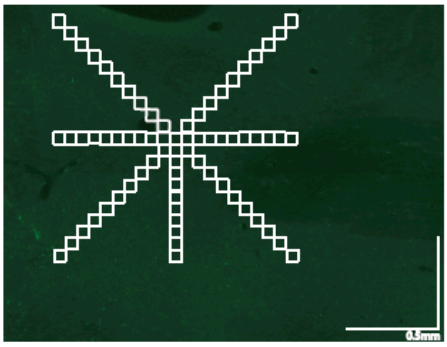
measures of feeding while simultaneously enhancing a fearful defensive behavior. These results point to a role for BNST in mediating both appetitive and aversive motivation. Future studies will be necessary to precisely characterize the psychological mechanism driving the observed increase in feeding behavior.

**Figure 2.1. Fos plumes in BNST.** *Target Region*: Highlights the location of BNST in a coronal slice, taken from Paxinos & Watson (2007). *Radial Arm Fos Sampling*: An example of the radial counting grid. Each grid square measures 68um x 68um, with 10 squares emanating from each radial arm. Grid squares falling in ventricles or over white matter tracts were excluded from quantification and analysis. *Normal BNST*: Representative image from uninjected, virgin tissue in BNST. *Vehicle BNST*: Representative image from vehicle microinjected tissue in BNST, showing a small area of increased Fos expression (relative to normal) near the injection site. *DAMGO (0.05ug), DAMGO (0.1ug)*: Representative plumes (relative to normal and vehicle controls) from DAMGO microinjected tissue. Note the presence of the small inhibitory regions near the excitatory plumes. *Muscimol (75ng), Muscimol (225ng)*: Representative plumes (relative to normal and vehicle controls) from muscimol treated tissue. All images shown are 2x2 tiled images taken at 10x magnification.

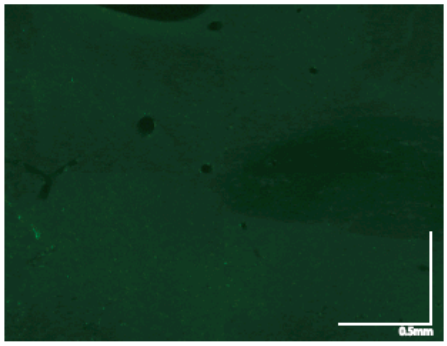
Target Region



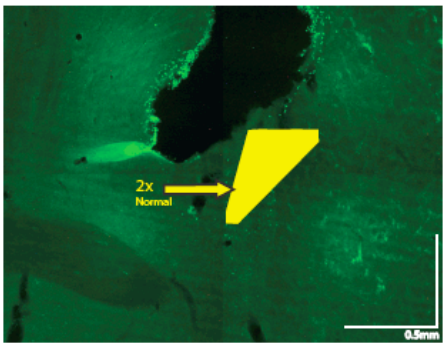

Radial Arm Fos Sampling



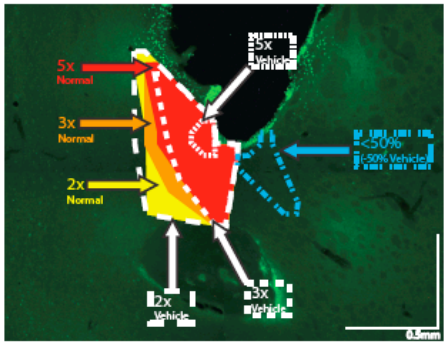
Normal BNST



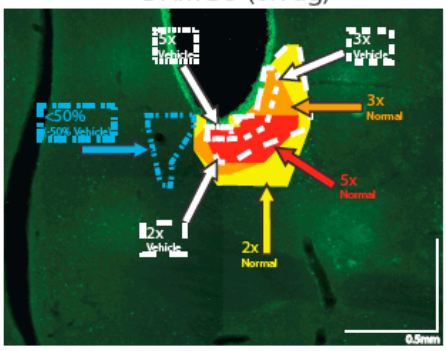
Vehicle BNST



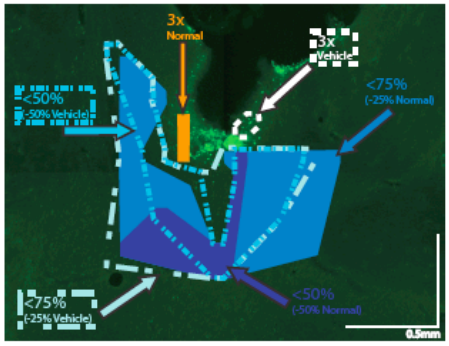

DAMGO (0.05ug)



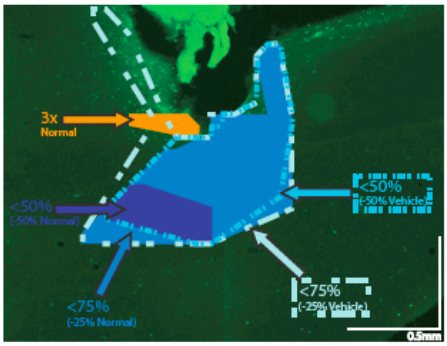

DAMGO (0.1ug)




Muscimol (75ng)




Muscimol (225ng)

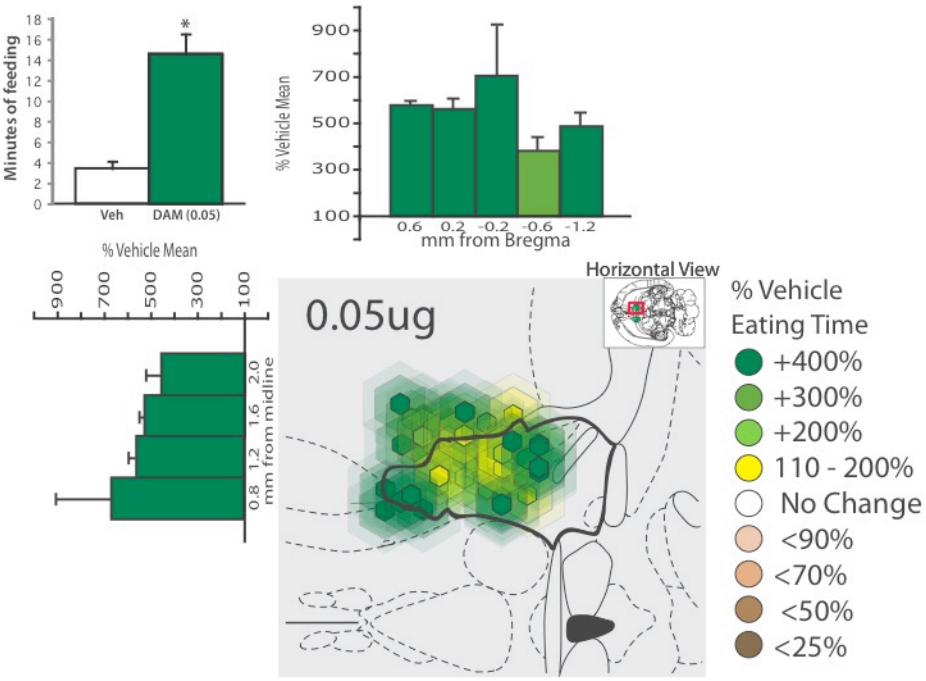


| | Mean Fos Plume Radius (mm) | | | Estimate Fos Plume Volume (mm3) | | |
|---|---|---|---|---|---|---|
| | Inner plume | Middle plume | Outer plume | Inner plume | Middle plume | Outer plume |
| | *500% control* | *300% control* | *200% control* | *500% control* | *300% control* | *200% control* |
| Vehicle | 0.07 | 0.16 | 0.31 | 0.001 | 0.017 | 0.120 |
| DAMGO (0.05ug) | 0.11 | 0.29 | 0.44 | 0.005 | 0.104 | 0.345 |
| DAMGO (0.1ug) | 0.11 | 0.25 | 0.35 | 0.006 | 0.062 | 0.175 |
| | *300% control* | *-50% control* | *-25% control* | *300% control* | *-50% control* | *-25% control* |
| Muscimol (75ng) | 0.10 | 0.45 | 0.59 | 0.005 | 0.380 | 0.840 |
| Muscimol (225ng) | 0.11 | 0.42 | 0.53 | 0.005 | 0.305 | 0.613 |

**Table 2.1. Fos plume radii and estimated volumes.** Mean radii (left) and volume (right) are listed for vehicle, DAMGO (0.05ug), DAMGO (0.1ug), muscimol (75ng), and muscimol (225ng) conditions. Plume sizes were calculated compared to normal tissue alone for the vehicle condition, and compared to both normal and vehicle control conditions for all other groups.

**Figure 2.2. DAMGO in BNST increases feeding time.** Feeding time enhancements following 0.05ug (top) and 0.1ug (bottom) DAMGO microinjections are mapped rat-by-rat onto a horizontal view of BNST, showing both the within-subject change in feeding time relative to vehicle day (color) and the functional spread of the microinjection based on Fos plume data (size). Each map symbol is composed of three nested hexagons; the inner symbol shows the average size of intense 5x activation, the middle symbol shows 3x activation, and the outer symbol shows 2x activation. Bar graphs along the rostro-caudal and medial-lateral axes show the average drug effect within each 0.4mm wide region. Bar graphs showing absolute comparisons of vehicle and drug feeding time (in minutes) can be found above and to the left of each anatomical map. * indicates differences from vehicle, $p<0.05$.

# BNST DAMGO Enhances Time Eating



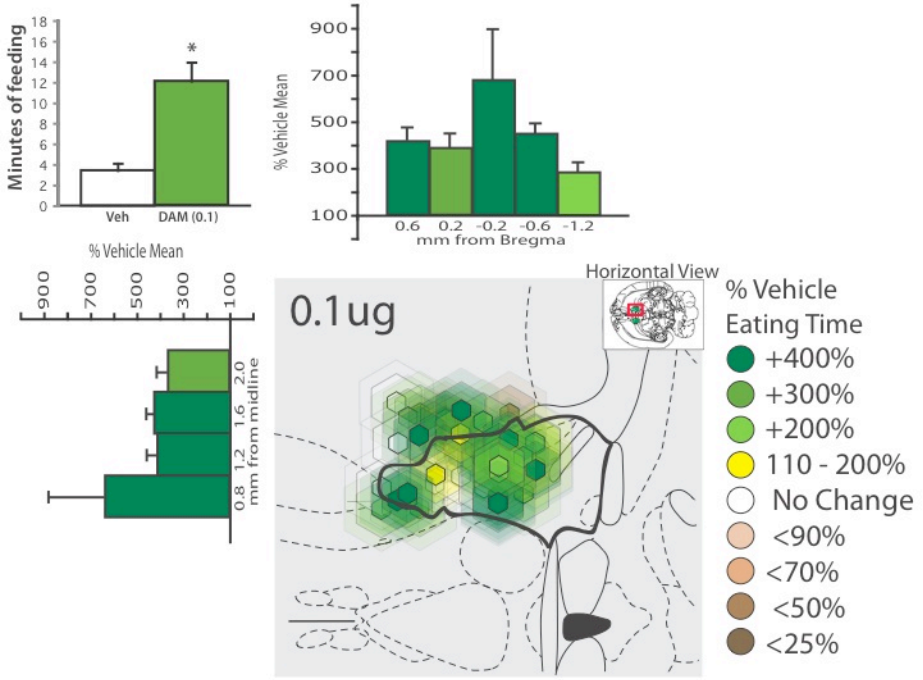
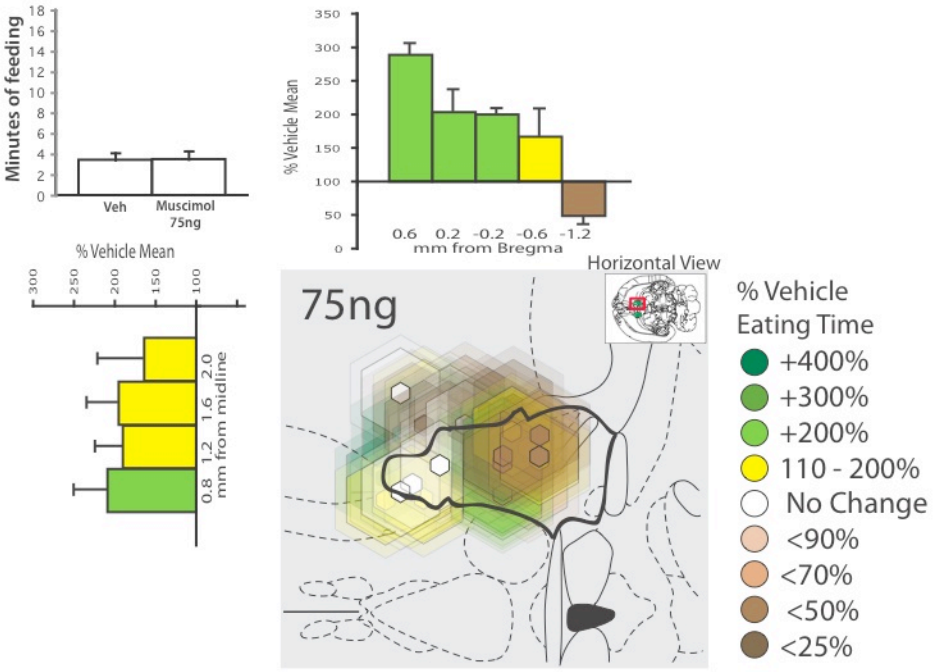
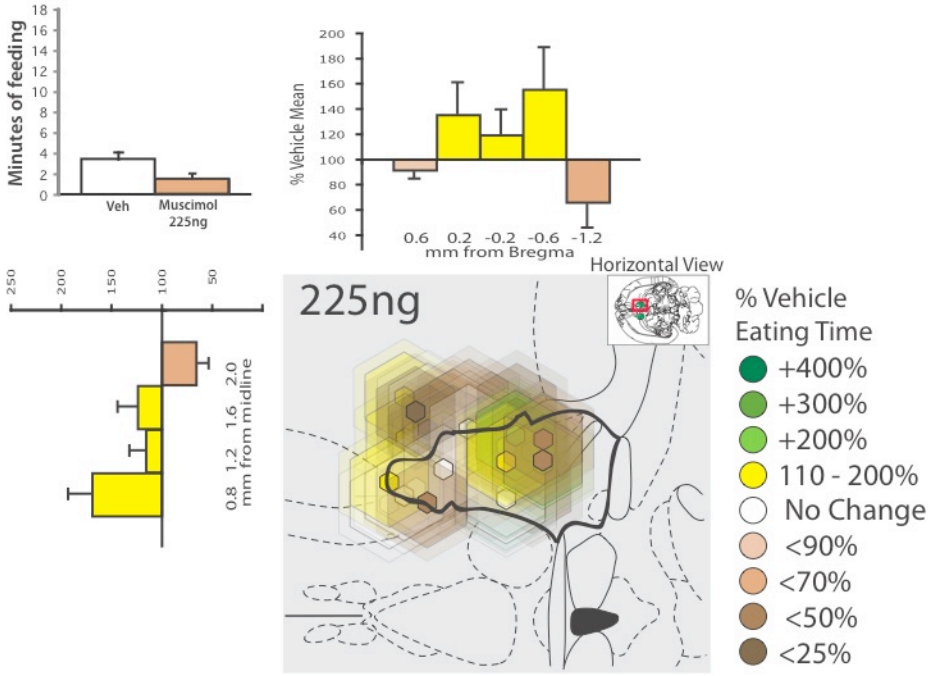



**Figure 2.3. Muscimol in BNST does not significantly change feeding time.** Anatomical maps of feeding time changes following intra-BNST muscimol at 75ng (top) and 225ng (bottom). For muscimol plumes, the inner symbol shows 3x control activation, the middle symbol shows -50% control inhibition, and the outer symbol shows -25% control inhibition.

# BNST Muscimol Does Not Affect Eating Time



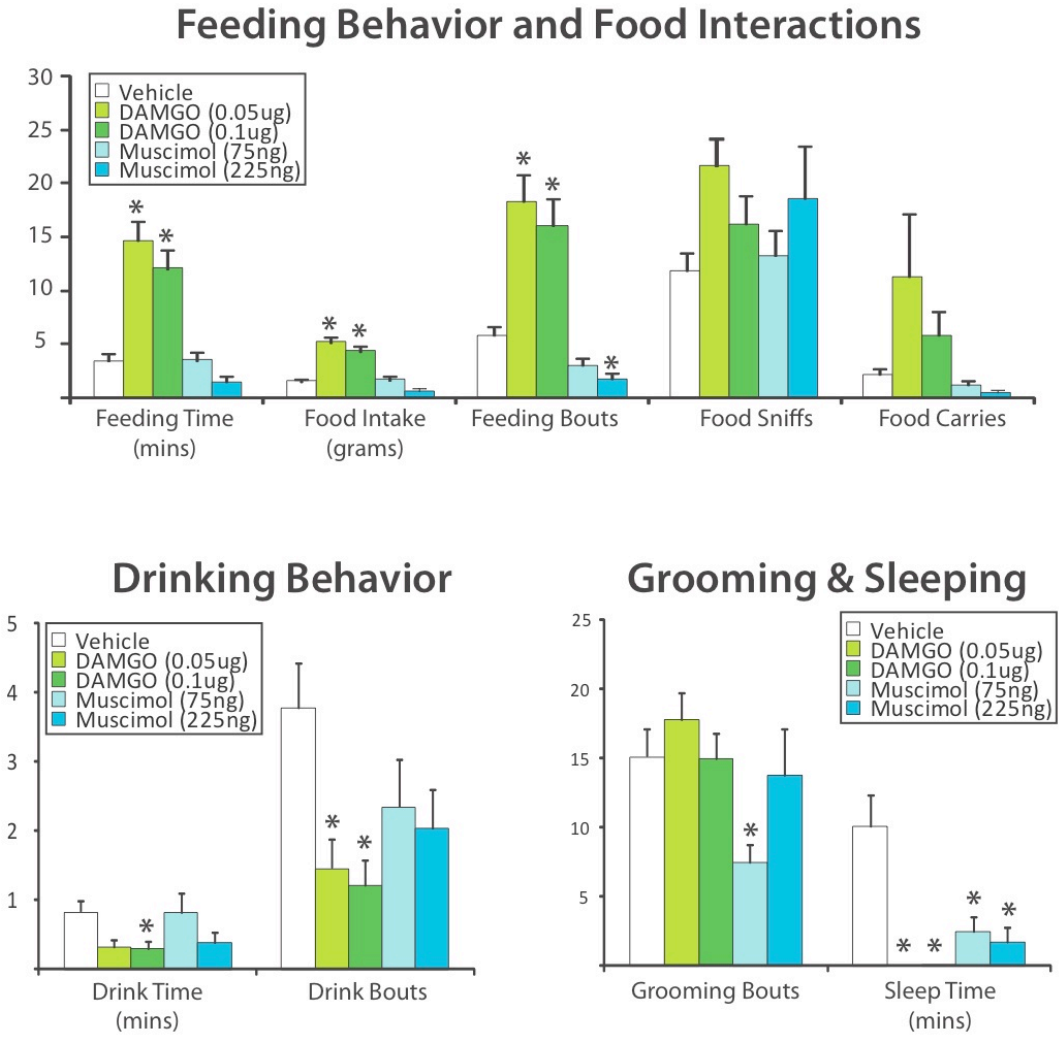



**Figure 2.4. Feeding, drinking, and other behavioral effects after intra-BNST DAMGO and muscimol.** Behavioral effects of vehicle (white bars), DAMGO 0.05ug (light green), DAMGO 0.1ug (dark green), muscimol 75ng (light blue), and muscimol 225ng (dark blue) on feeding, drinking, and other behaviors. * indicates differences from vehicle, $p<0.05$.

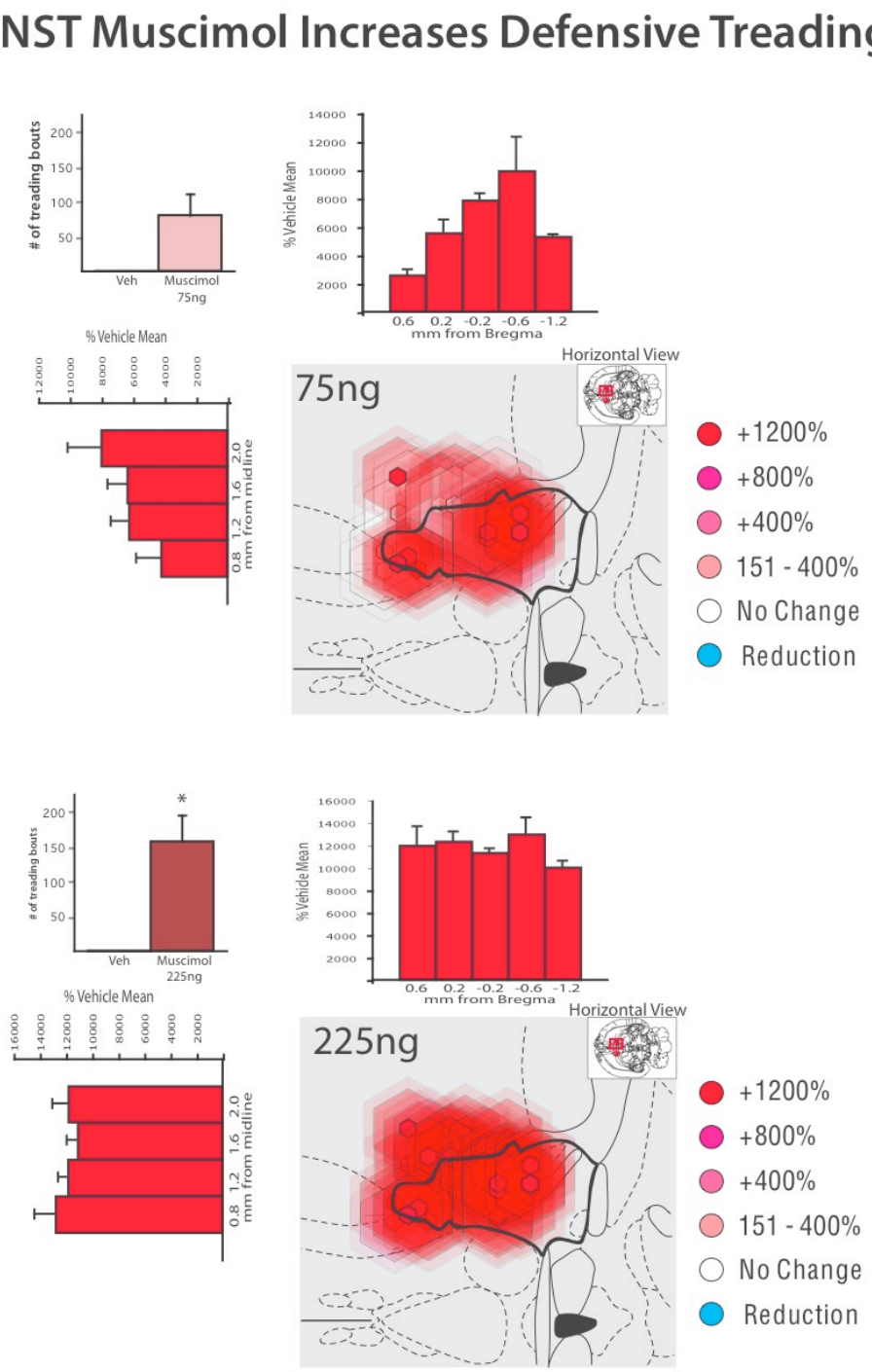



**Figure 2.5. Muscimol in BNST increases defensive treading behavior.** Anatomical maps of changes in defensive treading following intra-BNST muscimol at 75ng (top) and 225ng (bottom) doses. Defensive treading is robust throughout BNST, especially at the 225ng dose. * indicates differences from vehicle, $p<0.05$.

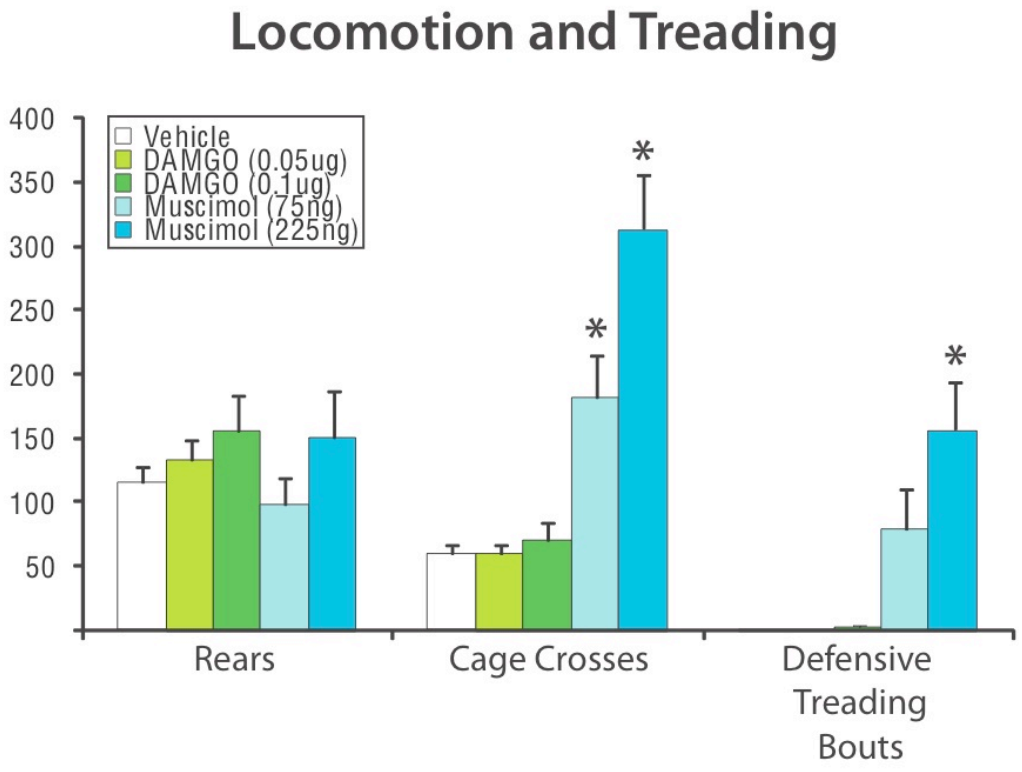



**Figure 2.6. Locomotor and defensive treading changes after intra-BNST DAMGO and muscimol.** Behavioral effects of vehicle (white bars), DAMGO 0.05ug (light green), DAMGO 0.1ug (dark green), muscimol 75ng (light blue), and muscimol 225ng (dark blue) on rearing, cage crosses, and defensive treading behavior. * indicates differences from vehicle, $p<0.05$.

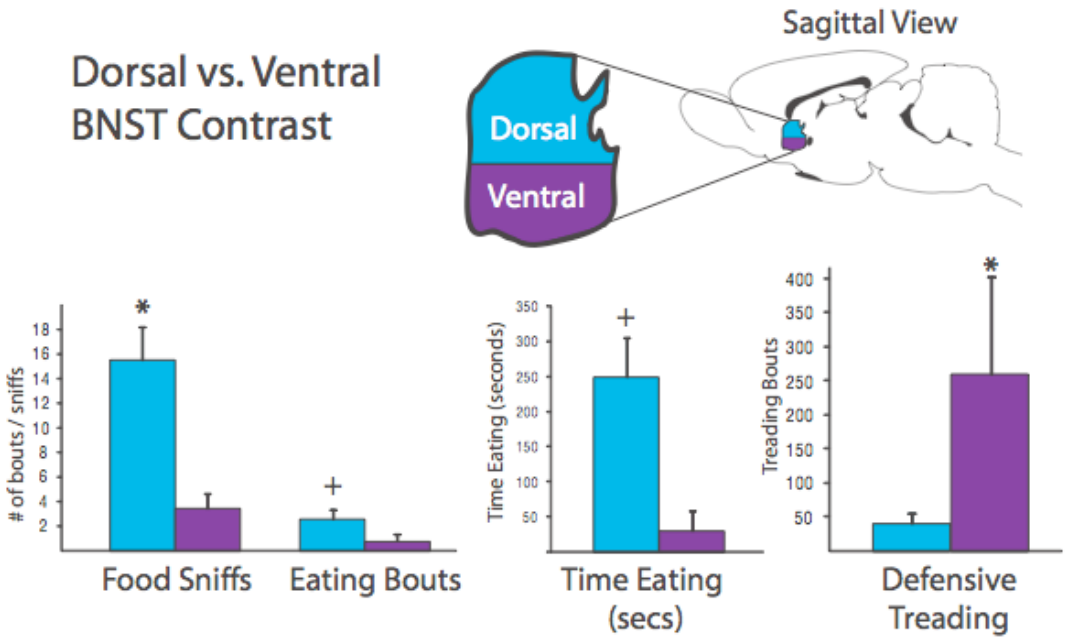



**Figure 2.7. Dorso-ventral gradients after intra-BNST microinjection of 75ng muscimol.** The 75ng dose of muscimol was more effective at disrupting feeding related behaviors and enhancing defensive treading at sites in the ventral portion of BNST compared to the dorsal portion. * indicates differences from vehicle, $p<0.05$; + indicates differences from vehicle, $p<0.1$.

# Chapter 3

## Does Opioid Activation in the Bed Nucleus of the Stria Terminalis and the Shell of the Nucleus Accumbens Directly Increase Incentive Salience 'Wanting:' Tests of Autoshaping and Conditioned Reinforcement

### *Introduction*

Conditional stimuli (CS) repeatedly associated with a rewarding unconditional stimulus (UCS) can have powerful and sustained effects on behavior. Conditional cues come to possess some of the motivational properties of the UCS (Berridge, 2001; Bindra, 1978; Toates, 1986), and can act as 'motivational magnets', attracting UCS-appropriate behaviors that are phase-locked with CS presentation. One explanation for the ability of CS's to attract appetitive behaviors comes from the incentive salience hypothesis of motivation, which suggests that the motivational magnet quality of the reward CS results from the attribution of incentive salience 'wanting,' a psychological phenomena that imbues the CS with incentive value (Berridge, 2001, 2007; Robinson & Berridge, 1993). This attribution of 'wanting' to Pavlovian CS's, thereby inbuing them with some of the incentive properties of the linked UCS reward and thus making the CS desireable in its own right, is particularly helpful in explaining why animals sometimes attempt to consume reward CS's (even inanimate objects like metal levers and cue lights) (Boakes, 1977; Flagel et al., 2009; Tomie, Grimes, & Pohorecky, 2008), or why crack addicts occasionally "chase ghosts," scrambling for and attempting to smoke small pebbles that superficially resemble crack cocaine (Berridge, 2007; Rosse et al., 1993).

CS-UCS relationships are deeply adaptive, allowing organisms to utilize a predictive stimulus to energize behavior toward receipt or pursuit of an impending reward, such as food (Holland & Petrovich, 2005) or sex (Pfaus, Kippin, & Centeno, 2001; Waddell, 2005), or, in cases where the UCS is aversive, to mobilize escape or avoidance behavior (Eilam, 2005; Fendt & Fanselow, 1999; Misslin, 2003). However, cues can also potentiate maladaptive behavior, such as the intake of drugs of abuse, by helping to sustain continued drug taking or even by triggering a relapse in drug seeking after a period of abstinence (Marlatt, 1990; Robinson & Berridge, 1993, 2000; See, 2002, 2005; Shaham et al., 1994). A significant challenge in the affective neurosciences – and one with potentially great clinical relevance – is to locate and characterize neural substrates involved in cue-triggered motivation.

Here we chose to focus on the role of pair of forebrain nuclei in autoshaping and conditioned reinforcement: bed nucleus of the stria terminalis (BNST) and the shell of the nucleus accumbens. Although BNST has primarily been linked to aversive motivational processes (M. Davis et al., 1997b; Erb & Stewart, 1999; Koob, 1999), it has recently been advanced as a component of a larger anatomical macrosystem, the extended amygdala (Alheid, 2003; de Olmos & Heimer, 1999; Heimer, 2003; Heimer & Van Hoesen, 2006). This exciting new macrosystem includes BNST, plus two particular components of the classic amygdala (the central nucleus and medial nucleus of amygdala), plus a few other components (sublenticular extended amydala and IPAC). With substantial inputs from limbic cortex and brainstem dopaminergic centers, the extended amygdala is well positioned to participate in the attribution and/or modulation of incentive salience. In particular, recent studies from our lab showed that opioid stimulation of the central

nucleus of amygdala potentiated the 'motivational magnet' properties of a rat's preferred CS in autoshaping (Mahler & Berridge, 2009). This suggests that other components of extended amygdala, BNST, might also play a role in incentive salience of CSs for reward.

Indeed, BNST has recently shown μ-opioid dependent increases in UCS 'wanting' for a food reward (see Chapter 2) at doses similar to those found to stimulate feeding in another extended amygdala nucleus, the central amygdala (CeA) (Gosnell, 1988; Mahler & Berridge, 2009). Additionally, lesions of the closely related CeA have been shown to disrupt conditioned orienting in autoshaping (also known as Pavlovian conditioned approach or sign-tracking) paradigms (Gallagher, Graham, & Holland, 1990; Holland et al., 2002) and to completely disrupt Pavlovian to instrumental transfer (PIT) (Holland & Gallagher, 2003). Yet it remains unclear whether increases in 'wanting' for rewards CS's within the extended amygdala are limited to CeA, or whether they extend to other structures within this macrosystem, such as the bed nucleus of the stria terminalis (BNST).

It can be noted that many other structures have been shown to participate in the development and expression of autoshaping behavior, including the core and shell of the nucleus accumbens (Blaiss & Janak, 2009; Flagel, Watson, Robinson, & Akil, 2007; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999; Parkinson, Willoughby, Robbins, & Everitt, 2000; Phillips, Setzu, & Hitchcott, 2003), cingulate and prefrontal cortex (Bassareo, De Luca, & Di Chiara, 2007; Parkinson et al., 2000), the subthalamic nucleus (Uslaner, Dell'Orco, Pevzner, & Robinson, 2008), and the both basolateral and central

nuclei of the amygdala (El-Amamy & Holland, 2007; Gallagher et al., 1990; Holland & Gallagher, 2003; Mahler & Berridge, 2009).

In accumbens shell, reversible inactivation prior to testing disrupts expression of autoshaping behavior (Blaiss & Janak, 2009), though excitotoxic lesions of accumbens shell do not (Parkinson et al., 1999; Parkinson et al., 2000). Permanent lesions of accumbens shell do, however, impair PIT (Corbit, Muir, & Balleine, 2001). It remains unclear, though, precisely how accumbens shell works to increase CS 'wanting.' Although opioid stimulation in CeA has been shown to focus 'wanting' on a previously learned and preferred CS, it has been hypothesized that accumbens shell may play a broader role in the generation of incentive salience. Stimulation of accumbens shell, therefore, might more readily be able to act as 'a rising tide that floats all boats,' elevating 'wanting' for all available reward CS targets in the environment (Berridge, 2007). By comparison, opioid stimulation of CeA may instead focus enhanced incentive salience upon a particular CS in a 'winner take all fashion' (Mahler & Berridge, 2009).

However, it is not yet entirely clear whether opioid agonists in accumbens shell also potentiate 'wanting' *directly* in a CS motivation paradigm, as dopamine has been shown to in medial shell (Wyvell & Berridge, 2000, 2001). Some pilot PIT results indicate that, indeed, medial shell opioids can directly enhance CS 'wanting' (Pecina & Berridge, In Preparation). However, no one has yet investigated the effects of opioid stimulation of accumbens shell in an autoshaping paradigm (Mahler & Berridge, 2009).

Here, we fill that accumbens gap, and compare its role in autoshaping to that of BNST, when stimulated by DAMGO. Specifically, I test the ability of microinjections of the μ-opioid agonist DAMGO into BNST or accumbens shell to modulate two measures

of CS incentive salience 'wanting' that remain to be explored with these manipulations: autoshaping and conditioned reinforcement. Although dopamine is perhaps the most clearly implicated neurotransmitter system in the attribution of incentive salience (Wyvell & Berridge, 2001), opioid neurotransmitter systems also appear able to modulate 'wanting' for reward CS's. Pre-exposure to systemic heroin increased the conditioned reinforcement value of a previously learned reward CS (Ranaldi, Egan, Kest, Fein, & Delamater, 2009). Microinjection of the μ-opioid agonist DAMGO into CeA enhances appetitive responding in both autoshaping (Mahler & Berridge, 2009) and PIT (Mahler & Berridge, 2007) testing, while DAMGO in accumbens (both core and shell) has been shown to enhance PIT (Pecina & Berridge, In Preparation).

It is possible that DAMGO in BNST might increase CS motivational magnet 'wanting' in autoshaping and enhance the conditioned reinforcement value of the autoshaping CS+ for those animals who preferentially interacted with this CS during prior autoshaping testing, similar to the effects of DAMGO in a closely related extended amygdala nucleus, the CeA. Alternatively, μ-opioids in BNST may only modulate 'wanting' for UCS reward (as previously demonstrated in Chapter 2), but fail to affect reward CS's, consistent with studies showing that BNST lesions only disrupt fearful responding to unconditioned, and not conditioned, cues (M. Davis, 1998). For accumbens shell, I predict that DAMGO will generate a broadly assigned incentive salience to CS's, elevating appetitive behavior toward both preferred and non-preferred CS's in autoshaping and enhancing the conditioned reinforcement value of the autoshaping CS+ for all animals (regardless of their cue preference during autoshaping testing).

***Methods***

*Subjects*

A total of 107 Sprague-Dawley rats were used (females, 250-400g at the time of surgery; individual experiments utilized n's of 57 [during testing], 37 [after testing], and 13 [fos plume mapping]). Rats were housed in pairs (~21˚C; 12hr light/dark cyle, lights on at 9am) with *ad libitum* access to food (Purina 5001 chow; Purina Mills, St. Louis, MO) and tap water, except during autoshaping and conditioned reinforcement testing when food was slightly restricted (~15g/day/rat). During food restriction, chow was always delivered immediately after training or testing. All phases of the estrous cycle were included in testing. All procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan in accordance with National Institute of Health guidelines.

*Autoshaping Paradigm*

Exposure to repeated pairings of a discrete CS+ metal lever with the subsequent delivery of a small UCS reward (such as a sucrose pellet) into a nearby food dish ($CS_{Cup}$) will induce most rats to approach, investigate and even attempt to consume one of these two available CS's (Boakes, 1977; Flagel et al., 2009; Hearst & Jenkins, 1974; Tomie et al., 2008). This motivational magnet quality of the rewards CS's, or the ability to attract motivated behaviors, is one of three principle components of incentive salience 'wanting' (Berridge & Robinson, 2003; Berridge et al., 2009).

Each CS possesses unique characteristics: the CS+ lever is maximally predictive of the UCS but is physically removed form the location of UCS delivery; conversely, the

$CS_{Cup}$ is spatially proximate to the site of UCS delivery but correlates poorly to the temporal availability of the UCS since it is present in the chamber throughout the entire test session. Interestingly, after several days of testing most rats will develop a preference for either the CS+ lever or the $CS_{Cup}$ that remains stable over the subsequent duration of autoshaping testing. This preferred CS elicits greater probability and frequency of approach and interaction during the CS+, and will be referred to as the 'prepotent CS.'

The ability of one of the reward CS's to act as a motivational magnet is phasic, with the CS+ presentation triggering approach and consummatory behaviors, which can sometimes reach near frenzied levels (especially after brain limbic stimulation, such as opioid stimulation of CeA) (Mahler & Berridge, 2009). Appetitive and consummatory behaviors arise and peak during the 8 sec CS+ duration, and quickly return to much lower baseline levels after CS+ termination and UCS delivery. During CS+ onset rats engage in a range of appetitive and consummatory behaviors toward the CS's. Both of the CS's were physically discrete objects that supported behaviors such as orientation, sniffing, nibbling, biting, and touching with forepaws. The latency, frequency, and probability of such behaviors all constitute measures of the incentive salience 'wanting' of a given CS. A third stimulus, a control lever identical in shape and size to the CS+ lever (but unlit), was always present in the chamber and had no predictive associations.

*Behavioral Videoscoring*

*Autoshaping:* All autoshaping test sessions were recorded using a digital video camera positioned beneath the chamber to provide a clear view of the entire chamber. For the autoshaping experiments where microinjections were delivered after training, a second camera was positioned on the side of the test chamber to provide a more detailed side-view of the CS's, in particular the $CS_{Cup}$. Videos were scored offline in slow motion (1/2 to $1/10^{th}$ speed) by an observer blind to the experimental condition. The observer recorded behaviors during the 8 seconds of the $5^{th}$, $10^{th}$, $15^{th}$, $20^{th}$, and $25^{th}$ cue presentations of each scored session (and also the 8 seconds prior to cue onset when microinjections were delivered after training).

Three CS-directed behaviors were hand-scored by the observer: *looks, nibbles and sniffs, and slow bites*. *"Looks"* were orientations of the head toward a CS where the nose was within ~3cm of the CS but no physical contact with the CS was made. *"Nibbles and sniffs"* were fast (<0.5sec) investigatory movements of the mouth and nose directed toward either CS, requiring physical contact with the CS and resembling initial investigatory contact with a UCS reward, such as a food pellet. *"Slow bites"* were longer duration (~0.5-1sec), discrete interactions that resembled consummatory-type actions toward the CS, involved grasping and clear biting of the CS+ lever or slow, discrete dips into the $CS_{Cup}$ resembling the movement required to retrieve a UCS pellet.

For autoshaping expression experiments, the observer also hand-scored the latency to the first physical interaction (either nibble/sniff or slow bite) with the CS+ lever. Latency was scored as the time between the first frame where the CS+ lever was visible and the first frame in which the initial CS+ lever nibble/sniff or slow bite began. Although latency to first computer-scored lever press was automatically recorded by the

accompanying software, hand-scoring was necessary to more accurately capture the latency of interactions (especially nibbles/sniffs) that were unlikely to be registered as discrete lever presses.

*Food intake:* Video recordings of food intake test sessions were scored offline by an observer blind to the experimental condition. The following behaviors were recorded: eating time (in seconds), eating bouts (triggered by interruptions of eating of more than 5 seconds), food sniffing, food carrying, drinking time (in seconds), drinking bouts (same criteria as eating bouts), grooming, cage crosses, sleeping, rearing, and defensive treading. Treading is a natural defensive behavior emitted by rodents, and involves rapid forelimb strokes away from the body that push debris (e.g. dirt or bedding) in the direction of a threat (see Chapter 2 for additional details).

*Conditioned Reinforcement Paradigm*

While autoshaping testing captures the motivational magnet qualities of incentive salience 'wanting,' we were also interested in the ability of the CS+ lever to serve as a conditioned reinforcer for a novel instrumental action. Here, we utilized a briefer (4 sec) presentation of the lit CS+ lever and accompanying tone from autoshaping testing to support acquisition of a novel nose-poking behavior. If, in fact, a cue possesses incentive salience, then it should be able to act as a conditioned reinforcer (Berridge & Robinson, 2003; Berridge et al., 2009), and changes in 'wanting' for the cue can be assessed by measuring differences in the effectiveness of the conditioned reinforcer under different neurobiological manipulations. All conditioned reinforcement testing occurred under

extinction conditions, with no UCS pellet rewards delivered after earned CS+ presentations.

*Surgery*

All animals were handled twice for a total of fifteen minutes prior to undergoing surgery. Rats were pretreated with atropine (0.04mg/kg) and then anesthetized with ketamine (80mg/kg) and xylazine (5mg/kg). Rats were then placed in a stereotaxic device and implanted with bilateral, chronic guide cannula (23 gauge, stainless steel), 14mm in length, aimed either so that the ventral tip would rest 2mm above the BNST (AP: +0.24 to -0.84mm; ML: +/-1.2 to 1.7mm; DV: -4.5 to -4.95mm; incisor bar: -3.3mm [flat skull]) or the shell of the nucleus accumbens (AP:+3.0 to 2.7mm; ML: +/-0.9mm; DV: -5.5mm to -5.8mm; incisor bar: +5.0mm). Guide cannula were secured to the skull using four stainless steel screws and dental acrylic, and fitted with stainless steel stylets to prevent occlusion.

All rats were given post-operative analgesic (0.3 mg/kg buprenorphine) and prophylactic antibiotic (50 mg/kg chloramphenicol), and allowed to recover for at least 7 days before the onset of behavioral testing.

*Drugs and Microinjections*

DAMGO (0.1µg, total of 0.2µg bilateral dose) was dissolved and diluted to dose in artificial cerebrospinal fluid (aCSF; Harvard Labs, Cambridge, MA). This dose was chosen for its ability to induce UCS 'wanting' in both BNST (see Chapter 2) and accumbens shell (Pecina & Berridge, 2005). aCSF alone was used for vehicle

microinjections. Microinjection schedules were counter-balanced across subjects when appropriate.

On test days where animals received intracranial microinjections, animals were gently handled as the stylets were removed. Rats then received bilateral microinjections (0.2μL per side, total bilateral volume) via 16mm stainless steel microinjection tips (29 gauge), which extended 2mm beyond the ventral tip of the guide cannula. Microinjection tips were attached via PE-20 surgical tubing to a microinfusion pump, which delivered the infusion over the course of 60 seconds. Microinjection tips were left in place for an additional 60 seconds after the infusion ended to allow for drug diffusion, after which the stylets were replaced and the rat placed immediately in the test chamber.

On test days where animals did not receive microinjections, the rats were gently handled for the same amount of time as it took to complete microinjections (~5 mins) and the stylets were removed, cleaned, and replaced before placing the animal in the test chamber.

*Autoshaping Testing Apparatus*

Autoshaping chambers were 30.5cm x 24.1cm x 21.0cm, with steel plates on front and back and clear plastic side walls, ceiling, and floor (Med Associates, Inc.; Vermont, USA). A red-tinted house light was mounted atop the back wall, and was illuminated throughout all test sessions. The back wall also contained the speaker for a tone generator utilized as part of the CS+ stimulus.

The front wall contained a central sucrose delivery cup (the $CS_{Cup}$) near the floor, flanked by two retractable levers on either side (the CS+ lever and the control lever). The

CS+ lever was periodically extended and retracted during test sessions, while the control lever remained extended throughout the autoshaping sessions. The CS+ lever also contained an embedded white LED light that was illuminated during lever extension and extinguished during lever retraction. An infrared beam was incorporated into the $CS_{Cup}$ to measure the number and duration of entries. A computer equipped with MED-PC software (Med Associates, Inc.; Vermont, USA) was attached to all test chambers, controlling sessions and recording all automated inputs (CS+ and control lever depressions, response latency, $CS_{Cup}$ entry frequency and duration).

*Conditioned Reinforcement Testing Apparatus*

Conditioned reinforcement testing occurred in the same chamber as autoshaping testing. The food cup was removed from the front wall and replaced with a solid steel plate. A retractable lever (identical to the CS+ lever) was installed in the center of the back wall, flanked by two infrared nose ports installed near the floor of the chamber. One of the nose ports was inactive, and although entries into this port were counted they did not generate a response. The other nose port was active, and entries into this port would generate a 4 sec presentation of the illuminated, retractable lever and the 2.9KHz tone (identical to the autoshaping CS+). During the CS+ presentation, the attached computer automatically recorded lever presses. Conditioned reinforcement sessions lasted 40 minutes.

*Behavioral Experiment Descriptions*

*Pre-training*: Rats were handled on 2 days for a total of fifteen minutes prior to training, and exposed to the UCS sucrose pellets in their home cage on the day prior to training. Rats then underwent 1 day of magazine training: "free" sucrose pellets on a variable interval (VI)-60 sec schedule for 20 minutes to habituate the rats to the process of retrieving pellets from the $CS_{Cup}$. During magazine training, the control lever was extended but the CS+ lever was never presented. Rats were considered to have successfully magazine trained if all pellets were retrieved and consumed. If pellets remained in the $CS_{Cup}$ or chamber after the initial magazine training session, the rat was exposed to another magazine training session 2-24 hours later. Most rats required only one magazine training session, and all rats included in this study successfully completed magazine training after one or two sessions.

*General autoshaping procedure*: Autoshaping sessions were composed of twenty five Pavlovian pairings of the CS+ lever (illuminated during extension by an embedded diode) and a 2.9KHz continuous tone (8 second duration, VI-90sec schedule) with one 45mg sucrose pellet delivered in the $CS_{Cup}$ immediately after CS+ lever offset. Testing was terminated 30 seconds after the $25^{th}$ cue, resulting in total autoshaping sessions lasting ~35-45 minutes.

*Autoshaping acquisition*: We first tested whether DAMGO microinjections throughout the duration of autoshaping testing (both initial learning and subsequent expression) would modulate incentive salience 'wanting.' Separate groups of rats received microinjections of either vehicle (BNST: n=3, CS+ lever prepotent; n=5, $CS_{Cup}$ prepotent; Accumben shell: n=3, CS+ lever prepotent; n=15, $CS_{Cup}$ prepotent) or DAMGO (BNST: n=6, CS+ lever prepotent; n=5, $CS_{Cup}$ prepotent; Accumben shell:

n=16, CS+ lever prepotent; n=4, $CS_{Cup}$ prepotent) into BNST or accumbens shell immediately prior to each of 6 days of autoshaping testing. Test days were spaced 24-48 hours apart.

*Autoshaping expression*: We also tested whether DAMGO would modulate incentive salience if delivered *after* the CS-UCS relationship had been learned and a prepotent cue was established. Rats in this experiment received 5 days of microinjection-free autoshaping training, followed by 2 days where rats received microinjection of vehicle and DAMGO (BNST: n=13, CS+ lever prepotent; n=2, $CS_{Cup}$ prepotent; Accumben shell: n=14, CS+ lever prepotent; n=8, $CS_{Cup}$ prepotent). Microinjection order was counterbalanced across subjects, and rats were given 48 hours between microinjection test days.

*Conditioned reinforcement*: Conditioned reinforcement was tested after the completion of autoshaping testing. For autoshaping acquisition, rats underwent 1 day of conditioned reinforcement testing and separate groups received either vehicle (BNST: n=3, CS+ lever prepotent; n=0, $CS_{Cup}$ prepotent; Accumben shell: n=12, CS+ lever prepotent; n=2, $CS_{Cup}$ prepotent) or DAMGO (BNST: n=4, CS+ lever prepotent; n=0, $CS_{Cup}$ prepotent; Accumben shell: n=2, CS+ lever prepotent; n=14, $CS_{Cup}$ prepotent) microinjections, consistent with what they received during acquisition testing, immediately prior to testing. For autoshaping expression, rats received 2 days of conditioned reinforcement testing with counterbalanced microinjection of vehicle and DAMGO (BNST: n=0, CS+ lever prepotent; n=9, $CS_{Cup}$ prepotent; Accumben shell: n=8, CS+ lever prepotent; n=14, $CS_{Cup}$ prepotent) immediately prior to testing.

*Food intake*: In the autoshaping expression experiments, rats were also tested for voluntary intake of a food UCS after the completion of autoshaping and conditioned reinforcement testing. Autoshaping acquisition animals were not tested for food intake due to the large number of microinjections already administered.

Prior to food intake testing, rats were returned to *ad lib* feeding for at least 48 hours, and then habituated to the test environment for three additional days. Rats were placed in clear plastic cages containing a pre-measured pile of standard lab chow (~25g), a water spout, and corncob bedding. On test days, food intake was tested for 1 hour immediately following microinjection of vehicle or DAMGO. The entire session was videotaped for subsequent offline analysis. After the test was completed, remaining chow (including crumbs) was carefully removed from the cage and weighed. Test days were always separated by at least 48 hours.

*Histology*

After testing was completed, subjects used for behavioral testing were deeply anesthetized with sodium pentobarbital (0.2mg/kg; Fatal-Plus) and decapitated. Brains were extracted and placed in a 10% paraformaldehyde solution for 24-48 hours, and then placed in a 30% sucrose solution for 3-5 days, until the brains sank. The brains were then sliced on a freezing microtome (Leica Microsystems; Illinois, USA) into 60µm coronal sections, mounted onto glass slides, allowed to dry for at least 24 hours, and then stained with cresyl violet. Stained slices were viewed under light magnification and used to map the microinjection centers in each hemisphere on coronal sections taken from a rat brain atlas. (Paxinos & Watson, 2007)

*Fos-Like Protein Immunohistochemistry*

Rats utilized for Fos plume analysis underwent identical procedures for cannula implantation (except sham surgery control animals, who underwent surgery but did not receive cranial guide cannula) and pre- and post-surgical handling. Here, we completed Fos plume analysis for intra-accumbens shell DAMGO (0.1µg); Fos plume analysis for this drug/dose in BNST was previously completed (see Chapter 2).

On the day of Fos plume testing, animals were given bilateral microinjections of vehicle (n=4) or 0.1µg DAMGO (n=6). Sham surgery animals (n=3) were handled gently for an amount of time equivalent to the animals receiving microinjections. Ninety minutes after microinjection, rats were transcardially perfused and their brains placed in 4% formaldehyde for 4-6 hours, then moved to a 30% sucrose solution for 3-4 days. Brains were then sliced on a freezing microtome in alternating 40µm coronal sections, with one series processed for Fos expression and the other retained for placement verification, if needed.

Fos activation following neurochemical manipulation was measured using immunohistochemistry and immunofluorescence (Faure et al., 2008; Reynolds & Berridge, 2008). Briefly, sections were immersed and gently agitated in successive baths of 0.1M sodium phosphate buffer (SPB) and 0.2% Triton containing (1) 5% normal donkey serum (NDS) for 30 minutes, (2) 5% NDS and goat anti-c-Fos (1:10) overnight at 4˚C, (3) 5% NDS and signal enhancer for 30 minutes and, (4) 5% NDS and donkey anti-goat Alexa Fluor 488 (excitation: 488nm; emission: 519nm; Invitrogen) for 1 hour.

Sections were then mounted, air dried for 2-4 hours, and then coverslipped with ProLong Gold antifade reagent (Invitrogen).

*Fos Plume Mapping of Neuronal Activation and Suppression*

Local Fos activation was visualized using a Leica microscope (DM 6000; Nussloch, Germany) equipped for both brightfield and fluourescent microscopy. A filter with an excitation band at 480-505nm and an emission band at 505-545 were used for fluorescent visualization, and images were captured at 10x magnification (2x2 tiled) using a Regita-SRV camera (Q-Imaging; Surrey, British Columbia) and MCID Elite software. Fos-labeled cells were individually counted by an observer blind to treatment condition within ten adjacent sampling squares (68µm by 68µm) along each of seven radial arms extending from the center of the drug microinjection (45˚, 90˚, 135˚, 180˚, 225˚, 270˚, and 315˚).

Baseline levels of Fos expression were established by quantifying expression in two control conditions, (1) normal tissue of sham surgery to assess expression in the absence of damage from guide cannula implantation and microinjector tip insertion and (2) following vehicle microinjection to assess expression following microinjection track and vehicle-induced Fos expression. These baseline values were compared to Fos densities in each of the four drug conditions to assess the functional spread of neural activation or inhibition following DAMGO or muscimol microinjection.

DAMGO Fos plumes were mapped as >500%, >300%, and >200% of Fos expression relative to vehicle microinjections and to normal tissue. In each case, the distance of each range of Fos expression was measured from the microinjection center

along each radial arm. Spread was considered to extend to the furthest sampling square that contained greater than or equal to the particular level of activation or suppression being evaluated. Finally, the distance was averaged across all seven radial arms to produce an average radius of elevation or suppression. This procedure was repeated for every level of activation and suppression. The resulting drug-induced change in Fos expression relative to controls was then mapped to visualize the plume of activation and/or suppression for each drug and dose.

In the final stage of mapping, the Fos plume data identifying functional drug spread was merged with the behavioral data. DAMGO excitatory plumes symbols were created based on the radius of intense (>500%), moderate (>300%), and low (>200%) changes in Fos expression compared to vehicle controls. The verified bilateral microinjection centers of each rat are indicated by a pair of these symbols, and then color-coded to indicate specific behavioral effects. Microinjection centers for each rat were mapped in the coronal, sagittal, and horizontal planes; for the latter two, bilateral placements were collapsed onto a single unilateral map. Separate maps were constructed for each drug treatment and dependent variable. Thus, each symbol conveys information for each individual subject about (1) the location of drug microinjection, (2) the functional spread of drug *in vivo* and (3) the behavioral effect of the drug treatment on the particular dependent variable being presented.

*Statistical Analyses*

Autoshaping acquisition results were analyzed using mixed ANOVAs, using the within-subjects factor of test day and the between subjects factors of drug (vehicle vs.

DAMGO) and prepotent cue (CS+ lever or $CS_{Cup}$). Autoshaping expression results were also analyzed using mixed ANOVAs, with within-subjects factors of drug and cue period (the 8 sec prior to each scored cue vs. the 8 sec duration of each cue presentation) and the between subjects factor of prepotent cue. Sidak corrected t-tests and one-way ANOVAs were used to assess interactions. No order effects were found for drug or day in autoshaping expression and food intake, so data was collapsed across days. Conditioned reinforcement data was analyzed using ANOVA with factors of nose port (active vs. inactive), drug, and prepotent cue. For food intake and other general behaviors, the effect of DAMGO versus vehicle was assessed using paired samples t-tests. When reporting percentage increases, a fixed value of 1 was added to all raw data to prevent division by 0.

***Results***

Activation of μ-opioid receptors in the shell of the nucleus accumbens broadly increased incentive salience 'wanting' in both autoshaping and conditioned reinforcement tests. That is, accumbens shell DAMGO enhanced looks and approach and of both autoshaping CS+s, and increased the reinforcement value of the autoshaping CS+ in conditioned reinforcement testing regardless of an animal's prepotent CS. Stimulation of μ-opioid receptors in accumbens shell also increased 'wanting' for a UCS food reward in the same animals. We suggest that this pattern of findings indicates that μ-opioid stimulation in accumbens shell has the ability to broadly elevate 'wanting' for all available reward cues, essentially acting as "a rising tide that floats all boats."

Conversely, DAMGO microinjection in BNST caused an even broader enhancement that was unfocused on the predictive CS+ lever, generating a temporal diffusion of appetitive and consummatory behaviors directed at a subject's prepotent cue that spilled into non-CS periods. BNST DAMGO enhanced these behaviors to the prepotent CS when the CS+ lever for sucrose reward was absent, and slightly decreasing them when the CS+ was present. DAMGO in BNST did not affect the conditioned reinforcement value of the compound CS+, but was able to enhance voluntary intake of a UCS food reward. This pattern of results suggests that BNST opioids directly increasing UCS 'wanting,' may diffusely and somewhat nonassociatively act to project 'wanting' to a preferred autoshaping reward CS, smoothing the normally sharp ebb and flow of incentive salience between cue and non-cue periods.

*Fos plumes and functional spread of drug microinjection*

Fos plumes estimating the functional spread of our neurochemical manipulations were constructed using histology of neural tissue from a separate group of rats. Analysis of Fos expression indicated that for the drugs/doses/volumes used above, neuronal modulation was primarily limited to within BNST and the medial shell of the nucleus accumbens (see Figure 1 and Table 1). Fos plume data for the 0.1µg dose of DAMGO in BNST were taken from a previous Fos analysis experiment using an identical dose, volume, and rate of infusion (see Chapter 2).

DAMGO microinjections were predominately excitatory in both BNST and accumbens shell. In BNST, Fos expression increased by five times relative to control tissue in a small, intense excitatory plume near the microinjection center (radius =

0.11mm). In a larger, intermediate zone of excitation, DAMGO tripled Fos expression (0.25mm), and an even larger zone of low excitation displayed double the Fos expression of control tissue (0.35mm). Assuming that these plumes are roughly spherical, the inner, intermediate, and outer plumes would have total volumes of approximately 0.006mm$^3$, 0.062mm$^3$, and 0.175mm$^3$. The estimated total volume of BNST is approximately 3mm$^3$ (~2mm rostro-caudal, 0.4 to 1.2mm medio-lateral, and 0.6 to 2mm dorso-ventral), meaning that the outer plume for the 0.1ug dose filled only about 12% of the BNST's total volume. The small, more intense inner plume filled only 4% of BNST.

In accumbens shell, DAMGO caused a similar pattern of intense (0.07mm), moderate (0.26mm), and low (0.43mm) Fos plumes. The Fos plumes for the accumbens shell were not significantly different in size from those in BNST [main effect of placement, interaction of placement x plume intensity level: all $F$'s <1.7, n.s.]. Assuming a roughly spherical shape yields estimated total volumes of 0.001mm$^3$, 0.076mm$^3$, and 0.338mm$^3$ for these intense, moderate, and low accumbens shell plumes, respectively. The total volume of the medial shell of the nucleus accumbens has been previously estimated at ~2.87 mm$^3$ (Pecina & Berridge, 2005), meaning that the intense inner plume would fill less than 1% of the total medial shell volume. Even the larger outer plume would occupy only ~12% of the medial shell volume, indicating that the functional spread of our microinjections was likely limited to our target structure.

It is worth noting briefly that the absolute Fos expression we observed in accumbens shell was less than the levels previously reported using a comparable dose, volume, and rate of infusion (Pecina & Berridge, 2005; K. S. Smith & Berridge, 2007). The primary difference between the methods used in those papers and the current

experiment is the method of Fos visualization (nickel diaminobenzidine glucose oxidation vs. immunofluorescence), indicating possible differences in sensitivity across the two methods. However, the overall structure and pattern of the Fos plumes observed here is consistent with prior reports.

*Classification of prepotent CS preference*

Each rat developed a preference for one of the two available reward cues (CS+ lever or $CS_{Cup}$), which they approached and attempted to consume much more than their non-prepotent cue [comparison of total prepotent vs. non-prepotent behaviors: BNST during training, days 4-6, $t(18)=6.3$, $p=0.001$; accumbens shell during training days 4-6: $t(38)=10.3$, $p=0.001$; BNST after training, $t(14)=8.3$, $p=0.001$; accumbens shell after training, $t(21)=6.5$, $p=0.001$] or other stimuli in the chamber. For rats tested during learning, the prepotent cue was approached and consumed on average 3x more than the non-prepotent cue during the CS+ period during days 4-6 of testing; for rats tested after learning, the prepotent cue was preferred to the non-propotent cue by nearly 5x. Individual rats in both during training and post-training experiments preferred either the CS+ lever (~35% for BNST, ~45% for accumbens shell) or the $CS_{Cup}$ (~65% for BNST, ~55% for accumbens shell).

We replicated previously published data showing markedly different patterns of behavior between CS+ lever (also called sign-tracking) and $CS_{Cup}$ preferring (also called goal-tracking) animals in their latency to approach each CS, probability of approaching each CS, and number of responses emitted at each CS (Boakes, 1977; Flagel et al., 2009; Flagel, Watson, Akil, & Robinson, 2008; Flagel et al., 2007). However, these two

behavioral phenotypes did not differ significantly in the amount of prepotent cue approaches and interactions [no effect of prepotent cue on total CS+ period prepotent cue behaviors: BNST during training, days 4-6, F(1,15)=0.1, n.s.; accumbens shell during training, days 4-6, F(1,35)=0.5, n.s.; BNST after training, F(1,9)=0.2, n.s.; accumbens shell after training, F(1,14)=0.2, n.s.]. So although CS+ lever and $CS_{Cup}$ animals directed their behaviors during the CS+ period at different targets, both phenotypes emitted similar numbers of motivated behaviors at their chosen prepotent cue.

**Accumbens shell microinjections**

*DAMGO in accumbens shell enhances and broadens incentive salience*

*DAMGO after autoshaping training.* When microinjected into accumbens shell after learning the autoshaping task, DAMGO broadened the attribution of incentive salience to each animal's non-prepotent cue. Behavioral "looks" (defined as orientations of the head toward a CS where the nose was within ~3cm of the CS but no physical contact with the CS was made) at the non-prepotent cue were elevated to ~150% of vehicle levels [interaction of cue period x drug: $F(1,20)=4.7$, $p=0.039$], indicating an increase in the incentive salience of the non-prepotent cue (Figure 2). The presence of this effect after autoshaping training was complete suggests that it is not simply the result of altered learning about the non-prepotent cue during training, and at least in part due to changes in the dynamic attribution of incentive salience after training.

Additionally, we also found that when DAMGO was microinjected into accumbens shell after learning the autoshaping task, 'wanting' of the prepotent cue was also enhanced, as evidenced by an increase in prepotent cue slow bites during the CS+

period (due to a camera malfunction, slow bites could not be scored for one $CS_{Cup}$ animal). Slow bites were increased by up to 270% above vehicle levels within the same animal (average increase = 130%) [$t(20)=2.4$, p=0.029] and this effect was consistent across both behavioral phenotypes [interaction of drug x cue preference: $F<1$, n.s.] (Figure 2). As terminal slow bites increased and began to occur earlier in the 8 sec CS+ period, there was an accompanying slight decrease in prepotent cue nibbles and sniffs to 87% of vehicle levels [interaction of cue period x drug: $F(1,20)=5.7$, p=0.027].

Although rats 'looked' at their non-preferred cue during CS+ more after DAMGO in accumbens shell, they did not approach the non-preferred cue more [main effect of drug, interaction of cue * drug: all $F$'s < 3.7, n.s.], possibly due to their increased slow bites on the preferred cue, which might compete with non-prepotent responding during the 8-sec CS+ period. In support of this possibility, slow bites are the longest duration cue interaction that we observed during autoshaping, and so an increase in that category of behavior would by necessity diminish the amount of time available to emit other behaviors, either at the prepotent or non-prepotent cue.

*DAMGO during autoshaping training*. When administered throughout training, DAMGO microinjections into accumbens shell broadened the attribution of incentive salience, which spilled over into the non-preferred cue. DAMGO elevated the number of appetitive and consummatory behaviors that rats directed at their non-prepotent cue, while preserving high levels of behaviors directed toward preferred-cue. In both behavioral phenotypes, looks at the non-prepotent cue increased by over 300% for all subjects over all 6 days of testing [$F(1,34)=5.6$, p=0.024] (Figure 3). Total hand-scored behaviors

emitted at the non-prepotent cue (looks + nibbles/sniffs + slow bites) were increased to over 175% of vehicle levels [$F(1,34)=7.5$, $p=0.010$]. These measures suggest a direct enhancement of the motivational magnet quality of the non-prepotent cue, which became more attractive and 'wanted' during the CS+ period following opioid stimulation of medial accumbens shell.

The broadening of 'wanting' was particularly strong for rats that initially preferred the CS+ lever. Most prominently, CS+ lever prepotent rats showed a significantly increased probability of approaching both reward cues (CS+ lever and $CS_{Cup}$) during a CS+ presentation [days 4-6 of testing: $F(1,17)=6.0$, $p=0.026$] (Figure 3). CS+ lever rats also showed an increased probability of approaching the non-propotent cue [days 4-6 of testing: $F(1,17)=5.6$, $p=0.031$]. Additionally, looks at the $CS_{Cup}$ were increased by an average of ~330% [$F(1,17)=12.1$, $p=0.003$] across all 6 days of testing. CS+ lever preferring animals also showed enhanced behavioral interactions with their non-prepotent cue. Total nibbles/sniffs + slow bites of the $CS_{Cup}$ across all 6 days increased by ~240% after DAMGO [$F(1,17)=6.1$, $p=0.025$]. Computer-scored entries into the food cup increased to 240% of vehicle levels during the CS+ period during days 4-6 for CS+ lever preferring animals [$F(1,17)=4.9$, $p=0.041$]. This enhancement of food cup entries spilled over into intervening non-CS+ periods, where entries increased to 208% of vehicle levels [$F(1,17)=4.5$, $p=0.049$], perhaps as a result of the unavailability of these animal's prepotent CS (the CS+ lever which was retracted in the ITI).

Rats preferring the $CS_{Cup}$ also displayed an increase (~400% relative to vehicle) in total interactions with non-prepotent cues over all 6 days of testing [$F(1,17)=5.5$, $p=0.032$], but did not show increased probability of approaching the non-prepotent cue

[main effect of drug on non-propotent approach and both approach: $F$'s<1.9, n.s.]. The latter finding is perhaps due to the significantly lower frequency of non-prepotent cue nibble/sniffs and slow bites in $CS_{Cup}$ subjects [main effect of prepotent cue preference on prepotent cue interactions: $F(1,34)=12.0$, p=0.001], which were used as the criteria for scoring whether a cue was approached.

$CS_{Cup}$ rats also showed a decrease in time spent in the food cup both during the CS+ period [$F(1,17)=8.9$, p=0.008] and also during the inter-trial intervals [$F(1,17)=15.8$, p=0.001] across all 6 days of testing, but no reduction in the total number of entries during either of these periods [main effect of drug on CS+ and non-CS+ food cup entries: $F$'s <1, n.s.]. This indicates that $CS_{Cup}$ rats microinjected with DAMGO were interacting more rapidly with their prepotent cue, which perhaps explains how these rats could increase their levels of non-prepotent cue behavior while still maintaining levels of prepotent cue behavior comparable to vehicle in spite of the constraints of an 8-sec CS+ period.

When DAMGO was administered during autoshaping training, we did not observe enhancements of prepotent cue behavior in either behavioral phenotype. It is unknown why we did not observe a potentiation of prepotent slow bites, as found when DAMGO was administered after training. One possible explanation is that the potentiation of prepotent slow bites may be uniquely linked to acute, rather than chronic, μ-opioid activation. If so, the failure to observe increased slow bites may simply be the product of their relative rarity during the early days of learning the autoshaping task. Additionally, it is worth noting that, due to the brevity of the autoshaping CS+ period, a potentiation of both prepotent and non-prepotent cue behavior would extremely difficult

to observe. It may be, then, that the especially robust broadening of 'wanting' that we observed when DAMGO was administered in accumbens shell during testing precluded a simultaneous increase in prepotent cue behaviors.

*DAMGO in accumbens shell increases the conditioned reinforcement value of a reward CS*

*Instrumental conditioned reinforcement after expression.* In rats that received microinjections after training (within subjects design), subjects again registered at least twice as many entries into the active nose port that delivered a brief CS+ presentation [$F(1,20)=46.3$, $p=0.001$], and DAMGO specifically enhanced entries into the active nose port to 250% of vehicle levels, without significantly increasing entries into the other port [no main effect of drug; interaction of nose port x drug: $F(1,20)=4.5$, $p=0.047$] (Figure 4). There was also a significant main effect of prepotent cue preference across both nose ports [$F(1,20)=23.1$, $p=0.001$], driven by higher responding in general by the CS+ lever prepotent animals Yet the effect of DAMGO on the conditioned reinforcement value of the CS+ was identical across prepotent cue preference [interaction of drug * prepotent cue, and drug * prepotent cue * nose port: all F's<1, n.s.], indicating that opioid stimulation in both CS+ lever and $CS_{Cup}$ animals can enhance the conditioned reinforcement value of the compound CS+. DAMGO did not alter the number of computer-recorded presses on the CS+ lever during its brief presentations in either prepotent cue phenotype [*t*'s<1.1, n.s.].

Across both behavioral phenotypes, there was a roughly 50% decay in entries into the conditioned reinforcement nose ports on the second day of testing [main effect of day:

F(1,21)=22.2, p=0.001] that was greater for the nose port yielding a brief CS+ presentation [interaction of day x nose port: F(1,21)=13.9, p=0.001]. This general decay in responding was likely the result of repeated testing under extinction conditions. However, there was no difference in the effect of DAMGO on responding for the autoshaping CS+ across the two days of testing [interaction of nose port x day of DAMGO treatment: F(1,19)=0.7, n.s], so data were collapsed across day.

*Instrumental conditioned reinforcement after acquisition.* In a between subjects design, conducted in rats that had previously received DAMGO or vehicle microinjections throughout autoshaping testing, rats worked almost twice as much for a brief presentation of the CS+ than they did to receive no response [main effect of nose port: F(1,27)=6.8, p=0.014]. DAMGO elevated the total number of nose pokes to ~130% of vehicle levels [main effect of drug: F(1,27)=4.6, p=0.04], but this elevation was applied to both nose holes [interaction of nose port x drug: F(1,27)<1.6, n.s.] (Figure 5).

Although we found only a marginal effect of prepotent cue preference on conditioned reinforcement behavior [F(1,27)=3.53, p=0.071], previous research has indicated that CS+ lever and $CS_{Cup}$ animals respond differently to instrumental conditioned reinforcement testing with the CS+ lever, when conducted after autoshaping training (Robinson & Flagel, 2008). When analyzed separately, CS+ lever prepotent rats showed a similar preference for the active nose port that earned the CS+ lever stimulus [main effect of nose port: F(1,12)=12.6, p=0.004]. DAMGO caused a ~200% increase in nose pokes for CS+ lever [main effect of drug: F(1,12)=8.2, p=0.014], but also similar ~200% increase for pokes in other port [interaction of nose port x drug: F<1, n.s.] and no

increase in CS+ lever presses [t(12)=0.7, n.s.]. $CS_{Cup}$ prepotent animals, conversely, did not display a preference for the active nose port, to begin with and were unaffected by DAMGO treatment [main effects of drug and nose port, interaction of nose port x drug: all F's<1.7, n.s.]. $CS_{Cup}$ animals also showed no change in pressing the CS+ lever during its brief extension [t(15)=0.6, n.s.].

However, it should be noted that there were marked inadvertent imbalances in the numbers of aninmal within DAMGO vs. vehicle treated groups within each of the CS+ lever (2 DAMGO vs. 12 vehicle) and $CS_{Cup}$ phenotypes (15 DAMGO vs. 2 vehicle). Of the two sub-groups with the largest *n*'s (CS+ lever prepotent animals receiving vehicle, n=12, and $CS_{Cup}$ prepotent animals receiving DAMGO, n=15), both showed greater than 2:1 preferences for the active nose port [*t*'s > 3.5, p<0.1]. This is consistent with prior reports of effective conditioned reinforcement of the autoshaping CS+ for CS+ lever animals (Robinson & Flagel, 2008), but we extend this finding to show that an autoshaping CS+ is also an effective conditioned reinforcer for $CS_{Cup}$ animals who have received DAMGO in accumbens shell. The effectiveness of the autoshaping CS+ as a conditioned reinforcer for $CS_{Cup}$ animals receiving DAMGO suggests a possible broad enhancement of incentive salience (similar to that observed in autoshaping testing), as these animals worked significantly more for a brief presentation of the autoshaping CS+ even though they did not prefer this cue during prior testing.

## BNST Microinjections

*DAMGO in BNST diffuses and disrupts incentive salience*

*DAMGO after autoshaping training*. Administering DAMGO in BNST after autoshaping caused an increase in approaches and consummatory behavior toward cup and CS lever, but primarily outside the CS+ period. However, during the CS+ period DAMGO in BNST actually suppressed approaches and consummatory behaviors of CSs, possibly because of a weaking of incentive motivation as 'wanting' diffused outside of the CS+ period.

During the non-CS+ period, food cup entries were increased in both behavioral phenotypes, reaching 175% of levels observed in the vehicle test day [$F(1,13)=6.4$, $p=0.025$] (Figure 6). Additionally, all rats showed a nearly 150% increase in prepotent cue nibbles and sniffs during the 8 seconds immediately prior to sampled CS+ periods [interaction of cue period x drug: $F(1,9)=8.5$, $p=0.017$; pre-CS+ period: $t(14)=2.8$, $p=0.016$]. For $CS_{Cup}$ animals, DAMGO elevated prepotent nibbles and sniffs of the autoshaping $CS_{Cup.}$ For CS+ lever animals DAMGO in BNST caused an increase in sniffing during the ITI of the recessed opening where the CS+ lever resided. These measures of enhanced appetitive responding for CS cup in all animals and for CS+ lever in rats that preferred it outside the appropriate CS+ window indicate that opioid stimulation in BNST facilitates a diffusion of 'wanting' for the $CS_{Cup}$ outside of the CS+ period and sometimes outside of the prepotent CS.

Interestingly, appetitive and consummatory behaviors were slightly diminished by BNST DAMGO toward the prepotent reward CS during the 8-sec CS+ window. Prepotent cue nibbles and sniffs during the CS+ period were reduced to 76% of vehicle levels [interaction of cue period x drug: $F(1,9)=8.5$, $p=0.017$; during CS+ period: $t(14)=2.2$, $p=0.042$] (Figure 6). This reduction in responding also extended to the non-

prepotent cue, where behaviors during the CS+ were reduced to 70% of vehicle levels [during CS+ period: $t(14)=2.2$, $p=0.049$].

However, it is important to note that DAMGO in BNST did not suppress responding during the CS+ period so far as to equalize the motivational magnet quality of an animal's prepotent CS throughout the test session. The rate of appetitive responding during the ITI was still markedly lower than during the presentation of the CS+ [main effect of cue period on prepotent nibbles and sniffs, prepotent slow bites, prepotent total interactions, and probability of approaching prepotent cue: all $F$'s >37, $p=0.001$], showing that DAMGO was not preventing subjects from detecting the CS+ or showing enhanced cue-triggered motivation. Additionally, latency to approach the prepotent cue was not changed for either $CS_{Cup}$ [$t(12)=0.7$, n.s.] or CS+ lever animals [$t(1)=0.04$, n.s.], indicating that rats were in proximity to their prepotent cue for similar amounts of time yet displayed a less vigorous appetitive response. The latency and probability of approach data also suggests that DAMGO-treated rats were not averse to the reward CS's, as they still approached them at similar rates and probabilities.

Diminished prepotent cue responding during the CS+ period could simply mean that DAMGO was a generally disruptive of locomotor activity, rather than specifically affecting appetitive motivation or incentive salience. However, as previously noted, intra-BNST DAMGO actually *enhanced* prepotent cue behaviors during the ITI. This suggests that intra-BNST DAMGO was not suppressing locomotor responding, but can instead disrupting and diffusing the attribution of incentive salience into moments when it would not normally be enhanced (due to absence of cue). This is especially clear in the $CS_{Cup}$ animals, where DAMGO disrupted the selectivity of food cup entries (calculated as [CS+

period entries into the food cup]/[sum of food cup entries in CS+ and non-CS+ periods]), reducing selectivity from 0.28 during vehicle treatment to 0.17 after DAMGO microinjection [$F(1,12)=30.3$, $p=0.001$]. In the two available CS+ lever prepotent animals, one showed a sharp decrease in CS+ lever selectivity from 0.98 during vehicle treatment to 0.50 during DAMGO treatment (calculated as [CS+ lever presses]/[sum of CS+ lever and control lever presses], while the other showed a modest increase in selectivity from 0.86 to 0.97.

We note that the distribution of $CS_{Cup}$ vs. CS+ lever animals was inadvertently imbalanced in our group of BNST animals ($CS_{Cup}$ n=13; CS+ lever n=2), and so our conclusions here are most robust for $CS_{Cup}$ animals. However, it is notable that both of the BNST CS+ lever prepotent animals showed a reduction in prepotent cue nibbles and sniffs during the CS+ period after microinjection of DAMGO (one animal dropped from 4.6 to 2.2, the other from 3.4 to 2.0). Future studies with a larger number of CS+ lever animals will be required to fully confirm that the effect of intra-BNST DAMGO is consistent across behavioral phenotypes.

In summary, BNST μ-opioid stimulation caused a moderate, but not complete, disruption of incentive salience during the CS+ period that resulted in diminished prepotent and non-prepotent appetitive responding, while at the same time leading to diffusion of incentive salience outside the appropriate CS+ window, resulting in increased inappropriate prepotent cue responding during the ITI.

*DAMGO during autoshaping training*. DAMGO in BNST, when administered throughout autoshaping training, again increased appetitive and consummatory responses

to the cup outside the CS+ period, possibly spilling elevation of incentive salience into non-CS+ moments and disrupting the normal requirement of synergy (brain limbic activation X cue presence) for enhancement of 'wanting'.

DAMGO in BNST caused a substantial increase in non-CS+ period entries into the food cup, which rose to ~140% above vehicle levels across all six days of testing for both behavioral phenotypes [main effect of drug: $F(1,15)=7.4$, $p=0.016$; main effect of prepotent cue and interaction of drug*prepotent cue preference: $F<1.8$, n.s.] (Figure 7). It would be of interest in future studies to investigate the time course of these non-CS+ food cup entries to clarify whether the effect is due to a slower decay of the motivational magnet qualities of the food cup after CS+ termination (in which case the increase in non-CS+ entries should cluster shortly after CS+ offset), to a steady growth of the incentive salience of the food cup as the time since the last CS+ period increases (non-CS+ entries cluster shortly before CS+ onset), or whether the attractiveness of the food cup is enhanced uniformly throughout the non-CS+ period.

Interestingly, DAMGO microinjection in BNST throughout autoshaping testing increased looks at the prepotent cue during the CS+ period for both behavioral phenotypes by an average of over 200% above vehicle levels across the first 3 days of testing [$F(1,15)=9.5$, $p=0.008$]. This suggests that some increases in appropriately timed incentive salience for reward cues may accompany the inappropriate enhancements observed here during the non-CS+ periods.

**DAMGO in BNST does not affect the conditioned reinforcement value of a reward CS**

*Instrumental conditioned reinforcement after expression.* As in accumbens shell, when conditioned reinforcement was conducted within subjects over the 2 days following autoshaping testing, responding in both nose ports was reduced [main effect of day: $F(1,7)=6.8$, $p=0.035$] with an greater absolute reduction in active nose port entries [interaction of nose port x day: $F(1,7)=9.4$, $p=0.018$], though a similar relative reduction of ~50% across both the active and inactive ports. Again, though, there was no impact of the day on DAMGO treatment [interaction of nose port x day of DAMGO treatment: $F<1.1$, n.s.], so data were collapsed across days. It should also be noted that, due to illness during the extended test schedule, only 9 rats with BNST placement completed both days of conditioned reinforcement testing, all of which were $CS_{Cup}$ prepotent animals.

The autoshaping CS+ was an effective conditioned reinforcer for $CS_{Cup}$ animals, with entries in the active nose port outnumbering inactive nose port entries by 2:1 [$F(1,8)=6.9$,$p=0.030$] (Figure 8). However, intra-BNST DAMGO did not affect the conditioned reinforcement value of the CS+ [main effect of drug, interaction of drug x nose port: both $F$'s <1, n.s.]. $CS_{Cup}$ animals recorded a very low number of lever presses during the brief CS+ presentation (~1 lever press per 40 minute test session), and the level of lever pressing was unaffected by DAMGO treatment [$t(8)=0.5$, n.s.]

*Instrumental conditioned reinforcement after acquisition.* A subset of BNST animals (total n=10, CS+ lever prepotent n=7, $CS_{Cup}$ prepotent n=3) were tested for conditioned reinforcement value of the autoshaping CS+ after receiving microinjections throughout

autoshaping testing. Due to the small number of $CS_{Cup}$ subjects tested in this between subjects design, these animals were not statistically analyzed.

The autoshaping CS+ served as an effective reinforcer for the CS+ lever animals tested, with the active nose port preferred over the inactive nose port by nearly 4:1 [$F(1,5)=16.5$, $p=0.01$]. However, DAMGO in BNST had no effect on this preference [main effect of drug, interaction of nose port x drug: all $F$'s < 1.2, n.s.] (Figure 8). DAMGO in BNST did, however, approach a marginal suppression of the number of lever presses during the brief CS+ presentation, which were reduced to 22% of vehicle levels [$t(5)=2.0$, $p=0.103$] (Figure 9).

**Food UCS consumption is enhanced by DAMGO in both accumbens shell and BNST**

Rats who received DAMGO or vehicle after autoshaping training also received separate testing for voluntary intake of a UCS food reward (rats that received microinjections during testing were not subjected to food intake testing due to the large number of microinjections administered during autoshaping). This testing occurred after both autoshaping and conditioned reinforcement testing was complete. A total of 26 animals were tested for voluntary intake (BNST n=6, all $CS_{Cup}$; accumbens shell n=20, CS+ lever=13, $CS_{Cup}$=7).

In accumbens shell, DAMGO microinjection nearly tripled each of the total amount of chow consumed [$F(1,18)=78.3$, $p=0.001$], total duration of eating [$F(1,22)=15.7$, $p=0.01$], and total number of feeding bouts [$F(1,22)=14.0$, $p=0.001$] (Figure 10). This feeding effect was greater in CS+ lever preferring animals [interaction

of prepotent cue x food intake in grams, feeding time, and feeding bouts: all $F$'s > 11.7, p<0.01].

In BNST, DAMGO similarly enhanced both total chow intake [$F(1,5)=23.7$, p=0.005] as well as total duration of eating [$F(1,5)=7.9$, p=0.037] to roughly three times vehicle levels (Figure 11). The magnitude of feeding effects was comparable across BNST and accumbens shell [interaction of drug*placement on food intake in grams, feeding time, feeding bouts: all $F$ values <3.2, n.s.].

Drinking behavior was unaffected by DAMGO microinjection in either accumbens shell or BNST (drinking bouts and drinking time: $t$ test values <1.6, n.s.), indicating that the increase in UCS 'wanting' was specific to the available food reward.

**Other behavioral effects of DAMGO in accumbens shell**

*Sleep, grooming, and locomotion*. Several additional behavioral changes were observed during food intake testing following microinjection of DAMGO into accumbens shell. The amount of time spent sleeping decreased from ~3 mins under vehicle conditions to ~0.5 mins [$F(1,22)=5.3$, p=0.031]. DAMGO also halved the number of observed grooming bouts [$F(1,22)=14.6$, p=0.001]. Conversely, accumbens shell DAMGO enhanced more general measures of locomotor activity, roughly doubling both the number of cages crosses [$F(1,22)=11.9$, p=0.002] and rears [$F(1,22)=13.2$, p=0.001] during food intake testing.

*Defensive treading behavior*. Defensive treading behavior was observed in 40% of rats after accumbens shell DAMGO, and was significantly elevated relative to vehicle controls [$F(1,22)=4.9$, p=0.038]. This treading was light to moderate in intensity – much

lower than the treading induced by either DNQX or muscimol in accumbens shell (Reynolds & Berridge, 2002, 2008) or by muscimol in BNST (see chapter 2) – and directed mainly at the corners and walls of the food intake chamber. Notably, DAMGO still increased feeding [food intake in grams, $t(7)=4.3$, $p=0.004$; feeding bouts, $t(7)=2.6$, $p=0.034$] in this sub-set of animals who displayed treading behavior. In fact, several animals were observed to tread briefly at food pellets they had been eating just moments prior.

**Anatomical gradients**

*Accumbens shell.* Microinjection sites in accumbens shell were classified according to their position along rostro-caudal and dorso-ventral axes according to previously published criteria (Faure et al., 2008; Mahler, Smith, & Berridge, 2007; Pecina & Berridge, 2005). For the rostro-caudal axis, a dividing line was placed at 1.4mm anterior to bregma, with placement anterior to this line classified as rostral (during training=13; after training=16) and placements posterior to this line classified as caudal (during training= 7; after training=6). For the dorso-ventral axis, the dividing line was placed at 7.4mm below skull surface, with microinjection sites above this line classified as dorsal (during training=8; after training=13) and sites below this line classified as ventral (during training=12; after training=9).

Although we observed slight trends towards increased feeding at more ventral sties in accumbens shell, none of these effects reached statistical significance [all F's < 2.8, n.s.]. For autoshaping and conditioned reinforcement testing, we also did not find any significant anatomical variance in the observed enhancement of 'wanting.'

*BNST*. Microinjection sites in BNST were classified according to their position along the rostro-caudal, medial-lateral, and dorso-ventral axes (see Chapter 2 for details).

For placements in BNST, there were no significant anatomical effects or gradients when microinjections were administered throughout autoshaping training, either on autoshaping or on subsequent conditioned reinforcement testing. When microinjections were administered after autoshaping training, the inhibitory effect of DAMGO on prepotent cue behaviors during the CS+ period was marginally stronger at ventral BNST sites [$t(13)=1.9$, $p=0.078$]. This may help explain why we observed significant disruption of prepotent cue behaviors after intra-BNST DAMGO only in the group that received microinjections after training (n=15, 80% ventral BNST placements) and not in the group that received intra-BNST DAMGO throughout testing (n=11, 27% ventral BNST placements). There was no significant anatomical effect on subsequent conditioned reinforcement testing in animals receiving microinjections after training.

Finally, no significant anatomical gradients were observed in BNST for food intake testing following autoshaping testing. This is consistent with our previous food intake testing in BNST using DAMGO (see Chapter 2).

***Discussion***

Here we compared the effect of μ-opioid stimulation in the shell of the nucleus accumbens and BNST on two measures of incentive salience 'wanting': autoshaping and conditioned reinforcement. As predicted, opioid stimulation of accumbens shell both broadened and enhanced incentive salience 'wanting' for reward CS's, increasing

approach and interaction with each animal's non-prepotent cue during autoshaping and increasing the reinforcement value of the autoshaping CS+ in subsequent conditioned reinforcement testing. In contrast, opioid stimulation in BNST diffusely enhanced approaches to cup and looks at preferred CS outside the temporal appearance of CS+, and robustly enhanced intake of food UCS itself, but if anything disrupted the focused 'wanting' to the most preferred reward CS's during autoshaping testing, and had little impact on responding during conditioned reinforcement. These studies afford an interesting comparison of the role of ventral striatal vs. extended amygdala opioid transmission in modulating the value of reward CS's.

A potential metaphor to assist in describing these varied effects is to imagine the attribution of incentive salience as similar to the beam of a flashlight. Under vehicle conditions, the beam is directed primarily at an animal's prepotent cue (once learning is complete) and turned on predominately during the CS+ period, with only weak and sporadic illumination in non-CS+ periods. After opioid stimulation in accumbens shell, the flashlight beam is broadened to more brightly illuminates the non-prepotent cue during CS+ periods and in some cases the illumination is intensified at the beams center, resulting in increased prepotent cue slow bites. In CeA, opioid stimulation causes an intensification, but not broadening, of the beam, resulting in a focused enhancement of prepotent cue 'wanting' (Mahler & Berridge, 2009). In BNST, μ-opioid stimulation appears to illuminate the flashlight beam *outside* the CS+ period, when it would normally be turned off, and perhaps as a result of this temporally redistributed intensity the beam fails to burn quite as brightly during the CS+ period. These effects produce three unique patterns of behavior, and present a striking contrast to the relative homogeneity of opioid

activity in ventral forebrain in other reward-related behaviors, such as feeding (Bakshi & Kelley, 1993a; Gosnell, 1988; Mahler & Berridge, 2009; Na, 2008; Zhang & Kelley, 2000).

***Broadening and enhancement of 'wanting' with accumbens shell opioids***

Opioid stimulation in accumbens shell has previously been shown to increase the hedonic impact, or 'liking', of pleasant food rewards like a sweet sucrose solution (Kelley et al., 2002; Pecina & Berridge, 2000, 2005; Pecina, Smith et al., 2006; K. S. Smith & Berridge, 2007) and also to increase 'wanting' for UCS food rewards (Bakshi & Kelley, 1993b; Zhang & Kelley, 2000), possibly as a result of increased 'liking.' Here we show that accumbens shell opioid stimulation can also *directly* enhance 'wanting' for Pavlovian CS's that predict a food reward. Our findings in autoshaping and conditioned reinforcement, together with the finding that intra-accumbens (both core and shell) opioid stimulation can also increase PIT for a food UCS (Pecina & Berridge, In Preparation), demonstrate that accumbens shell opioids can generate enhancements of 'wanting' in at least three separate behavioral tests of incentive salience (Berridge & Robinson, 2003).

In autoshaping, DAMGO in accumbens shell generated a broad enhancement of incentive salience that elevated non-prepotent cue responding. Opioid stimulation produced enhanced looks at their non-prepotent cue and also increased approach and interaction with the non-prepotent cue. This motivational broadening stands in contrast to the effects of intra-CeA DAMGO, which selectively increased responding only towards a subject's prepotent reward CS during an identical autoshaping test (Mahler & Berridge, 2009). This suggests divergent roles for CeA and accumbens shell in the attribution of

incentive salience: whereas opioid stimulation in CeA resulted in a 'winner take all' situation in which additional 'wanting' was exclusively directed at an animal's previously learned prepotent CS, accumbens shell opioid stimulation appeared to elevate 'wanting' for any and all available reward CS's, similar to a 'rising tide that floats all boats.'

The broadening and enhancement of 'wanting' following opioid stimulation in accumbens shell is also support by our finding that DAMGO microinjection increased the reinforcement value of the autoshaping CS+ during post-autoshaping conditioned reinforcement testing. In animals that preferred the CS+ lever during autoshaping testing, opioid stimulation enhanced the value of the lever/light/tone CS+ that was the primary motivational magnet for these animals during autoshaping. In $CS_{Cup}$ animals, intra-accumbens shell DAMGO also increased instrumental responding for the autoshaping CS+, even though their prepotent CS (the food magazine) was no longer available.

Our finding replicates earlier work reporting increased conditioned reinforcement following intra-accumbens DAMGO across a broad ranges of doses (from 0.1ug – which we used here – all the way down to 0.003ug) (Phillips et al., 1994). However, microinjections sites in Phillips et al. (2004) study were located in either accumbens core or along the boundary of accumbens core and shell. As such, the current results extend this prior finding by showing for the first time that opioid stimulation in accumbens shell alone is sufficient to support enhanced conditioned reinforcement.

We observed increased slow bites of the prepotent cue during autoshaping when DAMGO was microinjected into accumbens shell. Slow bites of the prepotent CS are a terminal consummatory behavior that mimics the biting action during consumption of a UCS food reward. Opioid stimulation in CeA, which shares direct connections to

accumbens shell, was recently reported to enhance anticipatory nibbles and sniffs of the prepotent autoshaping cue, a behavior that more closely resembles the early approach and investigation of a food reward (Mahler & Berridge, 2009). This interesting divergence suggests that, in addition to the differential focused vs. broad enhancements of incentive salience mediated by CeA and accumbens shell opioids, respectively, opioid neurotransmission in these structures may enhance different phases of the prepotent cue appetitive response. In support of this view, El-Amamy & Holland (1990) have suggested that different behavioral aspects of appetitive Pavlovian conditioning (UCS-dependent conditioned responses vs. CS-dependent orienting responses) may be linked to difference neural substrates, since CeA lesions disrupted CS-orienting responses but left UCS-dependent conditioned responses intact. Alternatively, emphasis on terminal slow bites in accumbens shell animals may be related to hedonic 'liking' enhancements that accompany μ-opioid stimulation in medial accumbens shell (Pecina, Smith et al., 2006), which could conceivably make the prepotent CS take on more of the palatable properties of the linked UCS reward and, subsequently, generate more terminal bites.

***Diffusion and disruption of 'wanting' with BNST opioids***

Contrary to our predictions, intra-BNST opioid stimulation did not increase 'wanting' for a reward CS. Instead, DAMGO microinjection during autoshaping appeared to cause a diffusion of incentive salience outside of the appropriate CS+ period. This was seen as increased responding for reward CS's during the intervening non-CS+ intervals and also reduced selectivity of prepotent cue responding during autoshaping testing (as measured by the proportion of total food cupe entries or CS+ lever presses that

occurred during CS+ period). The diffusion of incentive salience was also accompanied by a significant reduction in responding toward both the prepotent and non-prepotent cues.

The diffusion and disruption of 'wanting' following intra-BNST DAMGO is particularly interesting because it represents a dissociation between the effect of opioid stimulation in BNST and a closely related extended amygdala nuclei, the CeA, on behavior towards reward CS's. Mahler & Berridge (2009) found that DAMGO (at the same dose used in our experiment) potently *increased* the motivational magnet properties of a reward CS during autoshaping, focusing and enhancing 'wanting' for each animal's prepotent CS target. Thus, it appears that although 'wanting' for a UCS food reward is increased by DAMGO in both structures (Gosnell, 1988; Na, 2008), opioid stimulation has nearly opposite effects on appetitive behavior towards reward CS's. CeA opioid stimulation creates sharper, focused peaks of CS 'wanting' while BNST dulls these peaks while slightly elevating 'wanting' during the intervening ITI valleys.

Differences in the role of the BNST and CeA, in spite of their strong anatomical parallels, has been reported in other behavioral paradigms, including relapse of drug seeking and fear conditioning. For example, microinjection of the stress-related neuropeptides corticotropin-releasing factor (CRF) into the BNST, but not the CeA, is sufficient to induce relapse of cocaine seeking in rats; similarly, antagonism of CRF neurotransmission in BNST but not in CeA was sufficient to block relapse in cocaine seeking after a stressful foot shock (Erb & Stewart, 1999). Additionally, CRF microinjection into BNST potently reduced feeding in food-deprived rats, but had no effect on feeding when microinjected into CeA (Ciccocioppo et al., 2003). In fear

conditioning, Michael Davis and colleagues have convincingly argued for dissociation between the role of the BNST and CeA in mediating the potentiating effects of different types of aversive stimuli in an acoustic startle paradigm. Temporary lesions of the BNST inhibited potentiation of the startle response by long-duration, unconditioned stimuli (such as extended exposure to a bright lighting), but left intact startle potentiation by short-duration, conditioned stimuli; temporary lesions of the CeA had precisely the opposite effect, impairing response to short, conditioned cues but sparing potentiated startle to long, unconditioned cues (Walker & Davis, 1997). Davis and colleagues have argued that this distinction indicates a role for BNST in anxiety (a diffuse, long-duration and relatively unconditioned behavioral response), whereas the CeA is essential for the expression of focused, stimulus-specific fear (Davis, 1998; Davis & Shi, 1999; Davis et al., 1997a, 1997b; Walker et al., 2003).

It is possible, based on our current findings, that CeA and BNST play similar roles in their involvement in appetitive motivation, with CeA focusing and enhancing motivation for conditioned cues in close phase-lock with CS presentation, whereas BNST generates a more temporally diffuse appetitive motivation that results in enhanced responding outside of CS+ presentation periods. That the temporal diffusion of 'wanting' during autoshaping following DAMGO is in BNST, in some cases, accompanied by disruption of responding during the 8-sec CS+ window may be the result of direct inhibition of CeA by BNST. Indeed, a recent report suggests that excitation of neurons in the juxtacapsular region of BNST (a sub-nucleus in the lateral division and a likely target of many of our manipulations) sends inhibitory projections to CeA (Francesconi et al., 2009), which could explain the disruption in prepotent cue that we observed. This

interpretation is further supported by the finding that lesions of CeA (both temporary lesions with muscimol as well as permanent excitotoxic lesions) lead to a disruption of responding during the autoshaping CS+ (Gallagher et al., 1990; Holland & Gallagher, 2003; Mahler & Berridge, 2009). However, lesions in CeA did not increase responding during the ITI, suggesting that the diffusion of CS 'wanting' into the ITI observed following intra-BNST opioid stimulation is not simply the result of CeA inhibition.

In contrast to autoshaping, responding for the autoshaping CS+ during subsequent conditioned reinforcement testing was unaffected by intra-BNST DAMGO. This difference may be related to a difference in the temporal window for appropriate responding between autoshaping and conditioned reinforcement testing. During autoshaping, animals are presented with extended ITI's (here they averaged 90-sec) interspersed with short, 8-sec CS+ periods. In contrast, during conditioned reinforcement the active nose port (which earns a brief 4-sec CS+ presentation) is available throughout the session, effectively eliminating the requirement that appropriate responding be phase-locked to a limited temporal window. If, indeed, the primary effect of DAMGO in BNST is to blur the appropriate temporal assignment of 'wanting,' then these differences in the temporal nature of the two tasks suggests that a deficit in autoshaping is likelier than a deficit in conditioned reinforcement. It should be noted, however, that there is one behavioral measure in conditioned reinforcement that is only briefly temporally available: presses of the active lever during the CS+ presentations. Consistent with our interpretation, CS+ lever oriented animals that received DAMGO in BNST displayed marginally fewer lever presses during conditioned reinforcement testing than vehicle controls, suggesting that they may have been slightly disrupted in their ability to emit .

***Future directions***

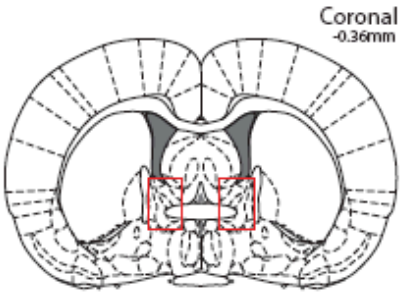
It will be important to test our hypothesis of broadly enhanced incentive salience after accumbens shell opioid stimulation in other behavioral paradigms. A primary candidate would be general PIT (Corbit et al., 2001; Glasner, Overmier, & Balleine, 2005). In this paradigm, animals are trained to associate instrumental responding on two or more levers with unique UCS rewards (e.g. banana flavored pellet for lever A, grape flavored sucrose solution for lever B, etc.), and then separately trained on the Pavlovian association of different cues (such as tones or lights) with the same UCS rewards used in instrumental training. In the critical transfer test, the Pavlovian cues are presented in the presence of the instrumental outcomes (though no UCS rewards are delivered). Pavlovian cues can normally energize instrumental responding on the lever that previously delivered the *same* UCS reward as the Pavlovian cue (Dickinson & Dawson, 1987; Rescorla & Solomon, 1967). However, our broadening interpretation also predicts that intra-accumbens shell opioid stimulation may also increase 'wanting' for *all* available reward cues, which could result in increased responding on all available reward-associated levers in response to a single Pavlovian cue.

In BNST, it will be of interest to explore the potential role of other neurochemical systems within BNST on 'wanting' for reward CS's. Although μ-opioids in BNST appear to mediate diffusion of 'wanting', it is possible that more focused incentive salience might be modulated via excitatory connections with midbrain dopaminergic cell populations (Georges & Aston-Jones, 2001; Massi et al., 2008), or even by dopamine release within BNST (Carboni et al., 2000; Kash et al., 2008).

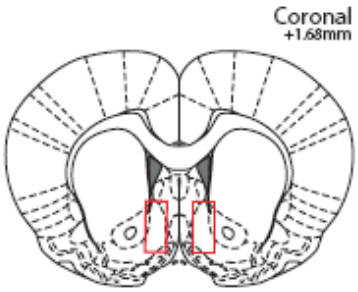
Additionally, it will be of interest to explore the role of other extended amygdala nuclei, such as the sublenticular extended amygdala (formerly caudal substantia inominata) and the interstitial nucleus of the anterior commissure, in incentive salience ‘wanting’ for reward cues, as well as circuit interactions between nodes within the extended amydala macrosystem.

**Figure 3.1. Fos plumes in BNST and accumbens shell.** *Target Region*: Highlights the location of BNST and accumbens shell in coronal slices, taken from Paxinos & Watson (2007). *Fos Sampling*: An example of the radial counting grid. Each grid square measures 68um x 68um, with 10 squares emanating from each radial arm. Grid squares falling in ventricles or over white matter tracts were excluded from quantification and analysis. *Normal*: Representative image from uninjected, virgin tissue in BNST and accumbens shell. *DAMGO (0.1ug)*: Representative plumes (relative to normal and vehicle controls) from DAMGO microinjected tissue. All images shown are 2x2 tiled images taken at 10x magnification.

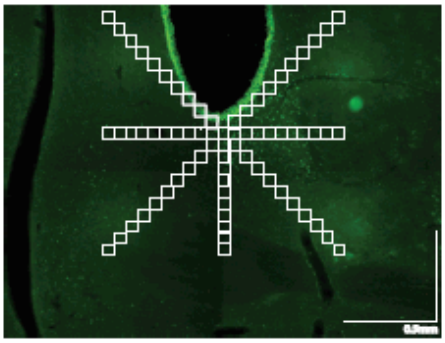
BNST Target Region



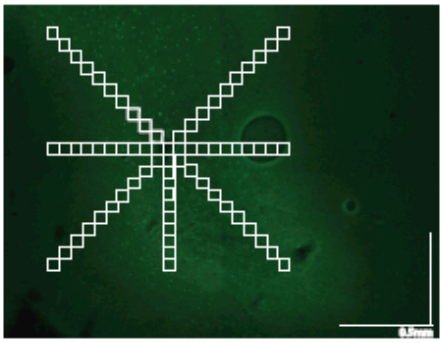

Accumbens Shell Target Region



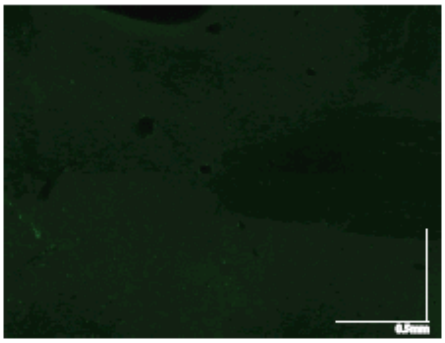

BNST Fos Sampling



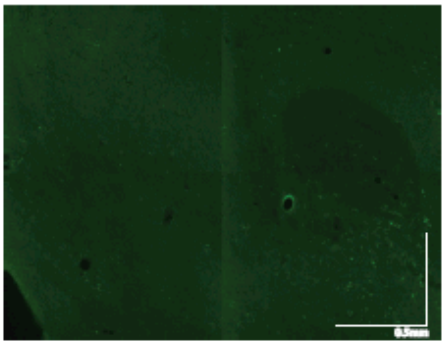
Nacc Shell Fos Sampling



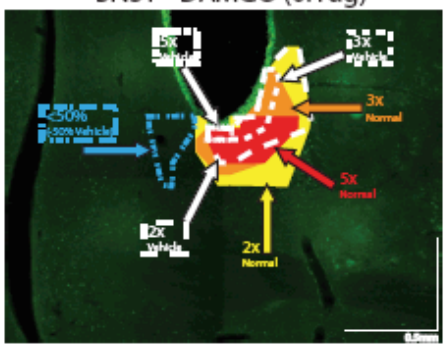
BNST - Normal



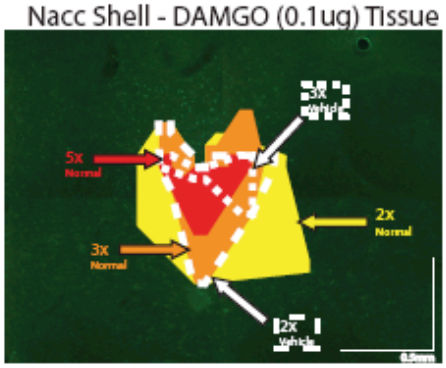
Nacc Shell - Normal Tissue



BNST - DAMGO (0.1ug)




Nacc Shell - DAMGO (0.1ug) Tissue



| | Mean Fos Plume Radius (mm) | | | Estimate Fos Plume Volume (mm3) | | |
|---|---|---|---|---|---|---|
| | Inner plume 500% control | Middle plume 300% control | Outer plume 200% control | Inner plume 500% control | Middle plume 300% control | Outer plume 200% control |
| Vehicle | 0.07 | 0.16 | 0.31 | 0.001 | 0.017 | 0.120 |
| BNST DAMGO (0.1ug) | 0.11 | 0.25 | 0.35 | 0.006 | 0.065 | 0.180 |
| Accumbens DAMGO (0.1ug) | 0.07 | 0.26 | 0.43 | 0.001 | 0.074 | 0.333 |

**Table 3.1. Fos plume radii and estimated volumes.** Mean radii (left) and volume (right) are listed for vehicle, BNST DAMGO (0.1µg), and accumbens shell DAMGO (0.1ug) conditions. Plume sizes were calculated compared to normal tissue alone for the vehicle condition, and compared to both normal and vehicle control conditions for all other groups.

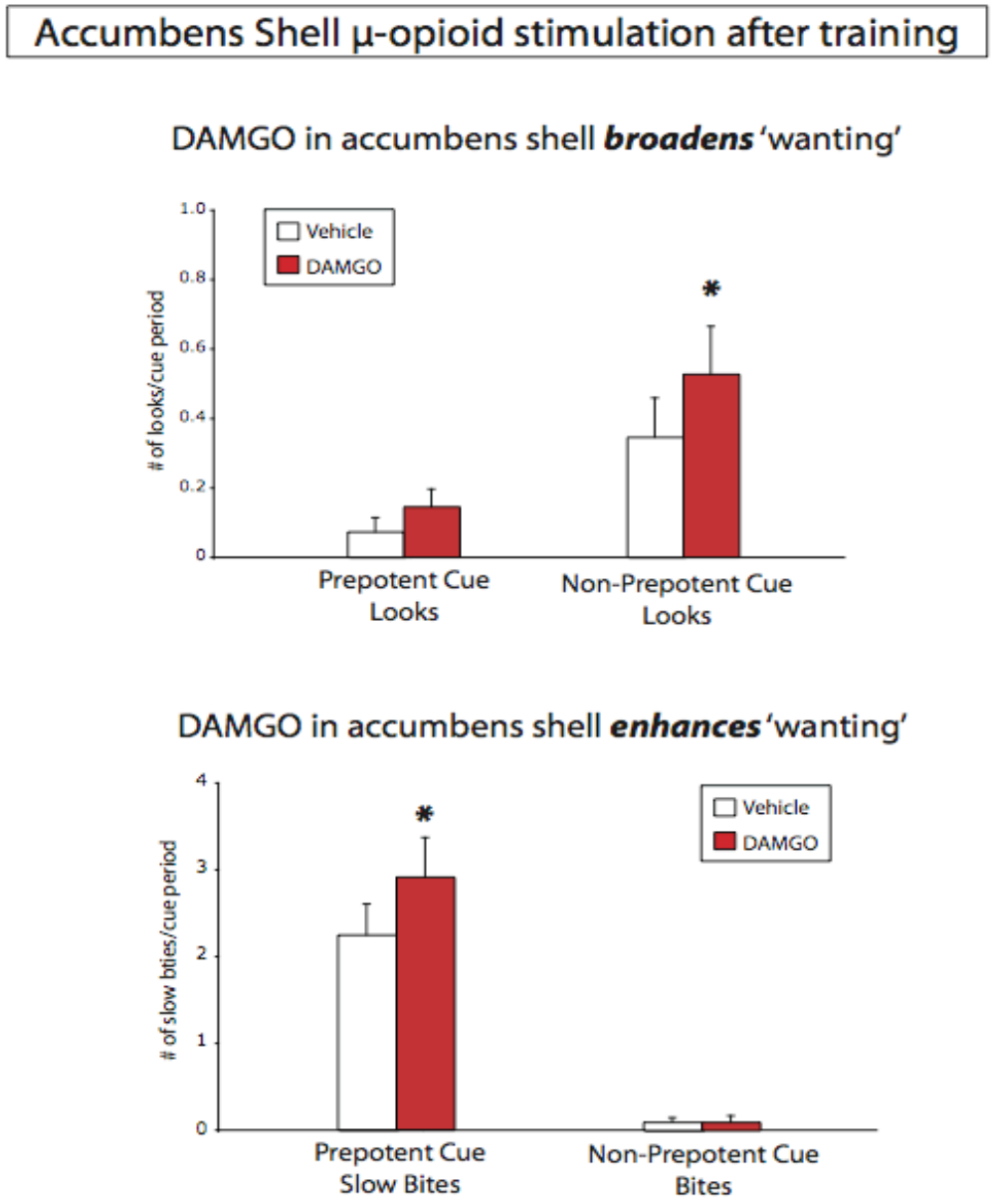



**Figure 3.2. DAMGO in accumbens shell broadens and enhances 'wanting' when delivered after autoshaping training.** * indicates difference from vehicle condition, $p<0.05$

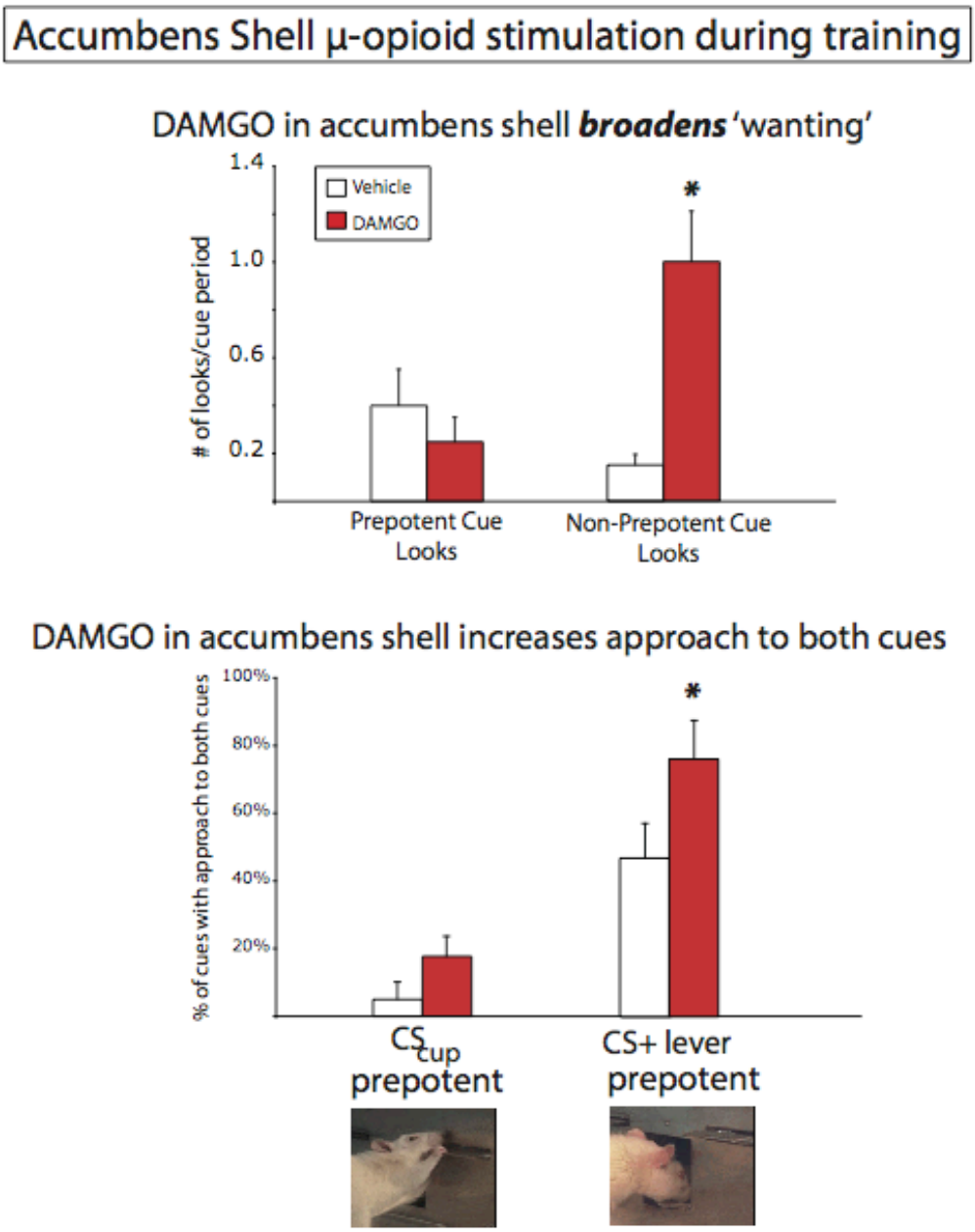



**Figure 3.3. DAMGO in accumbens shell broadens 'wanting' when delivered during autoshaping training.** * indicates difference from vehicle condition, *p<0.05*

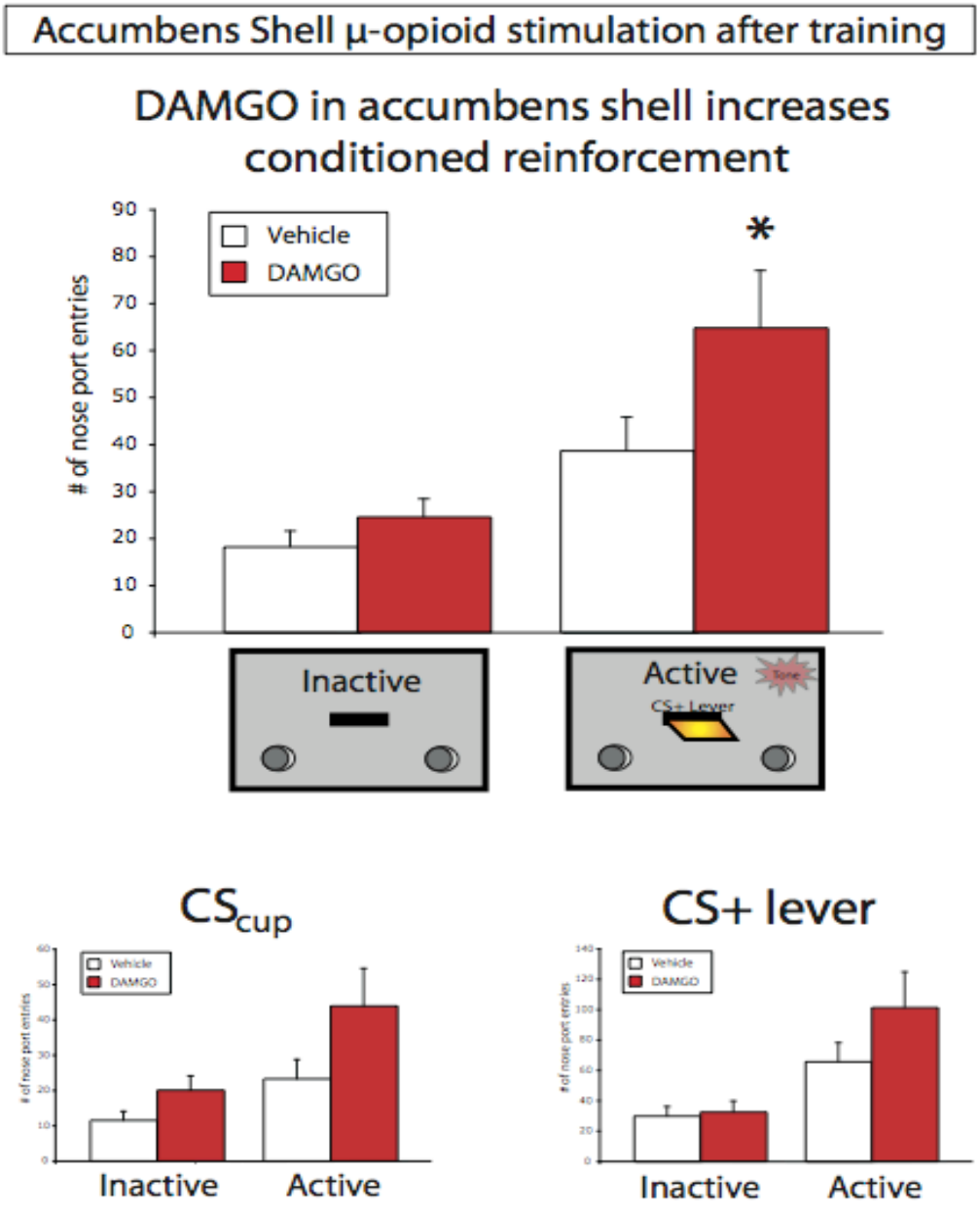



**Figure 3.4. DAMGO in accumbens shell increases conditioned reinforcement value of the autoshaping CS+.** *Top panel*: Entries into the active and inactive nose ports in the same animals under vehicle and DAMGO treatment. Both phenotypes are combined in this graph. * indicates significant drug*nose port interaction, $p<0.05$. *Bottom panel*: Data split to show the separate autoshaping phenotypes. Note the similar patterns of behavior, but higher overall responding for CS+ lever animals.

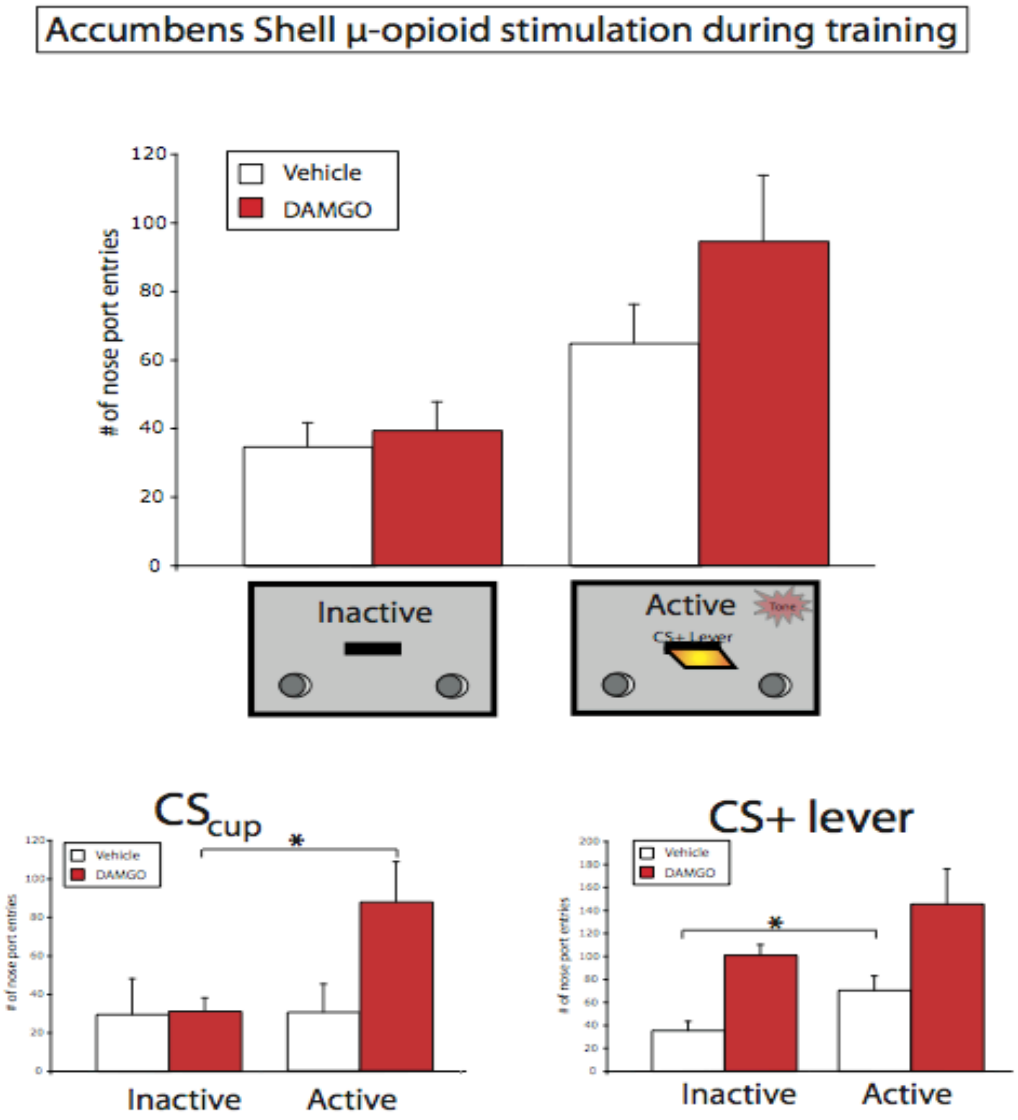



**Figure 3.5. DAMGO in accumbens shell increases instrumental responding during conditioned reinforcement testing.** *Top panel*: DAMGO increased instrumental responding, but this was applied to both the inactive and active nose ports. Both phenotypes are combined here. *Bottom panel*: Autoshaping phenotypes are separated. Due to inadvertently low n's in two conditions ($CS_{cup}$ vehicle and CS+ lever DAMGO groups), separate analysis was inconclusive, but did indicate significant conditioned reinforcement in $CS_{cup}$ DAMGO animals and CS+ lever vehicle animals. * indicates significant paired samples t-test, $p<0.05$.

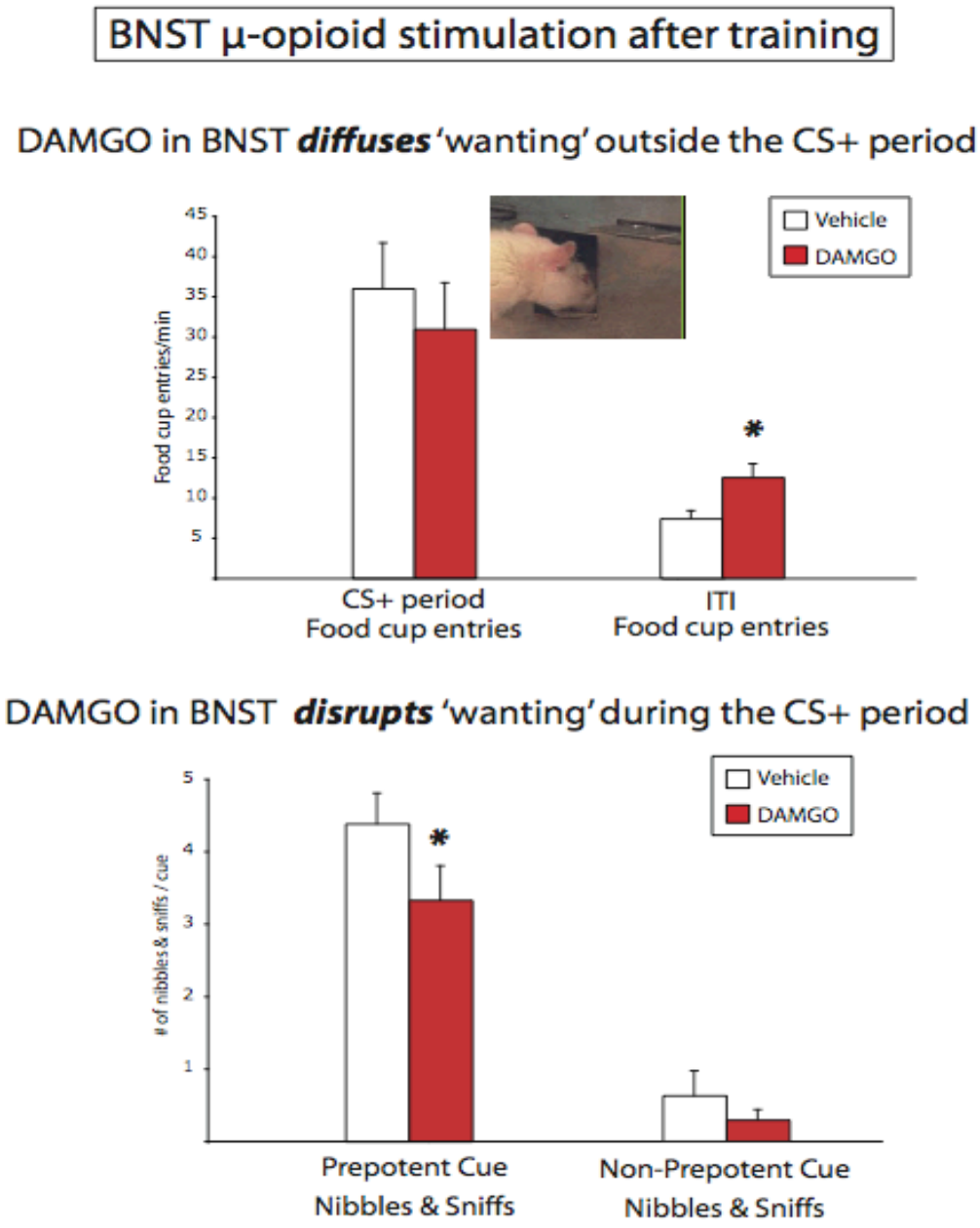



**Figure 3.6. DAMGO in BNST diffuses and disrupts 'wanting' when administered after training.** * indicates difference from vehicle condition, *p<0.05*

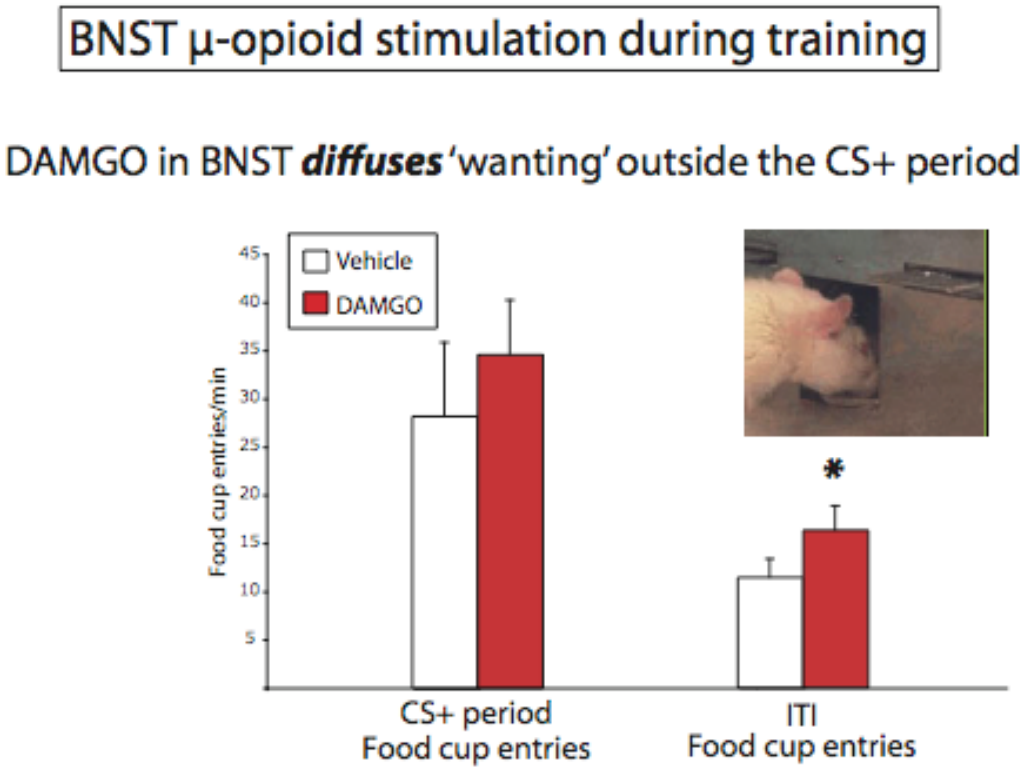



**Figure 3.7**. **DAMGO in BNST diffuse 'wanting' when administered during training.** * indicates difference from vehicle condition, *p<0.05*

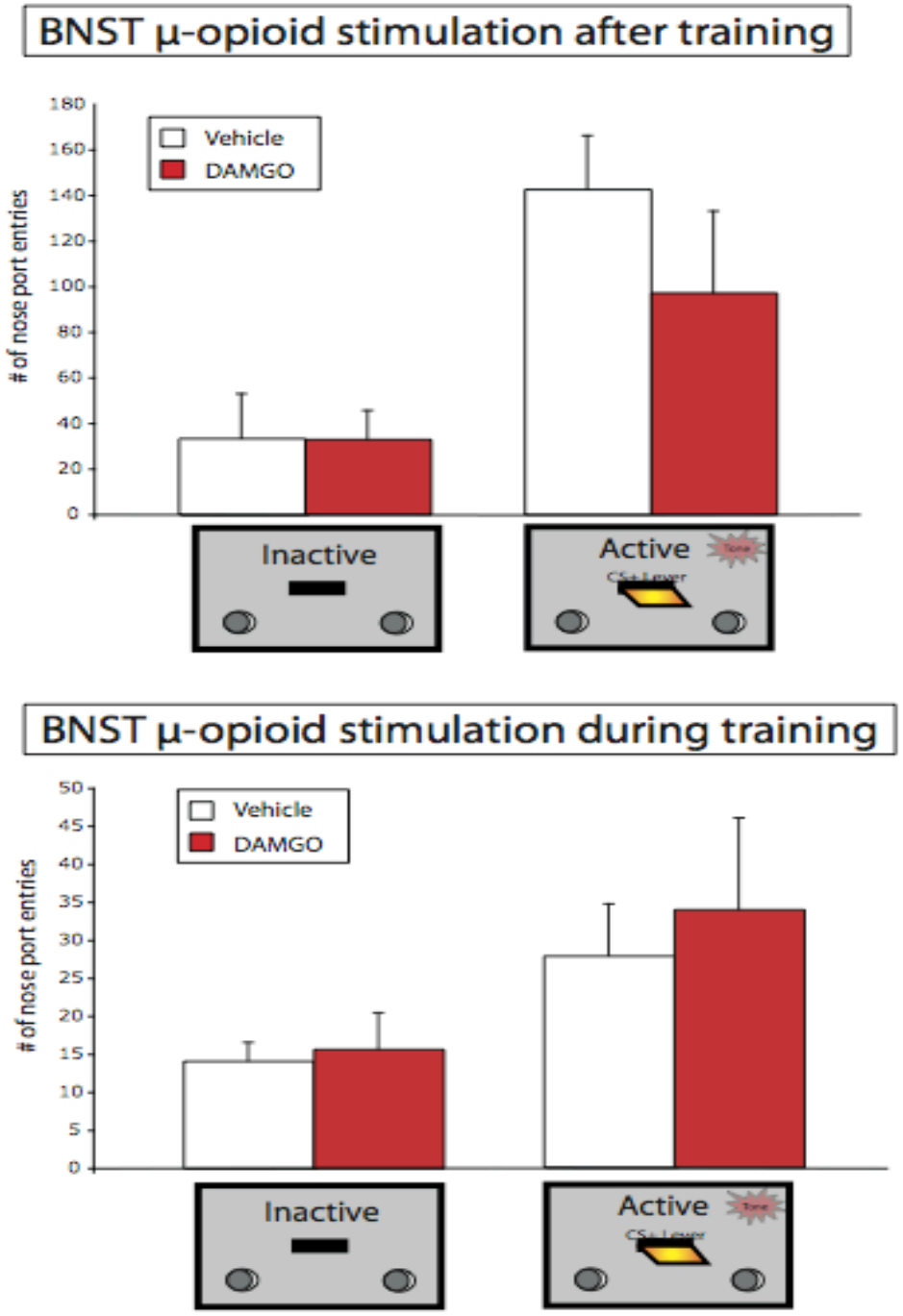



**Figure 3.8. Effects of BNST DAMGO on conditioned reinforcement testing.** *Top panel*: Conditioned reinforcement following autoshaping expression testing. Only CS+ lever animals were present in this group. *Bottom panel*: Conditioned reinforcement following autoshaping acquisition testing. Only $CS_{cup}$ animals were present in this group.

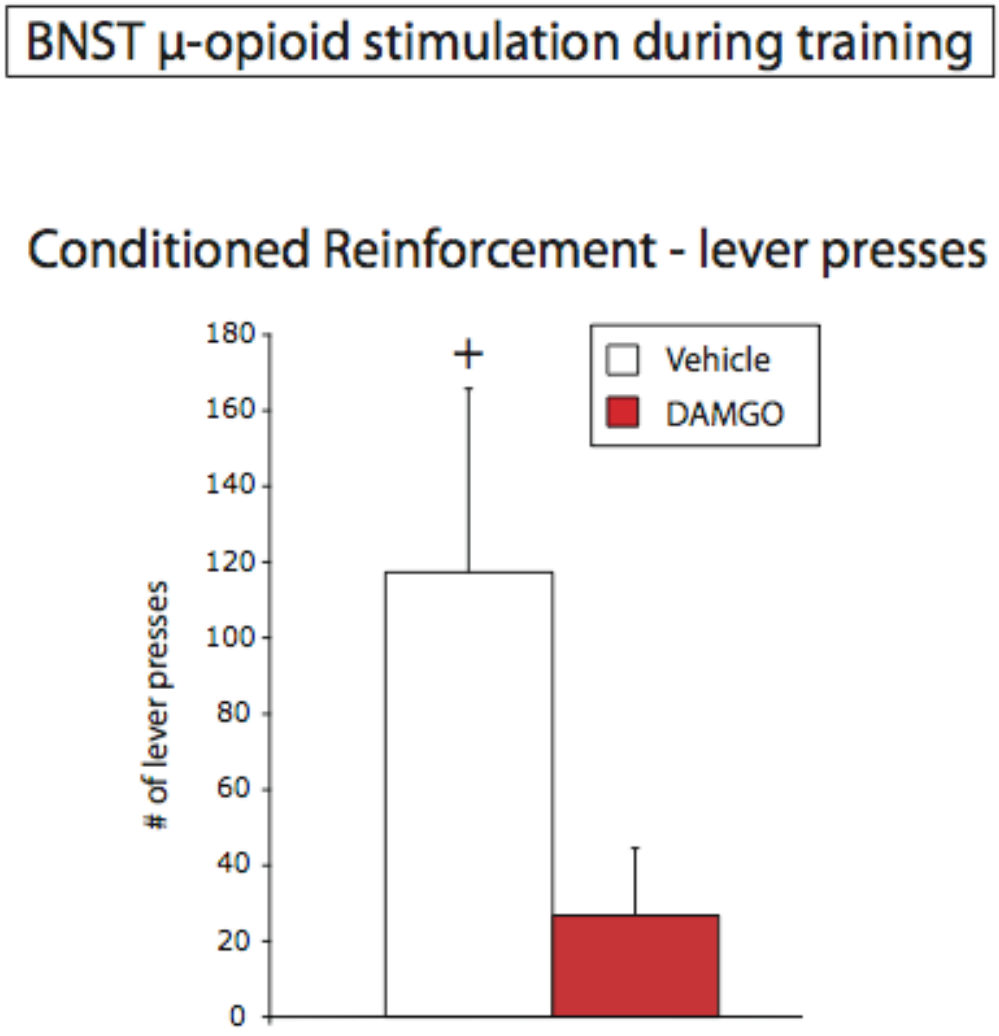



**Figure 3.9. DAMGO in BNST marginally reduced presses of the CS+ lever during conditioned reinforcement testing.** Only CS+ lever animals were present in this group. + indicates marginal difference from DAMGO group, $p<0.1$.

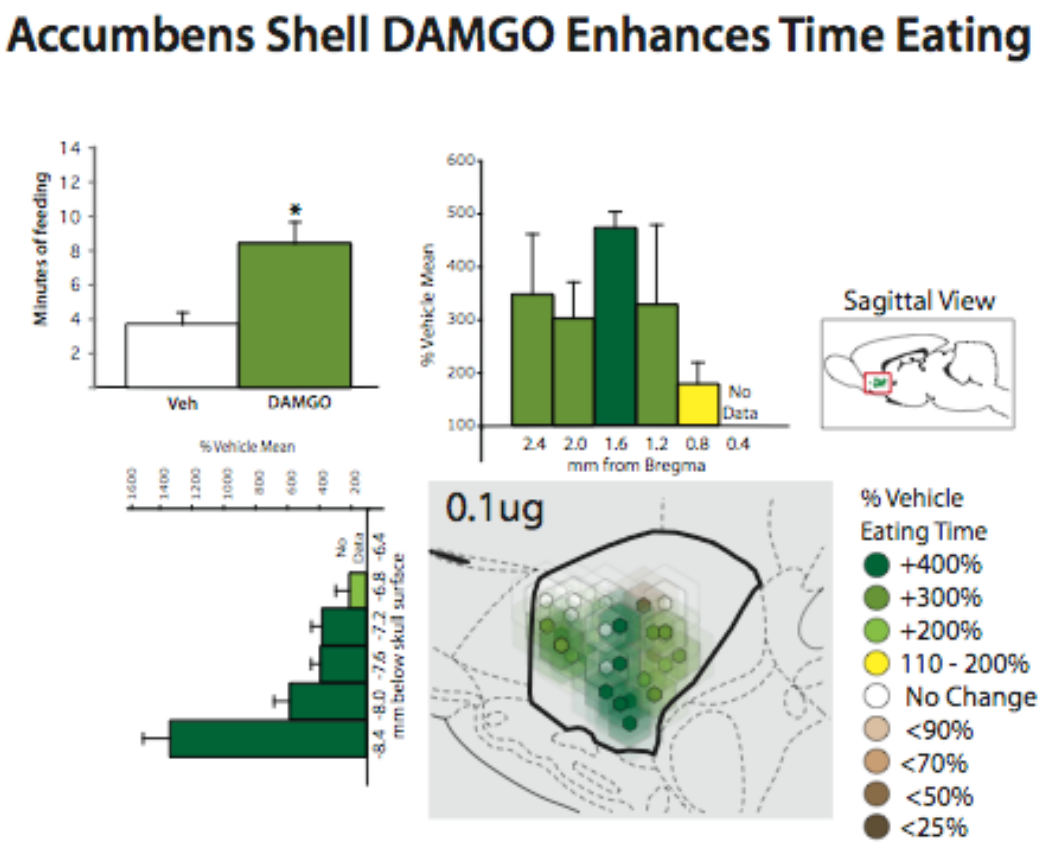
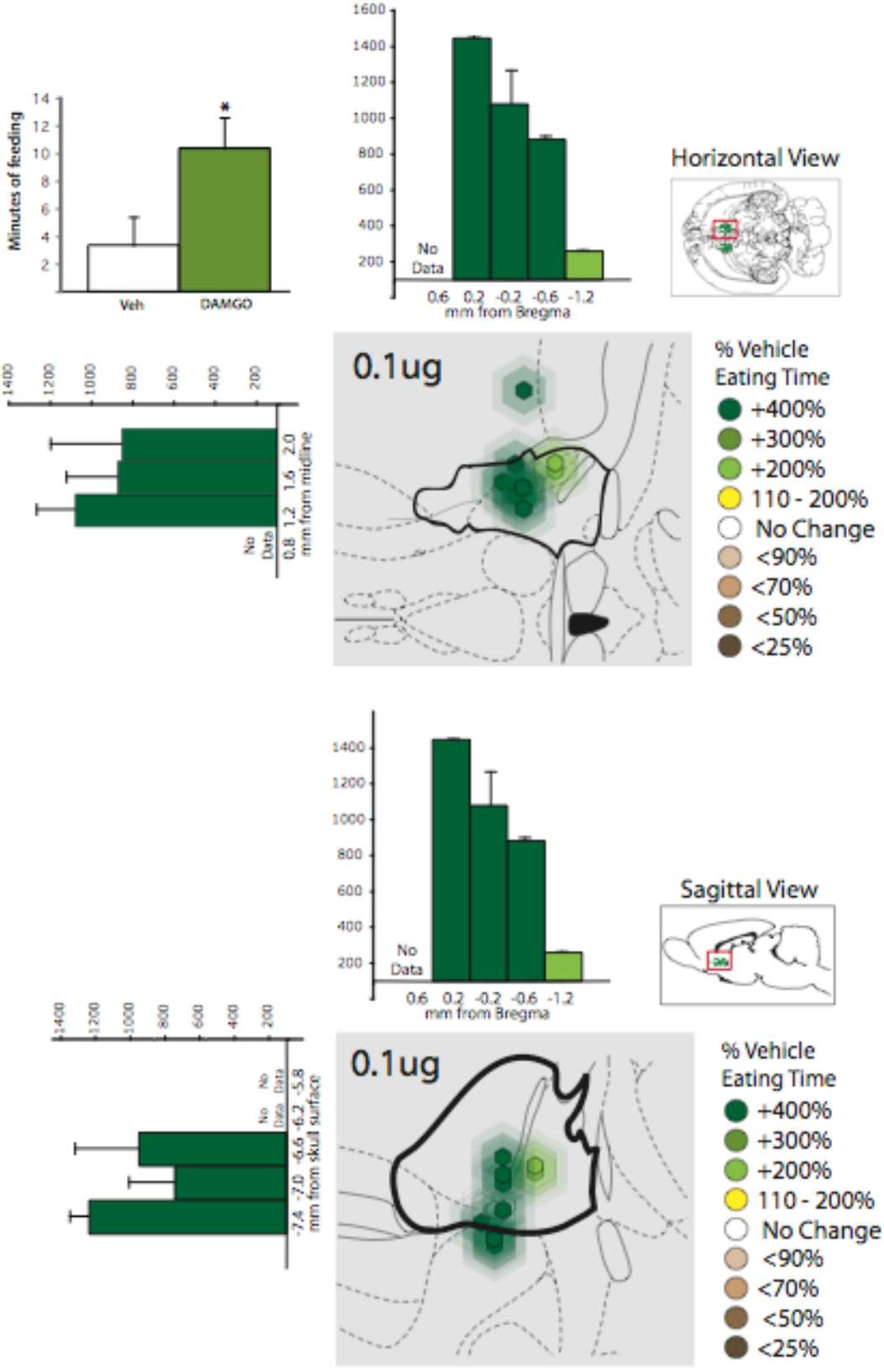



**Figure 3.10. DAMGO in accumbens shell increases feeding.** Feeding time enhancements for 0.1ug DAMGO microinjections are mapped rat-by-rat onto a sagittal view of accumbens shell, showing both the within-subject change in feeding time relative to vehicle day (color) and the functional spread of the microinjection based on Fos plume data (size). Each map symbol is composed of three nested hexagons; the inner symbol shows the average size of intense 5x activation, the middle symbol shows 3x activation, and the outer symbol shows 2x activation. Bar graphs along the rostro-caudal and medial-lateral axes show the average drug effect within each 0.4mm wide region. Bar graph showing absolute comparison of vehicle and drug feeding time (in minutes) can be found above and to the left of the anatomical map. * indicates differences from vehicle, $p<0.05$.

**Figure 3.11. DAMGO in BNST increases feeding.** Feeding time enhancements for 0.1ug DAMGO microinjections are mapped rat-by-rat onto a horizontal and sagittal views of BNST, showing both the within-subject change in feeding time relative to vehicle day (color) and the functional spread of the microinjection based on Fos plume data (size). Each map symbol is composed of three nested hexagons; the inner symbol shows the average size of intense 5x activation, the middle symbol shows 3x activation, and the outer symbol shows 2x activation. Bar graphs along the rostro-caudal and medial-lateral axes show the average drug effect within each 0.4mm wide region. Bar graph showing absolute comparisons of vehicle and drug feeding time (in minutes) can be found above and to the left the horizontal anatomical map. * indicates differences from vehicle, $p<0.05$.

# BNST DAMGO Enhances Feeding



# Chapter 4

## Opioid Activation in the Bed Nucleus of the Stria Terminalis Increases 'Wanting' Without Enhancement of Hedonic 'Liking'

### *Introduction*

Taste signals move through cranial nerves to the nucleus of the solitary tract (NST) in hindbrain medulla, then to the pontine parabrachial nucleus (PBN) in rodents, and then diverge in forebrain into a dorsal pathway to target gustatory thalamus for thalomo-cortical relay, and into a ventral or pathway to hypothalamus, amygdala, ventral pallidum and a variety of related limbic forebrain targets that include BNST (Spector & Travers, 2005). These ascending signals to the forebrain, as well as top-down modulation of taste by cortex (de Araujo et al., 2003; Rolls, 2006), are of interest to the neuroscience of reward because they influence how the brain dynamically and adaptively merges a sensory stimulus and information about an organism's current physiological or affective state in order to guide behavior.

Several sites and neurotransmitter systems in the forebrain have been shown to exert control over taste hedonics. The endogenous opioid system, and in particular the μ-opioid receptor, have frequently been linked to specific increases in the hedonic impact, or 'liking,' of food rewards (Berridge, 2000; Kelley et al., 2002; Pecina, Smith et al., 2006), including the recent identification of discrete μ-opioid hedonic hotspots within

rostro-dorsal accumbens shell and caudal ventral pallidum (Pecina & Berridge, 2000, 2005; Pecina, Smith et al., 2006; K. S. Smith & Berridge, 2005). The accumbens and pallidal hotspots appear to act in a cooperative circuit to enhance 'liking' (K. S. Smith & Berridge, 2007), and may further interact with a more recently identified endocannabinoid hotspot that is located in the dorsal half of the medial accumbens shell, roughly co-localized with the opioid hotspot (Mahler et al., 2007).

Here I address the role of the bed nucleus of the stria terminalis (BNST), another component of the extended amygdala, which receives direct projections from the pontine parabrachial nuclei (PBN) (Norgren, 1976; Spector & Travers, 2005); (Alden et al., 1994; Tokita, Inoue, & Boughter, 2009; Whitehead, Bergula, & Holliday, 2000), and sends efferent projections back to both PBN and NST, mostly from non-overlapping neuronal populations (Kang & Lundy, 2009). Electrophysiological studies have confirmed that these BNST efferents modulate firing of taste responsive neurons in both NST and PBN, and exert an almost exclusively inhibitory influence on cells in both brainstem regions (C. S. Li & Cho, 2006; D. V. Smith, Ye, & Li, 2005). The existence of such functional anatomical pathways suggests the hypothesis that previously observed increases in feeding after BNST opioid stimulation could be the result of changes in core taste processing. Yet it remains unclear precisely how BNST is acting to modulate gustatory information and which neurochemical systems are involved.

Within the nearby extended amygdala, the central nucleus of the amygdala (CeA) possesses reciprocal connections with gustatory PBN (Bernard, Alden, & Besson, 1993). However, although μ-opioid stimulation in CeA can increase voluntary food intake and 'wanting' for a reward CS (Gosnell, 1988; Mahler & Berridge, 2009), recent evidence

from our lab indicates that CeA opioid stimulation does not enhance 'liking' for a sucrose reward (Mahler & Berridge, 2006). In addition to highlighting the occasionally divergent nature of 'wanting' and 'liking', this finding also indicates a possible divergence between ventral-striatal-pallidal and extended amygdala opioid systems in the potentiation of hedonic 'liking'. That is, perhaps activation of NAc and ventral pallidal circuits potentiate 'liking', whereas activation of extended amygdala systems may not. However, so far in extended amygdala only the impact of opioid stimulation in CeA has been studied.

Here we test the prediction from the initial hypothesis that opioid and GABA stimulation in BNST are able to modulate primary taste reactions to experimenter infused oral tastants. We utilized the taste reactivity paradigm to evaluate rapid orofacial responses to taste stimuli (Berridge, 2000; Grill & Norgren, 1978), which avoids potential motivational confounds that arise when the subject is required to voluntarily consume the taste stimulus. We also tested whether our neurochemical manipulations affect subsequent voluntary food intake of both highly palatable (milk chocolate M&M's) and standard (lab chow) food sources.

***Methods***

*Subjects*: A total of 8 Sprague-Dawley rats (males, 250-500g at the time of surgery) were used for taste reactivity and food intake testing. All animals were housed in pairs (~21˚C; 12hr light/dark cyle, lights on at 9am) with *ad libitum* access to food (Purina 5001 chow; Purina Mills, St. Louis, MO) and tap water. All procedures were

approved by the University Committee on the Use and Care of Animals at the University of Michigan in accordance with National Institute of Health guidelines.

*Surgery*

All animals were handled twice for a total of fifteen minutes prior to undergoing surgery. Rats were pretreated with atropine (0.04mg/kg) and then anesthetized with ketamine (80mg/kg) and xylazine (5mg/kg). Rats were then placed in a stereotaxic device and implanted with bilateral, chronic guide cannulae (23 gauge, stainless steel), 14mm in length, aimed so that the ventral tip would rest 2mm above the BNST (AP: -0.35 to -0.4mm; ML: +/-1.4 to 1.6mm; DV: -4.75 to -5.3mm; incisor bar: -3.3mm [flat skull]). Guide cannulae were secured to the skull using four stainless steel screws and dental acrylic, and fitted with stainless steel stylets to prevent occlusion.

In the same surgery, all rats were implanted with bilateral oral cannulae (PE-100 tubing) to allow for oral infusion of liquid solutions during subsequent taste reactivity testing. Oral cannulae were attached to a 19-gauge needle, inserted lateral to the first maxillary molar, threaded behind the zygomatic arch, and emerged from the dorsal head. The 19-gauge needle was removed and replaced with a short section of 19-gauge stainless steel tubing. The pair of oral cannulae were loosely laced together with soldering wire and cemented to the skull screws with additional dental acrylic (Berridge, 2000; Grill & Norgren, 1978).

All rats were given post-operative analgesic (0.3 mg/kg buprenorphine) and prophylactic antibiotic (50 mg/kg chloramphenicol) immediately after surgery. Rats received a soft mash for 48 hours after surgery (Gerber oatmeal cereal; Michigan, USA),

and additional injections of antibiotic every 24 hours for at least 2 days to minimize infection. Rats were allowed to recover for at least 7 days before the onset of behavioral testing.

*Drugs and Microinjections*

All drugs were dissolved and diluted to dose in artificial cerebrospinal fluid (aCSF; Harvard Labs, Cambridge, MA). DAMGO was prepared at 0.05µg and 0.1µg doses (total bilateral dose of 0.1µg and 0.2µg, respectively), while muscimol was prepared at a 225ng dose (total bilateral dose of 450ng). These doses were chosen based on previous experiments showing successful modulation of feeding (see Chapter 2). aCSF alone was used for vehicle microinjections. Microinjection schedules were counter-balanced across subjects using a Latin Square design.

On test days, animals were gently handled as the stylets were removed. Rats then received bilateral microinjections (0.2µL per side, total 0.4µL bilateral volume) via 16mm stainless steel microinjection tips (29 gauge), which extended 2mm beyond the ventral tip of the guide cannula. Microinjection tips were attached via PE-20 surgical tubing to a microinfusion pump, which delivered the infusion over the course of 60 seconds. Microinjection tips were left in place for an additional 60 seconds after the infusion ended to allow for drug diffusion, after which the stylets were replaced and the rat was prepared for taste reactivity testing.

*Behavioral Taste Reactivity Tests*

Rats were habituated to the taste reactivity chamber for the 4 days immediately prior to the onset of taste reactivity testing. On habituation days, rats were placed in the taste reactivity for 30 minutes, and then moved to a food intake chamber for 60 minutes. On the final day of habituation, rats received a 60-sec oral infusion of distilled water in both oral cannulae to ensure patency.

On test days, rats received a microinjection of vehicle, DAMGO (0.05ug or 0.1ug), or muscimol (225ng), and then had tastant delivery tubes (PE-50 tubing attached to PE-10 nozzle with a short sleeve of EVA tubing to hold the delivery tube in place) attached to the oral cannulae immediately after the completion of drug microinjections, and finally the animals were placed in the taste reactivity chamber. The taste reactivity chamber consisted of a transparent plexiglass floor and a plexiglass cylinder (diameter 25cm), with an angled mirror below the transparent floor to allow for recording of orofacial reactions via a digital camcorder.

Rats received two oral infusions during taste reactivity testing: an infusion of a sweet 0.03M (1%) sucrose solution 20 minutes after drug microinjection (1mL volume infused over 1 minute), and an infusion of a bitter 3x10 M quinine (1mL volume infused over 1 minute) 10 minutes later at 30 minutes after drug microinjection. All solutions were diluted in distilled water, and brought to room temperature before infusion. The order of sucrose and quinine infusion was fixed to ensure that 'liking' reactions to the sucrose infusion were not contaminated by prior receipt of the bitter quinine solution (Mahler et al., 2007; Pecina & Berridge, 2005). All animals received a brief (~30 sec)

rinse with distilled water after each oral infusion to prevent lingering of the prior tastant in subsequent taste reactivity or food intake testing.

*Taste Reactivity Video Scoring*

Throughout the 1 minute oral infusions, rats were recorded with close-up video directed at the animal's mouth. An observer blind to the experimental condition subsequently analyzed this video off-line to measure hedonic, neutral, and aversive orofacial reactions. Video was scored in slow motion (1/4 to 1/10 speed) using The Observer XT 8.0 (Noldus; Netherlands) and following previously described criteria (Berridge, 2000). Positive hedonic 'liking' reactions included lateral tongue protrusions (extensions of the tongue away from the midline accompanied by a retraction of the lip), rhythmic midline tongue protrusions (smaller amplitude protrusions along the midline at roughly 6-8 Hz), and paw licking. Aversive responses included gapes (large openings of the mouth creating a triangular shape), forelimb flails (rapid waving of one or both forelimbs), head shakes, chin rubs (contact of the chin with the bottom or walls of the taste reactivity chamber), and face washing (cleaning of the face with both forepaws). Neutral reactions that cannot be classified as purely hedonic or aversive included mouth movements, passive drips of the solution out of the rats mouth, and bouts of grooming. Lateral tongue protrusions, gapes, forelimb flails, head shakes, chin rubs, and grooming bouts were counted as discrete actions each time they occurred. Other actions were counted as continuous and scored in the following time bins: midline tongue protrusions (2 sec bins), paw licking (5 sec bins), face washing (5 sec bins), mouth movements (5 sec bins), and passive drip (5 sec bins).

*Behavioral Food Intake Tests*

Immediately following complete of the second infusion on each day of taste reactivity testing, rats were placed in clear plastic cages containing a pre-measured pile of standard lab chow (~25g), a pre-measured pile of M&M chocolate candies (20 M&M's, ~17g), a water spout, and corncob bedding. Rats were habituated to the test environment for four days, as described above.

On test days, food intake was tested for 1 hour. The entire session was videotaped for subsequent offline analysis. When animals were removed from the test chamber after 1 hour, the experimenter would slowly insert one hand into the test chamber (~1ft/5sec). The response of rats to removal from the chamber was recorded, including the presence of distress vocalizations, dashing escape attempts, and attempted bites of the experimenter (Reynolds & Berridge, 2001).

After the test was completed, remaining chow and M&M's (including crumbs) were carefully removed from the cage and weighed. Test days were always separated by at least 48 hours.

*Food Intake Video Scoring*

Video recordings of food intake test sessions were scored offline by observers blind to the experimental condition. The following behaviors were recorded: eating time (in seconds), eating bouts (triggered by interruptions of eating of more than 5 seconds), food sniffing, food carrying, drinking time (in seconds), drinking bouts (same criteria as eating bouts), grooming, cage crosses, sleeping, rearing, and defensive treading. All

eating measures (time, bouts, total intake, sniffs, and carries) were scored separately for chow and M&M's. Treading is a natural defensive behavior emitted by rodents, and involves rapid forelimb strokes away from the body that push debris (e.g. dirt or bedding) in the direction of a threat.

*Histology*

After testing was completed, rats were deeply anesthetized with sodium pentobarbital (0.2mg/kg; Fatal-Plus) and decapitated. Brains were extracted and placed in a 10% paraformaldehyde solution for 24-48 hours, and then placed in a 30% sucrose solution for 3-5 days, until the brains sank. The brains were then sliced on a freezing microtome (Leica) into 60μm coronal sections, mounted onto glass slides, allowed to dry for at least 24 hours, and then stained with cresyl violet. Stained slices were viewed under light magnification and used to map the microinjection centers in each hemisphere on coronal sections taken from a rat brain atlas (Paxinos & Watson, 2007).

*Statistical analysis*

Taste reactivity data was analyzed using between subjects ANOVA followed by *post-hoc* comparisons where appropriate. Sucrose and quinine infusion data was analyzed separately. Due to difficulties with the oral infusions, three taste reactivity trials with the sucrose solution were excluded from analysis (one from 0.05ug DAMGO and two from muscimol). Food intake data was analyzed using a within subjects ANOVA (food type, drug) followed by *post-hoc* comparisons where appropriate. In all cases where percent

changes over vehicle are calculated or mapped, a fixed value of 1 was added to all data points to avoid division by 0.

***Results***

*DAMGO and muscimol in BNST suppress hedonic 'liking' reactions to sucrose*

Microinjection of both the μ-opioid agonist DAMGO and the $GABA_A$ agonist muscimol in BNST significantly decreased the total positive hedonic responses to a sweet sucrose solution in half, even though DAMGO microinjections stimulated increases in food intake $F(3,25)=3.3$, $p=0.037$]. This decrease in total hedonic responding was driven primarily by a reduction in the number of rhythmic midline tongue protrusions (MTP) [main effect of drug treatment: $F(3,25)=3.0$, $p=0.048$].

*DAMGO suppression of hedonic reactions to sucrose*

DAMGO (0.05ug and 0.1ug) reduced MTP by 50% [$F(2,20)=3.7$, $p=0.042$] and reduced total hedonic responses by 48% [$F(2,20)=3.8$, $p=0.041$] (Figure 1,2), suggesting that DAMGO treatment was sufficient to cause a reduction in 'liking' of a sucrose solution. Post-hoc tests indicated that both doses of DAMGO tested were equally effective and did not significantly differ from each other. DAMGO also appeared to slightly suppress hedonic lateral tongue protrusions, though this decrease did not reach the level of statistical significance. Hedonic paw licks were also not affected by DAMGO treatment.

Analysis of DAMGO also revealed a 180% increase in rhythmic mouth movements [$F(2,20)=4.0$, $p=0.035$], which has been suggested to be a relatively neutral

component of taste reactivity (neither positive hedonic nor negative aversive) (Berridge, 2000). The increase in mouth movements was highest for the 0.1ug dose ($p<0.02$) . However, the overall effect of DAMGO was specific to hedonic orofacial reactions, as neither total aversive nor total neutral expressions were significantly altered relative to vehicle treatment [main effect of drug treatment: all *F*'s<2.0, n.s.]. This suggests that μ-opioid stimulation in BNST specifically disrupts 'liking' of a sweet solution, while leaving neutral mouth movements and low levels of aversive responding to sucrose relatively unaffected (Figure 3).

*DAMGO does not change aversive reactions to quinine*

DAMGO microinjection in BNST did not significantly affect any of the measured orofacial reactions during the quinine infusion. This suggests that opioid simulation in BNST does not markedly alter 'disliking' for an unpleasant quinine solution (Figure 4).

*Muscimol suppression of hedonic reactions to sucrose*

Muscimol potently reduced total hedonic responding to a normally pleasant sucrose solution (Figure 5), primarily by reducing MTP to just 25% of vehicle levels [both *F*'s>10.0, $p<0.01$]. By contrast, muscimol modestly elevated the total number of neutral responses to almost 200% of vehicle levels [$F(1,14)=4.8$, $p=0.045$]. The typically low levels of aversive responding to sucrose were not affected by muscimol treatment (Figure 3).

*Muscimol in BNST may reorganize aversive responding to quinine*

When presented with an oral infusion of a bitter quinine solution, rats typically emit aversive reactions: gapes, forelimb flails, headshakes, and chin rubs. Muscimol may have marginally changed these aversive reactions (Figure 4).

Muscimol marginally alters the motor distribution of aversive components. Overall, muscimol did not change the total number of aversive reactions to quinine. However, it marginally reorganized the relative numbers of particular component reactions. Muscimol treatment caused a marginally significant increase of up to 3,000% during muscimol treatment (mean = 8.75, mean increase ~1100%). in aversive chin rubs [$F(1,14)=3.3$, $p=0.090$], which were rarely observed after vehicle control microinjections (mean = 0.75). Conversely, muscimol caused a slight decrease in aversive forelimb flails [$F(1,14)=3.3$, $p=0.093$]. Finally, muscimol also caused a small increase in passive dripping of the infusion fluid [$F(1,14)=3.6$, $p=0.080$].

*DAMGO increases food intake of both highly palatable M&Ms and standard chow pellets*

When animals were tested for intake of a highly palatable food (milk chocolate M&M's) and standard lab chow immediately after taste reactivity testing, there was a strong preference for intake of the highly palatable M&M's [main effect of food type on intake in grams, time eating, and eating bouts: all $F$'s$>10.9$, $p<0.015$], with animals consuming on average 10 times as many grams of M&M's than chow pellets across all days of testing. Rats were also more likely to carry the M&M's to other locations within the cage [$F(1,7)=8.6$, $p=0.022$].

Analysis indicated significant increases during drug treatment in total feeding time [F(1.6, 11.5)=5.2, p=0.029], feeding bouts [F(3,21)=10.0, p=0.001], investigatory sniffs of the two food options [F(2.1, 15.3)=4.1, p=0.036], and carrying of the food pellets within the cage [F(3,21)=4.1, p=0.019]. Post hoc analyses revealed that both DAMGO doses increased feeding time by nearly 300% over vehicle levels (Figure 6,7), while simultaneously increasing the number of feeding bouts by at least 200%. As with earlier testing in food intake (see chapter 2), there were no significant differences between the two DAMGO doses tested here.

Although we observed a significant increase in total food consumption (both chow and M&M's), we were also interested in whether this increase would be specific to the more palatable food option, as has been suggested for opioid stimulation of nearby regions of ventral striatum (Zhang & Kelley, 2000). However, no significant interactions between drug treatment and food type were found [all *F*'s < 2.9, n.s.], suggesting that the feeding potentiation we observed in response to opioid stimulation in BNST was not specific to the more palatable M&M's, but rather raised intake proportionately for both the M&M's and the standard lab chow.

*Muscimol does not affect intake of highly palatable M&M's*

Previously, muscimol at the dose tested here appeared to possibly disrupt the intake of normal lab chow (see chapter 2). Here, we found that muscimol treatment did not affect the total amount of food consumed [main effect of drug on food intake intake in grams, feeding time (Figure 8), and feeding bouts: all *F*'s <1, n.s.], nor did it disrupt the preference for the palatable M&M's [main effect of food type on food intake intake in

grams, feeding time, and feeding bouts: all *F*'s >7.5, p<0.05; interaction of food type x drug for food intake intake in grams, feeding time, and feeding bouts: all *F*'s <1, n.s.].

Closer examination of case-by-base feeding data revealed that 3 out of 8 animals consumed more M&M's after muscimol treatment than after vehicle (in one case the animal consumed all 20 M&M's in the hour-long test period), while the other 5 animals ate less following muscimol treatment (in several cases eating nothing at all). These different patterns of behavior were not anatomically segregated.

*Other behavioral effects of DAMGO and muscimol*

When analyzed together, all drug treatment groups markedly decreased sleeping during the food intake test, from an average of 20 mins during vehicle test days to an average of 3 mins or less during drug microinjection days [F(3,21)=18.6, p=0.001; post-hoc tests for each drug condition: p<0.05]. This is consistent with findings from our earlier food intake experiment (chapter 2), where we found similar reductions in sleep for both opioid and GABA stimulation in BNST.

DAMGO, when analyzed separately, increased cage crosses to nearly 200% above vehicle levels across both doses [*F*(1.3,9.4)=6.4, p=0.025], though only the lower 0.05ug dose rose to significance in post-hoc testing. An increase in locomotor behavior was not observed with DAMGO in our previous food intake experiment (see chapter 2). It is possible that the present increase was linked to the use of M&M's as a food source, as rats were more likely to pick up an M&M, located in the front of the cage, and retreat to the back of the cage to consume it before returning to pick up another M&M. With an average consumption of ~8 M&M's during DAMGO treatment sessions, this could result

in up to 16 cage crosses simply to retrieve and consume the M&M's, and may not reflect any broad enhancement of locomotor activity.

Muscimol, when analyzed separately, showed a 50% reduction in rearing relative to vehicle controls [$F(1,7)=12.1$, $p=0.01$], but no significant changes in defensive treading or cage crosses [all *F*'s<1.5, n.s.]. However, we did record high rates of distress vocalizations (75%) and dashes (25%) in muscimol-treated animals upon removal from the food intake chamber in the present experiment; by comparison, we observed no distress vocalizations or dashes during any of the vehicle or DAMGO treatment sessions. This provides some support for our earlier hypothesis that high doses of muscimol in BNST generate an aversive motivational state, despite the absence of a significant increase in defensive treading behavior.

Although both treading and cage crosses were elevated here relative vehicle controls, we did not observe the robust enhancements of both measures with muscimol in BNST that we previously reported (see Chapter 2). It is possible that the timeline of the current experiment, where animals did not enter food intake testing until roughly 30 minutes after microinjection due to taste reactivity testing, diminished the expression of treading and cage crossing.

*Anatomical gradients*

We found that musicmol microinjection at ventral sites in BNST (ventral to -6.6mm below skull surface) was more effective in suppressing hedonic midline tongue protrusion [$t(4)=4.8$, $p=0.008$] and total hedonic responses [$t(4)3.9$, $p=0.018$]. In dorsal regions of BNST, muscimol moderately reduced hedonic MTP to 71% of vehicle levels.

However, in ventral regions, MTP was nearly abolished by muscimol treatment, with only 4% of vehicle levels being expressed. This indicates that muscimol at ventral sites in BNST is more effective at suppressing 'liking' for a sucrose solution than similar injections in dorsal BNST.

Muscimol at ventral sites also increased the incidence of passive drip of the sucrose solution [$t(4)=4.0$, $p=0.016$]

DAMGO did not have any differential effects across the dorso-ventral gradient for responding to sucrose, and neither DAMGO nor muscimol microinjections had different anatomical effects during the infusion of quinine.

***Discussion***

Here I assessed food 'wanting' versus 'liking', comparing intake effects to taste reactivity testing combined with drug microinjections in BNST, which receives rich taste input from medial regions of PBN (Alden et al., 1994). Paradoxically, I found that stimulation of μ-opioid receptors of BNST with DAMGO decreased hedonic 'liking' reactions to a sweet sucrose solution, even though the same DAMGO microinjections caused increased food intake. That dissociation suggests that opioid stimulation in BNST can cause increased 'wanting' for a food reward without commensurate enhancement of reward 'liking' in the same animals, and in fact while actually suppressing 'liking' reactions. GABA-ergic inhibition with muscimol in BNST also decreased hedonic 'liking' reactions to a sweet solution, and sporadically suppressed food intake. Muscimol in BNST also caused some animals to emit distress vocalizations and escape dashes when handled by experimenters, supporting our previous hypothesis that GABA-ergic

inhibition in BNST generates an aversive motivational state in rodents. These findings further clarify the role of BNST, and the greater extended amygdala macrosystem, in appetitive motivation.

*BNST opioids do not amplify hedonic 'liking' of a food reward*

An increase in reward 'liking' was a plausible psychological mechanism that could have contributed to enhanced food intake following DAMGO in BNST, given the involvement of BNST with gustatory projections. Increasing the hedonic impact of a food item could make a merely palatable target like standard lab chow seem especially delicious, and a highly palatable item like milk chocolate almost irresistibly tasty. Increased 'liking' can feed back to cause parallel increase in reward 'wanting', thus driving increased food intake (Berridge et al., 2009; Lundy, 2008), potentially to maladaptive levels resulting in excessive weight gain or obsesity (Finlayson, King, & Blundell, 2007; Lutter & Nestler, 2009; Zheng & Berthoud, 2007). However, our results indicate that DAMGO in BNST is not increasing the hedonic impact of a pleasant food reward, and in fact actually decreased 'liking' for a sweet sucrose solution.

The current findings in BNST are in dramatic opposition to the effect of μ-opioid stimulation at a few other forebrain sites, which has frequently been shown to increase the hedonic impact of palatable food rewards (Barbano & Cador, 2007; Glass et al., 1999; Kelley et al., 2002). In a pair of recently identified hedonic hotspots in the medial shell of the nucleus accumbens and caudal ventral pallidum, DAMGO at doses similar to those used here in BNST potently increase 'liking' of a sweet sucrose infusion (Pecina & Berridge, 2005; K. S. Smith & Berridge, 2005). Our current findings for DAMGO in

BNST most closely resemble the effects of opioid stimulation in CeA and basolateral amygdala (BLA), where DAMGO stimulation also potently reduced 'liking' for a sucrose solution while simultaneously enhancing food intake in the same animals (Mahler & Berridge, 2006). The congruent behavioral effects of DAMGO in BNST and CeA in taste reactivity testing are similar to electrophysiological studies indicating that both structures exhibit an almost exclusively inhibitory influence on taste responsive cells in both PBN and NST (C. S. Li & Cho, 2006; D. V. Smith et al., 2005), suppressing firing to a range of taste stimuli.

Taken together, this dissociation between the effect of μ-opioid stimulation in BNST and CeA vs. accumbens shell and ventral pallidum may reflect a broader difference in the role of endogenous opioid systems in the anatomical macrosystems of the extended amygdala and ventral-striatal-pallidum. However, future studies will do well to continue probing the unique roles of nuclei within these macrosystems in taste processing, as they are unlikely to be fully homogeneous. For example, although BLA lesions dramatically impair the acquisition of condition taste aversion following lithium chloride illness (Yamamoto & Fujimoto, 1991), lesions of CeA and BNST have no effect on the development or expression of a taste aversion (Roman, Nebieridze, Sastre, & Reilly, 2006).

*BNST opioids enhance 'wanting' for food rewards, but do not favor palatable foods*

Although we show that DAMGO decreased 'liking' of a sweet sucrose solution, we also observed significant enhancements of food intake, a measure of reward 'wanting'. The DAMGO-induced increase in feeding is consistent with our previously

reported study of food intake (Chapter 2). Here, we are able to go further and show potently increased 'wanting' for a food reward in the same animals that only 10 minutes earlier had displayed reduced 'liking' under the same neurochemical manipulation, providing a noteworthy example of the occasional dissociation between 'liking' and 'wanting'. Similar dissociations have been reported with μ-opioids in CeA (Mahler & Berridge, 2006), and in regions of NAc shell outside the cubic-millimeter hedonic hotspot (Pecina & Berridge, 2005) as well as with dopamine in the nucleus accumbens (Wyvell & Berridge, 2000), but this is the first report of such a distinction within BNST.

In the current food intake test, we presented animals with two different sources of food: standard lab chow (which rats had *ad libitum* access to in their home cage throughout the experiement) and highly palatable chocolate candies (M&M's). Previous studies have strongly implicated opioids in the preferential potentiation of palatable food rewards, with opioid stimulation selectively increasing and opioid blockade selectively decreasing palatable food intake, while leaving a simultaneously available standard food source unaffected (Glass et al., 1999; Zhang & Kelley, 2000). Here, we found that although DAMGO in BNST did increase total food intake, this increase was equivalent for both the standard lab chow and the highly palatable M&M's. This suggests that opioid stimulation in BNST does not specifically boost intake of the most palatable food source available, but instead enhances feeding at all food sources proportional to the previously learned value of each food item.

*BNST muscimol disrupts reward 'liking' and reorganizes aversive motivation*

Like DAMGO, muscimol in BNST also decreased hedonic 'liking' reactions to a sweet sucrose taste, especially in ventral regions of BNST. However, muscimol also resulted in frequent distress vocalizations and occasional escape dashes when animals were removed from food intake testing; these latter aversive behaviors were never observed during vehicle or DAMGO treatment. These findings slightly resemble the aversive effects of muscimol in nearby caudal accumbens shell and in CeA (Reynolds & Berridge, 2001, 2002). However, our previous fos plume analysis suggests that our current microinjections remained almost entirely contained within BNST. Therefore, BNST is likely to be an independent forebrain site of aversive motivation following GABA-ergic inhibition. This is consistent with BNST's previously identified roles in the aversive motivational components of drug withdrawal (Koob, 1999, 2003), as well as its role in unconditioned fear responses (M. Davis & Shi, 1999).

In addition to increased aversive motivation, we previously reported that muscimol in BNST also appeared to reduce some measures of feeding overall, especially regular chow, but here we saw a wider variance for a highly palatable food, chocolate M&M candies (Chapter 2). We found a range of feeding outcomes after muscimol microinjection, ranging from complete suppression of feeding to consumption of twenty M&M's within a single hour of testing. It is possible that some animals responded to the aversive muscimol condition with enhanced intake of the highly palatable M&M's (which were not available in our previous food intake experiment) as a type of self-medication to reduce the stressful drug treatment (Dallman et al., 2003).

*Future directions*

Although BNST and CeA opioids have been shown to suppress 'liking', it remains unclear what role other extended amygdala nuclei, including the sublenticular extended amygdala and the interstitial nucleus of the posterior limb of the anterior commissure, play in reward 'liking'. Additionally, it would be of great interest to explore potential interactions between BNST and other brain sites that modulate reward 'liking', including CeA and accumbens shell. The opioid hedonic hotspots in accumbens shell and ventral pallidum appear to be jointly necessary to support enhanced 'liking', since opioid antagonism with naloxone in either hotspot can veto hedonic enhancement by μ-opioid stimulation of the other (K. S. Smith & Berridge, 2007). Future studies could be designed to evaluate whether the decrease in 'liking' generated by DAMGO in BNST or CeA can be overruled by opioid stimulation of accumbens or ventral pallidum, and also whether suppression of 'liking' by opioids in extended amygdala involved concerted or independent action of sub-nuclei within that emerging anatomical macrosystem.

**Figure 4.1. DAMGO (0.05μg) reduces 'liking' for a sweet sucrose solution.** Horizontal (top) and sagittal (bottom) maps showing within-subjects changes in total hedonic responding relative to vehicle treatment.

# BNST DAMGO (0.05µg) Decreases Sucrose 'Liking'

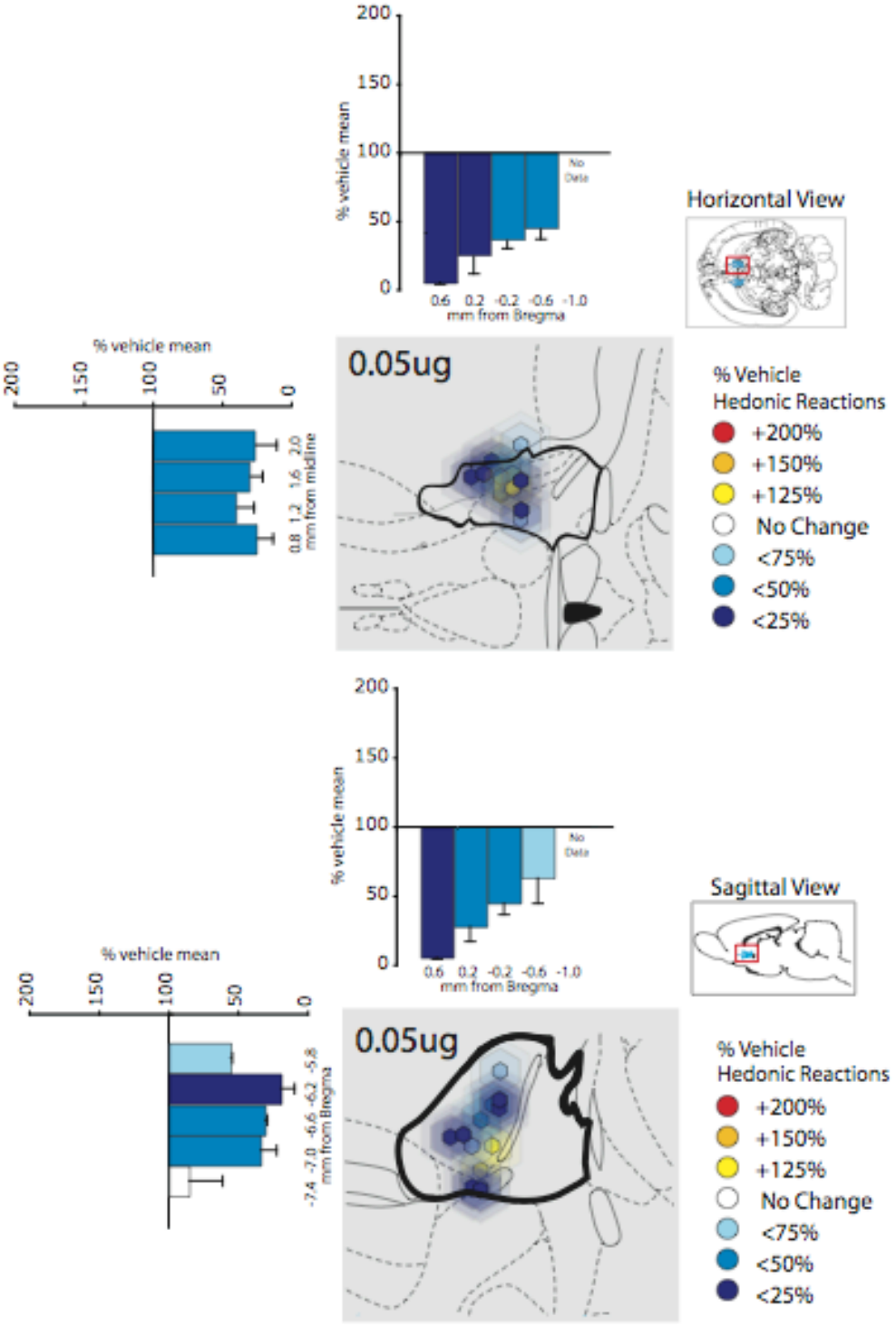


**Figure 4.2. DAMGO (0.1µg) reduces 'liking' for a sweet sucrose solution.** Horizontal (top) and sagittal (bottom) maps showing within-subjects changes in total hedonic responding relative to vehicle treatment.

# BNST DAMGO (0.1µg) Decreases Sucrose 'Liking'

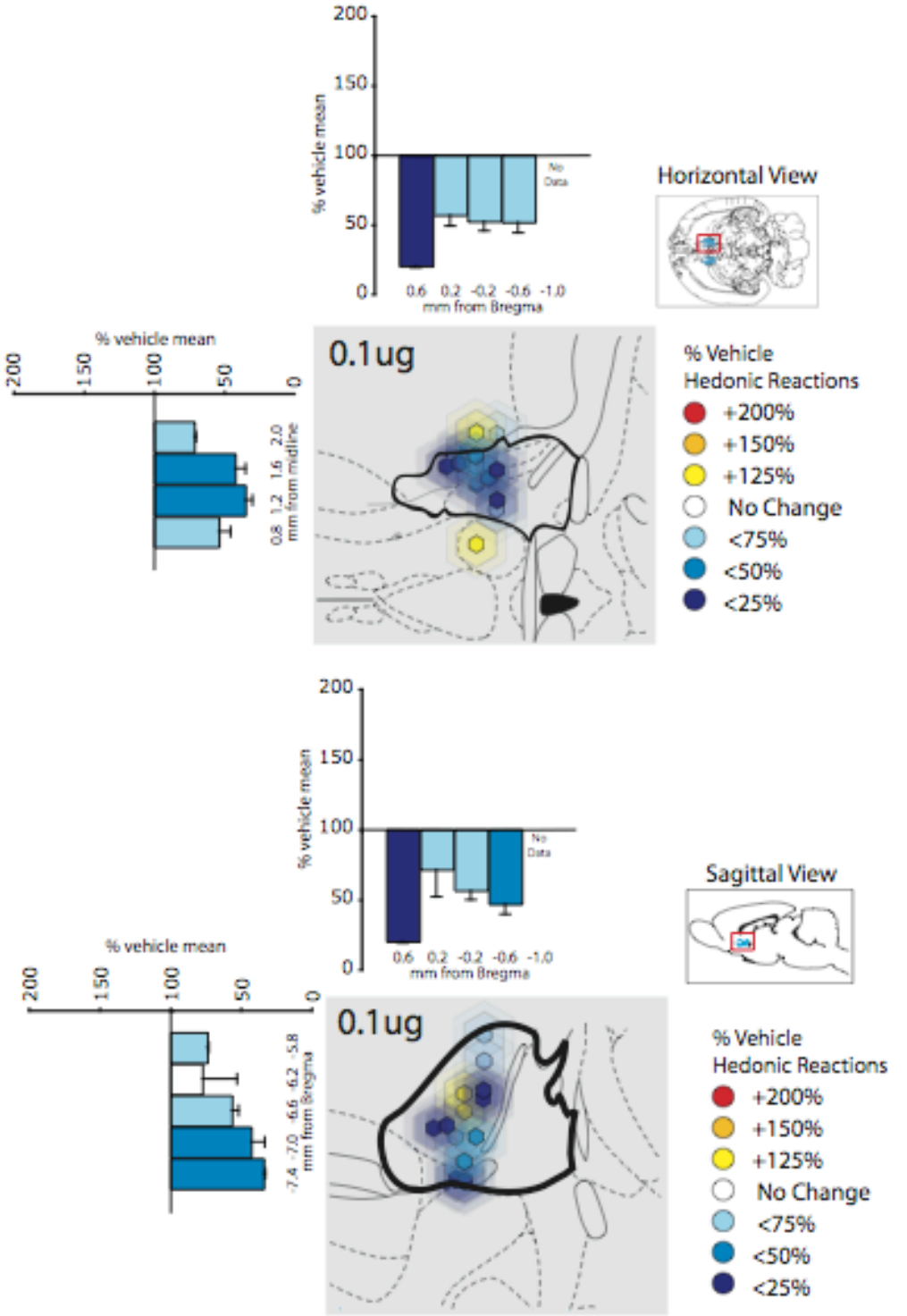


**Figure 4.3. Summary of taste reactivity responding for sucrose infusion.** Summary of all dependents variables (top) and the sum of each valence category (bottom) for all drug conditions. TP=midline tongue protrusion; LTP=lateral tongue protrusion; PL=paw licks; G=gapes; FF=forelimb flails; HS=head shakes; CR=chin rubs; FW=face washing; MM=mouth movements; PD=passive drip

# Sucrose Infusion

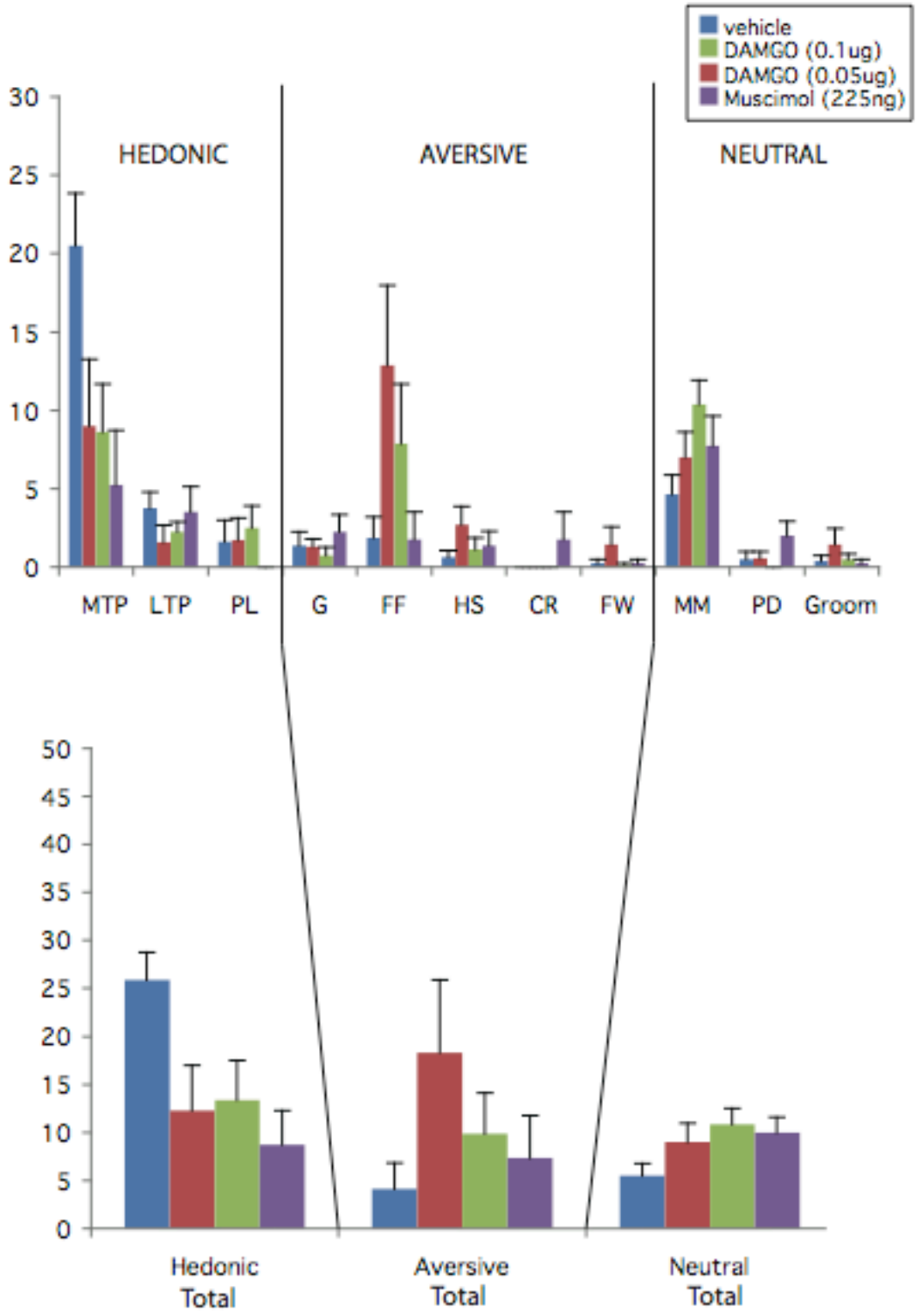


**Figure 4.4**. **Summary of taste reactivity responding for quinine infusion.** Summary of all dependents variables (top) and the sum of each valence category (bottom) for all drug conditions. TP=midline tongue protrusion; LTP=lateral tongue protrusion; PL=paw licks; G=gapes; FF=forelimb flails; HS=head shakes; CR=chin rubs; FW=face washing; MM=mouth movements; PD=passive drip

# Quinine Infusion

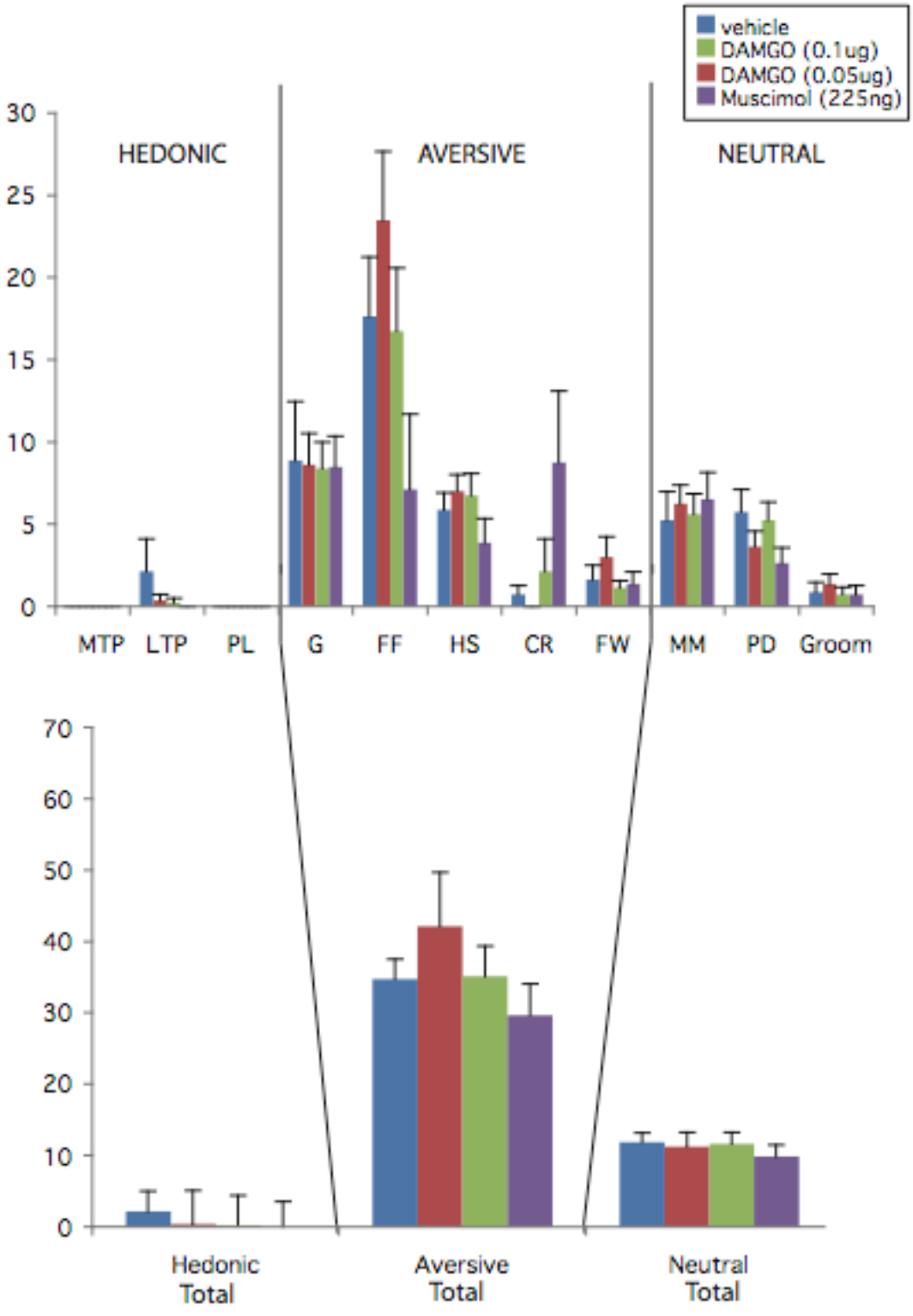


**Figure 4.5. Muscimol (225ng) reduces 'liking' for a sweet sucrose solution.** Horizontal (top) and sagittal (bottom) maps showing within-subjects changes in total hedonic responding relative to vehicle treatment.

# BNST Muscimol (225ng) Decreases Sucrose 'Liking'

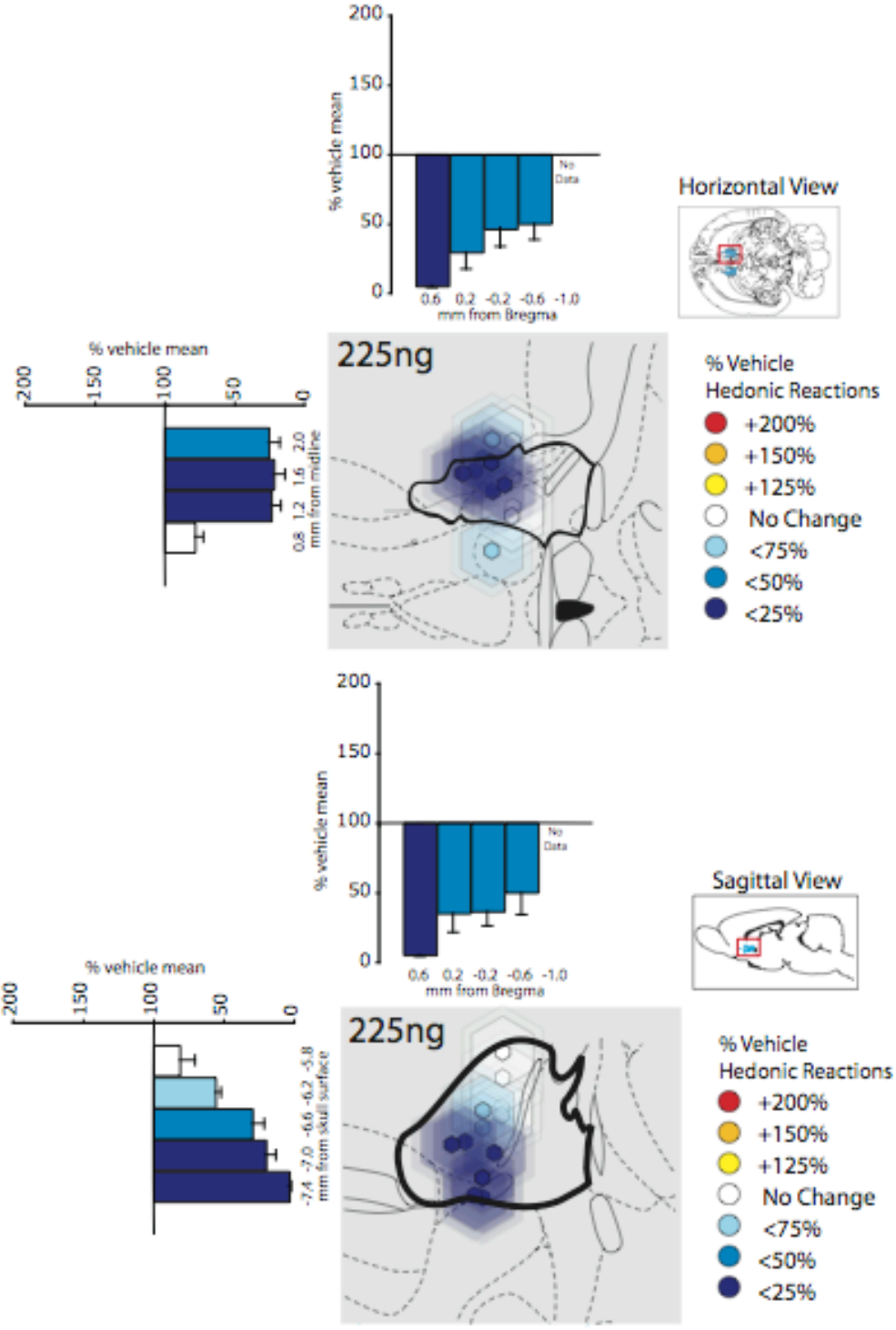


**Figure 4.6. DAMGO in BNST increases feeding.** Feeding time enhancements (combined M&M and chow) for 0.05ug DAMGO microinjections are mapped rat-by-rat onto a horizontal (top) and sagittal (bottom) views of BNST, showing both the within-subject change in feeding time relative to vehicle day (color) and the functional spread of the microinjection based on Fos plume data (size). Each map symbol is composed of three nested hexagons; the inner symbol shows the average size of intense 5x activation, the middle symbol shows 3x activation, and the outer symbol shows 2x activation. Bar graphs along the rostro-caudal and medial-lateral axes show the average drug effect within each 0.4mm wide region. Bar graph showing absolute comparisons of vehicle and drug feeding time (in minutes) can be found above and to the left the horizontal anatomical map. * indicates differences from vehicle, $p<0.05$.

# BNST DAMGO (0.05µg) Enhances Feeding

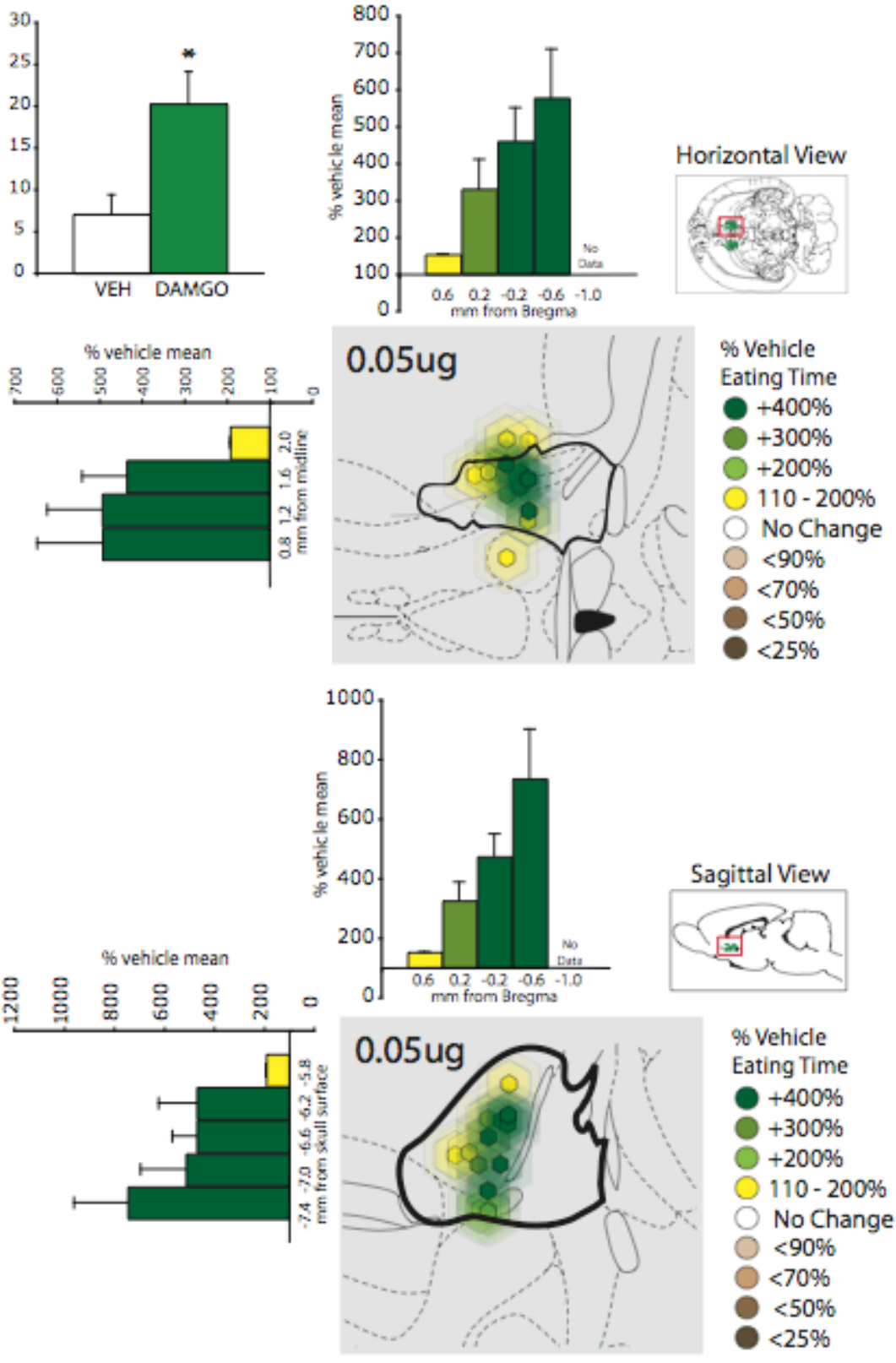


**Figure 4.7. DAMGO in BNST increases feeding.** Feeding time (combined M&M and chow) enhancements for 0.1ug DAMGO microinjections are mapped rat-by-rat onto a horizontal (top) and sagittal (bottom) views of BNST, showing both the within-subject change in feeding time relative to vehicle day (color) and the functional spread of the microinjection based on Fos plume data (size). Each map symbol is composed of three nested hexagons; the inner symbol shows the average size of intense 5x activation, the middle symbol shows 3x activation, and the outer symbol shows 2x activation. Bar graphs along the rostro-caudal and medial-lateral axes show the average drug effect within each 0.4mm wide region. Bar graph showing absolute comparisons of vehicle and drug feeding time (in minutes) can be found above and to the left the horizontal anatomical map. * indicates differences from vehicle, $p<0.05$.

# BNST DAMGO (0.1µg) Enhances Feeding

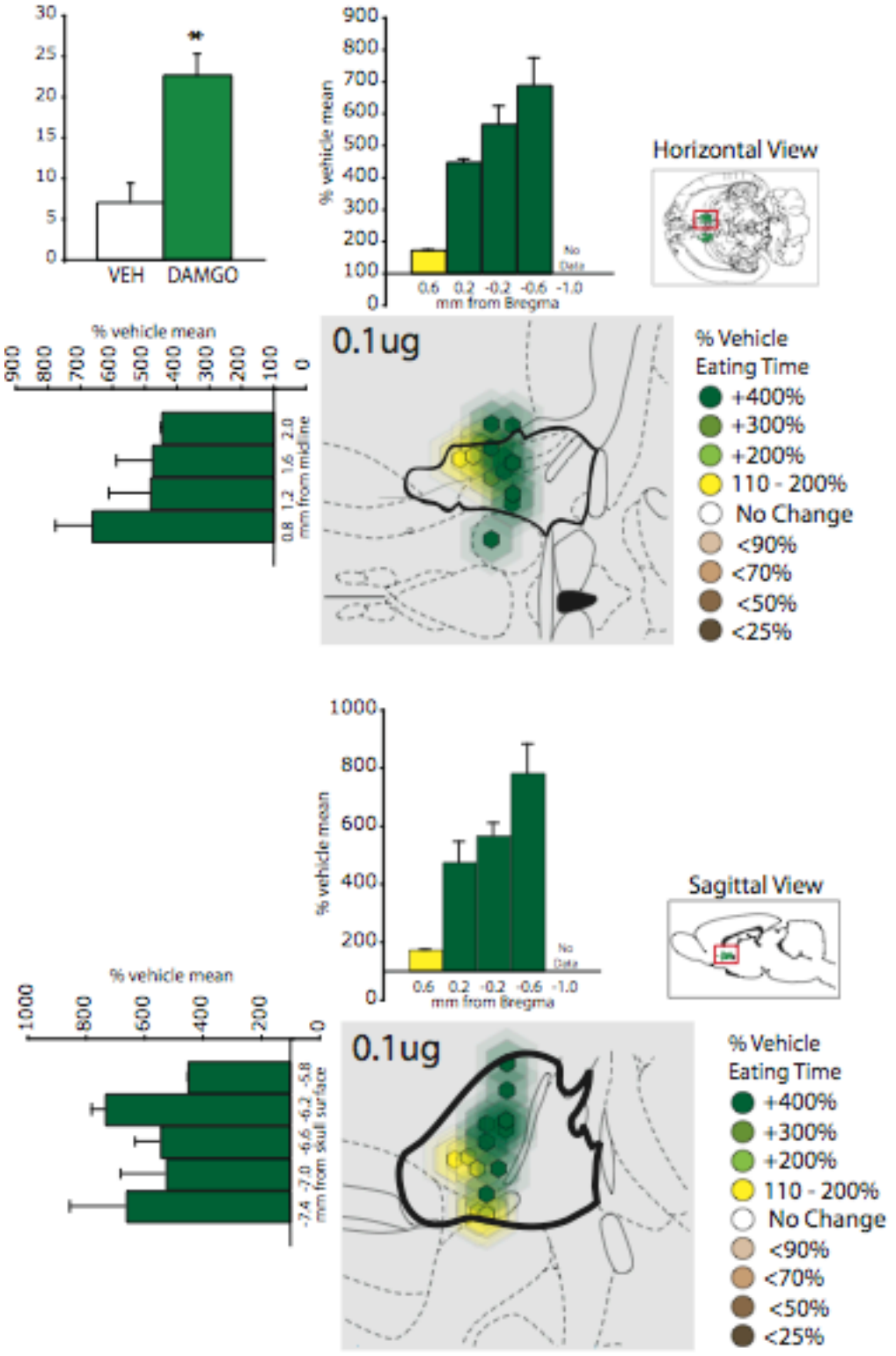
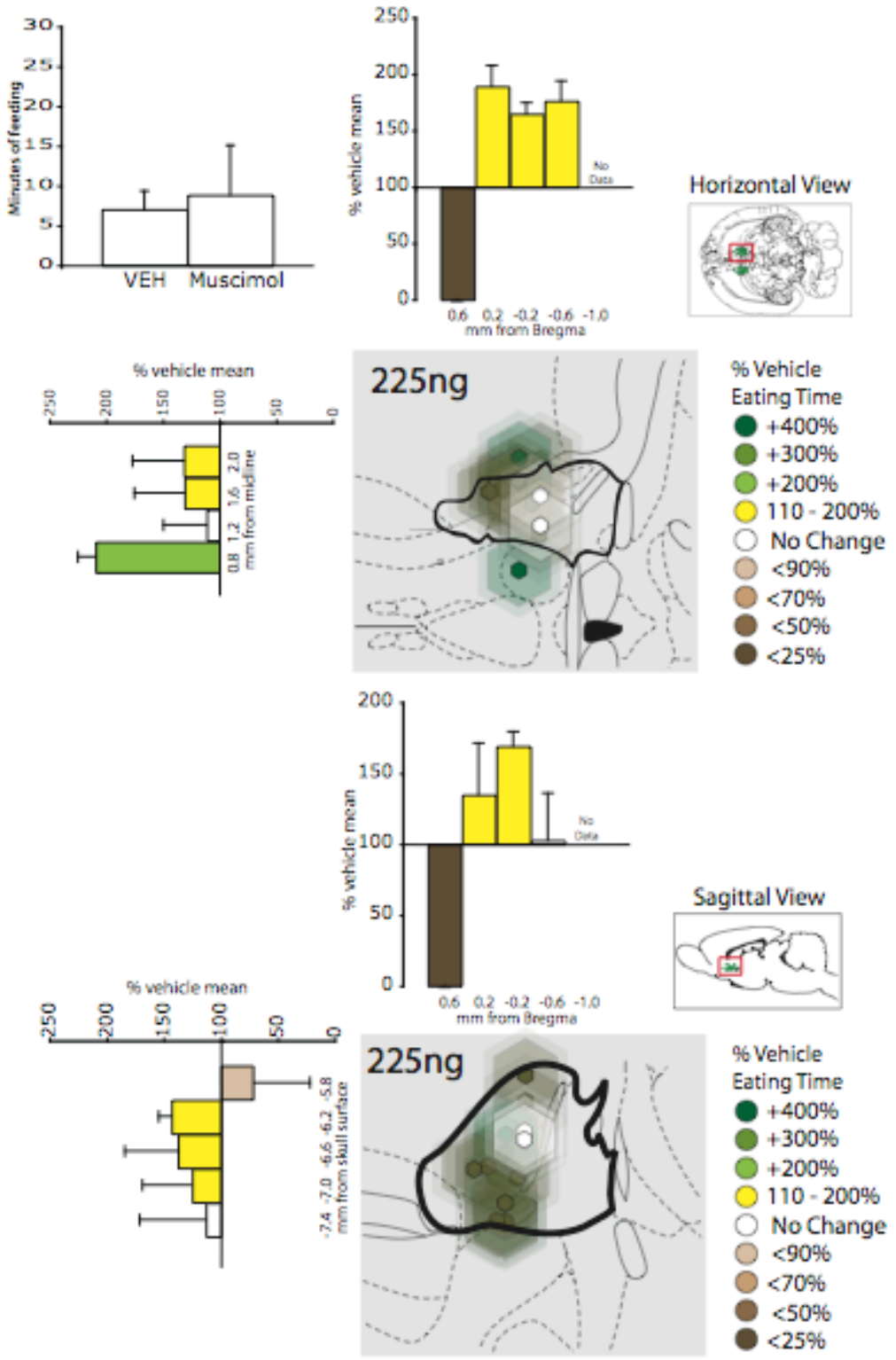


**Figure 4.8. Muscimol in BNST does not affect feeding.** Feeding time (combined M&M and chow) enhancements for 225ng muscimol microinjections are mapped rat-by-rat onto a horizontal (top) and sagittal (bottom) views of BNST, showing both the within-subject change in feeding time relative to vehicle day (color) and the functional spread of the microinjection based on Fos plume data (size). Each map symbol is composed of three nested hexagons; the inner symbol shows the average size of intense 3x activation, the middle symbol shows 50% inhibition, and the outer symbol shows 25% inhibition. Bar graphs along the rostro-caudal and medial-lateral axes show the average drug effect within each 0.4mm wide region. Bar graph showing absolute comparisons of vehicle and drug feeding time (in minutes) can be found above and to the left the horizontal anatomical map.

# BNST Muscimol (225ng) Does Not Affect Feeding



# Chapter 5

# Opioid Stimulation in the Bed Nucleus of the Stria Terminalis is Not Inherently Stressful

## *Introduction*

We have shown in three prior experiments that μ-opioid stimulation in BNST can potently increase voluntary food intake of standard chow pellets (see Chapters 2 & 3) and also of palatable M&M's (see Chapter 4), even in animals that are not food deprived. This increased feeding suggests a role for BNST in appetitive motivation, which is supported by the neuroanatomy of BNST. In addition to sharing reciprocal connections with a variety of sub-cortical sites implicated in taste and feeding, including the nucleus accumbens (de Olmos & Heimer, 1999), lateral hypothalamus (Alheid, 2003), and hindbrain taste nuclei (Alden et al., 1994; Bernard et al., 1993; C. S. Li & Cho, 2006), BNST also sends excitatory projections to midbrain dopaminergic centers that have been repeatedly implicated in appetitive motivational processes (Barbano & Cador, 2007; Berridge, 2007; Carlezon & Thomas, 2009; Heinz, Beck, Grusser, Grace, & Wrase, 2009). Further, the emerging anatomical concept of the extended amygdala links BNST with other forebrain nuclei, most notably the medial and central nuclei of the amygdala, that play prominent roles in the motivation for rewards such as food (Gosnell, 1988; Mahler & Berridge, 2009), social behavior and sexual partners (Kirkpatrick, Carter, Newman, & Insel, 1994), and drugs of abuse (Fattore, Fadda, Spano, Pistis, & Fratta,

2008; Rezayof, Golhasani-Keshtan, Haeri-Rohani, & Zarrindast, 2007). Taken together, these anatomical and behavioral findings seem to suggest a role for BNST in appetitive motivation.

Yet it could be questioned whether BNST truly mediates appetitive motivational processes. The issue is complicated because some appetitive behaviors can also be stimulated by aversive or stressful stimuli. Hedonic self-medication has sometimes been suggested as a mechanism for stress-induced appetitive behaviors (Kreek & Koob, 1998; Markou, Kosten, & Koob, 1998). That is, individuals in a dysphoric state might consume a hedonic reward such as food simply in order to escape the aversive state and return to a neutral hedonic baseline. Appetitive behavior motivated purely by escape from distress is not fully appetitive, in a positive incentive motivation sense of that term.

The BNST is one of a number of nuclei that compose the aforementioned network of stress-responsive nuclei (Dallman et al., 2003). It contains a high density of CRF-positive cell bodies (including both receptors and neurotransmitter pools) (Koob & Heinrichs, 1999), and receives significant CRF input from other limbic structures, including central amygdala (CeA) (Erb et al., 2001). BNST also contains the highest density of norepinephrine terminals in the forebrain (Aston-Jones et al., 1999; Aston-Jones & Harris, 2004), and these ascending projections interact with CRF in BNST to modulate the hypothalamic-pituitary-adrenal (HPA) axis (Forray & Gysling, 2004). Interestingly, and of great relevance to motivation, a robust link exists between BNST CRF and brainstem dopamine: not only does BNST send CRF projections directly to dopaminergic cells in the ventral tegmental area (Rodaros, Caruana, Amir, & Stewart, 2007), but ascending dopamine also enhances rapid excitatory transmission in BNST via

a CRF-dependent process (Kash et al., 2008). Due to the unique convergence of limbic inputs in BNST and its strong output channels to medial and lateral hypothalamus, some have argued that it serves as a critical relay between limbic brain regions and the HPA axis (Herman & Cullinan, 1997; Herman et al., 2005).

Much stress-induced behavior is ambiguous in this respect. Rats will run for miles on a running wheel after exposure to stressors such as food restriction (Altemus, Glowa, & Murphy, 1993; Uchiumi, Aoki, Kikusui, Takeuchi, & Mori, 2008). Changes in food intake are known have a particularly strong link to stress (Torres & Nowson, 2007). Acting via a network of central and peripheral nervous system substrates, glucocorticoids dramatically alter feeding strategies, shifting intake towards energy dense food options (high in fat and calories) and causing robust increases in abdominal fat stores (Dallman, Warne, Foster, & Pecoraro, 2007; Warne, 2009). High energy foods, in turn, help to inhibit central nervous system levels of stress-related neuropeptides such as CRF, and can act as "comfort food" to reduce the anxiety and dysphoria associated with enduring stress (Dallman et al., 2003).

On the other hand, even some 'stress components' can play a dual role in positive incentive motivation. Microinjection of the stress-related peptide corticotropin releasing factor (CRF) in BNST can increase drug-seeking behavior, and enhance cue-triggered 'wanting' for a food conditioned stimulus when delivered in the nearby nucleus accumbens (Erb et al., 2001; Erb & Stewart, 1999; Pecina, Schulkin, & Berridge, 2006). Similarly, Dallman and colleagues have suggested that stress may activate incentive brain systems to promote 'wanting' of incentives such as food (Dallman et al., 2007).

Here we test the hypothesis that μ-opioid stimulation of BNST has positive incentive motivation qualities. We test that idea against the alternative that BNST activation is an inherently aversive neural manipulation, which rats would avoid if they could, and which subsequently stimulates feeding to ameliorate this aversive motivational state. In order to assess the motivational valence of our μ-opioid manipulation, we will use the conditioned place preference paradigm. Though this testing procedure is perhaps best known as a broad measure of the rewarding properties of drugs and other neural manipulations (Bardo & Bevins, 2000), it can also be used as an effective assay of the aversive motivational properties of neurochemical manipulations. If μ–opioid stimulation in BNST is indeed stressful, we predict that pairing microinjection of the μ-opioid agonist DAMGO with a unique environmental context will result in the avoidance of that drug-paired environment in a subsequent drug-free test day. In contrast, if opioid stimulation of BNST promotes positive incentive motivation , we expect to find potentially a conditioned place preference.  I also compared the ability of DAMGO in BNST to establish a conditioned place preference or avoidance to its ability to promote food intake in the same rats.

***Method***

*Subjects*

A total of 7 Sprague-Dawley rats (females, 250-500g at the time of surgery) were used for conditioned place preference testing. All animals were housed in pairs (~21˚C; 12hr light/dark cyle, lights on at 9am) with *ad libitum* access to food (Purina 5001 chow; Purina Mills, St. Louis, MO) and tap water. All procedures were approved by the

University Committee on the Use and Care of Animals at the University of Michigan in accordance with National Institute of Health guidelines.

*Surgery*

All animals were handled twice for a total of fifteen minutes prior to undergoing surgery. Rats were pretreated with atropine (0.04mg/kg) and then anesthetized with ketamine (80mg/kg) and xylazine (5mg/kg). Rats were then placed in a stereotaxic device and implanted with bilateral, chronic guide cannulae (23 gauge, stainless steel), 14mm in length, aimed so that the ventral tip would rest 2mm above the BNST (AP: -0.15 to -0.45mm; ML: +/-1.6mm; DV: -4.8mm; incisor bar: -3.3mm [flat skull]). Guide cannulae were secured to the skull using four stainless steel screws and dental acrylic, and fitted with stainless steel stylets to prevent occlusion.

All rats were given post-operative analgesic (0.3 mg/kg buprenorphine) and prophylactic antibiotic (50 mg/kg chloramphenicol). Rats were allowed to recover for at least 7 days before the onset of behavioral testing.

*Drugs and Microinjections*

All drugs were dissolved and diluted to dose in artificial cerebrospinal fluid (aCSF; Harvard Labs, Cambridge, MA). DAMGO was prepared at a 0.1µg dose (total bilateral dose of 0.2µg). aCSF alone was used for vehicle microinjections. Microinjection schedules were counter-balanced across subjects using a Latin Square design.

On test days, animals were gently handled as the stylets were removed. Rats then received bilateral microinjections (0.2µL per side, 0.4µL total bilateral volume) via

16mm stainless steel microinjection tips (29 gauge), which extended 2mm beyond the ventral tip of the guide cannula. Microinjection tips were attached via PE-20 surgical tubing to a microinfusion pump, which delivered the infusion over the course of 60 seconds. Microinjection tips were left in place for an additional 60 seconds after the infusion ended to allow for drug diffusion, after which the stylets were replaced and the rat placed immediately in the conditioned place preference chamber.

*Conditioned Place Preference Procedure*

*Apparatus*: Testing was conducted using a three-chamber apparatus. Two large outer chambers (28 x 21 x 21 cm) were connected via a small middle "starting" chamber (12 x 21 x 21 cm). Each of the large outer chambers had different visual and tactile characteristics. One side had black walls, a wire grid floor, and was brightly illuminated (intensity 1,300 lux) using a Fiber-Lite MI-150 fiber optic surgical lamp (Dolan-Jenner Industries; Massachusetts, USA). The other side had white walls, a wire mesh floor, and was illuminated only by overhead lights in the testing room (intensity 550-650 lux). The middle chamber had solid gray walls and solid gray floor, and was only available to subjects on testing days. The three chambers were separated by removable divider walls, which remained in place on conditioning days and were removed on testing days. Each compartment had a clear Plexiglass lid that prevented the rats from escaping during conditioning and testing, but allowed an unobstructed view of the chamber to monitor the animal's position. Prior to the experiment, the effectiveness of the place conditioned apparatus and my procedures was verified in a separate group of rats using diazepam (1mg/kg, i.p.) (Spyraki, Kazandjian, & Varonos, 1985).

*Habituation and natural preference testing*: After recovery from surgery and two days of handling (5 mins the first day, 10 mins the second day), rats were habituated to the test apparatus for three consecutive days. During the habituation sessions, the divider walls were removed and animals were allowed to freely explore the test chamber for 30 mins under standard testing conditions, and then returned to their home cages. Animals received no microinjections during habituation days, but were briefly handled and their stylets were cleaned.

The third and final day of habituation was recorded using a digital camcorder mounted above the testing apparatus and centered on the crucial transition area of the chamber (the middle chamber that transitioned between the two larger chambers). This tape was later scored offline to establish each animal's natural preference for the two large conditioning chambers. After the final habituation session, each animal received a mock infusion of aCSF to accustom them to the microinjection procedure.

*Place conditioning training procedure*: After habituation and natural preference testing were completed, rats were assigned in a counterbalanced manner to have one large chamber of the testing apparatus paired with vehicle microinjection, and the other side paired with DAMGO microinjection. Rats then received four consecutive daily conditioning sessions, consisting of two vehicle microinjections (days 1 and 3) and two DAMGO microinjections (days 2 and 4). On each conditioning day, rats received the appropriate microinjection and then were immediately placed in the corresponding large chamber for 30 mins. On these conditioning days, the dividing walls were in place, so animals were confined solely to the appropriate large chamber. Between test sessions,

chambers were thoroughly cleaned with 70% ethanol to remove odors and excrement from the testing apparatus.

*Conditioned place preference test*: On the day immediately following the final conditioning session, rats were tested for conditioned place preference. As with habituation days, rats were gently handled and their stylets were cleaned, but they did not receive any microinjections. All dividing walls were removed, making all three chambers of the testing apparatus available on test day. After being briefly handled, rats were placed in the central starting chamber and allowed to freely explore the apparatus for 30 mins. This session was again recorded using a digital camcorder for offline analysis.

*Place preference video analysis*: Eat rat's natural preference and conditioned place preference videotape was scored offline for time spent in each of the three chambers, by an observer blind to the experimental condition. A rat was considered to be in a chamber of the apparatus whenever its head and both forelimbs were inside that chamber and on the ground for more than two seconds. Therefore, a rat that traveled directly from one large chamber to the other and spent less than two seconds in the central chamber was considered to have moved directly from one conditioning chamber to another, and no time in the central compartment would have been scored. After 30 minutes the total time (in seconds) spent in each chamber was recorded.

*Food intake testing*

*Apparatus*: Food intake testing was conducted in clear plastic cages (23 x 20 x 45 cm) containing a pre-measured pile of standard lab chow (~30g), a water spout, and

corncob bedding. Each was assigned the same food intake cage throughout habituation and testing.

*Food intake testing*: Rats were habituated to the food intake chamber for 3 consecutive days prior to testing. On habituation days, rats were briefly handled and had their stylets cleaned, and were then placed immediately into the food intake chamber for 1 hour.

On test days, food intake was tested for 1 hour immediately following microinjection of vehicle or DAMGO. The entire session was videotaped for subsequent offline analysis. After the test was completed, remaining chow (including crumbs) was carefully removed from the cage and weighed. Test days were always separated by at least 24 hours.

*Food Intake Video Scoring*: Video recordings of food intake test sessions were scored offline by observers blind to the experimental condition. The following behaviors were recorded: eating time (in seconds), eating bouts (triggered by interruptions of eating of more than 5 seconds), food sniffing, food carrying, drinking time (in seconds), drinking bouts (same criteria as eating bouts), grooming, cage crosses, sleeping, rearing, and defensive treading. Treading is a natural defensive behavior emitted by rodents, and involves rapid forelimb strokes away from the body that push debris (e.g. dirt or bedding) in the direction of a threat.

*Histology*

After testing was completed, subjects were deeply anesthetized with sodium pentobarbital (0.2mg/kg; Fatal-Plus) and decapitated. Brains were extracted and placed in

a 10% paraformaldehyde solution for 24-48 hours, and then placed in a 30% sucrose solution for 3-5 days, until the brains sank. The brains were then sliced on a freezing microtome (Leica) into 60μm coronal sections, mounted onto glass slides, allowed to dry for at least 24 hours, and then stained with cresyl violet. Stained slices were viewed under light magnification and used to map the microinjection centers in each hemisphere on coronal sections taken from a rat brain atlas. (Paxinos & Watson, 2007)

*Statistical analysis*

Analysis indicated that data for both conditioned place preference and food intake were distributed non-normally, so nonparametric Wilcoxon signed-rank tests were used to compare changes in place preference, and also to compare vehicle and drug conditions for all food intake dependent variables. Due to illness during testing, one animal was excluded from both conditioned place preference and food intake data analysis.

***Results***

*DAMGO in BNST generates a conditioned place preference*

When rats were conditioned to associate a unique environmental context with DAMGO microinjection in BNST, these rats subsequently expressed a strong preference for the drug-paired context. On average, rats more than doubled the amount of time they spent in the DAMGO-paired chamber, in comparison to their natural preference for that chamber ($Z$=1.99, p=0.05). Increased preference of the place associated with BNST stimulation was observed both in animals who initially preferred the chamber subsequently paired with DAMGO as well as in animals that initially preferred the *other*

chamber (the one *not* paired with DAMGO), indicating that our preference effect is not simply the result of either 1) a decay in the aversiveness of the non-preferred chamber with additional exposure or 2) the strengthening over time of initially established preferences. This result supports the hypothesis that opioid stimulation in BNST primarily triggers appetitive motivation, rather than a stressful or aversive response.

*DAMGO in BNST increases feeding*

In the same animals that displayed a conditioned place preference to a DAMGO-paired chamber, we also found that DAMGO increased feeding on standard lab chow in a 60-min voluntary feeding test. Consistent with our prior experiments (see Chapters 2, 3, and 4), opioid stimulation in BNST increased time spent feeding by an average of ~500% over vehicle controls ($Z$=2.02, p=0.04), and marginally increased the total intake of food in grams ($Z$=1.83, p=0.07) and investigatory sniffs of the chow pellets ($Z$=1.75, p=0.08). This demonstrates that, in the same animals that DAMGO generated a conditioned place preference, opioid stimulation also increased 'wanting' for a UCS food reward, and further supports the appetitive nature of opioid stimulation in BNST.

DAMGO did not significantly affect drinking, defensive treading, or locomotor behaviors such as cage crossing, rearing, or grooming. I also examined whether an animal's place preference score positively correlated with feeding behavior; however, there was no significant (or even trending) correlation between these two behaviors.

## ***Discussion***

*Synopsis*

Here we report that DAMGO in BNST actually caused a conditioned place *preference*, and not the conditioned place avoidance that an aversively stressful manipulation would be expected to generate. In addition to supporting our previous hypothesis that BNST is involved in appetitive motivational processes, this finding may also suggest a role for BNST in the acute rewarding effects of opioids.

*Opioid stimulation in BNST is not inherently stressful*

In this experiment we used conditioned place preference to examine the motivational valence of μ-opioid stimulation in BNST. Previous experiments in our lab using food intake have suggested that BNST opioids can increase appetitive motivation, but robust associations between BNST and brain stress networks raised the possibility that our observed increase in food intake was primarily the result of increased stress, which only secondarily enhanced appetitive desire to feed. Stress is well known to potentiate food intake, especially of energy dense and palatable food options.

However, we demonstrate here that opioid stimulation in BNST is not aversive, as evidenced by the absence of a conditioned place aversion. In fact, we found that DAMGO microinjection in BNST leads to the formation of a conditioned place preference, increasing the time spent in a drug-paired environment. Importantly, this finding supports our previously advanced hypothesis that BNST, in addition to its established roles in stress, anxiety, and drug withdrawal, is also associated with general appetitive motivation and reward. Although to date we have only shown a role for BNST in food reward, future studies could target other natural rewards, such as sex and social interaction.

*The role of BNST in reward and withdrawal of opiate drugs*

The current experiment also increases our understanding of the role of BNST as a substrate for the effects of opiate drugs. Previous studies have shown BNST to impact both the aversive and rewarding aspects of opiates. Lesions of noradrenergic cell populations in the A1 and A2 regions of caudal medulla (which richly innervate BNST), as well as direct disruption of norepinephrine in BNST, both dramatically reduce aversion to an environment paired with precipitated opiate withdrawal (Delfs et al., 2000). Additionally, opioid antagonism in BNST with methylnaloxonium reduced the self-administration of heroin, though only in rats previously made dependent on opiates with system morphine pellets (Walker et al., 2000). Both of these results may be linked by a later study showing that low doses of the opioid antagonist naltrexone can dramatically decrease norepinephrine efflux in the forebrain following opiate withdrawal, including in BNST (Van Bockstaele, Qian, Sterling, & Page, 2008). These findings suggest that, at least in dependent animals, BNST is an important node in mediating both reinforcing and aversive properties of opiate drugs. Taken together with reports of BNST's function in stress-induced relapse of cocaine seeking behavior, long after withdrawal symptoms have subsided (Erb et al., 2001; Erb & Stewart, 1999), BNST appears to play a role at all stages of drug use. The appetitive findings here in BNST and throughout out regions of the extended amygdala, including CeA, will necessitate additional studies to further characterize the role of the extended amygdala outside the late-stage processes of drug dependence, withdrawal and relapse (Koob & Le Moal, 2008)

Here we show a possible novel role for BNST in the positive motivation properties of μ-opioid receptor stimulation in drug naïve animals, as well as a role in potentiating appetitive motivation for a natural reward, food. Thus, regardless of the role of BNST in addiction, it seems also to be a mechanism that participates in generating true appetitive motivation for a range of potential incentives.

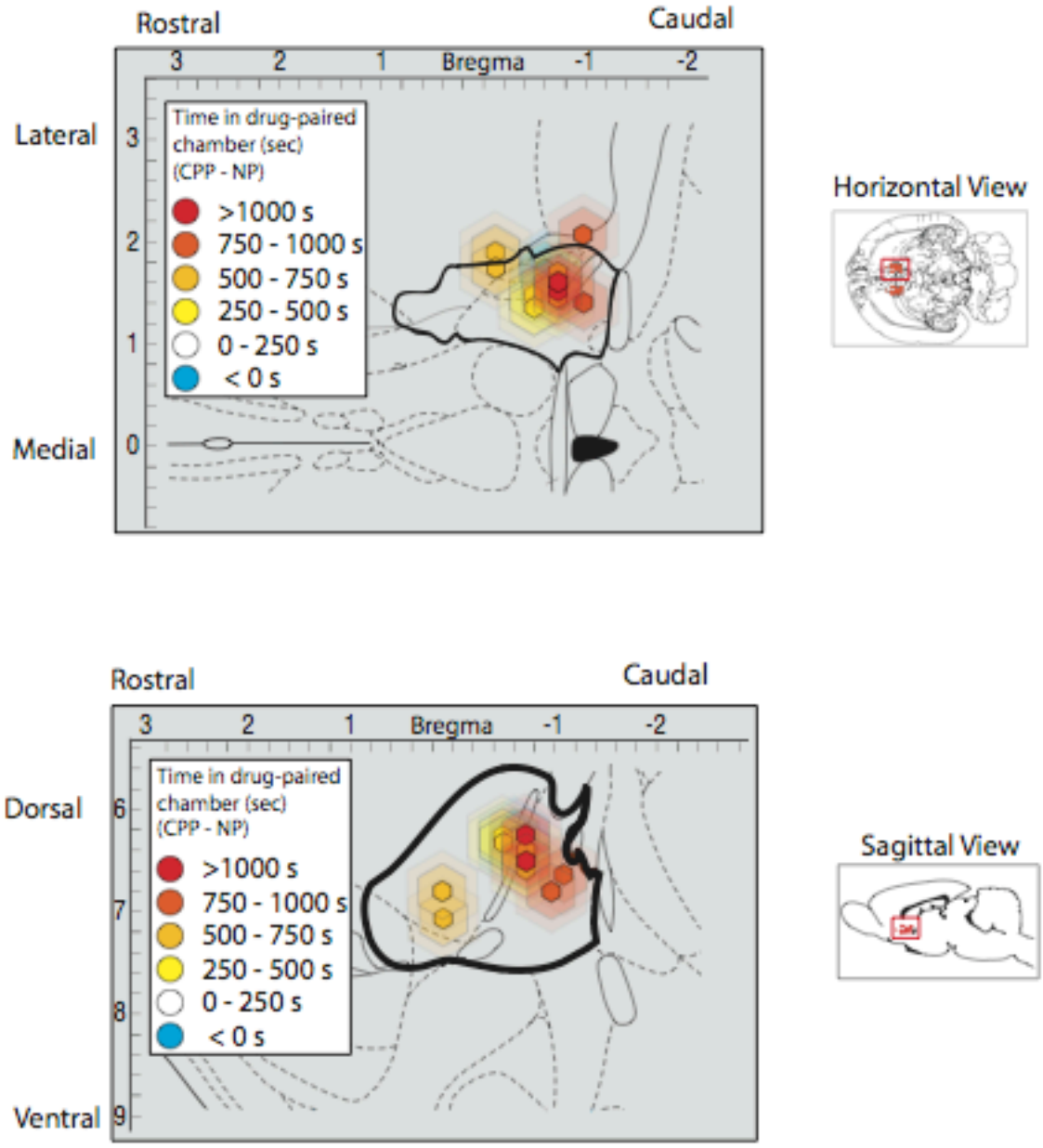



**Figure 5.1.** Anatomical maps showing the difference in time spent in the drug-paired side between the natural preference test and conditioned place preference test. Symbol placements are based on based on position of microinjection cannula in the BNST, shown in horizontal (top) and sagittal views (bottom). Fos plumes are adapted from previously reported values for this dose of DAMGO (0.1µg) in BNST (see Chapter 2).

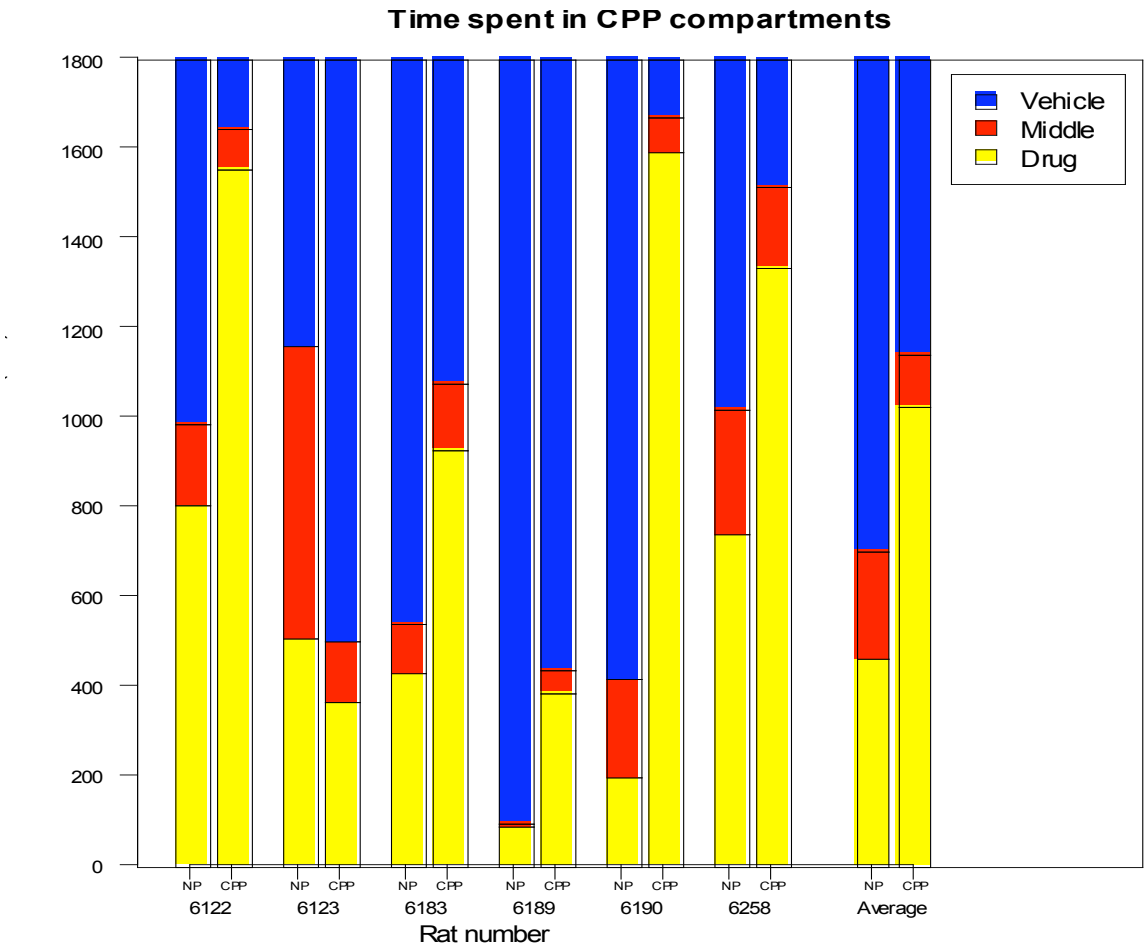



**Figure 5.2.** Stacked bar graph showing the distribution of time spent in each chamber for each rat during both the conditioned place preference (CPP) and natural preference (NP) test. The average for both natural preference and conditioned place preference tests is displayed as the final pair of bars. 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.

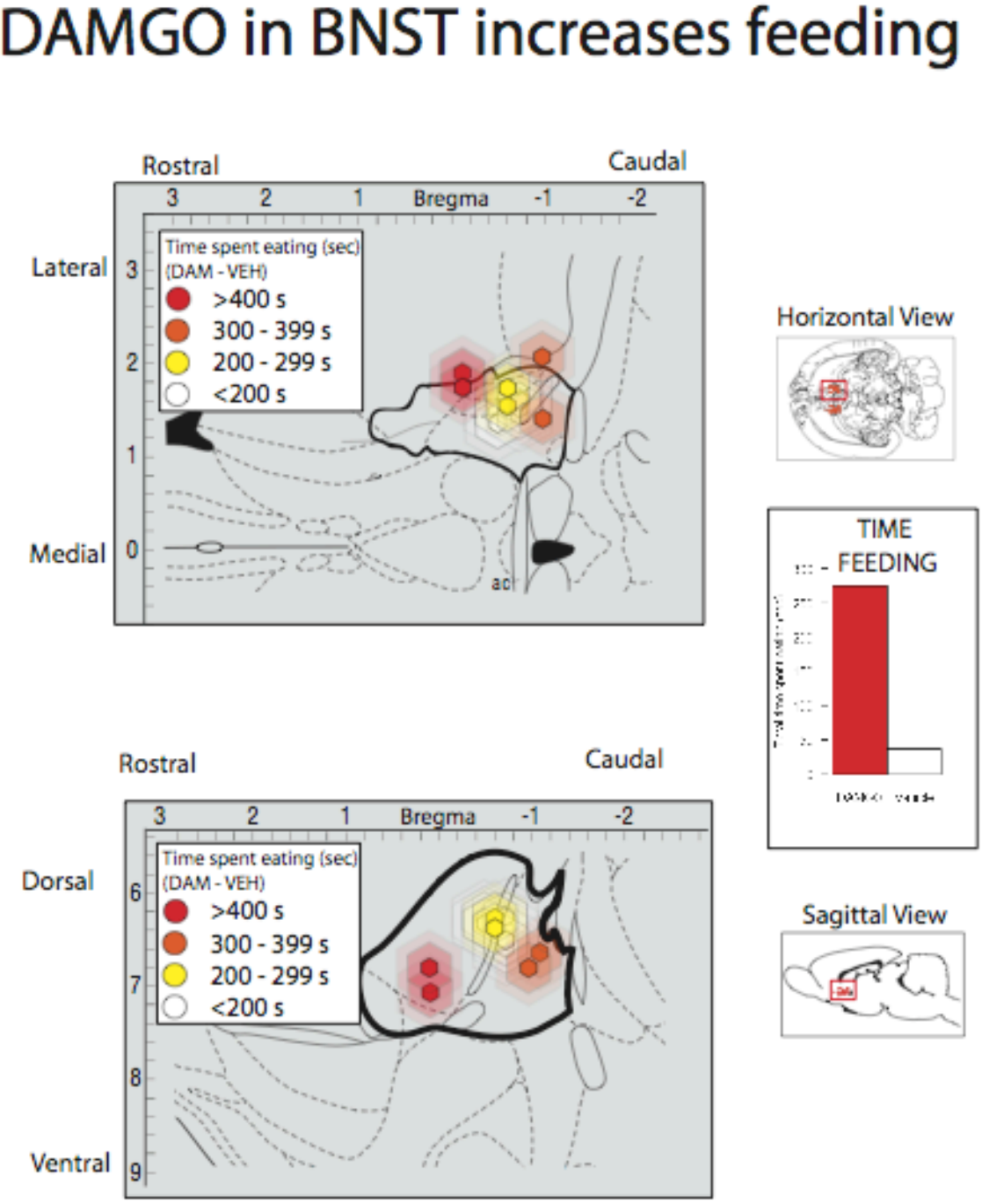



**Figure 5.3.** Anatomical maps showing within-subjects changes in feeding time relative to vehicle treatment day. Symbol placements are based on based on position of microinjection cannula in the BNST, shown in horizontal (top) and sagittal views (bottom). Fos plumes are adapted from previously reported values for this dose of DAMGO (0.1µg) in BNST (see Chapter 2).

# Chapter 6

# Conclusion

## *Summary of experimental findings*

In this series of studies, I first showed that BNST plays a role in appetitive motivation for natural rewards. I then subsequently explored some of the possible reward processes that BNST opioid stimulation may influence.

First, I showed that stimulation of μ-opioid receptors in BNST can potently increase voluntary food intake, a broad measure of appetitive motivation and incentive salience 'wanting.' Intake of standard laboratory chow was roughly tripled by both doses of DAMGO tested, even though the animals had not been food deprived in advance of testing. I also found that temporary inactivation of BNST with a high dose of the $GABA_A$ agonist muscimol disrupted some measures of feeding, while simultaneously stimulating robust increases in defensive treading and cage circling. These apparently aversive effects of muscimol are consistent with the BNST's role in aversive motivational processing, and together with the DAMGO feeding effect suggest that BNST may be able to generate oppositely valenced motivated behaviors in response to different types of neurochemical input. Although previous reports have shown inhibition of feeding following BNST microinjection with CRF (Ciccocioppo et al., 2003), to our knowledge this is the first demonstration of increased feeding following a manipulation of BNST.

Following the finding of increased feeding with μ-opioid stimulation in BNST, I conducted a series of experiments to explore specific psychological mechanisms that could contribute to enhanced food intake. One potential candidate was an increased attribution of incentive salience and so I tested the ability of DAMGO in BNST to increase incentive motivation in two separate tests of 'wanting': autoshaping and conditioned reinforcement. Additionally, I included a comparison group of animals with cannulae aimed at the medial shell of the nucleus accumbens, which allowed me to both 1) directly compare and contrast the effects of μ-opioid stimulation across extended amygdala and ventral striatal macrosystems and 2) test a novel hypothesis about the ability of accumbens shell opioids to amplify reward cues. I found that DAMGO in BNST caused a *diffusion* of incentive salience during autoshaping testing, resulting in increased 'wanting' for reward CS's during non-CS+ inter-trial intervals and a decrease in appetitive behaviors toward both prepotent and non-prepotent cues during the CS+ period. The effect of accumbens shell opioid stimulation, in contrast, was to *broadly enhance* incentive salience 'wanting,' resulting in increased looks at and approaches to the non-prepotent cue and sometimes also the prepotent cue. In conditioned reinforcement testing, I found that accumbens shell opioid stimulation again broadly enhanced 'wanting' for the autoshaping CS+, as evidenced by increased responding in a novel instrumental task; DAMGO in BNST did not affect responding during conditioned reinforcement. In summary, this experiment demonstrated that BNST opioid stimulation increases 'wanting' for reward cues in a temporally diffuse way, while accumbens shell opioids appeared to act as a "rising tide that floats all boats" by elevating 'wanting' for both prepotent and non-prepotent rewards CS's.

Another possible explanation for an increase in food intake is the enhancement of the hedonic impact, or 'liking,' of the food reward. If a neurochemical manipulation were to make food seem more palatable and delicious, intake of that food would be expected to rise. In order to test whether DAMGO in BNST increased reward 'liking,' I utilized the taste reactivity paradigm (Berridge, 2000; Grill & Norgren, 1978). By measuring the orofacial reactions elicited by direct oral infusion of taste solutions, I found that opioid stimulation in BNST actually decreased 'liking' for a sweet sucrose solution; similar results were observed following temporary inactivation of BNST with muscimol. In subsequent food intake testing, I found that DAMGO in BNST again potently increased feeding, but did not preferentially stimulate intake of a preferred palatable food source (M&M's) over standard lab chow. This experiment suggests that opioid stimulation in BNST does not increase feeding via an increase in hedonic 'liking,' in contrast to the effect of μ-opioids at nearby hedonic hotspots in accumbens shell and ventral pallidum (Pecina & Berridge, 2005; K. S. Smith & Berridge, 2005).

Finally, it was important to confirm that the apparently appetitive feeding effect following opioid stimulation of BNST was not instead the result of a direct increase in stress. Indeed, BNST has a well-established role in brain stress networks, and stressful manipulations can often stimulate appetitive behavior, including feeding. In order to investigate whether DAMGO in BNST was inherently stressful, I utilized a conditioned place preference/avoidance procedure, where animals were conditioned to associate a unique environmental context with opioid stimulation of BNST; a separate environment was paired with vehicle microinjection. If, indeed, opioid stimulation of BNST is stressful, then I predicted that animals would avoid the DAMGO-paired environment on

a subsequent drug-free test day. However, I found that rats actually preferred an environment associated with μ-opioid stimulation of BNST over an environment associated with vehicle microinjections, suggesting that DAMGO in BNST is not a stress-inducing manipulation and supporting our assertion that our initial feeding increase was primarily appetitive. In addition, our finding of a conditioned place preference also suggests a possible novel role for BNST in the acute reinforcing aspects of opiate drugs, in addition to its known role as a mediator of reinforcement and withdrawal in drug-dependent animals (Delfs et al., 2000; J. R. Walker et al., 2000).

These experiments are a first step in clarifying how the BNST, in addition to its established role in aversive motivational processes, might act as a link in the brain's vast reward network.

***BNST as a site of appetitive and aversive motivational building blocks***

It is important to emphasize that our findings, which suggest a relatively novel role for BNST in appetitive motivation, should not be considered as contradictory to the array of findings that implicate BNST in aversive motivation, including stress and anxiety. There is no reason to expect that the brain would be constructed of nuclei or macrosystems that only mediate emotions or motivation of a particular valence. Instead, the present findings, taken together with data from studies of anxiety and stress, suggest that BNST should be considered one of several sites in the brain capable of providing the motivational building blocks for both appetitive and aversive behaviors. These basic components of motivated behavior can then be dynamically combined with both internal

information (e.g. physiological state) and external information (e.g. presence of threatening or rewarding stimuli) to generate adaptive behavior.

The juxtaposition of appetitive and aversive motivation has been described in several other brain regions, including additional sub-cortical nuclei in the extended amygdala. Like BNST, CeA is perhaps best known for its role in aversive motivation, in particular its role in the expression of Pavlovian fear conditioning to discrete CS's such as a tone that has previously predicted an aversive footshock (Fendt & Fanselow, 1999; Goosens & Maren, 2001; D. L. Walker & Davis, 1997). However, CeA has also been shown to mediate a variety of appetitive motivational processes, such as the orientation response to conditioned stimuli that predict reward (Gallagher et al., 1990), the transfer of incentive motivation from Pavlovian reward cues to an instrumental action associated with the same reward (Corbit & Balleine, 2005; Mahler & Berridge, 2007), and food intake (Gosnell, 1988).

Outside the extended amygdala, the shell of the nucleus accumbens has also been shown to mediate both appetitive and aversive motivation. Rostro-caudal gradients have been discovered following GABA-ergic stimulation with muscimol, with microinjections in rostral shell eliciting robust feeding, conditioned place preference, and increased hedonic 'liking' (Reynolds & Berridge, 2001, 2002; Zhang et al., 2003) while identical injections in caudal shell result in suppressed feeding, conditioned place avoidance, increased 'disliking' of taste solutions, and dramatic increases in defensive treading (Reynolds & Berridge, 2002). A similar rostro-caudal gradient in accumbens shell has also been identified following disruption of glutamate (Reynolds & Berridge, 2003), and

more recently this glutamatergic gradient has been shown to be critically dependent upon dopamine signaling (Faure et al., 2008).

*Appetitive building blocks*

Although a variety of neuroanatomical evidence suggested that BNST would play a role in reward, including strong links to midbrain dopamine and upstream nuclei such as CeA, there was relatively little behavioral evidence strongly supporting this potential appetitive role. Data from c-Fos expression experiments showed enhanced activity in BNST following several appetitive activities, including exposure to a sexual reward CS, food intake, and stimulation of other food reward nuclei (B. H. Li et al., 1994; Mullett et al., 2000; Mungarndee et al., 2008; Park & Carr, 1998; Taziaux et al., 2008). Yet although c-Fos expression can provide useful data about circuit activity, it does not convey specific information about what role a nuclei might play in the elicited behavior, nor does it conclusively rule out the possibility of elevated activity due to a factor secondary to the target manipulation. Stronger support for the appetitive role for BNST comes from studies utilizing direct manipulation, such as permanent or temporary lesions, to tease apart the role of BNST in male sexual behavior (Newman, 1999). Yet even these studies only report *decreases* in appetitive motivation following disruption or destruction of BNST.

Here, using direct pharmacological stimulation, I show for the first time that BNST can *increase* appetitive motivation for a food reward and also cause diffuse 'wanting' for a reward cue. This suggests that, in addition to being considered a limbic relay for stressful and aversive information, BNST may also convey or directly stimulate

appetitive responses in downstream hypothalamic nuclei. As noted earlier, the lateral division of BNST projects robustly to regions of lateral hypothalamus, which has been strongly implicated in appetitive responding for food and other rewards (Harris, Wimmer, & Aston-Jones, 2005; Mullett et al., 2000; Zheng, Patterson, & Berthoud, 2007).

*Aversive building blocks*

In addition to the finding that BNST is directly involved in the generation of appetitive motivation, I also present further evidence of aversive motivational building blocks in BNST. Previous reports have suggested that BNST is part of a distributed network mediating the response to fearful environmental stimuli. In particular, some have argued that BNST is critically involved in the response to diffuse, unconditioned stimuli and corresponds most closely to the human state of anxiety, a nonspecific aversive response to an impending or suggested environmental threat (M. Davis, 1998; M. Davis & Shi, 1999). In support of this aversive role, I found that temporary inactivation of BNST with muscimol caused an intense increase in a defensive treading behavior (see Chapter 2). Interestingly, this aversive behavior was directed throughout the testing chamber and was accompanied by almost constant circling of the chamber perimeter, almost as if the animals were constantly monitoring their environment in anticipation of an impending threat.

I also found evidence that muscimol in ventral regions of BNST may be particular effective at evoking aversive motivational building blocks. During food intake testing, our lower dose of muscimol (75ng per side) in ventral regions of BNST showed increased defensive treading and reduced feeding relative to microinjections in dorsal BNST. In

taste reactivity testing, the highest dose of muscimol (225ng per side) was a more potent suppressor of hedonic 'liking' responses to sweet sucrose solutions when delivered in ventral regions. Several electrophysiological studies have suggested differences in the characteristics of dorsal and ventral neuronal populations (Egli & Winder, 2003), including higher responsivity in ventral areas to morphine, acetylcholine, and norepinephrine (Casada & Dafny, 1993). Interestingly, anatomical studies have also shown that ventral BNST receives dense norepinephrine from caudal medulla, and this catecholamine input has been repeatedly linked to stressful and aversive events (Aston-Jones et al., 1999; Delfs et al., 2000; Forray & Gysling, 2004; Leri et al., 2002; Pacak et al., 1995). It has been shown that norepinephrine in BSNT triggers $GABA_A$ inhibition (Dumont & Williams, 2004), suggesting the aversive motivational effects we observed following musicmol in ventral BNST may mimic the aversive characteristics of norepinephrine release.

*Overlap of appetitive and aversive building blocks with muscimol in anterior BNST?*

Most of the examples presented so far regarding elicitation of bivalent motivation involve either different neurochemicals delivered at the same location (such as DAMGO and muscimol in BNST) or the same neurochemical delivered at different locations (such as GABA/glutamate gradients in accumbens shell). Can the same drug also elicit both appetitive and aversive building blocks when delivered at the same location?

This may be the case when muscimol is delivered at different doses in anterior portions of BNST. At a lower dose (75ng per side), muscimol microinjection resulted in slightly increased feeding relative to vehicle, while a higher dose of muscimol (225ng per

side) delivered at the same location in the same rats resulted in decreased feeding (Chapter 2). Somewhat similar modulation at the same site has been reported in accumbens shell, where GABA-ergic and glutamatergic inhibition in the middle of the rostro-caudal axis can occasionally elicit both appetitive eating and fearful defensive treading during the same test session, sometimes within second of eachother (Reynolds & Berridge, 2001, 2003). The glutamatergic appetitive and aversive zones in accumbens shell have also shown some flexibility in the face of environmental manipulations, with appetitive regions expanding in the presence of a comfortable home environment while aversive zones increase in size when the environment is loud and bright (Reynolds & Berridge, 2008). It will be of interest in future studies to evaluate the sensitivity of BNST aversion to environmental context; early pilot data from our lab seem to indicate that testing in a home environment can, indeed, diminish some of the aversive measures that accompany muscimol microinjection, including the onset of defensive treading (Berridge lab, unpublished data).

***Homogeneity and heterogeneity in basal forebrain μ-opioid function***

Part of the reason for exploring appetitive behaviors in BNST was its anatomical relationship with a variety of forebrain nuclei linked to reward and motivation. Given my findings in BNST, it is of interest to re-evaluate the broad role of μ-opioids in basal forebrain. The result is an interesting pattern of results across feeding, incentive salience 'wanting,' and 'liking.'

*Food intake*

The distribution of sites where μ-opioids can stimulate feeding is the most homogeneous and widespread throughout basal forebrain. In addition to the already identified locations in nucleus accumbens (Bakshi & Kelley, 1993a; Zhang & Kelley, 2000), ventral pallidum (K. S. Smith & Berridge, 2005), and central amygdala (Gosnell, 1988), data presented here adds BNST to this list. Other data from our lab not presented here also implicates additional sites in extended amygdala, including SLEA and IPAC (Na, 2008), suggesting an almost unbroken corridor of opioid-sensitive feeding sites beginning at accumbens and stretching caudo-laterally all the way to central amygdala.

One slight divergence I observed in μ-opioid stimulation of feeding in BNST, as compared to nearby sites in accumbens or central amygdala (Glass et al., 1999; Zhang & Kelley, 2000), was the inability to selectively increase feeding for highly palatable foods. In BNST, I found that although palatable M&M's were strongly preferred to standard chow under both vehicle and DAMGO conditions, μ-opioid stimulation increased feeding on both foods and not just the M&M's.

*CS 'Wanting'*

Though food intake serves as a broad measure of 'wanting' for a UCS reward, I also present data here on 'wanting' for learned CS's that are associated with a sweet sucrose reward. In BNST, it appears that μ-opioid stimulation generates a temporally diffuse 'wanting' for rewards CS's that is not phase-locked to CS+ presentation. In accumbens shell, opioids appear to stimulate broad 'wanting' for all available reward

cues, while in central amygdala opioid stimulation generates focused 'wanting' for only an animal's prepotent CS (Mahler & Berridge, 2009). Thus, although μ-opioid receptors at several sites in basal forebrain appear to mediate 'wanting' for reward CS's, the precise structure of this CS motivation is dependent upon exactly which region is stimulated. The diversity of opioid-dependent CS 'wanting' is particularly interesting within extended amygdala, and may mirror dissociations between BNST and CeA in other behavioral contexts. Indeed, as previously noted, it has been suggested by some that BNST lesions appear to affect different classes of stimuli (primarily unconditioned, temporally diffuse) than CeA (primarily conditioned, temporally discrete) in fear conditioning and potentiated startle paradigms (M. Davis & Shi, 1999).

*'Liking'*

Forebrains sites where μ-opioids can act to increase the hedonic impact, or 'liking,' of food rewards appear to be the most scarce as well as the most anatomically restricted. So far, only two brain regions – the medial shell of the nucleus accumbens and ventral pallidum – have been shown to support μ-opioid enhancements of 'liking,' and even these hedonic hotspots are restricted to small sub-regions within these nuclei. In contrast, μ-opioid stimulation in CeA has been shown to decrease 'liking' for a sweet sucrose solution (Mahler & Berridge, 2006). Here I report that μ–opioid stimulation in BNST also suppresses 'liking' for a normally pleasant sucrose taste, similar to CeA. This suggests that although opioids within the ventral-striato-pallidum macrosystem can dynamically and transiently enhance 'liking' for a pleasant food reward, opioids within the extended amygdala macrosystem cannot make an already 'liked' reward even better,

and in fact actually diminish hedonic responding. Although the mechanism for this reduction in 'liking' after extended amygdala μ-opioid stimulation is currently unknown, in BNST at least it may be linked to descending, predominantly inhibitory connections to hindbrain taste nuclei (Kang & Lundy, 2009; C. S. Li & Cho, 2006; Lundy, 2008).

***Revisiting the Extended Amygdala Concept***

Prior to the current studies, BNST was a relatively unknown entity in reward and appetitive motivation. Outside of a limited series of studies that had implicated BNST as a necessary node in the network responsible for male sexual behavior (Newman, 1999), little direct evidence existed to link BNST with the generation of purely appetitive behavior. In fact some data, including studies of feeding, suggested that BNST might be involved in the *suppression* of appetitive behavior in response to stress-related neuropeptide signaling (Ciccocioppo et al., 2004; Ciccocioppo et al., 2003). Given our current findings that show a direct role for BNST μ-opioid receptor stimulation in the generation of appetitive behavior for food rewards, how does this impact the broader concept of the extended amygdala?

First, the current experiments provide further support for the hypothesis that the entire extended amygdala, and not just CeA, is involved in the generation and assignment of appetitive behaviors (Waraczynski, 2006). Indeed, pilot data from our laboratory suggests that all regions of the extended amygdala, including CeA, BNST, IPAC, and SLEA, can all support increased feeding in response to μ-opioid stimulation (Na, 2008). IPAC and SLEA are more unknown entities in reward and motivation than even BNST, and future studies will be required to clarify their specific roles in appetitive behavior.

The relative homogeneity of feeding and taste reactivity results in extended amygdala further supports the utility of this neuroanatomical macrosystem as a springboard for behavioral investigation, though it should also be noted that the appetitive role of each extended amygdala nuclei is unlikely to be redundant. For example, although previous research has shown that CeA μ-opioid stimulation yielded a focused enhancement of incentive salience on an animal's prepotent reward CS during autoshaping testing, I reported in Chapter 3 that BNST μ-opioid activation generated a more diffuse 'wanting' that was not linked to the phasic presentations of the autoshaping CS+.

Second, the current findings suggest that both the central (CeA through lateral BNST) and medial (MeA through medial BNST) divisions of extended amygdala are involved in appetitive motivation for a food reward. These parallel sub-systems within extended amygdala show sparse cross-connection relative to the dense interconnections within each of the central and medial divisions (Alheid, 2003; de Olmos & Heimer, 1999), and previous research strongly implicating CeA in appetitive motivation suggested that perhaps only more lateral regions of BNST would play a role in appetitive processes (Gallagher et al., 1990; Gosnell, 1988; Holland & Gallagher, 2003; Mahler & Berridge, 2009). However, though we did show increased feeding and diffuse 'wanting' following μ-opioid stimulation in lateral BNST, we also observed similar effects after stimulation in medial BNST regions (see Chapters 2, 3, and 4). This finding is again consistent with the observed role of medial extended amygdala regions in the generation of male sexual behavior (Newman, 1999), and also with the suggestion that the medial extended amygdala system may play a particular role in reward valuation more generally (Waraczynski, 2006). One notable limitation here to our ability to distinguish clearly

between the role of medial vs. central extended amygdala are the small size of these anatomical sub-regions; indeed, in spite of the relatively small Fos plumes observed in our experiments, many of the microinjections in the present study likely stimulated at least some receptors in both lateral and medial regions of BNST. Future studies could potentially use modified microinjection doses or volumes to further restrict the region of functional impact in order to more carefully tease apart the roles of central vs. medial extended amygdala.

Third, although the extended amygdala does appear to generate enhanced 'wanting' for both unconditioned rewards and also reward cues, the present studies and other related data suggest that this macrosystem does *not* play a role in increasing the hedonic impact of food rewards once they are received and consumed. In Chapter 4, I reported that μ-opioid stimulation in BNST potently reduced the number of hedonic orofacial responses to an oral infusion of a sweet sucrose solution, reducing the total number of hedonic reactions by almost 50%. Nearly identical behavioral results have been reported following μ-opioid stimulation in CeA (Mahler & Berridge, 2006). These results stand in contrast to the μ-opioid hedonic hotspots that have been indentified in rostro-dorsal accumbens shell and caudal ventral pallidum (Pecina & Berridge, 2000, 2005; Smith & Berridge, 2005), components of the nearby ventral striato-pallidal macrosystem.

Finally, although the extended amygdala has frequently been implicated in the *aversive* components of addiction and drug seeking (Aston-Jones & Harris, 2004; Delfs et al., 2000; Koob, 2003), the present studies and related experiments suggest that extended amygdala may also contribute to more purely appetitive drug-seeking. For

example, CeA opioid stimulation has recently been shown to enhance the motivational magnet quality of previously learned reward cues, focusing appetitive responses on a prepotent cue and offering a potential neural substrate for linking learning with motivation to approach and interact with cues that predict drug delivery. BNST opioid stimulation, in contrast, may act more broadly to enhance incentive motivation, perhaps making the entire environment seem more attractive and pleasant. Interestingly, as demonstrated in our autoshaping study in Chapter 3, this could potentially contribute to instances where 'wanting' appears to spill-over beyond learned boundaries, similar to rare cases of dopamine dysregulation disorder where patients not only develop addictive patterns of dopamine replacement but can also exhibit excessive motivation for other activities such as gambling (O'Sullivan, Evans, & Lees, 2009).

### *Future directions*

*Other neurochemical substrates for appetitive motivation in BNST*

The current series of experiments focuses primarily on appetitive motivation after μ-opioid stimulation within BNST. However, there are other potential neurochemical targets for reward and appetitive processes in BNST that deserve consideration in future studies. Foremost on the list is dopamine, a perennial target in affective neuroscience that has been implicated in several aspects of reward (Barbano & Cador, 2007; Berridge, 2007; Redish, 2004; Salamone, 2007; Schultz et al., 1997). BNST shares reciprocal connections with midbrain dopaminergic nuclei (Fudge & Haber, 2001; Georges & Aston-Jones, 2001), and both intrinsic stimulation of BNST as well as incoming stimulation from infralimbic cortex relayed via BNST can potently modulate firing in

VTA dopaminergic cell populations (Georges & Aston-Jones, 2001). Cannabinoid receptors, which have also been implicated in appetitive motivation (Fattore et al., 2008; Mahler et al., 2007), in BNST have recently been shown to modulate downstream activitiy in this infralimbic-BNST-VTA circuit, making them another potential target for future investigation.

*The role of BNST in mediating conditioned cues*

One particularly thorny issue worthy of future study is the role of BNST in the behavioral response to conditioned cues. Although some have argued that BNST is primarily involved in the response to unconditioned cues (especially in the context of anxiety) (M. Davis, 1998), a number of studies appear to show at least some role for BNST in the response to conditioned cues. For example, elevated c-Fos expression is found in BNST following exposure to a cue associated with a sexual reward (Taziaux et al., 2008), and presentation of a tone paired with stressful immobilization elicited increased responding in BNST neurons (Henke, 1984). Perhaps it could be argued in both these cases that activity in BNST was merely a relay node in a larger circuit, or was stimulated primarily by upstream activity in CeA, but it has also been shown that lesions of BNST, though they impair fear conditioning to a discrete tone stimulus, do not impact contextual fear expression for an environment previously paired with aversive shocks (Sullivan et al., 2004).

I presented evidence earlier that BNST opioid stimulation can increase 'wanting' for a reward CS in an autoshaping task (Chapter 3), though this 'wanting' was temporally diffuse and spread outside the CS+ periods where 'wanting' is normally expressed. The

temporal qualities of the cue may be particularly relevant to explaining the role BNST plays. For example, one key difference between the tone CS and environmental CS in the fear conditioning experiment described above are their temporal character: the former is brief and phasic, the latter extended and enduring. Perhaps, then, the key distinction between BNST and other regions involved in the processing of environmental cues (such as CeA) is not conditioned vs. unconditioned, but brief vs. extended or phasic vs. tonic.

*Role of BNST opioids in mediating other natural rewards*

In several experiments I showed that BNST μ-opioid stimulation could robustly increase 'wanting' for UCS food rewards, including both standard rat chow and more palatable M&M's. Can μ-opioid stimulation also increase the incentive motivational value of other natural rewards, like sex or social interaction?

As previously noted, there is considerable evidence to support a role for BNST in sexual motivation, especially in males (Newman, 1999), though direct infusion of beta-endorphin (an endogenous μ-opioid agonist) in BNST has been reported not to impact the performance of sexual behavior in male rats (Hughes, Everitt, & Herbert, 1987). In collaboration with an undergraduate honors student, I attempted to investigate in male rats whether DAMGO microinjection in BNST could enhance 1) appetitive investigation of a receptive female (and effect recently demonstrated in CeA – Berridge Lab, unpublished data) and 2) increase the preference for an opposite sex odor. Although we were able to replicate the finding of enhanced food reward, we did not find any increases in sexual or social odor preference. I believe that methodological issues (including problems with our ovariectomized females and subsequent hormone replacement) may

have contributed to this null finding, though based on the finding by Hughes et al. (1987) it is possible that BNST may not be sufficient to increase sexual behavior, even if BNST is a necessary part of the network that controls sexual behavior and reward.

*Circuit interactions within extended amygdala*

In this dissertation I have presented data showing that focused neurochemical manipulation of BNST can influence appetitive motivation, and referenced data suggesting that targeted manipulations of other extended amygdala nuclei, especially CeA, can do the same. However, as the anatomical concept of extended amygdala continues to grow and gain additional support, it will be important to begin investigating the circuit functions with this macrosystem. How do extended amygdala sub-regions act together to generate motivated behavior?

One such study has already been conducted examining the role of BNST and CeA in stress-induced relapse. Using asymmetrical bilateral lesions, it was shown that CeA acts in concert with BNST to generate the CRF-dependent relapse in extinguished drug-seeking that follows exposure to an acute stressor (Erb et al., 2001). This methodology could easily be adapted to evaluate the appetitive behaviors by, for example, testing whether the effect of DAMGO microinjection in CeA on feeding was influenced by simultaneous blockade of opioid function in the BNST, or vice versa. A similar design has recently revealed that hedonic hotspots in accumbens shell and ventral pallidum appear to work together in generating enhancements of 'liking.' Opioid blockade in one spot can veto hedonic enhancement normally caused by opioid activation in the other, though interestingly the effect of DAMGO on feeding is asymmetrical (K. S. Smith &

Berridge, 2007). Such studies will be necessary in extended amygdala to fully characterize how the extended amgydala macrosystem acts to generate appetitive (and aversive) motivation.

### *Conclusion*

In summary, I present here some of the first evidence that BNST opioid stimulation plays a direct role in the generation of appetitive motivation. This finding, together with its established role in aversive and stressful motivation processes, suggests that BNST should be considered a forebrain site of both appetitive and aversive motivational building blocks. Future studies will be required to fully characterize the role of BNST in various reward processes, though I do show here that it participates in UCS reward 'wanting,' diffuse 'wanting' for reward CS's, and possibly the acute rewarding effect of μ-opioid stimulation. BNST μ-opioid stimulation did not stimulate hedonic 'liking,' actually diminishing the pleasantness of a sweet sucrose solution in a manner similar to opioid stimulation in CeA. The possibility that these apparently appetitive effects were instead the result of stress was diminished following the discovery that opioid stimulation in BNST generated a conditioned place preference. Together, these findings support a role for BNST in appetitive motivational processes, and increase our knowledge of the function of μ-opioid stimulation in the emerging anatomical macrosystem of the extended amygdala.

## References


Alden, M., Besson, J. M., & Bernard, J. F. (1994). Organization of the efferent projections from the pontine parabrachial area to the bed nucleus of the stria terminalis and neighboring regions: a PHA-L study in the rat. *J Comp Neurol, 341*(3), 289-314.

Alheid, G. F. (2003). Extended amygdala and basal forebrain. *Ann N Y Acad Sci, 985*, 185-205.

Alheid, G. F., de Olmos, J. S., & Beltramino, C. A. (1995). Amygdala and extended amygdala. In G. Paxinos & C. Watson (Eds.), *The Rat Nervous System* (2nd ed.). San Diego: Academic Press.

Alheid, G. F., & Heimer, L. (1988). New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata. *Neuroscience, 27*(1), 1-39.

Altemus, M., Glowa, J. R., & Murphy, D. L. (1993). Attenuation of food-restriction-induced running by chronic fluoxetine treatment. *Psychopharmacol Bull, 29*(3), 397-400.

Aston-Jones, G., Delfs, J. M., Druhan, J., & Zhu, Y. (1999). The bed nucleus of the stria terminalis. A target site for noradrenergic actions in opiate withdrawal. *Ann N Y Acad Sci, 877*, 486-498.

Aston-Jones, G., & Harris, G. C. (2004). Brain substrates for increased drug seeking during protracted withdrawal. *Neuropharmacology, 47 Suppl 1*, 167-179.

Bakshi, V. P., & Kelley, A. E. (1993a). Feeding induced by opioid stimulation of the ventral striatum: role of opiate receptor subtypes. *J Pharmacol Exp Ther, 265*(3), 1253-1260.

Bakshi, V. P., & Kelley, A. E. (1993b). Striatal regulation of morphine-induced hyperphagia: an anatomical mapping study. *Psychopharmacology (Berl), 111*(2), 207-214.

Barbano, M. F., & Cador, M. (2007). Opioids for hedonic experience and dopamine to get ready for it. *Psychopharmacology (Berl), 191*(3), 497-506.

Bardo, M. T., & Bevins, R. A. (2000). Conditioned place preference: what does it add to our preclinical understanding of drug reward? *Psychopharmacology (Berl), 153*(1), 31-43.

Bassareo, V., De Luca, M. A., & Di Chiara, G. (2007). Differential impact of pavlovian drug conditioned stimuli on in vivo dopamine transmission in the rat accumbens shell and core and in the prefrontal cortex. *Psychopharmacology (Berl), 191*(3), 689-703.

Been, L., & Petrulis, A. (2008). *The role of the posterior bed nucleus of the stria terminalis in opposite-sex odor preference and sexual odor processing in male*

*Syrian hamsters*. Paper presented at the Annual Meeting of the Society for Neuroscience.
Bernard, J. F., Alden, M., & Besson, J. M. (1993). The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: a Phaseolus vulgaris leucoagglutinin (PHA-L) study in the rat. *J Comp Neurol, 329*(2), 201-229.
Berridge, K. C. (2000). Measuring hedonic impact in animals and infants: microstructure of affective taste reactivity patterns. *Neurosci Biobehav Rev, 24*(2), 173-198.
Berridge, K. C. (2001). Reward learing: Reinforcement, Incentives, and Expectations. In D. L. Medin (Ed.), *The Psychology of Learning and Motivation* (Vol. 40, pp. 223-278). New York City: Academic Press.
Berridge, K. C. (2007). The debate over dopamine's role in reward: the case for incentive salience. *Psychopharmacology (Berl), 191*(3), 391-431.
Berridge, K. C., & Robinson, T. E. (1998). What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain Res Brain Res Rev, 28*(3), 309-369.
Berridge, K. C., & Robinson, T. E. (2003). Parsing reward. *Trends Neurosci, 26*(9), 507-513.
Berridge, K. C., Robinson, T. E., & Aldridge, J. W. (2009). Dissecting components of reward: 'liking', 'wanting', and learning. *Curr Opin Pharmacol, 9*(1), 65-73.
Bindra, D. (1978). How adaptive behavior is produced: a perceptual-motivation alternative to response reinforcement. *Behav Brain Sci*(1), 41-91.
Blaiss, C. A., & Janak, P. H. (2009). The nucleus accumbens core and shell are critical for the expression, but not the consolidation, of Pavlovian conditioned approach. *Behav Brain Res, 200*(1), 22-32.
Boakes, R. (1977). Preformance on learning to associate as sitmulus with positive reinforcement. In H. Davis & H. Hurwitz (Eds.), *Operant-pavlovia interactions*. Hillsdale, NJ: Lawrence Erlbaum Associates.
Carboni, E., Silvagni, A., Rolando, M. T., & Di Chiara, G. (2000). Stimulation of in vivo dopamine transmission in the bed nucleus of stria terminalis by reinforcing drugs. *J Neurosci, 20*(20), RC102.
Carlezon, W. A., Jr., & Thomas, M. J. (2009). Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. *Neuropharmacology, 56 Suppl 1*, 122-132.
Casada, J. H., & Dafny, N. (1991). Restraint and stimulation of bed nucleus of the stria terminalis produce similar stress-like behaviors. *Brain Res Bull, 27*(2), 207-212.
Casada, J. H., & Dafny, N. (1993). Responses of neurons in bed nucleus of the stria terminalis to microiontophoretically applied morphine, norepinephrine and acetylcholine. *Neuropharmacology, 32*(3), 279-284.
Cassell, M. D., Freedman, L. J., & Shi, C. (1999). The intrinsic organization of the central extended amygdala. *Ann N Y Acad Sci, 877*, 217-241.
Cecchi, M., Capriles, N., Watson, S. J., & Akil, H. (2007). ß1 adrenergic receptors in the bed nucleus of stria terminalis mediate differential responses to opiate withdrawal. *Neuropsychopharmacology, 32*, 589-599.
Choi, D. C., Evanson, N. K., Furay, A. R., Ulrich-Lai, Y. M., Ostrander, M. M., & Herman, J. P. (2008). The anteroventral bed nucleus of the stria terminalis

differentially regulates hypothalamic-pituitary-adrenocortical axis responses to acute and chronic stress. *Endocrinology, 149*(2), 818-826.

Choi, D. C., Furay, A. R., Evanson, N. K., Ostrander, M. M., Ulrich-Lai, Y. M., & Herman, J. P. (2007). Bed nucleus of the stria terminalis subregions differentially regulate hypothalamic-pituitary-adrenal axis activity: implications for the integration of limbic inputs. *J Neurosci, 27*(8), 2025-2034.

Choi, D. C., Furay, A. R., Evanson, N. K., Ulrich-Lai, Y. M., Nguyen, M. M., Ostrander, M. M., et al. (2008). The role of the posterior medial bed nucleus of the stria terminalis in modulating hypothalamic-pituitary-adrenocortical axis responsiveness to acute and chronic stress. *Psychoneuroendocrinology, 33*(5), 659-669.

Ciccocioppo, R., Cippitelli, A., Economidou, D., Fedeli, A., & Massi, M. (2004). Nociceptin/orphanin FQ acts as a functional antagonist of corticotropin-releasing factor to inhibit its anorectic effect. *Physiol Behav, 82*(1), 63-68.

Ciccocioppo, R., Fedeli, A., Economidou, D., Policani, F., Weiss, F., & Massi, M. (2003). The bed nucleus is a neuroanatomical substrate for the anorectic effect of corticotropin-releasing factor and for its reversal by nociceptin/orphanin FQ. *J Neurosci, 23*(28), 9445-9451.

Corbit, L. H., & Balleine, B. W. (2005). Double dissociation of basolateral and central amygdala lesions on the general and outcome-specific forms of pavlovian-instrumental transfer. *J Neurosci, 25*(4), 962-970.

Corbit, L. H., Muir, J. L., & Balleine, B. W. (2001). The role of the nucleus accumbens in instrumental conditioning: Evidence of a functional dissociation between accumbens core and shell. *J Neurosci, 21*(9), 3251-3260.

Crane, J. W., Buller, K. M., & Day, T. A. (2003). Evidence that the bed nucleus of the stria terminalis contributes to the modulation of hypophysiotropic corticotropin-releasing factor cell responses to systemic interleukin-1beta. *J Comp Neurol, 467*(2), 232-242.

Dallman, M. F., Pecoraro, N., Akana, S. F., La Fleur, S. E., Gomez, F., Houshyar, H., et al. (2003). Chronic stress and obesity: a new view of "comfort food". *Proc Natl Acad Sci U S A, 100*(20), 11696-11701.

Dallman, M. F., Warne, J. P., Foster, M. T., & Pecoraro, N. C. (2007). Glucocorticoids and insulin both modulate caloric intake through actions on the brain. *J Physiol, 583*(Pt 2), 431-436.

Daunais, J. B., Letchworth, S. R., Sim-Selley, L. J., Smith, H. R., Childers, S. R., & Porrino, L. J. (2001). Functional and anatomical localization of mu opioid receptors in the striatum, amygdala, and extended amygdala of the nonhuman primate. *J Comp Neurol, 433*(4), 471-485.

Davis, C., Strachan, S., & Berkson, M. (2004). Sensitivity to reward: implications for overeating and overweight. *Appetite, 42*(2), 131-138.

Davis, M. (1998). Are different parts of the extended amygdala involved in fear versus anxiety? *Biol Psychiatry, 44*(12), 1239-1247.

Davis, M., & Shi, C. (1999). The extended amygdala: are the central nucleus of the amygdala and the bed nucleus of the stria terminalis differentially involved in fear versus anxiety? *Ann N Y Acad Sci, 877*, 281-291.

Davis, M., Walker, D. L., & Lee, Y. (1997a). Amygdala and bed nucleus of the stria terminalis: differential roles in fear and anxiety measured with the acoustic startle reflex. *Philos Trans R Soc Lond B Biol Sci, 352*(1362), 1675-1687.

Davis, M., Walker, D. L., & Lee, Y. (1997b). Roles of the amygdala and bed nucleus of the stria terminalis in fear and anxiety measured with the acoustic startle reflex. Possible relevance to PTSD. *Ann N Y Acad Sci, 821*, 305-331.

de Araujo, I. E., Rolls, E. T., Kringelbach, M. L., McGlone, F., & Phillips, N. (2003). Taste-olfactory convergence, and the representation of the pleasantness of flavour, in the human brain. *Eur J Neurosci, 18*(7), 2059-2068.

de Olmos, J. S., & Heimer, L. (1999). The concepts of the ventral striatopallidal system and extended amygdala. *Ann N Y Acad Sci, 877*, 1-32.

Delfs, J. M., Zhu, Y., Druhan, J. P., & Aston-Jones, G. (2000). Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. *Nature, 403*(6768), 430-434.

Dickinson, A., & Dawson, G. (1987). Pavlovian processes in the motivational control of instrumental performance. *Q J Exp Psychol B, 39*, 201-213.

Dong, H. W., Petrovich, G. D., & Swanson, L. W. (2000). Organization of projections from the juxtacapsular nucleus of the BST: a PHAL study in the rat. *Brain Res, 859*(1), 1-14.

Dong, H. W., Petrovich, G. D., & Swanson, L. W. (2001). Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res Brain Res Rev, 38*(1-2), 192-246.

Dong, H. W., Petrovich, G. D., Watts, A. G., & Swanson, L. W. (2001). Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. *J Comp Neurol, 436*(4), 430-455.

Dong, H. W., & Swanson, L. W. (2003). Projections from the rhomboid nucleus of the bed nuclei of the stria terminalis: implications for cerebral hemisphere regulation of ingestive behaviors. *J Comp Neurol, 463*(4), 434-472.

Dong, H. W., & Swanson, L. W. (2004a). Organization of axonal projections from the anterolateral area of the bed nuclei of the stria terminalis. *J Comp Neurol, 468*(2), 277-298.

Dong, H. W., & Swanson, L. W. (2004b). Projections from bed nuclei of the stria terminalis, posterior division: implications for cerebral hemisphere regulation of defensive and reproductive behaviors. *J Comp Neurol, 471*(4), 396-433.

Dong, H. W., & Swanson, L. W. (2006a). Projections from bed nuclei of the stria terminalis, anteromedial area: cerebral hemisphere integration of neuroendocrine, autonomic, and behavioral aspects of energy balance. *J Comp Neurol, 494*(1), 142-178.

Dong, H. W., & Swanson, L. W. (2006b). Projections from bed nuclei of the stria terminalis, dorsomedial nucleus: implications for cerebral hemisphere integration of neuroendocrine, autonomic, and drinking responses. *J Comp Neurol, 494*(1), 75-107.

Dong, H. W., & Swanson, L. W. (2006c). Projections from bed nuclei of the stria terminalis, magnocellular nucleus: implications for cerebral hemisphere regulation of micturition, defecation, and penile erection. *J Comp Neurol, 494*(1), 108-141.

Drewnowski, A., Krahn, D. D., Demitrack, M. A., Nairn, K., & Gosnell, B. A. (1995). Naloxone, an opiate blocker, reduces the consumption of sweet high-fat foods in obese and lean female binge eaters. *Am J Clin Nutr, 61*(6), 1206-1212.

Dumont, E. C., Mark, G. P., Mader, S., & Williams, J. T. (2005). Self-administration enhances excitatory synaptic transmission in the bed nucleus of the stria terminalis. *Nat Neurosci, 8*(4), 413-414.

Dumont, E. C., Rycroft, B. K., Maiz, J., & Williams, J. T. (2008). Morphine produces circuit-specific neuroplasticity in the bed nucleus of the stria terminalis. *Neuroscience, 153*(1), 232-239.

Dumont, E. C., & Williams, J. T. (2004). Noradrenaline triggers GABAA inhibition of bed nucleus of the stria terminalis neurons projecting to the ventral tegmental area. *J Neurosci, 24*(38), 8198-8204.

Egli, R. E., & Winder, D. G. (2003). Dorsal and ventral distribution of excitable and synaptic properties of neurons of the bed nucleus of the stria terminalis. *J Neurophysiol, 90*(1), 405-414.

Eilam, D. (2005). Die hard: a blend of freezing and fleeing as a dynamic defense--implications for the control of defensive behavior. *Neurosci Biobehav Rev, 29*(8), 1181-1191.

El-Amamy, H., & Holland, P. C. (2007). Dissociable effects of disconnecting amygdala central nucleus from the ventral tegmental area or substantia nigra on learned orienting and incentive motivation. *Eur J Neurosci, 25*(5), 1557-1567.

Epping-Jordan, M. P., Markou, A., & Koob, G. F. (1998). The dopamine D-1 receptor antagonist SCH 23390 injected into the dorsolateral bed nucleus of the stria terminalis decreased cocaine reinforcement in the rat. *Brain Res, 784*(1-2), 105-115.

Erb, S., Salmaso, N., Rodaros, D., & Stewart, J. (2001). A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl), 158*(4), 360-365.

Erb, S., & Stewart, J. (1999). A role for the bed nucleus of the stria terminalis, but not the amygdala, in the effects of corticotropin-releasing factor on stress-induced reinstatement of cocaine seeking. *J Neurosci, 19*(20), RC35.

Everitt, B. J., Parkinson, J. A., Olmstead, M. C., Arroyo, M., Robledo, P., & Robbins, T. W. (1999). Associative processes in addiction and reward. The role of amygdala-ventral striatal subsystems. *Ann N Y Acad Sci, 877*, 412-438.

Fattore, L., Fadda, P., Spano, M. S., Pistis, M., & Fratta, W. (2008). Neurobiological mechanisms of cannabinoid addiction. *Mol Cell Endocrinol, 286*(1-2 Suppl 1), S97-S107.

Faure, A., Reynolds, S. M., Richard, J. M., & Berridge, K. C. (2008). Mesolimbic dopamine in desire and dread: enabling motivation to be generated by localized glutamate disruptions in nucleus accumbens. *J Neurosci, 28*(28), 7184-7192.

Fendt, M., Endres, T., & Apfelbach, R. (2003). Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *J Neurosci, 23*(1), 23-28.

Fendt, M., & Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neurosci Biobehav Rev, 23*(5), 743-760.

Finlayson, G., King, N., & Blundell, J. E. (2007). Liking vs. wanting food: importance for human appetite control and weight regulation. *Neurosci Biobehav Rev, 31*(7), 987-1002.

Flagel, S. B., Akil, H., & Robinson, T. E. (2009). Individual differences in the attribution of incentive salience to reward-related cues: Implications for addiction. *Neuropharmacology, 56 Suppl 1*, 139-148.

Flagel, S. B., Watson, S. J., Akil, H., & Robinson, T. E. (2008). Individual differences in the attribution of incentive salience to a reward-related cue: influence on cocaine sensitization. *Behav Brain Res, 186*(1), 48-56.

Flagel, S. B., Watson, S. J., Robinson, T. E., & Akil, H. (2007). Individual differences in the propensity to approach signals vs goals promote different adaptations in the dopamine system of rats. *Psychopharmacology (Berl), 191*(3), 599-607.

Forray, M. I., & Gysling, K. (2004). Role of noradrenergic projections to the bed nucleus of the stria terminalis in the regulation of the hypothalamic-pituitary-adrenal axis. *Brain Res Brain Res Rev, 47*(1-3), 145-160.

Francesconi, W., Berton, F., Repunte-Canonigo, V., Hagihara, K., Thurbon, D., Lekic, D., et al. (2009). Protracted withdrawal from alcohol and drugs of abuse impairs long-term potentiation of intrinsic excitability in the juxtacapsular bed nucleus of the stria terminalis. *J Neurosci, 29*(17), 5389-5401.

Fudge, J. L., & Haber, S. N. (2001). Bed nucleus of the stria terminalis and extended amygdala inputs to dopamine subpopulations in primates. *Neuroscience, 104*(3), 807-827.

Gallagher, M., Graham, P. W., & Holland, P. C. (1990). The amygdala central nucleus and appetitive Pavlovian conditioning: lesions impair one class of conditioned behavior. *J Neurosci, 10*(6), 1906-1911.

Georges, F., & Aston-Jones, G. (2001). Potent regulation of midbrain dopamine neurons by the bed nucleus of the stria terminalis. *J Neurosci, 21*(16), RC160.

Giraudo, S. Q., Billington, C. J., & Levine, A. S. (1998). Effects of the opioid antagonist naltrexone on feeding induced by DAMGO in the central nucleus of the amygdala and in the paraventricular nucleus in the rat. *Brain Res, 782*(1-2), 18-23.

Glasner, S. V., Overmier, J. B., & Balleine, B. W. (2005). The role of Pavlovian cues in alcohol seeking in dependent and nondependent rats. *J Stud Alcohol, 66*(1), 53-61.

Glass, M. J., Billington, C. J., & Levine, A. S. (1999). Opioids and food intake: distributed functional neural pathways? *Neuropeptides, 33*(5), 360-368.

Goosens, K. A., & Maren, S. (2001). Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. *Learn Mem, 8*(3), 148-155.

Gosnell, B. A. (1988). Involvement of mu opioid receptors in the amygdala in the control of feeding. *Neuropharmacology, 27*(3), 319-326.

Gosnell, B. A., Morley, J. E., & Levine, A. S. (1986). Opioid-induced feeding: localization of sensitive brain sites. *Brain Res, 369*(1-2), 177-184.

Goudriaan, A. E., Oosterlaan, J., de Beurs, E., & Van den Brink, W. (2004). Pathological gambling: a comprehensive review of biobehavioral findings. *Neurosci Biobehav Rev, 28*(2), 123-141.

Grant, J. E., Brewer, J. A., & Potenza, M. N. (2006). The neurobiology of substance and behavioral addictions. *CNS Spectr, 11*(12), 924-930.

Gray, T. S., Piechowski, R. A., Yracheta, J. M., Rittenhouse, P. A., Bethea, C. L., & Van de Kar, L. D. (1993). Ibotenic acid lesions in the bed nucleus of the stria terminalis attenuate conditioned stress-induced increases in prolactin, ACTH and corticosterone. *Neuroendocrinology, 57*(3), 517-524.

Grill, H. J., & Norgren, R. (1978). The taste reactivity test. I. Mimetic responses to gustatory stimuli in neurologically normal rats. *Brain Res, 143*(2), 263-279.

Harris, G. C., & Aston-Jones, G. (2007). Activation in extended amygdala corresponds to altered hedonic processing during protracted morphine withdrawal. *Behav Brain Res, 176*(2), 251-258.

Harris, G. C., Wimmer, M., & Aston-Jones, G. (2005). A role for lateral hypothalamic orexin neurons in reward seeking. *Nature, 437*(7058), 556-559.

Hearst, E., & Jenkins, H. (1974). Sign tracking: the stimulus-reinforcer relation and directed action. In *Monograph of the Psychonomic Society*. Austin, TX: The Psychonomic Society.

Heimer, L. (2003). A new anatomical framework for neuropsychiatric disorders and drug abuse. *Am J Psychiatry, 160*(10), 1726-1739.

Heimer, L., Trimble, M., Van Hoesen, G. W., & Zahm, D. S. (2007). *Anatomy of Neuropsychiatry: The New Anatomy of the Basal Forebrain and its Implications for Neuropsychiatric Illnes*: Academic Press.

Heimer, L., & Van Hoesen, G. W. (2006). The limbic lobe and its output channels: implications for emotional functions and adaptive behavior. *Neurosci Biobehav Rev, 30*(2), 126-147.

Heinz, A., Beck, A., Grusser, S. M., Grace, A. A., & Wrase, J. (2009). Identifying the neural circuitry of alcohol craving and relapse vulnerability. *Addict Biol, 14*(1), 108-118.

Henke, P. G. (1984). The bed nucleus of the stria terminalis and immobilization-stress: unit activity, escape behaviour, and gastric pathology in rats. *Behav Brain Res, 11*(1), 35-45.

Herman, J. P., & Cullinan, W. E. (1997). Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci, 20*(2), 78-84.

Herman, J. P., Ostrander, M. M., Mueller, N. K., & Figueiredo, H. (2005). Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog Neuropsychopharmacol Biol Psychiatry, 29*(8), 1201-1213.

Holland, P. C., & Gallagher, M. (2003). Double dissociation of the effects of lesions of basolateral and central amygdala on conditioned stimulus-potentiated feeding and Pavlovian-instrumental transfer. *Eur J Neurosci, 17*(8), 1680-1694.

Holland, P. C., Han, J. S., & Winfield, H. M. (2002). Operant and Pavlovian control of visual stimulus orienting and food-related behaviors in rats with lesions of the amygdala central nucleus. *Behav Neurosci, 116*(4), 577-587.

Holland, P. C., & Petrovich, G. D. (2005). A neural systems analysis of the potentiation of feeding by conditioned stimuli. *Physiol Behav, 86*(5), 747-761.

Hughes, A. M., Everitt, B. J., & Herbert, J. (1987). Selective effects of beta-endorphin infused into the hypothalamus, preoptic area and bed nucleus of the stria terminalis on the sexual and ingestive behaviour of male rats. *Neuroscience, 23*(3), 1063-1073.

Hyytia, P., & Koob, G. F. (1995). GABAA receptor antagonism in the extended amygdala decreases ethanol self-administration in rats. *Eur J Pharmacol, 283*(1-3), 151-159.

Johnson, A. K., de Olmos, J., Pastuskovas, C. V., Zardetto-Smith, A. M., & Vivas, L. (1999). The extended amygdala and salt appetite. *Ann N Y Acad Sci, 877*, 258-280.

Johnston, J. B. (1923). Further contribution to the study of the evolution of the forebrain. *J Comp Neurol, 35*, 337–481.

Kang, Y., & Lundy, R. F. (2009). Terminal field specificity of forebrain efferent axons to brainstem gustatory nuclei. *Brain Res, 1248*, 76-85.

Kash, T. L., Nobis, W. P., Matthews, R. T., & Winder, D. G. (2008). Dopamine enhances fast excitatory synaptic transmission in the extended amygdala by a CRF-R1-dependent process. *J Neurosci, 28*(51), 13856-13865.

Kelley, A. E., Bakshi, V. P., Haber, S. N., Steininger, T. L., Will, M. J., & Zhang, M. (2002). Opioid modulation of taste hedonics within the ventral striatum. *Physiol Behav, 76*(3), 365-377.

Kirkpatrick, B., Carter, C. S., Newman, S. W., & Insel, T. R. (1994). Axon-sparing lesions of the medial nucleus of the amygdala decrease affiliative behaviors in the prairie vole (Microtus ochrogaster): behavioral and anatomical specificity. *Behav Neurosci, 108*(3), 501-513.

Knutson, B., & Cooper, J. C. (2005). Functional magnetic resonance imaging of reward prediction. *Current Opinion in Neurology, 18*, 411-417.

Koob, G. F. (1999). The role of the striatopallidal and extended amygdala systems in drug addiction. *Ann N Y Acad Sci, 877*, 445-460.

Koob, G. F. (2003). Neuroadaptive mechanisms of addiction: studies on the extended amygdala. *Eur Neuropsychopharmacol, 13*(6), 442-452.

Koob, G. F. (2006). The neurobiology of addiction: a neuroadaptational view relevant for diagnosis. *Addiction, 101 Suppl 1*, 23-30.

Koob, G. F., & Heinrichs, S. C. (1999). A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res, 848*(1-2), 141-152.

Koob, G. F., & Le Moal, M. (2008). Addiction and the Brain Antireward System. *Annu Rev Psychol, 59*, 29-53.

Kreek, M. J., & Koob, G. F. (1998). Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend, 51*(1-2), 23-47.

Le Moal, M., & Koob, G. F. (2007). Drug addiction: pathways to the disease and pathophysiological perspectives. *Eur Neuropsychopharmacol, 17*(6-7), 377-393.

Leri, F., Flores, J., Rodaros, D., & Stewart, J. (2002). Blockade of stress-induced but not cocaine-induced reinstatement by infusion of noradrenergic antagonists into the bed nucleus of the stria terminalis or the central nucleus of the amygdala. *J Neurosci, 22*(13), 5713-5718.

Li, B. H., Xu, B., Rowland, N. E., & Kalra, S. P. (1994). c-fos expression in the rat brain following central administration of neuropeptide Y and effects of food consumption. *Brain Res, 665*(2), 277-284.

Li, C. S., & Cho, Y. K. (2006). Efferent projection from the bed nucleus of the stria terminalis suppresses activity of taste-responsive neurons in the hamster parabrachial nuclei. *Am J Physiol Regul Integr Comp Physiol, 291*(4), R914-926.

Lundy, R. F., Jr. (2008). Gustatory hedonic value: potential function for forebrain control of brainstem taste processing. *Neurosci Biobehav Rev, 32*(8), 1601-1606.
Lutter, M., & Nestler, E. J. (2009). Homeostatic and hedonic signals interact in the regulation of food intake. *J Nutr, 139*(3), 629-632.
Mahler, S. V., & Berridge, K. C. (2006). *Amgydala opioids amplify 'wanting' but not 'liking' of a sucrose reward*. Paper presented at the Society for Neuroscience.
Mahler, S. V., & Berridge, K. C. (2007). *Fos plume mapping of amygdala opioid 'wanting'*. Paper presented at the Society for Neuroscience, San Diego.
Mahler, S. V., & Berridge, K. C. (2009). Which cue to "want?" Central amygdala opioid activation enhances and focuses incentive salience on a prepotent reward cue. *J Neurosci, 29*(20), 6500-6513.
Mahler, S. V., & Berridge, K. C. (In press). Which cue to 'want?' Central amygdala opioid activation enhances and focuses incentive salience on a prepotent reward cue. *J Neurosci*.
Mahler, S. V., Smith, K. S., & Berridge, K. C. (2007). Endocannabinoid Hedonic Hotspot for Sensory Pleasure: Anandamide in Nucleus Accumbens Shell Enhances 'Liking' of a Sweet Reward. *Neuropsychopharmacology*.
Mansour, A., Fox, C. A., Akil, H., & Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci, 18*(1), 22-29.
Markou, A., Kosten, T. R., & Koob, G. F. (1998). Neurobiological similarities in depression and drug dependence: a self-medication hypothesis. *Neuropsychopharmacology, 18*(3), 135-174.
Marlatt, G. A. (1990). Cue exposure and relapse prevention in the treatment of addictive behaviors. *Addict Behav, 15*(4), 395-399.
Massi, L., Elezgarai, I., Puente, N., Reguero, L., Grandes, P., Manzoni, O. J., et al. (2008). Cannabinoid receptors in the bed nucleus of the stria terminalis control cortical excitation of midbrain dopamine cells in vivo. *J Neurosci, 28*(42), 10496-10508.
McElligott, Z. A., & Winder, D. G. (2009). Modulation of glutamatergic synaptic transmission in the bed nucleus of the stria terminalis. *Prog Neuropsychopharmacol Biol Psychiatry*.
Misslin, R. (2003). The defense system of fear: behavior and neurocircuitry. *Neurophysiol Clin, 33*(2), 55-66.
Mullett, M. A., Billington, C. J., Levine, A. S., & Kotz, C. M. (2000). Hypocretin I in the lateral hypothalamus activates key feeding-regulatory brain sites. *Neuroreport, 11*(1), 103-108.
Mungarndee, S. S., Lundy, R. F., Jr., & Norgren, R. (2008). Expression of Fos during sham sucrose intake in rats with central gustatory lesions. *Am J Physiol Regul Integr Comp Physiol, 295*(3), R751-763.
Na, S. (2008). The extended amygdala modulates food intake and other behaviors. University of Michigan.
Naleid, A. M., Grace, M. K., Chimukangara, M., Billington, C. J., & Levine, A. S. (2007). Paraventricular opioids alter intake of high-fat but not high-sucrose diet depending on diet preference in a binge model of feeding. *Am J Physiol Regul Integr Comp Physiol, 293*(1), R99-105.

Nesse, R. M., & Berridge, K. C. (1997). Psychoactive drug use in evolutionary perspective. *Science, 278*(5335), 63-66.
Newman, S. W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. *Ann N Y Acad Sci, 877*, 242-257.
Norgren, R. (1976). Taste pathways to hypothalamus and amygdala. *J Comp Neurol, 166*(1), 17-30.
O'Sullivan, S. S., Evans, A. H., & Lees, A. J. (2009). Dopamine dysregulation syndrome: an overview of its epidemiology, mechanisms and management. *CNS Drugs, 23*(2), 157-170.
Olive, M. F., Koenig, H. N., Nannini, M. A., & Hodge, C. W. (2002). Elevated extracellular CRF levels in the bed nucleus of the stria terminalis during ethanol withdrawal and reduction by subsequent ethanol intake. *Pharmacol Biochem Behav, 72*(1-2), 213-220.
Owings, D. H., & Coss, R. G. (1977). Snake Mobbing by California Ground Squirrels - Adaptive Variation and Ontogeny. *Behaviour, 62*, 50-69.
Pacak, K., McCarty, R., Palkovits, M., Kopin, I. J., & Goldstein, D. S. (1995). Effects of immobilization on in vivo release of norepinephrine in the bed nucleus of the stria terminalis in conscious rats. *Brain Res, 688*(1-2), 242-246.
Park, T. H., & Carr, K. D. (1998). Neuroanatomical patterns of fos-like immunoreactivity induced by a palatable meal and meal-paired environment in saline- and naltrexone-treated rats. *Brain Res, 805*(1-2), 169-180.
Parkinson, J. A., Olmstead, M. C., Burns, L. H., Robbins, T. W., & Everitt, B. J. (1999). Dissociation in effects of lesions of the nucleus accumbens core and shell on appetitive pavlovian approach behavior and the potentiation of conditioned reinforcement and locomotor activity by D-amphetamine. *J Neurosci, 19*(6), 2401-2411.
Parkinson, J. A., Willoughby, P. J., Robbins, T. W., & Everitt, B. J. (2000). Disconnection of the anterior cingulate cortex and nucleus accumbens core impairs Pavlovian approach behavior: further evidence for limbic cortical-ventral striatopallidal systems. *Behav Neurosci, 114*(1), 42-63.
Paxinos, G., & Watson, C. (2007). *The Rat Brain in Stereotaxic Coordinates* (7th ed.). Amsterdam: Elsevier.
Pecina, S., & Berridge, K. C. (2000). Opioid site in nucleus accumbens shell mediates eating and hedonic 'liking' for food: map based on microinjection Fos plumes. *Brain Res, 863*(1-2), 71-86.
Pecina, S., & Berridge, K. C. (2005). Hedonic hot spot in nucleus accumbens shell: where do mu-opioids cause increased hedonic impact of sweetness? *J Neurosci, 25*(50), 11777-11786.
Pecina, S., & Berridge, K. C. (In Preparation). *Increased cue-triggered 'wanting' by opioid microinjections into the nucleus accumbens core and shell: a novel psychological mechanism of action.*Unpublished manuscript.
Pecina, S., Schulkin, J., & Berridge, K. C. (2006). Nucleus accumbens corticotropin-releasing factor increases cue-triggered motivation for sucrose reward: paradoxical positive incentive effects in stress? *BMC Biol, 4*, 8.
Pecina, S., Smith, K. S., & Berridge, K. C. (2006). Hedonic hot spots in the brain. *Neuroscientist, 12*(6), 500-511.

Petrovic, P., Pleger, B., Seymour, B., Kloppel, S., De Martino, B., Critchley, H., et al. (2008). Blocking central opiate function modulates hedonic impact and anterior cingulate response to rewards and losses. *J Neurosci, 28*(42), 10509-10516.

Pfaus, J. G., Kippin, T. E., & Centeno, S. (2001). Conditioning and sexual behavior: a review. *Horm Behav, 40*(2), 291-321.

Phillips, G. D., Robbins, T. W., & Everitt, B. J. (1994). Mesoaccumbens dopamine-opiate interactions in the control over behaviour by a conditioned reinforcer. *Psychopharmacology (Berl), 114*(2), 345-359.

Phillips, G. D., Setzu, E., & Hitchcott, P. K. (2003). Facilitation of appetitive pavlovian conditioning by d-amphetamine in the shell, but not the core, of the nucleus accumbens. *Behav Neurosci, 117*(4), 675-684.

Ranaldi, R., Egan, J., Kest, K., Fein, M., & Delamater, A. R. (2009). Repeated heroin in rats produces locomotor sensitization and enhances appetitive Pavlovian and instrumental learning involving food reward. *Pharmacol Biochem Behav, 91*(3), 351-357.

Redish, A. D. (2004). Addiction as a computational process gone awry. *Science, 306*(5703), 1944-1947.

Rescorla, R. A., & Solomon, R. L. (1967). Two-process learning theory: Relationships between Pavlovian conditioning and instrumental learning. *Psychol Rev, 74*(3), 151-182.

Reynolds, S. M., & Berridge, K. C. (2001). Fear and feeding in the nucleus accumbens shell: rostrocaudal segregation of GABA-elicited defensive behavior versus eating behavior. *J Neurosci, 21*(9), 3261-3270.

Reynolds, S. M., & Berridge, K. C. (2002). Positive and negative motivation in nucleus accumbens shell: bivalent rostrocaudal gradients for GABA-elicited eating, taste "liking"/"disliking" reactions, place preference/avoidance, and fear. *J Neurosci, 22*(16), 7308-7320.

Reynolds, S. M., & Berridge, K. C. (2003). Glutamate motivational ensembles in nucleus accumbens: rostrocaudal shell gradients of fear and feeding. *Eur J Neurosci, 17*(10), 2187-2200.

Reynolds, S. M., & Berridge, K. C. (2008). Emotional environments retune the valence of appetitive versus fearful functions in nucleus accumbens. *Nat Neurosci, 11*(4), 423-425.

Rezayof, A., Golhasani-Keshtan, F., Haeri-Rohani, A., & Zarrindast, M. R. (2007). Morphine-induced place preference: involvement of the central amygdala NMDA receptors. *Brain Res, 1133*(1), 34-41.

Robinson, T. E., & Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev, 18*(3), 247-291.

Robinson, T. E., & Berridge, K. C. (2000). The psychology and neurobiology of addiction: an incentive-sensitization view. *Addiction, 95 Suppl 2*, S91-117.

Robinson, T. E., & Flagel, S. B. (2008). Dissociating the Predictive and Incentive Motivational Properties of Reward-Related Cues Through the Study of Individual Differences. *Biol Psychiatry*.

Rodaros, D., Caruana, D. A., Amir, S., & Stewart, J. (2007). Corticotropin-releasing factor projections from limbic forebrain and paraventricular nucleus of the

hypothalamus to the region of the ventral tegmental area. *Neuroscience, 150*(1), 8-13.
Rolls, E. T. (2006). Brain mechanisms underlying flavour and appetite. *Philos Trans R Soc Lond B Biol Sci, 361*(1471), 1123-1136.
Roman, C., Nebieridze, N., Sastre, A., & Reilly, S. (2006). Effects of lesions of the bed nucleus of the stria terminalis, lateral hypothalamus, or insular cortex on conditioned taste aversion and conditioned odor aversion. *Behav Neurosci, 120*(6), 1257-1267.
Rosin, D. L., Robeva, A., Woodard, R. L., Guyenet, P. G., & Linden, J. (1998). Immunohistochemical localization of adenosine A2A receptors in the rat central nervous system. *J Comp Neurol, 401*(2), 163-186.
Rosse, R. B., Fay-McCarthy, M., Collins, J. P., Jr., Risher-Flowers, D., Alim, T. N., & Deutsch, S. I. (1993). Transient compulsive foraging behavior associated with crack cocaine use. *Am J Psychiatry, 150*(1), 155-156.
Rutters, F., Nieuwenhuizen, A. G., Lemmens, S. G., Born, J. M., & Westerterp-Plantenga, M. S. (2009). Acute stress-related changes in eating in the absence of hunger. *Obesity (Silver Spring), 17*(1), 72-77.
Salamone, J. D. (2007). Functions of mesolimbic dopamine: changing concepts and shifting paradigms. *Psychopharmacology (Berl), 191*(3), 389.
Salamone, J. D., Correa, M., Farrar, A., & Mingote, S. M. (2007). Effort-related functions of nucleus accumbens dopamine and associated forebrain circuits. *Psychopharmacology (Berl), 191*(3), 461-482.
Schultz, W., Dayan, P., & Montague, P. R. (1997). A neural substrate of prediction and reward. *Science, 275*(5306), 1593-1599.
See, R. E. (2002). Neural substrates of conditioned-cued relapse to drug-seeking behavior. *Pharmacol Biochem Behav, 71*(3), 517-529.
See, R. E. (2005). Neural substrates of cocaine-cue associations that trigger relapse. *Eur J Pharmacol, 526*(1-3), 140-146.
Shaham, Y., Rodaros, D., & Stewart, J. (1994). Reinstatement of heroin-reinforced behavior following long-term extinction: implications for the treatment of relapse to drug taking. *Behav Pharmacol, 5*(3), 360-364.
Smith, D. V., Ye, M. K., & Li, C. S. (2005). Medullary taste responses are modulated by the bed nucleus of the stria terminalis. *Chem Senses, 30*(5), 421-434.
Smith, K. S., & Berridge, K. C. (2005). The ventral pallidum and hedonic reward: neurochemical maps of sucrose "liking" and food intake. *J Neurosci, 25*(38), 8637-8649.
Smith, K. S., & Berridge, K. C. (2007). Opioid limbic circuit for reward: interaction between hedonic hotspots of nucleus accumbens and ventral pallidum. *J Neurosci, 27*(7), 1594-1605.
Smith, K. S., Tindell, A. J., Aldridge, J. W., & Berridge, K. C. (2009). Ventral pallidum roles in reward and motivation. *Behav Brain Res, 196*(2), 155-167.
Smith, R. J., & Aston-Jones, G. (2008). Noradrenergic transmission in the extended amygdala: role in increased drug-seeking and relapse during protracted drug abstinence. *Brain Struct Funct, 213*(1-2), 43-61.
Spector, A. C., & Travers, S. P. (2005). The representation of taste quality in the mammalian nervous system. *Behav Cogn Neurosci Rev, 4*(3), 143-191.

Spyraki, C., Kazandjian, A., & Varonos, D. (1985). Diazepam-induced place preference conditioning: appetitive and antiaversive properties. *Psychopharmacology (Berl), 87*(2), 225-232.
Stewart, J. (2000). Pathways to relapse: the neurobiology of drug- and stress-induced relapse to drug-taking. *J Psychiatry Neurosci, 25*(2), 125-136.
Stratford, T. R. (2005). Activation of feeding-related neural circuitry after unilateral injections of muscimol into the nucleus accumbens shell. *Brain Res, 1048*(1-2), 241-250.
Sullivan, G. M., Apergis, J., Bush, D. E., Johnson, L. R., Hou, M., & Ledoux, J. E. (2004). Lesions in the bed nucleus of the stria terminalis disrupt corticosterone and freezing responses elicited by a contextual but not by a specific cue-conditioned fear stimulus. *Neuroscience, 128*(1), 7-14.
Swanson, L. W. (2000). Cerebral hemisphere regulation of motivated behavior. *Brain Res, 886*(1-2), 113-164.
Swanson, L. W. (2003). The amygdala and its place in the cerebral hemisphere. *Ann N Y Acad Sci, 985*, 174-184.
Swanson, L. W. (2005). Anatomy of the soul as reflected in the cerebral hemispheres: neural circuits underlying voluntary control of basic motivated behaviors. *J Comp Neurol, 493*(1), 122-131.
Taziaux, M., Kahn, A., Moore, J., 3rd, Balthazart, J., & Holloway, K. S. (2008). Enhanced neural activation in brain regions mediating sexual responses following exposure to a conditioned stimulus that predicts copulation. *Neuroscience, 151*(3), 644-658.
Thorpe, A. J., & Kotz, C. M. (2005). Orexin A in the nucleus accumbens stimulates feeding and locomotor activity. *Brain Res, 1050*(1-2), 156-162.
Tindell, A. J., Berridge, K. C., Zhang, J., Pecina, S., & Aldridge, J. W. (2005). Ventral pallidal neurons code incentive motivation: amplification by mesolimbic sensitization and amphetamine. *Eur J Neurosci, 22*(10), 2617-2634.
Toates, F. (1986). *Motivational systems*. Cambridge, UK: Cambridge University Press.
Tokita, K., Inoue, T., & Boughter, J. D., Jr. (2009). Afferent connections of the parabrachial nucleus in C57BL/6J mice. *Neuroscience, 161*(2), 475-488.
Tomie, A., Grimes, K. L., & Pohorecky, L. A. (2008). Behavioral characteristics and neurobiological substrates shared by Pavlovian sign-tracking and drug abuse. *Brain Res Rev, 58*(1), 121-135.
Torres, S. J., & Nowson, C. A. (2007). Relationship between stress, eating behavior, and obesity. *Nutrition, 23*(11-12), 887-894.
Treit, D., Aujla, H., & Menard, J. (1998). Does the bed nucleus of the stria terminalis mediate fear behaviors? *Behav Neurosci, 112*(2), 379-386.
Treit, D., Pinel, J. P., & Fibiger, H. C. (1981). Conditioned defensive burying: a new paradigm for the study of anxiolytic agents. *Pharmacol Biochem Behav, 15*(4), 619-626.
Uchiumi, K., Aoki, M., Kikusui, T., Takeuchi, Y., & Mori, Y. (2008). Wheel-running activity increases with social stress in male DBA mice. *Physiol Behav, 93*(1-2), 1-7.
Uslaner, J. M., Dell'Orco, J. M., Pevzner, A., & Robinson, T. E. (2008). The influence of subthalamic nucleus lesions on sign-tracking to stimuli paired with food and drug

rewards: facilitation of incentive salience attribution? *Neuropsychopharmacology, 33*(10), 2352-2361.
Van Bockstaele, E. J., Qian, Y., Sterling, R. C., & Page, M. E. (2008). Low dose naltrexone administration in morphine dependent rats attenuates withdrawal-induced norepinephrine efflux in forebrain. *Prog Neuropsychopharmacol Biol Psychiatry, 32*(4), 1048-1056.
Volkow, N. D., Wang, G. J., Fowler, J. S., & Telang, F. (2008). Overlapping neuronal circuits in addiction and obesity: evidence of systems pathology. *Philos Trans R Soc Lond B Biol Sci, 363*(1507), 3191-3200.
Waddell, S. (2005). Courtship learning: scent of a woman. *Curr Biol, 15*(3), R88-90.
Walker, D. L., & Davis, M. (1997). Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. *J Neurosci, 17*(23), 9375-9383.
Walker, D. L., Toufexis, D. J., & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *Eur J Pharmacol, 463*(1-3), 199-216.
Walker, J. R., Ahmed, S. H., Gracy, K. N., & Koob, G. F. (2000). Microinjections of an opiate receptor antagonist into the bed nucleus of the stria terminalis suppress heroin self-administration in dependent rats. *Brain Res, 854*(1-2), 85-92.
Wang, G. J., Volkow, N. D., Thanos, P. K., & Fowler, J. S. (2004). Similarity between obesity and drug addiction as assessed by neurofunctional imaging: a concept review. *J Addict Dis, 23*(3), 39-53.
Waraczynski, M. A. (2006). The central extended amygdala network as a proposed circuit underlying reward valuation. *Neurosci Biobehav Rev, 30*(4), 472-496.
Warne, J. P. (2009). Shaping the stress response: interplay of palatable food choices, glucocorticoids, insulin and abdominal obesity. *Mol Cell Endocrinol, 300*(1-2), 137-146.
Whitehead, M. C., Bergula, A., & Holliday, K. (2000). Forebrain projections to the rostral nucleus of the solitary tract in the hamster. *J Comp Neurol, 422*(3), 429-447.
Wise, R. A., & Bozarth, M. A. (1985). Brain mechanisms of drug reward and euphoria. *Psychiatr Med, 3*(4), 445-460.
Wise, R. A., & Rompre, P. P. (1989). Brain dopamine and reward. *Annu Rev Psychol, 40*, 191-225.
Wyvell, C. L., & Berridge, K. C. (2000). Intra-accumbens amphetamine increases the conditioned incentive salience of sucrose reward: enhancement of reward "wanting" without enhanced "liking" or response reinforcement. *J Neurosci, 20*(21), 8122-8130.
Wyvell, C. L., & Berridge, K. C. (2001). Incentive sensitization by previous amphetamine exposure: increased cue-triggered "wanting" for sucrose reward. *J Neurosci, 21*(19), 7831-7840.
Yacubian, J., & Buchel, C. (2009). The genetic basis of individual differences in reward processing and the link to addictive behavior and social cognition. *Neuroscience*.
Yamamoto, T., & Fujimoto, Y. (1991). Brain mechanisms of taste aversion learning in the rat. *Brain Res Bull, 27*(3-4), 403-406.

Zahm, D. S. (1998). Is the caudomedial shell of the nucleus accumbens part of the extended amygdala? A consideration of connections. *Crit Rev Neurobiol, 12*(3), 245-265.

Zhang, M., Balmadrid, C., & Kelley, A. E. (2003). Nucleus accumbens opioid, GABaergic, and dopaminergic modulation of palatable food motivation: contrasting effects revealed by a progressive ratio study in the rat. *Behav Neurosci, 117*(2), 202-211.

Zhang, M., & Kelley, A. E. (2000). Enhanced intake of high-fat food following striatal mu-opioid stimulation: microinjection mapping and fos expression. *Neuroscience, 99*(2), 267-277.

Zheng, H., & Berthoud, H. R. (2007). Eating for pleasure or calories. *Curr Opin Pharmacol, 7*(6), 607-612.

Zheng, H., Patterson, L. M., & Berthoud, H. R. (2007). Orexin signaling in the ventral tegmental area is required for high-fat appetite induced by opioid stimulation of the nucleus accumbens. *J Neurosci, 27*(41), 11075-11082.