Role of Central Amygdala Opioids in Incentive Motivation: Translating Learning into Focused and Amplified 'Wanting'

by

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To Vincent and Mary Mahler

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Abstract

Wanting a reward like food is different from liking it, and the brain processes these sensations separately. Components of the limbic system mediate both, and here I examined how different neurochemical signals (opioids and cannabinoids) in particular limbic structures [central amygdala (CeA), basolateral amygdala (BLA), and nucleus accumbens (NAc)] process Pavlovian 'wanting,' and hedonic 'liking.'

Experiment 1 examined how CeA activation targets incentive motivational 'wanting' upon particular cues (e.g., an extending lever that signals sucrose reward delivery), to make them stronger 'motivational magnets.' Microinjecting the drug DAMGO to stimulate μ opioid receptors in CeA of rats enhanced approach and food consummatory behaviors directed at cues.

Experiment 2 examined how amygdala mediates cue-induced motivation to obtain rewards themselves (cue-triggered reward 'wanting'). Opioid activation of CeA, but not BLA enhanced such cue-triggered motivation, integrating Pavlovian and instrumental learning to create sharper bursts of 'wanting' for the predicted reward.

'Wanting' is distinguishable from 'liking,' so Experiment 3 used taste reactivity procedures to examine if amygdala opioid stimulation also altered the hedonic impact of food when it is consumed. Strikingly, opioid stimulation *reduced* hedonic 'liking' reactions to sweetness, even when it enhanced food 'wanting.' This points to a specialized and unique role for amygdala opioids in enhancing reward 'wanting,' in a manner separate from 'liking' of the same reward.

Experiment 3 raised the question of what brain substrates *do* enhance reward 'liking' (and made it important for me to show that I can identify 'liking' enhancement when it does occur using the present methodologies). The nucleus accumbens shell is known to process food 'liking,' and so in experiment 4, I found that microinjection of the endocannabinoid anandamide within the dorsomedial shell enhances hedonic 'liking.'

This is the first demonstration of a brain endocannabinoid 'hotspot' for stimulation of sensory pleasure.

Altogether, these experiments demonstrated that amygdala opioids play a specialized role in amplifying and focusing 'wanting' based on prior learning, while nucleus accumbens endocannabinoids amplify hedonic 'liking.' These findings expand our knowledge of how the limbic system produces distinct components of reward and motivation, and carries implications for understanding appetitive disorders including addiction and obesity.

Chapter 1
Introduction

Studying Psychological Components of Reward

'Reward' is not a simple concept in neuroscience research, yet it is often used to simply describe the hedonic affect produced by a reward (Ikemoto and Wise, 2004; Koob and Le Moal, 2008). However, in order for animals to actually recognize and efficiently pursue these pleasurable and usually evolutionarily advantageous rewards, they must also learn which rewards in the environment are best, and how to get them. Natural selection solved these problems by evolving at least three separate brain systems, each specialized to perform a specific function in reward pursuit and learning. Some unconditioned stimuli (UCSs) are 'liked,' or hedonically pleasurable. When these UCSs are encountered in the future, they will be 'wanted,' and animals will pursue them. If particular environmental stimuli are reliably associated with a 'liked' UCS, animals will pair them using stimulus-stimulus (S-S) associations, and they too will come to be 'wanted' conditioned stimuli (CSs). Although these three processes normally work together seamlessly, and animals learn to 'want' what they 'like,' they can in some cases become separated. One such instance may be drug addiction, in which mesolimbic dopamine systems are sensitized by addictive drugs, which may result in elevated 'wanting,' but not 'liking' or learning (Robinson and Berridge, 1993; Berridge, 2007; Robinson and Berridge, 2008). Neuroscientists have begun to parse the differing neural substrates of 'wanting,' 'liking,' and learning, and found that they rely upon overlapping, but anatomically and neurochemically distinct, pathways (Berridge and Robinson, 2003).

Measuring Reward 'Wanting'

'Wanting' refers to a motivational state that provides the psychological "fuel" for appetitive behaviors in pursuit of a reward. This 'wanting' state can be triggered by Pavlovian CSs that have previously been associated with rewards, and thereby attain some of properties of the associated reward. One of these properties has been called incentive salience, or a quality of noticeability and attractiveness (Bolles, 1972; Robinson and Berridge, 1993; Berridge, 2007). CSs with incentive salience are attractive in the same way as the rewards associated with them, and draw in similar approach and even attempted consummatory behavior (Bindra, 1978; Toates, 1986; Berridge, 2007; Flagel et al., 2007; 2008b). In natural environments, reward cues such as smell or visual appearance would often be co-localized with rewards themselves, and therefore ascribing incentive salience to reward cues is a useful evolutionary strategy to promote the attainment of fitness-enhancing rewards.

Autoshaping: Measuring the Motivational Magnet Properties of Reward Cues

In the laboratory, we can experimentally remove reward cues from physical coincidence with rewards themselves, and thereby examine how animals target incentive salience onto either reward-predictive or reward-adjacent stimuli. Autoshaping, which is also called sign tracking, is one paradigm used to explore these issues (Hearst and Jenkins, 1974; Boakes, 1977). In this paradigm, animals are trained to associate the presentation of a particular physical stimulus, usually one that is localized and physically discrete, with a reward delivered in another area of the testing environment. Upon learning this association, many animals will come to approach and interact with the reward predictive CS or the site of reward delivery, as if they have become 'motivational magnets' through association with reward (Berridge, 2001). Interestingly, the phenotype of behaviors directed at these reward cues differs based on the associated reward. For example, pigeons display unique 'eating pecks' and 'drinking pecks' during consumption of grain or water. When a cue (extension of a key into the testing chamber) is associated with food or water rewards, pigeons peck it, but only using the type of peck appropriate to the associated reward (Brown and Jenkins, 1968). Similarly, rats will nibble, sniff, and bite discrete food-associated cues (Boakes, 1977; Davey and Cleland, 1982; Flagel et al., 2007; 2008a; Tomie et al., 2008), but only approach and investigate cues predicting rewards that have no associated consummatory behaviors such as rewarding brain

stimulation and intravenous cocaine infusions (Peterson et al., 1972; Uslaner et al., 2006). Similarly, quails and pigeons will attempt to copulate with cloth stimuli predicting sexual access to female birds (Burns and Domjan, 1996; Koksal et al., 2004). In human crack cocaine addicts, CS motivational magnets may become excessively strong, and cause compulsive searching and examination of small items resembling crack cocaine, even when they know the objects are almost certainly merely pebbles or detritus (Rosse et al., 1993; Berridge, 2007).

One way to conceptualize these seemingly bizarre reward-consummatory behaviors directed at non-reward stimuli is the concept of incentive salience. The incentive salience hypothesis (Berridge and Valenstein, 1991; Robinson and Berridge, 1993), describes a psychological process by which reward predictive stimuli come to attain incentive properties of the rewards they are associated with—eliciting approach, exploration, and even attempts at consumption (Bolles, 1972; Bindra, 1978; Toates, 1986; Robinson and Berridge, 1993; Berridge, 2001; Flagel et al., 2008a). Through S-S Pavlovian association, reward cues come to elicit similar appetitive motivational states that are otherwise elicited by UCS rewards themselves. When such cues are encountered, therefore, animals are likely to notice them and be attracted to them—they attain incentive salience. Not only are animals drawn into these cues, but they often even attempt to consume them as if they actually *were* the associated reward. In this way, the nature of the UCS determines the type of CS-directed consummatory behavior, but CS features are also important in determining behavioral responses to cues. For example, food CSs that are discrete and roughly food-sized are 'eaten,' while diffuse auditory food CSs are merely approached and oriented toward (Wasserman, 1973; Lajoie and Bindra, 1976; Holland, 1980; Tomie et al., 2008). Similarly, rats will groom and sniff a conspecific that predicts food, but will not display these social behaviors toward a similarly food-predictive block of wood (Timberlake and Grant, 1975).

One interesting feature of typical autoshaping paradigms is that while only the Pavlovian CS+ (such as a metal lever extending into a chamber) is predictive of reward (such as a sucrose pellet), the reward delivery area (such as a metal dish) can also be targeted with incentive salience, eliciting approach and consummatory interactions. Individual animals seem to vary in which of these two stimuli they target incentive

salience upon, and after a few days of training will predominantly approach, nibble, sniff and bite either the sucrose predictive CS+ lever, or the dish in which sucrose is delivered (here, the CS$_{source}$)(Zener, 1934). Rats that preferentially approach and interact with these stimuli are often called 'sign trackers' and 'goal trackers,' respectively (Boakes, 1977; Flagel et al., 2007; 2008a; 2008b).

Comparing these stimuli, the CS+ lever is associated in a predictive sense—when it occurs, a reward will follow. The CS$_{source}$ dish is instead associated physically and temporally with the reward that must be consumed there, but is not predictive of reward delivery itself. The exact nature of the differences between animals that attribute incentive salience to the reward predictive CS+ and the reward adjacent CS$_{source}$ is unknown, but Shelly Flagel, Terry Robinson, and colleagues have reported several interesting differences between these populations. For instance, sign trackers have higher accumbens D$_1$ mRNA than goal trackers after a single autoshaping session, while further training results in higher tyrosine hydroxylase, dopamine transporter, and dopamine D$_2$ receptor mRNA levels in goal trackers (Flagel et al., 2007). Responses to cocaine injections are also different between the groups, with goal trackers showing greater locomotor responses to acute cocaine, while sign trackers have greater propensity to sensitize upon repeated doses (Flagel et al., 2008b). These results suggest that pre-existing individual differences exist in the dopamine systems of rats that preferentially target incentive salience upon one prepotent reward-associated stimulus—the CS+ or the CS$_{source}$. Furthermore, autoshaping may be a useful tool for examining brain systems that target incentive salience upon certain reward-associated stimuli, at the expense of others.

Measuring UCS 'Wanting:' Food Intake

Although autoshaping is a useful paradigm for examining the targeting of cue-directed incentive salience, Pavlovian 'wanting' may also be targeted upon cue-triggered representations of reward themselves. The most straightforward way to measure UCS reward-directed incentive motivational 'wanting' is to examine intake of a reward. For this reason, neuroscientists sometimes examine food intake effects of brain manipulations as a rough estimate of reward 'wanting' (Peciña and Berridge, 2005; Smith and Berridge, 2005; Finlayson et al., 2008). Food intake is certainly susceptible to control by Pavlovian cues (Holland and Petrovich, 2005), including presumably those olfactory, visual, and

texture cues inherent in food itself. This said, other non-Pavlovian processes also affect food intake, so a more precise measure of cue-driven 'wanting' directed at rewards themselves is often desirable for measuring Pavlovian incentive motivation in particular.

Measuring Cue-Triggered UCS 'Wanting:' Pavlovian to Instrumental Transfer

When a Pavlovian reward cue is experienced, a psychological representation of the associated reward is activated in the brain (Bolles, 1972; Holland and Rescorla, 1975; Bindra, 1978). This representation is ascribed with a certain level of incentive salience, based on several factors including the current value of the reward to the animal, the correlation of the cue with the reward, and the animal's current physiological state (Zener, 1934; Timberlake and Grant, 1975; Boakes, 1977; Dickinson and Mackintosh, 1978; Toates, 1986). In animals, such cue-triggered, but reward-targeted incentive motivation can be measured with a paradigm called Pavlovian to Instrumental Transfer, or PIT (Walker, 1942; Estes, 1943; Rescorla and Solomon, 1967; Hall et al., 2001; Holland and Gallagher, 2003; Corbit and Balleine, 2005). PIT measures this cue-triggered, but UCS-focused 'wanting' by examining instrumental reward seeking that is elicited by Pavlovian CS for the same rewards. That is, instead of cues triggering motivated approach and interaction with themselves, they are also capable of triggering 'wanting' that is directed toward a reward representation, and expressed as instrumental seeking of the same reward. Crucially, animals never actually receive rewards during PIT testing, eliminating potential effects of hedonic or reinforcement processes in producing reward seeking behavior. In addition, while increased lever pressing during CSs demonstrates increased 'wanting,' this behavior competes with the previously learned stimulus-response (S-R) habit of entering the sucrose delivery cup during CSs. Therefore, effects of brain manipulations on incentive motivational (S-S) vs. habitual (S-R) behavior can be also parsed with PIT.

In drug addiction, this type of cue-triggered, UCS-directed motivation may put addicts at risk for relapse when drugs are not immediately available, as would be the case for many attempting to quit. For example, an alcoholic who passes a bar he formerly frequented might experience a strong craving for alcohol triggered by Pavlovian cues of the bar name, neon signs, etc. If he doesn't have money in his pocket, simply being drawn into the cues themselves will not be enough to yield a drink from the bartender

(unless perhaps the bartender knows he's about to relapse, and is a smart businessman). The potential relapser will also need money, and to get this he must perform some type of instrumental action—ranging from finding an ATM to robbing a convenience store.

Synopsis of 'Wanting' Measures

In sum, the brain substrates of incentive motivation can be usefully measured using behavioral paradigms aimed at revealing three main characteristics of reward 'wanting.' Brain manipulations that affect incentive salience 'wanting' would be expected to A) affect consumption of UCS rewards themselves, B) affect the propensity of Pavlovian reward-associated stimuli to pull in motivated and consummatory-like behaviors like a 'motivational magnet,' and C) affect the ability of Pavlovian cues to elicit motivational 'wanting' states that can be directed into particular reward seeking behaviors.

Measuring Reward 'Liking:' Taste Reactivity

'Liking' refers to the hedonic impact of a reward itself once it is received. "Liking' is an affective evaluation of a reward, which is dissociable from both 'wanting' and the sensory aspects of reward receipt. For ingestive taste reward, particular tastes have both sensory properties (such as sweetness) and an affective value reflecting their hedonic evaluation (what Frijda (2006) calls a 'pleasure gloss'). Sensory and hedonic properties of tastes are often dissociated, as tastes remain relatively constant in their sensory properties, but often vary in their hedonic value. A classic example of this is a phenomenon called alliesthesia, coined by Cabanac (1971) to describe the phenomenon that the same taste, smell, or thermal stimulus can be perceived as pleasant or unpleasant, depending upon physiological conditions at the time. For example, a taste of cake may be very pleasant on an empty stomach, but the very same cake may be much less so after eating two slices.

The ways in which the brain ascribes hedonic value to rewards is poorly understood, and cannot be easily studied in humans. Fortunately, a method exists for measuring hedonic evaluations of tastes in animals: taste reactivity (Grill and Norgren, 1978; Berridge, 2000). Humans, other primates, and rats all evolved to eat similar foods—with strong preferences for foods high in sugar and fat, and aversions to bitter

tastes that are often associated with poisons. Similar behavioral responses to such pleasant and unpleasant tastes are shown in these species, with rhythmic tongue protrusions and licking to sweet and other pleasant tastes, and gaping, head shaking, and other reactions to bitter and other unpleasant tastes. These characteristic reactions were evolutionarily conserved across this wide range of mammals in phenotypic appearance and timing, with body size being strongly negatively correlated with orofacial reaction speed (Berridge, 2000; Steiner et al., 2001). These reactions respond predictably to alliesthesia (Berridge, 1991; Grill et al., 1996), and a classic demonstration of this is shown using a salt appetite paradigm (Berridge et al., 1984). When rats are infused intraorally with a very salty solution, they respond primarily with aversive reactions such as gapes. However, if they are then pharmacologically deprived of body salt and tested again, their reactions flip from aversive gapes to hedonic tongue protrusions. This indicates that the very same taste can have opposite hedonic valence depending upon physiological state. More recently, Tindell et al (2006) showed that both hedonic valence and its electrophysiological representation in ventral pallidum reflect the aversive-to-hedonic flip in valence of salt solution after salt deprivation. These findings strongly suggest that taste reactivity measures affective hedonic 'liking,' rather than sensory or other properties the taste experience.

Reward Learning: Informing What is 'Wanted' With What Was 'Liked'

Learning is a third psychological process that influences reward, and plays a crucial role in guiding animals' future behavior based on past experiences. In fact, the necessity for animals to learn is likely the basis for the evolution of both incentive salience 'wanting' and hedonic 'liking' mechanisms. If animals were not able to learn from experience, an ability to evaluate rewards as 'liked' or 'disliked' would have little use, as this experience would not be used in future encounters with the stimulus. Similarly, the ability to target motivational salience at CSs associated with rewards would be useless without the ability to feed learned information about reward value into 'wanting' mechanisms.

Learning is the crucial mediator between 'liking' and 'wanting,' in that rewards that are 'liked' (and the cues that predict them) are ordinarily thereafter 'wanted.'

Similarly, when stimuli that are 'disliked' are encountered, experience of this encounter can be incorporated in the decision to avoid the stimulus and its predictors in future. Although appetitive learning is highly integrated with 'wanting' and 'liking,' the brain substrates of each of these processes are dissociable under certain circumstances. For example, drug-induced sensitization of dopamine projections from midbrain to nucleus accumbens likely potentiates only reward 'wanting,' rather than learning or 'liking' (Robinson and Berridge, 1993; Wyvell and Berridge, 2001; Robinson et al., 2005; Cagniard et al., 2006; Berridge, 2007).

Like 'reward,' 'learning' is a term referring to a complex amalgam of brain processes and circuits that underlie various ways in which the brain incorporates experience into behavior. Animals can learn that reward occurs in a particular area (a context cue), in temporal association with a particular sensory stimulus (a Pavlovian cue), or as a consequence of a particular action taken by the animal (an instrumental relationship). Each of these memories is mediated by slightly different brain circuits. Even for the seemingly simple case of Pavlovian conditioning, animals may learn different Pavlovian associations under different circumstances, and multiple Pavlovian associations during the same experience. For example imagine a rat that encounters a grape for the first time in its life. The rat sniffs the grape, handles it, and finally tastes it—it is good (in a hedonic 'liking' sense), and in the future he will eat other grapes without hesitation. During that meal, the animal learned that the grape is good, and that smell, visual, and other cues associated with the grape are also good. These cues became 'good' and 'wanted' via the formation of S-S associations with the 'goodness' experienced by eating the grape. When he smells grape on another rat, this S-S association will be activated, and he will experience a sudden state of incentive 'wanting.' This 'wanting' will be the motivational fuel for his abrupt trip to the vine for a grape of his own. When the rat encounters such cues many, many times, however, a new Pavlovian association begins to form, and control behavior. This is a S-R association, meaning that smelling grape may no longer elicit 'wanting,' but instead a particular behavioral response, such as running out of the burrow, taking a left at the rock, climbing the trellis and grabbing a grape. S-R associations are mediated by slightly different brain circuits than S-S associations, and have some different characteristics including increased

automaticity, and insensitivity to devaluation of the associated UCS reward (Pavlov, 1927; Skinner, 1938; Hull, 1943; Robbins et al., 1989; Graybiel, 1998).

Pavlovian learning (and particularly S-S learning) is thought to interact with 'liking' as well as 'wanting.' For example, conditioned taste aversion refers to a conditioned negative affective state that is elicited by a taste previously associated with illness or nausea. My father got sick after eating popcorn as a child. To this day, even smelling popcorn will cause him to become nauseous—the same affective illness state he experienced 50 years ago is felt again today because of this strong S-S association. Rats form similar associations, and pairing tastes with drug-induced illness can powerfully affect future 'liking' responses to otherwise pleasant or neutral tastes (Garcia and Ervin, 1968; Berridge et al., 1981)

"The Amygdala"

First Hints of a Role for the Amygdala in Reward

The amygdala (meaning almond in Latin) was first named by Karl Burdach (1826), after the nut-like appearance of its basolateral nucleus in the human brain. As early as 1888, the temporal lobe (which contains the amygdala) was posited to be important for emotional reactivity and food intake behavior. Brown and Schafer (1888) reported the following unusual food ingestive behaviors after large bilateral temporal lobe lesions in a monkey (p. 311):

> He appears no longer to discriminate between the different kinds of food; e.g., he no longer picks out the currants from a dish of food, but devours everything just as it happens to come. He still, however, possesses the sense of taste, for when given a raisin which has been partly filled with quinine he shows evident signs of distaste, and refuses to eat the fruit.

Brown and Schafer also reported deficits in emotional reactivity and memory, such as the following (p. 311);

> A strange monkey, wild and savage, was put into the common cage. Our Monkey immediately began to investigate the newcomer . . . but his attentions were repulsed, and a fight resulted, in which he was being considerably worsted. The animals were, however, separated and tied up away from one another, but our Monkey soon managed to free himself, and at once proceeded, without any signs of fear or suspicion, again to investigate the stranger, having apparently already entirely forgotten the result of his former investigation.

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The authors were intrigued by these findings, but not were primarily interested in the sensory deficits caused by lesions, and therefore instead emphasized their other findings of dramatic visual deficits after occipital cortex lesions. These strange emotional behaviors resulting from large bilateral temporal lesions therefore received little attention for nearly 50 years.

Kluver and Bucy (1937) rediscovered unusual emotional and motivational changes following temporal lobectomy in a more systematic series of tests. Their descriptions of unusual food intake-related behaviors were similar to Brown and Schafer's, and they describe the emergence of "a strong tendency to examine all objects by mouth," consisting of "putting the object into the mouth, biting gently, chewing, licking, touching with the lips, and 'smelling' by holding the object before the nostrils" (Kluver and Bucy, 1939). These exploratory behaviors seemed compulsive, and were directed at all available objects whether animals had learned that they were affectively significant or not. "The monkey seems to be just as eager to examine the tongue of a hissing snake, the mouth of a cat, feces, a wire cage, or a wagon as a piece of food" (or stimuli that predict food; p.610). In addition, Kluver and Bucy also reported unusual hypersexual behavior in their male monkeys including excessive masturbation, unusually extended and repeated sex with female conspecifics, and sexual behavior directed at other males and inanimate objects. In sum, Kluver and Bucy described a syndrome in which animals seemed to be unable to focus motivated attention on appropriate targets, instead rapidly moving from stimulus to stimulus as if they had never seen it before.

This suite of unusual motivational, affective, and memory effects of temporal lobe lesions helped gain the amygdala a prominent spot in Paul MacLean's (1952) concept of the limbic system, or 'visceral brain.' This idea elaborates upon Broca's (1878) description of a 'limbic lobe,' which forms a ring in the medial surface of the telencephalon, and Papez' (1937) description of a similar looping emotion circuit. MacLean added several structures to prior brain emotion circuit conceptions, and explicitly described a role for amygdala in the "visceral brain that interprets and gives expression to its incoming information in terms of *feeling*" (MacLean, 1952)(p. 415). To this day, the concept of the limbic system (and its dopamine-containing relative the

mesolimbic system) remains useful to neuroscientists for generally describing brain circuits of emotion and reward, though some dislike the concept because of its lack of clear definition or list of brain components (LeDoux, 2002).

Amygdala Anatomy: Brain System Context and Subdivisions

Although often conceived of as a unitary structure, in reality the amygdala is a cluster of ten or more interconnected nuclei that play complex and varied roles in motivation, learning, and emotion. Broadly speaking, the amygdala can be divided into two main groupings of nuclei, first described by Johnston (1923) as the corticomedial and basolateral portions. Johnston noted that the corticomedial portion appeared relatively unchanged across many mammalian species, and is related to olfactory processing areas in fish and other vertebrates. The basolateral nucleus in mammals (and especially primates) is greatly expanded compared to simpler vertebrates, and is therefore likely to have undergone more recent evolutionary change.

In addition to their different phylogenetic relationships, central (CeA) and basolateral (BLA) amygdala nuclei are also markedly different in many other ways—to the extent that many have questioned whether the amygdala exists as a functional unit at all. The chief difference between the amygdala subnuclei is that BLA is essentially a cortical structure, while central CeA is essentially subcortical and striatum-like (Alheid and Heimer, 1988; Swanson and Petrovich, 1998). BLA has much in common with other cortical substrates of emotion, and thus is sometimes considered part of the 'limbic lobe,' or the band of cortical structures at the medial surface of the brain, first proposed by Broca (1878). In contrast, CeA has much more in common with striatal areas, and is often considered part of a looping subcortical macrosystem called the extended amygdala (Alheid and Heimer, 1988; Swanson and Petrovich, 1998; Zahm, 2006).

Like other cortex, BLA receives substantial thalamic sensory input, and sends heavy glutamatergic projections both to other cortical sites (eg. cingulate and orbitofrontal cortices), and subcortical nuclei (eg. striatum)(Maren and Fanselow, 1996; Pitkanen et al., 1997; Pitkanen, 2000). BLA is usually considered to consist of the lateral, basolateral, and basomedial nuclei. Lateral amygdala is the main input structure for sensory thalamic afferents, and Pavlovian fear memories are known to be formed and stored there (Fanselow and LeDoux, 1999; Maren and Quirk, 2004; Schafe et al., 2005).

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Lateral amygdala projects heavily to CeA and other amygdala nuclei directly or via the intercalated amygdala nucleus, and this BLA-CeA pathway is known to be important for the expression of conditioned fear (Maren and Quirk, 2004; Pare et al., 2004; Fanselow and Poulos, 2005). Rostral and caudal BLA differ in both cell size (magnocellular and parvocellular, respectively), and anatomical connectivity, with rostral BLA preferentially projecting to anterior cingulate cortex and NAcC, and caudal BLA projecting to posterior cingulate and NAcS (Alheid, 2003).

BLA projections to reward-related regions like nucleus accumbens and medial prefrontal cortex are especially likely to be important for the amygdala's role in reward. Functionally, BLA inactivation causes changes in dopamine levels in mPFC (Ahn and Phillips, 2003), and the BLA projection to NAcC controls accumbens cell firing in relation to incentive motivational behavior (Ambroggi et al., 2008), NAc dopamine release (Howland et al., 2002), and cocaine seeking behavior (Di Ciano and Everitt, 2004). Finally, BLA may also affect reward-related processes via its previously described direct and indirect projections to CeA (Maren and Quirk, 2004; Pare et al., 2004).

In contrast, CeA seems to have much in common with a group of subcortical structures, roughly forming a ring around the internal capsule, termed the 'extended amygdala' (Alheid and Heimer, 1988; Alheid, 2003; Zahm and Trimble, 2008). The extended amygdala consists of the central and medial amygdaloid nuclei (CeA and MeA) as well as several basal forebrain structures including sublenticular extended amygdala (SLEA), interstitial nucleus of the posterior limb of the anterior commissure (IPAC), and bed nucleus of the stria terminalis (BNST). These structures are very similar in several ways, including their parallel sensory and cortical afferents, and their prominent efferents to midbrain and brainstem behavioral control and autonomic nuclei. Extended amygdala structures are also similar in their neurochemical content (such as high levels of opioid peptides), and their composition consisting of medial and lateral portions (such as MeA and CeA in the amygdala). 'Macrosystems' such as the extended amygdala may specialize in processing certain types of information, and interact with other brain systems to influence behavior. For example, extended amygdala may be specialized to identify emotionally relevant situations and stimuli from cortical and other inputs, and

accordingly activate brainstem behavioral activation circuits such as reticular formation and midbrain dopamine populations (Zahm, 2006).

Some have argued that CeA is a key brain site for the integration of sensory information with learned emotional information to produce appropriate behavioral responses. Accordingly, CeA receives processed sensory and other information from sensory thalamus (Ledoux et al., 1987; Linke et al., 2000) and insular and prefrontal cortex (McDonald et al., 1999 Davis, 1999), as well as BLA (Pare et al., 1995; Pitkanen et al., 1995; Petrovich and Swanson, 1997). In fact, the majority of cortical inputs to extended amygdala are to CeA and MeA (McDonald et al., 1999). CeA also receives primary sensory information from brainstem taste processing nuclei including opioidergic and other inputs from nucleus of the solitary tract and parabrachial nucleus (Zardetto-Smith and Gray, 1990; Bernard et al., 1993). Many CeA inputs are onto GABAergic interneurons (Sun et al., 1994), leading some to suggest that CeA is a main site for the integration of sensory and learned affective inputs, and the use of these convergent inputs in the production of coordinated emotional and motivational behavior via its GABAergic projections to hypothalamic, midbrain and brainstem nuclei controlling arousal and autonomic components of emotional responses (eg. periaquaductal grey (PAG), parabrachial nucleus, (PBN), lateral hypothalamus (LH))(Sun et al., 1994; Pitkanen, 2000; Rodrigues et al., 2004; Maren, 2005).

The central amygdala contains central (CeAC), medial (CeAM), lateral (CeAL), and lateral capsular (CeALC) portions, each with somewhat different patterns of anatomical connections and segregated peptide contents (Cassell et al., 1999). For example, CeAM receives preferential afferent from PBN taste areas and sends unique efferents to ventrolateral BNST, IPAC, and posterior LH, while CeALC sends unique projections to caudal ventral pallidum, and receives special inputs from PBN autonomic areas (Bourgeais et al., 2001).

The medial amygdala (MeA) is also considered to be part of the extended amygdala, and has a similar pattern of connectivity and neurochemical content as CeA. This structure is known to be important for social (especially sexual) behavior, as well as olfactory processing (Newman, 1999; Ferguson et al., 2002). Although it likely plays a

unique and important role in reward itself, the scope of this dissertation unfortunately does not allow detailed investigation of its role in natural reward 'wanting' vs. 'liking.'

In addition to its well known efferents controlling brainstem and hypothalamic autonomic and arousal processes, CeA also interacts with the mesocorticolimbic dopamine system, likely via direct or indirect projections to the midbrain ventral tegmental area and substantia nigra (Krettek and Price, 1978; Fudge and Haber, 2000; Pitkanen, 2000; Zahm, 2006). Functionally, CeA can influence basal dopamine levels and food-evoked dopamine release in nucleus accumbens and prefrontal cortex (primary targets of addiction-related dopamine release in the forebrain) via projections to the ventral tegmental area (VTA) or vicinity (Ahn and Phillips, 2002; Phillips et al., 2003).

In sum, both basolateral and central amygdala are crucial hubs within brain circuits of sensory processing, emotion, learning, and reward. Their unique patterns of anatomical connections suggest distinct but complementary roles in behavior, necessitating careful analysis of the exact psychological processes each mediate. In the following section, we review some of the evidence for the roles of CeA and BLA in reward 'wanting,' 'liking,' and learning.

Amygdala Functions in Incentive Motivational 'Wanting'

As discussed above, reward 'wanting' can be measured in a variety of ways. Central and basolateral amygdala nuclei play important and distinct roles in appetitive and aversive motivation and emotional memory. Several types of evidence have been reported implicating both subnuclei in processing slightly different aspects of reward 'wanting' and learning. The majority of experiments conducted examining the role of amygdala in reward employ specific lesions of the CeA or BLA, then examine which reward-related processes are compromised. Similarly, some researchers have also used drugs to temporarily inactivate amygdala nuclei during particular phases of experiments, allowing them to test the animals again when the drugs have worn off, and the amygdala is again intact.

Food Reward 'Wanting'

As described above, one way to measure 'wanting' is to examine intake of a rewarding UCS such as food. Neither CeA nor BLA lesions have profound effects on

spontaneous food intake (Kemble et al., 1972; King et al., 1996), although CeA lesions can reduce preferences for sweet solutions or other foods (Touzani et al., 1997; Ganaraj and Jeganathan, 1998), and BLA lesions can sometimes increase food intake (Ganaraja and Jeganathan, 2000), especially of otherwise unpalatable foods (Machado and Bachevalier, 2007). On the other hand, pharmacologically inactivating the CeA (but not the BLA) with either muscimol or lidocaine profoundly reduces food intake (Ahn and Phillips, 2002; Baldo et al., 2005). In addition, both CeA and BLA muscimol inactivation reduce feeding elicited by opioid stimulation of the NAc (Will et al., 2004), while only CeA inactivation blocks NAc GABA agonist-induced feeding (Baldo et al., 2005). Feeding behavior can be elicited in sated rats by exposure to a food-associated CS, but this effect is specifically blocked by BLA, but not CeA lesions.

The CeA seems to be particularly important for the ability of Pavlovian reward CSs to elicit conditioned approach responses directed at themselves, or the 'motivational magnet' properties of cues. For example when a diffuse tone is played to a rat, it will often orient toward the noise, rear, and sniff. When this auditory stimulus is associated with reward, much more orienting is displayed toward it, and this conditioned orienting response requires an intact CeA, but not BLA (Gallagher et al., 1990). Similarly, rats will approach and investigate a visual stimulus on a computer screen that is associated with a food reward, and the CeA, but not BLA is necessary for these CSs to come to elicit approach (Parkinson et al., 2000; Cardinal et al., 2002b). Unfortunately, we are not aware of any published experiments examining the role of amygdaloid nuclei in approach and consummatory-like behaviors directed at discreet, localized Pavlovian reward CSs. It is possible that approach and attempted consumption would go hand in hand, and both require CeA in particular, but it is also possible that BLA would play a role in these post-approach consummatory attempts.

Another feature of cue-triggered 'wanting' is that it can be directed onto representations of a UCS, fueling instrumental reward-seeking behavior in the PIT paradigm. Interestingly, Corbit and Balleine (2005; 2007) have shown that instrumental reward-seeking can be triggered either by cue-triggered 'wanting' directed either toward a specific reward (such as sucrose solution vs. food pellets), or toward all available rewards. When a CS that predicts chow occurs, rats will press primarily on a lever they

learned would deliver chow, and sucrose CSs primarily induce pressing on a different sucrose lever. However, both cues also elicit some pressing on the 'wrong' lever, a phenomenon they attribute to general behavioral activation or non-specific reward seeking. Interestingly, BLA is required for a cue to elicit reward-specific PIT, while CeA is necessary only for cue triggered 'wanting' not directed at any particular reward (Corbit and Balleine, 2005).

BLA, but not CeA, is also necessary for several other processes depending upon the use of learned S-S associations between CSs and the cognitive representation of the specific reward they predict. For example, animals without BLAs are unable to reduce their seeking of a UCS that is suddenly less valuable due to its being paired with lithium chloride-induced illness (Hatfield et al., 1996). Similarly, BLA-lesioned animals cannot reduce their approach to a visual CS that predicts a reward they have just consumed to satiety (Blundell et al., 2003).

While it is clear that CeA and BLA play distinct roles in motivation and reward, the exact nature of these differences is still not completely understood. Some have interpreted these findings to mean that BLA is involved in producing motivated behavior based on representations of specific rewards and their sensory properties, while CeA mediates Pavlovian motivation based more on affective value of CSs than the particular rewards these cues predict (Blundell et al., 2001; Everitt et al., 2003; Corbit and Balleine, 2005; Balleine and Killcross, 2006). In both cases, however, it *is* clear that CeA and BLA are likely to be involved in reward 'wanting' triggered by, and targeted upon Pavlovian CSs.

'Wanting' for Drug and Sex Rewards:

Many brain substrates of food 'wanting' are similarly involved in 'wanting' for other types of rewards such as addictive drugs and sex, and the amygdala is no exception. Both CeA and BLA show Fos or Jun markers of neuronal activation in relation to contextual CSs associated with morphine and alcohol (Harris and Aston-Jones, 2003; Radwanska et al., 2008). BLA is required (with hippocampus) for context cues to reinstate cocaine seeking (Fuchs et al., 2007), and for the acquisition and expression of morphine conditioned place preference (Zarrindast et al., 2004). CeA may show even greater Jun activation to discrete Pavlovian CSs for alcohol (Radwanska et al., 2008) and

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it and BLA are required for discrete cocaine and heroin CSs to reinstate drug seeking behavior (Kruzich and See, 2001; Rogers et al., 2008). Conversely, stimulating BLA either electrically or pharmacologically reinstates cocaine seeking behavior (Hayes et al., 2003). Interestingly, neither nucleus is required for cocaine itself to reinstate drug seeking (McFarland and Kalivas, 2001), yet at least BLA is necessary for heroin to do so (Fuchs and See, 2002), perhaps suggesting a special role for amygdala opioids in 'wanting.'

For sexual behavior, the medial amygdala seems to be the most important player in the amygdala, likely as a function of its role as a node in larger systems regulating social behavior and memory (Newman, 1999). This said, other amygdala nuclei are involved in various aspects of sexual behavior (van Furth et al., 1995), and it is likely that CeA and BLA play similar roles in regulating responses to CS associated with sex rewards as they do for other rewards. For example, BLA is not necessary for sexual behavior itself in male rats, but is required for sex-associated CSs to reinforce a new instrumental behavior (Everitt, 1990). In humans, amygdala may also be involved in processing CSs for sex, as sexual stimuli produce similar amygdala activation to cocaine cues in cocaine-addicted subjects (Childress et al., 2008).

CeA and BLA in Reward Hedonics:

Little is known about how the amygdala is involved in unconditioned hedonics. While some have argued that CeA and other regions of extended amygdala are involved in negative hedonic states associated with drug withdrawal (Harris and Aston-Jones; Koob and Le Moal), it is unclear to what extent these negative affective states are related to hedonic 'liking' or disliking of primary rewards. In the taste reactivity paradigm, lesions of the CeA do not affect unconditioned hedonic 'liking' or 'disliking' reactions to sweet, salty, or sour tastes, or enhancement of hedonic evaluations of a NaCl solution after physiological sodium depletion. Interestingly the same CeA lesions did prevent sodium depletion from enhancing voluntary NaCl intake, potentially suggesting a dissociation of CeA's role in hedonics and motivation (Galaverna et al., 1993; Seeley et al., 1993). Rana and Parker (2008) recently found using specific excitotoxic lesions that neither CeA nor BLA is necessary for normal hedonic responding to intraoral saccharine, or for conditioned 'disliking' for a taste paired with LiCl-induced illness. These findings

suggest that while the CeA and BLA are crucial for reward learning and 'wanting,' they may be less important for regulating hedonic responses to primary rewards, or for adjusting these responses based on learning.

Amygdala and Pavlovian Learning

Both CeA and BLA are well known to be crucial for appetitive and aversive emotional learning, in animals as well as humans (LaBar et al., 1995; Killcross et al., 1997; LeDoux, 2000; Maren, 2005). Fear memories are formed and stored in the lateral amygdala, and have even been associated with synapse-specific changes within BLA (Doyere et al., 2007). There is little better evidence in neuroscience for a brain area being the site for formation and storage of experience-dependent plasticity. Recently, it has become clear that that similar plasticity exists in central as in basolateral synapses from sensory thalamic inputs (Maren, 2005; Samson and Pare, 2005), opening the possibility that CeA too may be a brain substrate of Pavlovian learning and memory itself, in addition to motivation and emotion resulting from learning.

For reward, both BLA and CeA are necessary brain substrates of appetitive Pavlovian learning as well. For example, CeA lesions block only the acquisition of conditioned approach to a visual CS, but not the expression of this approach in animals that learned the task with an intact brain (Cardinal et al., 2002b). BLA is required for the formation and consolidation of Pavlovian associations between CSs and drugs, as BLA inactivation following formation of these associations blocked the ability of the CSs to reinstate cocaine-seeking behavior (Fuchs et al., 2006). Similarly, protein synthesis inhibition in BLA blocks the reconsolidation of morphine conditioned place preferences (Milekic et al., 2006). BLA is also involved in the extinction of Pavlovian memories and their consolidation (Marsicano et al., 2002; Fuchs et al., 2006; Feltenstein and See, 2007; Boccia et al., 2008).

BLA and especially CeA receive heavy dopamine afferents (Kilts et al., 1988; Freedman and Cassell, 1994; Asan, 1997), and some evidence points to a role for these projections in Pavlovian learning and memory consolidation. For example, dopamine D1 and D2 antagonists in BLA block the acquisition of Pavlovian fear conditioning and fear potentiated startle, respectively (Lamont and Kokkinidis, 1998; Guarraci et al., 2000). For reward learning, CeA D1 and D2 antagonists block, and agonists facilitate the

acquisition of morphine conditioned place preferences (Rezayof et al., 2002; Zarrindast et al., 2004). D3 receptors may be particularly important for consolidating Pavlovian associations, as post-training D3 stimulation of CeA enhances learning of a CS-UCS association for food and intra-accumbens amphetamine rewards (Hitchcott and Phillips, 1998a, b). Interestingly, when the same D3 agonist is infused prior to testing, it *inhibits* expression of Pavlovian conditioned approach in CeA, and conditioned reinforcement in BLA (Hitchcott and Phillips, 1998b), potentially suggesting a unique role for amygdala D3 receptors in learning and memory as opposed to motivation.

Summary of Amygdala Functions in Reward

Clearly, BLA and CeA are both major players in learning about stimuli in the world that predict emotionally significant events, as well as in producing conditioned affective states and motivated behaviors. At least for appetitive motivation, CeA may mediate Pavlovian incentive salience that is directed upon reward CSs themselves, and that is not based on particular sensory features of rewards, or directed toward one particular reward among several options. The BLA may instead allow CSs to be associated with representations of particular rewards and their sensory features and current hedonic value, allowing incentive 'wanting' directed at one reward in particular among several.

Amygdala μ Opioids and Reward

Opioids as a Reward Transmitter

μ opioids have been linked to reward since the addictive drug morphine was discovered to be the active ingredient in opium (Sertürner, 1817). It was long known that morphine and related opiates, prized for their pain relief properties, are also highly addictive, and their ingestion results in euphoria. For example, Thomas de Quincey (1821) wrote of opium's psychoactive effects, "here was the secret of happiness, about which philosophers had disputed for so many ages, at once discovered; happiness might now be bought for a penny, and carried in the waistcoat pocket."

Pert & Snyder (1973) first described the CNS opioid receptor, and it soon became apparent that it was the main psychoactive target of both plant-based opiate drugs, and endogenous ligands such as ß endorphin and enkephalin. Functionally, opioids are well

known to be involved in reward, with agonists producing pleasure and euphoria, as well as potent stimulation of 'wanting' for these drugs and the cues that predict them. Opioid agonists (especially μ agonists) can also stimulate 'wanting' for other rewards, and potently stimulate food intake when administered systemically or directly into brain areas including the amygdala, striatum, ventral pallidum, hypothalamus, and nucleus of the solitary tract (Gosnell, 1988; Stanley et al., 1988; Kotz et al., 1997; Zhang and Kelley, 2000; Smith and Berridge, 2005).

More recently, Susana Peciña and Kent Berridge (2005) discovered that the dorsomedial nucleus accumbens shell contains a $1mm^3$ 'hotspot' in which the μ opioid agonist DAMGO more than doubles hedonic 'liking' of sucrose compared to control levels. Food intake 'wanting' is also stimulated by DAMGO, but throughout a much larger region of ventral and dorsal striatum (Zhang and Kelley, 2000; Peciña and Berridge, 2005). Similarly, DAMGO throughout the entire accumbens stimulates cue-triggered UCS 'wanting' in a PIT paradigm (Peciña and Berridge, 2008). In the ventral pallidum, a major source of reward-related accumbens output, opioids similarly stimulate 'liking' in a restricted $0.8mm^3$ region of caudal VP, while enhancing food intake wanting in a larger area of rostral and caudal VP (Smith and Berridge, 2005).

Amygdala Opioid Anatomy and Physiology

Both CeA and BLA contain relatively high levels of μ opioid receptor mRNA and immunoreactivity (Sharif and Hughes, 1989; Mansour et al., 1994; Mansour et al., 1995). Mu receptors are largely located in axonal processes in CeA and BLA, but are also present on postsynaptic neurons there (Mansour et al., 1995; Zhu and Pan, 2004, 2005; Finnegan et al., 2006). Presumably, these receptors are endogenously activated by enkephalin and endorphin, which are located and released in CeA and BLA (Mansour et al., 1988; Stein, 1993; Lam et al., 2008). The specific μ agonist DAMGO acts on μ opioid receptors in both CeA and BLA (Levine et al., 2004; Shin and Helmstetter, 2005). In CeA, DAMGO inhibits most (60% of) cells, including both GABAergic interneurons and projection neurons (Chieng et al., 2006). DAMGO acts in CeA both by presynaptically inhibiting glutamate (Zhu and Pan, 2005) and GABA release (Finnegan et al., 2005), and by directly inhibiting projection cells (Zhu and Pan, 2004; Chieng et al., 2006). Less is known about how opioid agonists function at the cellular level in BLA,

but again their effects are complex, and modulated by the basal activity of cells they act on (McGaraughty and Heinricher, 2002). Effects of μ agonists in BLA may differ from those in CeA, in that GABA but not glutamate release is inhibited presynaptically in BLA by DAMGO *in vitro* (at least in CeA-projecting cells)(Finnegan et al., 2006).

In sum, μ opioids are likely to play an extremely complex modulatory role in amygdala, and their actions in a given region of CeA or BLA are likely to depend on the local predominance of pre or post synaptic μ receptors, the neurotransmitters co-released with endogenous opioids, and the presence or absence of afferent stimulation.

Amygdala Opioid Functions in Reward

Although limbic opioids have been suggested to be involved in addiction since at least the 1970s (Wikler et al., 1972), μ opioid receptors in amygdala first received attention for their role in reward 'wanting' in the late 1980s, when two groups nearly simultaneously discovered that μ opioid agonist injections into amygdala stimulate food intake (Gosnell, 1988; Stanley et al., 1988). Most research since has focused on μ stimulation of feeding in CeA, with few or no reports of similar BLA opioid agonist-induced feeding published. CeA opioids may act in association with activation of other brain reward systems, as μ activation there stimulates both feeding and Fos expression in the nucleus accumbens shell (Levine et al., 2004). CeA DAMGO-induced feeding can be blocked by simultaneous blockade of opioid receptors with naltrexone in accumbens shell, paraventricular hypothalamus, or nucleus of the solitary tract (Giraudo et al., 1998a; Giraudo et al., 1998b; Kim et al., 2004). Conversely, CeA naltrexone blocks NAcSh and PVN DAMGO-induced feeding, suggesting reciprocal interactions between these structures in the control of feeding. Less is known about how or if BLA opioid receptors modulate feeding, although μ agonist binding in BLA is related to weight gain in obese rats, suggesting a potential involvement for them as well (Smith et al., 2002).

A role for amygdala μ opioids has also been examined with several measures of reward 'wanting' other than food intake. Systemic morphine produces robust conditioned place preferences (CPPs), and several reports have indicated that BLA acetylcholine and $GABA_A$ receptors (Zarrindast et al., 2004; Zarrindast et al., 2005), and CeA NMDA receptors modulate formation of associations between morphine and contextual cues (Zarrindast et al., 2007). Interestingly, neither intra-CeA nor intra-BLA

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morphine seems to form CPPs (van der Kooy et al., 1982; Olmstead and Franklin, 1997), but morphine is voluntarily self-administered by animals directly into amygdala (to sites primarily in the BLA)(David and Cazala, 1994). The experience of rewards themselves also involves amygdala opioids, as acute morphine modulates amygdala neuronal activity (Bot and Chahl, 1996), ethanol injections induce β-endorphin release in CeA (Lam et al., 2008), and self-administration of VTA electrical stimulation is associated with decreased opioid agonist binding, suggesting an increase in endogenous opioid activity (Stein, 1993).

With regard to tasks measuring non-reward learning, amygdala opioids are generally inhibitory of memory formation. For example, CeA (but not caudal BLA) morphine injection reduces inhibitory avoidance learning in a task measuring latency to step down from a platform onto a floor where a shock had occurred before (Ragozzino and Gold, 1994; Good and Westbrook, 1995). Tone-shock and context-shock Pavlovian fear learning is also impaired by CeA morphine injections (Good and Westbrook, 1995). In a non-Pavlovian working memory task, neither CeA inactivation nor β endorphin injection affected learning.

Other Functions of Amygdala Opioids

In addition to their role in reward, opioids in amygdala (and particularly central amygdala) also seem to play an important role in more purely aversive processes such as pain perception and negative affective states such as those associated with withdrawal from addictive drugs (Drolet et al., 2001; Harris and Aston-Jones, 2007; Koob and Le Moal, 2008). A review of these large literatures is beyond the scope of the present dissertation, but clearly amygdala opioids are an important player in several types of behavioral and psychological processes, some of which may overlap with their reward functions and some which may not. Much is yet to be learned about the function of opioids in BLA and CeA, and this dissertation approaches only one small set of questions in this larger aim: how does amygdala opioid stimulation modulate natural reward learning, 'wanting,' and 'liking?'

Summary of the Present Experiments

The experiments described in this dissertation seek to assess the role or roles played by opioids and endocannabinoids in mesolimbic brain structures in reward 'wanting' and 'liking.' To do so, we stimulated μ opioid receptors in amygdala and cannabinoid receptors in accumbens and examined the behavioral and neural consequences. We discovered both a specific opioid 'wanting' hotspot in central amygdala, and a cannabinoid 'liking' hotspot in dorsomedial accumbens shell.

Chapter 2: Which Cue to 'Want?' Central Amygdala Opioid Activation Enhances and Focuses Incentive Salience on a Prepotent Reward Cue

In this chapter, we explore how stimulating opioid receptors in the central nucleus of amygdala affects incentive motivational 'wanting' directed at reward-associated stimuli. We use an autoshaping paradigm, in which a sucrose reward was associated with a CS+ presentation, and animals come to approach and attempt to consume one of two available reward-associated stimuli. CeA opioid stimulation enhanced incentive salience targeted upon whichever stimulus an individual animal learned to preferentially approach prior to testing. CeA DAMGO also enhanced food intake, further supporting enhancement of incentive salience. In addition, Fos plume analysis suggests that these effects were chiefly mediated by CeA, rather other nearby structures.

Chapter 3: Which Cue to 'Want?' Central Amygdala Opioid Activation Enhances and Focuses Incentive Salience on a Prepotent Reward Cue

In this chapter, we ask whether CeA or BLA opioid stimulation enhances cue-triggered 'wanting' for a UCS reward itself using a PIT paradigm. Therefore, we expand upon the previous chapter by asking whether CeA DAMGO enhances incentive salience attribution to a UCS reward representation as well as to a reward CS itself, and whether BLA opioid stimulation has similar or distinct effects on 'wanting' from CeA. We found that, as expected, CeA DAMGO enhanced cue triggered UCS 'wanting' in PIT, primarily by focusing reward seeking behavior into cue periods.

Chapter 4: A Specific Role for Central Amygdala in 'Wanting:' Lack of Hedonic Enhancement by Amygdala Opioid Activation

In this chapter, we examine the effects of CeA and BLA opioid stimulation on hedonic 'liking' of a received reward, as well as 'disliking' of an aversive taste. We administered intra-amygdaloid DAMGO or vehicle prior to intraoral infusions of sucrose

or quinine solutions, and measure resulting hedonic and aversive taste reactivity to these stimuli. Animals were then tested for food intake on the same day, in order to compare 'liking' and 'wanting' effects of the same microinjections. We found that although DAMGO robustly stimulated food intake 'wanting,' sucrose 'liking' was potently reduced in the same animals in both CeA and BLA. These results indicate specific enhancement of food 'wanting,' with concurrent deficits in food 'liking' after amygdala opioid stimulation.

Chapter 5: Endocannabinoid Hedonic Hotspot for Sensory Pleasure: Anandamide in Nucleus Accumbens Shell Enhances 'Liking' of a Sweet Reward

In the previous chapters, we demonstrate an amygdala opioid substrate for integrating reward 'wanting' with prior learning about rewards. In order to expand these findings to also demonstrate a novel brain substrate for 'liking,' we turned to the nucleus accumbens shell (NAcSh), where opioids are well-known to process reward hedonics. Given the suspected role played by cannabinoids in appetitive and hedonic reward processing, and the known molecular and systems-level interactions of brain cannabinoids and opioids (which enhance 'liking' in a dorsomedial NAcSh 'hotspot'), we asked whether stimulating endocannabinoid systems in nucleus accumbens would also increase hedonic 'liking.' Indeed, intra-accumbens shell anandamide microinjections robustly enhanced sucrose 'liking,' without affecting quinine 'disliking.' This enhancement was particularly strong in a ~1.6mm^3 'hedonic hotspot' in the dorsomedial NAcSh. Anandamide also enhanced food intake 'wanting' in some of the same animals. These findings stand in stark contrast to the reduction in hedonics seen after DAMGO opioid stimulation of amygdala, demonstrating that we can identify 'hotspots' for both 'wanting' and 'liking' where they exist in the brain.

Chapter 2

Which Cue to 'Want?' Central Amygdala Opioid Activation Enhances and Focuses Incentive Salience on a Prepotent Reward Cue

Introduction

Discrete cues or Pavlovian conditioned stimuli (CSs) that predict rewards (unconditioned stimuli/ UCSs) are well known to attract attention, potentiate ongoing action, and elicit approach behavior (H. Liddel, cited in Timberlake and Grant (1975; Holland, 1977; Robbins and Everitt, 1996). Additionally, sometimes a reward CS also itself becomes a powerful incentive stimulus that is worked for and 'wanted,' almost like the reward it predicts (Bindra, 1978; Toates, 1986; Gallagher and Schoenbaum, 1999; Berridge, 2001; Di Ciano and Everitt, 2005). This can lead to bizarre consummatory-like behaviors directed at CSs. For example, crack cocaine addicts are known to 'chase ghosts,' or compulsively pick up pebbles on the ground that resemble crack rocks (Rosse et al., 1993; Berridge, 2007), rats eagerly sniff a lever CS that predicts intravenous cocaine or other noningestive rewards (Peterson et al., 1972; Uslaner et al., 2006), quail copulate with a terrycloth CS that predicts a sex partner (Burns and Domjan, 1996), and rats and pigeons approach and interact with food or water CSs as if they were the associated ingestive rewards themselves (Jenkins and Moore, 1973; Boakes, 1977; Tomie et al., 2007).

An explanation for such peculiar consummatory behaviors focused upon a reward CS is provided by the *incentive salience* hypothesis of motivation, which posits mesocorticolimbic activation to cause particular Pavlovian reward cues to become

attractive, interesting, and 'wanted' incentive stimuli that pull behavior towards themselves and associated goals (Robinson and Berridge, 1993; Berridge, 2001; Berridge, 2007). Since multiple reward cues are often available in an environment, associative learning or other factors must be used to target incentive salience upon one cue at a time, making it more 'wanted' than others (Berridge, 2007; Flagel et al., 2007).

The amygdala is an important mesocorticolimbic structure for interfacing learning and motivation (Morris and Dolan, 2001; Di Ciano and Everitt, 2004; Holland and Gallagher, 2004; Phelps and LeDoux, 2005; Balleine and Killcross, 2006; Beaver et al., 2006; Phillips et al., 2008), and its central nucleus (CeA) may help integrate Pavlovian learning with appetitive motivation (Cardinal et al., 2002b; Balleine and Killcross, 2006; Phillips et al., 2008), perhaps involving μ opioid activation (Gosnell, 1988; Kim et al., 2004; Scott et al., 2007). We predicted that activating CeA opioids would enhance targeted incentive salience, making selected CSs more 'wanted' than others.

One way to measure the targeting of incentive salience onto a particular CS is the Pavlovian autoshaping or sign-tracking paradigm, in which CS consummatory behaviors described above can be observed. For example, if a metal CS+ lever is repeatedly inserted through a wall to predict a sucrose pellet delivered into a metal dish (which we will call CS_{source}), rats will approach, nibble, sniff, and bite *either* the predictive lever CS+ *or* the CS_{source} sucrose dish (Flagel et al., 2007; 2008b). Here, we used CeA opioid activation and muscimol inactivation to test whether CeA controls these CS-directed appetitive-consummatory behaviors. Our results indicate that central amygdala enhances and focuses incentive salience, making thereby guiding reward-directed behavior.

Materials and Methods

Overview

We hypothesized that CeA opioid activation by DAMGO microinjection would intensify appetitive-consummatory behaviors directed towards a CS, and that this intensification would be selectively targeted at whichever cue animals came to preferentially approach and interact with during early training (the prepotent CS).

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Conversely, we hypothesized that inactivating the CeA with muscimol microinjections would suppress appetitive-consummatory behaviors toward the prepotent CS. These predictions were confirmed, using CeA microinjections of DAMGO or muscimol, coupled with detailed video analyses of consummatory behaviors toward CSs in an autoshaping paradigm, and a Fos plume mapping technique to confirm CeA anatomical responsibility. Finally, we measured food intake in the same animals, and confirmed that motivation for the UCS was increased similarly as for the CS by CeA opioid activation, and decreased similarly by CeA inactivation.

Subjects

Sprague Dawley rats were used (n=105 females, 250-350 g; different experiments used ns of 24, 64, and 17 as described below, and every estrous phase was included). Rats were housed in a reverse 12h light/12h dark cycle, ~21°C room (housed in pairs until surgery, and alone thereafter). Chow and water were available *ad libitum* at all times, except during autoshaping procedures when food was restricted to 20-25g/day/rat (delivered daily following training or testing). To measure maximize effects of DAMGO and muscimol on behavior as well as local Fos plumes, separate groups were used to measure a) the attribution or expression of food consummatory-like behaviors targeted at CSs (tested during or after learning in different groups), b) motivation to consume UCS reflected in food intake, and c) local spread of neuronal modulation mapped by local Fos plumes around sites of DAMGO or muscimol microinjection.

Autoshaping Paradigm

To assess if central amygdala activation/inactivation altered appetitive and consummatory behaviors directed toward a reward CS, we measured cue-triggered consumption-like behaviors directed towards a metal lever CS+ and metal dish CS$_{source}$ that were associated with sucrose pellet delivery. Consummatory behaviors were sniffing, nibbling, and biting movements directed to the CS+ lever or CS$_{source}$ dish. Each CS was a discrete and localized physical object that can be grasped and partly 'eaten' (i.e., roughly the size of a chow pellet, located in one place, and easily noticed) (Tomie, 1996). The sequence of ingestive behaviors targeted at CSs is similar to the sequence of

unconditioned ingestive movements directed toward UCS food pellets at onset of eating (described below).

The CS+ was a metal 4.5 x 2cm lever containing an illuminated diode that was physically inserted through the chamber wall for 8sec immediately prior to each UCS pellet delivery, and accompanied by a 2.9KHz continuous tone during lever insertion (Flagel et al., 2007; 2008b). The CS+ presentation therefore was a phasic event which predicted the UCS with 100% correlation, and the physical lever was discrete, graspable and bitable. The CS$_{source}$ was a recessed dish (3cm diameter) with raised metal edges (0.7cm high) that rats could also approach, sniff, insert their head into, nibble, and bite on. Contrasting the two sucrose-associated stimuli, the CS+ lever was the most predictive CS, having the highest correlation with presentations of the UCS reward (but spatially segregated from it by 5cm), whereas the CS$_{source}$ dish was more immediately proximal in space and more contiguous in time to the UCS pellet (because a rat had to retrieve each UCS directly from the dish at the moment of reward), but less well correlated in a predictive sense because the dish was present during the entire session, including between CS+ and UCS presentations. The CS+ lever was the information-containing, predictive CS+ that triggered cue approach and consummatory behaviors regardless of which CS was the target. A second lever was always present as a control CS that had no specific excitatory or inhibitory association with UCS presentations.

After a few days of autoshaping training, each individual rat develops its own prepotent target for its appetitive and consummatory behaviors, and this difference in choice is correlated with individual mesocorticolimbic markers such as D$_1$ receptor mRNA levels in nucleus accumbens and neuropharmacological markers such as susceptibility to sensitization, and level of psychomotor response to cocaine (Flagel et al., 2007; Flagel et al., 2008b). Some rats reliably approach and interact with the CS+ lever as if it were food, directing appetitive-consummatory behaviors toward it when it is extended (sometimes called 'sign tracking'). Other individual rats reliably approach and similarly interact with the CS$_{source}$ (sucrose dish) whenever the lever CS+ is extended (sometimes called goal tracking). In both cases, CS interactions appear markedly similar to food intake, both in the sequencing and timing of behavioral elements. We will call whichever CS target a rat reliably chose its 'prepotent CS' (Valenstein et al., 1970), and

this choice of a particular prepotent CS is so reliable that a rat can be categorized as either 'CS+ prepotent' or 'CS$_{source}$ prepotent' within several days of training.

Here we capitalized on individual differences in CS focus by measuring changes in appetitive and consummatory behaviors directed at CS+ versus CS$_{source}$ cues after CeA microinjections. We hypothesized that amygdala opioid activation would focus enhanced incentive salience based on that rat's previously learned bias, and therefore enhance motivated behavior directed toward a rat's prepotent CS more than toward any alternative CS.

Surgery

Prior to behavioral testing or Fos staining, all rats were anesthetized with ketamine (80mg/kg), xylazine (7mg/kg), and atropine (0.04mg/kg) and surgically implanted with chronic, bilateral, 14mm microinjection guide cannulae (23ga) positioned 2mm above CeA sites. Cannulae were anchored to the skull with bone screws and acrylic cement, and steel stylets were inserted to prevent their occlusion. Stereotaxic placement coordinates for cranial cannulae were calculated based on Paxinos and Watson (2007). Cannulae were aimed at multiple sites in the CeA ranging from (relative to Bregma): -1.8 to -3.0 AP; ±3.0 to ±4.2 ML; and -5.8 to -6.5 DV), or control sites in the BLA (-3.0 to -3.12 AP, +4.5 to ±5.3 ML, and -9.0 to -9.4 DV) or the interstitial nucleus of the posterior limb of the anterior commissure (IPAC; -1.2 to -1.56 AP, ±3.5 to 4.2 ML, and 7.5 to 8.5 DV). All rats were allowed 1 week to recover from surgery before any habituation or testing procedures were started.

Behavioral Experiment Descriptions

DAMGO enhancement of previously learned CS incentive salience: The first experiment tested whether central amygdala μ opioid stimulation with DAMGO on a test day *after CS-UCS learning sessions were completed* would enhance approach and consummatory-type interactions with a previously learned reward CS. This group of rats was trained drug-free on an autoshaping task for 5 days after surgery. Beginning on day 6, the rats were given bilateral microinjections of DAMGO and vehicle in randomized order into CeA (n=22) or control sites including the caudal BLA (n=2), and IPAC (n=2) on each of the next two days (0.1μg/0.5μl, 48hrs between test days), immediately before

testing in the same autoshaping paradigm. For all microinjections, rats were gently held while stylets were removed, and a microinjector was inserted extending 2mm beyond the end of each guide cannulae. Vehicle or drugs were bilaterally infused in 0.5μl volume over 90sec using an automated syringe pump. The microinjector was held in place for 1 min after each injection to allow for drug diffusion from the injector tip.

DAMGO enhancements during learning: To test whether amygdala opioid stimulation would similarly enhance incentive salience during initial learning, separate rats were given daily microinjections of either vehicle (n=26) or DAMGO (0.1μg/0.5μl, n=30) into CeA before each of 6 autoshaping training sessions.

GABA suppression of prepotent CS incentive salience: In order to test the opposite prediction that amygdala *inactivation* would suppress approach and consummatory transactions with a prepotent cue, a second set of experiments was run using microinjections of the GABA$_A$ agonist muscimol (0.25μg/0.5μl; dose chosen to inactivate amygdala based on previous reports of behavioral suppression or learning interference (Wilensky et al., 2000; Maren et al., 2001)). The same rats (n=24) used above to test DAMGO effects on post-learning cue approach and consummatory behaviors were given 7 additional drug free training days after DAMGO tests (days 8-14), and then tested after microinjections of either muscimol (0.25μg/0.5μl) or vehicle in randomized order on days 15 & 16. Finally, in order to test whether muscimol inactivation of CeA would similarly prevent a CS from being attributed with incentive salience during learning, a separate group of rats received microinjections of muscimol (0.25μg/0.5μl; n=4) or vehicle (n=4) prior to each of the first 4 days of autoshaping training.

Opioid stimulation and GABA suppression of UCS intake: The effects of DAMGO and muscimol on unconditioned motivation to consume a food reward itself were subsequently tested independently in a subset of the above rats after completion of autoshaping tests (total n=31). Each rat received a microinjection of vehicle and either DAMGO (0.1μg, n=22) or muscimol (0.25μg, n=9) on separate days, immediately before being placed for 1 hr in a tub cage that contained 15-20g of pre-measured food pellets,

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water, and bedding. The amount of food consumed was measured, and eating, drinking and other behaviors were video recorded for subsequent quantification of behavior.

Autoshaping Testing Apparatus

Autoshaping chambers were 30.5cm x 24.1cm x 21.0cm, with steel front and back plates, and clear plastic sides, ceiling, and floor. A red house light was mounted on the top of the back wall, which was lit during all training sessions. Two retractable levers were present on either side of the front of the chamber, one which extended periodically during autoshaping sessions (the CS+ lever), and another that was always extended during autoshaping sessions (the control CS- lever). The CS+ lever also contained a white light that illuminated during cue presentations, and the box was equipped with a tone generator. A sucrose delivery dish was located between the levers near the floor of the front of the box. An infrared beam was incorporated into the sucrose dish to measure number of entries. A computer equipped with MED-PC software (Med Associates, Inc.) controlled all events and recorded behavior during training sessions.

Testing Procedures

General autoshaping procedures: Prior to training, rats were handled for 3 days, and exposed to 20 sucrose pellets/rat in their home cage overnight before autoshaping training. Rats then received 1 day of magazine training: 'free' sucrose pellets on a variable interval (VI)-60sec schedule for 20 min to habituate them to taking pellets from the sucrose dish. The control lever was extended throughout this session, but the CS+ lever was never extended.

All autoshaping sessions consisted of twenty-five Pavlovian pairings of the CS+ (8sec, VI-90sec schedule) with one 45mg sucrose pellet UCS delivered into the sucrose dish. Sessions ended 90sec after the 25th cue (35-45 min). Rats were handled for ~5 min prior to each session, during which time microinjections were given, or stylets were removed, cleaned, and replaced to approximate microinjection procedures.

Effects of amygdala opioid stimulation and inactivation on approach to previously learned CSs: Following recovery from surgery, rats were trained on autoshaping procedures for 5 days without microinjections. On the 6th day, rats received a

microinjection of either vehicle or 0.1µg DAMGO in counterbalanced order, then immediately placed into autoshaping chambers for testing. Forty-eight hours later they received the other microinjection, and were tested again. After 7 additional days of drug-free training, rats then received muscimol and vehicle in counterbalanced order on the next 2 testing days at least 48 hours apart (days 15&16).

CS+ preferring rats were classified as those that approached, and initiated an average of at least 2 consummatory interactions (nibbles, sniffs, and bites) per cue directed at the CS+ lever on any of the first 5 drug-free days [lever interactions/cue on day 5, m(SEM)=5.2(0.4)], and that displayed >5X more consummatory behaviors toward the CS+ lever than the sucrose dish during cue periods by the final day (FC interactions m=0.8(0.2); n=15). CS_{source} preferring rats were classified as those that approached and attempted to consume the sucrose dish >2 times/cue [dish consummatory interactions/cue on day 5, m=3.0(0.5)], and that interacted with the sucrose dish >5X more times than the CS+ lever [m=0.3(0.1)] during cues. All rats met classification criterion for at least one CS. Based on these individual differences, we will use the phrase 'prepotent CS' to denote the CS+ lever for rats that preferentially approached and attempted to consume the CS+ lever during cues, and to denote CS_{source} for rats that preferentially approached and attempted to consume the sucrose dish instead. Conversely, the phrase 'non-preferred CS' will refer to the other cue (CS_{source} in CS+ preferring rats, and CS+ lever in CS_{source} preferring rats).

Effects of amygdala opioid stimulation and inactivation during learning on CS appetitive interactions: Rats were randomly assigned to receive vehicle (n=30), DAMGO (n=30), or muscimol (n=4) prior to each of the first 6 days of autoshaping training (4 days in the muscimol experiment). Procedures were otherwise identical to above. Rats were classified as 'CS+ preferring' or 'CS_{source} preferring' based on their performance on the first three days of training as described above.

Effects of amygdala opioid stimulation and inactivation on food UCS consumption: It seemed important to confirm that modulation of CS-directed food consummatory-like behaviors was due to changes in incentive salience by testing whether motivation to consume a UCS reward was also altered in the same directions. Therefore, 31 rats from

the above experiments were subsequently tested for food intake and UCS consumption-related behaviors on separate trials after either DAMGO or muscimol microinjections. Food intake tests were carried out in transparent plastic tub cages with pre-weighed food (Purina chow pellets), *ad lib* water, and corn cob bedding. Each rat was always tested for 1hr intake in the same cage after microinjections into amygdala of vehicle and either DAMGO (0.1μg, n=22), or muscimol (0.25μg, n=9) in counterbalanced order on separate days 48 hrs apart. Food and water intake was measured in grams, and behavior was videotaped for subsequent analysis of eating, locomotion, and other behaviors.

Behavioral Videoscoring

All video analyses were scored in slow motion (1/10th to ½ actual speed) by observers blind to experimental conditions. For autoshaping experiments, a video camera positioned under the transparent floor of the autoshaping chamber provided a clear view of the rat's head and body. In addition, presses on the CS+ lever and control CS- lever were automatically recorded, and nose pokes into the sucrose dish were recorded by sucrose dish photobeam breaks. Behavior for the 8 seconds of the 5th, 10th, 15th, 20th and 25th CS+ presentations of each scored session (and the 8 seconds prior to each cue in the autoshaping expression experiments) was also coded. Consumption-like nibbles and sniffs of the CS+ lever and CS$_{source}$ were the primary measures of attempted cue consumption. *"CS Nibbles and sniffs"* were small-amplitude, short duration (<0.5sec) exploratory movements of the mouth or nose upon the lever or sucrose dish, in physical contact with it, typically occurring in bouts of 1-2sec each. Nibbles and sniffs were quite frenzied in appearance and speed (1-3Hz), and resembled exploratory nibbling and sniffing of chow pellets that initiate normal food intake behavior. Most rats transitioned from a preliminary nibbling and sniffing period to a period of *"CS slow bites,"* where rats grabbed the lever with one or both paws, and clearly opened and closed their mandibles upon it over 0.5-1sec. Slow biting typically occurred from 1-6 times/CS+ period, and resembled bites of chow taken during food normal food intake behavior in rats.

In food intake tests, scored behaviors included time spent eating, number of eating bouts, latency to eat, food sniffing and nibbling bouts, time drinking, number of drinking

bouts, occurrences and seconds of defensive paw treading, bouts of digging in bedding, cage crosses, and rears.

Statistical Analyses

Autoshaping results were analyzed with mixed ANOVAs, using within-subjects factors of drug (DAMGO vs. vehicle, muscimol vs. vehicle) and period (the 8sec prior to each scored cue vs. the 8sec of each cue), and between-subjects factor of preferred cue (CS+ or CS_{source}). Training day was also a within-subjects factor in acquisition experiments. Bonferroni corrected t-tests and repeated measures ANOVAs were used to assess interactions. No order effects were found between drug and vehicle days in autoshaping expression and food intake experiments, so data were collapsed across days for all analyses. Food intake and other general behavioral effects of DAMGO and muscimol were analyzed with paired samples t-tests comparing drug to vehicle days. When percentage increases were reported to describe the magnitude of DAMGO and muscimol effects, raw data were adjusted by adding 1 to every score, to avoid the problem of calculating percentage increases over 0 for behaviors with low baselines.

Anatomical Specificity of Microinjection Effects: Fos Plumes and Anatomical Controls

In order to map behavioral effects of CeA microinjections, and assign responsibility to the relevant anatomical sites, we measured local plumes of neuronal modulation caused by microinjections of DAMGO, muscimol, or controls (total n=17). Local Fos plumes were measured, and plume maps of effects were constructed as described previously (Peciña and Berridge, 2005; Mahler et al., 2007; Smith and Berridge, 2007). Fos plumes were mapped in a separate group of naïve rats to ensure maximal estimation of Fos plumes, which are likely to be largest for initial microinjections on the first test day in naïve rats due to progressive gliosis and related factors that might impede microinjection impact and shrink plumes after multiple microinjections. By measuring Fos plumes in separate rats, and then mapping observed average plume sizes onto behavioral microinjection data as in previous studies, we aimed to avoid underestimation of plume size. Similarly, by measuring plumes in naïve rats we avoided baseline elevation of Fos in limbic structures due to training and reward anticipation that might

impose ceiling effects on the magnitude of drug-induced plume elevations in Fos, and thus lead to underestimation of drug plumes. Avoiding underestimation of plume size seemed important to avoid the danger of false inference of overly precise localization of function, which could result if plume estimates were smaller than actual plumes. This procedure follows similar methods in previous studies (Peciña and Berridge, 2005; Mahler et al., 2007; Smith and Berridge, 2007).

Vehicle or drugs were bilaterally infused identically to above (μ opioid agonist DAMGO, 0.1μg dose, n=6; GABA$_A$ agonist muscimol, 0.25μg dose, n=3; or sterile isotonic saline vehicle, n=4). Additional control rats received sham surgeries but no cannula implantation or microinjections in order to assess spontaneous Fos levels in the absence of any cannula-associated gliosis (n=4). Rats were deeply anesthetized, and 75 min later brains were removed and processed for Fos-like immunoreactivity (see Peciña & Berridge, 2005 for details). DAMGO and muscimol Fos plumes were mapped based on the percentage change in Fos-like immunoreactivity surrounding injections sites after DAMGO or muscimol vs. controls, measured in blocks along each radial arm (excitatory plume = 2X and 3X elevations above control levels; inhibitory anti-plume = >50% decline from control levels). Baselines were measured in central amygdala and the surrounding structures of intact brains to assess normal expression, and around the site of vehicle microinjections similar to drug microinjections (Figure 2.1). Nearby slices were stained for Substance P to identify landmarks for comparison to a brain atlas (Paxinos and Watson, 2007).

Fos immunoreactivity was visualized with the ABC method, and plume radii were measured and plotted using procedures described elsewhere (Peciña and Berridge, 2000, 2005; Smith and Berridge, 2005; Mahler et al., 2007; Smith and Berridge, 2007). Although group sizes were relatively low, observed plume sizes were consistent with these previous reports. The size of plume symbols was assigned based on the average radii of Fos plumes for that drug. The color of each plume symbols was coded to show the change in behavioral effects produced by drug microinjection at the corresponding site. The bilateral cannulae for each rat were plotted separately to depict every placement (2 sites per rat). For rats that received drug and vehicle after training, symbol color was calculated based on comparisons with each animal's own vehicle day. For rats that

received microinjections during training, drug rats were plotted with plume symbols, and colors based on comparison with the average of vehicle data. Cannulae placements for animals receiving vehicle were marked with 'Xs.' Maps were always plotted separately in sagittal, coronal, and horizontal planes to construct a 3-dimensional map set.

Results

Synopsis

Activation of μ opioid circuits in central amygdala by DAMGO microinjection enhanced appetitive and consummatory behaviors directed toward each rat's own prepotent CS, more than toward the alternative CS or other stimuli in the chamber. The CS+ for sucrose reward (insertion of a metal lever through the wall into the chamber, accompanied by a tone) elicited appetitive and consummatory behaviors directed either at the CS+ lever itself, or at the metal dish where sucrose was delivered (CS$_{source}$) within several days of training. When the CS+ was presented, rats began to nibble and sniff, then bite their prepotent CS (either the CS+ lever or the CS$_{source}$ sucrose dish). These bouts of intense consummatory behaviors were time-locked to the 8sec duration of the lever CS+. The effect of DAMGO microinjections in CeA was to increase the number and intensity of appetitive and early-phase consummatory behaviors of the preferred CS (exploratory nibbles and sniffs; Figures 2.2&2.3). Appetitive and consummatory behaviors directed towards the prepotent CS were reliably enhanced whether DAMGO was administered during learning or after learning. DAMGO also subsequently enhanced food intake or UCS 'wanting' in the same rats, consistent with the conclusion that it enhanced the incentive salience of both food and food cues. Conversely, inactivating the CeA with muscimol during or after learning suppressed approach and consummatory behaviors directed toward the prepotent CS, and suppressed UCS food intake.

Fos Plumes and Functional Spread of Drug Microinjections

Fos plume maps indicated that microinjection effects were chiefly mediated by the CeA. Vehicle microinjections induced only tiny plumes of moderate elevation with no zone of intense elevation. DAMGO microinjections produced roughly spherical Fos

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plumes in the central nucleus of amygdala, with a total volume of tissue affected being about 0.43mm^3 (0.47mm mean radius, used for mapping symbols). Muscimol microinjections caused small central Fos-excitatory plumes near the microinjection center, surrounded (especially ventrally) by larger inhibitory zones or 'antiplumes' that had only half the Fos density of normal tissue (and even less compared to vehicle-injected tissue). Overall, the total volume of muscimol-induced neuronal modulation (central Fos plume + outer Fos anti-plume) was 0.21mm^3, which was used for mapping symbols (0.37mm total radius). Example Fos plumes are shown in Figure 2.1, and complete Fos plume radii and estimated volumes are listed in Table 1.

The entire volume of the unilateral CeA nucleus was estimated to be roughly 1.27mm^3 (~2mm AP x between 0.4-1.3mm DV and ML), and we calculated the average DAMGO plume center of intense Fos elevation to fill about 2.4% of CeA volume, whereas its outer plume of moderate Fos filled about 43% of CeA volume. The largest muscimol outer plume (actually, an inhibitory antiplume) similarly filled about 36% of CeA volume. Beyond the CeA, the larger amygdala complex (including basolateral, medial and basal nuclei as well as central nucleus) was estimated to be ~8.7mm^3, which implied that each outer DAMGO or muscimol plume/antiplume filled about 5-6% of the entire amygdala complex (amygdala between AP levels 1.32 and 3.36 caudal of Bregma; ~2mm AP x 1.4-2.9mm DV and ML).

Anatomical Containment of Plumes in CeA

Over 94% of rats had moderate or greater Fos plumes contained largely within the CeA (n=79), and most cannula placements were located in the central nucleus (88% of rats had at least unilateral CeA placement, n=74). Intense DAMGO Fos plumes were entirely contained within the CeA on at least one side for 69% of rats (n=58), and on both sides for 57% of rats (n=48). On average, 74% of each CeA-centered DAMGO plume stayed entirely within the boundaries of CeA, including nearly all of the most intense Fos activation zones. The remainder of peripheral plumes penetrated the basolateral (BLA), basomedial (BMA), or medial (MeA) nuclei of amygdala, or the interstitial nucleus of the posterior limb of the anterior commissure (IPAC; Figure 2.1). 25% of rats (n=21) had at least moderate Fos plume penetration into BMA as well as CeA, a structure that is also

involved in emotional learning and food intake (Goosens and Maren, 2001; Smith et al., 2002). Behavioral effects of DAMGO and muscimol in these rats were no different than in the remainder of rats that had no plume penetration into BMA, however, suggesting that drugs primarily acted in CeA to modify appetitive behavior. Only 33% of rats (n=28) had any plume penetration of their BLA, and only 11% had injection centers or intense Fos plumes in BLA. Therefore we selected rats with moderate Fos plumes that were at least 50% contained within CeA for primary analyses of behavioral effects (n=79), and contrasted them to rats with plumes in other structures.

Classification of Prepotent CS Preference:

All rats developed a prepotent CS, which they approached and 'consumed' during CS+ presentations on average >7 times more than the alternative CS, and at least three times more often than the other CS [($F_{(4,88)}=8.3$, $p<0.001$ for group tested after learning; $F_{(2,90)}=5.4$, $p<0.01$ for group tested during training). Approach and consummatory sniffing, nibbling, licking, and biting movements were directed specifically to the prepotent CS. Individual rats in the drugs post-training or drugs during training experiments preferred either the CS+ (~70% of rats), or the CS_{source} (sucrose dish; ~30% of rats). Over 70% of all rats preferred to approach and attempt to consume their prepotent CS nearly exclusively (over 9 of 10 cue interactions were with the preferred CS). Only about 10% of rats interacted with both cues roughly equally (though always slightly more on one or the other), and 20% of rats interacted with the both cues on a 2:1 basis.

Peaks of consummatory behavior toward the prepotent CS were always time-locked to insertions of the lever CS+ through the wall into the chamber ($F_{(1,22)}=78.6$, $p<0.001$). Each rat's preference for a particular CS stimulus remained stable across days, and CS prepotency was not redirected by DAMGO, but merely intensified in the sense of increasing consummatory behaviors toward that CS, but not changing behaviors directed toward other CSs ($\chi^2=1.8$, *n.s.*; no interaction of type x drug on CS+ nibbles and sniffs: $F_{(2,90)}=2.4$, *n.s.*).

DAMGO Enhances Incentive Salience of Previously-Learned Prepotent CSs

DAMGO microinjections ($0.1\mu g/0.5\mu l$) specifically enhanced by up to 220% over vehicle levels the number of nibbles and sniffs directed toward a rat's prepotent CS ($F(1,21)=9.2$, $p<0.01$). DAMGO increased appetitive and consummatory behaviors toward the preferred CS only, and not toward the non-preferred CS (interaction of cue type x drug on during cue nibbles and sniffs: $F(1,21)=6.7$, $p<0.05$). Thus, in CS+ preferring rats, DAMGO specifically enhanced consummatory nibbles and sniffs of the CS+ lever by up to 218% of vehicle day levels (mean increase=146%, $t=4.7$, $p<0.001$). Conversely in CS_{source} preferring rats, DAMGO microinjections in anterior CeA increased nibbles and sniffs of the sucrose dish to up to 200% of vehicle levels (mean increase=120%, $t=10.1$, $p<0.01$) when the CS+ was present. As DAMGO enhanced appetitive/consummatory nibbles and sniffs that typically initiated bouts of ingestive-type behaviors, it simultaneously excluded other more instrumental and terminal behaviors, thus reducing paw pressing of the lever (computer-scored lever presses 47% of vehicle; $F(1,14)=14.8$, $p<0.01$) and slower, discrete bites (lasting over ~0.5sec in duration) that tended to terminate ingestive sequences (79% of vehicle day, $F(1,14)=12.4$, $p<0.05$), in a manner suggesting response competition.

DAMGO During Training Also Enhanced CS Consummatory Behavior: Similarly, the separate group of rats that received opioid stimulation of central amygdala while they were initially learning CS/UCS associations (days 1-6) showed enhancements of appetitive and consummatory behaviors directed toward their prepotent CS, but only beginning around day 4 $F(1,47)=7.9$, $p<0.01$) (Figure 2.3&2.7). On the initial 3 training days, DAMGO did not increase cue nibbles or sniffs during CS+ presentations ($F(1,47)=2.6$, *n.s.*), nor advance the day of preference acquisition ($t=0.26$, n.s.), though it did increase sucrose dish entries in all animals during non-cue periods (main effect of drug in precue period for days 1-3, $F(1,47)=8.6$, $p<.01$).

Detailed Behavioral Topography of DAMGO Effect: Consummatory behaviors directed toward preferred CSs were usually observed to follow a predictable sequence, which was similar to behaviors observed in UCS food intake. When eating a UCS food pellet, rats approach and vigorously nibble and sniff it for 2-10 sec, then pick up the pellet and subsequently transition into slower, more discrete bites that terminate the consummatory sequence with actual ingestion of food. Food cue-oriented behaviors in

autoshaping followed a similar sequence, with 2-3 intense, frenzied bouts of sniffs and nibbles, which are repeated several times in rough alternation during each 1-2sec bout. Thus nibbles and sniffs appear to be a transitional appetitive-consummatory phase of behavior, reflecting initial moments of intense exploration and motivated interest in the CS, similar to what would be expected of an attractive, salient stimulus. Sniffs and nibbles were usually followed by a period of slower, discrete bites lasting ~0.5sec each, more similar to the terminal stereotyped biting and swallowing movements of ingesting actual food, which often terminated the CS consummatory sequence, and competed with the preliminary period of nibbling and sniffing for expression within 8sec cue periods. For the prepotent CSs, the main effect of DAMGO in CeA was to prolong the initial phase of intense exploratory nibbling and sniffing, postponing or even displacing the terminal bites because the lever disappeared before the rat stopped showing nibbles and sniffs (Figure 2.2). DAMGO specifically potentiated the initial phase of vigorous nibbles and sniffs, as if the CS+ lever had suddenly become a more attractive and salient stimulus worth of intense investigation. In short, DAMGO did not simply enhance all pre-existing cue-directed behaviors, or cause rats to confuse the CS with food or actually try to eat the metal object (with terminal bites), but specifically potentiated a pattern of appetitive approach and intense initial consummatory behaviors that are plausibly linked to incentive salience.

Specificity of DAMGO Effects: DAMGO selectively enhanced appetitive and consummatory behaviors that were triggered by appearance of the lever CS+, and did not enhance these nibbles or sniffs of a CS in intervening periods in absence of CS+. Even for the CS$_{source}$ dish that was always present, DAMGO failed to enhance approach or consummatory behaviors in the absence of CS+ lever (interaction of cue period x drug on preferred cue nibbles and sniffs: $F(1,21)=9.0$, $p<0.01$). Thus for all CS consummatory behaviors, enhancements were time-locked to the presence of the CS+, occurring as phasic peaks that typically decayed to baseline within 1-2 sec of the end of CS+ presentation.

As mentioned above, a few rats approached and attempted to consume the CS+ and CS$_{source}$ cues roughly equally during the CS+ presentation. Even for these rats (n=3), DAMGO selectively enhanced approach and attempted consumption of only one cue

(CS+ for 2 rats, and CS_{source} for the remaining rat). Thus, even in rats that are ordinarily attracted to both CSs, CeA DAMGO enhanced the incentive salience of only one CS at a time. Similarly, DAMGO never enhanced the near-zero levels of consummatory behaviors directed toward the immobile control CS lever which was always present (during or pre-cue effect of DAMGO on control lever nibbles and sniffs, ts<1, *n.s.*).

DAMGO enhancement of appetitive interactions with the CS faded after the drug had cleared: when rats were tested drug-free 48-96 hours after DAMGO (day 8 of training, following DAMGO administration on day 6 or 7), DAMGO enhancement of consummatory behaviors directed toward the preferred CSs totally disappeared [cue nibbles and sniffs on day 8 (m=3.8(0.3)) were reduced from the previous DAMGO day for the same rats (m=5.0(0.4); $F_{(1,21)}=8.1$, p=0.01) and were no longer different than vehicle day (m=3.8(0.4); $F_{(1,21)}=0.1$, *n.s.*)]. This result indicates that enhancement of appetitive and consummatory behaviors directed toward a prepotent CS is transient and reversible, and depends on current opioid levels in CeA.

Anatomical Ccontainment Within CeA: DAMGO injections had the strongest effects on prepotent CS consummatory behaviors when microinjections were centered directly into the CeA, as opposed to just outside it (179% enhancement of preferred cue nibbles and sniffs from bilateral CeA injections, 155% for unilateral, and 118% for injections centered bilaterally within 0.4mm from, but just outside the borders of CeA, vehicle=100%; Figure 2.3). By contrast, DAMGO in neither the caudal BLA nor rostral IPAC (total n=4) increased CS consummatory behaviors. When data were pooled across these extra-CeA control sites, a trend for DAMGO to slightly *decrease* nibbling and sniffing of their prepotent CS was observed (t=2,4, p=0.095), suggesting that DAMGO enhancements of incentive salience were likely to be due to CeA activation and not to surrounding structures. In addition, CeA itself may have had subregional differences. Only sites in the anterior half of the central amygdala produced DAMGO enhancements of consummatory sniffs and nibbles of the CS_{source} sucrose dish in CS_{source} preferring rats (between -1.4 and -2.04mm caudal of Bregma; 132% of vehicle day consummatory behavior). Further, DAMGO at sites in the posterior half of the CeA actually decreased consummatory behaviors toward the CS_{source} (sites between -2.04 and -3.4mm caudal of Bregma: 87% of vehicle day, t=2.9, p<0.05; anterior vs. posterior difference: $F_{(1,7)}=7.7$,

p<0.05). By contrast, the entire CeA appeared to support enhancements of CS+ lever interactions in rats that preferred this CS, suggesting interaction between cue prepotency and subregional differences that deserves further exploration.

Baseline Effect of DAMGO in the Absence of Cues

DAMGO appeared to produce a general locomotor enhancement in the absence of the CS+. In the periods between CS+ presentations, DAMGO increased the number of head entries into the sucrose dish for both CS+ and CS_{source} preferring rats (main effect of drug in precue period, $F_{(1,22)}=331.6$, $p<.001$), especially CS+ preferring rats whose preferred CS+ was not then present (interaction of rat cue preference x drug, $F_{(1,22)}=6.0$, $p<0.05$). Touches of the control CS- lever, as well as the number of rears were also increased by DAMGO during between-CS+ periods (interaction of drug x period, CS-lever: $F_{(1,22)}=6.0$, $p<0.05$; rears: $F_{(1,22)}=13.8$, $p=0.001$). DAMGO had no effect on rears and control lever touches during CS+.

DAMGO Enhances Food UCS Consumption

In separate food intake tests, opioid stimulation of central amygdala doubled UCS chow intake compared to vehicle levels (grams eaten: $t=6.1$, $p<.001$). DAMGO increased the cumulative duration of eating behavior to >350% of vehicle levels: ($t=2.9$, $p<0.01$), and tripled the number of eating bouts initiated (358% of vehicle: $t=3.1$, $p<0.01$; Figure 2.5). DAMGO also increased the number of sniffs of food pellets (165% increase in frequency; $t=2.4$, $p<0.05$), and pellet pickups (234% frequency increase; $t=2.1$, $p=0.05$), though the total number of sniffs displayed toward food per bout was always far lower than was directed by the same rats toward their prepotent CS in autoshaping testing (0.5-2sec of UCS sniffing, 4-7sec prepotent CS sniffing and nibbling). In contrast, DAMGO did not alter drinking behavior (drinking bouts, drinking time, drinking time/bout: ts<1, *n.s.*; Figure 2.6), or chewing of non-food objects in the cage, such as control lever or lights, pieces of bedding or excrement (ts<1, *n.s.*).

Muscimol Inactivation of CeA Suppresses Incentive Salience of Prepotent CSs

Inactivation of the CeA by muscimol microinjections dramatically suppressed CS+ triggered consummatory behaviors directed at the prepotent CS, reducing levels to 10-

30% of vehicle levels (t=3.3, p<0.01). Muscimol did not affect the already low levels of consummatory behaviors directed toward the non-preferred CS (prepotent vs. non-preferred cue: $F_{(1,21)}=64.7$, $p<0.001$; no effect of muscimol on non-preferred cue: $t=2.0$, $n.s.$; Figure 2.4). Muscimol microinjection reduced cue-triggered consummatory nibbles and sniffs of the preferred CS in both CS+ and CS_{source} preferring rats, and CS+ slow bites (cue period x drug interaction on nibbles and sniffs: CS+ rats: $F_{(1,22)}=25.3$, $p<0.001$; CS_{source} rats: $F_{(1,22)}=12.12$, $p<0.001$; period x drug interaction for slow bites: $F_{(1,14)}=9.0$, $p=0.01$). Muscimol similarly reduced prepotent CS approach and consummatory interactions when administered during training, with lever consummatory behaviors being decreased by over 70% from vehicle levels by day 4 (interaction of drug x test day for CS+ nibbles and sniffs: $F_{(3,18)}=4.3$, $p<0.05$, t test on day 4: $t=3.5$, $p<0.05$). However, rats still developed cue-triggered approach responses to the sucrose dish during the lever CS+ (main effect of pre vs. during cue period: $F_{(1,6)}=7.3$, $p<0.05$), and did not significantly differ from vehicle rats in CS_{source} entries (vehicle m(SEM)=0.84(0.25)), muscimol m=0.77(0.34), $F_{(1,6)}=0.01$, $n.s.$; Figure 2.8). Thus overall, inactivation of CeA prevented rats from attributing strong incentive salience to a discrete CS+, whether they were still learning its motivational significance during training, or had already learned on previous days before the motivational expression was suppressed.

Suppression of CS appetitive-consummatory behaviors by muscimol appeared to be stronger at anterior sites in central amygdala (defined as between 1.4-2.04mm behind Bregma: 31% of vehicle levels) than at posterior sites (2.04-3.4mm behind Bregma: 46% of vehicle; main effect of cannula placement on prepotent CS nibbles and sniffs ($F_{(1,13)}=9.5$, $p<.01$). The rostrocaudal difference again suggested that anterior central amygdala may be more sensitive than posterior CeA for modulating the incentive salience of prepotent CSs. In contrast, animals with cannulae outside CeA did not show a significant muscimol suppression of consummatory behaviors ($t=1.97$, $p=0.14$), though the two animals with sites in rostral basomedial nucleus or basolateral nucleus of amygdala did show signs of some muscimol suppression of interactions with the prepotent CS.

In baseline periods between CS+ presentations, muscimol decreased corner sniffing and rearing behavior (interaction of period x drug; sniffing: $F_{(1,22)}=23.6$, $p<0.001$; rears:

F(1,22)=19.3, p<0.001), and conversely increased the duration spent totally immobile (F(1,22)=8.4, p<0.01).

CeA Muscimol Reduces UCS Food and Water Intake

During autoshaping test sessions muscimol decreased eating behavior and suppressed the retrieval and consumption of UCS sucrose pellets to 30-40% of vehicle levels (t=7.8, p<0.001 for post-learning test; F(1,6)=13.31, p<.01 for during-training test; however sucrose pellet eating rebounded to >85% from the second test day on in the during-training DAMGO experiment).

In the separate free intake test, using normal chow pellets that may have been less palatable than sucrose, muscimol microinjections in CeA completely abolished food intake to zero (t=2.8, p<0.01). Muscimol also reduced video scored eating behaviors, including the number of eating bouts (t=3.5, p=0.001), cumulative duration of time spent eating (Figure 2.5; t=3.0, p<0.01), and the total number of food sniffs (51% of vehicle, t=2.5, p<0.05). CeA muscimol also reduced water intake bouts (t=4.2, p<0.001) and time spent drinking (t=4.0, p<0.001; Figure 2.6), but did not affect the number of bouts or total time spent chewing objects such as bedding or excrement (ts<1, *n.s.*).

In contrast to CeA muscimol suppression of eating, and somewhat surprisingly, we note that muscimol in caudal basolateral nucleus of amygdala robustly stimulated eating behavior (between 3.0 and 3.24mm caudal, and 9.0 to 9.4mm ventral to Bregma) in both rats that had sites there. These BLA sites were too distant from CeA for their Fos plumes to have penetrated the CeA (Figure 2.9). Muscimol at these caudal BLA sites more than quadrupled food intake (409% of vehicle, t=6.55, p<0.001), the number of eating bouts (614% vehicle, t=5.6, p<0.001), time eating (376% vehicle, t=3.1, p<0.01), and the incidence of carrying food pellets (627% vehicle, t=5.3, p<0.001). Eating stimulation by muscimol in caudal BLA is a novel preliminary finding that may depend upon dose and location of injection within BLA, as a very low dose (0.02µg; less than 1/12 of our 0.25µg dose) in BLA is reported to not induce eating (Will et al., 2004; Baldo et al., 2005).

Other General Behaviors Elicited by Amygdala Opioid Stimulation or Muscimol Inactivation

Defensive Treading Behavior Elicited by DAMGO and Muscimol: Beyond reward-related behavior, the central amygdala is well known for involvement in fear-related behavior. Our CeA manipulations may have additionally modulated defensive, as well as appetitive motivation. Defensive treading is an innate fearful response in rodents; elicited in the wild by rattlesnakes, scorpions or similar threats, and elicited in the lab by discrete threats like shock prods (Owings and Coss, 1977; Treit et al., 1981; Rodgers et al., 1997). Treading involves forward motions of the forepaws that push and throw dirt or bedding forcefully in the direction of the perceived threat (predator, laboratory shock prod in chamber, or experimenters nearby), and microinjections of GABA/glutamate agents in limbic brain structures can sometimes elicit treading behavior in the absence of an identifiable threatening stimulus (Reynolds and Berridge, 2001; Smith and Berridge, 2005).

Defensive paw treading behavior was observed in about 20% of rats after CeA DAMGO in chambers containing corn cob bedding, but only very rarely observed after vehicle microinjections (<4% of rats tread more than once/1hr session; main effect of drug: $F(1,22)=5.9$, $p<0.05$; Figure 2.10), and was never observed in autoshaping chambers that had bare floors. DAMGO-induced treading was moderate in intensity ($m=4.5(2.5)$), and was oriented toward the corners and walls of the testing cage, especially the front wall through which the experimenters and cameras could be seen. Defensive treading generally alternated with periods of food intake. Rats were sometimes even observed to sniff and pick up a piece of food, quickly drop it and tread at it for 1-2 seconds, then immediately return to the food pellet and begin eating again. DAMGO-induced treading occurred most frequently in animals with placements centered in the ventral half of the CeA, bordering on BLA and BMA (8.4-9.8mm ventral of Bregma) compared to rats with dorsal CeA sites (7.0-8.4mm ventral of Bregma; drug x dorsal/ventral placement interaction: $F(1,22)=4.3$, $p=0.05$; Figure 2.10).

Inhibition of CeA by muscimol microinjections elicited even stronger defensive treading behavior than CeA DAMGO ($m=34.6(12.8)$ seconds treading/session; 71% of animals; $t=2.7$, $p<0.05$; Figure 2.10). Again, treading only occurred in testing chambers that contained bedding. Muscimol-induced treading was more prolonged and did not alternate with periods of food intake as with DAMGO-induced treading, and was very

rarely oriented toward food pellets. CeA muscimol-induced treading was comparable to that seen after AMPA antagonist microinjections in the caudal accumbens shell under comparable testing conditions (Reynolds and Berridge, 2008). In control sites, muscimol did not cause significant defensive treading behavior overall (t=1.3, *n.s.*), though one animal caudal BLA cannulae placements did exhibit a moderate level of treading (20 seconds/60min session).

Other Behavioral Effects of DAMGO and Muscimol: DAMGO moderately enhanced general locomotor behaviors in food intake chambers, including cage crosses (147% of vehicle; t=2.2, p<0.05) and rears (146% vehicle; t=2.2, p<0.05). However, DAMGO decreased the number of grooming bouts (t=2.4, p<0.05), and did not affect the amount of time spent sleeping (t=1.3, *n.s.*).

Muscimol generally decreased locomotor activities, including rearing (54% of vehicle, t=2.6, p=0.01) and grooming (17% of vehicle levels, t=3.7, p=0.001). Cage crosses were not affected, however (96% of vehicle, t=0.2, *n.s.*), indicating that rats still moved around the chamber after muscimol but specifically reduced their grooming behavior and vertical exploration.

Most rats (83%) also displayed unusual spontaneous digging movements after CeA and control site muscimol in both autoshaping and food intake testing situations (Baldo et al., 2005), punctuated by brief (5-30sec) periods of total immobility, which were never seen after vehicle or DAMGO microinjections (main effect of muscimol on digging in autoshaping boxes: F(1,22)=20.4, p<0.001; t-test on muscimol vs. vehicle in food intake chambers: t=2.4, p=0.05; Figure 2.6). These spontaneous digging behaviors were different from defensive treading in movement morphology and orientation, including directional thrust of paw movements and position of the paws during the thrust (digging scooped or pulled bedding toward the body, treading pushed bedding away), length of bouts (digging occurred in bouts of 10-240sec, treading occurred in bouts of ~1sec), orientation (treading was directed at cage corners or other discrete stimuli, digging was not directed at any stimulus), and ongoing behavior (digging occurred during linear or circular forward locomotion, while treading occurred while the animal was stationary or moving backwards).

Discussion

Here we demonstrated that central amygdala (CeA) is a key brain substrate of appetitive motivation directed at reward cues. Pavlovian cues for reward elicit approach, but can also become attractive and salient, and elicit consummatory behaviors normally directed at rewards. Our results show that activation of CeA μ opioid mechanisms increases the amplitude of incentive salience and focuses it on a particular CS. Stimulating CeA with DAMGO microinjections potentiated intense exploratory and nibbling and sniffing behaviors that are usually directed at food itself, but here were directed at the starkly artificial metal lever or dish that had become a prepotent CS. CeA thus increased motivated behavior focused upon already prepotent cues, a process which would normally guide behavior toward rewards.

Each rat had its own individual 'prepotent CS:' the incentive target it preferentially approached and seemed to begin to consume with intense nibbling and sniffing behaviors. For some rats, the prepotent target of cue-triggered incentive salience was the sucrose-predictive CS+ lever, whereas for others it was the sucrose-delivering CS_{source} dish. In either case, CeA opioid activation made the already prepotent CS even more powerfully able to pull in appetitive and consummatory behavior like a 'motivational magnet.' The sharpened focus of incentive salience upon a single CS was further illustrated by the observation that DAMGO amplified appetitive-consummatory behavior toward only one CS, even in rats that ordinarily had split their interactions between both cues. Thus CeA opioid activation appears to focus and intensify incentive salience upon one CS at a time, consistent with roles for CeA in specific Pavlovian-guided motivations (Cardinal et al., 2002b; McDannald et al., 2004; Corbit and Balleine, 2005).

DAMGO intensified approach and nibbling/sniffing behaviors of the prepotent CS that were similar to UCS-directed behaviors seen when rats begin to consume food pellets (Jenkins and Moore, 1973; Davey and Cleland, 1982). However, DAMGO-intensified behaviors remained sensitive to the physical features of whatever stimulus they were directed toward (e.g., CS+ metal lever versus UCS food pellet). For example, DAMGO stimulated appetitive and initial consummatory nibbles and sniffs of a prepotent

CS (to higher levels than are usually ever displayed toward actual food), but enhanced both these behaviors and actual consumption bites of UCS food. Such observations suggest that DAMGO does not simply activate stereotyped motor programs of approach or oral interactions, nor cause the metal CS to fully substitute for food, but rather takes on only incentive salience features that make the cues more attractive and interesting. Physical features of the CS remain important in determining in how motivated behavior manifests—just as physical features of the associated UCS determine whether a CS becomes an ingestive, social, sexual or other type of incentive (Jenkins and Moore, 1973; Lajoie and Bindra, 1976; Timberlake and Lucas, 1985; Tomie, 1996; Uslaner et al., 2006).

Conversely, inactivation of CeA with muscimol dramatically reduced appetitive and consummatory behaviors directed toward the prepotent reward CS, and similarly suppressed food UCS intake. These findings suggest that CeA circuits can bidirectionally control the amplitude of incentive salience either targeted at particular reward cues, or rewards themselves.

CeA Opioids Enhance Phasic Temporal Peaks of 'Wanting'

Although microinjected DAMGO remained in CeA at relatively constant or gradually declining levels during the half-hour after a microinjection, incentive salience enhancements generally were visible as transient peaks that were closely time-locked to coming and going of the reward-predictive CS+ (8-sec duration). This pattern of phasic enhancement was not simply due to CS availability, since it applied even to the CS_{source} dish, which was constantly present throughout the session. This interaction between DAMGO and the CS+ is similar to the pattern of phasic enhancements of cue-triggered 'wanting' for reward by mesolimbic dopamine stimulation (Wyvell and Berridge, 2001; Tindell et al., 2005). Phasic peaks of 'wanting' in both cases indicate that tonic mesocorticolimbic activation amplifies the in-the-moment incentive salience of reward stimuli as they are encountered, not by producing a steady drive or similarly constant state.

Anatomical Localization of Function

Fos plume maps indicated that local neuronal modulation caused by microinjections were largely contained within the CeA. Basolateral and basomedial amygdala subnuclei are also known to be important in reward learning and motivation (Nishijo et al., 1988; Wolinsky et al., 1994; Smith et al., 2002; Everitt et al., 2003; See et al., 2003; Corbit and Balleine, 2005; Petrovich and Gallagher, 2007), but Fos plumes rarely penetrated BLA, and so direct modulation of neurons there was not likely to have mediated observed behavioral effects here.

Some evidence indicated that a 'hotspot' for CS-directed consummatory effects might be localized in an anterior subregion of the CeA. DAMGO at sites in the rostral one-third of the CeA ($\sim$0.4mm^3 volume) nearly doubled consummatory behaviors directed toward a prepotent CS, whereas microinjections at more caudal CeA sites actually reduced CS consummatory behaviors below control levels. Similarly, muscimol inhibition of incentive salience was 15% more potent at rostral than at caudal sites in CeA. We note that the rostral subregion contains a greater portion than the caudal subregion of the medial subdivision of CeA, and therefore an anterior hotspot may receive greater gustatory projections and other afferents from parabrachial nucleus and solitary tract nucleus in brainstem, some of which are enkephalinergic (Zardetto-Smith and Gray, 1990; Bernard et al., 1993; Shammah-Lagnado et al., 2001). The rostral subregion also projects to a lesser extent to basal forebrain than other areas of CeA (Jolkkonen et al., 2002), and contains fewer GABAergic neurons, but many GABA terminals from other areas of CeA (Sun and Cassell, 1993).

Of course, we stress that incentive salience is not mediated by mechanisms contained solely within the amygdala. The CeA is embedded in larger mesocorticolimbic reward circuits, and connections with other structures in those circuits are likely to be involved in the effects described here (Cardinal et al., 2002a; Ahn and Phillips, 2003; Kelley, 2004; Kim et al., 2004; Levine et al., 2004).

Fear Versus Feeding Effects in CeA

Central amygdala manipulations appeared sometimes to elicit fearful or defensively-motivated behavior in addition to appetitive motivation. For example, defensive treading, associated with fearful motivation (Owings and Coss, 1977; Treit et al., 1981; Reynolds

and Berridge, 2008), is forceful pushing of bedding forward, typically directed here toward the experimenter visible through the transparent wall of the chamber (or its light-reflecting corners). Inactivation of CeA with muscimol caused defensive treading in most rats when they were tested in chambers that contained crushed corn-cob bedding suitable for throwing and pushing, though never in the autoshaping chamber with a bare plastic floor. Even opioid activation of CeA elicited defensive behavior from ~20% of rats tested in food intake cages. Fearful behavior appeared somewhat independent of appetitive motivation (i.e., defensive treading accompanied increased food intake after DAMGO, but accompanied suppressed food intake after muscimol). In a few rats after DAMGO, treading movements were even directed toward the same food pellet the rat ate a few seconds before or later. This mixture of fear and feeding motivation may reflect ambivalence in motivational salience generated by DAMGO, consistent with the possibility that the generation of desire and dread may share particular underlying limbic mechanisms (Faure et al., 2008; Reynolds and Berridge, 2008).

Addiction and Targeted Incentive Salience

Drug addiction and other compulsive motivational disorders often involve excessive motivational attraction pulled toward Pavlovian CSs. For example, some cocaine addicts 'chase ghosts,' drawn by their attraction to small white pebbles (Rosse et al., 1993), which has been suggested to result from sensitized levels of 'wanting' targeted on stimuli that resemble cocaine CSs (Robinson and Berridge, 1993). The incentive-sensitization theory of addiction posits that excessive motivational salience of drug cues contributes to cue-induced craving and relapse, even after long abstinence from drugs (Robinson and Berridge, 1993; 2003; 2008). Our results open a way to explain the puzzle of how incentive-sensitization could ever specifically amplify 'wanting' just for a drug-related CS while leaving CSs for other rewards unchanged (Vanderschuren and Everitt, 2005). At least in principle, our findings indicate that particular reward cues can be selectively targeted with enhanced incentive salience in a 'winner take all' fashion by activation of CeA opioids. We note that drug cues are reported to elicit amygdala activation (including opioid activation) in human addicts (Grant et al., 1996; Franklin et al., 2007; Scott et al., 2007). Further, we note the incentive salience of prepotent drug cues might also be primed additionally by heroin or any drug that produces amygdala

opioid activation, just as DAMGO microinjections enhanced prepotent cue 'wanting' here, thereby promoting increase in inclination to prolong a binge upon trying "just a little" drug. Similar CeA mechanisms, if endogenously activated, could participate in relapse to drug use or other addictions. In short, amygdala enhancement of focused incentive salience might contribute to aberrant Pavlovian motivation in drug addiction, binge eating, and other compulsive motivations.

Figure 2.1: Fos Plumes and CeA Anatomical Staining. *Fos Sampling Method:* Illustrates the method used to sample Fos expression around injection sites, in which radial arms for sampling extend from the center of microinjection sites, viewed in a coronal plane. Fos-expressing neurons are counted in 62.5 x 62.5μm blocks on arms spaced at 62.5μm intervals; 5x magnification. Insets show sample tissue blocks from equivalent sites in brains injected with nothing, vehicle alone, DAMGO, or muscimol. *Vehicle Plume:* Example changes in Fos following vehicle microinjection alone, compared to uninjected tissue. *DAMGO and Muscimol Plumes:* Example DAMGO and muscimol-induced plumes of Fos modulation. Colors indicate areas of Fos elevation by 2x (yellow) or 3x (red) over uninjected control levels for the relevant structure. Lines represent areas of Fos elevation by 2x (dashed line) or 3x (dotted line) over vehicle microinjection levels at equivalent sampling boxes and brain areas. Inhibitory muscimol 'anti-plumes' began on average 0.36mm from microinjection centers. *%Δ Fos by Distance From Injection:* Fos activation was plotted as percent change from uninjected tissue levels, as a function of distance from the center of microinjection. Data were averaged across rats for the vertical sampling arm, extending ventrally from the injection site, and compared to uninjected control Fos levels in equivalent brain areas. *Map Backgrounds: CeA Anatomical Stain:* Anatomical borders of the CeA were visualized in a sagittal view with a Substance P stain (left), and the inset shows the background used for mapping Figures 2.3-2.5 within the larger brain and atlas boundaries (right; adapted from (Paxinos and Watson, 2007), maximum rostrocaudal and dorsoventral extent of CeA depicted). Injection sites in behavioral and Fos animals were identified in coronal sections with adjacent slices stained for Substance P to identify the CeA, then transferred to a sagittal view for presentation. CeA=Central Amygdala, BLA=Basolateral Amygdala, MeA=Medial Amygdala, BMA=Basomedial Amygdala, I=intercalated nuclei, ic=internal capsule, GP=Globus Pallidus, PLH=Posterior Lateral Hypothalamus.

Fos Sampling Method

Vehicle Plume

DAMGO Plume

%Δ Fos by Distance From Injection

Muscimol Plume

Map Backgrounds: CeA Anatomical Stain

Substance P Stain

CeA Inset

Atlas Boundries
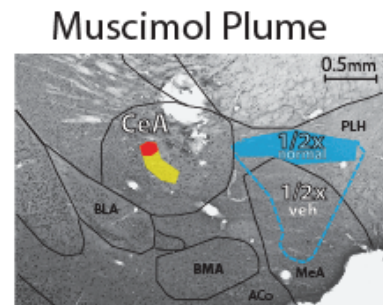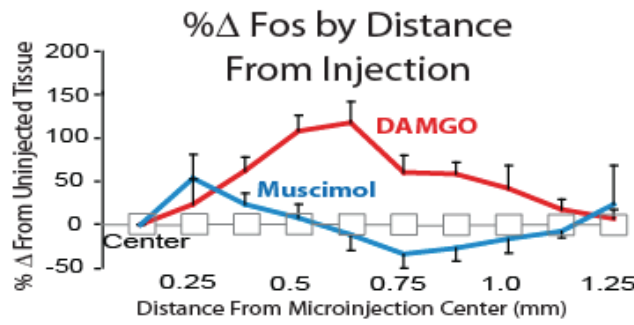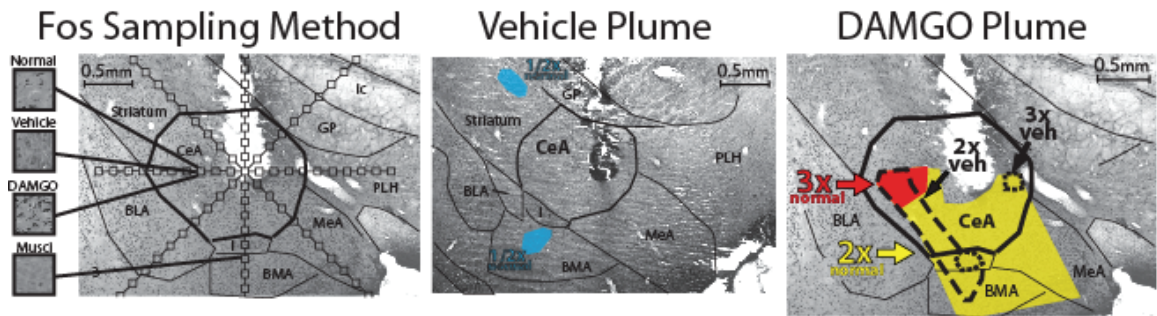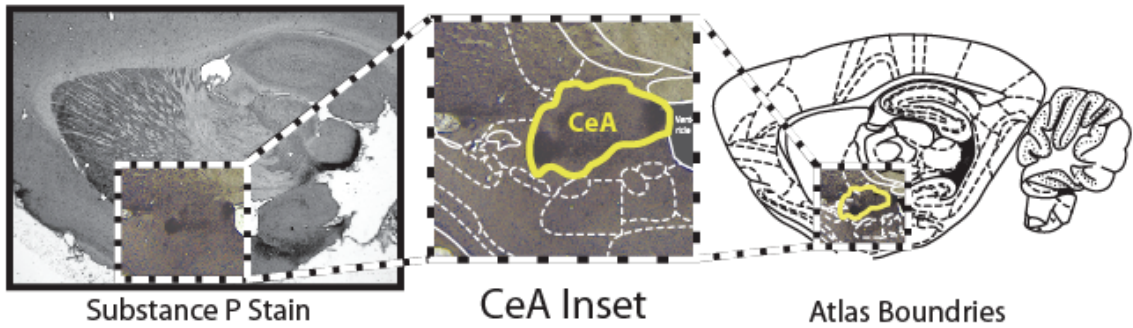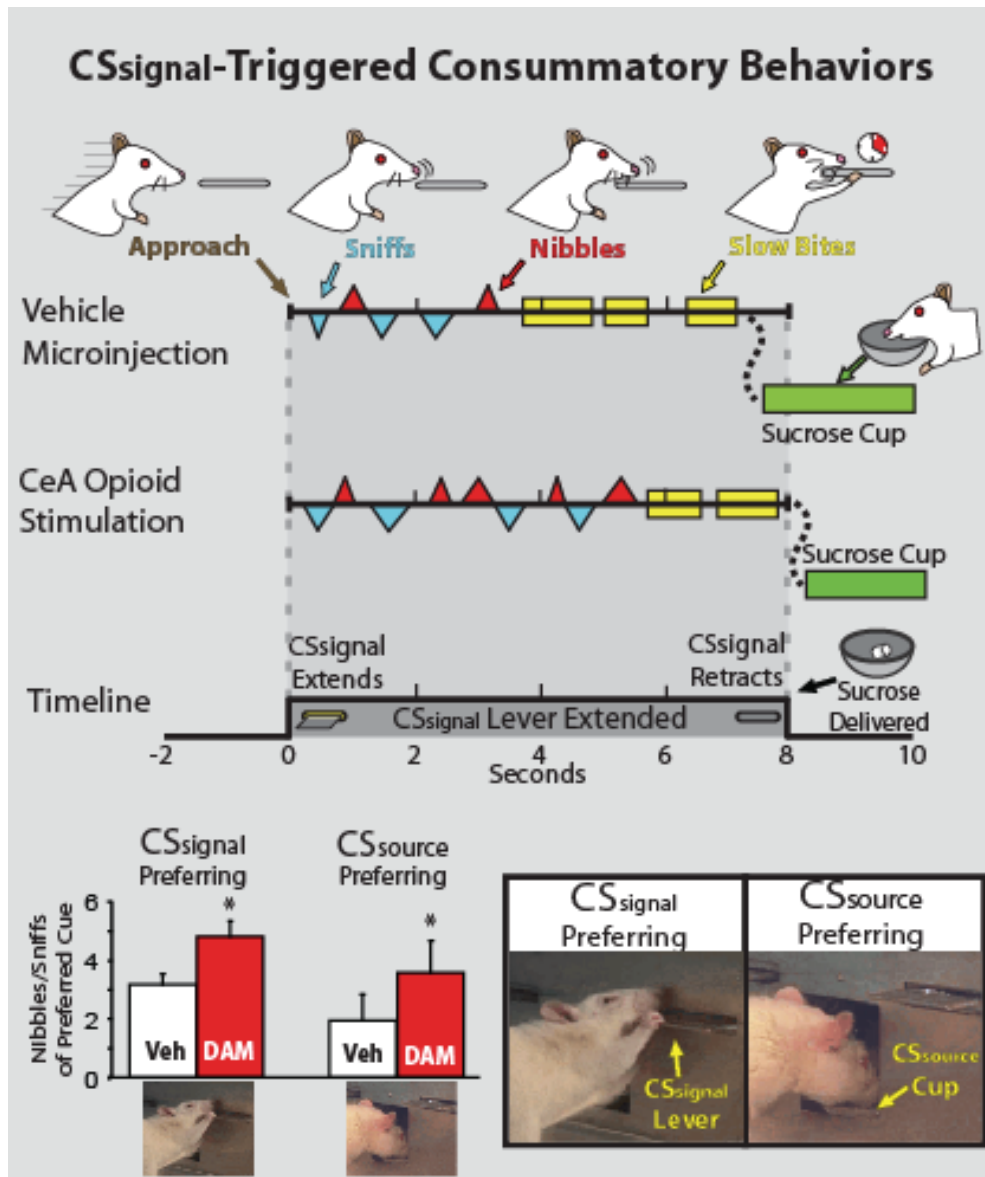
Figure 2.2: Effects of DAMGO on the Microstructure of CS Appetitive-Consummatory Behavior. The behavior of a typical rat with CeA cannulae following vehicle and DAMGO microinjections on separate days following acquisition of preference for the CS+ lever is shown in the top section. Following vehicle or no microinjections, CS+ lever-prepotent rats typically approach, nibble, and sniff the CS+ lever when it extends, then transition to a period of slower, discrete bites. When DAMGO is administered in CeA, the nibbling and sniffing period is extended at the expense of the slow biting period. Bar graphs at the bottom show that anterior CeA DAMGO enhances consummatory behaviors directed at the CS+ or CS_{source} over vehicle levels in CS+ or CS_{source} preferring rats, respectively.

Figure 2.3: Stimulating CeA Opioids After or During Autoshaping Training Specifically Stimulates Appetitive-Consummatory Interactions with the Prepotent CS. DAMGO enhancement of 'wanting' for preferred and non-preferred cues are mapped rat-by-rat based on the intensity of DAMGO effects at the mapped microinjection sites (color) and average DAMGO Fos plumes measured at similar sites (size). *CeA μ Opioid Stimulation After Training* (Left Column): After animals learned to preferentially approach and interact with one of the 2 CSs, DAMGO enhanced nibbles and sniffs only of the prepotent, but not the alternative CS during cues. *CeA μ Opioid Stimulation During Training* (Right Column): When DAMGO was administered while animals were learning the autoshaping task, again it enhanced CS consummatory behavior after a cue preference had been established (average cue nibbles and sniffs for the last 3 training days shown). Hexagonal symbol colors denote DAMGO modulation of prepotent CS nibbles and sniffs, calculated as percent change from vehicle day in the same animals, or the mean of the vehicle animals in the DAMGO during training experiment. Inner symbols represent average diameter of 3x Fos enhancement over uninjected tissue levels, surrounded by semitransparent halos that show 2x Fos enhancement zones. 'X' symbols indicate cannulae placements for rats receiving vehicle microinjections in the between-subjects design, DAMGO-during-training experiment. At the upper left corner of each map, bar graphs show overall data for CeA animals after vehicle and DAMGO. Bars along rostrocaudal and dorsoventral axes show the intensity of DAMGO effects (mean ± SEM percent of vehicle levels) within each 0.4mm-wide level, centered on the labeled coordinate; a plume symbol can contribute to more than one bar when it straddles multiple levels). Bar colors reflect mean percentage change from vehicle in that zone. * indicates difference from vehicle for all CeA animals, $p < 0.05$.

CeA μ Opioid Stimulation After Training   CeA μ Opioid Stimulation During Training

Preferred CS+

Non-Preferred CS+

% Control Nibbles/Sniffs
181%+
160-180%
140-159%
110-139%
90-109%
<90%
<70%
<50%
<25%
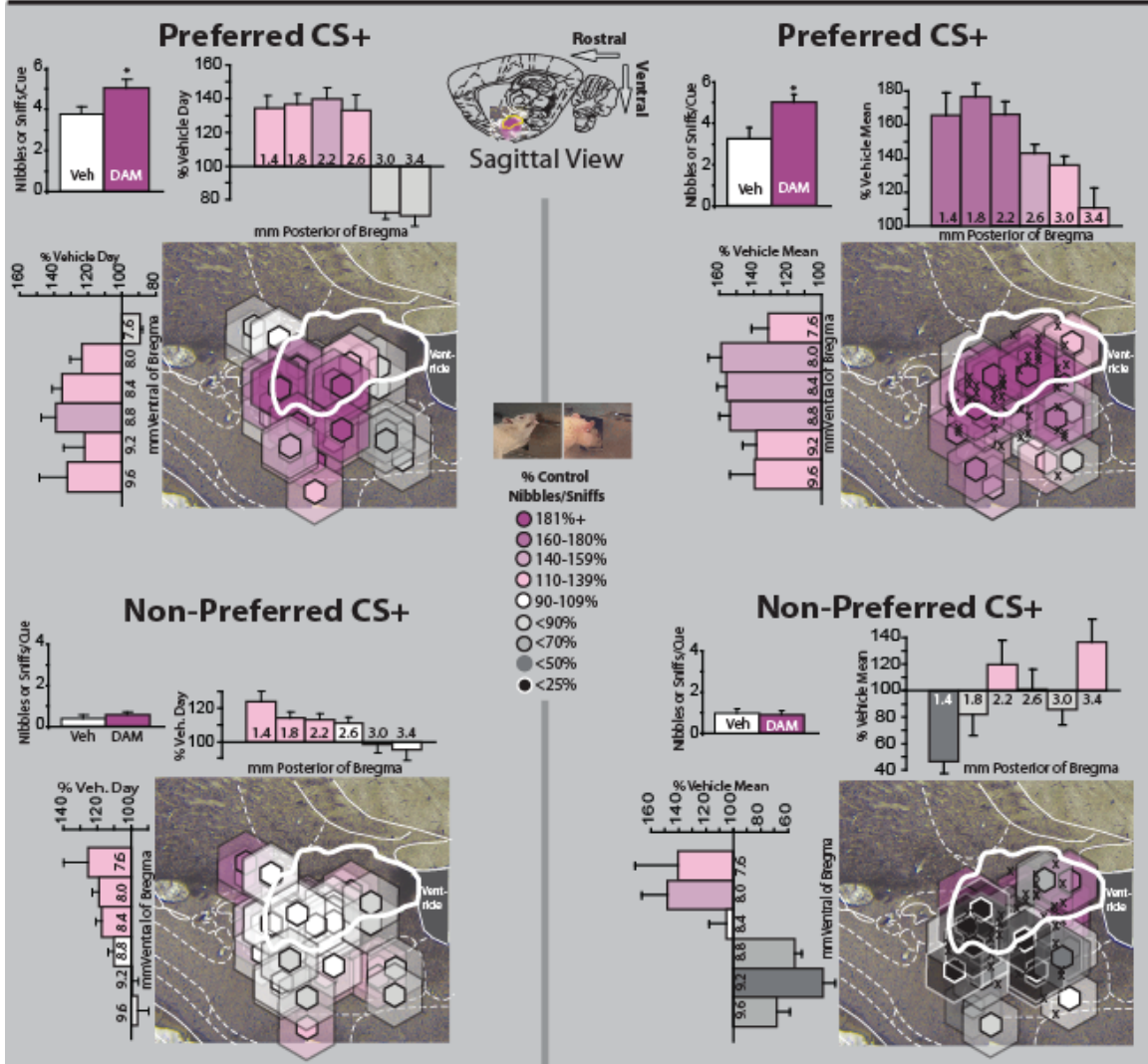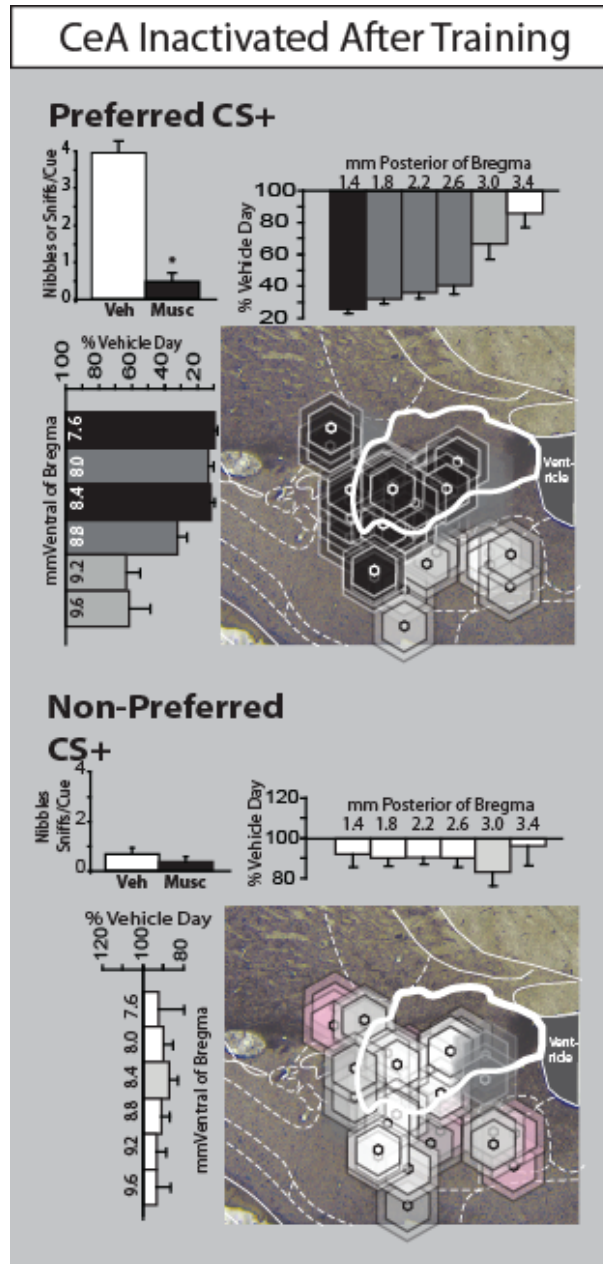
Figure 2.4: Inactivating CeA Following Autoshaping Training Specifically Blocks Appetitive-Consummatory Interactions with the Prepotent CS. Inactivating the CeA with muscimol following training (percent change from vehicle day in the same rats dramatically reduces prepotent cue nibbling and sniffing, but does not consistently affect these appetitive-consummatory interactions with the non-preferred CS. * indicates difference from vehicle for all CeA animals, p<0.05.
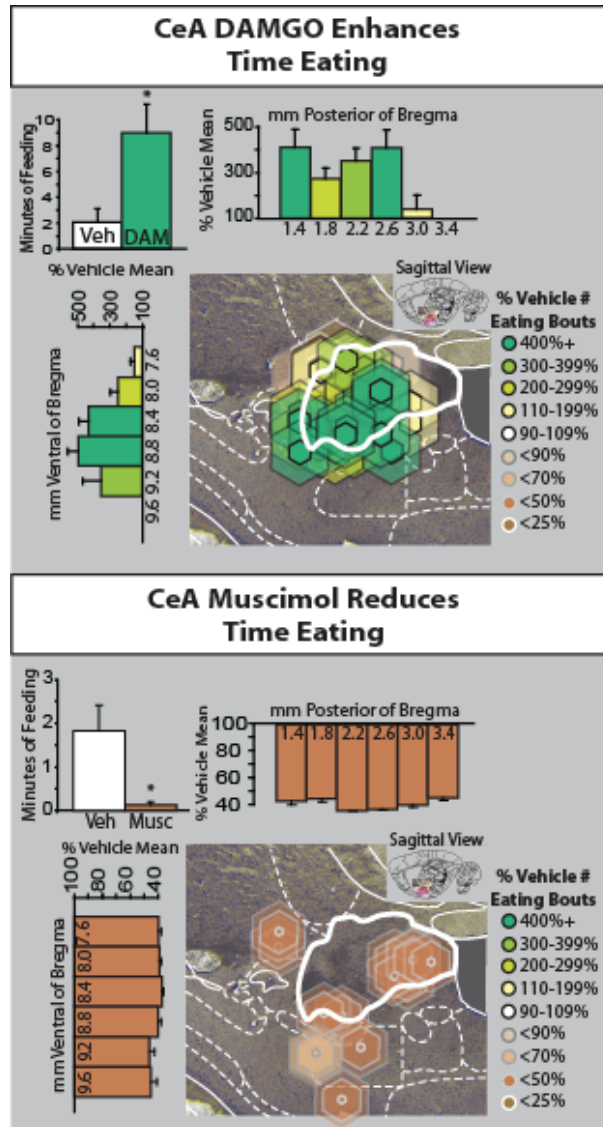
Figure 2.5: Opposite Effects of CeA Opioid Stimulation and Temporary Inactivation on Food Intake. DAMGO enhances, and muscimol reduces the time spent eating in a 1 hour period following microinjections into the CeA and immediate vicinity. * indicates difference from vehicle for all CeA animals, p<0.05.
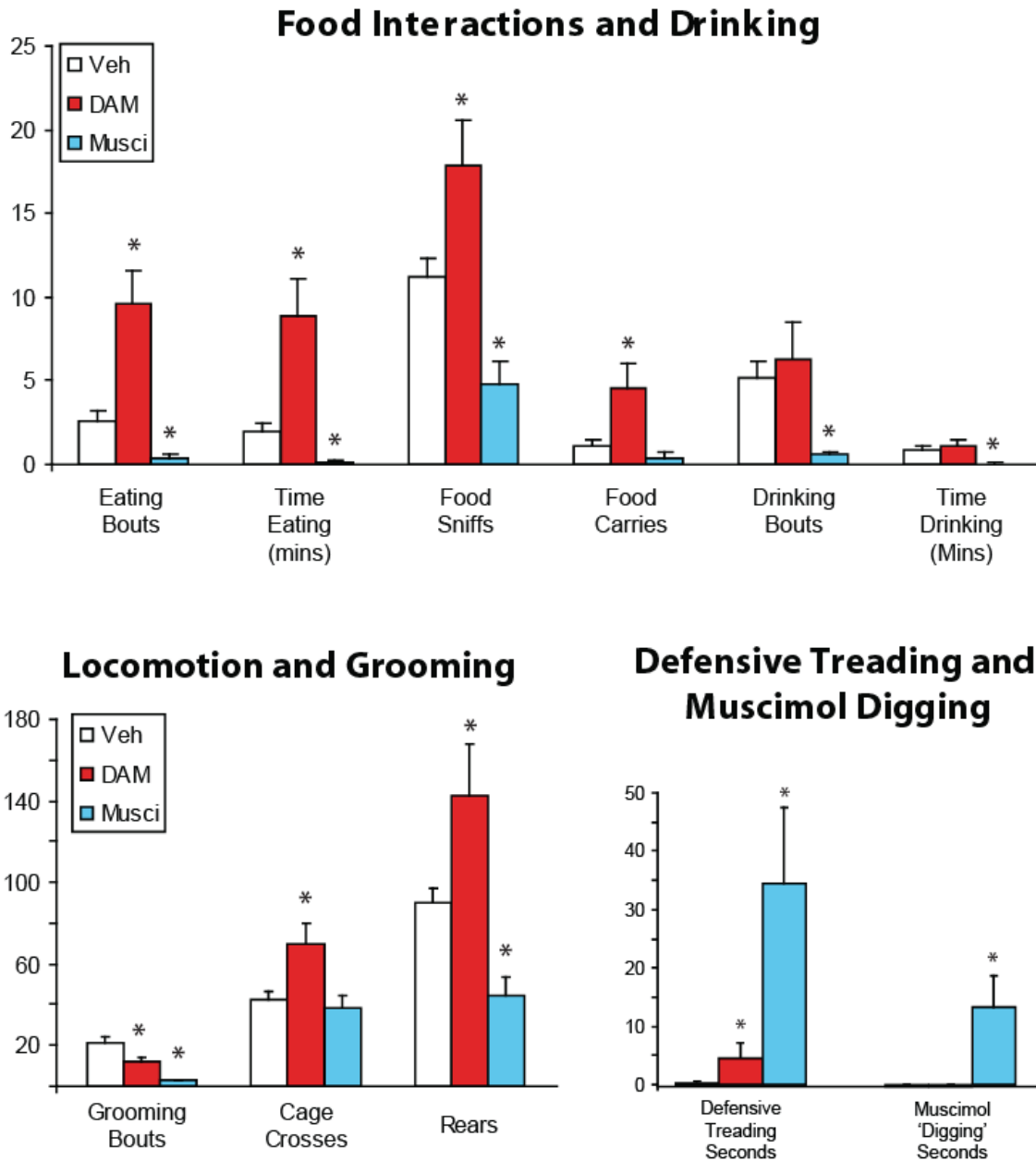
Figure 2.6: Food-Oriented and Other Behavioral Effects of DAMGO and Muscimol. Behavioral effects of microinjections of vehicle (white bars), 0.1µg DAMGO (red bars), and 0.25µg muscimol (blue bars) on interactions with food pellets and drinking behaviors (top), locomotor behavior and grooming (bottom left) and defensive treading and muscimol-induced aimless digging (bottom right). * indicates difference from vehicle, $p < 0.05$.
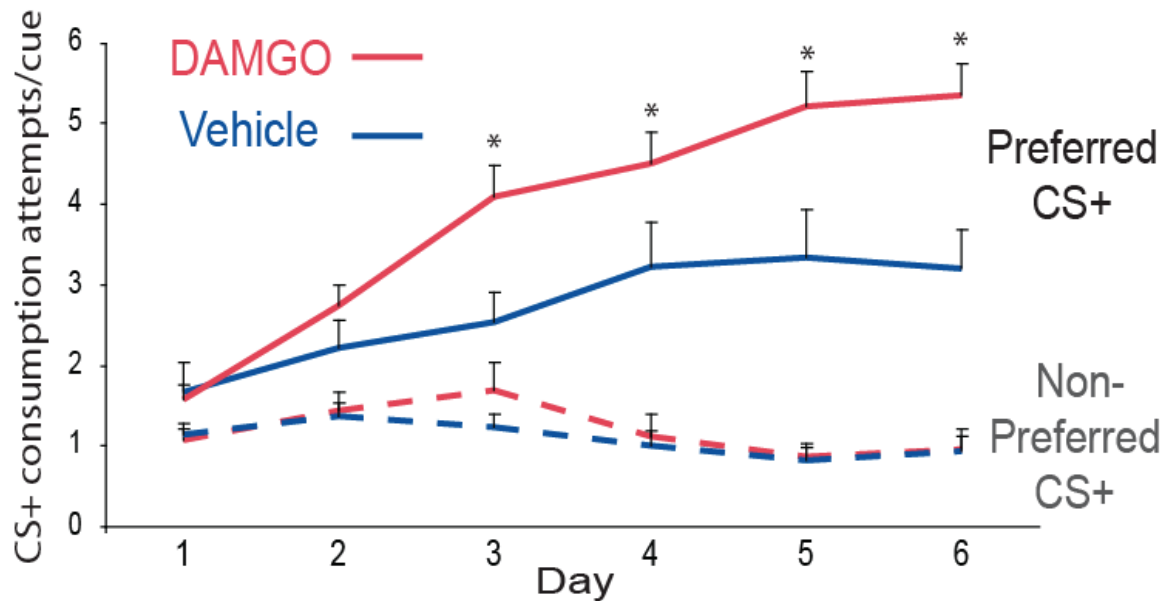
Figure 2.7: Effects of DAMGO on Autoshaping Acquisition Day-by-Day. DAMGO (0.1µg; maroon lines, vehicle=green lines), administered before each of 6 days of autoshaping testing, only enhances consummatory nibbles and sniffs of whichever cue becomes preferred. CS preference for either the CS+ lever or CS_{source} food dish was determined on day 3, by which time all animals had developed a stable cue preference. No differences in cue approach due to DAMGO were found on days 1 or 2, before cue preference had been consistently established. * indicates difference from vehicle in preferred cue nibbles and sniffs, $p<0.05$.
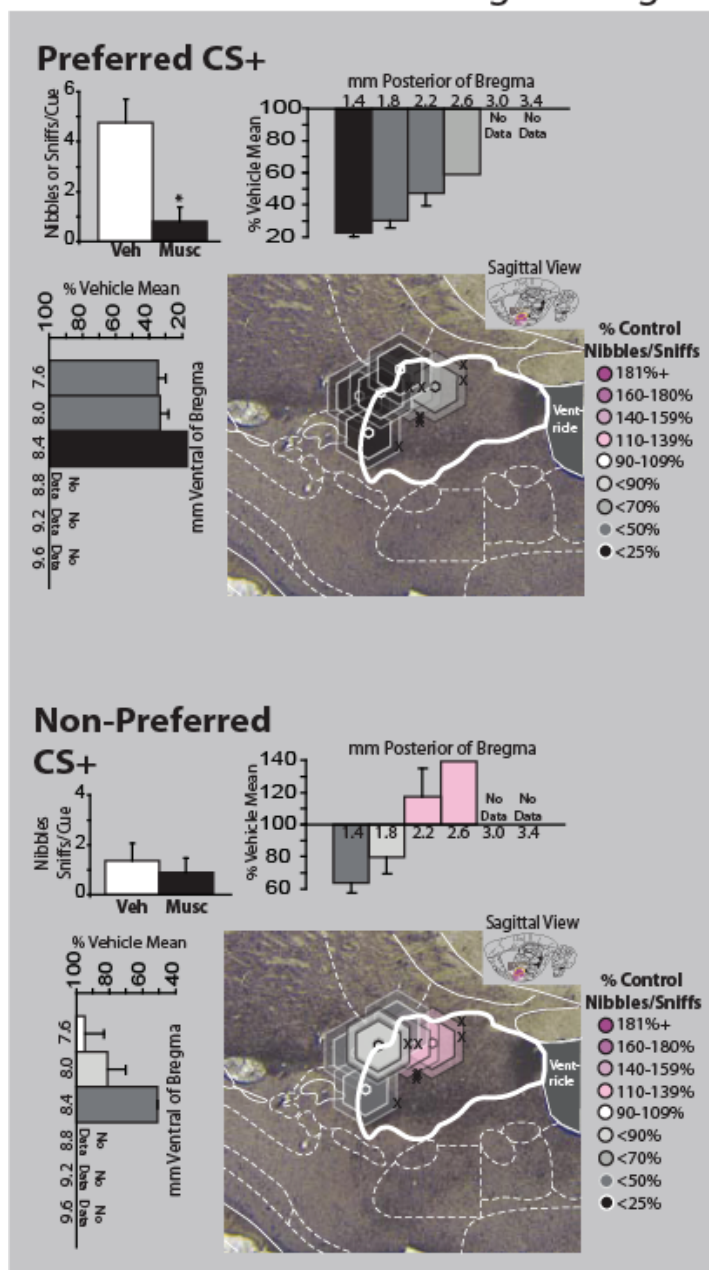
Figure 2.8: Muscimol Inactivation of CeA Blocks Autoshaping Acquisition.
Compared to rats receiving vehicle ('X's mark cannulae sites), animals receiving muscimol (concentric hexagons) displayed fewer consummatory nibbles and sniffs directed at the CS+, but such interactions with the non-preferred cue was not affected. Symbol logic follows other mapping figures.

Map of Muscimol Effects on Initiations of Eating
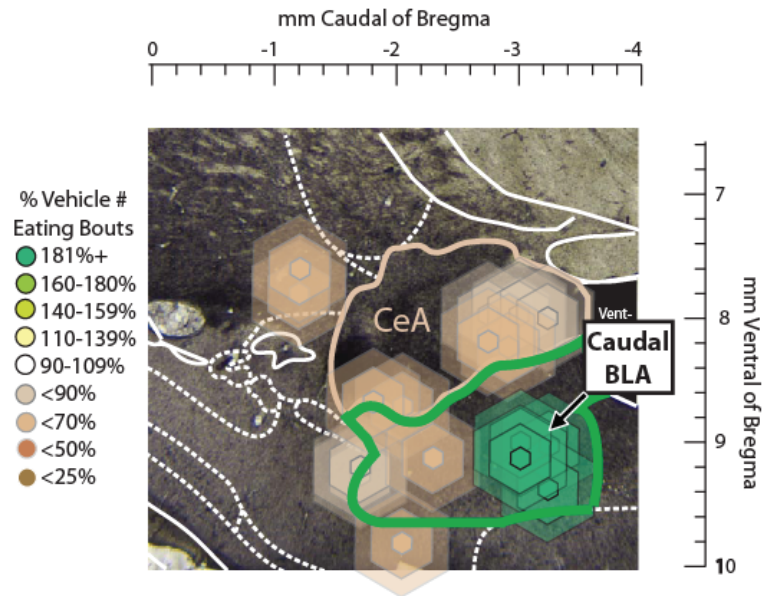
Eating Initiations Following CeA or Caudal BLA Muscimol
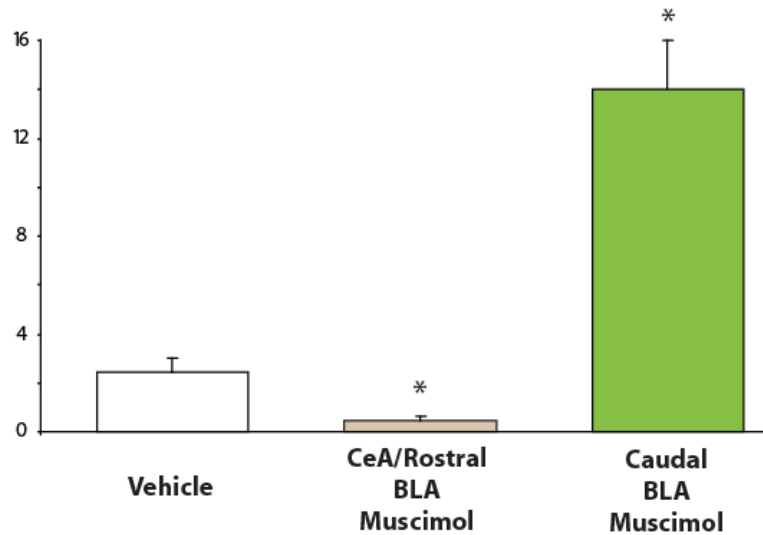
Figure 2.9: Caudal BLA Inactivation Stimulates Feeding. *Map of Muscimol Effects on Eating Initiations:* Muscimol (0.25µg) microinjections in CeA or anterior BLA suppress the number of initiations of food intake relative to vehicle, while caudal BLA injections dramatically increase food intake initiations in the two animals tested. Symbol logic follows that of Figures 4-6. Rostrocaudal and dorsoventral coordinates are relative to Bregma. *Eating Initiations Following CeA or Caudal BLA Muscimol:* Vehicle (white), CeA/anterior BLA muscimol (tan), and caudal BLA (green) eating bouts per 1hr session are shown. * indicates difference from vehicle, $p<0.05$.
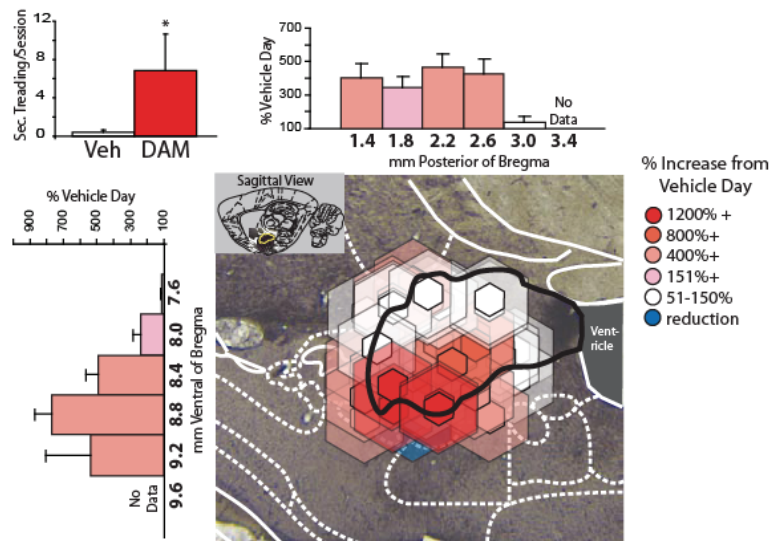
Figure 2.10: DAMGO and Muscimol-Induced Treading Maps. DAMGO (0.1µg) and Muscimol (0.25µg)-induced defensive treading is shown. Symbol logic is identical to Figures 4-6. *DAMGO-Induced Defensive Treading:* DAMGO, particularly in the ventral half of the CeA near the BLA border, stimulates defensive treading. *Muscimol-Induced Defensive Treading.* Muscimol throughout the CeA and BLA induces robust defensive treading. * indicates difference from vehicle, $p<0.05$.

Normal Tissue-Relative

	Mean Fos Plume Radii (mm) mean(SEM)			Estimated Fos Plume Volumes (mm³)		
Vehicle	0.02 (0.02)	0.14 (0.09)	0.03 (0.02)	0.00002	0.01	0.007
DAMGO	0.15 (0.04)	0.47 (0.06)	0 (0)	0.015	0.43	0
Muscimol	0.08 (0.04)	0.27 (0.05)	0.09 (0.04)	0.003	0.09	0.12
	Intense Plume >300% Normal	Moderate Plume >200% Normal	Inhibition Anti-Plume <50% Normal	Intense Plume >300% Vehicle	Moderate Plume >200% Vehicle	Inhibition Anti-Plume <50% Vehicle

Vehicle Microinjection-Relative

	Mean Fos Plume Radii (mm) mean(SEM)			Estimated Fos Plume Volumes (mm³)		
DAMGO	0.1 (0.02)	0.32 (0.05)	0 (0)	0.007	0.13	0
Muscimol	0.06 (0.026)	0.18 (0.55)	0.14 (0.04)	0.006	0.025	0.03
	Intense Plume >300% Normal	Moderate Plume >200% Normal	Inhibition Anti-Plume <50% Normal	Intense Plume >300% Vehicle	Moderate Plume >200% Vehicle	Inhibition Anti-Plume <50% Vehicle

Table 2.1: DAMGO and Muscimol Fos Modulation Radii and Volumes. Mean(SEM) Fos plume radii (left) and volumes (right) are listed for 0.1µg/0.5µl DAMGO, 0.25µg/0.5µl muscimol, and 0.5µl 0.9% saline vehicle. Plume sizes were calculated compared to uninjected, normal tissue (top), and to microinjection of vehicle alone (bottom).

Chapter 3
Focusing of Cue-Triggered 'Wanting:' Central Amygdala Opioid Activation
Focuses Cue-Triggered Instrumental Sucrose Seeking

Introduction

Pavlovian reward cues (or conditional stimuli: CSs) can powerfully modulate motivated behavior. Activation of mesocorticolimbic brain systems can amplify the motivational effects of CSs, sometimes to an excessive extent as in appetitive disorders such as addiction and binge eating (Robinson and Berridge, 1993; Kelley and Berridge, 2002; Robinson and Berridge, 2008; Volkow et al., 2008). Incentive salience conceptions of appetitive Pavlovian motivation posit that cues for rewards come to attain motivational properties of the rewards they predict, or incentive salience. In contrast to particular cue-triggered motor behaviors elicited by stimulus-response (S-R) learning, incentive salience is thought to be the product of stimulus-stimulus (S-S) associations between cues and a psychological representation of reward and its attractive and desirable qualities (Bindra, 1978; Berridge, 2001). When CSs are encountered following learning, they can therefore elicit a conditioned 'wanting' state that can motivate flexible behaviors directed either at the CSs themselves (Chapter 2), or at a predicted UCS reward. In addiction, this flexible cue-triggered motivation may explain the ability of drug CSs to put recovering addicts at risk for relapse. For example, an alcoholic who sees a liquor ad may suddenly feel highly motivated to find the nearest liquor store, even in an unfamiliar neighborhood (Robinson and Berridge, 1993).

A useful paradigm to specifically measure such cue-triggered, but UCS-directed 'wanting' in animals is known as Pavlovian to Instrumental Transfer, or PIT (Walker, 1942; Estes, 1943; Wyvell and Berridge, 2001; Corbit and Balleine, 2005). In this

paradigm, animals learn a Pavlovian association between a CS and a reward UCS, then separately learn to press a lever for the same UCS in the absence of discrete Pavlovian cues. The excitatory influence of Pavlovian cues on instrumental behavior is then tested under extinction conditions following the completion of all learning. Crucially, since animals do not receive rewards during testing sessions, brain manipulations that affect cue-triggered lever pressing must do so based on real-time modulation of the remembered incentive value of a UCS triggered by a CS, rather than changes in the hedonic or reinforcing properties of a reward itself. Additionally, no levers are present during Pavlovian training, so animals learn to approach the reward delivery area during CS presentations. Therefore, stimulation of lever pressing by a CS during PIT testing cannot be due to enhancements of previously learned stimulus-response (S-R) habits, but must instead result from a motivational state elicited by cues, and transferred into a particular UCS reward-seeking behavior.

Katherine Walker (1942), a student of B.F. Skinner, first demonstrated that a tone signaling the availability of food at the end of a long runway (a discriminative stimulus) was capable of eliciting lever pressing when played in a totally distinct operant chamber in the absence of reward delivery. One year later, another Skinner student published a similar report that a purely Pavlovian tone cue can also elicit lever pressing that was separately trained (Estes, 1943). Remarkably, Estes interpreted this effect as the "conditioning of an anticipatory state to the tone" (p. 155), which on face value seems hard to interpret in terms of the purely behaviorist views of Skinner.

Today, neuroscientists still use PIT for investigating the neural substrates of UCS directed Pavlovian incentive motivation, in isolation from S-R habits, hedonics, and learning itself. Midbrain dopamine projections to nucleus accumbens are thought to mediate the magnitude of this cue-triggered UCS 'wanting' (Wyvell and Berridge, 2000, 2001; Murschall and Hauber, 2006; Lex and Hauber, 2008; Peciña and Berridge, 2008), but less is known about how prior learning is integrated with motivation to create directed, focused 'wanting' specifically triggered by reward-associated stimuli.

The amygdala is a key player in learning about the motivational significance of Pavlovian CSs, and generating directed motivated behavior in response to them. In particular, the amygdala may tag particular sensory stimuli with motivational valence and

meaning based on learned associations between them and rewards or punishers. When these stimuli are encountered again, the amygdala is also important for generating the appropriate motivated responses, as well as focusing and directing this motivation into particular behaviors and onto particular environmental targets (Morris and Dolan, 2001; Everitt et al., 2003; Maren, 2003; Holland and Gallagher, 2004; Phelps and LeDoux, 2005; Balleine and Killcross, 2006; Phillips et al., 2008).

The central nucleus of the amygdala (CeA) may be specialized for generating incentive motivation based on Pavlovian learning. CeA is necessary for incentive salience to be targeted upon reward-associated CSs in an autoshaping paradigm (Parkinson et al., 2000; Cardinal et al., 2002b), as well as for Pavlovian CSs to trigger UCS 'wanting' in the PIT paradigm (Hall et al., 2001; Holland and Gallagher, 2003; Corbit and Balleine, 2005). The basolateral amygdala (BLA) is also necessary for Pavlovian CSs to elicit reward seeking based on the sensory properties of particular UCSs (Blundell et al., 2001; Everitt et al., 2003; Corbit and Balleine, 2005), suggesting that it may also modulate certain types of cue triggered motivation.

Little is known about the role of particular neurochemical systems in amygdala in mediating incentive motivation. Some evidence suggests that μ opioid receptors in central amygdala may play a special role in this process. For example, μ opioid stimulation in CeA enhances intake of an unconditioned food reward (Gosnell, 1988; Levine et al., 2004). In Chapter 2, we also demonstrate that CeA opioid stimulation can amplify and target incentive salience upon particular learned reward-associated stimuli, opening the possibility that food intake enhancement might be due to modulation of the incentive salience of food cues in particular. We know of no published reports of specific BLA opioid enhancement of UCS or CS 'wanting,' though opioid receptors are dense in the structure (Mansour et al., 1995; Poulin et al., 2006), and they may modulate certain aspects of reward (Stein, 1993; Smith et al., 2002; Zarrindast et al., 2004; Zarrindast et al., 2005).

We tested effects of microinjections of the μ opioid agonist DAMGO (0.05 & 0.1μg) in CeA or BLA on the focusing of UCS 'wanting' triggered by reward CSs in a Pavlovian to instrumental transfer paradigm. By examining Fos plumes of neuronal activation surrounding injection sites, we examined the specific sites within amygdala in

which DAMGO modulated sucrose 'wanting' triggered by exposure to learned CSs associated with the same sucrose reward. We found that CeA, but not BLA DAMGO (0.1μg) enhanced CS+ triggered sucrose seeking relative to pre-CS+ levels and vehicle controls. We also found evidence that CeA DAMGO focused 'wanting' to CS+ periods, and reduced generalization of CS+ induced pressing to the CS-. In addition, CeA but not BLA DAMGO enhanced intake of a chow reward in the absence of explicitly trained Pavlovian stimuli, further indicating enhancement of UCS 'wanting' by CeA opioid stimulation.

Materials and Methods

Here we asked whether stimulating μ opioid receptors with the specific agonist DAMGO (0.1&0.05μg) throughout the amygdala would affect cue-triggered 'wanting,' as measured with the Pavlovian to Instrumental Transfer (PIT) paradigm, or consumption of a UCS food reward. To examine the precise localization of opioid effects in amygdala, we measured Fos plumes of neuronal activation caused by DAMGO to estimate drug spread from injection sites in CeA and BLA, and examined the anatomical localization of injections in relation to behavioral effects observed at comparable sites in separate animals. We predicted that CeA DAMGO would enhance and focus the incentive value of a Pavlovian cue-triggered reward representation, leading to increased cue-triggered 'wanting' in the Pavlovian to Instrumental Transfer paradigm and stimulation of food intake. We confirmed this hypothesis, and determined that these effects were specific to the CeA, but not BLA.

Animals and Design

Sprague Dawley rats (n=45 female; 250-350g) were pair housed in a reverse 12h light/12h dark cycle, ~21°C room. During PIT training and testing, chow was restricted to 20-25g/day, delivered daily following testing, except during surgery recovery and food intake testing when food unlimited. Water was available *ad libitum* at all times. Following all PIT training and testing procedures, animals underwent surgery to implant amygdala cannulae. Following recovery, instrumental refresher sessions and an instrumental extinction session, testing sessions were conducted over three days, during which animals received vehicle and DAMGO (0.05&0.1μg/0.2μl) in randomized order.

Next, animals were tested for food intake and general behavioral effects of these doses of DAMGO and vehicle in additional sessions. The spread of DAMGO from injection sites was examined in a separate group of rats by quantifying Fos plumes of neuronal activation surrounding injection sites of DAMGO. Only animals with bilateral CeA or BLA cannulae placements were included in analyses (PIT/food intake: CeA n=12, BLA n=11; Fos: CeA n=9, BLA n=10, uninjected n=3)

Measurement of DAMGO's maximal impact on 'wanting,' food intake, and Fos plumes was achieved by a split-and-recombine design, in which rats were assigned upon surgical implantation to either a behavioral test group (n=23) or a Fos group (n=22). Cannulae placements in CeA and BLA were similar for both groups. Therefore, Fos plumes were assessed under conditions similar to the first day of behavioral testing, allowing examination of the maximal impact of DAMGO on both 'wanting' and Fos. In order to project observed behavioral 'wanting' and food intake effects onto the precise locations where DAMGO was likely to have acted, we then recombined data from Fos and behavioral animals in mapping Figures 3.2&3.4.

Microinjections & Drugs

For all microinjections, rats were gently held while stylets were removed, and a microinjector was inserted extending 2mm beyond the end of each guide cannulae. DAMGO (0.1& 0.05μg) and its vehicle (artificial cerebrospinal fluid (ACSF)) were then bilaterally infused in a volume of 0.2μl over 90sec using an automated syringe pump, and the microinjector was held in place for 1 min after each injection to allow for drug diffusion from the injector tip. DAMGO doses were chosen based on robust enhancements of food intake, and cue-directed consummatory behaviors in a Pavlovian autoshaping paradigm [Chapter 1 and (Levine et al., 2004)]. For comparison of DAMGO Fos plumes with uninjected baseline Fos levels, 3 additional animals were implanted with a cement skullcap, but no indwelling cannulae.

Training and Testing Apparatus

Pavlovian to Instrumental Transfer Training and Testing: Chambers were 30.5 cm x 24.1cm x 21.0 cm, with steel front and back plates, and clear plastic sides, ceiling, and floor, enclosed in a sound attenuating box with ventilation fans to mask external

noise. A red house light was mounted on the top of the back wall, which was lit during all training sessions. Two retractable levers were present on either side of the front of the chamber, which were extended at the beginning of instrumental training and PIT testing sessions. A sucrose delivery cup was located between the levers near the floor of the front of the box, in which an infrared beam was incorporated to measure number of head entries. Tone, white noise, and clicker CS stimuli were presented diffusely throughout the chamber from speakers near the back of the box (opposite levers and sucrose cup) during Pavlovian training and PIT testing. A computer equipped with MED-PC software (Med Associates, Inc.) controlled all events and recorded lever presses and sucrose cup entries during training and testing sessions, and a video camera under the box recorded behavior for later videoanalysis of cue interactions and other behaviors.

Food Intake: Food intake tests were carried out in transparent plastic tub cages with pre-weighed food (Purina chow pellets), *ad lib* water, and corn cob bedding. Food and water intake was measured in grams, and behavior was videotaped for subsequent slow-motion analysis of eating duration, drinking duration, locomotion, and other behaviors in a subset of animals.

Pavlovian and Instrumental Training Procedures

Habituation and Magazine Training: Rats were handled for 5min/day for 3 days prior to commencement of instrumental training, then were given 20 sucrose pellets/rat in their home cage overnight prior to magazine training to overcome neophobia. On day 1, rats received sucrose pellets from the sucrose cup in the training box on a variable interval 60sec (VI-60) schedule for 20 min, to habituate them to taking sucrose from the sucrose dish.

Instrumental Training: Rats were trained to press one of the two available levers many times for each delivered sucrose pellet over the course of instrumental training. Daily instrumental training sessions were 30min long, began with the extension of lever(s) and illumination of a red houselight, and ended with the retraction of the lever(s) and the houselight turning off. On days 1-3, rats received one sucrose pellet per press on the 'active' lever (FR-1). On days 1 and 2, only the active lever was extended, but on day 3 and thereafter in instrumental training, a control lever was also extended at the start

of the session. Control lever presses were recorded, but never yielded sucrose rewards. After establishment of a FR-1 schedule by day 3, the reinforcement schedule was incrementally increased up to VI-45 over the next 12 days: days 4-6=VI-5, days 7-9=VI-15, days 10-12=VI-30, days 13-15=VI-45. By the last day of instrumental training, all rats pressed the active lever many more times than the inactive lever (active lever presses/session: m(SEM)=767.2(47.2), inactive lever presses: m=35.7(6.4)).

Pavlovian Training: Following instrumental training, rats were subjected to 12 days of Pavlovian training, during which they learned to associate an auditory stimulus with the same sucrose reward delivered during instrumental training. Both levers were retracted at all times during Pavlovian training to prevent adventitious reinforcement of lever pressing. Rats were first assigned a CS+ and CS- stimulus, which were tone (CS+: n=5, CS-: n=5), click (CS+: n=5, CS-: n=5), or white noise (CS+: n=10, CS-: n=4). Over 8 days, rats received 4 CS+/sucrose pairings per day for 30sec each, followed immediately by delivery of 3 sucrose pellets into the sucrose cup. Following CS+ training, rats received an additional 4 days of CS+/CS- discrimination training, in which they received alternate presentations of their CS+ (followed by sucrose) and CS- (not followed by sucrose). Rats were considered to have met Pavlovian learning criteria if they entered the sucrose cup more than twice as often during CS+ periods than in the 30sec before them.

Surgery

Following Instrumental and Pavlovian training, rats were anesthetized with ketamine (80mg/kg), xylazine (7mg/kg), and atropine (0.04mg/kg) and surgically implanted with chronic, bilateral, 14mm microinjection guide cannulae (23ga) positioned 2mm above CeA sites. Cannulae were anchored to the skull with bone screws and acrylic cement, and steel stylets were inserted to prevent their occlusion. Placement coordinates for cranial cannulae were calculated based on Paxinos and Watson (2007), and lowered into place with a stereotaxic apparatus. Cannulae were aimed at multiple sites in the CeA ranging from (relative to Bregma): -1.8 to -3.0 AP; ±3.4 to ±4.6 ML; and -5.8 to -6.5 DV), or BLA (-1.8 to -3.2 AP; ±4.6 to 5.2; -8.2 to 9.2 DV). A separate group of rats used to measure DAMGO Fos plumes were implanted with cannulae at equivalent sites in

CeA and BLA to behaviorally tested animals, or a cement skull cap only without cannulae. All animals were allowed 1 week to recover from surgery before any further behavioral testing was conducted.

Instrumental Retraining and Extinction

Following recovery from surgery, rats were given 3 additional VI-45 instrumental training sessions to ensure that instrumental learning was maintained following the intervening period of Pavlovian learning, surgery, and recovery (~3 weeks). On the next day, rats underwent a final instrumental extinction session on which both active and inactive levers were extended, but neither produced rewards. This session was intended to habituate animals to extinction conditions, and to partially extinguish non cue-triggered lever pressing behavior. All animals showed significant extinction of sucrose lever pressing during this session, and went from a mean (SEM) of 25.5(2.8) presses during the first 5min of the session, to 2.8(0.1) during the final 5min.

Pavlovian to Instrumental Transfer Testing

Rats received microinjections of DAMGO (0.1 or 0.05μg/0.2μl) or vehicle in randomized order, 15min prior to each of three testing sessions, conducted 48hrs apart. Following microinjections, animals were returned to their home cages for 13min, then placed in the darkened testing box for 2min to habituate them. 15min after injection, the house light was illuminated, the levers were extended, and the testing session began. PIT testing was conducted under extinction conditions, and no sucrose rewards were ever delivered on testing sessions. Following a 3.5min baseline period, the 30sec CS+ and CS- cues were presented 4 times each in alternating order, 3min apart (CS+ presented first: n=15; CS- presented first: n=8). Lever presses, sucrose cup entries, and time spent in the sucrose cup were recorded during the 30sec of each cue, and the 30sec immediately prior as a precue baseline. Sessions were also recorded with a video camera positioned below the transparent floor of the testing chamber, for later coding of behavior.

Food Intake and General Behavior Habituation and Testing

Following completion of all PIT testing days, food intake and other behavioral effects of DAMGO (0.1&0.05μg) and vehicle were assessed in some of the same rats.

Rats were handled and placed in clear tub cages with corncob bedding and ad lib food and water for 3 days prior to testing to habituate them to testing procedures. Following testing, rats were injected with DAMGO (0.1&0.05µg) and vehicle in randomized order over three test days held 48hrs apart. Food was weighed before and after sessions to determine intake, and all other behaviors were recorded via videotape for later coding.

General Histology

After the completion of testing, rats used for behavioral experiments were deeply anesthetized with sodium pentobarbital (0.2g/kg), microinjected with 0.2µl black ink, and their brains were extracted. Brains were sectioned with a freezing microtome into 60µm coronal slices, stained with cresyl violet, and mapped for microinjection center locations according to Paxinos and Watson (2005).

Fos-Like Protein Immunohistochemistry

Rats in the Fos plume group (n=22) were handled for 3 days for 10 min each (like behaviorally tested groups), then microinjected bilaterally in the CeA or BLA with DAMGO (n=15; 0.1µg/0.2µl) or vehicle (n=5; 0.2µl) as described above, or handled equivalently but not injected for rats with skullcaps but no cannulae (n=3). Cannulae placements were located throughout the CeA and BLA, similarly to the behavioral group, and it was later confirmed that every behavioral group site was within 1mm of a corresponding Fos group site. Fos-like protein expression was therefore harvested under conditions similar to the first day of testing for the behavioral group.

Fos staining procedures were identical as those employed in Chapter 2, except that animals were sacrificed 90, rather than 75min following DAMGO microinjection to accommodate the addition of a 15min post-injection period before testing began in this experiment. Again, cannulae placements were localized by comparing the image from a low magnification light microscope with a standard rat brain atlas (Paxinos and Watson, 2007). In addition, every 5th slice was stained for Substance P to help localize the borders of CeA and other nuclei, and localize Fos plumes within amygdala subnuclei.

Analysis of DAMGO-Induced Fos Plumes of Neuronal Activation

Our procedure for measuring drug-induced Fos plumes immediately surrounding a microinjection site followed procedures described in Chapter 2, and previously (Peciña and Berridge, 2005; Smith and Berridge, 2005; Mahler et al., 2007). Mapping figures

employed averaged plume radii projected onto the microinjection centers from the behavioral group to depict extent of activation spread. Behavioral data from each microinjection site were used to assign the color of its symbol on the map that coded intensity of 'liking' or 'wanting' effects produced by microinjection at that site.

Again, DAMGO Fos plumes (0.1μg/0.2μl) were mapped in two ways: (1) as 2X and 3X increases in Fos expression over normal, uninjected tissue levels, and (2) as 2X and 3X increases in Fos over vehicle microinjection levels. The distance from the injection site for 2X and 3X spread was then averaged for all seven radial arms, providing a final mean radius of 2X and 3X elevation, and allowing computation of estimated volumes of tissue activated by drug, assuming a spherical shape. Thus, DAMGO-induced increases in Fos were compared to normal and vehicle Fos levels at equivalent sites within CeA and BLA, and maps were created based on average moderate (2X uninjected levels) and intense (3X uninjected levels) Fos elevation zones (Figure 3.1, see Table 3.1 for complete CeA and BLA Fos plume radii and volumes).

In the next mapping stage, Fos data were recombined with behavioral data to create Figures 3.2&3.4, which depict behavioral effects of DAMGO relative to the anatomical localization of drug action. Each site shows two concentric hexagon symbols, representing the average size of plume zones of Fos elevation: inner hexagon=intense Fos elevation; outer hexagon=moderate Fos elevation. Each hexagon is color coded for the magnitude of DAMGO's behavioral effects on measured behaviors (Peciña and Berridge, 2005; Smith and Berridge, 2005; Mahler et al., 2007). Separate maps were plotted in sagittal, coronal, and horizontal planes to construct a three-dimensional database of the position of Fos plumes in the brain and the location of functional hotspots (Paxinos and Watson, 2007)

Behavioral Videoscoring

All video analyses were scored in slow motion (1/10th to ½ actual speed) by observers blind to experimental conditions. For PIT testing sessions, a video camera positioned under the transparent floor of the testing chamber provided a clear view of the rat's head and body. In addition to computer scored lever pressing and sucrose cup entries, lever and sucrose cup looks (orientation of the head toward the lever or cup, and the nose coming within 1cm of it but not touching), rearing, bouts of corner sniffing, and

sudden orientation shifts (sudden movement of the head and body at least 90° within 1sec) were coded for the 30 sec before, and 30sec of each CS+ and CS- presentation.

In food intake tests, scored behaviors included time spent eating, number of initiations of eating behavior, food sniffing initiations, time drinking, number of drinking bouts, occurrences of defensive paw treading, front-back cage crosses, and rears.

Statistical Analyses

PIT results were analyzed with repeated measures ANOVAs in animals with bilateral CeA (n=12), or BLA (n=11) cannulae placements. Primary analyses were conducted on data reflecting during-CS behaviors relative to pre-CS baselines (percentage increases from precue levels of behavior). These data reflect cue-triggered increases in lever pressing compared to behavior in the absence of CSs, to specifically examine the impact of the CS+ and CS- compared to ongoing levels of behavior. 3 (DAMGO (0.1 & 0.05µg) and vehicle) x 2 (CS+ vs. CS-) x 2 (sucrose vs. control lever) ANOVAs were used to determine DAMGO effects in CeA or BLA. DAMGO effects on baseline (30sec precue period) and 30sec cue period levels of computer scored, and hand scored behaviors were also examined with repeated measures ANOVAs with drug as the within subjects factor. No significant order effects over the 3 days of PIT or food intake testing were found, so data was combined across days. Likewise, the particular CS+ and CS- assigned to each animal (click, tone, or white noise) had no significant effects on behavior or DAMGO effects, so were similarly combined. Behaviors displayed in 30sec precue periods were representative of the entire 3min inter-cue intervals, so only precue data are reported. Food intake and other behavioral effects of DAMGO were analyzed with oneway ANOVAs and t-tests comparing drug and vehicle. Bonforroni corrected t-tests were used to conduct post-hoc tests when applicable. When data were expressed as percentage of baseline or vehicle levels, raw data were adjusted by adding 1 to every score, to avoid the problem of calculating percentage increases over 0 for behaviors with low baselines.

Results

Synopsis

CeA, but not BLA DAMGO enhanced Pavlovian to Instrumental Transfer and food intake, demonstrating a specific role for CeA opioids in incentive motivational 'wanting.' CeA DAMGO robustly elevated cue-triggered pressing only on the lever that previously delivered sucrose but not a control lever, indicating that sucrose seeking in particular was enhanced. In addition, only sucrose seeking triggered by presentations of an auditory CS that was associated with sucrose reward was enhanced by DAMGO, indicating that enhanced 'wanting' was based upon prior Pavlovian learning. Finally, DAMGO did not enhance sucrose seeking in the absence of the CS+, and in fact pressing at these times was reduced by CeA DAMGO. In this way, CeA opioid stimulation seemed to focus sucrose seeking into cue periods, when S-S associations were activated by the Pavlovian sucrose cue, possibly in a 'winner take all' manner at the expense of non-cue periods. All these effects were specific to CeA, but not BLA DAMGO, as determined by microinjection site-specificity, and Fos plume analysis of functional drug spread.

Fos Plumes of Local Neuronal Activation Caused by Intra-Amygdala DAMGO

Consistent with Chapter 2 and previous reports (Peciña and Berridge, 2005; Smith and Berridge, 2005), DAMGO caused local Fos plumes of neuronal activation around injection sites in CeA. To establish baselines with which to compare Fos activation elicited by DAMGO, we measured Fos expression in animals injected with the ACSF vehicle alone, and animals treated equivalently but not microinjected at all. Baseline Fos levels were measured for all structures in the vicinity of the amygdala, and DAMGO and vehicle injected tissue were compared to these baselines structure-by-structure.

Consistent with previous reports, vehicle microinjections produced small Fos elevations in the immediate vicinity of injection sites in both CeA and BLA (radius 2X normal-relative zone m(SEM)=0.03(0.03)mm), presumably related to damage or mechanical pressure associated with microinjections. No differences were observed between the size or intensity of Fos plumes caused by CeA vs. BLA vehicle injections (Table 3.1), so data for these groups were combined for comparison of CeA and BLA DAMGO-induced Fos plumes to vehicle controls.

When injected in the CeA, DAMGO induced robust Fos plumes relative to normal tissue and vehicle microinjections. CeA DAMGO plumes generally produced

small zones of intense Fos enhancement (>3X controls) immediately around injection sites (m(SEM) radius= 0.12(0.05)mm, volume= 0.007mm^3), surrounded by larger zones of moderate (>2X controls) Fos activation (radius= 0.31(0.04)mm, volume= 0.12mm^3; complete Fos plume radii and volumes shown in Table 3.1). These plumes are smaller than those previously reported to be induced by the same dose of CeA DAMGO in a larger, 0.5µl volume of vehicle (0.47(0.06)mm radius; Chapter 2), indicating that microinjection volume affects the functional spread of DAMGO microinjections in amygdala.

In contrast, identical microinjections of DAMGO in BLA did not produce consistent plumes of elevated Fos around injection sites (Figure 3.1; Table 3.1). Since BLA DAMGO did have observable effects on both behavior and distant Fos activation (described below), it is likely that DAMGO did modulate neuronal activity in BLA, but this modulation did not result in consistent local elevation of Fos protein. A lack of BLA DAMGO Fos plumes therefore likely reflects differences in the localization of µ opioid receptors between CeA and BLA (e.g. pre vs. postsynaptic localization), or other aspects of local circuitry that differ between the structures (Zhu and Pan, 2004, 2005; Finnegan et al., 2006). For this reason, we used estimates of Fos plume size derived from CeA microinjections for mapping behavioral effects of DAMGO injected in both BLA and CeA, in order to avoid underestimating functional drug spread for BLA microinjections (Figures 3.1, 3.2, 3.4).

CeA Opioid Stimulation Enhances Cue-Triggered Sucrose 'Wanting'

As expected, Pavlovian CSs for sucrose robustly stimulated lever pressing during the 30sec CS+ periods, compared to the 30sec immediately prior to these CSs (lever x period interaction: $F(1,21)=7.9$, $p=0.01$). Since testing was conducted under extinction conditions, lever pressing in inter-cue periods was relatively low, and CS+ presentations elicited phasic peaks of pressing that rapidly decayed after the cue ended. CeA DAMGO (0.1µg) potentiated these baseline-relative peaks, enhancing cue-triggered sucrose lever pressing by over 375% compared to vehicle day, indicating a robust enhancement in cue-triggered sucrose 'wanting' (main effect of drug on CS+ triggered sucrose lever pressing, $F(2,22)=6.1$, $p<0.01$; 0.1ug: $t=2.7$, $p<0.05$). Low dose CeA DAMGO (0.05µg) did not similarly affect CS+ elicited lever pressing ($t=0.2$, *n.s.*).

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In general, the CS+ elicited more sucrose lever pressing than the CS- (main effect of CS type on cue-triggered (percent of precue) lever pressing: $F_{(1,21)}=8.4$, $p<0.01$). DAMGO only affected this sucrose lever pressing that was elicited by the CS+, but not the CS- ($F_{(2,22)}=6.1$, $p<0.01$; 0.1ug: $t=4.1$, $p<0.01$), indicating that 'wanting' enhancement was dependent upon activation of a sucrose UCS representation elicited by Pavlovian CS exposure.

Throughout test sessions, animals pressed much more on the sucrose lever than the control lever ($F_{(1,21)}=14.7$, $p=0.001$). This was particularly the case during CS+ presentations, compared to CS- presentations or the pre cue period (CS type x lever x period interaction: $F_{(1,21)}=8.2$, $p<0.01$), indicating that sucrose cues elicited sucrose 'wanting' in particular, rather than general locomotor behavior. CeA DAMGO (0.1μg) only stimulated this sucrose lever pressing, and did not affect control lever pressing during CS+ periods ($F_{(2,22)}=4.9$, $p<0.05$; 0.1ug: $t=4.5$, $p=0.001$). This again indicates the specificity of CeA DAMGO 'wanting' stimulation rather than general locomotor behavior, and shows that DAMGO did not affect the ability of animals to transfer sucrose 'wanting' into an appropriate instrumental target.

BLA Opioid Stimulation Does Not Enhance 'Wanting'

In contrast to DAMGO microinjected into CeA, BLA opioid stimulation did not similarly enhance sucrose 'wanting.' BLA DAMGO did not affect CS+ triggered sucrose lever pressing at either dose ($F_{(2,20)}=0.4$, *n.s.*; 0.1ug: $t=0.6$, *n.s.*; 0.05ug: $t=0.7$, *n.s.*). BLA DAMGO also had no effect on control lever pressing stimulated by the CS+ (no drug x lever interaction, $F_{(1,20)}=0.2$, *n.s.*), indicating that BLA opioid stimulation did not change general locomotor responses to the cue. Similarly, lever pressing elicited by the CS- was also not significantly affected ($F_{(2,20)}=1.0$, *n.s.*), indicating that BLA DAMGO did not cause animals to respond abnormally to the CS-. These results indicate that DAMGO has few effects on sucrose 'wanting,' as measured with the Pavlovian to instrumental transfer paradigm.

CeA DAMGO Sharpens Focus of Pavlovian Cue-Triggered 'Wanting'

As described above, DAMGO (0.1μg) robustly enhanced Pavlovian to instrumental transfer as it is traditionally measured, relative to baseline levels of pressing (Hall et al., 2001; Holland and Gallagher, 2003). However, upon further inspection of

the absolute number of presses during and prior to cue periods, we found that CeA DAMGO (0.1µg) did not affect the intensity of cue-triggered sucrose seeking *per se* ($F_{(2,22)}=0.25$, *n.s.*), but instead caused animals to press the sucrose lever *more exclusively* during CS+ presentation periods (Figure 3.3). In the absence of the CS+ (for example in the 30sec periods prior to CS+ presentations), CeA DAMGO (0.1µg) reduced pressing on the sucrose lever ($F_{(2,22)}=5.2$, $p<0.05$; 0.05ug: $t=2.1$, $p=0.06$; 0.1ug: $t=2.7$, $p<0.05$). This focusing of sucrose lever pressing to CS+ presentation periods suggests a specific deficit in sucrose seeking that is not triggered by Pavlovian a discrete reward-paired CS+, since control lever pressing ($F_{(2,22)}=0.9$, *n.s.*) and other scored behaviors were not affected or non-significantly increased by DAMGO (rears: $F_{(2,22)}=2.0$, *n.s.*; orientation shifts $F_{(2,22)}=1.6$, *n.s.*; corner sniffing: $F_{(2,22)}=0.03$, *n.s.*). This pattern of effects could represent a deficit in responses to contextual cues less reliably associated with sucrose delivery than the CS+, or other factors such as habitual lever pressing learned during instrumental training, or a facilitation of extinction of lever pressing in the absence of the CS+. Whatever its cause, however, it is clear that CeA DAMGO focused the sucrose seeking behavior displayed during sessions more specifically to CS+ presentation periods, and robustly enhanced the signal-to-noise ratio of lever pressing in response to a Pavlovian cue.

In addition to a focusing of sucrose seeking to cue periods, CeA DAMGO (0.05&0.1µg) also marginally reduced sucrose lever pressing during CS- presentations compared to vehicle ($F_{(1,22)}=3.0$, $p=0.07$; 0.05ug: $t=1.8$, $p=0.1$; 0.1ug: $t=1.8$, $p=0.1$). After vehicle, the CS- elicited a fair amount of sucrose lever pressing due to stimulus generalization (Figure 3.3). After CeA DAMGO, however, the contrast between the stimuli was greatly enhanced, and sucrose lever pressing was only enhanced when the sucrose-paired cue was presented. Again, these results suggest that CeA DAMGO focused sucrose 'wanting' elicited by S-S associations, but not other, non-Pavlovian factors.

When effects of BLA DAMGO were similarly analyzed separately for pre and during cue periods, again few effects were found. Sucrose lever pressing was not affected during CS+ periods ($F_{(2,20)}=2.0$, *n.s.*), or pre-cue periods ($F_{(2,20)}=0.2$, *n.s.*). BLA opioid stimulation also did not affect pressing during the CS- ($F_{(2,20)}=1.0$, *n.s.*),

and did not affect inactive lever pressing at any time ($F(2,20)=0.2$, n.s.; precue period: $F(2,20)=0.7$, *n.s.*; during CS+: $F(2,20)=2.5$, *n.s.*; during CS-: $F(2,20)=1.2$, *n.s.*)

DAMGO Effects on Sucrose Cup Approach

During Pavlovian training, animals learned only to approach the sucrose cup during CS+ presentations, since levers were not present at the time. During PIT testing, CS+ presentations also enhanced sucrose cup approach relative to precue and CS- levels (CS type x period interaction: $F(1,22)=59.0$, $p<0.001$), in addition to the previously mentioned increase in sucrose lever pressing. Neither CeA nor BLA opioid stimulation affected the number of food cup entries in pre cue periods (CeA: $F(2,22)=0.2$, *n.s.*; BLA: $F(2,20)=1.6$, *n.s.*), indicating that locomotion and habitual food cup entries were not significantly affected by DAMGO. As expected, given the enhancement of sucrose lever pressing, CS+ triggered sucrose cup approach was reduced by CeA DAMGO, but unexpectedly only at the lower dose (percent precue sucrose cup entries; 0.05µg: $t=2.4$, $p<0.05$; 0.1µg: $t=1.0$, *n.s.*). Also unexpectedly, BLA DAMGO reduced sucrose cup entries during CS+ periods at both doses ($F(2,20)=6.2$, $p<0.01$; 0.05ug: $t=2.4$, $p<0.05$; 0.1ug: $t=3.0$, $p=0.1$), though it did not enhance lever pressing as described above. Therefore, both CeA and BLA DAMGO decreased cue-triggered conditioned approach of the sucrose cup at one or both doses, in a manner seemingly unrelated to competition with DAMGO-enhanced lever pressing. These results potentially suggest attenuated cue-triggered habitual sucrose cup approach, independent of concurrent DAMGO effects on sucrose seeking.

Food Intake and Other Behavioral Effects of DAMGO

In a subset of the same animals tested on PIT, CeA DAMGO (0.1µg) subsequently robustly stimulated voluntary chow intake to over 450% of vehicle levels (Figure 3.4; $t=3.4$, $p<0.01$). Similar to previous findings in Chapters 2&4, CeA DAMGO (0.1µg) also increased time eating to an average of over 900% of vehicle levels ($t=2.7$, $p<0.05$), and marginally increased eating initiations (430% of vehicle; $t=1.8$, $p=0.1$), and food sniffing initiations (160%; $t=1.9$, $p=0.08$). Low dose DAMGO (0.05µg) in CeA increased eating in ~40% of tested rats to an average of ~300% of vehicle, although overall food intake and eating behavior were not significantly enhanced at this dose (Figure 3.4; intake: $t=1.4$, *n.s.*; eating initiations: $t=0.1$, *n.s.*; time eating: $t=1.3$, *n.s.*).

Low dose CeA DAMGO did increase the number of food sniffs across animals, however (0.05ug: t=2.4, p=0.04). Similar to Chapter 2, drinking behavior was not affected by either dose in CeA (drinking initiations and time: Fs <1.1, *n.s.*).

Across animals, BLA DAMGO did not affect food intake, eating behavior, or drinking at either dose (intake: F(2,30)=0.4, *n.s.*; eating initiations: F(2,16)=0.7, *n.s.*; eating time: F(2,16)=0.9, *n.s.*; drinking initiations: F(2,16)=2.3, *n.s.*; drinking time: F(2,16)=0.6, *n.s.*). This said, about 1/3 of BLA animals did show some enhanced food intake after 0.1μg DAMGO, and about 20% after 0.05μg. Only one animal showed enhanced intake at both doses. These findings suggest that BLA opioid stimulation can increase feeding, but this effect is inconsistent and may depend upon currently unknown intervening variables.

CeA and BLA DAMGO also had several other behavioral effects in food intake testing chambers. Both doses of DAMGO in CeA marginally increased cage crossing, rearing, and food sniffing (crosses: F(2,16)=2.5, p=0.1, 0.05ug: t=1.9, p=0.08, 0.1ug: t=2.0, p=0.07; rears: F(2,16)=2.9, p=0.09, 0.05ug: t=3.6, p=0.004, 0.1ug: t=2.0, p=0.075), but affected neither defensive treading (F(2,16)=0.8, *n.s.*) nor grooming (F(2,16)=0.5, *n.s.*). BLA DAMGO reduced the time spent sleeping at the high dose, and marginally at the low dose (F(2,16)=18.0, p<0.001; 0.05ug: t=1.8, p=0.1; 0.1ug: t=7.1, p<0.001), but no other measured behaviors (defensive treading, grooming, cage crossing, rearing, or food sniffing: Fs <1, *n.s.*).

Discussion

These results demonstrate that stimulating opioid receptors in the central nucleus of amygdala (but not the nearby basolateral nucleus) with the μ agonist DAMGO (0.1μg) potently enhances phasic peaks of sucrose 'wanting' elicited by a Pavlovian sucrose CS. This enhancement specifically enhanced 'wanting' triggered by a Pavlovian CS+, and was appropriately transferred only into pressing on a lever that previously delivered sucrose. In addition, CeA DAMGO seemed to channel sucrose seeking more specifically into CS+ periods, and reduced the generalization of incentive motivation to a control stimulus unpaired with sucrose. CeA, but not BLA DAMGO also robustly and consistently stimulated food intake, suggesting enhancement of 'wanting' for both a cue-

triggered UCS representation, and the UCS itself. Based on Fos plume analysis of functional drug spread, we determined these enhancements to be due to opioid stimulation within CeA in particular. These data therefore suggest that CeA opioids function to target and focus cue-triggered incentive motivation and 'wanting.'

CeA Opioids Focus Incentive Salience

CeA DAMGO (0.1µg) robustly enhanced sucrose 'wanting' triggered by Pavlovian cues by over 375% compared to vehicle levels. This enhancement was specific to cue-triggered 'wanting,' as neither responses to control cues, nor was pressing on a control lever that never delivered sucrose were affected.

CeA opioid stimulation seemed to focus 'wanting' based on activation of S-S representations of sucrose by a Pavlovian cue for the reward. After CeA DAMGO, sucrose seeking occurred more specifically during CS+ periods, as opposed to precue periods (Figure 3.2&3.3). In fact, in the absence of the Pavlovian CS+ DAMGO actually *reduced* sucrose lever pressing, though animals still explored the chamber and entered the sucrose delivery area to an equivalent extent as after vehicle. In this way, CeA opioid stimulation seems to have enhanced the signal to noise ratio of the CS+, immediately focusing behavior upon sucrose seeking when relevant S-S associations are activated by the best Pavlovian predictor of reward, the CS+.

In PIT, animals generally press the sucrose lever to some extent in the absence of the CS+. Presumably, this sucrose seeking also involves 'wanting,' for example triggered by contextual cues of the chamber, cognitive expectancies, and/or motor habits. CeA DAMGO seemed to inhibit these processes, instead shifting 'wanting' more exclusively to the CS+ triggered UCS representation. The CS+ was the one stimulus that most reliably predicted sucrose during training. In this way, 'wanting' was focused upon the best Pavlovian predictor of reward, possibly in a 'winner take all' manner. In some ways, this is similar to the pattern of results described in Chapter 2, when 'wanting' was focused only on the 'best' learned cue, at the expense of other stimuli also associated with reward.

In PIT, it is not uncommon for a control auditory CS that was never associated with sucrose to elicit some sucrose lever pressing (Wyvell and Berridge, 2000, 2001), presumably because the loud auditory control stimulus is similar enough to the CS+ that

animals generalize the stimuli. Here, animals did show such generalization of the CS+ and CS- under vehicle conditions. However, CeA opioid stimulation very specifically enhanced only pressing triggered by the CS+, and this enhanced 'wanting' was not generalized to the control stimulus. Therefore, in addition to focusing sucrose seeking more specifically into CS+ periods, CeA DAMGO also focused enhanced 'wanting' directly triggered by previously formed Pavlovian associations. Presumably this means that whichever brain mechanisms that allow the CS- to access the motivational value of the CS+ by generalization were not affected by CeA opioid stimulation. Instead, DAMGO only potentiated sucrose 'wanting' that was directly elicited by the activation of an S-S Pavlovian association, as would be predicted by incentive salience theories of Pavlovian motivation (Bindra, 1978; Berridge and Valenstein, 1991).

Finally CeA DAMGO also focused 'wanting' in yet another way; shifting behavior away from the expression of the S-R conditioned approach of the sucrose delivery cup learned during Pavlovian training. Curiously, this was only the case at the low dose of CeA DAMGO, which did not concurrently enhance cue-triggered sucrose lever pressing. In addition, BLA DAMGO also reduced cue-triggered sucrose cup approach without affecting cue-triggered lever pressing. These results may indicate that amygdala opioid stimulation may independently enhance cue-triggered 'wanting' (CeA only), and reduce cue-triggered S-R habits (CeA and BLA). Such dissociations are not unprecedented with amygdala manipulations in the PIT paradigm, as CeA lesions attenuate cue-triggered lever pressing, but not sucrose cup approach in a PIT paradigm (Holland and Gallagher, 2003). In any case, the present results provide additional support for the idea that CeA is a key site for the generation of 'wanting' in particular, rather than S-R habits triggered by Pavlovian stimuli.

CeA Opioids Play a Specific Role in 'Wanting'

Both Pavlovian to instrumental transfer and food intake stimulation by DAMGO was specific to the central nucleus, but not the basolateral nucleus. In fact, BLA DAMGO had few effects during PIT testing at all. BLA has previously been reported to play a role in PIT, but only in a particular a type of PIT that depends upon linking the sensory properties of specific rewards with their current motivational value, and generating appropriate instrumental behaviors as a result (Blundell et al., 2001; Corbit

and Balleine, 2005). The type of PIT measured here does not require animals to use sensory features of UCSs to produce 'wanting,' and accordingly CeA seemed to be the only amygdala structure involved in opioid 'wanting' enhancement. An obvious future direction would be to examine whether BLA DAMGO would enhance sensory-specific PIT in a paradigm designed to measure this.

DAMGO Fos Plumes in CeA and BLA

DAMGO microinjection into the CeA demonstrated a similar pattern of functional spread of drug around injection sites as previously reported in Chapter 2. DAMGO was injected in a lower volume of vehicle in the present experiment than in the prior chapter (0.2µl vs. 0.5µl), and DAMGO plumes were accordingly more localized (mean radii ~0.3mm here, ~0.5mm in Chapter 2).

Interestingly, DAMGO failed to produce consistent plumes when injected in BLA. BLA contains high levels of µ opioid receptors (Mansour et al., 1995; Poulin et al., 2006), and neurons there respond *in vitro* with changes in firing (Finnegan et al., 2006). In addition, we observed distinct behavioral effects of BLA DAMGO, including decreases in sleeping and sucrose cup approach at both 0.05& 0.1µg, and (in Chapter 4) increases in Fos-like immunoreactivity in nucleus accumbens and other structures. Therefore, it is likely that DAMGO actions in BLA failed to produce Fos plumes due to differences in the local circuitry, or differences in the predominant localization of µ opioid receptors (e.g. pre vs. postsynaptic). Less is known about the actions of opioid agonists in BLA compared to CeA, and the nature of its differences and similarities with CeA is worthy of future investigation.

Summary

The present data provide the first clear evidence that µ opioid receptors in the central amygdala help target and focus cue-triggered 'wanting' for a UCS reward. We show both that these effects are exclusively targeted upon an S-S sucrose representation activated by the 'best' Pavlovian cue for the 'wanted' reward, and appropriately targeted into a particular instrumental action which previously delivered that reward. In addition, the same CeA opioid stimulation also robustly enhanced intake of a UCS reward itself, further supporting enhancement of 'wanting.' Finally, we demonstrate that these effects were chiefly mediated by the central, rather than the nearby basolateral amygdala

nucleus, suggesting that CeA may be an opioid hotspot for targeted and focused cue-triggered 'wanting.'
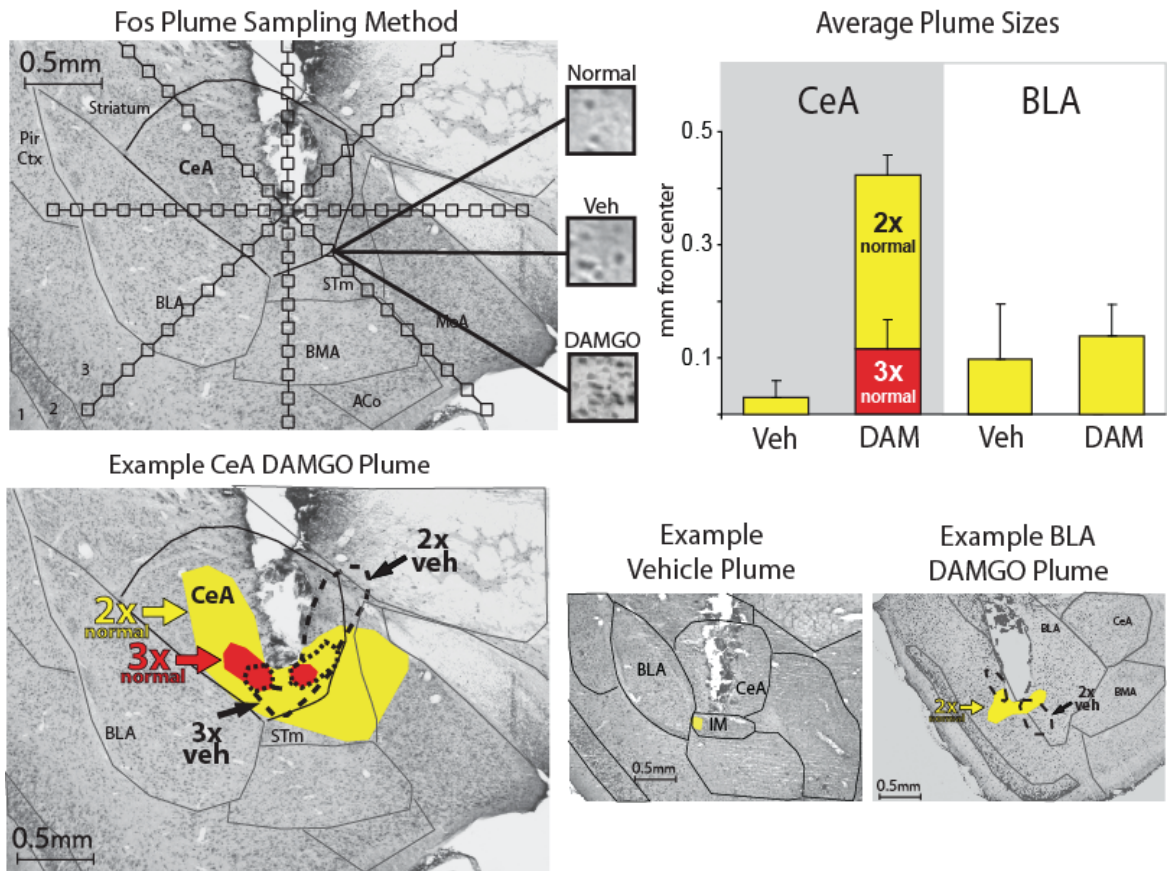
Figure 3.1: CeA and BLA DAMGO Fos Plumes. *Fos Plume Sampling Method:* The method used to sample Fos expression around injection sites is illustrated, in which radial arms for sampling extend from the center of microinjection sites, viewed in a coronal plane. Fos-expressing neurons are counted in 62.5 x 62.5μm blocks on arms spaced at 62.5μm intervals; 5x magnification. Insets show sample tissue blocks from equivalent sites in uninjected brains, or those injected with vehicle or DAMGO (0.1μg). *Example CeA DAMGO Plume:* Example CeA DAMGO-induced plume of Fos modulation. Colors indicate areas of Fos elevation by 2x (yellow) or 3x (red) over uninjected control levels for the relevant structure. Lines represent areas of Fos elevation by 2x (dashed line) or 3x (dotted line) over vehicle microinjection levels at equivalent sampling boxes and brain regions. *Average Plume Sizes:* Mean(SEM) plume radii are shown for vehicle and DAMGO microinjections in CeA and BLA. 2x normal levels of Fos elevation are pictured in yellow, while inner 3x normal plume radii are in red, (only seen after CeA DAMGO). *Example Vehicle Plume:* Example small ACSF vehicle plume, compared to uninjected tissue. *Example BLA DAMGO Plume:* Example small, inconsistent plume observed after BLA DAMGO.

Figure 3.2: CeA DAMGO (0.1μg) Enhances Cue-Triggered 'Wanting:' DAMGO enhancement of cue-triggered 'wanting' in the Pavlovian to instrumental transfer paradigm is mapped rat-by-rat based on the intensity of DAMGO effects at the mapped microinjection sites (color) and average DAMGO Fos plumes measured at similar sites (size). The left column depicts 0.1μg DAMGO effects in CeA (top row) and BLA (bottom row), mapped in a horizontal view. The right column depicts 0.05μg DAMGO effects in CeA and BLA. DAMGO (0.1μg) only enhanced cue-triggered 'wanting' in CeA, as shown on top left. Hexagonal symbol colors denote DAMGO modulation of cue-triggered sucrose lever pressing (% precue pressing), calculated as change from vehicle (DAMGO-vehicle) in the percent of precue pressing during CS+ presentations. Inner symbols represent average diameter of 3x Fos enhancement over uninjected tissue levels, surrounded by semitransparent halos that show 2x Fos enhancement zones. To the left of maps, bar graphs show aggregate % of precue sucrose lever pressing after vehicle, 0.05μg & 0.1μg DAMGO for all CeA (top) and BLA (bottom) animals. Bars along rostrocaudal and mediolateral axes show the intensity of DAMGO effects (mean ± SEM percent of vehicle levels) within each 0.4mm-wide level, centered on the labeled coordinate; a plume symbol can contribute to more than one bar when it straddles multiple levels. Bar colors reflect mean percentage change from vehicle in that zone. * indicates difference from vehicle, $p < 0.05$.

0.1μg

0.05μg

CeA

BLA
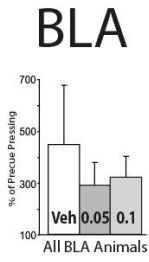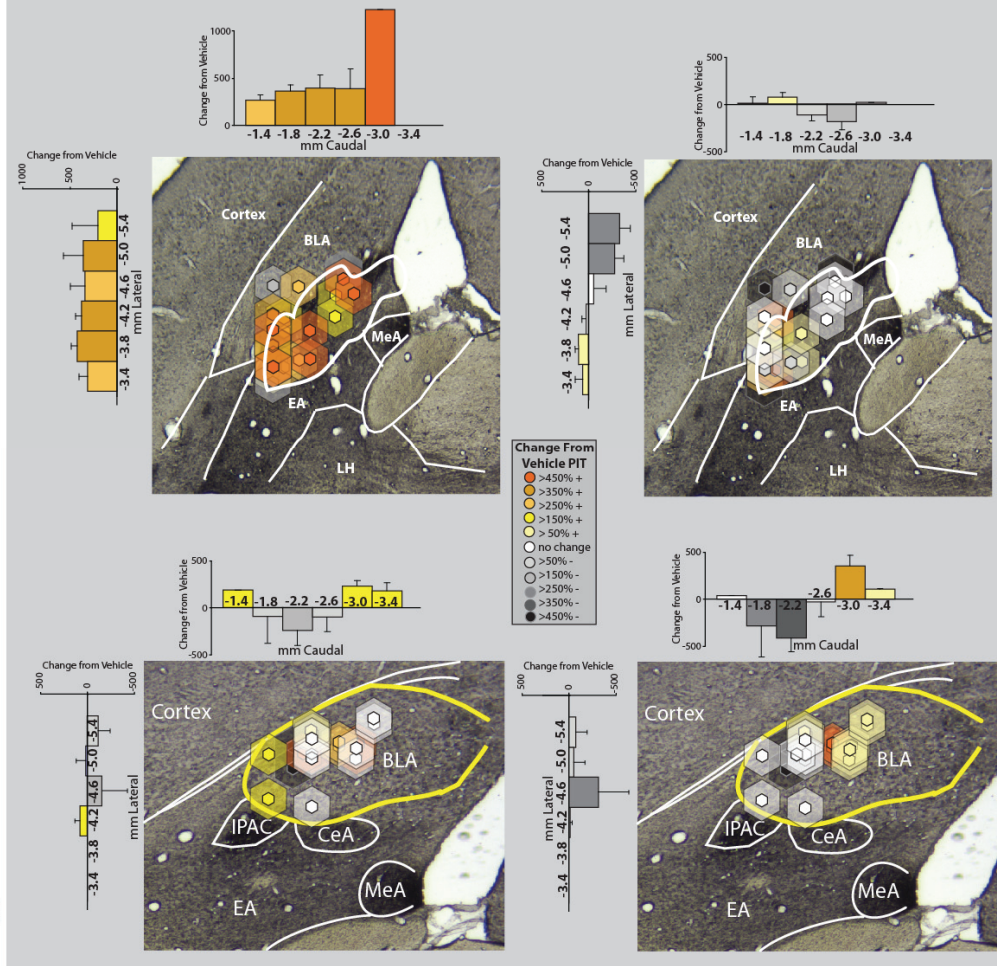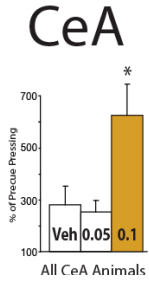
Figure 3.3: CeA DAMGO Sharpens Cue-Triggered Peaks of Sucrose Seeking: Total pressing on the sucrose lever is shown after DAMGO (0.1µg: Red line) and vehicle (Blue line). Mean (SEM) presses on the sucrose lever (y-axis) are shown during the 30sec prior to (Pre CS), and 30sec of each of the four successive CS+ and CS- presentations during PIT sessions. DAMGO enhanced the focus of cue-triggered 'wanting,' in that CS+ triggered peaks in pressing were sharpened relative to precue levels, compared to vehicle day. CS- presentations did not cause increased sucrose lever pressing, especially after DAMGO.
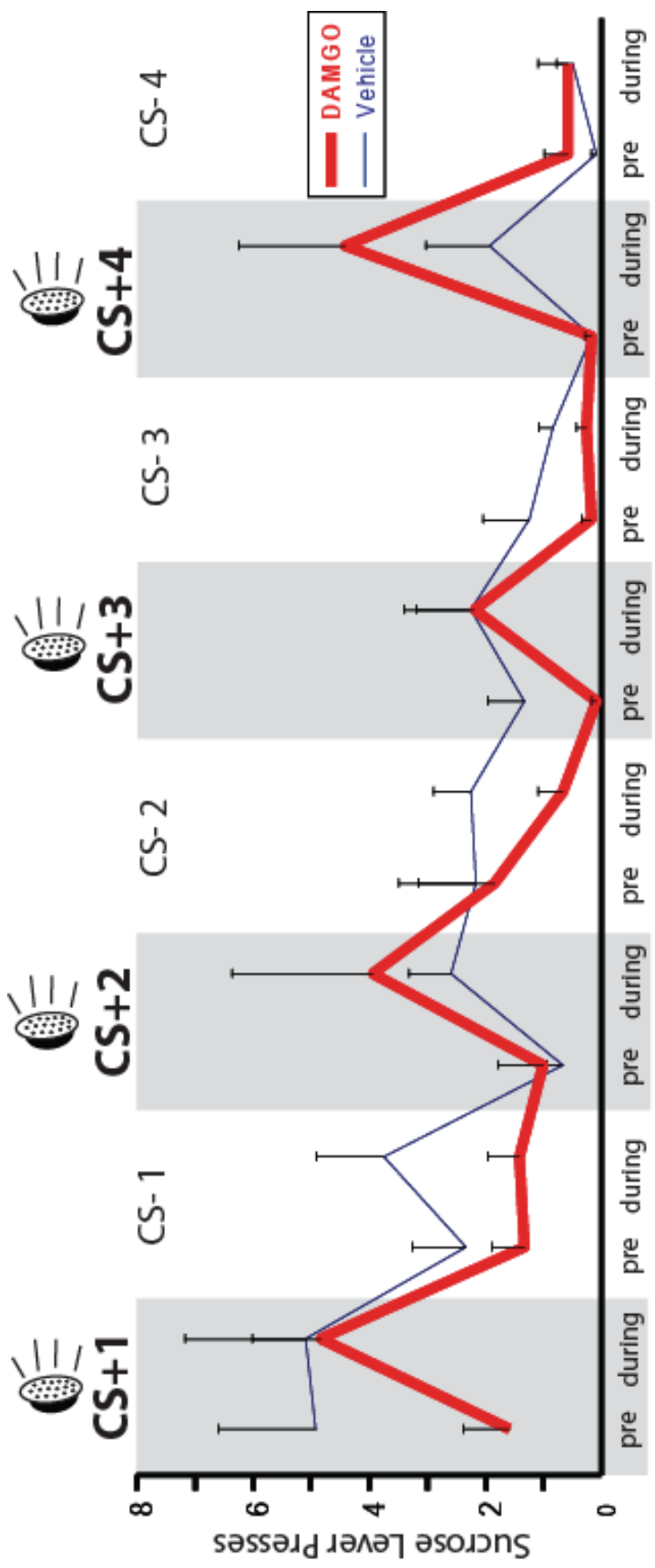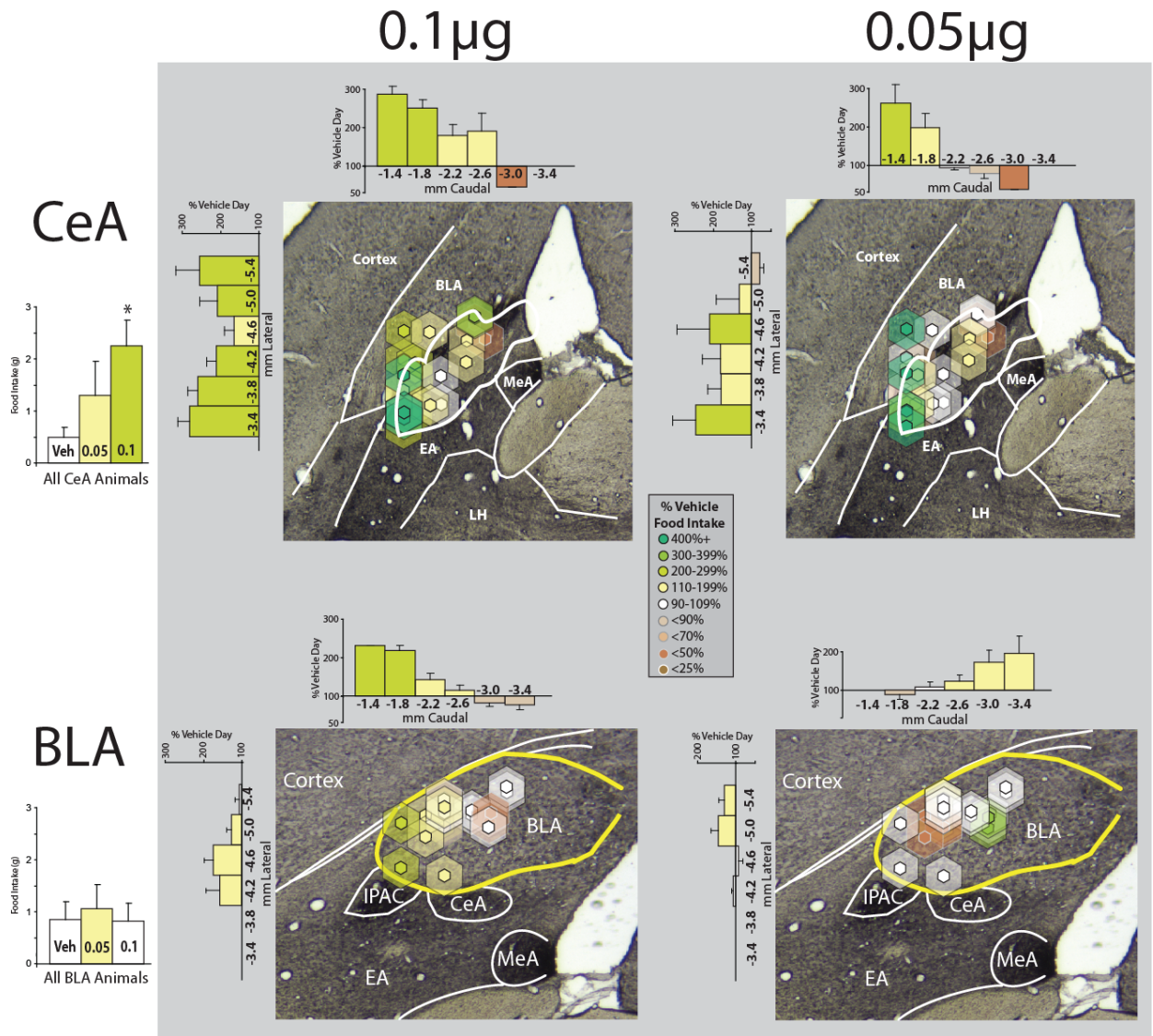
Figure 3.4: CeA DAMGO Enhances Food UCS Consumption: Maps of food intake after DAMGO (0.1µg: left column, 0.05µg: right column) are shown for CeA (top row) and BLA (bottom row), shown in a horizontal view using symbol logic identical to Figure 3.2. CeA DAMGO (0.1µg) enhanced intake of chow over the 1hr session, but neither 0.05µg DAMGO nor BLA DAMGO did so. * indicates increase in food intake (p<0.05) compared to vehicle.

Normal Tissue-Relative

	Mean Fos Plume Radii (mm) mean(SEM)			Estimated Fos Plume Volumes (mm^3)		
CeA Vehicle	0 (0)	0.03 (0.03)	0.03 (0.02)	0	0.0001	0.0001
CeA DAMGO	0.12 (0.05)	0.31 (0.04)	0.05 (0.02)	0.007	0.121	0.0007
BLA Vehicle	0 (0)	0.1 (0.1)	0 (0)	0	0.004	0
BLA DAMGO	0 (0)	0.08 (0.03)	0.06 (0.03)	0	0.002	0.001
	Intense Plume >300% Normal	Moderate Plume >200% Normal	No Change <50% Change	Intense Plume >300% Vehicle	Moderate Plume >200% Vehicle	No Change <50% Change

Vehicle Microinjection-Relative

	Mean Fos Plume Radii (mm) mean(SEM)			Estimated Fos Plume Volumes (mm^3)		
CeA DAMGO	0.07 (0.04)	0.19 (0.04)	0.07 (0.03)	0.001	0.028	0.0014
BLA DAMGO	0.03 (0.01)	0.05 (0.02)	0.11 (0.04)	0.0001	0.0004	0.006
	Intense Plume >300% Normal	Moderate Plume >200% Normal	No Change <50% Change	Intense Plume >300% Vehicle	Moderate Plume >200% Vehicle	No Change <50% Change

Table 3.1: DAMGO Fos Modulation Radii and Volumes. Mean(SEM) Fos plume radii (left) and volumes (right) are listed for 0.1μg/0.2μl DAMGO or 0.2μl ACSF vehicle alone injected in CeA or BLA. Plume sizes were calculated compared to uninjected normal tissue (top), or to vehicle microinjections (bottom).

Chapter 4
**A Specific Role for Central Amygdala in 'Wanting:' Lack of Hedonic Enhancement
by Amygdala Opioid Activation**

Introduction

Brain reward systems may participate in reward 'wanting' (incentive motivation
targeted at rewards or reward cues), 'liking' (the hedonic impact of a reward itself), or
both. Fortunately, it is possible to specifically measure these psychological components
of food reward separately with specific behavioral paradigms in the rat, in order to parse
the exact role played by particular brain structures or neurochemical systems in reward.

When rewards are experienced by an animal, its affective value is determined by
the brain, based on species-specific evolved predispositions (e.g. calorically rich sweet
tastes are 'liked' by most omnivores), and the animal's current physiological state (e.g.
foods are 'liked' more when the animal is hungry). The taste reactivity paradigm allows
examination of this affective evaluation of taste stimuli in animals by examining
characteristic orofacial reactions displayed to 'liked' and 'disliked' tastes. Rats, humans,
and other primates exhibit characteristic hedonic (e.g. tongue protrusions) or aversive
(e.g. gapes) reactions to nice or nasty tastes, which reflect affective 'liking' or 'disliking'
evaluations.

μ opioid receptors have frequently been linked to hedonic 'liking' throughout the
forebrain and brainstem. For example, opioid stimulation of brain areas including
nucleus accumbens, ventral pallidum, and parabrachial nucleus of the brainstem have all
been proposed to enhance palatability and 'liking' of pleasant rewards (Berridge, 2003).
In fact, ~1mm^3 'hedonic hotspots' within dorsomedial accumbens shell and caudal
ventral pallidum have been discovered (Peciña and Berridge, 2005; Smith and Berridge,

2005), in which μ opioid stimulation with the specific agonist DAMGO robustly enhances 'liking' of an intra-orally administered sucrose solution. Interestingly, DAMGO also robustly enhances food intake 'wanting' in accumbens and ventral pallidum, but do so in much larger regions of these structures.

The amygdala, consisting of several subregions including the central (CeA) and basolateral (BLA) nuclei, is well known to modulate appetitive and aversive learning and motivation (Morris and Dolan, 2001; Di Ciano and Everitt, 2004; Phelps and LeDoux, 2005; Balleine and Killcross, 2006). Some have argued that these amygdala nuclei may also be involved in hedonic evaluations, as well as in learning and motivation for rewards. For example, a recent positron emissions tomography imaging study in humans reports endogenous opioid release when positive affective states are induced by a funny movie, favorite music, and unexpected receipt of a monetary reward (Koepp et al., 2008).

Some have proposed that amygdala may also process negative affective states produced by drug withdrawal. For example, morphine withdrawal causes increased Fos expression in CeA, and this increase is negatively correlated with the degree of preference for a food-associated environment in these animals, but not those which were not withdrawn from morphine (Harris and Aston-Jones, 2007). Koob and colleagues have similarly proposed that withdrawal-induced negative affective states are related to changes in various neurochemical systems in CeA and other extended amygdala structures (Menzaghi et al., 1994; Merlo Pich et al., 1995; Roberts et al., 1996; Olive et al., 2002), including opioids (Schulteis et al., 1994; Gracy et al., 2001).

For food reward, amygdala (and particularly CeA) opioids have also been tied to hedonic affective processes. For example, Pomonis et al. (2000) found that systemic naloxone injections enhanced Fos expression in CeA, which was enhanced by concurrent palatable sucrose drinking, which they interpreted to mean endogenous release of opioids in CeA is involved in palatability. Will et al. (2004) have also proposed that amygdala may be involved in hedonic processing of food rewards. They found that CeA inactivation with the GABA agonist muscimol blocked intake of a fat reward on its own, as well as fat intake that is otherwise preferentially enhanced by intra-accumbens infusion of DAMGO. BLA inactivation, on the other hand, only blocked accumbens DAMGO-induced fat feeding, without affecting basal levels of intake.

Clearly, some evidence supports a potential role for opioids in CeA and/or BLA in the hedonic processing of rewards. Here we tested this possibility by examining effects of μ opioid stimulation with the specific agonist DAMGO (0.1μg) on hedonic 'liking,' and aversive 'disliking' reactions to intraorally administered sweet and bitter solutions in a taste reactivity paradigm. In addition, we examined Fos expression in 5 mesolimbic structures induced by CeA and BLA opioid stimulation, in order to examine the brain potential circuits potentially involved in the observed behavioral effects.

We found that instead of enhancing taste 'liking,' as in other brain reward structures, CeA and BLA μ opioid stimulation actually decreased 'liking' of an otherwise pleasant sucrose taste. In contrast, the very same injections increased subsequent food intake 'wanting' on the same day. Overlapping, but distinct patterns of brain activation were found in distant reward structures after CeA and BLA DAMGO. These results provide a dramatic dissociation of the brain substrates of 'wanting' and 'liking,' and in conjunction with previous chapters, point to a specific role for amygdala opioids in enhancing incentive salience targeting but not hedonics.

Materials and Methods

Design

Here we tested effects intra CeA or BLA microinjection of the μ opioid agonist DAMGO (0.1μg/0.2μl) on 'liking' and 'wanting' of food rewards. Rats were tested on four separate days in counterbalanced order, on which they received all combinations of DAMGO and vehicle microinjections, and sucrose and quinine intraoral infusions (15 & 30min after microinjection). Following taste reactivity testing, rats were transferred to food intake chambers for an additional hour of testing. To investigate the neural circuits recruited due to amygdala DAMGO microinjections and/or the concomitant behavioral effects, we also measured Fos in several reward structures that interact with amygdala in a separate group of animals.

Subjects

Sprague Dawley rats (n=34, female, 250-350g, pair housed, all estrous phases tested) were implanted with cannulae aimed at the central (CeA) or basolateral amygdala (BLA), assigned to behavioral (n=22) or distant Fos activation groups (n=12), and given

microinjections of DAMGO (0.1μg/0.2μl) or vehicle (0.2μl) to observe effects on sucrose and quinine taste reactivity 'liking,' food intake, and distant Fos expression.

Surgery

Rats were anesthetized with ketamine (80mg/kg), xylazine (5mg/kg), and pretreated with atropine (0.04mg/kg). Using a stereotaxic device, rats were implanted with bilateral 23ga cannulae, 14mm in length, aimed 2mm dorsal to CeA (-1.8 to -3.0 AP; ±3.4 to ±4.6 ML; and -5.8 to -6.5 DV), or BLA (-1.8 to -3.2 AP; ±4.6 to 5.2; -8.2 to 9.2 DV). Additional animals were also implanted with cannulae outside the amygdala, centered in the endopiriform cortex lateral of BLA (-1.6 AP; ±5.6 ML; -9.1 DV). A separate group of rats used to measure DAMGO effects on distant Fos were implanted with cannulae at equivalent CeA and BLA sites as behaviorally tested animals. Cannulae were secured with skull screws and dental cement, and occluded with steel stylets.

In the same surgery, behaviorally tested rats were also implanted with bilateral oral cannulae (PE-100 tubing) to allow oral infusions of sucrose (1%) or quinine (3 x 10^{-4} M) solutions during taste reactivity testing. Oral cannulae were inserted lateral to the first maxillary molar, threaded behind the zygomatic arch, and exited through the dorsal head where they were cemented to skull screws (Grill and Norgren, 1978; Berridge et al., 1984). All rats were allowed to recover before testing for at least 7 days after surgery, and were habituated to their taste reactivity and food intake test chambers for 30 min on 4 consecutive days prior to the first test. On the last day of habituation, all rats received one 0.2μl artificial cerebrospinal fluid (ACSF) microinjection following procedures below to acclimate them to microinjections themselves.

Drugs and Microinjections

DAMGO (Sigma) was dissolved in ACSF to a dose of 0.1μg/0.2μl/side. ACSF (0.2μl) alone was used for vehicle microinjections. On test days, rats were gently hand-held while stylets were removed. Rats then received bilateral microinjections over 60sec of vehicle or DAMGO via stainless steel injector cannulae (29ga), which extended 2mm beyond the guide cannulae into the target sites. Microinjector cannulae were left in place for an additional 60sec to allow for drug diffusion, then stylets were replaced and rats were immediately placed into testing chambers (or home cages for the "distant Fos" group).

Behavioral Taste Reactivity Tests

After rats received bilateral brain microinjections of DAMGO or vehicle on test days, a tastant delivery tube was connected to their oral cannulae, and rats were placed in the taste reactivity test chamber. To elicit taste reactivity patterns, 1ml sucrose or quinine solutions were infused over 60sec through the oral cannula at 15 and 30min following brain microinjection (sucrose and quinine were tested on separate days, with the same tastant delivered at 15 and 30min after microinjections on each day). A digital video camera recorded orofacial reactions to all infusions, via an angled mirror under the transparent taste reactivity chamber floor. Sessions were conducted in counterbalanced order on separate days, separated by at least 48hrs.

Food Intake and General Behavioral Tests

In order to compare hedonic effects of amygdala opioid stimulation with effects on appetitive 'wanting' of a food reward, we tested food intake in the same rats following taste reactivity on each test day. Following the second intraoral infusion of sucrose or quinine on each test day, infusion tubes were removed, and rats were allowed to remain in the testing cylinders for 15 additional minutes to allow dissipation of administered tastes. They were then transferred to food intake testing chambers, which were clear tub cages with corncob bedding, pre-weighed food pellets, and *ad lib* water. They were videotaped for one hour (45-105min following DAMGO or vehicle microinjections). Food intake was recorded in grams, and tapes were later scored offline for eating and other spontaneous behaviors.

Taste Reactivity Scoring

Hedonic, aversive, and neutral response patterns were later scored off-line in slow motion (frame by frame to $1/10^{th}$ actual speed) by a trained observer who was blind to experimental condition, using time bin scoring procedures developed to assess hedonic vs. aversive taste valuations (Berridge et al., 1984; Berridge, 2000). Hedonic responses included rhythmic midline tongue protrusions, lateral tongue protrusions, and paw licks. Aversive responses included gapes, head shakes, face washes, forelimb flails, and chin rubs. Neutral responses, which are less consistently linked to hedonic/aversive taste valuation, included passive dripping of solution out of the mouth, ordinary grooming, and rhythmic mouth movements. All video analyses were conducted blind to the

microinjection contents and cannula placements using Observer software (Noldus, Netherlands).

A time bin scoring procedure was used to ensure that taste reactivity components of different relative frequencies were balanced in their contributions to the final affective hedonic/aversive totals (Berridge, 2000). For example, rhythmic mouth movements, passive dripping of solution, paw licking, and grooming reactions typically occur in long bouts, and were thus scored in 5sec time bins (up to 5sec continuous bout duration equaled one occurrence). Tongue protrusions, which occur in shorter bouts, were scored in 2sec time bins. The other behavioral components (lateral tongue protrusions, gapes, forelimb flails, head shakes, chin rubs) typically occur as discrete events and were therefore scored as single occurrences each time they occurred (e.g. one gape equaled one occurrence). Individual totals were calculated for hedonic vs. aversive categories for each rat by adding all response scores within an affective category for that rat. Hedonic 'liking' was defined as the sum of scores for lateral tongue protrusions, rhythmic tongue protrusions, and paw licks. Similarly, aversive 'disliking' was the sum of gapes, head shakes, face washes, forelimb flails, and chin rubs.

Food Intake and Other Behavioral Scoring

Food intake and general behavior were scored offline in slow motion by observers blind to experimental conditions for a subset of tested animals (food intake in grams was recorded in all animals). The number of initiations of eating, drinking, sleeping, food sniffing, food carrying, front/back cage crossing, rearing, grooming, and defensive paw treading behaviors were recorded, as was time spent eating, drinking, and sleeping.

Histology

After the completion of testing, rats were deeply anesthetized with sodium pentobarbital (0.2g/kg), microinjected with 0.2μl black ink, and their brains were extracted. Brains were sectioned with a freezing microtome into 60μm coronal slices, stained with cresyl violet, and mapped for microinjection center locations according to Paxinos and Watson (2007).

Fos Immunohistochemistry

Rats in the "distant Fos" group were handled for 3 days for 10 min each (like behaviorally tested groups), then microinjected bilaterally in the CeA or BLA with

DAMGO (n=8; 0.1μg/0.2μl) or vehicle (n=4; 0.2μl) as described above. Cannulae placements were located throughout the CeA and BLA, similarly to the behavioral group, and it was later confirmed that every behavioral group site was within 1mm of a corresponding Fos group site. Fos-like protein expression in several brain structures was therefore harvested under conditions similar to the first day of testing for the behavioral group, as described below.

Fos staining procedures were identical as those employed in Chapter 2. Again, cannulae placements were localized by comparing the image from a low magnification light microscope with a standard rat brain atlas (Paxinos and Watson, 2007). In addition, every 5th slice from near the injection site, and every other slice at distant sites was stained for Substance P to help localize the borders of CeA and other nuclei, and to better localize injection sites within amygdala.

Fos Expression In Structures Distant From Injection Sites

To assess DAMGO activation of Fos expression in distant mesolimbic or extended amygdala structures with substantial anatomical connections to CeA or BLA, we sampled the number of Fos immunoreactive neurons in several sites within each structure of interest after CeA and BLA vehicle (CeA n=2, BLA n=2) or DAMGO (CeA n=5, BLA n=3) microinjections. Fos sampling followed standard procedures (Smith and Berridge, 2007). Only animals with bilateral cannulae placements in either CeA or BLA were included in analyses, and Fos was only measured in structures at least 1mm from microinjection sites, to exclude Fos activation resulting from direct actions of drugs on measured tissue. Fos was counted by placing a microscope eyepiece grid (composed of 5x5, 0.05 x 0.05mm boxes at 20x magnification) within a structure of interest (e.g. dorsal medial accumbens shell), so that all four corners of the grid were entirely within the structure. Fos was then counted in the dorsomedial, dorsolateral, ventrolateral, ventromedial, and central boxes of the grid for each measured AP and DV level of each structure, so each sampling box was 0.15mm apart mediolaterally and dorsoventrally. These sampling boxes were averaged for each region of each structure on each slice, and again averaged to yield a per-hemisphere mean for that portion of each structure. At least two slices were sampled from each hemisphere for each subregion of each structure of interest. Anatomical placement of sampling grids was determined by comparison of Fos-

immunostained slices with adjacent Substance P-immunostained slices and the Paxinos & Watson (2007) brain atlas.

Nucleus accumbens core, medial shell, and lateral shell were measured in 3 rostrocaudal bins (coordinates relative to Bregma): rostral: +2.28-2.76mm, medial: 1.68-2.16mm, caudal: 0.84-1.32mm. Medial shell and core were sampled at dorsal (shell: -5.9 to -7.0mm; core: -5.8 to -6.6mm) and ventral sites (shell: -7.2 to -8.3mm, core: -6.6 to -7.8mm) at each rostrocaudal level. Ventral pallidum (VP) was sampled separately at rostral and caudal levels (~ -0.5 and -0.5mm, respectively), while lateral hypothalamus (LH), interstitial nucleus of the posterior limb of the anterior commissure (IPAC), and ventral tegmental area (VTA) averages were computed from 3 levels/structure/rat between -1.44 and -3.1mm (LH), 0.5 and 0 (IPAC), and -4.9 and -5.5mm (VTA) caudal of Bregma.

Statistical Analyses

All behavioral analyses were two-tailed, and significance was always set at p<0.05. Since DAMGO effects on taste reactivity were stable between 15-30min timepoints, and hedonic and aversive responding to tastants did not differ between infusions, average reactions for each drug and tastant were used for primary taste reactivity analyses (no effects of infusion time on sucrose or quinine reactivity, or interactions of drug with time (Fs<1.5, *n.s.*)). Mixed within/between subjects ANOVAs were used for primary analyses of taste reactivity, food intake, and other behaviors. Drug (DAMGO and vehicle) and taste (sucrose and quinine) were within subjects factors, and injection site (CeA or BLA) was the between subjects factor. Additional Bonforroni-corrected ANOVAs and t-tests were used for post-hoc analyses of significant main effects and interactions. Sucrose vs. quinine taste reactivity did not affect subsequent food intake or other spontaneous behaviors unless otherwise noted in results, so although this variable was included in statistical analyses, it is not reported unless relevant. To describe DAMGO behavioral effects as a percentage increase over vehicle levels, a constant value of 1 was added to every datum to avoid the problem of calculating percentage increases over zero for rats with low baselines.

For analysis of CeA and BLA DAMGO effects on Fos in distant structures, Fos counts from within each structure of interest were averaged for each hemisphere of each

animal, resulting in a mean Fos/$0.05mm^2$ estimate for each structure, and each subregion within accumbens core (dorsal and ventral), and shell (dorsal, ventral, and lateral). For analysis of distant Fos effects on whole structures, t-tests comparing DAMGO and vehicle for equivalent regions were conducted. For examining Fos gradients within accumbens, 2 (drug) x 2 (dorsal vs. ventral core or shell), or 2 (drug) x 3 (rostral, medial, and caudal core or shell) ANOVAs were employed. To compare Fos activation by CeA vs. BLA DAMGO, 2 (drug) x 2 (injection site) ANOVAs were performed for each structure of interest.

Results

Synopsis

We tested how stimulating μ opioid receptors in the central or basolateral amygdala would affect 1) 'liking' and 'disliking' of rewarding sucrose, and aversive quinine tastes, and 2) food intake 'wanting' and other spontaneous behaviors, and 3) Fos activation of distant reward-related structures. We found that DAMGO injection in either CeA or BLA reduced hedonic reactivity to sucrose without consistently affecting reactivity to quinine. In contrast, the same microinjections increased food intake and feeding behavior, indicating simultaneous potentiation of food 'wanting.' Finally, CeA and BLA DAMGO induced overlapping, but somewhat distinct patterns of Fos activation in distant reward-related structures.

Amygdala DAMGO Effects on Hedonic and Aversive Taste Reactivity

Intraoral infusions of sucrose and quinine solutions produced characteristic taste reactivity responses: predominately hedonic reactions to sucrose (e.g. tongue protrusions) with few or no aversive reactions (e.g. gapes), and predominately aversive reactions to quinine with few or no hedonic reactions. DAMGO reduced hedonic reactivity whether it was injected in CeA (79% of vehicle day hedonics) or BLA (60% vehicle hedonics; main effect of DAMGO across structures: $F_{(1,18)}=13.4$, p=0.002; no interaction of CeA vs. BLA x drug: $F_{(1,18)}=1.1$, *n.s.*; Figure 4.1). No similar hedonic decrease was seen for rats with cannulae outside the amygdala, in the adjacent endopiriform cortex ($F_{(1,1)}=1$, *n.s.*), indicating that hedonic decreases by DAMGO were specific to the amygdala.

Manipulations affecting hedonics should modulate each type of hedonic reaction similarly, and accordingly DAMGO in CeA and BLA significantly reduced hedonic rhythmic tongue protrusions ($F(1,18)=6.2$, $p<0.05$) and paw licks ($F(1,18)=9.9$, $p<.01$), and trended toward reducing lateral tongue protrusions ($F(1,18)=2.3$, *n.s.*). DAMGO also increased affectively neutral mouth movements ($F(1,18)=40.7$, $p<0.001$), presumably replacing hedonic reactions that were reduced by DAMGO.

Amygdala opioid stimulation did not significantly increase the low number of aversive reactions to sucrose (Figure 4.1; $F(1, 18)=1.4$, *n.s.*), although ~40% of CeA rats, and ~70% of BLA rats did show some increased aversion (with only 15% of both groups showing any aversive decreases), suggesting a potential shift in sucrose responding from less hedonic to more aversive in some rats, and a relatively specific reduction of hedonics in others.

In contrast to the hedonic shift from 'liking' to 'disliking' for a sucrose taste, DAMGO had little effect on aversive reactivity to a quinine taste in either CeA or BLA (Figure 4.2; no main effect of DAMGO: $F(1,15)=0.01$, *n.s.*; no interaction of structure x drug: $F(1,15)=1.5$, *n.s.*). None of the measured aversive reactions were affected by DAMGO, including gapes, chin rubs, forelimb flails, head shakes, face washes, or defensive treading ($Fs<1.5$, *n.s.*). The low levels of hedonic reactivity to quinine were also not consistently affected by DAMGO (main effect of DAMGO: $F(1,15)=0.1$, *n.s.*; no interaction of CeA vs. BLA and drug: $F(1,15)=0.2$, *n.s.*), nor were any of the individual hedonic reactions ($Fs<1.4$, *n.s.*).

These results demonstrate that stimulating μ opioid receptors in either CeA or BLA (but not nearby control sites) robustly and specifically decreases hedonic reactivity to sucrose. In some animals, this hedonic decrease was accompanied by a corresponding increase in aversion to sucrose, and in others hedonic responding was replaced instead by affectively neutral mouth movement reactions. Neither hedonics nor aversion to a bitter quinine solution was affected by CeA DAMGO, suggesting a specific effect of amygdala opioid stimulation on responses to a rewarding taste.

Amygdala DAMGO Modulation of Food Intake and other Spontaneous Behaviors

Following taste reactivity testing, we transferred rats to food intake chambers to examine effects of amygdala DAMGO on food 'wanting' as well as 'liking' in the same

rats after the same microinjections. CeA and BLA DAMGO increased feeding to an equivalent extent, by up to 650% of vehicle day levels (Figure 4.3). Amygdala DAMGO increased grams of food intake to 180% of vehicle levels (main effect of drug: $F_{(1,20)}=23.1$, $p<0.001$), eating initiations to over 330% ($F_{(1,20)}=14.9$, $p=0.001$), and time spent eating to over 650% of vehicle levels ($F_{(1,20)}=32.0$, $p<0.001$). No significant differences were found between the intensity of food intake or eating behavior by CeA vs. BLA DAMGO (no structure x drug interactions ($Fs<1.4$, *n.s.*). Food intake stimulation was specific to amygdala, as DAMGO in the nearby endopiriform cortex reduced food intake to less than 60%, and eating initiations and eating time to ~50% of vehicle levels. CeA and BLA DAMGO both increased the occurrence of food pellet carrying ($F_{(1,20)}=4.4$, $p<0.05$), but only CeA DAMGO enhanced the time spent sniffing food pellets ($F_{(1,20)}=3.5$, $p<0.05$).

CeA and BLA DAMGO also enhanced drinking initiations ($F_{(1,20)}=9.6$, $p<0.01$) and time drinking ($F_{(1,20)}=35.4$, $p<0.001$) to over 150% of vehicle levels. Both CeA and BLA DAMGO reduced sleeping (initiations: $F_{(1,20)}=11.1$, $p<0.01$; time: $F_{(1,20)}=22.7$, $p<0.001$). CeA DAMGO increased grooming ($F_{(1,14)}=10.3$, $p<0.01$), while BLA DAMGO reduced it ($F_{(1,20)}=11.5$, $p<0.01$). DAMGO did not significantly affect rears, cage crosses, or treading behavior in either CeA or BLA ($Fs <1.3$, *n.s.*).

Fos Activation of Distant Structures by Amygdala DAMGO Microinjection

In a second experiment, we examined activation of distant mesolimbic structures associated with CeA or BLA opioid stimulation, to explore the brain circuits recruited by microinjections of DAMGO. To do so, we examined Fos expression in 5 reward-related structures following CeA or BLA DAMGO and controls. Fos was measured in medial accumbens shell (NAcSh; dorsal and ventral, rostral-caudal), lateral NAcSh (rostral-caudal), accumbens core (NAcC; dorsal and ventral, rostral-caudal), VP (rostral and caudal), IPAC, LH, and VTA. Since the distant regions measured were much further from injection sites (>1mm) than the maximal spread of local Fos plumes ever extended, it is likely that these effects were due to circuit interactions of CeA with these structures, rather than direct effects of DAMGO diffusing there and directly inducing Fos. Results of CeA and BLA effects on distant Fos are summarized in Figure 4.4.

Amygdala DAMGO Effects on Accumbens Fos: DAMGO microinjected in CeA and BLA produced similar, but not identical patterns of Fos activation in nucleus accumbens (Figure 4.5). Both CeA and BLA DAMGO increased Fos overall in both NAcC and NAcSh to over 170% of control levels (CeA DAMGO vs. vehicle: NAcSh: t=3.4, p<0.01, NAcC: t=3.0, p<0.05; BLA DAMGO vs. vehicle: NAcSh: t=3.9, p<0.01, NAcC: t=5.1, p=0.001).

Within medial accumbens shell, CeA DAMGO more than doubled Fos in dorsal shell (262% of control levels; t=3.2, p<0.01), and increased Fos to a lesser extent in ventral shell (154%; t=2.5, p<0.05; drug x dorsal/ventral medial shell interaction: F(1,11)=4.9, p=0.05). BLA DAMGO also strongly increased Fos in dorsal medial shell (250% control; t=3.1, p<0.05) and weakly in ventromedial shell (154%; t=2.1, p=0.07). BLA, but not CeA DAMGO also increased Fos in lateral accumbens shell (CeA: t=0.3, *n.s.;* BLA: t=3.6, p<0.01; CeA vs. BLA effect on lateral shell Fos after DAMGO: t=3.1, p=0.01). No significant rostrocaudal gradients in Fos activation within accumbens shell were found after CeA or BLA DAMGO (Fs<1.2, *n.s.*).

CeA DAMGO increased dorsal NAcC Fos to 240% of vehicle levels (t=3.0, p<0.05), but did not affect ventral NAcC Fos (119%; t=0.8, *n.s.*). NAcC Fos enhancement did not vary by rostrocaudal level (no drug x AP level interaction: F(2,22)=1.1, *n.s.*). BLA DAMGO induced Fos in dorsal core sites to over 270% of vehicle levels (t=6.4, p<0.001), and also in ventral core to a lesser extent, to about 150% of control (t=2.5, p<0.05; BLA drug x dorsal/ventral interaction: F(1,8)=10.6, p<0.05). BLA DAMGO effects on NAcC Fos was greater at medial and caudal sites in core (>250% control) compared to rostral sites (130%; drug by AP level: F(2,43)=8.1, p=0.001; rostral vs. caudal: t=2.8, p=0.01; rostral vs. medial: t=2.1, p<0.05).

Amygdala DAMGO Effects on Distant Fos in Other Structures: CeA and BLA DAMGO caused Fos enhancement in IPAC, to over 170% of control (CeA: t=2.8, p<0.05; BLA: t=3.1, p<0.05). Both CeA and BLA DAMGO increased Fos in the rostral half of the VP (CeA: t=2.2, p<0.05; BLA: t=3.4, p=0.01), but only BLA DAMGO significantly increased Fos in the caudal VP (CeA: t=1.7, *n.s.*; BLA: t=2.5, p<0.05). LH Fos was also increased to a greater extent by BLA vs. CeA DAMGO (CeA vs. BLA effect on LH fos after DAMGO: t=2.6, p<0.05; CeA: t=0.9, *n.s.*; BLA: t=2.1, p=0.07).

DAMGO did not significantly affect VTA Fos when injected in either CeA (t=0.9, *n.s.*) or BLA (t=0.9, *n.s.*).

Discussion

These results provide the first demonstration that stimulation of amygdala μ opioid receptors does not enhance 'liking' of a UCS reward in the taste reactivity paradigm, even when the same injections enhance food UCS consumption minutes later. These findings demonstrate both a specific and unique role for amygdala opioids in facilitating 'wanting,' as well as a dramatic dissociation of 'wanting' and 'liking' effects of the same brain manipulation.

Specificity of DAMGO Effects

Amygdala opioid stimulation robustly and consistently decreased hedonic 'liking' of a sucrose taste, compared to vehicle microinjections in the same rats. This hedonic suppression was not due to a general behavioral inhibition, as neither quinine reactivity, nor affectively neutral mouth movement reactions to sucrose were decreased by amygdala DAMGO. In addition, the very same injections that suppressed hedonic reactivity enhanced food intake, a measure of reward 'wanting,' only minutes later.

Dissociation of 'Wanting' and 'Liking' by Amygdala Opioid Stimulation

Both CeA and BLA opioid activation caused consistent reductions in hedonic reactivity to sucrose, indicating a specific suppression of 'liking' by amygdala opioids. This pattern of hedonic suppression stands in stark contrast to the effects of opioid stimulation in other reward-related forebrain structures. For example, DAMGO microinjection in nucleus accumbens enhances hedonic 'liking,' particularly in a 1mm^3 'hotspot' in the dorsomedial nucleus accumbens shell. Similarly, a 0.8mm^3 hotspot for DAMGO enhancement of 'liking' exists in the caudal ventral pallidum. In fact, enhancement of hedonics and palatability by opioid agonists is so consistent throughout many brain areas that some have proposed that opioids might function as a hedonic signal throughout the brain, specifically modulating palatability-related features of reward (Koob, 1992; Kelley et al., 2005; Cota et al., 2006; Barbano and Cador, 2007).

Clearly, the present data provide a demonstration that at least in amygdala, this is not the case. Instead, amygdala opioids seem to specifically modulate appetitive aspects

of reward, such as the focus and enhancement of incentive salience 'wanting' triggered by Pavlovian reward-associated cues (Chapters 2&3). This interpretation supports conceptions of the central amygdala as a structure that is specialized for integrating Pavlovian learning with motivation (Everitt et al., 2003; Balleine and Killcross, 2006), and creating directed and focused 'wanting.'

It is worth noting that this is not the first demonstration of specific involvement of a neurochemical system in enhancing reward 'wanting,' without similar enhancement of reward 'liking.' Mesolimbic dopamine activation by microinjection of amphetamine into nucleus accumbens shell, or knockdown of the dopamine transporter in mutant mice also specifically enhances reward 'wanting' without similar enhancement of taste reactivity 'liking' (Wyvell and Berridge, 2000; Pecina et al., 2003). CeA is known to modulate dopamine release in accumbens in relation to reward consumption and anticipation (Ahn and Phillips, 2002), opening the possibility that CeA opioid and accumbens dopamine systems interact in enhancing reward 'wanting' in particular. In fact, we also provide evidence that CeA and BLA DAMGO indirectly stimulates Fos in accumbens, which potentially could be related to increased dopamine release there. Clearly, the functional interaction of amygdala opioids with dopamine systems is worthy of future study.

Overlapping Effects of CeA and BLA Opioid Stimulation in Reward

Although CeA and BLA opioid stimulation often have distinct effects on incentive salience 'wanting' (Chapters 2&3), here we found that DAMGO similarly reduces hedonics in both structures. Interestingly, we also found here that food (and water) intake behavior was stimulated by opioid activation of both CeA and BLA. Previously, we reported that BLA DAMGO only enhances food intake in a minority of animals, and that water intake is not affected by CeA or BLA DAMGO (Chapter 3). The reasons for these differences with the present experiment are unknown, but they could be related to methodological differences such as the time after DAMGO animals were tested, the number of injections animals received prior to food intake testing, the presence of oral cannulae, or the prior taste reactivity testing earlier on the test day.

CeA and BLA DAMGO also had largely similar effects on Fos activation of distant reward-related brain structures, including the rostral ventral pallidum, the interstitial nucleus of the posterior limb of the anterior commissure, and the nucleus

accumbens as a whole. This said, only BLA DAMGO enhanced Fos in ventral nucleus accumbens core, lateral accumbens shell, caudal ventral pallidum, and lateral hypothalamus, and only CeA DAMGO enhanced Fos in the dorsomedial medial accumbens shell. These results suggest some commonalities, and some differences in the neural circuits recruited after stimulation of these amygdala nuclei. The meaning of the overlapping pattern of Fos activation of by CeA and BLA DAMGO is unclear, however, as similarities in Fos activation does not imply similarities in neuronal firing patterns, populations of cells involved, or neurochemical involvement within these structures. For example, it is known that accumbens contains segregated populations of neurons that display distinct patterns of firing in response to rewards and cues that predict them (Carelli and Wondolowski, 2006; Wightman et al., 2007). Each of the structures activated by CeA and BLA DAMGO also contain heterogeneous populations of neurons that vary in their neurochemical profile, receptor content and localization, afferents and efferents, and many other factors. Clearly, the present results are only a preliminary step for understanding the similarities and differences in the brain circuits recruited by opioid stimulation of CeA and BLA opioid stimulation.

Conclusions

These results demonstrate that amygdala opioid stimulation enhances 'wanting' for food rewards, while simultaneously causing these rewards to be less 'liked' when they are actually received. This surprising pattern of effects demonstrates that amygdala plays a unique role in interfacing learning with motivation, and that opioids there play a different role there than in other brain reward structures in which they are well known to modulate hedonics.

Figure 4.1: Amygdala DAMGO Reduces Hedonic 'Liking' of Sucrose: DAMGO effects on hedonic 'liking' (left) and aversive 'disliking' reactions (right) to sucrose are mapped rat-by-rat based on the intensity of DAMGO effects at the mapped microinjection sites (color) and average DAMGO Fos plumes measured at similar sites (size). All maps are displayed in a horizontal view, with a background stained for Substance P to define CeA. The top row depicts CeA DAMGO (0.1µg), and the bottom row depicts BLA DAMGO (0.1µg) reductions in sucrose 'liking,' and marginal increases in sucrose 'disliking,' compared to vehicle microinjections. Hexagonal symbol colors denote DAMGO modulation of 'liking' or 'disliking,' calculated as percent change from vehicle day for the equivalent condition in that animal. Inner symbols represent average diameter of 3x Fos enhancement over uninjected tissue levels, surrounded by semitransparent halos that show 2x Fos enhancement zones (from Chapter 3). Bar graphs to the top and left of each map display mean hedonic or aversive reactions after vehicle and DAMGO for all animals included in the maps. Bars along rostrocaudal and mediolateral axes show the intensity of DAMGO effects (mean(SEM) percent of vehicle levels) within each 0.4mm-wide level, centered on the labeled coordinate; a plume symbol can contribute to more than one bar when it straddles multiple levels. Bar colors reflect mean percentage change from vehicle in that zone. * indicates difference from vehicle, p<0.05.
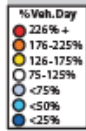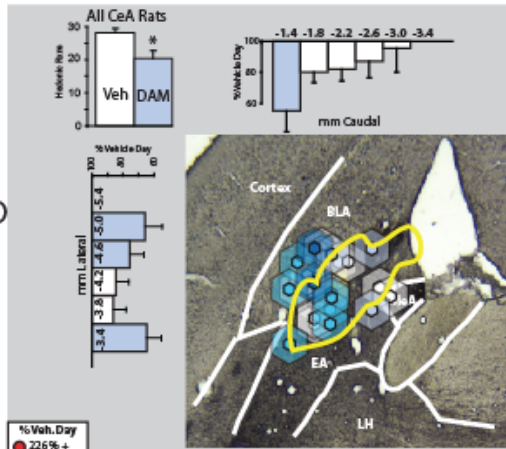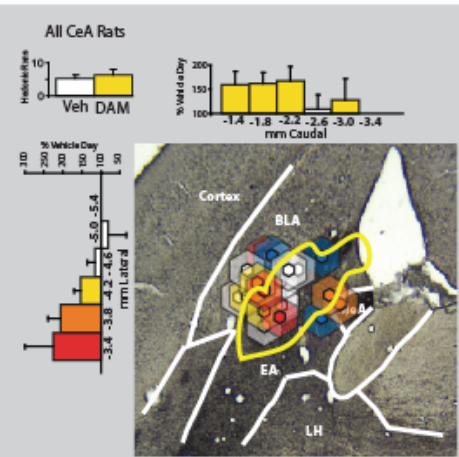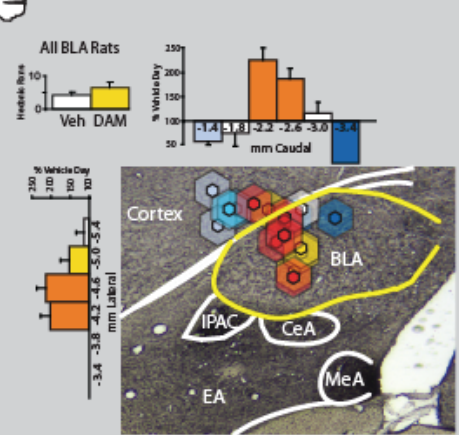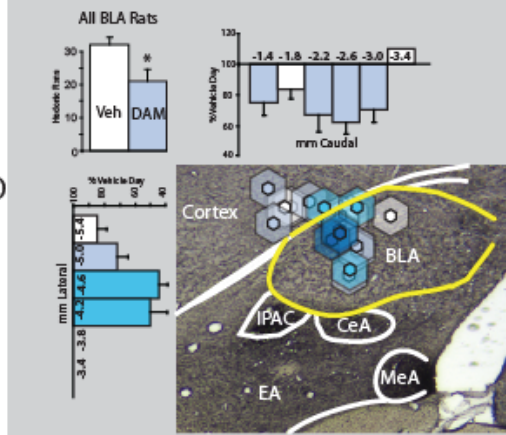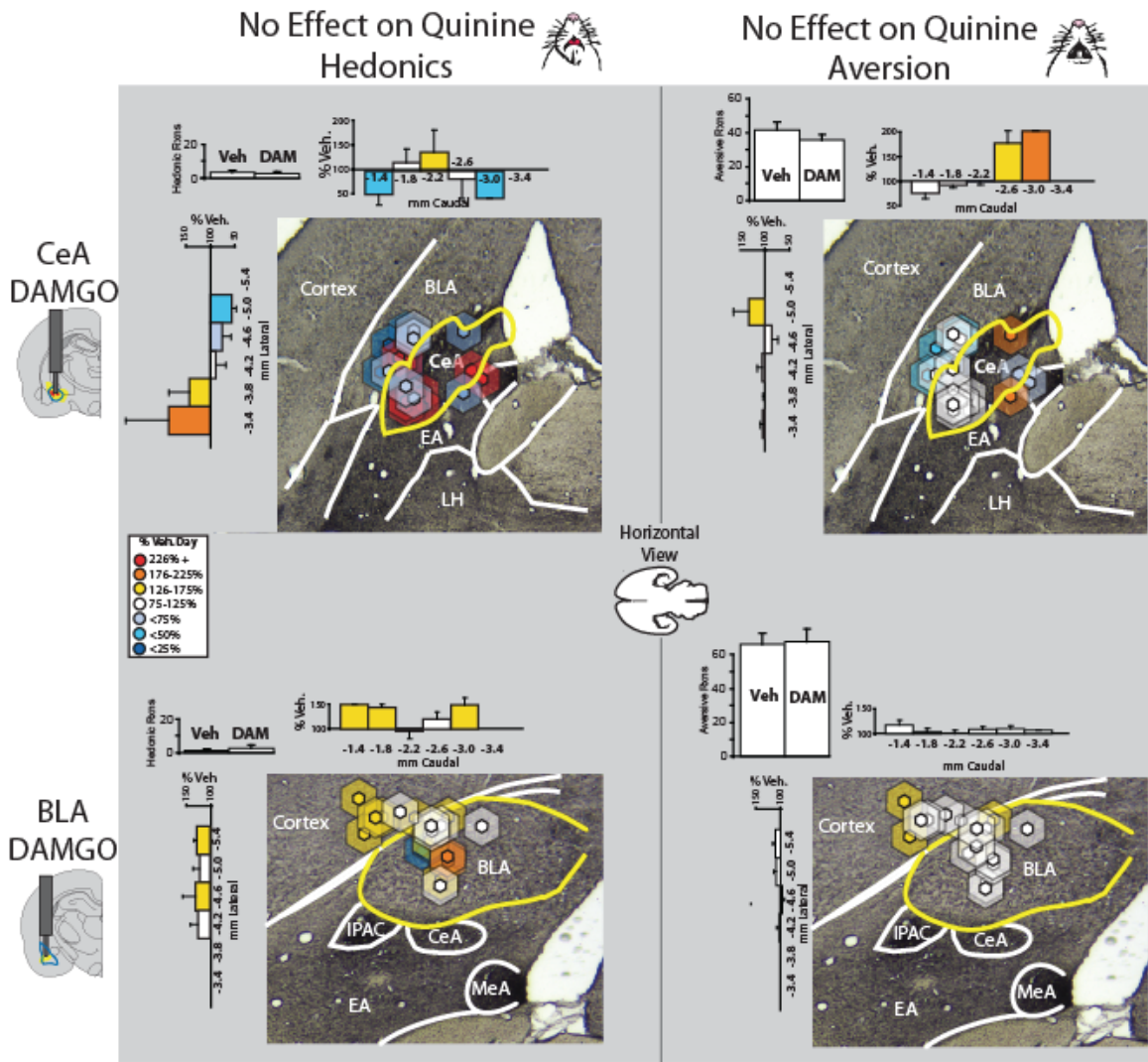
Figure 4.2: Amygdala DAMGO Does Not Affect Reactivity to Quinine: Neither CeA (top row), nor BLA (bottom row) DAMGO (0.1µg) affected hedonic (left column) or aversive (right column) reactivity to intraoral quinine infusions. Mapping procedures and symbol meanings are identical to Figure 4.1.
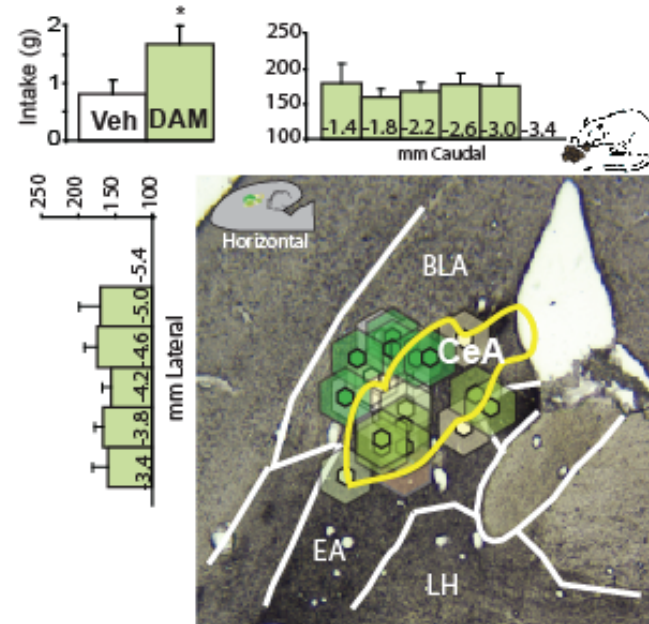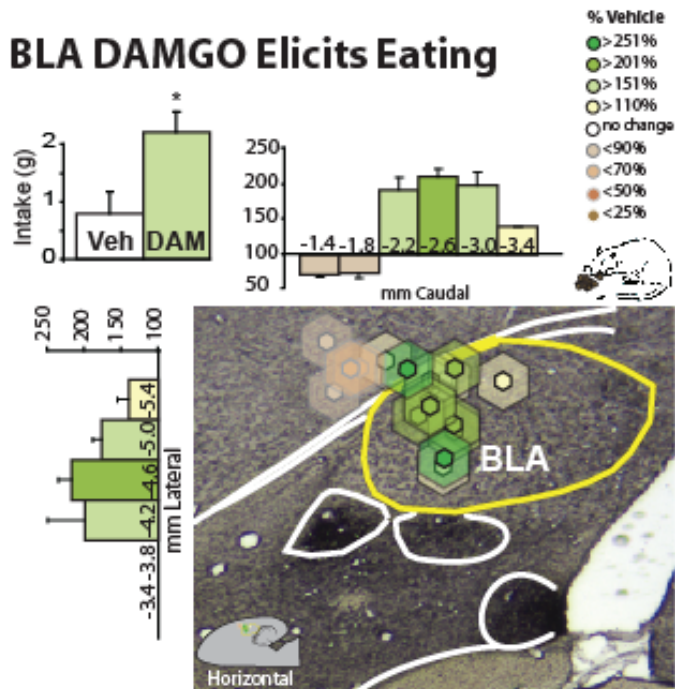
Figure 4.3: Amygdala Opioid Stimulation Following Taste Reactivity Testing Enhances Food Intake 'Wanting:' Both CeA and BLA DAMGO (0.1µg) enhance chow intake following taste reactivity testing on the same days. Mapping procedures and symbol logic follow those used in Figure 4.1.

Figure 4.4: CeA and BLA Cause Overlapping Patterns of Fos Activation in Distant Reward Structures: CeA (red bars and brain structures) and BLA (blue bars and brain structures) DAMGO (0.1µg) effects on Fos in distant structures are summarized at top, compared to vehicle injections at similar sites (brain structures Fos activated by both CeA and BLA DAMGO are colored with blue and red stripes). Bars represent mean(SEM) Fos/0.05mm^2 across all sampled portions of the listed structures. CeA elicited significant Fos activation of nucleus accumbens (NAc) core, NAc shell, the rostral half of the ventral pallidum (VP), and the interstitial nucleus of the posterior limb of the anterior commissure (IPAC). BLA DAMGO injections activated Fos in NAc Core, NAc Shell, Rostral VP, IPAC, and the lateral hypothalamus (LH). DAMGO did not induce Fos activation of caudal VP or the ventral tegmental area (VTA) when injected in either CeA or BLA.

Distant Fos Activation
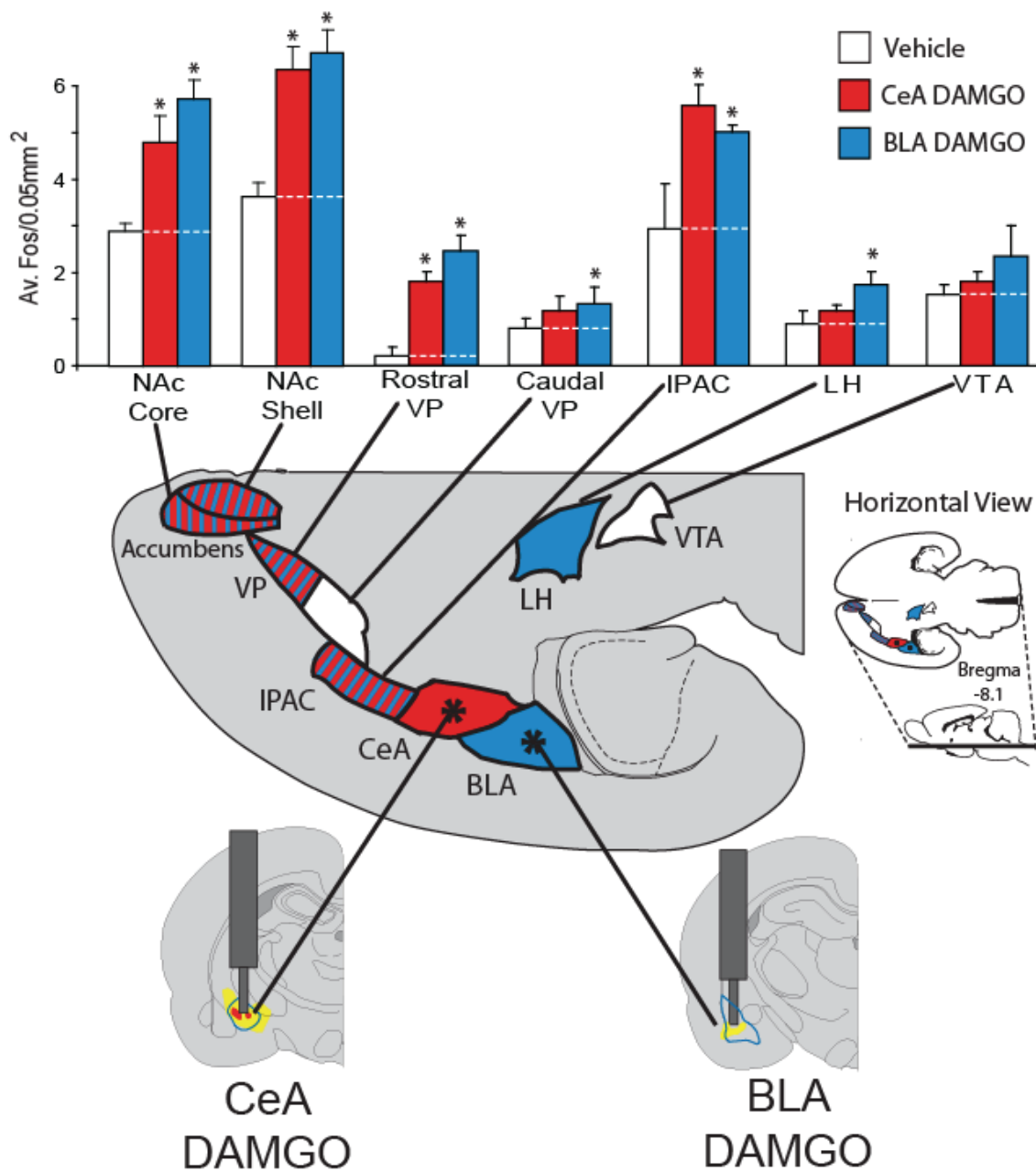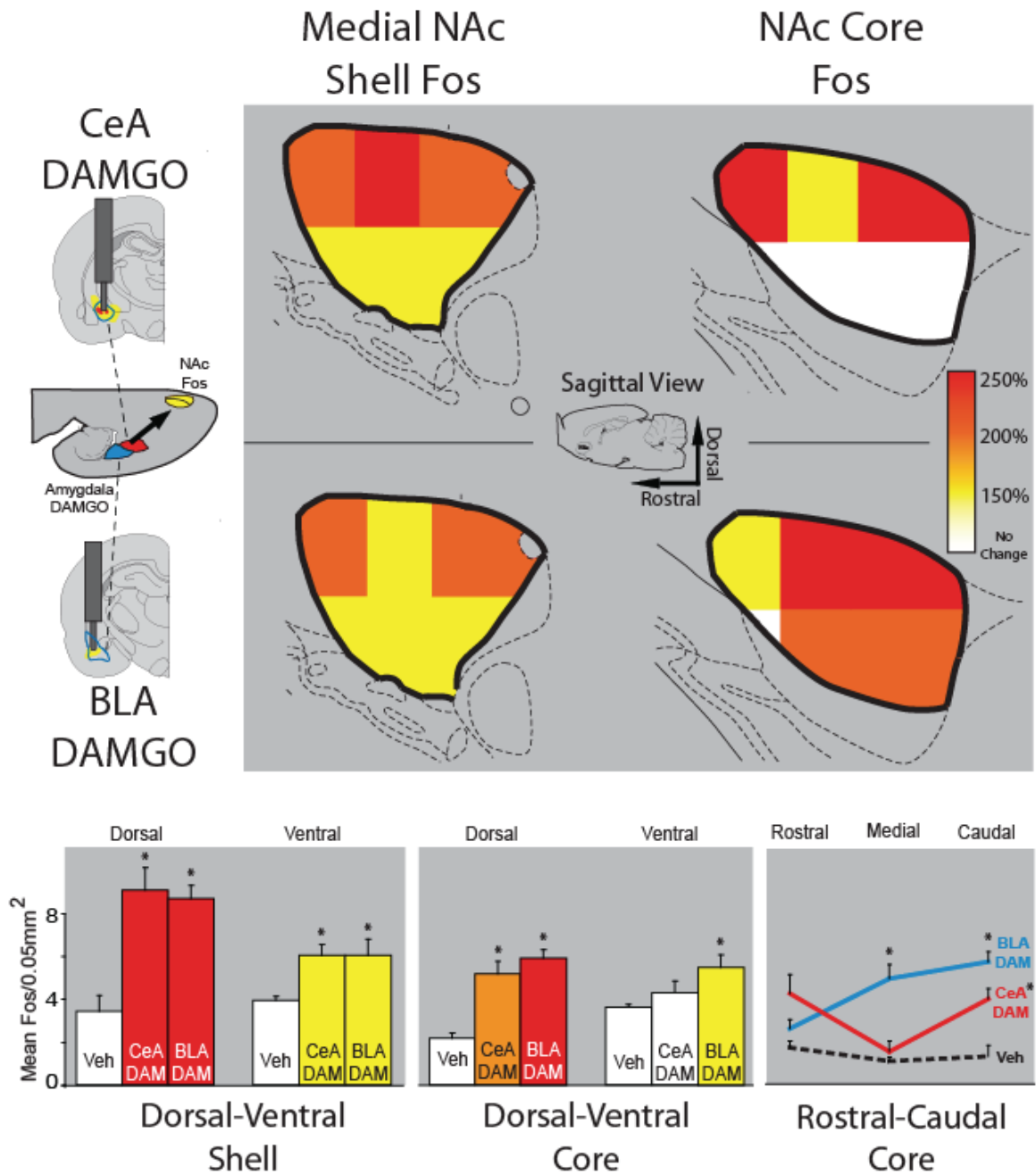
Figure 4.5: Intra-Accumbens gradients in Fos Activation After Amygdala DAMGO: Top panels represent distant Fos activation in nucleus accumbens shell (left column) and core (right column) after CeA (top row) or BLA (bottom row) DAMGO (0.1µg) microinjections. Bar and line graphs at bottom represent mean(SEM) Fos expression/0.05mm^2 within dorsal vs. ventral accumbens shell (left), dorsal vs. ventral accumbens core (middle) and rostral, medial, and caudal accumbens core after vehicle microinjections, CeA DAMGO, or BLA DAMGO. * indicates significant difference from vehicle microinjections in Fos at equivalent sites (p<0.05).

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Chapter 5
Endocannabinoid Hedonic Hotspot for Sensory Pleasure: Anandamide in Nucleus Accumbens Shell Enhances 'Liking' of a Sweet Reward

Introduction

As described above, the brain processes reward 'wanting,' 'liking,' and learning with integrated, but dissociable neural substrates. The previous chapters described a specific 'hotspot' in central amygdala for μ opioid enhancement 'wanting' focused based upon prior learning. In the present chapter, we aim to describe a novel brain substrate of hedonic aspects of reward processing, or 'liking.' In doing so, this dissertation will describe novel neural substrates for both appetitive and hedonic aspects of reward, thereby presenting a more complete picture of how the brain processes rewards at all stages of their pursuit and attainment.

To do so, we turned to the medial nucleus accumbens shell, which is well known for its participation in reward 'liking' (Peciña and Berridge, 2000, 2005; Smith and Berridge, 2007). Brain cannabinoids are known for their pleasurable rewarding effects, and for their ability to stimulate increases in food intake (e.g., the marijuana 'munchies'). Therefore, it seems likely that they play a role in the processing of reward. Here we asked whether this role involves stimulating 'liking' and/or 'wanting' via actions in nucleus accumbens shell.

In animal studies of appetite, infusions of cannabinoid drugs such as Δ^9-THC, or of endogenous cannabinoid neurotransmitters such as anandamide stimulate eating behavior and food intake (Williams et al., 1998; Williams and Kirkham, 1999; Di Marzo and Matias, 2005; Pagotto et al., 2006). In human clinical applications, cannabinoid agonist drugs stimulate appetite in hypophagic patients, and conversely, cannabinoid

antagonist drugs are currently of interest for potential therapeutic roles to suppress consumption as dieting aids and addiction treatments (Di Marzo and Petrocellis, 2006).

Cannabinoid receptors (CB1 and/or CB2) are present throughout the limbic forebrain, including the striatum and nucleus accumbens (Herkenham et al., 1991; Moldrich and Wenger, 2000; Fusco et al., 2004; Gong et al., 2006). Many functional effects of endocannabinoids in brain are thought to occur via CB1 receptors (Piomelli, 2003). CB1 receptors are located on GABAergic presynaptic axons in the nucleus accumbens shell (Matyas et al., 2006), and are often colocalized with μ opioid receptors at the same synapses and in the same cells in striatum (Pickel et al., 2004; Schoffelmeer et al., 2006). CB2 receptors have also been reported to occur on glial cells and neurons in ventral striatum, though less is known about their synaptic localization or function (Gong et al., 2006).

Regarding the relation between reward and appetite effects, a promising hypothesis is that cannabinoid drugs might act in the brain to increase hedonic impact or palatability of the taste of foods, as part of the mechanism by which they increase appetite and food intake (Cooper, 2004; Jarrett et al., 2005; Kirkham, 2005). Systemic and ICV Δ^9-THC potently increase intake of sweet foods more than less palatable foods (Koch and Matthews, 2001), and enhance voluntary licking bouts at a sucrose spout in a manner consistent with palatability enhancement (Higgs et al., 2003). Microinjections of the endocannabinoid 2-AG directly into the medial shell of nucleus accumbens similarly increases food intake in rats (Kirkham et al., 2002). Conversely, food-related manipulations such as deprivation and satiety, or access to a palatable diet produce changes in CB1 receptor density and in dialysate levels of endogenous anandamide and 2-AG in nucleus accumbens and other brain areas, and modulate appetite stimulation by cannabinoids (Di Marzo et al., 2001; Harrold et al., 2002; Kirkham et al., 2002). Most relevant to this study, systemic administration of Δ^9-THC in rats is reported to cause eventual increase in affective orofacial 'liking' reactions elicited by the taste of sucrose, suggesting enhancement of taste palatability (Jarrett et al., 2005).

The brain substrates of cannabinoid hedonic effects are so far unknown, but such observations give rise to the hypothesis examined here: that endogenous cannabinoid neurotransmission in limbic brain structures such as nucleus accumbens mediates the

hedonic impact of natural rewards like sweetness. The nucleus accumbens is an especially likely candidate for cannabinoid mediation of hedonic impact because it is known to contribute to the generation by other neurotransmitters of hedonic affect ('liking') and appetitive motivation ('wanting') for food and drug rewards (Berridge and Robinson, 2003).

The medial shell region of nucleus accumbens appears particularly important for amplifying the hedonic impact of rewarding incentives. For example, a 1mm^3 'hedonic hotspot' was recently found in the medial shell where μ opioid receptor activation by DAMGO microinjections tripled positive 'liking' orofacial reactions that are elicited by sucrose taste in rats (Peciña and Berridge, 2005), and stimulated food intake (though the intake 'wanting' site extended further) (Bakshi and Kelley, 1993; Zhang and Kelley, 2000; Peciña and Berridge, 2005). Opioid and endocannabinoid neurotransmission are known to positively interact (Tanda et al., 1997; Kirkham and Williams, 2001; Navarro et al., 2001; Rowland et al., 2001; Williams and Kirkham, 2002a; Verty et al., 2003; Solinas and Goldberg, 2005; Vigano et al., 2005; Caille and Parsons, 2006; Cota et al., 2006), raising the possibility that endocannabinoid activation might increase 'liking' for natural rewards in the same hedonic hotspot of the medial accumbens shell where opioids do so.

Based on these considerations, we tested whether endogenous cannabinoid neurotransmission in the medial shell of nucleus accumbens mediates the hedonic impact of a natural sensory pleasure, sweetness. Microinjections of anandamide into the medial shell multiplied positive 'liking' reactions to sucrose taste (a measure based on homologous orofacial expressions of affective 'liking' versus 'disliking' consummatory reactions that are elicited by tastes in human infants, apes, monkeys, and rats (for a review see Berridge (2000)). To assess where in the brain anandamide acted to enhance 'liking' for sweet hedonic impact, we mapped the substrate responsible for hedonic enhancement, using local Fos plumes produced by comparable anandamide microinjections to identify where microinjections acted. Our results indicate that natural 'liking' reactions to sweetness, as well as eating behavior, are amplified by endogenous cannabinoid signals in nucleus accumbens, especially within a hedonic hotspot in the dorsal medial shell.

Materials and Methods:

Subjects: Sprague Dawley rats (*n*=62, male, 250-400g, pair housed) were given microinjections into the medial accumbens shell or control structures of vehicle or anandamide doses. Groups of rats were subsequently tested for taste reactivity to sucrose or quinine infusions into the mouth and for voluntary feeding behavior. Each behaviorally tested rat received vehicle and every dose for its group in counterbalanced order spaced at least 48 hrs apart. Different groups were used to test 1) time course of anandamide 'liking' effects (*n*=11; anatomical control sites: *n*=5), 2) balance of anandamide effects on sucrose 'liking' versus quinine 'disliking' (*n*=19), and 3) food and water intake effects (*n*=11), and 4) Fos plume measurements under conditions equivalent to day 1 of behavioral testing (n=16). These groups were separated in order to ensure that no rat received more than 4 microinjections (to avoid damage accumulation), and to ensure that Fos and behavior were measured under identical conditions when drug impact was maximal, as explained below.

Measurement of anandamide's maximal impact on 'liking,' food intake, and Fos plumes was achieved by a split-and-recombine design, in which rats were assigned upon surgical implantation to either a behavioral test group (*n*=46 behavioral animals, which then also received oral cannulae) or a Fos plume test group (*n*=16 Fos animals). Placements in medial shell were similar for both groups, which were treated identically after surgery. Fos plumes were assessed under conditions similar to the first test day of the behavioral group to allow maximal impact of anandamide microinjections. The reason for the split was to ensure measurement of initial maximum drug impact, and avoid the diminishment in efficacy of drug that might occur after several repeated microinjections. The possibility that repetition may reduce drug impact on local tissue creates a type of 'uncertainty principle' regarding measurement of maximum impact: one can measure either the behavioral maximum or the Fos plume maximum in a repeated-measures experiment but not both. The reason for the recombination was to project the observed behavioral 'liking' and 'wanting' effects onto the precise locations where anandamide microinjections were likely to have acted based on observed Fos plumes.

Splitting allowed measurement of both maximums, while still allowing repeated-measures comparison in the same rat of anandamide and vehicle effects on hedonic

impact or intake. Recombination allowed integration of behavioral and Fos data, obtained under similar conditions, into the same Fos plume maps of anandamide effects on 'liking' reactions and on food intake.

Surgery: Rats were anesthetized with ketamine (80mg/kg), xylazine (5mg/kg), and pre-treated with atropine (0.04mg/kg). Using a stereotaxic device, rats were implanted with bilateral 23ga microinjection guide cannulae, 14mm in length, aimed at a level 2.5mm anterodorsal to the accumbens shell. A slanted cannula track was used to avoid penetrating the lateral ventricles (slanted skull = incisor bar: +5.0mm; coordinates: AP: +3.4—+2.4mm anterior to Bregma, ML: $\pm$ 1.0mm, DV: 4.7—6.0mm below skull surface (Paxinos and Watson, 2005). To provide anatomical control sites for behavioral studies, an additional 5 rats were implanted with guide cannulae in other brain structures outside the accumbens, including sites along the cannulae tracks that were dorsal or anterior to accumbens. Anatomical control sites were in anterior prelimbic cortex (*n*=2, AP: +3.1, ML: $\pm$ 1.0, DV: -2.0), dorsal striatum (*n*=1, AP: +1.6, ML: $\pm$ 3.0, DV: -3.0), or anterior ventral pallidum (caudal to nucleus accumbens; *n*=2, AP: +0.4, ML: $\pm$1.1, DV: -5.5). Microinjection guide cannulae were secured with skull screws and dental cement, and occluded with steel stylets.

In the same surgery, all rats used for taste reactivity testing were also implanted with bilateral oral cannulae (PE-100 tubing) to allow oral infusions of sucrose or quinine solutions during taste reactivity testing (rats used for Fos did not receive oral cannulae). Oral cannulae were inserted lateral to the first maxillary molar, threaded behind the zygomatic arch, and exited the dorsal head where they were cemented to skull screws (Grill and Norgren, 1978; Berridge et al., 1984). All rats were allowed to recover before testing for at least 7 days after surgery, and were habituated to their taste reactivity or food intake test chambers for 30 min on 4 consecutive days prior to the first test. On the last day of habituation, all rats received one 0.5µl saline microinjection following procedures described above to acclimate them to microinjections themselves.

Drugs and Microinjections: Anandamide in a bioavailable aqueous soya suspension (Tocrisolve, Tocris) was diluted to dose with 0.9% saline solution. Tocrisolve vehicle was similarly diluted for control vehicle microinjections. On test days, rats were gently hand held while stylets were removed. Rats then received bilateral microinjections (0.5µl

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volume per side over a 90 sec period) of vehicle or anandamide via a stainless-steel injector cannula (29ga), which extended 2.5mm beyond the guide cannulae into the target site. Microinjector cannulae were held in place for an additional 1 min to allow for drug diffusion, then stylets were replaced and rats were immediately placed into their taste reactivity or food intake test chamber.

Behavioral Taste Reactivity Tests: For taste reactivity tests, after rats received a bilateral microinjection into nucleus accumbens or a control structure, a tastant delivery tube was connected to their oral cannulae, and rats were placed in the test chamber. To elicit taste reactivity patterns, 1ml sucrose or quinine solutions were infused over 1 min through the oral cannula at various times after brain microinjection, as described below. A digital video camera recorded orofacial reactions to all infusions, via an angled mirror under the transparent taste reactivity chamber floor.

Rats in the time-course group received microinjections of vehicle or anandamide (0, 25 and 50ng) in counterbalanced order over 3 test days separated by at least 48 hrs, and were tested for sucrose reactivity at 15, 30, & 45 min after microinjection (*n=16*). Rats in the sucrose versus quinine comparison group similarly received microinjections of vehicle or anandamide (0, 12.5, 25, or 50ng; *n=19*) over 4 test days spaced 48 hrs apart. Because initial time-course results indicated that anandamide effects were maximal and constant 30-45 min after microinjection, rats in the comparison group received an oral infusion of 1% sucrose solution (1ml volume, 1 min duration) at 30 min after drug, and received a second oral infusion of 3×10^{-4} M quinine (1ml volume, 1 min duration) 15 min later at 45 min after the drug. This order of testing was used to ensure that 'liking' reactions to sucrose were always pure and uncontaminated by any prior taste on that day, because positive hedonic 'liking' reactions are generally more vulnerable to disruption than negative 'disliking' reactions, and because it is identical to the procedure used in a previous mapping study of the accumbens hedonic hotspot (Peciña and Berridge, 2005).

Taste reactivity video scoring: Hedonic, aversive, and neutral response patterns were later scored off-line in slow motion (frame by frame to 1/10th actual speed) by a trained observer who was blind to experimental condition, using time bin scoring procedures developed to assess hedonic versus aversive taste valuations (Berridge et al., 1984;

Berridge, 2000). Hedonic responses included rhythmic midline tongue protrusions, lateral tongue protrusions, and paw licks. Aversive responses included gapes, head shakes, face washes, forelimb flails, and chin rubs. Neutral responses, which are less consistently linked to hedonic/aversive taste valuation, included passive dripping of solution out of the mouth, ordinary grooming, and rhythmic mouth movements. All video analyses were conducted blind to the microinjection contents and cannula placement using Observer software (Noldus, Netherlands).

A time bin scoring procedure was used to ensure that taste reactivity components of different relative frequency were balanced in their contributions to the final affective hedonic/aversive totals (Berridge, 2000). For example, rhythmic mouth movements, passive dripping of solution, paw licking, and grooming reactions typically occur in long bouts, and were thus scored in 5 sec time bins (up to 5 sec continuous bout duration equaled one occurrence). Tongue protrusions, which occur in shorter bouts, were scored in 2 sec time bins. The other behavioral components (lateral tongue protrusions, gapes, forelimb flails, head shakes, chin rubs) typically occur as discrete events and were therefore scored as single occurrences each time they occurred (e.g., one gape equaled one occurrence). Individual totals were calculated for hedonic versus aversive categories for each rat by adding all response scores within an affective category for that rat. Finally, the hedonic "liking" reaction total was defined as the sum of scores for lateral tongue protrusions, rhythmic tongue protrusions, and paw licks. Similarly, the aversive "disliking" total was the sum of gapes, head shakes, face washes, forelimb flails, and chin rubs.

Histology: After the completion of testing, rats used for behavioral testing were deeply anesthetized with sodium pentobarbital (0.2g/kg), microinjected with 0.2μl black ink, and their brains were extracted. Brains were sectioned with a freezing microtome into 60μm coronal slices, stained with cresyl violet, and mapped for microinjection center locations according to Paxinos & Watson (2005).

Behavioral Eating Tests: To confirm that anandamide selectively increased food intake, behavioral eating and drinking effects were assessed in an additional group of rats in a voluntary food and water intake test (*n*=11). After vehicle or anandamide (25ng) microinjections, rats were placed in clear cages that contained food, corncob bedding and

a water spout. Pre-measured chow pellets and water were freely available for a 1 hr voluntary feeding test. Rats were videotaped during the test for subsequent off-line scoring of eating, drinking, and other behaviors during the 1 hr test. Videotapes were later scored in slow motion for time spent eating and drinking, object sniffing, gnawing, and carrying, sleeping, and other behaviors by observers blind to the experimental condition.

Fos-like Protein Immunohistochemistry: Rats in the Fos plume group (n=16) were handled for 3 days for 10 min each, similar to the behavioral groups, and then microinjected bilaterally in the accumbens shell with one of three doses of anandamide or vehicle as described above (12.5ng, *n=3*; 25ng, *n=4*; 50ng, *n=3*; veh, *n=6*; normal uninjected tissue, *n=4*). Placements were bracketed throughout the medial shell similarly to the behavioral group, and it was later confirmed that every behavioral group site was within 1mm of a corresponding Fos group site. Fos-like protein expression was harvested under conditions identical to the first day of testing for the behavioral group.

Rats were deeply anesthetized with sodium pentobarbital (0.2g/kg) 75 min after microinjection, since translation of c-fos mRNA to Fos protein is maximal between 60-120 min (Muller et al., 1984). After transcardial perfusion, brains were removed and placed in 4% paraformaldehyde for 2 hrs, 30% sucrose overnight, and then sectioned at 40μm and stored in 0.2M NaPb, pH 7.4. To visualize Fos-like immunoreactivity, we used the avidin–biotin procedure (Hsu et al., 1981). Brain sections were immersed in blocking solution [3% normal goat serum (NGS) and 0.3% Triton X-100 in Tris PBS (TPBS)] for 1 hr and then incubated at room temperature for 24 hrs with a rabbit polyclonal antiserum directed against the N-terminal region of the Fos gene (dilution of 1:5000 in TPBS, 1% NGS, and 0.3% Triton X-100; Sigma). To reduce background staining, the antiserum was preabsorbed with acetone-dried rat liver powder overnight at 4°C. After the primary antibody incubation, tissue was exposed to goat anti-rabbit, biotinylated secondary IgG (diluted 1:200; Santa Cruz Biochemicals, Santa Cruz, CA) and then to avidin–biotin–peroxidase complex for 1 hr at room temperature. A nickel diaminobenzidine (Nickel-DAB) glucose oxidase reaction was used to visualize Fos-like immunoreactive cells. Finally, sections were washed in Tris buffer, mounted from PBS, air-dried, dehydrated in alcohol, cleared in xylene, and coverslipped. Fos-like

immunoreactivity was visualized using a Leica (Nussloch, Germany) microscope coupled to a SPOT RT slider (Diagnostic Instruments, Sterling Heights, MI) using SPOT software (SPOT version 3.3). Cannulae placements were localized by superimposing the image from a low-magnification light microscope onto a computerized brain atlas (Paxinos and Watson, 2005).

Some additional brains were sliced on the sagittal plane and stained for Substance P to help localize the position of mapped Fos plumes within the borders of nucleus accumbens shell (Berridge et al., 1997) (Figure 5.1). The procedures for Substance P staining were identical to Fos immunohistochemistry except that the primary antibody was for Substance P (Immunostar; 1:2000 concentration).

Fos Plume Maps of Anandamide-Induced Neuronal Activation Spread: Our procedure for measuring drug-induced Fos plumes immediately surrounding a microinjection site followed procedures described previously (Peciña and Berridge, 2000, 2005; Smith and Berridge, 2005). Observed Fos plume measurements from the Fos group were averaged to determine mean volumes for each Fos intensity zone, assuming a spherical shape of functional drug spread. Averaged plume radii for each zone were projected onto the microinjection center from the behavioral group to depict extent of activation spread, and used to assign symbol sizes for maps. Behavioral data from each microinjection site were used to assign the color of its symbol on the map that coded intensity of 'liking' or 'wanting' effects produced by microinjection at that site (details below).

To quantify spread of drug-induced neuronal activation, Fos-labeled cells on tissue surface near the microinjection site were visualized with 5X–40X magnification and counted individually within blocks (125μm X 125μm) at locations spaced at 125μm intervals along each of seven radial arms emanating from the center of the microinjection site (45, 90, 135, 180, 225, 270, 315°; see Figure 5.1). To establish baselines for comparison to drug plumes, control values for Fos densities were measured 1) in normal nucleus accumbens shell tissue of intact brains to assess normal baseline expression in 'virgin tissue' that was not damaged by surgical intrusion or gliosis, and 2) around the site of vehicle microinjections to assess cannula track and vehicle-induced baseline Fos expression. Fos densities were also measured around the site of anandamide microinjections to assess drug-induced elevations of local Fos expression (Figure 5.1). In

this study, we improved the sensitivity of detection of elevated Fos expression, mapping 2X and 3X elevations over control levels in addition to higher levels, compared to previous studies that detected >5X as the lowest increase over control levels (Peciña and Berridge, 2005; Smith and Berridge, 2005) .

Anandamide Fos plumes (12.5, 25 & 50ng) were mapped in two ways: (1) as 2X, 3X, 5X, and 10X vehicle-relative increases caused by anandamide, and (2) as absolute 2X, 3X, 5X and 10X increases above normal uninjected tissue. In both cases, the distance of 2X spread from the microinjection site was measured on each radial arm. Spread extended to the last box expressing greater-than or equal-to 2X elevation. The distance from the injection site for 2X spread was then averaged for all 7 radial arms, providing a final radius-cubed zone of 2X elevation. The same procedure was followed for 3X, 5X, and 10X zones (distance from center in which boxes expressed greater-than or equal-to the elevation). Thus, anandamide-evoked increases in Fos were compared to normal and vehicle Fos levels at the same site within medial accumbens shell, and maps were created of lowest, low, moderate, and high Fos elevation zones for each of the three anandamide doses.

Fos data were recombined with behavioral data in the next mapping stage. The radii of Fos plumes were averaged for each elevation zone, and each zone was assigned a hexagon map symbol of a size based on Fos plume radii. Behavioral effects of each microinjection site were represented by the colors of plume-derived symbols (Figures 5.2, 5.4, 5.5; 5.7, 5.8). Each plume symbol in a map illustrates three important pieces of information: the location of microinjection in a particular rat tested for behavior, the intensity of behavioral effects of anandamide on food reward 'liking' or 'wanting' for that rat, and the size of the local neuronal modulation implicated in anandamide's effects at that site (based on average Fos plume radii). The bilateral cannulae for each rat were collapsed into one unilateral map and plotted separately to depict every placement (2 sites per rat). Each site shows three concentric hexagon symbols, representing the average size of plume zones of Fos elevation: inner hexagon = intense Fos elevation; intermediate hexagon = moderate; outer hexagon = low or lowest Fos elevation. Each hexagon is color-coded for the magnitude of anandamide's behavioral effects on 'liking' reactions to sucrose, 'disliking' reactions to quinine, or food intake (Peciña and Berridge, 2000, 2005;

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Smith and Berridge, 2005). Separate maps were plotted in sagittal, coronal, and horizontal planes to construct a 3-dimensional database of the position of Fos plumes in the brain and the location of functional hotspots (Paxinos and Watson, 1998) (Figure 5.2). *Statistical Analyses:* All behavioral analyses were two-tailed and alpha was always set at $p<0.05$. Repeated measures ANOVAs were used for the time-course taste reactivity data (0, 25 & 50ng doses X 15, 30, 45 min time points after drug), sucrose-quinine taste reactivity data (0, 12.5, 25 &50ng dose; analyzed separately for sucrose and quinine tastes), and food and water intake data (0 & 25ng doses; analyzed separately for food and water). To describe anandamide behavioral effects as percentage increase over vehicle levels, a constant value of 1 was added to every datum to avoid the problem of calculating percentage increases over zero for rats with low baselines. Between-subjects ANOVAs were used to determine anatomical location effects of microinjection sites (dorsal vs. ventral). For anatomical localization and hedonic component analyses, all 30 min time-point sucrose taste reactivity data were combined because preliminary analysis showed no differences existed between groups in anandamide effects on hedonic taste reactivity 30 min after 25 & 50ng anandamide. Paired samples t-tests were used to test anandamide (0 & 25ng) effects on eating behavior. Bonferroni corrected, paired samples t-tests and Tukey post hoc tests were employed to determine the nature of main effects and interactions after significant ANOVA outcomes.

Results

Fos plume mapping: Fos plume maps helped identify where a drug microinjection acted on surrounding neural tissue, and where it stopped acting, as described in previous studies (Peciña and Berridge, 2000; Ikemoto and Wise, 2004; Peciña and Berridge, 2005; Smith and Berridge, 2005). Andandamide microinjections may cause local Fos induction either directly by stimulating cannabinoid or other receptors on the neurons that express Fos, or indirectly by modulating neighboring neurons that in turn activate adjacent Fos-expressing neurons via local circuits. In either case, drug-induced Fos plumes reflected local spheres of modulated neuronal function. Therefore, plumes indicated the functional zones likely to be responsible for behavioral effects of anandamide microinjections, even if functionally inert levels of drug drifted farther.

Microinjections of anandamide produced roughly spherical and concentric zones of Fos enhancement: small inner zones of intense elevations of Fos expression, surrounded by larger zones of moderate, low, and lowest elevations (Figure 5.1). The inner intense plume was defined as the zone in which Fos expression was increased by >10X above control 'normal' accumbens levels from uninjected rats, or above vehicle microinjection levels. A moderate Fos activation zone was defined as >5X over normal levels or vehicle levels, a low Fos elevation zone was defined as >3X over normal accumbens or vehicle levels, and the lowest Fos elevation zone was defined as >2X over normal or vehicle levels. Vehicle injections induce a small area of Fos expression immediately around the center of the injection site (mean radius 0.27±0.06mm of 2X Fos elevation over normal uninjected tissue levels), perhaps due to vehicle injection pressure, or cannula-related damage. The ability of vehicle to produce Fos plumes, even though small and low in intensity, places a ceiling on drug-induced Fos increases relative to levels measured around the tip of a vehicle microinjection.

The lowest 12.5ng dose of anandamide produced mean plume volumes in medial shell ranging from an intense center sphere, $4189 \pm 4189 \mu m^3$ in volume (or 4.189×10^{-6} mm^3 at 1 billion cubic microns per mm^3; mapped by >10X normal tissue criterion) to an outer plume volume of $2.95 \pm 0.01 mm^3$ (mapped by >2X increase over normal-tissue criterion; $1.5 \pm 0.07mm$ for >2X increase over vehicle). The 25 and 50ng doses produced slightly larger Fos plume volumes, having intense >10X Fos elevation centers of $0.017 \pm 0.0001 mm^3$ [25ng dose] to $0.024 \pm 0.024 mm^3$ [50ng dose], and outer total volumes of up to $3.05 \pm 0.001 mm^3$ (50ng; mapped by >2X normal tissue criterion; $1.91 \pm 0.002 mm^3$ relative to vehicle: see Table 1 for complete plume radius and volume data). We estimated the entire unilateral accumbens medial shell to be roughly $3mm^3$ in tissue volume, and so these Fos plume volumes meant that the intense center of a typical anandamide plume filled about 1% of medial shell volume (center = where Fos was at least 10X above normal), whereas the outer Fos plume (where Fos was doubled above vehicle plume or above normal tissue, respectively) spread through nearly 70% to 100% of medial shell volume.

Anandamide Enhances Sucrose Hedonic Impact: Anandamide microinjections in medial shell caused overall increases of 130% to 210% in the number of positive hedonic

reactions to sucrose compared to control levels after vehicle microinjections (vehicle=100%; мain effect of drug: $F(2,16)=13.35$, $p<0.001$ in time-course group; $F(3,41)=4.11$, $p<0.05$ in sucrose/quinine group). All three doses of anandamide increased hedonic reactions, and at all three time points tested in the 45 min after microinjection (details below; Figure 5.3). Sucrose taste elicited only positive hedonic reactions and hardly any aversive reactions, even after vehicle microinjections. Anandamide selectively amplified the number of positive hedonic reactions elicited by sucrose, and never induced aversive reactions to sucrose.

Hedonic hotspot maps for 'liking' enhancement: Plume-sized hexagons matching the sizes of intense, moderate, and low Fos plumes were color-coded to reflect the quality and magnitude of behavioral effects produced by anandamide microinjection at that site, and plotted onto each corresponding site location (Mapping figures represent anandamide plumes relative to lower, normal tissue baselines, to avoid underestimation of drug spread (Peciña and Berridge, 2005; Smith and Berridge, 2005). Anandamide increased hedonic reactions to sucrose at most microinjection sites, within approximately $2.75mm^3$ volume that extended over most of the medial shell. The effective hedonic enhancement zone extended from an anterior level just rostral to the far anterior genu of the corpus callosum in medial shell (Bregma + 2.52mm) to posterior level at the caudal end of the lateral accumbens shell (Bregma +1.08; Figures 5.2, 5.4).

Hedonic hotspot focus in dorsal shell: A particularly effective hedonic hotspot for enhancing 'liking' reactions to sucrose was found to be concentrated within the dorsal portion of medial shell (Figure 5.3). Sites centered in the dorsal half of the medial shell (dorsal to 7.4mm DV) produced stronger anandamide enhancements of positive hedonic reactions to sucrose taste than sites in the ventral half of medial shell ($F(3,17)=4.01$, $p<0.05$; Figure 5.3).

The dorsal hot spot focus was approximately $1.6mm^3$ in volume (defined as the zone where anandamide reliably produced increases in positive hedonic reactions >150% compared to after vehicle microinjection at the same site). For example, in dorsal shell, injections of 25ng anandamide more than doubled hedonic reactions relative to vehicle levels (210%), while the same dose in ventral shell yielded increases of only 127% ($t=2.5$, $p<0.05$). Similarly, 50ng anandamide had greater hedonic effects in the dorsal

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half of shell (160%) than in ventral shell (101%; t=2.2, $p<0.05$), and at a few ventral sites actually seemed to suppress hedonic reactions below vehicle levels (Figure 5.4). The same dorsoventral pattern appeared as a trend for 12.5ng anandamide (t=1.5, *n.s.*; dorsal: 180%, ventral: 120%).

Hedonic enhancement was not produced by anandamide at any anatomical control sites in brain structures outside the nucleus accumbens tested here, including dorsal and anterior sites along cannula tracks. Although the outer penumbra of a number of Fos plumes penetrated into accumbens core, limbic cortex, or septum (lowest 2X and 3X Fos elevation), 91% of hedonic Fos plume centers (intense >10X Fos elevation) were entirely contained within the medial shell, and 79% of middle plume zones (moderate >5X Fos elevation) were likewise completely contained within the medial shell. While future studies will be needed to identify the Fos threshold for hedonic enhancement, the present results at least indicate that medial shell contained well over 90% of the total local Fos expression caused by microinjections that enhanced hedonic impact. Finally, no hedonic enhancement was detectable at control sites in dorsal striatum or in anterior prelimbic cortex, nor in anterior ventral pallidum. In fact, when all anatomical control sites were pooled for statistical analysis, anandamide in those structures actually *decreased* hedonic sucrose reactions to below vehicle levels (t=3.13, $p<0.05$).

This overall pattern of Fos plumes and anatomical control sites indicates that anandamide acted primarily in the medial accumbens shell to cause amplification of positive hedonic 'liking' reactions elicited by the taste of sucrose.

Dose-Response Effects for Hedonic Impact Enhancement: All doses of anandamide tested here produced hedonic increases over vehicle control levels (one-way ANOVA on all rats at 30 min time point, $F(3,73)=5.7$, $p=0.001$). Effects of the three doses were of similar magnitudes, although the middle, 25ng dose was more effective at increasing positive hedonic reactions (146% of vehicle levels) than the highest, 50ng dose (134%; t=2.45, $p<0.05$; Figures 5.2-5.3). Further, nearly half of rats (46%, mostly with ventral cannulae placements) showed no change in positive hedonic reactions after the 50ng dose (25% for 12.5ng, 33% for 25ng). This pattern of results suggests that anandamide dose-response effects on hedonic enhancement might have an "inverted U" shape, similar to

cannabinoid drug effects reported for food intake. Further work would be necessary to confirm potential dose-response effects on hedonic impact.

Time Course of Hedonic Enhancements: Hedonic enhancement was detected at the first taste reactivity test conducted 15 min after anandamide microinjections ($F_{(2,20)}=3.52$, $p<0.05$), and there was no difference in the magnitude of hedonic enhancement across the time points tested at 15 min, 30 min and 45 min after microinjection (no main effect of time after infusion ($F_{(2,16)}=0.788$, *n.s.*, or interaction between dose and time of test ($F_{(4, 32)}=0.316$, *n.s.*); Figures 5.3&5.6). Thus anandamide-induced hedonic enhancements appear to occur early within 15 min after microinjection and remain robust and stable at least across the entire ensuing 45 min.

Analysis of Hedonic Components of Taste Reactivity: When positive hedonic reactions were broken into orofacial components, anandamide significantly increased midline tongue protrusions ($F_{(2,28)}=14.9$, $p<0.001$; individual doses: 25ng: t=4.0, $p<0.01$; 50ng: t=2.7, $p<0.05$) and lateral tongue protrusions elicited by sucrose ($F_{(2,28)}=5.1$, $p<0.05$; 25ng: t=1.6, p=0.12; 50ng: t=2.0, p=0.06). Paw licks were additionally increased by the 25ng dose ($F_{(2,28)}=2.6$, $p=0.09$; 25ng: t=3.0, $p<0.01$; 50ng: t=1.1, *n.s*; Figure 5.7).

Anandamide Does Not Affect Aversive Reactivity to Quinine: In contrast to the robust hedonic enhancement of positive reactions elicited by sucrose taste, anandamide had no significant effect on aversive reactions to quinine taste ($F_{(3,27)}=1.7$, *n.s.*; at any dose: 12.5ng: t=1.2, *n.s*; 25ng: t=1.8, *n.s*; 50ng: t=0.2, *n.s.*; Figures 5.8&5.9). Oral infusions of bitter quinine elicited primarily aversive reactions, and never more than a few positive reactions. Anandamide did not change the low level of positive reactions to quinine ($F_{(3.27)}=0.4$, *n.s.*). After 15–30 sec of quinine infusion, rats typically switched from active aversive reactions (e.g., gapes) to passive dripping of the quinine solution from their mouths (often accompanied by forelimb flailing and head shaking, possibly elicited as a grooming response by solution dripping on animals' paws and fur). Since the transition to passive dripping may have obscured more active aversive responses, we also examined the initial 10 sec of responding, before passive dripping began. Again, anandamide did not alter negative aversive reactions at any dose (aversive reactions: $F_{(3,18)}=1.6$, *n.s.*; Figure 5.9). Thus, anandamide microinjections in medial shell of nucleus accumbens failed to alter the negative aversive impact of the taste of quinine,

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even when the same microinjections enhanced the positive hedonic impact of the taste of sucrose for the same rats.

Anandamide Increases Eating Behavior and Food Intake: Anandamide (25ng) increased spontaneous voluntary eating behavior and food intake (Figures 5.2&5.5). Anandamide microinjections in the medial shell more than doubled the cumulative duration of time spent eating (254% of vehicle levels, t=2.36, *p*<0.05; Figure 5.5). Intake was stimulated by anandamide at sites in dorsal shell that also enhanced 'liking,' and possibly extended into a few ventral shell sites as well. However, we caution that it would be premature to draw conclusions about site differences for 'wanting' versus 'liking,' since the effects were tested in different rats, and there were not many discrepancies between groups. Overall for the entire group, anandamide similarly doubled the number of eating bouts (203% of vehicle, t=2.52, *p*<0.05), and produced 600% increase in chow intake for rats who ate at all on either day (t=2.6, *p*<.05). Anandamide did not produce detectable changes in the amount of uneaten crumbs or latency to begin eating, changes in water intake or time spent drinking, or in non-ingestive behaviors such as object gnawing, object sniffing, food carrying, and sleeping (eating latency: t=1.1, *n.s.*; time drinking: t=0.13, *n.s.*; drinking bouts: t=0.10, *n.s.*; sniffing: t=1.4, *n.s.*; food carrying: t=0.4, *n.s.*; sleeping: t=0.15, *n.s.*). Overall, these results regarding spontaneous behaviors confirm that delivery of anandamide to a hedonic hotspot in medial shell of nucleus accumbens stimulates food eating in most rats, but does not increase water drinking or various other behaviors.

Discussion

Intra-Accumbens Shell Anandamide Specifically Enhances Taste 'Liking'

These results demonstrate that anandamide stimulation of a roughly 2.75mm^3 volume of medial shell of nucleus accumbens (and especially a 1.6mm^3 hotspot in its dorsal half) acts to amplify the hedonic impact of a natural sensory reward, sweetness. Anandamide microinjections in the dorsal hotspot more than doubled the number of hedonic 'liking' reactions elicited by the taste of a sucrose solution that was infused into the mouth (compared with reaction levels after vehicle microinjections). Hedonic enhancement appeared rapidly within 15 min after anandamide microinjection, persisted

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at all tests throughout the ensuing 45 min, and was robust after all anandamide doses tested here.

Anandamide did not alter aversive 'disliking' reactions to a bitter quinine taste, even when the same microinjections enhanced 'liking' reactions to sucrose on the same day. This selective enhancement pattern indicates that endocannabinoids specifically amplify the positive hedonic impact of reward without changing the negative aversive impact of unpleasant stimuli. In other words, endocannabinoid stimulation appeared to selectively make sweetness become even more positively 'liked,' but did not reliably improve or worsen an unpalatable bitter taste. This suggests a 'rich get richer' form of reward amplification by endocannabinoid action in the nucleus accumbens, in which the most pleasant stimuli gain even greater hedonic value while other stimuli that are less liked to begin with remain relatively unchanged. If so, that might help explain why cannabinoid-stimulated increases in intake in rats and humans appear to be targeted specifically toward already palatable foods (e.g., sweet or high-fat) more than to other less-palatable foods (Foltin et al., 1988; Koch and Matthews, 2001).

Anandamide Hedonic Hotspot is within Nucleus Accumbens

It seems reasonable to conclude from our results that the medial shell of nucleus accumbens, especially its dorsal region, contained the hedonic hotspot responsible for the observed endocannabinoid enhancement of 'liking' for sweetness. Intense Fos plumes for our hedonic sites were essentially contained within the nucleus accumbens shell, and the vast majority did not protrude significantly into other brain structures. In addition, anatomical control sites in striatum, ventral pallidum, and prelimbic cortex did not produce any detectable anandamide enhancement of hedonic impact outside the nucleus accumbens, further supporting anatomical localization of hedonic endocannabinoid mechanisms in the accumbens shell. This does not mean that no other cannabinoid hedonic hotspots will eventually be found in other brain structures, but does mean that the endocannabinoid hedonic effects reported here are likely to be mediated by a hotspot within the medial shell of nucleus accumbens.

The present data further indicate that anandamide microinjections in accumbens that enhance taste 'liking' may also promote appetitive 'wanting' of food, as measured by

food consumption behavior, consistent with previous reports of cannabinoid-modulation of appetitive motivation (Williams and Kirkham, 2002b; Thornton-Jones et al., 2005).

Hottest Spot in Dorsal Medial Shell

We found evidence that the most intense hedonic enhancement by anandamide was produced in a localized hotspot contained specifically within the dorsal half of the medial shell. Here we operationally defined a hedonic hotspot as an anatomical concentration of sites where anandamide produced >150% increases in 'liking' reactions to sucrose. Microinjection sites in a 1.6mm^3 hotspot of the dorsal shell were significantly more potent than sites in the ventral shell for amplifying positive 'liking' reactions to sucrose. For example, within that dorsal spot the most effective anandamide dose (25ng) reliably caused a greater than doubling of the number of 'liking' reactions elicited by sucrose, whereas such enhancements rarely occurred at more ventral sites. In addition, high dose anandamide at some ventral sites in medial shell actually appeared to *suppress* sucrose 'liking' reactions below control levels, whereas hedonic suppression was never observed at dorsal accumbens sites. The 1.6mm^3 dorsal hottest spot composed approximately 50% of total medial shell volume (medial shell = approximately 3mm^3). Overall, this suggests that the dorsal medial shell contains an especially potent hotspot for endocannabinoid magnification of the hedonic impact of sweetness (and tentatively that ventral shell could contain an opposite 'coldspot' where the same endocannabinoid stimulation can sometimes even dampen reactions to a sensory pleasure).

The 1.6mm^3 hedonic hotspot for anandamide enhancement of hedonic impact intriguingly overlaps with a roughly 1mm^3 opioid hedonic hotspot that was previously mapped in the dorsal rostral quadrant of medial shell by another taste reactivity study in our laboratory [where the μ opioid agonist DAMGO amplified 'liking' reactions (Peciña and Berridge, 2005)]. The anandamide hedonic hotspot identified here completely covered that opioid hedonic hotspot in the dorsal rostral quadrant, and possibly extended beyond it caudally throughout most of the dorsal half of medial shell. We did not observe rostrocaudal differences for anandamide effects in medial shell here, unlike for previously reported reward-related effects of opioid, GABA, glutamate, and Δ^9-THC microinjections in medial shell (Reynolds and Berridge, 2002, 2003; Peciña and Berridge, 2005; Zangen et al., 2006).

However, we caution that it may be premature to draw strong conclusions about precise relative boundaries of endocannabinoid versus opioid hotspots, or about the existence or lack of endocannabinoid rostrocaudal effects, because we mapped larger Fos plumes here than in the previous opioid mapping study. One reason is that to maximize the chance of successful endocannabinoid hedonic amplification, we deliberately chose large microinjection volumes (0.5μl) that were over twice the volume used for DAMGO microinjections in the previous study (0.2μl) (Peciña and Berridge, 2005). The larger volumes used here might therefore have led to relative over-estimation of endocannabinoid hotspot borders, and obscured some finer details of its inner structure. These issues could be resolved by future direct comparisons of endocannabinoid and opioid hedonic hotspots using smaller drug microinjection volumes, and the same rats.

It also may be premature to draw conclusions about the specific receptor mechanisms of hedonic hotspot effects of anandamide. Reward-related effects of anandamide are often viewed to be mediated by CB1 receptors (Piomelli, 2003; Cheer et al., 2004; De Vries and Schoffelmeer, 2005; Gardner, 2005; Kirkham, 2005; Thornton-Jones et al., 2005; Zangen et al., 2006). However, other CB receptor and even nonreceptor neuronal targets have been proposed for anandamide, some of which are present in the accumbens shell [see (Di Marzo et al., 2002) and (Pertwee, 2005) for reviews]. Additional experiments, perhaps involving the use of selective CB1 and CB2 agonists or antagonists, will be required in order to confirm the role of CB1 receptors in the effects of anandamide reported here.

At present, it seems safe to conclude simply that endocannabinoid and opioid hedonic hotspots anatomically overlap in the dorsal medial shell, and that CB1 receptors in the accumbens hotspot are the leading candidate to mediate hedonic enhancement by anadamide. Anatomical overlap suggests the possibility that both endocannabinoid and opioid signals might act on the same local circuits to amplify hedonic impact. It is of interest that CB1 receptors and opioid receptors are reported to interact, can occur in the same striatal synapses, and even be co-localized on the same neurons in nucleus accumbens shell and core (Tanda et al., 1997; Hohmann and Herkenham, 2000; Rodriguez et al., 2001; Pickel et al., 2004; Caille and Parsons, 2006). If co-localization occurs in hotspot neurons, this would support the possibility that endocannabinoid and

opioid neurochemical signals in nucleus accumbens might interact to enhance 'liking' reactions to the sensory pleasure of sucrose.

Conclusions

These results provide the first demonstration that endocannabinoids in the nucleus accumbens specifically amplify the hedonic impact of a prototypical sensory pleasure, sweetness. Anandamide acted especially in a dorsal hotspot of medial shell in nucleus accumbens to enhance positive 'liking' reactions to a rewarding sucrose taste. It would be of interest to know whether other types of sensory pleasure besides sweetness can be enhanced by the endocannabinoid hedonic hotspot described here, and whether the rewarding and euphoric effects of exogenous cannabinoid drugs such as Δ^9-THC are mediated by the same endocannabinoid hedonic hotspot that amplifies taste 'liking.' Food intake was also stimulated by anandamide microinjections that amplified hedonic 'liking,' suggesting that magnifying the pleasurable impact of food reward might be part of the mechanism for cannabinoid promotion of appetite or incentive motivation, likely in conjunction with the 'wanting' hotspots described in the previous chapters.

Therefore, these findings provide evidence for different roles in reward for accumbens cannabinoids and central amygdala (CeA) opioids. Here, we show that cannabinoid stimulation of accumbens enhances 'liking' as well as 'wanting' of food reward. In previous chapters, we showed that opioid stimulation of CeA enhances only appetitive behavior, without similarly potentiating 'liking' of rewards once they are received. Together, these findings reinforce the idea that 'wanting' and 'liking' are processed by dissociable neural substrates, and that it is important to know exactly which reward components are affected by brain manipulations such as local drug microinjections. Such careful parsing of reward components is the only way we can begin to untangle the vastly complex neural substrates of reward-related behaviors, and know for sure that we are, as Plato advised, 'carving nature at its joints.'

Figure 5.1: Anandamide Fos Plume Examples. *Radial Arm Fos Sampling:* Illustrates the Fos sampling method, in which radial arms for sampling extend from center of microinjection, viewed in the coronal plane (Fos-expressing neurons are counted in 125 X 125 μm blocks on arms spaced at 125μm intervals; 5X magnification). Insets show sample tissue blocks from anandamide or vehicle plumes and from a normal uninjected brain. *Vehicle Plume:* Small vehicle-induced Fos plume, mapped as low (2X) Fos elevation relative to normal tissue. *Sample Fos Expression:* Neurons expressing Fos-like activity in medial shell of nucleus accumbens after anandamide (25ng) or vehicle microinjection (5X magnification, contrast enhanced in both panels). *Anandamide Fos Plumes:* Fos plume examples for each dose (12.5ng, 25ng, 50ng; brains taken 75 min after microinjection); color denotes plume as mapped by Fos elevation over normal expression (percentage increase over vehicle levels); lines denote plume as mapped by Fos elevation over vehicle microinjection levels at equivalent boxes and sites.

Sample Fos Expression

Vehicle

Anandamide (25ng)

Radial Arm Fos Sampling

Vehicle Plume

2x
Normal

Anandamide

Vehicle

Normal

12.5ng Plume

5x
Normal

3x
Normal

2x
Normal

3x
Vehicle

2x
Vehicle

5x
Vehicle

0.5mm

25ng Plume

10x
Normal

5x
Normal

3x
Normal

2x
Normal

2x
Vehicle

3x
Vehicle

0.5mm

50ng Plume

5x
Vehicle

3x
Vehicle

2x
Vehicle

2x
Normal

3x
Normal

10x
Normal

5x
Normal

0.5mm

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Figure 5.2: Summary Fos Plume Maps for Hedonic 'Liking' and Food Intake Enhancements Produced by Anandamide in Medial Shell. Anandamide hotspots are mapped based on behavioral 'liking' or eating effects elicited at the mapped microinjection site (color) and on average Fos plumes measured at similar sites (size). Anatomical borders are visualized with *Substance P Stain*, and inset shows mapping area within larger brain (top). Lower columns show hotspots in sagittal, horizontal and coronal planes of nucleus accumbens shell. *Sucrose 'Liking' Summary* (middle)*:* Anandamide amplifies 'liking' reactions to sucrose taste especially in dorsal hotspot (all doses collapsed; all at 30 min time point). Symbol colors denote intensity of increase in number of positive 'liking' reactions, calculated as percent change from control vehicle injections at the same site. Inner symbols show average diameter of uninjected tissue-relative Fos plume intense centers (10X), surrounded by semitransparent halos that show moderate elevation zone (>5X), and lowest zone (>2X). *Food intake* (bottom): Anandamide stimulates food intake (grams consumed in 1 hr). Each unilateral cannula placement is represented with a symbol (25ng only). Symbol colors similar to above, and symbol size logic same as above. Note that eating effects were strong throughout the shell, whereas 'liking' sites were strongest in the dorsal half of shell.

Substance P Stain

Accumbens Inset

Sagittal

Horizontal

Coronal
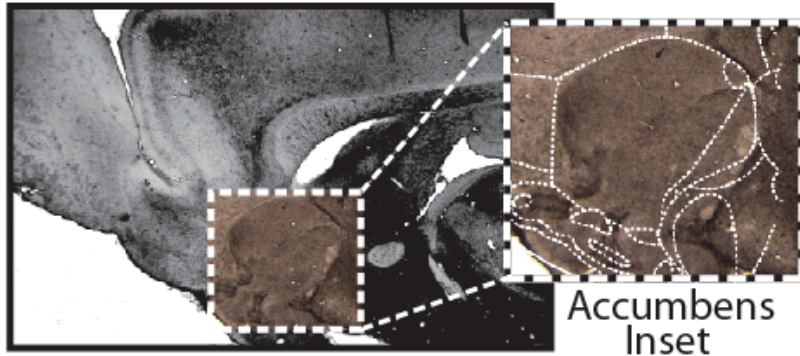
Sucrose 'Liking'

Intake 'Wanting'

>250%
>200%
>135%
No change
<75%
<50%

>250%
>200%
>150%
>110%
No Change

Shell
VP
Core
Shell
Core
Shell

Bregma +0.9

Bregma -7.6

Bregma +2.16

138
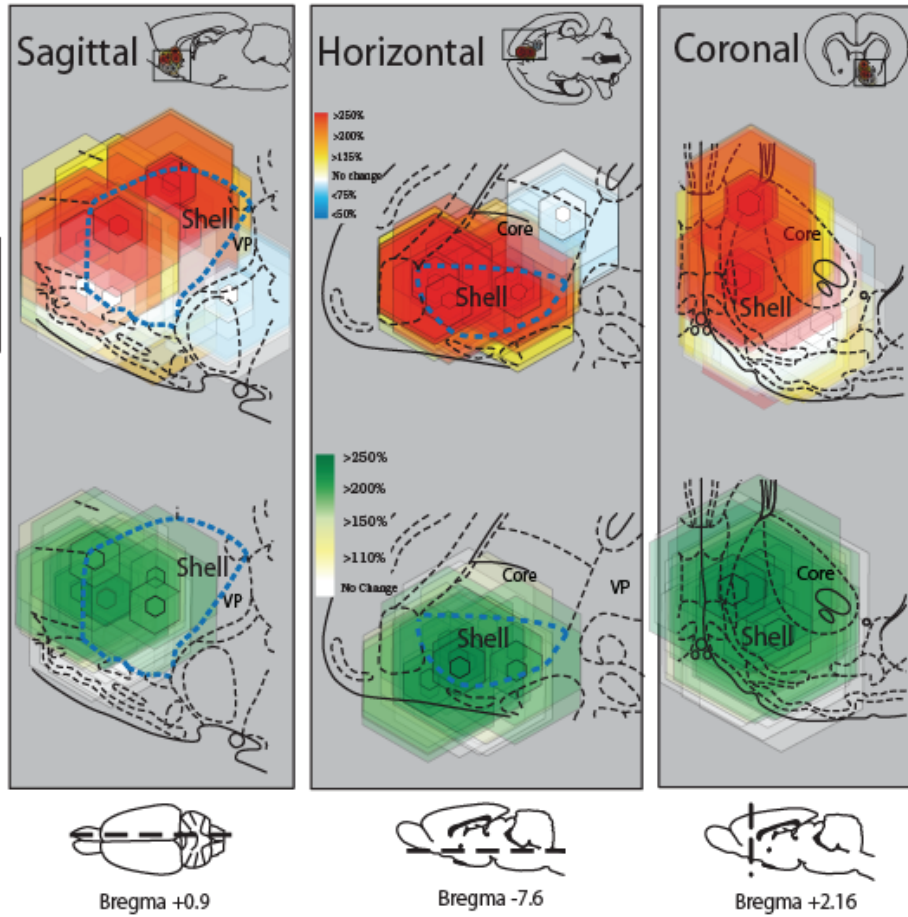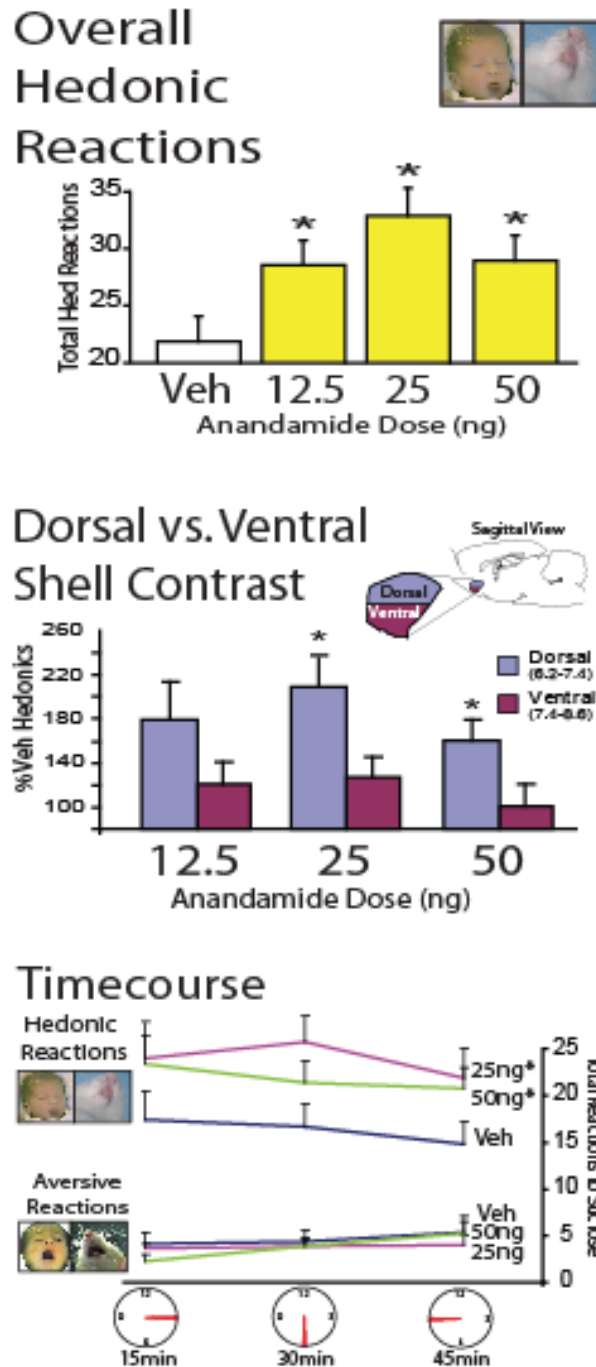
Figure 5.3: Anandamide Enhances Positive Hedonic Reactions to Sucrose, Particularly in Dorsal Accumbens Shell: Dose-response effect for hedonic enhancement: All doses (12, 25, 50ng) of anandamide amplified hedonic 'liking' reactions elicited by a sucrose taste compared to vehicle microinjection at same sites (30 min timepoint; *$p<.05$). *Dorsal vs. Ventral Shell Contrast:* Anandamide (25&50ng) increased hedonic reactions more at cannulae sites in the dorsal half of the accumbens shell than at sites in the ventral half (*$p<.05$). *Timecourse:* Anandamide enhancement was similar at all time points tested (15, 30, and 45 min after microinjection) *$p<.05$.

Figure 5.4: Anandamide Hedonic Enhancement: Dose Maps. Anandamide-induced increases in hedonic reactions to sucrose are shown for each dose separately (12.5ng, 25ng, 50ng; all at 30 min after microinjection). Hedonic enhancement is expressed as within-subject percentage change from vehicle levels for each rat, represented by symbol color. Map symbol size represents uninjected-relative intense (10X), moderate (5X), and low (3X) zones of Fos elevation, similar to Fig 2. *Dorsal shell advantage:* Bars along rostrocaudal and dorsoventral axes show intensity of anandamide effects within each 0.4mm-wide level (mean ± SEM percent of vehicle levels); a plume symbol can contribute to more than one bar when it straddles multiple levels). Bar colors reflect mean percentage change from vehicle. Backgrounds stained for Substance P.

12.5ng

25ng

50ng

% Vehicle Hedonics

>250%
>200%
>125%
No
<75%
<50%

Sagittal View
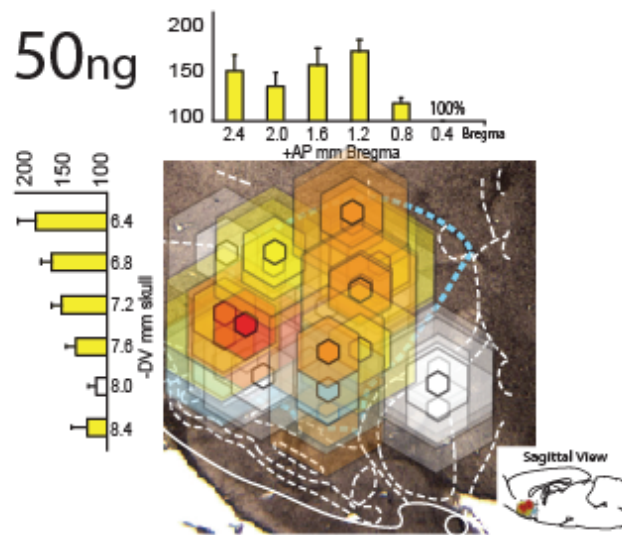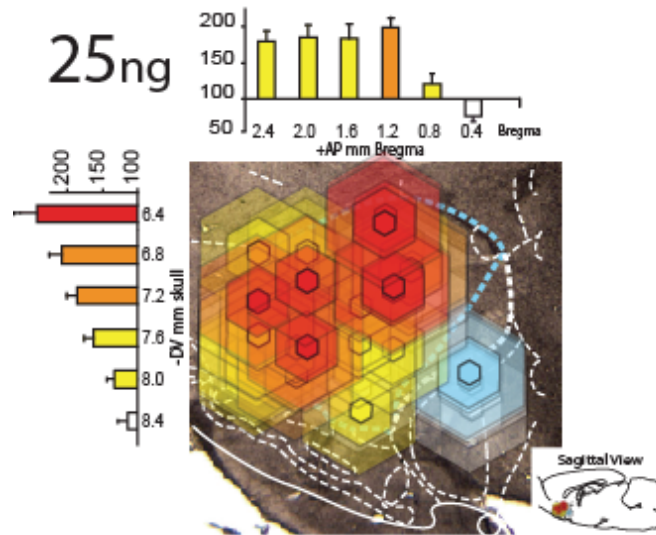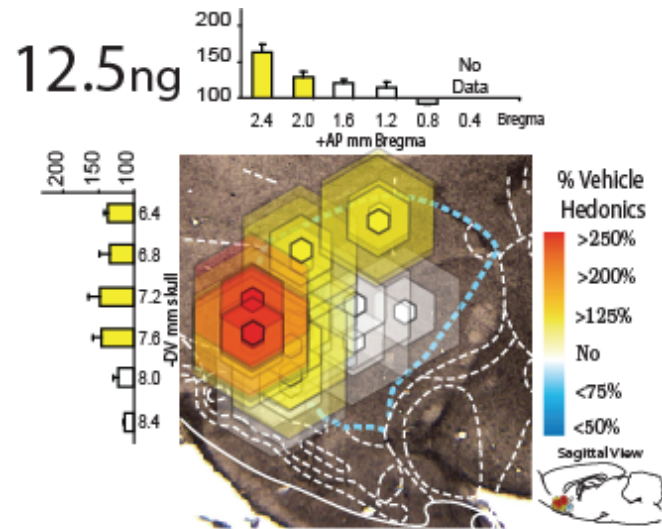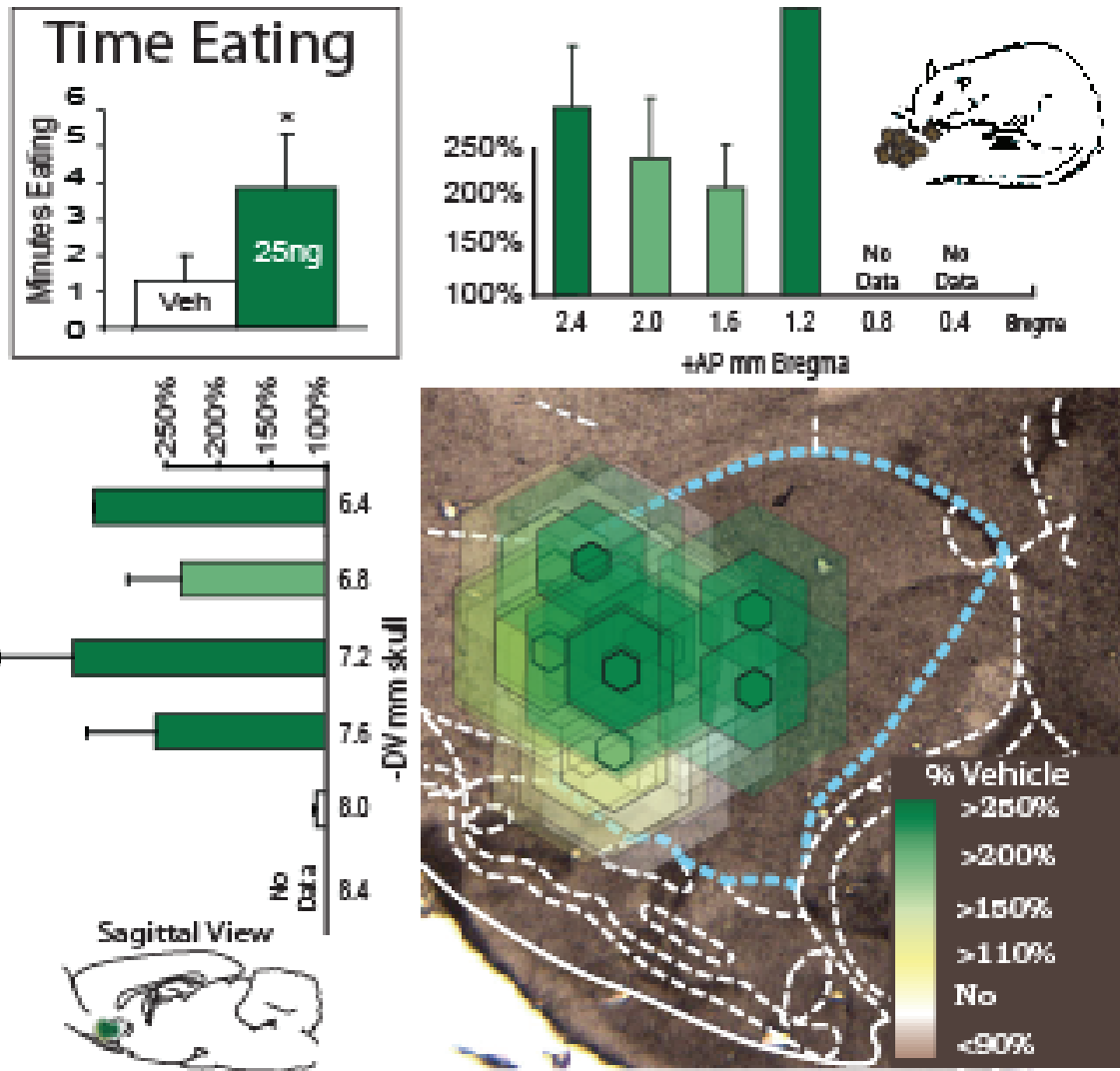
Figure 5.5: Anandamide Stimulates Voluntary Eating: *Time Eating:* Anandamide microinjections increased the cumulative duration of eating bouts (measured during 1 hr). *Time Eating Map:* Eating duration increases for each rat were mapped onto symbols as in Fig. 4 (25ng dose anandamide compared to vehicle at same sites). Anandamide stimulated food intake at most sites throughout the medial shell.

Figure 5.6: Anandamide Hedonic Enhancement: Timecourse Maps.
Anandamide-induced increases in hedonic reactions to sucrose are shown for 25&50ng anandamide at 15, 30, and 45 min after microinjection. Hedonic enhancement is expressed as within-subject percentage change from vehicle levels for each rat, represented by symbol color. Map symbol size represents uninjected-relative intense 10X), moderate (5X), and low (3X) zones of Fos elevation, similar to Figs. 5.2, 5.4&5.5. Bars along rostrocaudal and dorsoventral axes show intensity of anandamide effects within each 0.4mm-wide level (mean ± SEM percent of vehicle levels); a plume symbol can contribute to more than one bar when it straddles multiple levels). Bar colors reflect mean percentage change from vehicle. Backgrounds stained for Substance P.
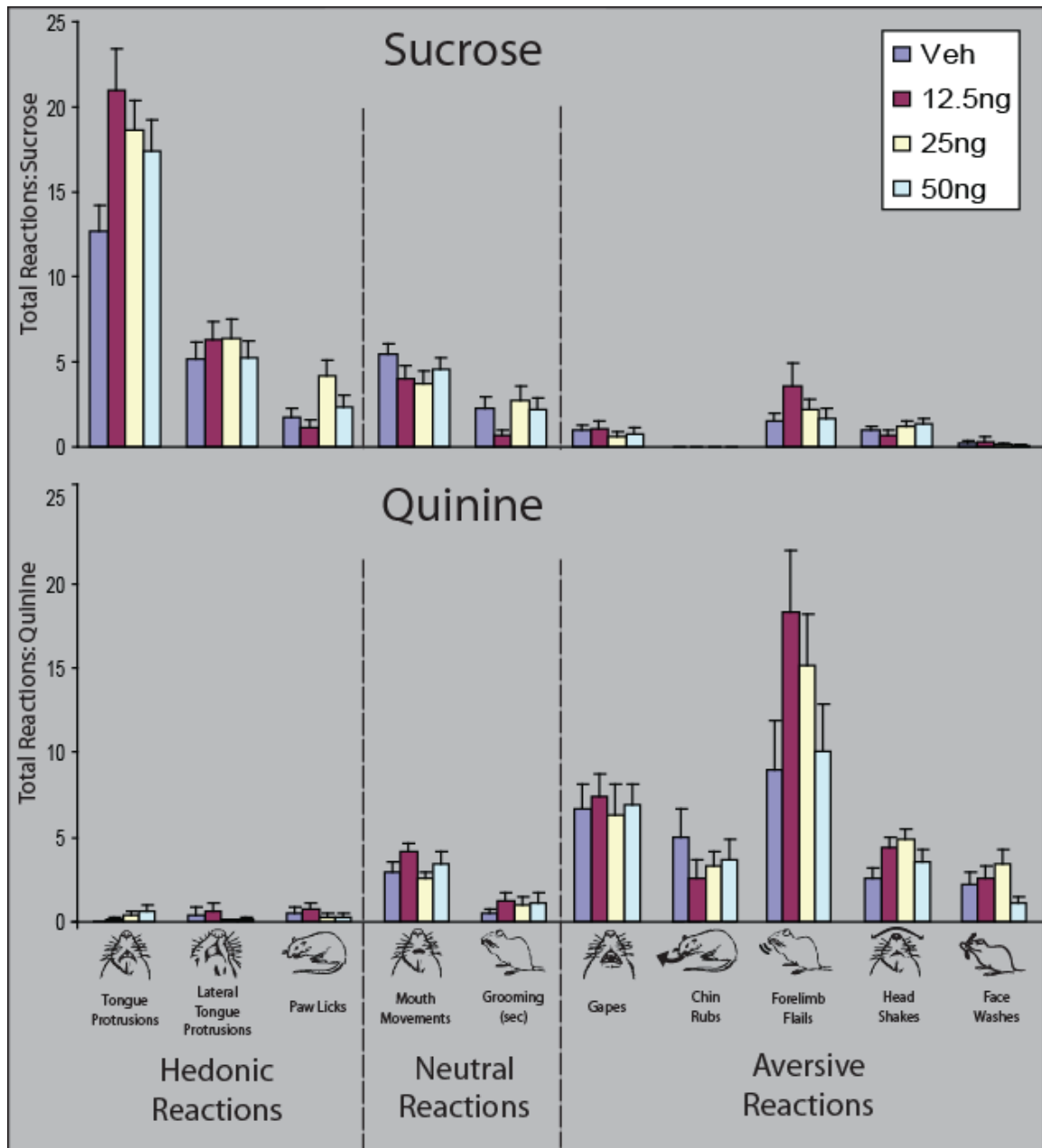
Figure 5.7: Individual Components of Hedonic and Aversive Reactivity to Sucrose and Quinine. Mean (SEM) occurrences of observed hedonic, neutral, and aversive reactions during 1 min infusions of sucrose (top panel) and quinine (bottom panel), 30 min after microinjection of anandamide (12.5, 25&50ng) or vehicle.

Figure 5.8: Anandamide Does Not Alter Aversive Reactivity to Intraoral Quinine.
No dose of anandamide significantly affected aversive 'disliking' reactions elicited by quinine taste. Changes in the number of hedonic reactions produced by anandamide is expressed as within-subject percentage changes from control levels after vehicle microinjections at the same site (vehicle = 100%). Fos plume symbols show functional effect at each site (color coded) mapped onto diameters representing intensity levels of Fos elevation induced by anandamide in separate animals (centers show zone of strong (10X) elevation above normal Fos expression, halos show moderate (5X) and low elevations (3X)). Colors indicate changes in 'disliking' from anandamide, expressed as a percentage of vehicle-induced 'liking' levels. Single unilateral view contains collapsed data from bilateral sites.

Quinine 'Disliking'
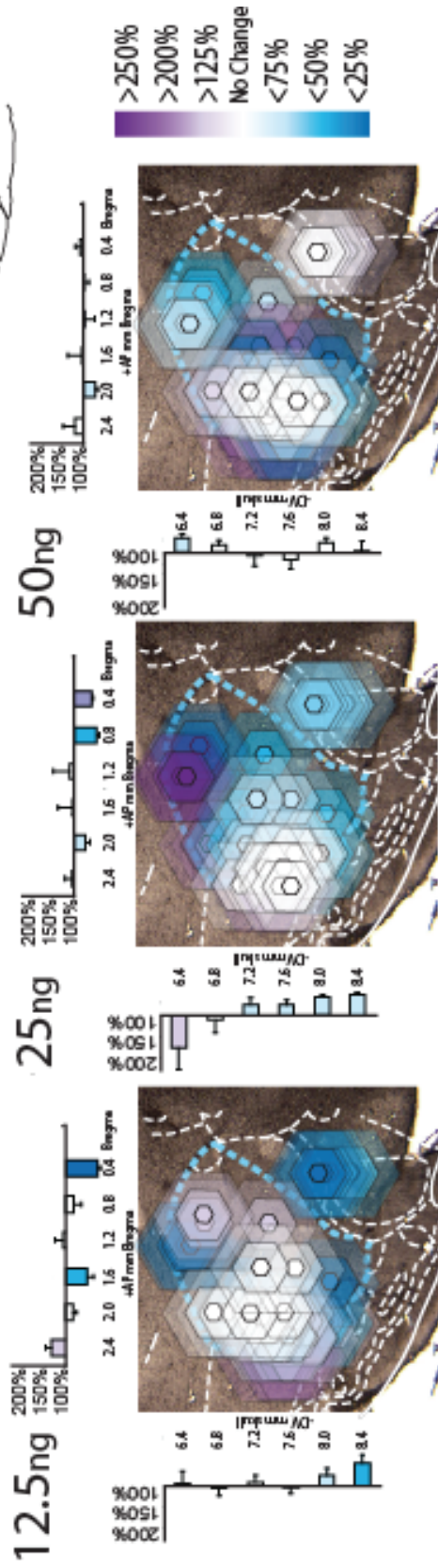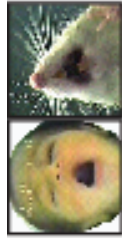
Figure 5.9: Initial and Overall Reactivity to Sucrose and Quinine after Anandamide.
Left panels show hedonic reactions, neutral mouth movements, and aversive reactions to sucrose (top row) and quinine (bottom row). *significantly different than vehicle, $p < 0.05$.

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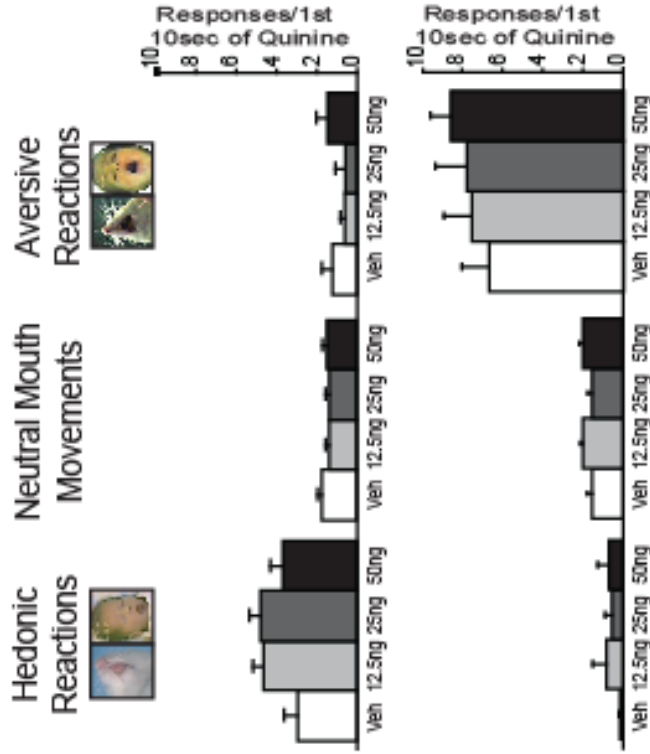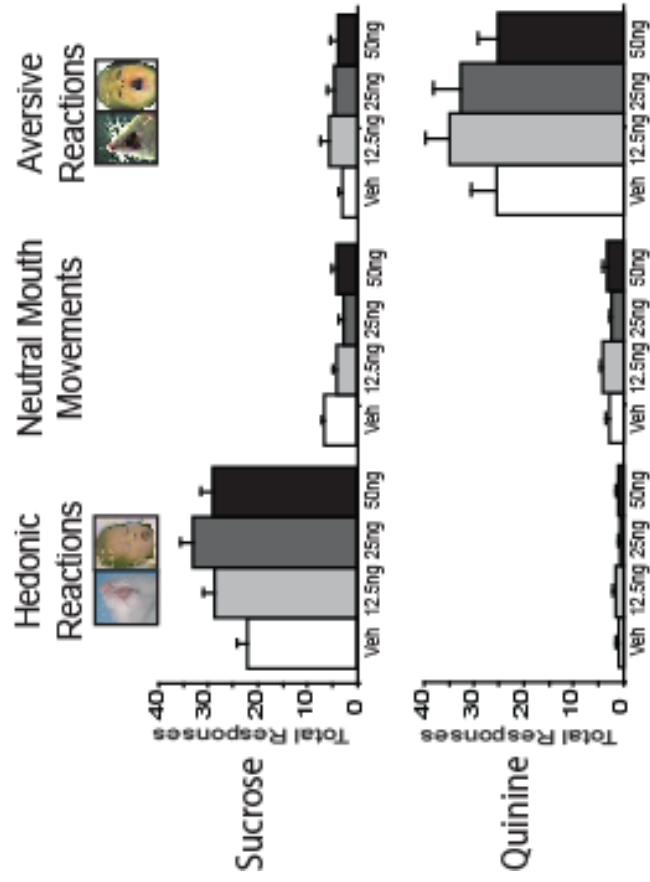
	Anandamide Dose (in 0.5µl)	Vehicle-Relative				Normal-Relative			
		Intense	Moderate	Low	Lowest	Intense	Moderate	Low	Lowest
radius mm	12.5ng	0±0	.08±.04	.33±.12	.71±.27	.01±.01	.42±.13	.69±.18	.89±.15
	25ng	0±0	.09±.06	.36±.09	.78±.07	.16±.03	.35±.09	.64±.16	.90±.10
	50ng	0±0	.18±.18	.53±.23	.77±.08	.13±.11	.51±.21	.80±.10	.90±.07
volume cubic mm	12.5ng	0±0	.002±.0003	.15±.007	1.5±.08	4189±4189*	.31±.009	1.38±.02	2.95±.01
	25ng	0±0	.024±.024	.20±.003	1.99±.001	.017±.0001	.18±.003	1.1±.017	3.05±.004
	50ng	0±0	.024±.024	.62±.62	1.91±.002	.009±.006	.56±.04	2.14±.004	3.05±.001

*=units in cubic micrometers (1cubic mm=1 billion cubic micrometers)

Table 5.1: Radii and Volumes of Anandamide Fos Plumes. Mean (SEM) increases in radii (top row, in mm) and total volume (bottom row, in mm^3 and µm^3, calculated assuming spherical plume shapes) of anandamide Fos plumes (12.5ng *n*=3; 25ng *n*=4; 50ng *n*=3) over controls (vehicle *n*=6; normal accumbens *n*=4) are shown. Left panels show anandamide plume values relative to vehicle (*Tocrisolve* and saline) microinjections at equivalent sites in medial shell of nucleus accumbens. Right panels show plume values relative to 'normal' accumbens tissue from uninjected rats. Intense zones are defined as the area surrounding anandamide in which levels of in Fos-like immunoreactivity are at least 10X over vehicle or normal tissue levels. Moderate, low and lowest zones have mean Fos increases of at least 5X, 3X, or 2X, respectively, over vehicle or normal tissue levels.

Chapter 6
Conclusion

Synopsis

These experiments demonstrate novel neurochemical substrates within the forebrain limbic system for modulating appetitive 'wanting' and hedonic 'liking' components of reward. We show that central amygdala μ opioids help translate past Pavlovian learning into focused and enhanced incentive salience 'wanting' for rewards and their cues. Using a variety of pharmacological, behavioral, immunohistochemical, and functional-anatomical 'mapping' techniques, we demonstrate that stimulation of μ opioid receptors in the central amygdala robustly enhances 'wanting' of both rewards and the environmental stimuli that predict them. Unlike the well-known hedonic enhancements caused by opioid stimulation in other brain areas, opioid stimulation of amygdala *reduced* hedonic reward 'liking.' In contrast, similar techniques were used to demonstrate a novel cannabinoid 'hotspot' in the dorsomedial nucleus accumbens shell for enhancement of 'liking' of a sweet reward. These findings demonstrate both a specific role for amygdala opioids in transforming learning into directed motivation, and a novel neurochemical substrate of reward 'liking' in the dorsomedial nucleus accumbens shell.

Central Amygdala Opioids Amplify 'Wanting' Targeted by Prior Learning

Reward CSs often attract approach and consummatory interactions usually reserved for the rewards they predict, which can result in seemingly bizarre behaviors such as nibbling and biting of a food-predictive lever CS in rats, or 'chasing ghosts' in human crack cocaine addicts. These behaviors are difficult to interpret in terms of simple

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habitual approach motivated by stimulus-response learning. One way in which these behaviors can be explained, however, is with an incentive salience account of Pavlovian motivation (Bindra, 1978; Berridge and Valenstein, 1991; Berridge, 2001). This theory proposes that stimuli associated with rewards come to attain similar motivational properties as the rewards they predict via stimulus-stimulus associations. Following learning, these conditioned stimuli (CSs) become reward-like in that they are attractive, noticeable, and liable to be approached and even consumed like the rewards they predict (Lajoie and Bindra, 1976; Boakes, 1977). When learned CSs are later encountered, they are attributed with incentive salience, and animals are drawn to them as if they were 'motivational magnets.'

We examined this property of reward CSs with an autoshaping paradigm. We paired extension of a lever into a chamber with delivery of a sucrose reward into a cup elsewhere in the chamber. Animals come to preferentially approach, nibble, sniff, and bite *either* the lever CS+ itself *or* the cup (CS_{source}) during cue presentations. We also exploited this individual difference in the targeting of Pavlovian incentive motivation to examine how stimulation of μ opioid receptors in the central amygdala would affect the targeting and degree of incentive motivational behavior displayed.

We found that microinjection of the μ opioid agonist DAMGO (0.1μg) into the CeA following or during training enhanced appetitive-consummatory interactions with the specific reward-associated stimulus upon which an animal had previously learned to target incentive salience. DAMGO only facilitated the motivational magnet properties of this prepotent CS, without affecting approach or interaction with the alternative reward-associated stimulus. Conversely, inactivating CeA with the $GABA_A$ agonist muscimol (0.25μg) potently reduced approach and interaction with the prepotent CS, but did not affect interactions with the alternative stimulus. CeA DAMGO and muscimol had parallel effects on food intake (enhancing and reducing, respectively), confirming modulation of 'wanting.' Fos plume analysis confirmed that these effects were primarily due to actions of DAMGO in CeA.

These findings point to a unique role for central amygdala μ opioids in targeting incentive salience upon prepotent reward-associated stimuli based on previously learned Pavlovian associations. Instead of globally enhancing the incentive salience of all

reward-associated cues, CeA preferentially targeted enhanced 'wanting' in a manner dependent upon previously learning, indicating that it may play a special role in interfacing prior learning with in-the-moment motivation resulting from exposure to a conditioned stimulus.

Central Amygdala Opioid Activation Focuses Cue-Triggered UCS 'Wanting'

This experiment examined whether, in addition to targeting incentive salience upon reward CSs, amygdala opioid stimulation would also enhance Pavlovian cue-triggered 'wanting' for a UCS reward.

The Pavlovian to instrumental transfer (PIT) paradigm allows examination of cue-triggered 'wanting' for a UCS reward that is triggered by Pavlovian cues, but is directed into seeking of a UCS reward. This is done by examining the impact of an auditory Pavlovian CS upon instrumental lever pressing that previously yielded the same reward.

PIT is designed to specifically measure 'wanting' that is triggered by Pavlovian cues, but expressed as an increase in instrumental lever pressing for the same reward under extinction conditions. Pavlovian and instrumental tasks are trained separately, so cue-triggered lever pressing is necessarily motivated by a remembered representation of the UCS reward that is triggered by the CS. The sucrose representation is 'wanted,' and animals are able to transfer this Pavlovian motivation into a particular reward-seeking action aimed at attaining the predicted reward. Since this particular instrumental sucrose-seeking response was not possible during Pavlovian training (levers were not present at that time), increased lever pressing must be due to an activated S-S associations between the cue and the represented reward, rather than S-R associations between the cue and a particular action such as entering the sucrose delivery cup. Importantly, PIT testing is conducted in the absence of rewards themselves (under extinction conditions). Therefore, all cue-triggered instrumental behavior during testing is driven by incentive salience attributed to the cue-elicited reward representation, rather than response reinforcement or the hedonic impact of a reward once it is received. In this way, PIT allows examination of cue-triggered UCS 'wanting' in isolation from other reward-related processes.

We examined how stimulating opioid receptors in the central or basolateral amygdala with the μ agonist DAMGO (0.05&0.1μg) would affect cue-triggered UCS

'wanting' in the PIT paradigm. We found that CeA, but not BLA DAMGO enhanced baseline-relative lever pressing during CS+ presentations. This effect was specific to the CS+ but not a control CS-, and was expressed as increases in cue-triggered pressing on the sucrose lever, but not a control lever. Therefore, CeA DAMGO enhancements were dependent upon prior Pavlovian learning, and were specifically directed into sucrose seeking, but not other behaviors. Food intake was also enhanced by CeA, but not BLA DAMGO, similar to Chapter 2.

In addition to enhancing cue-triggered sucrose seeking as it is usually measured in PIT experiments (Hall et al., 2001; Holland and Gallagher, 2003), CeA DAMGO also seemed to enhance the focus and cue-specificity of 'wanting.' Sucrose lever pressing in the absence of CSs was actually reduced by CeA DAMGO compared to vehicle levels, indicating that opioid stimulation shifted 'wanting' to the best Pavlovian predictor of reward—the CS+. This effect suggests that CeA DAMGO focused sucrose seeking, so that it occurred more exclusively in response to a stimulus that was explicitly paired with sucrose. When Pavlovian S-S associations were not directly activated by the CS+ (such as during non-cue periods and during the CS-), animals showed much less sucrose seeking, and only rarely pressed the sucrose lever. This reduction was not due to mere decreases in locomotor behavior generally, as pressing on the inactive lever and exploratory behavior were not affected. These findings again suggest that stimulating CeA opioids causes preferential targeting of incentive salience upon certain prepotent Pavlovian stimuli (such as the CS+), at the expense of others (such as contextual cues of the testing chamber).

In contrast to CeA DAMGO, opioid stimulation of BLA had few effects upon behavior here. Cue-triggered sucrose seeking, precue sucrose seeking, and cue-triggered food cup approach were all unaffected by BLA DAMGO. Food intake was also not significantly affected by BLA DAMGO, although a minority of animals did show some enhancement of food intake.

These results demonstrate that CeA opioids powerfully enhance cue-triggered 'wanting,' and focus reward seeking based on previously learned Pavlovian associations. They also demonstrate that this focusing of Pavlovian 'wanting' by DAMGO is specific

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to the CeA, and that BLA opioid stimulation does not have strong effects on the modulation of cue-triggered, UCS-targeted 'wanting.'

A Specific Role for Central Amygdala Opioids in 'Wanting' but not 'Liking'

In the prior experiments, we demonstrated that CeA opioid stimulation potently enhanced and focused incentive salience 'wanting' directed at both rewards and particular cues that are associated with them. Opioids in other mesolimbic reward structures such as nucleus accumbens and ventral pallidum also modulate cue-triggered 'wanting,' but are probably best known for their role in palatability and hedonic 'liking' of rewards once they are received (Peciña and Berridge, 2005; Smith and Berridge, 2005). Some have proposed that amygdala opioids also modulate hedonic states such as the negative affect induced by drug withdrawal (Koob and Le Moal, 2008). Here we asked whether CeA or BLA DAMGO (0.1μg) would enhance affective hedonic 'liking' in a taste reactivity paradigm. This paradigm allows examination of the neural substrates of hedonics by measurement of evolutionarily conserved orofacial reactions characteristically produced in reaction to pleasant and unpleasant tastes.

We found that opioid stimulation of both CeA and BLA markedly decreased hedonic 'liking' of a rewarding sweet taste, without affecting aversive 'disliking' of a bitter quinine taste. In contrast, the very same injections that suppressed 'liking' robustly enhanced food intake 'wanting' only minutes later. These results demonstrate a dramatic dissociation of the role of amygdala μ opioid receptors in 'wanting' vs. 'liking.' In addition, they provide novel evidence that opioids play a specialized role in amygdala, different from the role they play in other mesolimbic brain areas.

An Endocannabinoid Hedonic Hotspot in Dorsomedial Accumbens Shell

The previous experiments describe a specific role for CeA μ opioid receptors in enhancing incentive salience 'wanting,' but not hedonic 'liking.' Reward 'liking,' therefore, must be processed in other ways by the brain, and we sought to describe one of these as well. In addition, our finding that CeA opioid stimulation reduces hedonics was surprising, and we desired verification that we could observe hedonic increases using the present methodologies when are altered by experimental manipulations.

Cannabinoid receptors in nucleus accumbens have been proposed to modulate reward-related behavior (Cooper, 2004; Jarrett et al., 2005; Kirkham, 2005), so we asked

whether stimulating them with the endogenous cannabinoid anandamide would increase hedonic 'liking' in the taste reactivity paradigm. We found that anandamide did enhance 'liking' of a sucrose taste, particularly in a 1.6mm^3 dorsal medial shell 'hotspot' that intriguingly overlaps with a known accumbens hotspot for μ opioid agonist stimulation of 'liking.' In addition, we found that anandamide also enhanced food intake 'wanting' in similar areas of accumbens. These results are both novel in their own right, and contrast starkly with the results of opioid stimulation of amygdala. They also enhance confidence that the surprising suppression of hedonics by amygdala opioid stimulation is reliable, and not due to methodological issues or other factors particular to this researcher.

Comparison of Amygdala Opioid Stimulation with Amygdala Lesions: Necessity vs. Sufficiency

Many previous experiments examining the role of brain nuclei in reward motivation, learning, and hedonics have examined behavioral effects of lesions or pharmacological inactivation of reward structures like CeA or BLA. These techniques are very useful for examining the *necessity* of a brain structure for a particular behavior or psychological process. In other words, when a structure is destroyed or inactivated, can an animal still perform a particular behavior? A somewhat different question is that of sufficiency of a brain reward substrate for a behavior. Can stimulation of a structure or a particular neurochemical therein result in increased levels of the behavior of interest?

Although these questions are related, they are not synonymous. In some cases a neural system may be both necessary and sufficient for behavior, and in others it may be one and not the other. For example, opioid stimulation of the dorsomedial nucleus accumbens shell increases hedonic reactions to a sweet taste in the taste reactivity paradigm (Peciña and Berridge, 2005), suggesting that opioid stimulation there is sufficient for enhancing taste 'liking.' However, microinjection of the opioid antagonist naloxone does not prevent animals from reacting normally to 'liked' sweet tastes (unpublished data from the Berridge Lab). This suggests that accumbens opioids are sufficient for enhancing 'liking' above normal levels, but are not necessary for normal 'liking.' In contrast, the ventral pallidum seems to be both necessary for normal 'liking,' and sufficient for opioid enhancement of 'liking' (Cromwell and Berridge, 1993; Smith

and Berridge, 2005, 2007) Therefore, it is always important when determining the role of a brain structure or system in behavior to consider whether it is necessary, sufficient or both.

The experiments in this dissertation primarily focus upon the sufficiency of opioid and cannabinoid systems in limbic structures for enhancing reward 'wanting' and 'liking.' Much previous work has been conducted upon the necessity of these structures in these processes. From these lesion and pharmacological inactivation experiments, a consensus seems to be emerging that CeA and BLA play slightly different roles in reward learning and motivation. For example, CeA but not BLA lesions prevent animals from acquiring conditioned approach to cues that predict food rewards (Parkinson et al., 2000), and for generating reward seeking triggered by Pavlovian reward cues (Pavlovian to instrumental transfer)(Hall et al., 2001; Holland and Gallagher, 2003; Corbit and Balleine, 2005). Both these properties rely upon animals accessing the current motivational value of a reward predicted by a cue, and using this value to guide behavior directed at cues and cue-elicited representations of rewards.

In contrast, BLA, but not CeA lesions seem to block the ability of animals to form and utilize representations of particular rewards to guide instrumental behavior and new learning. For example, BLA lesions prevent animals from reducing their instrumental seeking of a food reward that has been devalued by feeding the animal to satiation on that particular reward prior to testing (Hatfield et al., 1996; Balleine et al., 2003), and from forming new representations of a reduced-value reward after it has been paired with lithium chloride-induced illness (Pickens et al., 2003). BLA lesions also block the ability of Pavlovian cues to serve as reinforcers of new instrumental behavior, often called conditioned reinforcement, again suggesting an impairment in the ability of animals to use cue-triggered reward representations to guide instrumental behavior (Robbins et al., 1989; Hatfield et al., 1996; See et al., 2003). Another effect of BLA lesions is to block the ability of Pavlovian cues associated with food delivery to trigger food intake in the absence of physiological hunger (Holland et al., 2002; Petrovich et al., 2002). Presumably, in this case animals are unable to produce the instrumental act of feeding based upon a cue-elicited representation of food triggered by the Pavlovian stimulus. Finally, BLA lesions also prevent animals from directing instrumental behavior

appropriately based upon cue-triggered representations of particular rewards and their distinct sensory properties (reward-specific PIT). When intact animals are trained that a Pavlovian cue predicts one of several available rewards (e.g. dextrose), playing this cue primarily elicits seeking of the predicted reward (pressing a lever that once delivered dextrose) but not other equally valuable rewards available during training (e.g. sucrose) (Balleine et al., 2003; Corbit and Balleine, 2005). When BLA is lesioned, animals can no longer appropriately direct cue-triggered motivation toward the predicted reward only, and indiscriminately seek all rewards when the cue is encountered (Corbit and Balleine, 2005). Again, this might be a sign that BLA facilitates a linkage of a cue with a particular cognitive representation of the reward it predicts, allowing appropriate instrumental behavioral output.

In sum, these findings suggest that CeA and BLA are necessary for related, but subtly different aspects of reward. The CeA may be specialized for imbuing a cue or reward with incentive salience, causing it to be approached, investigated, and 'wanted.' The BLA may instead allow cues to trigger representations of specific predicted rewards, and to produce instrumental behavior and new learning based upon them.

In the present experiments, we focused upon examine the effects of stimulating amygdala opioids on incentive motivational 'wanting,' and primarily found CeA-dependent effects. In several respects, the present findings complement previous amygdala lesion experiments, in that CeA opioid stimulation had opposite effects from CeA lesions in similar behavioral paradigms measuring 'wanting.' Here, CeA but not BLA opioid stimulation enhanced cue-triggered 'wanting' directed at either cues (autoshaping, Chapter 2) or in pursuit of rewards themselves (PIT, Chapter 3). CeA lesions block these same processes (Parkinson et al., 2000; Hall et al., 2001; Holland and Gallagher, 2003; Corbit and Balleine, 2005).

For BLA, we provide little evidence that μ opioid stimulation enhances incentive salience 'wanting.' Here, a BLA opioid agonist failed to enhance either autoshaping or PIT, suggesting a different role for BLA and CeA opioids in stimulating incentive salience 'wanting.' So if BLA opioids don't stimulate 'wanting,' what do they do? One possibility (based on the lesion literature) is that they simply facilitate the processes that are attenuated in animals with BLA lesions: the formation and use of cue-triggered

representations of particular rewards, and their use in instrumental behavior and new learning. If so, then BLA DAMGO might enhance reward-specific PIT, second order conditioning, and cue-potentiated feeding, just as BLA lesions block these behaviors.

Alternatively, it is possible that BLA opioids may play a more complex and precise role than this—a role that would be difficult to examine with the present methodologies. For example, BLA participation in cue-responses may depend upon activation of particular synapses and groups of neurons that allow incorporation of specific sensory features of a reward representation to inform motivated behavior. If so, incoming information about a cue might selectively activate a particular pattern of synapses that represent the predicted reward's sensory characteristics. Lesions might destroy these crucial synapses, and thus attenuate this process. While many other synapses and neurons are also destroyed by lesions, if they are not activated by incoming cues, their destruction may be inconsequential to the measured task.

In contrast, stimulating BLA opioid receptors with local microinjections might indiscriminately activate many receptors and neurons, which could obscure the specific pattern of activation mediating the relevant cue/reward association. One could explore this question by examining the effects of BLA opioid stimulation on a task known to require BLA, such as reward-specific PIT (Corbit and Balleine, 2005). If no effect of BLA DAMGO on specific PIT were found, this might argue for such a possibility.

Role of Amygdala Opioids in Learning

The amygdala is a crucial brain structure for both appetitive and aversive Pavlovian learning. Here we demonstrated that in addition, CeA also participates in attributing particular environmental stimuli with incentive salience, based upon prior learning. In Chapter 2, we found that the 'motivational magnet' properties of cues were enhanced whether CeA DAMGO was administered either during initial learning or following it. In Chapter 3, we showed that CeA DAMGO also enhanced cue-triggered 'wanting' for rewards even after all Pavlovian and instrumental learning had already occurred. Both these findings suggest that CeA opioid stimulation can enhance 'wanting' based upon prior learning, even without enhancing learning itself.

This said, it is possible that CeA opioids may also affect learning itself, as well as 'wanting.' While we present no clear evidence in these studies to support this possibility, previous findings suggest that CeA opioid stimulation can sometimes attenuate learning, for example of Pavlovian fear responses (Ragozzino and Gold, 1994; Good and Westbrook, 1995; Westbrook et al., 1997). If appetitive learning is similarly inhibited by CeA opioids, this decrement was not apparent here when DAMGO was administered during autoshaping training in Chapter 2 (e.g. Figure 2.6). Instead, CeA DAMGO animals generally learned to approach their prepotent CS just as quickly as control animals. Once this association was learned, however, DAMGO animals displayed more vigorous appetitive-consummatory interactions directed at the cue. This seeming contradiction might point to a different role for opioids in aversive and appetitive Pavlovian learning, with inhibition of the former but not the latter. Alternatively, DAMGO could have simultaneously enhanced reward 'wanting' while decreasing learning efficiency itself, resulting in no change in learning rate in the present experiment. The present experiments were not designed to dissociate the potentially independent effects of CeA opioid stimulation upon learning itself in the absence of motivation, but future studies should be able to address this potentially theoretically important question.

Another point regarding the role of CeA in appetitive Pavlovian learning *per se* is the finding that CeA lesions block the acquisition of conditioned approach to a visual stimulus predicting food reward, but not the expression of this conditioned approach following learning (Cardinal et al., 2002b). Here, we found that CeA opioids enhanced the expression of approach and appetitive/consummatory interaction with a food-predictive lever cue even when stimulated following prior acquisition of the task. This difference may depend upon several factors. CeA opioid stimulation may be sufficient for enhancing incentive salience, but not necessary for its expression in the absence of an intact CeA. Alternatively, CeA opioids might be specifically recruited in autoshaping tasks in which cues become true 'motivational magnets,' taking on attributes of the associated reward including liability to be consumed (as with the physical lever and cup cues here), but not tasks in which only approach and mere investigation of cues is possible (as with a visual CS on a monitor used in the lesion study)(Cardinal et al.,

2002b). In order to fully explain the nature of this apparent difference, further experiments designed to address these questions will be required.

Amygdala Opioids in Hedonics

Chapter 4 describes a novel role for forebrain opioids in specifically enhancing reward 'wanting,' without affecting reward 'liking.' As previously discussed, opioids are well-known to modulate reward hedonics, making tasty foods even tastier (Baldo and Kelley, 2007), and some have even argued that they play a specialized role in this process throughout the brain (Koob, 1992; Kelley et al., 2005; Cota et al., 2006; Barbano and Cador, 2007). Some have also made a case for opioids in CeA and other extended amygdala structures modulating negative affect characteristic of withdrawal, again suggesting a role for them in hedonic processes (Schulteis et al., 1994; Gracy et al., 2001; Harris and Aston-Jones, 2007; Koob and Le Moal, 2008).

Although CeA and BLA lesions do not seem to affect hedonic reactivity to primary tastes in taste reactivity (Galaverna et al., 1993; Rana and Parker, 2008), amygdala has been proposed to play a role in hedonics, especially in cases when an animal must learn what to 'like,' and what to 'dislike.' An example comes from conditioned taste aversion (CTA), in which neutral or hedonic tastes are associated with the experience of illness (induced with lithium chloride injections). Intact animals quickly learn to avoid these illness-paired tastes when they are offered, and to respond with aversive orofacial reactions (like gapes) when they are involuntarily administered in a taste reactivity paradigm. The exact role of CeA and BLA in the formation and expression of CTAs is controversial, though emerging consensus suggests that CeA is unlikely to mediate CTAs (Yamamoto et al., 1995; Touzani et al., 1997; Reilly and Bornovalova, 2005; Rana and Parker, 2008). The BLA, although initially proposed to be necessary for expression of CTAs (Yamamoto, 1993; Schafe et al., 1998), might instead be necessary for processes other than taste aversion learning *per se*, such as neophobia (Reilly and Bornovalova, 2005). This said, BLA shows enhanced Fos expression in animals exposed to Pavlovian stimuli associated with sickness-paired taste (Kerfoot et al., 2007), and BLA may be required for animals to reduce voluntary consumption of an illness-paired taste (Rana and Parker, 2008).

Here, we find that CeA opioid stimulation can actually *reduce* hedonic responses to an otherwise 'liked' sucrose taste, even when it simultaneously makes these less 'liked' rewards more 'wanted.' Therefore, opioids in amygdala appear to play a different role in reward than in other brain structures examined to date.

Brain Substrates of 'Wanting:' Amygdala Opioids and Accumbens Dopamine

Amygdala opioids seem to enhance 'wanting,' but not to enhance (and even reduce) 'liking' of rewards. This pattern of results is reminiscent of the effects of stimulating the mesolimbic dopamine system including projections from ventral tegmental area to the nucleus accumbens. For example, enhancing dopamine with either microinjection of amphetamine into accumbens, or sensitization of the system by repeated systemic amphetamine injections strongly enhances the *intensity* of instrumental behavior by Pavlovian cues in the PIT paradigm, but not cue-independent levels of lever pressing (Wyvell and Berridge, 2000, 2001).

The present experiments demonstrate that in addition to increasing the intensity of cue-triggered 'wanting' (Chapter 2), CeA microinjection of an opioid agonist also targets incentive salience upon only some cues (the CS+ in PIT, the prepotent reward-associated stimulus in autoshaping, or food itself in intake tests), at the expense of others (contextual cues in PIT, and the non-preferred cue in autoshaping). In other words, CeA opioids seem to channel incentive salience toward the motivational 'path most travelled,' in a 'winner take all' fashion rather than as a 'rising tide' that floats all motivational 'boats.'

These findings may point to an important difference in the role of CeA opioids and mesolimbic dopamine in terms of their respective roles in 'wanting.' CeA opioids may specifically modulate only the most affectively significant reward cues, and focus resulting incentive motivation upon particular targets. In contrast, it is possible that dopamine release in accumbens is specialized to enhance the amplitude of incentive motivation that is already targeted by other systems, such as amygdala. Both CeA and BLA are known to be capable of modulating mesolimbic dopamine systems (Phillips et al., 2003; Ambroggi et al., 2008), as well as dopamine activity in response to rewards and their cues (Ahn and Phillips, 2002), providing a mechanism for such a hierarchical reward processing pathway (Phillips et al., 2008).

Several hypotheses can be generated from the hypothesis that CeA opioids interface learning with 'wanting' to target incentive salience, while accumbens dopamine then amplifies this 'wanting' in a less directed manner. For example, stimulating dopamine in accumbens might enhance approach and consummatory behaviors directed at all available cues in an autoshaping paradigm (the lever and the sucrose cup) instead of only the cue that was already prepotent, as described in Chapter 2. This might be particularly evident if amygdala targeting of incentive salience was compromised by inactivation or lesions of CeA in combination with dopamine stimulation in accumbens.

Another way of testing the relative roles of CeA opioids and accumbens dopamine in 'wanting' would be to explore the effects of stimulating or inactivating amygdala opioids, accumbens dopamine, or combinations thereof in a PIT paradigm that allows examination of reward-specific 'wanting' (Blundell et al., 2001; Corbit and Balleine, 2005; Corbit et al., 2007). In this paradigm, animals are taught to associate three different auditory CSs with three equally valuable, but distinct rewards. They are also trained that pressing one lever delivers one of these rewards, and another lever delivers a second one. When animals are exposed to a CS that was associated with a reward that was also delivered by pressing a particular lever, they primarily press on the appropriate lever. When they are exposed to the CS for a reward that has no corresponding lever, they press equally upon both available inappropriate levers. Based on the present findings, we might predict that stimulating CeA opioids would enhance only targeted cue-triggered incentive motivation directed at the 'appropriate' lever for the 'wanted' reward. In contrast, dopamine stimulation might enhance 'wanting' of all available rewards regardless of the particular reward that is predicted by a given cue. In line with this hypothesis, Corbit et al. (2007) report that inactivation of ventral tegmental area dopamine cells indiscriminately attenuates both reward-specific and reward-independent PIT.

Relevance of CeA Incentive Salience Targeting to Drug Addiction

Robinson and Berridge (1993) first proposed that sensitization of mesolimbic dopamine systems is involved in elevated 'wanting' or craving in response to drug cues, thus putting recovering addicts at risk for relapse. In the last 15 years, this theory has

gained in prominence among addiction neuroscientists, owing to mounting evidence that dopamine plays a specific role in appetitive and motivational aspects of reward (Wise and Colle, 1984; Robinson and Berridge, 1993; Wyvell and Berridge, 2001; Robinson et al., 2005; Cagniard et al., 2006; Baldo and Kelley, 2007; Berridge, 2007; Di Chiara and Bassareo, 2007; Salamone et al., 2007). Sensitization of dopamine systems by repeated drug administration seems to be a rather global phenomenon, however, and results in widespread changes in dopamine systems in many neurons in many brain areas. These non-specific changes caused by sensitization can sometimes result in 'spillover' of enhanced 'wanting' for other rewards. For example, sensitized addicts can become addicted to more than just drugs, and are for example unusually likely to be sexually compulsive (Washton and Stone-Washton, 1993; Fiorino and Phillips, 1999).

This said, drug addicts are primarily addicted to drugs, and while motivation for other rewards might sometimes also be enhanced, the fundamental problem in addiction is abnormal motivation for drugs in particular. This problem has caused some to argue that sensitization of dopamine systems cannot be the fundamental cause of drug addiction, since dopamine also mediates 'wanting' of other rewards, including food, sex, drugs, and even pair bonding in monogamous rodents (Fiorino and Phillips, 1999; Wyvell and Berridge, 2001; Nocjar and Panksepp, 2002; Aragona et al., 2006; Curtis et al., 2006). Why then should globally sensitized dopamine systems result in the relatively specific enhancements of drug 'wanting' characteristic of addiction, where addicts come to more and more exclusively put drugs ahead of all other available rewards (Vanderschuren and Everitt, 2005)?

In animal models, there is some evidence that sensitization can result in increased 'wanting' of some rewards but not others. For example, Nocjar and Panksepp (2002) demonstrate that amphetamine sensitization induced *either* enhanced place preference for amphetamine *or* enhanced pursuit of a food reward, but not both. Similarly, Tindell et al. (2005) found that amphetamine sensitization increases 'wanting'-related firing of ventral pallidal cells in response to some reward cues and not others.

The present findings may be relevant to how sensitization can sometimes result in targeted 'wanting' for some rewards and not others, and for drug rewards in particular in addiction. In the present experiments, we demonstrate that amygdala opioid stimulation

facilitates incentive motivation based on prior Pavlovian learning about the association of rewards with particular stimuli. This associative specificity of cue-triggered 'wanting' is an essential feature of the Bindra-Toates model of incentive salience (Bindra, 1978; Toates, 1986; Berridge, 2001), and the present findings suggest that amygdala opioids may be a brain substrate for the interface of particular learned Pavlovian associations with sensitized drug-targeted motivation in addiction. It is worth noting that amygdala activation is one of the most consistent findings in brain imaging experiments examining neural responses to drug-associated cues in human addicts (Grant et al., 1996; Bonson et al., 2002; Franklin et al., 2007; Childress et al., 2008). In fact, Scott et al (2007) even recently reported enhanced μ opioid release in response to smoking cues in smokers, further supporting a similar role for opioids in humans as in the present experiments in rats. Finally, drugs of abuse such as heroin show 'priming' effects in animals and humans, where small doses of drugs promote enhanced drug seeking. To the extent that heroin or any other drug directly or indirectly causes opioid activation in amygdala, the present findings suggest that this could result in targeted 'wanting' for drugs, and contribute to the risk of further relapse after taking 'just as taste.'

Amygdala Functions in a Wider Context

The Amygdala as an Affective Watchdog

The amygdala is a crucial for forming associations between emotionally significant events and the sensory stimuli that are associated with them, as well as producing appropriate emotional responses to these stimuli when they are encountered again. For example, the ability to predict situations that are likely to be dangerous has obvious evolutionary value, and therefore amygdala plays an important and ancient role in self-preservation. When a rat is exposed to a novel auditory stimulus like a tone, and then given a mild shock immediately thereafter, it will thereafter come to 'fear' the tone CS, and display a species-typical threat response—freezing. Both CeA and BLA are involved in forming and using these emotional Pavlovian memories, by virtue of their sensory inputs from thalamic nuclei and projections to hypothalamic, midbrain, and brainstem regions important for arousal, autonomic, and behavioral aspects of the fear response (Maren, 2001; LeDoux, 2003; Fanselow and Poulos, 2005).

Amygdala is likely to play a parallel role in learning about and motivation for hedonic rewards (Cardinal et al., 2002a; Gabriel et al., 2003; Balleine and Killcross, 2006), although fewer details are known about the circuitry and mechanisms of this function of amygdala. One way to think of amygdala's role in general is as an affective watchdog—constantly monitoring incoming sensory stimuli for emotionally relevant information that can be learned about or reacted to based on prior learning, and alerting other brain 'wanting' systems accordingly.

Two Sides of the Affective Coin: Fear vs. 'Wanting'

We have much to learn about the way the brain processes motivation and emotion of opposite valences, but considerable evidence supports a great deal of overlap in the neural substrates of reward and fear. For example, nucleus accumbens is well known to be crucial for reward, but it is also important for producing aversive motivation (Reynolds and Berridge, 2001, 2002, 2003). Intriguingly, the very same neurons in accumbens may modulate appetitive or aversive motivation, depending on contextual information such as the pleasantness of the environment in which the animal finds itself (Reynolds and Berridge, 2008).

Although most researchers acknowledge a role for amygdala in reward and reward learning, most emphasize its role in fear and negative emotion. In fact, in Chapter 2 we report that CeA manipulations can cause both appetitive and defensive behaviors. Is the amygdala structure in fact specialized to detect threats, or does it instead play a more general role in affective motivation and learning? One issue relevant to this question is that there are different stakes involved in identifying potential threats and potential rewards. Lack of immediate coordinated reaction to a threat such as a predator could result in immediate death, while failing to identify a potential reward could result merely in a lost meal or sexual liaison. While both these situations are bad, it is clear that in a long-lived species, failure to eat or mate is less so, as tomorrow will often bring additional opportunities for attaining these rewards. Failure to identify a predator, on the other hand, could be deadly to both the animal and its genes. Therefore, amygdala and related brain systems are likely to be 'tuned' to detect potential threats and their predictors when there is even a slight possibility they may occur (LeDoux, 2002), while maintaining a higher standard for detecting reward-related stimuli.

This difference in the stakes involved in fear and reward learning is relevant to the paradigms neuroscientists use to examine the functions of amygdala. For example, tone-shock pairing can be learned by rats in as little as one trial, while association of the same tone with food takes many, many trials to learn. Therefore, even if amygdala plays exactly the same role in reward and fear learning, its 'tuning' for identifying threat-associated stimuli might sometimes give the appearance that it is especially involved in negative emotion.

One hypothesis stemming from this issue is that if the evolutionary stakes were different for appetitive vs. aversive learning and motivation, then the function of amygdala in these processes would differ accordingly. It is common knowledge that animals learn food-reinforced tasks faster when they are hungry (as were the animals in the present studies). Does the amygdala respond differently to food rewards and cues in a hunger state than in a sated one? This hypothesis could also be tested with comparative psychology techniques. For example, do species with very short lives or infrequent breeding opportunities have amygdale (or homologous structures) that are 'tuned' to detect these rewards more rapidly than rats or humans?

Future Directions

As is typical in neuroscience research, the present findings raise nearly as many questions as they provide answers. Broadly, these experiments provide preliminary evidence that central amygdala μ opioid stimulation recruits brain incentive salience circuits, allowing certain stimuli to be more likely to be noticed, and organizing other psychological and behavioral outputs in a manner leading animals to be more likely to approach and interact with them. CeA opioids seem to selectively potentiate the incentive salience of certain stimuli and not others. Presumably, other brain systems incorporate this information about the 'direction' of incentive salience attribution into producing relevant behaviors aimed at attaining the predicted reward or its cue. The nature and substrates of these 'downstream' incentive motivational systems is presently unclear. One hypothesis is that mesolimbic dopamine systems, which receive input from CeA and are modulated by it, may be one such brain system. If so, the functional interactions between amygdala and mesolimbic dopamine targets are worthy of

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examination using a variety of methodologies from selective lesions to immunochemical and electrophysiological measures of neuronal activation. For example, CeA could be lesioned or stimulated, and immunoreactive or electrophysiological consequences of this could be studied in structures like nucleus accumbens (preferably in conjunction with simultaneous measurements of behavior).

Another major question left unanswered by these experiments regards the interaction of CeA and BLA processing of cue information and reward seeking behavior. While it is clear that BLA processes information about reward cues, directs specific types of motivated behavior, and contains high levels of µ opioid receptors, the present data do not support a role for BLA in the measures of incentive salience 'wanting' studied here. Clearly, future research testing BLA opioid functions using behavioral paradigms known to be sensitive to BLA manipulations (including second order conditioning, and tasks in which animals are required to couple cues with sensory features of predicted rewards to produce appropriate behavior) are likely starting points. In addition, CeA and BLA are highly interconnected and influence each others' functioning. The nature of this interaction in controlling behavior, and the role of opioids in this action, is another promising area for future investigation.

Another question presented by these findings regards the function of nucleus accumbens cannabinoids in incentive salience 'wanting.' Given that anandamide stimulation of accumbens enhanced voluntary food intake (Chapter 5), such a role seems likely. This said, the degree to which accumbens cannabinoids specifically modulate responses to Pavlovian cues predictive of reward (for example in autoshaping or PIT paradigms) is unclear. In addition, the anatomical localization within accumbens of cannabinoid 'wanting' modulation is also unknown. Do cannabinoid agonists enhance food intake or more specific measures of incentive salience 'wanting' in large areas of accumbens shell and core, as do opioid agonists? Additionally, are cannabinoid and opioid enhancement of 'wanting' and 'liking' in similar areas of accumbens indicative of a molecular or synaptic interdependence of the neuromodulators? For example, would cannabinoid antagonists block opioid agonist behavioral effects, or vice versa?

It is our hope that ongoing studies in the Berridge Lab and elsewhere will answer some of these questions. These answers will further advance our understanding of how

the amygdala is involved in natural reward motivation, and how this motivation can go awry in addiction.

Conclusions

The present studies provide the first demonstrations of a specific role for central amygdala μ opioid receptors in targeting and focusing cue-triggered incentive salience upon food rewards and the Pavlovian stimuli that predict them, without affecting hedonic evaluation of these rewards. In contrast, we also show that cannabinoid receptors in a dorsomedial nucleus accumbens shell 'hedonic hotspot' play a very different role in reward, enhancing 'liking' of a sweet reward. Therefore, these studies provide a demonstration of a novel function for CeA opioids in targeting incentive salience, and provide a potential brain substrate for the excessive 'wanting' and craving for certain rewards and not others in addiction and other appetitive disorders.

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