Cortical and Subcortical Mechanisms of Pleasure and Motivation

by

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DEDICATION

This dissertation is dedicated to my best friend Tselil Schramm.

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ABSTRACT

So far, investigations into the neural basis of affect have revealed discrete, anatomically localized sites called hedonic "hotspots" in nucleus accumbens (NAc) and ventral pallidum (VP) that are able to modulate the hedonic impact of a reward. Here, I further examined the localization and neurochemical specificity of the hotspots, as well as explored new potential sites of interest to more clearly establish a hedonic circuit. In Chapter 2, I focused on the NAc hotspot, testing the effects of mu, delta, or kappa opioid receptor stimulation on hedonic and motivated behaviors. I found that while all three opioid receptor subtypes could reliably enhance hedonic 'liking' in the hotspot, they all had unique effects on motivated food intake. In Chapter 3, I extended the neurochemical investigation to include orexin and acetylcholine systems within NAc. I found that like the opioids, orexin stimulations only enhanced hedonic impact in the rostral hotspot, but enhanced food intake throughout shell. By contrast, blockade of acetylcholine muscarinic receptors caused a broad shift toward 'disgust', reducing appetitive behaviors and enhancing aversive ones. In Chapter 4, I sought to determine whether any cortical sites were capable of modulating hedonic impact or motivation, especially orbitofrontal cortex (OFC) and insula. I found that both of these areas contained localized hedonic hotspots that were sensitive to mu or orexin stimulations. The OFC hotspot was localized to the rostral 2/3 of OFC, and the insula hotspot was localized to the far caudal 1/3. I also found a single large hedonic coldspot that started in caudolateral OFC and extended into anterior and mid insula. Lastly, in Chapter 4, I found that selectively stimulation of lateral hypothalamic inputs into the VP hotspot could

enhance hedonic ‘liking’ reactions to sucrose, as well as intake of palatable M&Ms. By contrast, local VP stimulation only increased hedonic ‘liking’ reactions, and local LH stimulation only increased food intake. Collectively, these experiments expand the neurochemical repertoire of the hotspots, as well as expand the hedonic circuit to include cortical and hypothalamic mechanisms for generating affect, carrying important implications for the treatment of affective disorders like depression or bipolar.

CHAPTER 1

GENERAL INTRODUCTION

Affect, or the experience of positive and negative valence, contributes to and guides motivated and learned behaviors. Though often working in tandem with motivation and learning, affect remains a unique and independent process that is not reducible to either process (T. E. Robinson & Berridge, 1993, 2008). Affect is also not an embedded feature of a sensory stimulus, but must be generated and subsequently integrated into the sensory properties of a stimulus. Evidence for affect's independence stems from studies that demonstrate its resilience after the loss or absence of motivation or learning (K. C. Berridge, Venier, & Robinson, 1989; Galaverna et al., 1993; Kaczmarek & Kiefer, 2000; M. J. Robinson & Berridge, 2013), as well as affect's ability to flexibly transform for a stimulus in response to learning or motivation (e.g., shifting a stimulus's valence from positive to negative for the same sensory stimulus) (Parker, Kwiatkowska, Burton, & Mechoulam, 2004; Spector, Breslin, & Grill, 1988).

If affect is a unique psychological component of reward, then it follows that unique neural circuits likely mediate its production. Below, I will assert that the affective taste reactivity test effectively measures the affective component of tastes (good versus bad), independent of the taste quality itself (e.g., sweet, bitter) and independent of the neural mechanisms that generate the motivation to consume palatable stimuli. This behavioral measure of affect can be combined with various brain manipulations, including electrical stimulation, drug microinjection, or

optogenetic/pharmacosynthetic simulations to identify and localize the neural mechanisms mediating affect. Broadly, I am interested in answering the question: how does any stimulus, whether it be an internal emotional state or external cue/reward/punisher, become valenced at all? Toward that end, and for the purposes of this dissertation, I will seek to more specifically answer the following questions: 1) where in the brain does the generation of a valenced signal occur? And 2) what neurochemical/neuroanatomical systems are involved in generating that valenced signal?

Identifying Neural Substrates of 'Wanting' and 'Liking'

To begin evaluating the neural mechanisms of affect, we can turn to several influential studies from the 1950's and 60's. These studies largely focused on food intake because it is a measureable and objective behavioral phenotype. Food intake provides a means to test a number of neuropsychological questions, including how different brain areas may be involved in palatability (affective value of food) or motivation (instant transformations or amplifications of food intake behaviors). During this era of experimentation, electrolytic lesions or electrical stimulation techniques were used to address two fundamental questions: 1) what brain areas are necessary for normal motivated food intake, such that damage to that area would cause a reduction in eating behavior and food reward, and 2) what brain areas, when stimulated, are sufficient to cause increases in food intake? These, and many of the studies detailed below, will focus on experiments using food/taste rewards. As will be discussed in "Taste reactivity as a measure of hedonic impact" below, food stimuli provide a useful mechanism for teasing apart the neural mechanisms of motivation and affect.

In 1951, Anand and Brobeck first showed that lesions of the lateral hypothalamic area (LH) would cause intense aphagia and adipsia; animals would completely fail to eat or drink, and

would starve to death if not fed intragastrically (Anand & Brobeck, 1951). These findings were extended in the following decade by Teitelbaum and Epstein, and by many other researchers (Boyle & Keesey, 1975; Oltmans & Harvey, 1976; Schallert, Whishaw, & Flannigan, 1977; Teitelbaum & Epstein, 1962). Meanwhile, studies using electrical stimulation showed that activation of LH caused increases in food or water intake (Coons & Cruce, 1968; Delgado & Anand, 1953; Mogenson & Stevenson, 1967). These findings contributed to LH becoming known as a "feeding center" (Anand & Brobeck, 1951; Stellar, 1954).

In 1962, Teitelbaum and Epstein provided the first evidence that LH was also involved in applying the affective feature of palatable food (Teitelbaum & Epstein, 1962). Specifically, they found that lesions of LH caused rats to react with intense aversive reactions, such as disgust-type gapes, to palatable, sweetened milk. These results also occurred in tandem with consistently observed aphagia. In their own words:

[The rat] actively resists having milk placed in its mouth by a medicine dropper, and it does not swallow the milk once it is there, but rather allows it to dribble out the side of the mouth. Ordinarily a normal rat does not show such behavior…This suggests that mouth contact with food and water is highly aversive to a rat with lateral lesions during this stage (Teitelbaum and Epstein, 1962, pp. 75-76).

Their finding of aversive gapes after LH lesions, in addition to aphagia, extended the role of LH to now include palatability, or the affective component of food reward.

Partially based on these findings, some investigators proposed that LH electrode stimulation might enhance a hedonic taste signal to effectively make food taste better via direct LH projections to brainstem gustatory nuclei (Hoebel, 1988). Hedonic enhancement was suggested as a psychological mechanism for producing increases in food intake, and might also

contribute to electrode self-stimulation effects. For example, Hoebel suggested that "…lateral hypothalamic stimulation is like palatable food in inducing both appetite and reward" (Hoebel, 1988, pp. 583-584). Regarding rewarding effects of LH stimulation, he further speculated: "Where is reinforcement? …One component of reinforcement could be in LH cells that enhance sweet taste input in the NST (nucleus of the solitary tract) … What is reinforcement? To this question we can now answer that one aspect of reinforcement could be taste-induced stimulation of LH cells which enhance the taste" (Hoebel, 1988, p. 594).

In other words, LH stimulation was posited to enhance sweet taste signals as part of the mechanism of LH stimulation-induced reward.

Taste Reactivity as a Measure of Hedonic Impact

To ascertain whether or not LH stimulation did in fact enhance a taste signal, a more direct measure of taste hedonic impact was needed, such as the affective taste reactivity test. Below I will outline the development of the taste reactivity test, which measures the affective orofacial reactions that are elicited by different taste stimuli in animals and human infants. I will go on to explain how this test reliably and specifically tracks the hedonic component of a food reward (rather than the motivational incentive value), and contrast this behavioral measure to other popular (albeit less definitive) measures of hedonic impact or hedonic 'liking'.

The taste reactivity test and its component affective facial expressions were first described for human infants in the early 1970's by Jacob Steiner (Steiner, 1973). The test was soon adapted for rats using an intra-oral cannula administration of taste solutions by Harvey Grill and Ralph Norgren (Grill & Norgren, 1978a), and has been shown to be applicable to a number of mammalian species, including chimpanzees, orangutans, horses, mice, rats, and humans (K. C. Berridge, 2000; Jankunis & Whishaw, 2013; Kiefer, Hill, & Kaczmarek, 1998; Steiner, Glaser,

Hawilo, & Berridge, 2001). Affective orofacial reactions include three classes of responses: 1) positive hedonic or 'liking' reactions (e.g. tongue protrusions, paw licking) typically elicited by sucrose; 2) negative aversive or 'disgust' reactions (e.g. gapes, chin rubs, face washing) typically elicited by bitter quinine; and 3) neutral reactions (e.g. passive drip, mouth movements) that are elicited by water and many other tastes that are relatively neutral, as well as by palatable (mouth movements) or unpalatable tastes (dripping) (Steiner et al., 2001).

Importantly, these orofacial reactions are not merely sensory reflexes to a sweet versus bitter sensation, but reflect the hedonic impact of that taste sensation. One line of evidence for this comes from the observation that quite different taste sensations can elicit the same pattern of hedonic reactions, suggesting they have similar palatability (Steiner et al., 2001). For example, sucrose and dilute sodium chloride elicit similar positive patterns of 'liking' reactions, despite being dissimilar sensations, and both are preferred over water by rats in intake tests (Flynn & Grill, 1988; Grill & Norgren, 1978a). Conversely, bitter tasting quinine or highly concentrated sodium chloride both elicit similar aversive 'disgust' reaction patterns, and both are avoided compared to water (K. C. Berridge, Flynn, Schulkin, & Grill, 1984; Seeley, Galaverna, Schulkin, Epstein, & Grill, 1993). Another line of evidence supporting a hedonic rather than a sensorimotor interpretation comes from observations that a single taste sensation can evoke quite different orofacial reactions under different conditions relevant to its palatability. For example, orofacial 'liking' reactions to sucrose are increased by physiological hunger and suppressed by satiety states (termed alliesthesia), and orofacial reactions to concentrated NaCl (or to associated gustatory labels of sourness or bitterness paired as conditioned stimuli with NaCl as UCS) are changed from negative 'disgust' to positive 'liking' by hormonal induction of salt appetite (K. C. Berridge & Schulkin, 1989; Clark & Bernstein, 2006; Seeley et al., 1993). Learned shifts in

palatability will similarly change reaction patterns to a particular taste that has been used as a conditioned stimulus, based on Pavlovian associations formed between tastes, or associations between a taste and its ensuing physiological consequence (e.g., visceral illness; caloric satiety; salt appetite). A dramatic example is Pavlovian conditioned taste aversion, in which sucrose or a similar sweet taste is associatively paired with nausea induced by a LiCl injection (Garcia, McGowan, Ervin, & Koelling, 1968). The learned aversion changes orofacial reactivity to the paired sweet taste from positive 'liking' to negative 'disgust', guided by forebrain circuitry (K. Berridge, Grill, & Norgren, 1981; Grill & Norgren, 1978b; Schafe, Seeley, & Bernstein, 1995; Spector et al., 1988). In short, affective orofacial reactions reflect the hedonic impact of a taste stimulus, and so are determined not only by the taste itself, but also by relevant physiological states and Pavlovian associations that influence its palatability. Finally, these affective reactions can also be dramatically changed by specific brain manipulations (e.g., lesions or stimulations), which help to reveal brain mechanisms of hedonic impact, as discussed below.

Importantly, I note that affective taste reactivity patterns still faithfully track hedonic impact, even in situations when other behavioral measures of palatability, such as voluntary food intake, or consummatory behavioral measures such as lick-pattern microstructure (i.e., lickometers), diverge from hedonic impact (K. C. Berridge, 2000). This divergence may occur because all appetitive tests and most other consummatory tests of palatability require the animal to engage in spontaneous appetitive approach toward the food stimulus and voluntarily ingest it. Relying upon voluntary intake or appetitive behavior and choice makes the test depend additionally on brain mechanisms of incentive motivation, in addition to hedonic impact. This is problematic, as incentive motivation and hedonic impact can sometimes change independently. By comparison, in the taste reactivity measure, a taste is delivered directly into the mouth to

elicit orofacial reactions without any need for appetitive behavior or even voluntary ingestion. Several examples of divergence of lickometer/intake/choice measures versus taste reactivity come from manipulations of mesolimbic dopamine systems, which change incentive motivation and consequently free intake and lickometer measures, but may not truly change hedonic impact as reflected in affective orofacial reactions to tastes (G. P. Smith, 1995). Such dopamine manipulations include neurotoxin 6-OHDA lesions of mesolimbic dopamine systems and pharmacological blockade of dopamine receptors. These manipulations suppress food intake, or even cause complete aphagia in the case of 6-OHDA lesions (similar to electrolytic LH lesions), and reduce lickometer measures of sucrose motivation/palatability (e.g., shorter lick bursts) (K. C. Berridge et al., 1989; Higgs & Cooper, 2000; Oltmans & Harvey, 1976; E. T. Rolls et al., 1974; Schneider, Davis, Watson, & Smith, 1990; G. P. Smith, 1995; Zis & Fibiger, 1975). However, the same dopamine-suppressing manipulations do not alter hedonic impact, as reflected in taste 'liking' patterns of taste reactivity (K. C. Berridge et al., 1989; Pecina, Berridge, & Parker, 1997; Pecina, Cagniard, Berridge, Aldridge, & Zhuang, 2003; Wyvell & Berridge, 2000).

Other brain manipulations can further dissociate hedonic impact from other consummatory behaviors that do not depend on voluntary ingestion of an external food, such as intra-oral intake, or the passive swallowing of a taste substance already in the mouth. For example, lesions of central nucleus of the amygdala suppress the expression of salt appetite in consummatory intra-oral intake as well as in appetitive approach and voluntary intake tests, but do not suppress the increase in 'liking' for intense NaCl taste that is reflected in affective taste reactivity patterns (Galaverna et al., 1993; Seeley et al., 1993). Under normal conditions, rats respond negatively with 'disgust' reactions (e.g., gapes) to a hypertonic 3% NaCl solution and

avoid consumption. However, in a salt depleted state, rats approach, consume, and even show 'liking' reactions to the same salty taste (K. C. Berridge et al., 1984; Krieckhaus & Wolf, 1968; Schulkin, Arnell, & Stellar, 1985). After central amygdala lesions, rats no longer seek or ingest the salty solution, and no longer increase their passive intra-oral intake when the salty solution is infused into the mouth (Galaverna et al., 1993; Seeley et al., 1993). Yet, when rats with central amygdala lesions are tested in taste reactivity, they still show an affective change in orofacial reactions from 'disgust' to 'liking' patterns, even as the solution drips out of their mouths. This dissociation indicates that the hedonic alliesthesia shift remains intact, but no longer controls any appetitive or consummatory aspect of ingestion (Galaverna et al., 1993; Seeley et al., 1993). Altogether, these studies indicate that taste reactivity specifically reflects hedonic impact, and under some conditions, captures hedonic shifts even more reliably than appetitive or other consummatory measures.

Regarding the neural substrates of taste reactivity behaviors, the basic orofacial reactions can be generated by brainstem circuitry alone (Grill & Norgren, 1978b). However, it is important to note that many hedonic modulations by psychological or physiological factors require forebrain control, suggesting that these orofacial reactions are more than simple reflexes (Grill, 2006; Grill & Kaplan, 2001; Grill & Norgren, 1978a; Kaplan, Roitman, & Grill, 2000). For example, decerebrate rats that have received transections at the level of the midbrain are still able to generate orofacial movements to sweet or bitter tastes, but no longer show sensitivity to hunger alliesthesia and are unable to learn a conditioned taste aversion (Grill & Norgren, 1978b; Kaplan et al., 2000; Schafe et al., 1995). Finally, and most important, discrete forebrain manipulations sites in nucleus accumbens, ventral pallidum, and neocortex (e.g., drug microinjections, excitotoxic lesions or other manipulations) can powerfully enhance positive

'liking' reactions to sweetness, whereas other manipulations at some of the same forebrain sites abolish 'liking' reactions (described further below). Thus, although the basic circuitry needed to generate oromotor facial patterns is contained within the brainstem, the forebrain imposes hierarchical control over affective aspects of these reaction patterns.

Lateral Hypothalamus and Hedonic Impact?

Returning to the question of whether or not LH stimulation did in fact enhance the hedonic impact of tastes as a neuropsychological mechanism to increase eating posed above, Berridge and Valenstein (1991) electrically stimulated LH and measured its effect on affective orofacial responses elicited by sucrose and quinine using the taste reactivity test, as well as measuring stimulation-evoked eating behavior. Using this measure of taste hedonic impact, Berridge and Valenstein (1991) found that electrical stimulation of LH completely failed to enhance positive hedonic reactions to sucrose, despite making the rats eat over four times as much food. If anything, 'disgust' reactions to sweetness were increased during LH stimulation, providing evidence against the hedonic hypothesis for LH stimulated eating.

However, if LH is not able to generate a hedonic signal, why did LH lesions cause rats to respond aversively to the taste of milk, as described by Teitelbaum and Epstein? The answer actually appears to be that LH lesions *per se* do not produce aversion. Rather, damage to a nearby structure, the ventral pallidum, is primarily responsible for the positive to negative shift in affective reactions. Careful mapping of lesion-induced effects on eating behavior is crucial for understanding the underlying neural circuitry (Khan, 2013). Several subsequent mapping studies following Teitelbaum and Epstein's findings indicated that LH damage was not the cause of the 'disgust' reactions. First, it is important to note the electrolytic lesions produced by Teitelbaum and Epstein (1962) were large by contemporary standards, damaging structures outside the LH,

as well as the LH itself. Those lesions extended as far rostral as to include ventral pallidum, and as far caudal as to include premammillary nucleus, thereby damaging ventral pallidum, lateral preoptic area, subthalamic nucleus, and portions of the sublenticular extended amygdala. Morgane (1961) independently showed that large electrolytic lesions of ventral globus pallidus produced aphagia comparable to LH lesions, and while his study did not report whether aversive reactions to taste were induced by the globus pallidus lesions, it can be noted that the globus pallidus in brain atlases at the time extended ventrally into what is now recognized as ventral pallidum. Further localization of function came from an anatomically-detailed study by Schallert and Whishaw (1978) which separated LH into anterior and posterior regions, and demonstrated that 'disgust' induction was not simply due to damage of LH as a whole. Schallert and Whishaw showed that only in the anterior half of LH were electrolytic lesions capable of causing the 'disgust' reactions to sucrose, as well as aphagia. By contrast, posterior LH lesions only induced passive aphagia without inducing any active aversion or disgust to palatable foods. In our view, inspection of the histology figures of Schallert and Whishaw suggests that their anterior LH lesions also extended rostrally into the ventral pallidum (VP) and the lateral preoptic area (LPO) (pg. 736, Figure 7).

To more thoroughly localize the site of 'disgust' release, Cromwell and Berridge (1993) made smaller excitotoxic lesions, comparing lesion sites in VP, rostral LH, LPO, and nearby regions of striatum, globus pallidus, extended amygdala, or substantia innominata. Cromwell mapped consequent changes in motivated food intake and hedonic taste reactivity on to the sites of damage, and found only lesions that damaged VP produced the change in affective responses from 'liking' to 'disgust'. That is, even anterior LH/LPO lesions did not produce greater numbers of 'disgust' reactions to the taste of sucrose if VP was spared, even though aphagia was produced

by all lesion sites in LH, LPO and VP. Later studies found that transient inhibition of VP neurons via hyperpolarizing muscimol microinjections was enough to cause aversive 'disgust' reactions to sucrose, even without destroying neurons (Ho & Berridge, 2014; Shimura, Imaoka, & Yamamoto, 2006).

Recently, to more accurately map the site in VP responsible for production of disgust, Chao-Yi Ho compared the effects of small excitotoxic lesions to localized pharmacological inactivation in LH, VP, and extended amygdala (Ho & Berridge, 2014). Ho and Berridge found that the posterior half of ventral pallidum was the primary site for inducing 'disgust', both by temporary muscimol inactivations and by excitotoxic lesions. Posterior VP sites of lesions/inactivations were more effective at inducing 'disgust' reactions than anterior VP sites, or than any sites outside VP, such as in LH, LPO or extended amygdala. Ho and Berridge's findings suggest that the posterior subregion of VP is especially important for normal hedonic impact, because posterior VP is the only site in the brain known so far in which lesions or inactivations replace positive hedonic reactions to sweetness with aversive 'disgust' reactions.

Ventral Pallidum Hedonic Hotspot

Given the apparent importance of VP for generating normal hedonic impact, a related question is: does any region of VP induce increases or boost the hedonic impact of a taste? In a first demonstration that VP contains a "hedonic hotspot", namely, a site capable of increasing the number of positive 'liking' reactions elicited by sweetness, Smith and Berridge (2005) performed a microinjection mapping study of VP using the mu opioid receptor agonist DAMGO. Smith found that mu opioid receptor stimulation specifically at sites in the caudal half of VP caused robust increases in the number of hedonic 'liking' reactions elicited by sucrose taste, as well as increases in food intake. This site was roughly $0.8mm^3$ in volume, and localized to the

posterior half of VP (and the same site as described above where neuronal damage/inhibition causes 'disgust'). By contrast, the same opioid stimulation in the rostral half of VP suppressed 'liking' reactions to sucrose (i.e., a hedonic coldspot), and suppressed food intake. Lastly, and in contrast to the hedonic effects of mu opioid receptor stimulation, microinjections of the GABA antagonist bicuculline stimulated food intake throughout the entire VP, but never altered taste reactivity orofacial patterns at any VP site (K. S. Smith & Berridge, 2005).

Ventral Pallidum Orexin Hotspot

Ho and Berridge recently confirmed that the posterior VP contained an opioid-mediated hedonic hotspot, and additionally found that orexin stimulation in the same caudal VP site, via microinjections of orexin-A, similarly increased hedonic 'liking' reactions to sucrose taste (Ho & Berridge, 2013). Orexin (also known as hypocretin) is a peptide implicated in hunger and arousal that is only produced in hypothalamus (Baldo, Daniel, Berridge, & Kelley, 2003; Peyron et al., 1998; Swanson, Sanchez-Watts, & Watts, 2005). A hypothalamic subregion localized to the perifornical and mid-tuberal region of lateral hypothalamus contains orexin neurons that appear especially important for food and drug reward (Aston-Jones et al., 2010; Harris, Wimmer, & Aston-Jones, 2005). Orexin neurons also extend medially to dorsomedial hypothalamus and other hypothalamic subregions, where orexin influences arousal, sleep/wake cycles and attention (Adamantidis, Carter, & de Lecea, 2010; Espana, Baldo, Kelley, & Berridge, 2001). LH orexin neurons project to numerous sites throughout the brain, reaching targets as far caudal as the brainstem parabrachial nucleus of the pons, and as far rostral as orbitofrontal cortex (Baldo et al., 2003; Peyron et al., 1998). Importantly, orexin neurons in LH also appear to project to VP, and immunoreactivity for orexin receptors has been found in caudal VP (Marcus et al., 2001), suggesting connectivity and receptor mechanisms for functional hedonic effects of orexin in VP.

Ho and Berridge compared the effects of orexin microinjections at sites distributed in VP and in anterior LH, and found that orexin only increased hedonic 'liking' reactions within caudal VP (Ho & Berridge, 2013). In that posterior VP hotspot, orexin stimulation selectively increased positive 'liking' reactions to sucrose, without altering negative 'disgust' reactions elicited by quinine. Orexin microinjections in nearby lateral hypothalamus or extended amygdala failed to alter orofacial responses to either sucrose or quinine. These results collectively show that the hotspot of posterior VP can use either orexin or opioid signals to similarly increase the positive hedonic impact of a sweet taste.

Anatomical Basis for the VP hotspot

The larger anatomical zone in which VP is located has been traditionally called the substantia innominata (SI), or unnamed substance. This was due to its lack of distinguishing features (as far as was then known), and the confusing nature of what constituted its borders. The term substantia innominata, however, was later criticized as too vague (Heimer, Harlan, Alheid, Garcia, & de Olmos, 1997). The VP boundaries reveal themselves when tissue is stained for enkephalin or substance P; VP produces more enkephalin and substance P than other nearby SI regions, and has distinct afferent and efferent patterns from that of the dorsally positioned globus pallidus (Groenewegen & Russchen, 1984; Haber & Nauta, 1983), marking it as a relatively distinct structure within SI.

In addition to being anatomically distinct zone, caudal VP has several unique characteristics that differ from other VP subregions that may contribute to its hedonic function. For example, Kupchik and Kalivas (2013) showed that the electrophysiological signature of the neurons in VP change, depending on where they recorded along a rostrocaudal axis. Neurons in anterior VP included a mix of "Type I" and "Type II" neurons, whereas posterior VP was

characterized solely by Type I neurons. Type I neurons are tonically active and easily excited, while Type II neurons have low basal firing rates, and require more stimulation to elicit an action potential. In addition to this, Type II neurons morphologically resemble the accumbens medium spiny neurons, whereas Type I neurons that predominate in posterior VP are relatively aspiny and are somewhat larger than Type II. Although it is still unclear how Type I and II neurons differ functionally, it is interesting to note that the change in neuron type follows the rostrocaudal functional difference between caudal VP hotspot and rostral VP coldspot sites.

Nucleus Accumbens Hedonic Hotspot

Similar to the VP, the NAc also contains an opioid-mediated hedonic hotspot where opioid stimulation can increase 'liking' reactions to sucrose taste. The NAc hotspot is approximately one cubic millimeter in volume, and is located in the rostrodorsal quadrant of medial shell (Castro & Berridge, 2014b; Pecina & Berridge, 2005). This NAc opioid hotspot was discovered using a similar microinjection and functional mapping technique that was used for the VP hotspot. Microinjections of the mu agonist DAMGO were compared at many sites throughout the medial shell of NAc. Results showed that only stimulation within the rostrodorsal quadrant of NAc medial shell caused a three-fold increase of 'liking' reactions to sucrose (Pecina & Berridge, 2005). By contrast, mu receptor stimulation in a similarly-sized hedonic coldspot located in the caudal half of NAc medial shell actually reduced hedonic reactions to sucrose. At all other locations in medial shell, DAMGO microinjections induced no hedonic change in taste reactivity to sucrose. However, DAMGO microinjections at all sites in NAc medial shell produced increases in food intake two- to eight-times higher than baseline intake, even at caudal sites (Pecina & Berridge, 2005). This pattern demonstrated an anatomical distinction between NAc mu opioid stimulation of 'liking' (i.e., specific to the rostral hotspot) versus NAc mu

stimulation of ‘wanting’ for food (i.e., anywhere in NAc medial shell). The stimulation of ‘wanting’ also confirmed previous reports that DAMGO microinjections in NAc increased food intake at virtually all sites in the NAc, including both shell and core (Bakshi & Kelley, 1993; Zhang & Kelley, 2000). Thus, opioid neurocircuitry for enhancing motivated ‘wanting’ to consume food rewards is more widely distributed in NAc than opioid circuitry for enhancing hedonic ‘liking’ in the same structure. Indeed, opioid mechanisms for ‘wanting’ without ‘liking’ extend to several other structures, including regions of amygdala, neostriatum, and prefrontal cortex (DiFeliceantonio, Mabrouk, Kennedy, & Berridge, 2012; Mahler & Berridge, 2009; Mena, Sadeghian, & Baldo, 2011; Zhang & Kelley, 2000).

Anatomical Basis for the NAc Hotspot

What anatomical basis might help explain the functional existence of a functionally unique hotspot for opioid amplification of sensory pleasure, and why is it uniquely able to enhance hedonic impact to tastes, compared to other regions of NAc shell?

Two independent groups of neuroanatomists have evaluated the anatomical connectivity patterns of the NAc rostrodorsal quadrant of medial shell, and found that this hotspot region differs from other subregions of medial shell (e.g., caudal shell). Using a double injection of anterograde and retrograde tracers, Thompson and Swanson (2010) revealed that the rostrodorsal quadrant appears to belong to a different striato-pallido-hypothalamo-thalamo-cortical closed circuit loop from other subregions of medial shell. In other words, if one follows the projections from the rostrodorsal quadrant of medial shell along a point to point axis, one will end up back in the hotspot. This loop travels from the NAc hotspot to particular subregions of pallidum or hypothalamus, up to paraventricular nucleus of the thalamus, next passing through the infralimbic region of prefrontal cortex, and finally projecting back again to rostrodorsal medial

shell. The subregions of each of these structures are distinct from the subregions visited by other parallel loops that pass through more posterior regions of medial shell. Exactly how many parallel loops pass through medial shell of NAc remains to be elucidated, but it seems clear now that there are at least two (visiting rostral vs caudal shell) and possibly additional loops that more finely dissect NAc shell into further subregions, each belonging to its own loop (Thompson & Swanson, 2010).

Similarly, Zahm and colleagues (Zahm, Parsley, Schwartz, & Cheng, 2013) recently found a related pattern of distinct connectivity that distinguishes the rostral hotspot from more caudal subregions of NAc medial shell. Those authors suggest that the rostral hotspot projects to particular regions of lateral preoptic area and lateral hypothalamus, and receives inputs from infralimbic cortex (analogous to Brodmann's area 25) and other nearby regions of prefrontal cortex such as prelimbic and orbitofrontal cortex. They also suggest that the projection patterns of NAc rostral shell are similar to those of lateral septum, compared to the caudal shell, and that the rostral zone of medial shell is a unique transition region between NAc and lateral septum. In contrast, they suggest the caudal zone is a different transition region blending features of NAc and extended amygdala. While the Zahm et al. and the Thompson and Swanson studies differ on some points, the overall anatomical scheme presented by the two studies seems to agree that the circuitry belonging to the rostrodorsal hotspot quadrant of NAc medial shell is fundamentally different compared to the connectivity patterns of the rest of the medial shell, and these anatomical differences may in part contribute to the hotspot's unique abilities to amplify hedonic impact of taste sensations.

In addition to differences in projection patterns, there may also be other local neurobiological features of neurons in NAc medial shell that are relevant to hedonic

contributions compared to other NAc components such as core. Meredith et al. (2008) suggest that the local characteristics of neurons in NAc medial shell are different from other regions of NAc and striatum. For example, the projecting medium spiny neurons (MSNs) within medial shell are less spiny and smaller compared to NAc core or dorsal striatum. Furthermore, the distinction between different MSNs belonging to a D1/dynorphin/direct pathway versus a D2/enkephalin/indirect pathway (a hallmark of dorsal striatum), is somewhat diluted in NAc medial shell, where at least 17% of MSNs harbor both D1 and D2 receptors (Bertran-Gonzalez et al., 2008; Humphries & Prescott, 2010). Intriguingly, volume ratios of patch/matrix compartments in dorsal striatum (as delineated by mu opioid or calbindin binding) may also be flipped, or at the very least are not as cleanly split, in nucleus accumbens (Jongen-Relo, Groenewegen, & Voorn, 1993; Meredith, Pattiselanno, Groenewegen, & Haber, 1996). Although the roles of these neurobiological features is still unclear, some of these unique anatomical or cellular features of NAc medial shell might be relevant to its ability to generate hedonic functions that are fundamentally different from other regions of striatum.

A Functional Circuit for Hedonic Processing

The existence of multiple hedonic hotspots allows for the possibility that the hotspots interact and work together within a coordinated hedonic circuit. A functional circuit would not necessarily imply that the hotspots are all directly connected anatomically, since intermediary stops could be equally effective in creating a functional circuit. To determine whether at least a functional interaction existed, Smith and Berridge unbalanced the circuit by infusing DAMGO into one hotspot (e.g. NAc), while simultaneously infusing naloxone, an opioid antagonist, into another hotspot (e.g. VP) (K. S. Smith & Berridge, 2007). The guiding hypothesis was that if the simultaneous opioid neurotransmission is required in both hotspots, essentially creating

unanimous opioid votes for enhancement of 'liking' in both sites, then blocking endogenous opioid signals in one hotspot should prevent exogenous opioid stimulation by DAMGO microinjection in the other from causing any hedonic enhancement. The results supported this hypothesis: opioid blockade in either the VP or NAc hotspot prevented DAMGO enhancement of positive 'liking' reactions in the other hotspot. Further supporting the functional relationship between the NAc and VP hotspots, it was also found that DAMGO activation in one hotspot enhanced Fos activity both locally and in the other hotspot, demonstrating their functional interactions could be detected via neural markers of genomic transcription. It should be noted that although naloxone in VP prevented DAMGO-enhanced 'liking' in the NAc hotspot, enhancements of eating by NAc DAMGO were still robustly generated, suggesting again that there are independent controls for hedonic 'liking' versus motivated 'wanting' of the same food reward.

In a further electrophysiological demonstration of NAc-VP hotspot interactions, Smith et al. (2011) recorded taste reactivity responses and extracellular neuronal firing patterns in the VP hotspot during an intraoral infusion of sucrose. They found that neurons in the VP hotspot appeared to encode impact of sucrose in neuronal firing, correlating with behavioral 'liking' reactions. This hedonic pattern manifested itself by steadily increasing the neural firing rate in a slow-onset but sustained burst of action potentials, becoming evident during the first 1.5 sec after the sweet taste was introduced, and sustaining this elevation in firing for the duration of the 10 sec sucrose infusion. DAMGO microinjection into the NAc hotspot elevated both behavioral orofacial reactions to sucrose as well as the hedonically correlated neural signal associated with the sweet taste. In behavioral contrast, amphetamine microinjections that potentiated dopamine transmission in the NAc hotspot only increased food intake and a more transient VP neural

signal burst that encoded cue-triggered ‘wanting’, and correlated with amount of food eaten, but had no effect on behavioral taste reactivity ‘liking’ patterns or on the hedonic-encoding VP neural response to sucrose. Altogether, these results show that the VP and NAc hotspots interact to form a larger functional circuit that mediates the hedonic reaction to a palatable taste.

Summary of the Present Experiments

Presented within this dissertation are a series of experiments that seek to extend our understanding of the neural mechanisms of hedonic ‘liking’ and aversive ‘disgust’. Specifically, I manipulated the known hotspots in nucleus accumbens and ventral pallidum via drug microinjection or optogenetic stimulation, and pharmacologically mapped two novel hotspots in the orbitofrontal cortex and insula. I found that opioid or orexin stimulation appear to generate overlapping anatomical zones in NAc, OFC and insula within which the hedonic impact of palatable sucrose can be amplified, independent of these neurochemical stimulations’ ability to generate intense ‘wanting’ for food. I also found that optogenetic stimulation of the VP hotspot or LH inputs to the VP hotspot could likewise enhance hedonic ‘liking’ reactions to sucrose, further implicating the LH-orexin system in reward and affect.

Chapter 2: Opioid Hedonic Hotspot in Nucleus Accumbens Shell: Mu, Delta, and Kappa Maps for Enhancement of Sweetness ‘Liking’ and ‘Wanting’.

Previous work in our lab has pinpointed the rostrodorsomedial shell of NAc as a site for mu opioid stimulated enhancement of sucrose ‘liking’. By contrast, these same microinjections increased food intake at any site in NAc, even at caudal sites that concurrently appeared to decrease affective ‘liking’ to sucrose. Here, I replicate these initial findings, and extend my investigation to include delta and kappa opioid receptor subtypes. I found that stimulation of any of the three receptors was sufficient to enhance hedonic ‘liking’ for sucrose, so long as the sites

were within the previously mapped rostral hotspot. By contrast, stimulations in caudal NAc actually suppressed 'liking' reactions. The positive effects observed in rostral shell were corroborated by a conditioned place preference paradigm in a separate group of rats. Unlike the consistent effects observed after opioid receptor stimulation during taste reactivity, the three opioid subtypes differed on their effects on food intake. Mu stimulation increased food intake at all sites, delta stimulation only enhanced food intake in the rostral hotspot, and kappa stimulation never consistently enhanced intake at any rostrocaudal site. Altogether, these results confirm the existence of the rostral hotspot, and posit a role for delta and kappa receptors in reward function.

Chapter 3: Orexin in Rostral Hotspot of Nucleus Accumbens Enhances Sucrose 'Liking' and Intake but Scopolamine in Caudal Shell Shifts 'Liking' Toward 'Disgust' and 'Fear'.

Recently our lab found that orexin stimulation in the caudal VP hotspot could amplify 'liking' similarly to opioid enhancement. I sought to extend these findings by similarly mapping the hedonic and motivated effects of orexin stimulation in NAc medial shell. I found that orexin generated a nearly identical profile to mu opioid stimulation, in that rostral stimulations enhanced hedonic impact, and simulations throughout medial shell enhanced food intake of palatable M&Ms. In the same rats, I also examined the effects of acetylcholine muscarinic blockade. Previous work by other labs has shown that muscarinic blockade via scopolamine robustly reduces food intake (even after a concurrent injection of the mu agonist DAMGO) and generates intense taste and place avoidances, suggesting that basal acetylcholine may be important for reward function. I found that muscarinic blockade decreased hedonic sucrose 'liking', increased bitter quinine 'disgust', enhanced fearful-defensive treading, and decreased food intake. Collectively, these results indicate that both orexin and acetylcholine neurochemical systems are capable of modulating hedonic and motivated behaviors in NAc medial shell.

Chapter 4: Cortical Maps of Hedonic Impact and Motivation: Distinct Roles for Orbitofrontal cortex and Insula

So far, only subcortical mechanisms mediating hedonic impact have been explored. To determine what role cortex plays in 'liking' and motivation, I stimulated mu opioid or orexin receptors throughout OFC and insula and measured consequent changes in taste reactivity or food intake behaviors. I found that rostromedial OFC and caudal insula each contain a localized hedonic hotspot, and that a hedonic coldspot spanned the transition zone between caudolateral OFC and rostral insula, continuing unbroken until the caudal insula hotspot. The maps generated by the two drugs were nearly identical, suggesting common sites of action. By contrast, food intake was limited to rostral OFC, with the effects becoming weaker in caudolateral OFC. Insula did not consistently increase food intake at any rostrocaudal site.

Chapter 5: Ventral Pallidum Hotspot for Sweetness 'Liking' is Modulated by Lateral Hypothalamus Circuitry for 'Wanting' to Eat.

Collectively, there is now evidence for at least four hedonic hotspots, and each of these hotspots can also be activated by orexin. Because orexin is only produced within a localized zone in lateral hypothalamus, it is likely that LH orexin-containing neurons functionally project to the hotspots and directly activate or inhibit their processing online. To test this hypothesis, I injected a viral vector carrying channelrhodopsin protein into either lateral hypothalamus or the ventral pallidum hotspot, and implanted fibers in LH (LH-LH) or VP (VP-VP; LH-VP). Animals were then tested on taste reactivity and food intake while directly stimulating LH (LH-LH), the VP hotspot (VP-VP), or lateral hypothalamic projections that terminate in the VP hotspot (LH-VP). I found that direct stimulation of the VP hotspot exclusively amplified hedonic 'liking' reactions to sucrose by 300% without altering orofacial reactions to quinine, whereas direct stimulation of

LH solely amplified motivated food ‘wanting’ by increasing intake of palatable M&M candies by 150%. Interestingly, stimulation of LH terminals in the VP hotspot increased *both* hedonic reactions to sucrose and food intake. These findings suggest that although LH itself may not contain a hedonic hotspot, it is able to specially recruit and activate the VP hotspot.

CHAPTER 2

OPIOID HEDONIC HOTSPOT IN NUCLEUS ACCUMBENS SHELL: MU, DELTA, AND KAPPA MAPS FOR ENHANCEMENT OF SWEETNESS 'LIKING' AND 'WANTING'

Introduction

The medial shell of nucleus accumbens (NAc) contributes to the hedonic impact of sensory pleasures (e.g., 'liking' reactions to sweetness), as well as incentive motivation to consume foods, drugs and other rewards (e.g., 'wanting') (Grueter, Rothwell, & Malenka, 2012; Michaelides et al., 2013; Resendez et al., 2013; Zhang, Balmadrid, & Kelley, 2003). Disorders such as drug addiction, binge eating or depression may involve mesocorticolimbic dysfunction of 'liking' or 'wanting' processes (Berner, Bocarsly, Hoebel, & Avena, 2011; K. C. Berridge, Ho, Richard, & DiFeliceantonio, 2010; Ito, Robbins, & Everitt, 2004; Koob, 2000; Nathan & Bullmore, 2009).

An anatomically-localized "hedonic hotspot" has been found within the rostrodorsal quadrant of NAc medial shell: a roughly cubic-millimeter sized subregion where mu opioid stimulation via microinjection of DAMGO can double the hedonic impact of sweet tastes (Pecina & Berridge, 2005; K. S. Smith & Berridge, 2005, 2007; K. S. Smith et al., 2011). By contrast, the same mu opioid stimulation in other subregions of medial shell fails to enhance 'liking'

reactions to sucrose (Pecina and Berridge, 2005). As part of a larger functional circuit,the NAc hotspot interacts with a second anatomical hotspot in the ventral pallidum to amplify 'liking' reactions tosweetness (K. S. Smith & Berridge, 2007; K. S. Smith et al., 2011).

Separable from hedonic impact, mu opioid stimulation also increases the motivation to eat ('wanting') both in the shell hotspot and at many additional sites distributed throughout the entire NAc, and in several limbic or striatal structures beyond (with or without also enhancing 'liking') (DiFeliceantonio et al., 2012; Gosnell, Levine, & Morley, 1986; Katsuura & Taha, 2010; Mahler & Berridge, 2012; Mena et al., 2011; Pecina & Berridge, 2005, 2013; Ragnauth, Moroz, & Bodnar, 2000; K. S. Smith et al., 2011; Woolley, Lee, & Fields, 2006; Zhang & Kelley, 2000; Zheng et al., 2010).

In contrast to mu stimulation, delta or kappa stimulation in NAc has mixed or negative effects on food intake. For delta stimulation, several studies reported increases in consumption (Bakshi & Kelley, 1993; Majeed, Przewlocka, Wedzony, & Przewlocki, 1986; Ragnauth, Znamensky, Moroz, & Bodnar, 2000; Zhang & Kelley, 1997), whereas other studies reported no change (Katsuura & Taha, 2010). For kappa stimulation in NAc, most studies report no change in intake (Bakshi & Kelley, 1993; Zhang & Kelley, 1997) (although a few early systemic administration studies reported increased intake (Cooper, Jackson, & Kirkham, 1985; Jackson & Cooper, 1985)), and dynorphin/kappa stimulation at most sites in the brain is generally associated with aversive effects (Bals-Kubik, Ableitner, Herz, & Shippenberg, 1993; Knoll & Carlezon, 2010; Land et al., 2008; Wee & Koob, 2010).

Here we aimed to better identify and map mu, delta or kappa stimulation effects in NAc medial shell, and to compare potential localizations of function for sensory pleasure versus motivation to eat, as well as the inducement of a conditioned place preference.

Materials & Methods

Subjects

Male Sprague Dawley rats, weighing 300-450g at surgery (total, $n = 84$; behavioral taste reactivity and intake groups, $n = 21$; behavioral conditioned place preference test groups, $n = 39$; dedicated Fos plume radius groups, $n = 24$) were housed in pairs at ~21°C on a reverse 12 h light/dark cycle. All rats had *ad libitum* access to both food and water in their home cage. All experimental procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Surgery

Rats were anesthetized with ketamine hydrochloride (80mg/kg, i.p.) and xylazine (5mg/kg, i.p.), pretreated with atropine (0.05 mg/kg, i.p.) to prevent respiratory distress, and placed in a stereotaxic apparatus (David Kopf Instruments), with the incisor bar set at 5.0mm above intra-aural zero to avoid cannula penetration of lateral ventricles. Rats received bilateral NAc implantation of permanent microinjection guide cannulae (14mm, 23-gauge, stainless steel) individually aimed at sites staggered throughout the rostro-caudal extent of medial shell. Sites were located between +3.1 and +2.7mm anterior from bregma (AP), bilateral ±0.9mm mediolateral (ML), and between -5.7 and -6.2mm dorsoventral (DV). Site coordinates were staggered across individuals so that targets for the group as a whole filled the entire dorsal two-thirds of NAc medial shell, but for a given rat coordinates were bilaterally symmetrical and as nearly identical as possible in placement on both sides. Microinjection guide cannulae were anchored to the skull using surgical screws and dental acrylic, and stainless-steel obturators (28 gauge) were inserted to avoid occlusion except for during behavioral tests.

For rats in the taste reactivity testing group, bilateral oral cannulae were additionally

implanted in the same surgery to permit oral infusions of sucrose solutions [polyethylene (PE)-100 tubing]. Oral cannulae entered the mouth in the upper cheek pouch lateral to the first maxillary molar, ascended beneath the zygomatic arch, and exited the skin at the dorsal head cap (Grill & Norgren, 1978a). Oral cannulae did not disrupt normal eating. After surgery, each rat received subcutaneous injections of chloramphenicol sodium succinate (60mg/kg) to prevent infection and carprofen (5mg/kg) for pain relief. Rats received carprofen again 24 h later and were allowed to recover for one week before any behavioral testing occurred.

Drugs and NAc microinjections

Rats were gently cradled by hand in the experimenter's lap during NAc microinjections. PE-20 polyethylene tubing was connected to stainless-steel microinjection cannulae (16mm, 29 gauge) extending 2mm beyond the guide cannulae to reach NAc targets. On test days, drug or vehicle solutions were brought to room temperature (~21ºC), and inspected to confirm the absence of precipitation prior to microinjection. Drugs were prepared at the beginning of each test series, and then either frozen (ACSF, DAMGO, DPDPE) or kept refrigerated (U50488H) across consecutive test days. All drugs were dissolved in a vehicle of artificial cerebrospinal fluid (ACSF) and microinjected over a 1min period at a volume of 0.2µl per side at a speed of 0.2µl/min by syringe pump. Injectors were left in place for 1min following microinjection to allow drug diffusion, after which obturators were replaced, and rats were immediately placed in the testing chamber. Microinjection solutions contained one of the following: 1) DAMGO, a selective mu receptor agonist at a dose of 0.05µg/0.2µl per side; 2) DPDPE, a selective delta receptor agonist at a dose of 3.1µg/0.2µl per side; 3) U50488H, a selective kappa receptor agonist at a dose of 0.186µg/0.2µl per side, or 4) ACSF vehicle alone in a volume of 0.2µl per side (vehicle control condition). Order of drugs and vehicle were counterbalanced across rats.

Rats received only one drug or vehicle microinjection per day. Drug doses were chosen based on previous studies by Zhang and Kelley (1993) and by Smith and Berridge (2011).

Taste reactivity testing

The taste reactivity test (Grill & Norgren, 1978a; Steiner, 1973; Steiner et al., 2001) was used to measure affective orofacial reactions of rats to a 1 ml volume of sucrose solution infused into the mouth via oral cannula. Tests occurred during 1min infusions administered at times when peak pharmacological effects could be expected (25min for vehicle, DAMGO, U50488H, or 15min for DPDPE) after drug microinjection (Bakshi & Kelley, 1993). To infuse sucrose solution into the mouth, a syringe containing sucrose in a syringe pump (1.0%; 0.029M; 1ml per test) was attached via hollow tubing (PE-50 connected to a PE-10 delivery nozzle) to a rat's oral cannula. A 1ml volume of sucrose was infused evenly over a period of 1min duration. Orofacial taste reactivity responses were video recorded via close-up lens and an angled mirror placed underneath the transparent floor for subsequent slow-motion video analysis.

Taste reactivity video scoring

Hedonic, aversive, and neutral taste reactivity patters were scored off-line in slow motion (1/30 s frame-by-frame to 1/10th actual speed). Hedonic responses were classified as rhythmic midline tongue protrusions, lateral tongue protrusions, and paw licks (K. C. Berridge, 2000). Aversive responses were classified as gapes, head shakes, face washes, forelimb flails, and chin rubs. Neutral responses were classified as passive dripping of solution out of the mouth, ordinary grooming, and rhythmic mouth movements. A time-bin scoring procedure was used to ensure that taste reactivity components of different relative frequencies still contribute equally to final affective hedonic/aversive totals, and that frequent components such as rhythmic tongue protrusions do not swamp rare but equally informative components, such as lateral tongue

protrusions (K. C. Berridge, 2000). Specifically, rhythmic mouth movements, passive dripping, and paw licking reactions, which occur in long bouts, were scored in 5 s time bins (e.g., 5 s continuous paw licking behavior equals one bout occurrence). Rhythmic midline tongue protrusions and chin rubs, which occur in shorter bouts, were scored in 2 s time bins. Lateral tongue protrusions, gapes, forelimb flails, and head shakes, which typically occur as discrete events, were scored as single occurrences each time they occurred (e.g., one gape equals one occurrence). Individual totals were calculated for hedonic and aversive categories. A hedonic reaction total was quantified as the sum of scores for lateral tongue protrusion, rhythmic tongue protrusion, and paw lick scores. An aversive reaction total was quantified as the sum of gape, head shake, face wash, forelimb flail, and chin rub scores.

Food intake testing

Spontaneous eating behavior was video-recorded and voluntary food consumption measured in a 1hr free intake test that began 1min after the taste reactivity test on each microinjection day. Rats were habituated to the food intake testing chamber for 3 daily 1hr sessions prior to the first microinjection day. Each food intake chamber (23 x 20 x 45 cm) contained 1 cm depth of corncob bedding, and piles of two foods: palatable chocolates (M&Ms, ~20 g) and standard chow pellets (Purina, ~20 g); a water bottle was also always available. Amounts of M&Ms and chow were weighed before each test and again after the test to calculate amount eaten (chamber was inspected for spillage, and incorporated into amount remaining). All behavior in the chamber was also video recorded during the 60min test, and scored later offline for video analysis by a researcher blind to the drug microinjection condition. Videos were scored for eating behavior (duration in seconds), water drinking behavior (in seconds), grooming behavior (in seconds), and for number of bouts of food sniffs, food carrying (grasping and transport of

food by 2 or more steps), cage crosses, and rears (each counted separately).

Conditioned place preference test

Conditioned place preference/avoidance was trained and tested using a three-compartment apparatus (Bardo & Bevins, 2000; Reynolds & Berridge, 2002; Tzschentke, 1998). Two large side chambers (28 × 21 × 21 cm) surrounded a smaller central compartment (12 × 21 × 21 cm). To allow distinctive sensory cues, one side compartment had an overhead light, black-colored walls, a wire grid floor (~1 cm spacing), and scent of soap, whereas the other side compartment had no extra lighting, white walls, a wire mesh floor (~ 2mm spacing), and scent of 70% ethanol.

To first assess any pre-existing preference, rats were pre-tested by being placed in the center compartment and allowed to freely explore all three chambers for 30 m while behavior was video recorded. Preference was calculated by the ratio of cumulative time spent in each side compartment. Whichever compartment an individual rat spent more time in was designated its originally-preferred compartment. Opioid agonist microinjections were paired against each rat's original preference (biased procedure), so that DAMGO (0.05mg/0.2µl per side), U50488H (0.186mg/0.2µl per side) or DPDPE (3.1mg/0.2µl per side) microinjections were paired with a rat's unpreferred compartment, and vehicle microinjections were paired with the originally-preferred compartment (Guo, Garcia, Taylor, Zadina, & Harlan, 2008; Tzschentke, 1998; Wang et al., 2012). Each rat received only one type of opioid agonist, which was paired twice with the same chamber (and vehicle paired twice with the other chamber) in counter-balanced order. Between-group comparisons were made to compare strength of conditioned preferences across the three types of opioid agonist.

On the preference test day (6^{th} day), rats did not receive any microinjection, and instead were placed directly into the central compartment and allowed to freely explore the entire

apparatus for 30min. Animal behavior during test sessions was videotaped and subsequently scored for cumulative time (seconds) spent in each compartment location. A rat was scored as being inside a particular compartment whenever its head and both forelimbs were within the boundary.

Histology and Fos-like protein immunohistochemistry

Following behavioral testing, rats were deeply anesthetized with an over-dose of sodium pentobarbital. Rats in which Fos plumes were measured were perfused and brains treated as described previously (Reynolds & Berridge, 2008; Richard & Berridge, 2011b). Fos plumes surrounding microinjection tips were assessed in rats that had been behaviorally tested in taste reactivity and food intake (n = 10), and additionally in a separate dedicated Fos group (n = 14) that was included in order to detect the maximal spread of neuronal impact after a single drug microinjection (Richard & Berridge, 2011b). In the behavioral group, Fos was assessed after a final (sixth) drug or vehicle microinjection. A dedicated group was also used for Fos because previous results suggested that repetition of up to six serial microinjections during behavioral tests can lead to gradual plume shrinkage by the 7th microinjection, possibly due to gliosis or necrosis around the microinjection tip (Richard & Berridge, 2011b). If used alone, shrunken plumes could give rise to overly-precise estimates of localization of function in brain maps (due to underestimation of the spread of drug impact). In the dedicated group, which could be expected to have maximum drug spread, a single drug or vehicle microinjection in NAc medial shell was administered under conditions identical to the first day of testing for behavioral rats. Rats were then anesthetized and transcardially perfused 90min after their final or sole bilateral microinjection of vehicle (n = 4), DAMGO (n = 6), U50488H (n = 6), or 80 minutes after their final or sole bilateral microinjection of DPDPE (n = 6). Rats with no surgery were also included

in a “normal” group (n=2) to assess counts in naïve brains. Brain slices were processed for Fos-like immunoreactivity using normal donkey serum, goat anti-c-fos (Santa Cruz Biotechnology), and donkey anti-goat Alexa Fluor 488 (Invitrogen). Sections were mounted, air-dried, and coverslipped with Prolong Gold antifade reagent (Invitrogen). Zones where the local expression of fluorescent Fos was elevated in neurons surrounding microinjection sites (“Fos plumes”) were assessed via microscope as described previously (Reynolds & Berridge, 2008). Behaviorally-tested rats had brains removed and fixed in 10% paraformaldehyde for 1-2 d and in 25% sucrose solution (0.1M NaPB) for 3 d for assessment of microinjection site locations. Brains were sliced at 60µm on a freezing microtome, mounted, air-dried, and stained with cresyl violet for verification of microinjection sites. Bilateral microinjection sites for each rat were placed on coronal slices from a rat brain atlas (Paxinos & Watson, 2007), which were used to extrapolate the position of each site on a sagittal map of medial shell.

Mapping behavioral effects of NAc shell microinjections in the sagittal plane allows for the presentation of all sites on the same map of the entire rostrocaudal and dorsoventral extents of NAc medial shell. Functional effects on hedonic and motivated behaviors were mapped using color coding to express the intensity changes in affective and motivated behaviors for individual behaviorally tested rats. Map symbols were sized to match the maximal diameter of Fos plumes measure as described below. For statistical comparison of rostral versus caudal behavioral effects, NAc sites were classified as being in rostral half of shell if located more anterior than +1.5mm ahead of bregma (between +1.5 to +2.4), and as in caudal half of shell if located posterior to +1.5 (i.e., between +0.4 to +1.5 bregma).

Regions for statistical analysis

Anatomical regions of interest in medial shell were defined *a priori* for statistical

comparisons. For each behavioral effect, first, *rostral versus caudal halves* of medial shell were contrasted. This was followed by a finer-grained contrast of *dorsal versus ventral quadrants* within each half, in order to separately compare all 4 quadrants (e.g., rostrodorsal, rostroventral, caudodorsal, caudoventral quadrants). Nonparametric comparisons were made using Friedman's two-way ANOVA or Kruskal-Wallis ANOVA, followed by appropriate Wilcoxon or Mann-Whitney U tests to make paired comparisons.

Results

Overview

Overall, each of the three opioid agonists produced enhancements of 200% to 400% for positive orofacial reactions elicited by the hedonic impact of sucrose taste infusions when microinjections were made at NAc sites within the rostrodorsal hotspot of medial shell. But none of the opioid agonists enhanced hedonic reactions at any other medial shell sites that were outside the hotspot (e.g., anywhere in caudal shell) (Figures 2.2 & 2.4). When microinjection sites were located within the rostrodorsal quadrant of medial shell, the number of positive hedonic orofacial reactions elicited by the taste of sucrose was doubled to quadrupled by microinjections of mu, delta, or even kappa opioid agonists (DAMGO, DPDPE, or U50488H) compared to within-subject control levels measured after vehicle microinjections in the same rats (Friedman's Two-Way ANOVA, $X^2 = 12.573$, $p = 0.006$). No change was statistically detectable for sites in the ventral half of the rostral shell (i.e., rostroventral quadrant) (Friedman's Two-Way ANOVA, $X^2 = 5.57$, $p = 0.134$). In contrast, when microinjection sites were located more posteriorly in the caudal half of NAc medial shell, each of the three agonists suppressed hedonic reactions to sucrose, cutting levels to roughly one-half of vehicle control levels. The hedonic suppression sites in posterior medial shell were anatomically clustered together sufficiently to

reveal a caudal 'hedonic coldspot' for reducing positive hedonic impact (Friedman's Two-Way ANOVA, $X^2 = 10.675$, $p = 0.014$).

Conditioned place preference results confirmed that rostrodorsal hotspot sites induced positive reward effects for both mu and kappa agonists (Kruskal-Wallis Test, $X^2 = 16.599$, $p = 0.0001$), and a similar trend towards a positive rostrodorsal cluster for the delta agonist (Figure 2.3). Sites in the caudal half of medial shell tended to produce a trend towards place avoidance for kappa stimulation, and no consistent place preference effects for mu or delta stimulation.

Finally, opioid stimulations had varied effects on food intake depending on agonist and location, described below ($X^2 = 11.796$, $p = 0.008$).

Fos plume measures: anatomical spread of local drug impact

Measurements of Fos plume sizes indicated first, that repetition of several serial microinjections for behavioral induced shrinkage in the diameter of final Fos plume produced by a drug in the same rat (i.e., a rat's 6th drug microinjection) compared to a 1st microinjection diameter for that drug in naïve rats. For all three opioid agonists, the spread of impact shrunk by 15% to 75% in rats that had been previously behaviorally-tested, compared to the diameter of plumes induced by the same drug in the dedicated Fos groups, which received only a single microinjection (Figure 2.1; DAMGO mu = 33% shrinkage, $F_{(1,585)} = 8.251$, $p = 0.004$; DPDPE delta = 75% shrinkage, $F_{(1,781)} = 120.437$, $p = 0.0004$; U50488H = 15%, $F_{(1,821)} = 7.877$, $p = 0.005$). Shrinkage induced by serial repetition confirmed our supposition that a dedicated Fos group provides more accurate measures of the maximum spread of drug impact, induced by each opioid agonist at the doses and volumes used here, than measuring final-microinjection plumes in a previously-tested behavioral group. For this reason, all plume sizes described below and used for mapping symbols come from the dedicated Fos groups that were histologically assessed

after receiving a single microinjection of their drug or vehicle, and which had the maximum Fos plume diameters.

The functional spread of each opioid agonist microinjection had a radius between 0.15 – 0.2mm, and a total local volume between 0.014– 0.034mm^3, as assessed by Fos plumes (Figure 2.1). Fos expression was elevated by opioid agonist microinjections within those zones compared to expression levels after vehicle microinjections, or compared to similar sites in normal control brains. Microinjections of the mu agonist DAMGO generated Fos plumes with a total radius of 0.15mm (0.014mm^3 volume), defined as an elevation >150% over normal tissue in the number of neurons expressing Fos protein at points surrounding the microinjector tip ($F_{(4,2776)} = 28.193$, $p = 0.0001$). These contained a smaller central plume with a radius of 0.08mm (0.002mm^3 volume), defined as >150% elevation over vehicle microinjection levels at corresponding points. Vehicle microinjections and cannula implants evoke modest Fos elevations on their own, and therefore a higher drug-induce elevation is required to exceed 150% of vehicle-control levels than is required to exceed 150% of normal-control levels measured in unoperated tissue. For that reason, the inner intense zone of >150% vehicle elevation in Fos is smaller than the outer zone of >150% elevation over normal spontaneous levels of Fos expression. Delta agonist DPDPE microinjections produced >150% normal radius of 0.20mm (0.034mm^3 volume), and again a slightly smaller radius of 0.15mm compared to vehicle control levels. Kappa agonist U50488H microinjections produced a radius of 0.15mm (0.014mm^3 volume) >150% elevation over normal tissue, and a slightly smaller radius of 0.13mm compared to control vehicle plumes. The averaged diameters of Fos plumes measured for each agonist were used to set its symbol size for maximal impact spread in functional maps (Figures 2.2-2.4). These measurements were also used therefore to calculate the volume of rostrodorsal hedonic hotspot and caudal hedonic

coldspot derived from the functional maps. All other information in function maps shows behavioral effects of drug microinjections on taste reactivity and food intake at depicted sites (symbol colors depict effect intensities; symbol placements depict site locations; bar graphs depict behavioral intensities at AP or DV stereotactic levels).

Rostrodorsal localization of hedonic enhancement in medial shell

At sites in the rostrodorsal quadrant of medial shell, the number of positive hedonic reactions elicited by the taste of sucrose was more than doubled by DAMGO, DPDPE or U50488H microinjections (Figure 2.2) (Friedman's Two-Way ANOVA, $X^2 = 12.573$, $p = 0.006$; described separately below). Hedonic enhancements produced within the rostrodorsal quadrant were always greater than produced by the same drugs in any of the other three quadrants of medial shell ($X^2 = 24.723$, $p = 0.0001$), which all failed to produce any increase over vehicle levels in the number of positive reactions elicited by sucrose ($X^2 = 5.57$, $p = 0.134$).

Mu localization in rostral hotspot: Mu receptor stimulation by DAMGO microinjections at sites within the rostrodorsal quadrant nearly quadrupled the number of hedonic reactions elicited by sucrose taste (median = 400%, Z = -3.059, $p = 0.002$). That localized mu enhancement was significantly greater compared to the other three quadrants of medial shell (Z = -3.114, $p = 0.001$).

Delta localization in rostral hotspot

Delta stimulation by DPDPE microinjections in the rostrodorsal quadrant nearly tripled hedonic reactions elicited by the taste of sucrose compared to vehicle levels in the same rats (median = 292%; Z = -2.346, $p = 0.019$). Again, the rostrodorsal delta enhancement was significantly higher compared to the other three quadrants of medial shell (Z = -2.926, $p = 0.002$).

Kappa localization in rostral hotspot

Perhaps most surprisingly, even kappa stimulation by U50488H microinjections inside the rostrodorsal quadrant more than doubled positive hedonic reactions to sucrose (median = 225%, Z = -2.703, $p = 0.007$). Kappa enhancement in the rostrodorsal quadrant was significantly higher compared to the other three quadrants (Z = -2.744, $p = 0.004$). Although this is the first report to our knowledge that kappa opioid stimulation can enhance sensory pleasure impact in any brain region, we emphasize that kappa hedonic enhancement was observed only for sites within the rostrodorsal hotspot.

Thus, within the rostrodorsal quadrant, all three opioid agonists produced increases that were valence-specific to positive hedonic orofacial reactions to sucrose (e.g., rhythmic and lateral tongue protrusions, and paw licks). The number of aversive reactions (e.g., gapes) remained near zero and never was altered, even by opioid agonist microinjections in the rostrodorsal quadrant (Friedman's Two-Way ANOVA, $X^2 = 5.567$, $p = 0.135$). Finally, although the magnitude of hedonic enhancement nominally varied from two-fold for kappa to three-fold for delta and up to four-fold for mu within the rostrodorsal hotspot, those magnitudes of increase did not differ statistically from each other across agonists (Friedman's Two-Way ANOVA, $X^2 = 3.455$, $p = 0.178$), suggesting that within the hotspot boundaries all three types of opioid stimulation produce hedonic enhancements of comparable intensity.

Defining anatomical boundaries for opioid hedonic enhancement and suppression

To more precisely map its anatomical boundaries, we operationally defined the hotspot as a spatial region containing contiguous plume-diameter sites which produced hedonic reaction elevations of at least 150% (compared to vehicle-control levels in the same rat). Defined that way, the rostrodorsal hotspot extended dorsally to the top of NAc shell, and was anatomically

bounded by lateral septum and the lateral ventricles. Ventrally, the hotspot extended just past the dorsoventral midpoint in medial shell, and touched the islands of Calleja. Medially, the hotspot extended to the medial edge of shell, and laterally extended to the edge of the NAc core. Relative to stereotaxic placements, the entire rostrocaudal extent of the hotspot ran approximately from +2.20mm anterior to bregma (none of our sites hit further rostral portions of NAc) posteriorly to +1.5mm bregma, or almost to the AP midpoint of the medial shell. The ventral boundary of the hotspot in our map was not as clear as we would have wished because our microinjection sites the most ventral strip of the medial shell. Sparseness of ventral sites make it more difficult to have full confidence in the exact placement of the ventral boundary for the hedonic hotspot. However, there appeared to be a trend in the rostral bar graphs of Figure 2.2 suggesting a decline in the intensity of DAMGO hedonic enhancement as hotspot sites moved ventrally in shell, potentially matching the ventral boundary originally outlined for the mu hotspot (Pecina & Berridge, 2005). The centers of microinjection sites within the hotspot ranged within approximately 1.1mm DV X 0.5mm ML X 0.8mm AP (dorsoventral (DV): -6.2 to -7.3mm; mediolateral (ML): ±0.4 to ±0.9mm; anteroposterior (AP): +2.4 to +1.6mm). Given that DAMGO produced Fos plumes with radii extending to 0.15mm at its maximum extent (defined as >150% elevation over normal unoperated NAc tissue), that implies that the range of Fos elevation produced by those microinjection sites would extend along a DV axis of 1.4mm, ML of 0.8mm, and AP of 1.1mm.

Regarding cubic volume, the coordinates above were used to calculate a total volume of ~1.2mm^3 for the mu hotspot where >150% enhancements in hedonic reactions were produced. Using a smaller Fos plume based on Fos elevation >150% over Fos levels produced by vehicle microinjections (for the same points surrounding the microinjection tip) as a more conservative

estimate of DAMGO spread of impact, the mu hotspot volume would decline slightly to ~0.8mm^3. These values of 1.2mm^3 and 0.8mm^3 essentially bracket the original 1.0mm^3 estimate of the volume of the NAc mu hotspot (Pecina & Berridge, 2005). For DPDPE enhancements, the delta hotspot volume was estimated between 1.2mm^3 and 1.62mm^3. For U50488H enhancements, the kappa hotspot volume was estimated between 1.2mm^3 and 1.1mm^3. Altogether, these values indicate that the entire opioid NAc hedonic hotspot has a total volume of approximately 1mm^3 or slightly larger. By comparison, the volume of the entire medial shell is about 3mm^3, and the volume of the entire NAc is about 10mm^3 (shell and core combined).

Posterior 'coldspot' for hedonic suppression

In contrast to rostral enhancements above, a suppressive 'coldspot' was observed for all three opioid agonists in the caudal half of medial shell. In the posterior half of medial shell microinjections of DAMGO, DPDPE or U50488H generally reduced positive hedonic reactions to sucrose taste to about one-half of vehicle control levels (Friedman's Two-Way ANOVA, X^2 = 10.675, p = 0.014). A rostral versus caudal comparison for hedonic effects in medial shell shows that mu, delta, or kappa stimulation all had opposite effects in anterior vs posterior halves (X^2 = 37.899, p = 0.0001).

However, the magnitude of suppression produced at caudal sites differed somewhat across the three drugs (X^2 = 6.867, p = 0.032).

Delta caudal coldspot

DPDPE in the caudal half of medial shell suppressed positive reactions to only 40% of control vehicle levels (Z = -2.380, p = 0.017). This posterior suppression was again opposite from anterior enhancements caused by DPDPE microinjections (Z = -3.514, p = 0.0001).

Kappa caudal coldspot

Kappa stimulation suppressed hedonic reactions to sucrose in caudomedial shell by about 50% compared to vehicle controls (Z = -2.035, p = 0.042). This kappa suppressive hedonic effect seems compatible with previous reports on kappa function in reward. For example, Land et al. (2008) reported that kappa receptor activation in NAc and basolateral amygdala encodes the dysphoric component of a stressful event. Similarly, Wee and Koob (2010) suggested that kappa activation may be responsible for the aversive or dysphoric component of drug withdrawal. Our results suggest that kappa suppression of positive hedonic impact may be localized in medial shell to primarily the caudal half, similarly to the localization of hedonic suppression effects for mu and delta stimulation found here.

Mu caudal coldspot

In the caudal half of medial shell, mu stimulation only non-significantly suppressed 'liking' reactions elicited by sucrose to 66% of control levels (Z = -1.863, p = 0.063). Still, this trend toward posterior suppression was significantly different from the anterior enhancements caused by microinjections of the same DAMGO drug in the rostrodorsal shell (Z = -3.626, p = 0.0001).

Anatomical borders and volume of caudal suppressive coldspot

The caudal coldspot penetrated both the dorsal and ventral quadrants of the caudal half of medial shell. Like the hotspot, the dorsal border appeared to touch the lateral septum and the lateral ventricles. The medial border changes based on the anteroposterior plane, beginning with the island of Calleja (AP +1.56) at the rostral edge of the caudal coldspot, then lateral septum (AP +0.96), and finally rostral ventral pallidum at the most caudal edge of the coldspot (AP +0.72). The ventral border appeared to extend to rostral ventral pallidum (raising the possibility that the NAc coldspot may be continuous with another opioid hedonic coldspot previously mapped within the rostral half of the ventral pallidum itself) (K. S. Smith & Berridge, 2005). The

lateral border touched predominantly the NAc core at most points, but posteriorly transitions to bed nucleus of the stria terminalis at is most caudal points.

Mapping the coldspot borders with the same technique as for the hotspot, the caudal hedonic coldspot (sites producing 30% or greater suppression of hedonic reactions) extended from AP +1.5 to +0.8mm, ML ±0.4 to ±0.9mm, and DV -6.3 to -7.9mm (caudal medial shell shrinks in the dorsoventral plane as it becomes posterior). For the mu coldspot, the trapezoidal volume was approximately ~1.3mm^3 to ~0.9mm^3. For the delta hedonic coldspot, the corresponding volume was between ~1.8mm^3 and ~1.3mm^3. For the kappa coldspot, the volume of was between ~1.3mm^3 and ~1.2mm^3. Altogether, these overlapping results suggest that the anatomically localized hedonic coldspot in caudal accumbens slightly exceeds one cubic millimeter in volume, and is therefore comparable in size to the rostrodorsal hedonic hotspot.

Food intake and eating behavior

In 1-hr free intake tests conducted immediately after taste reactivity tests with sweet M&M chocolates and ordinary chow available, opioid stimulations significantly altered the total amount eaten in ways that depended on the particular agonist and precise site (Figure 2.3) (Friedman's Two-Way ANOVA, $X^2 = 11.796$, $p = 0.008$). In all cases, consumption was almost exclusively of palatable M&Ms.

The three agonists produced very different NAc anatomical patterns within medial shell of effects on food intake. For mu stimulation, DAMGO microinjections at all sites throughout the entire medial shell homogeneously enhanced the consumption of food by nearly 140% above vehicle ($Z = -3.250$, $p = 0.001$). Consumption of palatable chocolate M&Ms rose from ~6.5g after vehicle microinjection to 8.0g after DAMGO ($Z = -2.698$, $p = 0.007$). The mu increase in consumption was comparable in magnitude at all sites throughout the entire medial shell, and did

not differ between rostral and caudal halves (Z = -0.362, p = 0.750). This broad anatomical distribution for mu-stimulated sites of hyperphagia is consistent with earlier reports that DAMGO throughout entire NAc, as well as in other structures, including central amygdala and parts of neostriatum, can increase consumption of palatable food (DiFeliceantonio et al., 2012; Mena et al., 2011; Pecina & Berridge, 2005; Ragnauth, Moroz, et al., 2000; Zhang & Kelley, 2000).

For delta stimulation of intake, DPDPE microinjections also enhanced eating in medial shell, specifically enhancing consumption of palatable M&M chocolates (Z = -2.129, p = 0.033). However, an anatomical analysis revealed that sites for the observed delta increase in eating were not broadly distributed across medial shell, but rather were confined within the rostrodorsal hotspot (Z = -2.866, p = 0.004, median = 125% compared to vehicle). By comparison, in the posterior half of medial shell, delta stimulation had no detectable effect on intake (total eaten: Z = -0.070, p = 0.944), and the elevation of eating behavior in the rostrodorsal hotspot quadrant was larger than produced in the other three quadrants (Z = -2.034, p = 0.044). Thus, delta stimulation enhanced eating, but only within the rostrodorsal hotspot.

By contrast, kappa receptor stimulation did not produce a clear enhancement of intake at any anatomical site in medial shell (Z = -1.064, p = 0.287, median = 110%), although there was a nonsignificant trend toward >150% increase in several sites concentrated in the rostrodorsal hotspot (Z = -1.824, p = 0.68). An inspection of kappa stimulation in rostral hotspot versus caudal coldspot effects on eating showed no difference between anterior vs posterior halves of medial shell (Z = -0.926, p = 0.384). However, we did observe a strikingly large individual variance in kappa stimulated eating, which was nearly three times as large as vehicle variance and twice as large as any other opioid agonist type (vehicle variance, 8.799; DAMGO variance,

12.7; DPDPE, 10.882; U50488H, 23.473). That is, some rats intensely increased their consumption after U50488H microinjection (more than 10g above vehicle), but other rats were either not affected at all or even ate ~ 30% less compared to after vehicle (~1.5g less). The lack of a consistent kappa change in intake seems consistent with previous reports of no overall change in food consumption after kappa stimulation in NAc medial shell (Bakshi & Kelley, 1993; Zhang & Kelley, 1997).

Conditioned place preference

Conditioned place preference (CPP) results indicated that the rostral NAc hotspot more effectively supported establishment of a positive conditioned place preference for all three types of opioid stimulation than other regions of medial shell (Friedman Two-Way ANOVA, X^2 = 9.80, p = 0.002). Within the rostral half of medial shell, microinjection sites in the dorsal two-thirds induced a conditioned place preference of 216% for mu stimulation (Z = -2.201, p = 0.28), a 168% preference for kappa stimulation (Z = -2.197, p = 0.028), and a 139% trend towards preference for delta stimulation, although the last did not reach statistical significance. However, the strength of delta conditioned preference was not statistically lower than either mu or kappa preference (Z = -1.387, p = 0.183), consistent with the possibility of a delta preference. (Z = 1.69, p = 0.091). Inside the rostrodorsal hotspot, mu and kappa stimulations produced comparable magnitudes of positive place preference of ~170% to ~215% , and did not differ from each other (Z = -1.359, p = 0.181).

Anatomical comparison of sites across medial shell indicated that only inside the rostrodorsal hotspot did kappa or mu microinjections produce positive place preferences, whereas the same drug microinjections in other regions of medial shell outside the hotspot failed to produce any significant preference (e.g., no preference induced in the entire caudal half of

medial shell) (Figure 2.4) (U5088H, Z= -2.714, $p = 0.005$; DAMGO, Z = -2.429, $p = 0.014$). That is, even for mu stimulation, DAMGO microinjections in the posterior half of medial shell failed to establish a significant preference (Z = -0.507, $p = 0.612$). These results appear similar to a previous report of failure to produce a conditioned place preference by DAMGO microinjections into NAc core, lateral hypothalamus, or medial prefrontal cortex (Bals-Kubik *et al.*, 1993). However, as a caveat, our data were inadequate to draw a strong conclusion about mu stimulation in the most ventral one-third of medial shell, leaving some uncertainty for mu stimulation in that subregion. For kappa sites outside the hotspot, we found a trend toward establishing negative place avoidance of 33% (Z = -1.782, $p = 0.075$). A conditioned place avoidance for most of shell would be similar to reports that kappa stimulation in the adjacent core of NAc produced a conditioned place avoidance (Bals-Kubik et al., 1993).

Discussion

Our results confirm that the rostrodorsal quadrant of medial shell in NAc contains a specialized opioid hedonic hotspot, roughly $1mm^3$ in volume, where mu stimulation generates 200-400% enhancements of 'liking' reactions to sweetness. We report that delta and kappa stimulations also amplify hedonic impact similarly within the hotspot. The functional uniqueness of the hotspot in rostrodorsal shell contrasts to lack of capacity to generate comparable hedonic enhancement observed for at least all sites in the remaining two-thirds of medial shell mapped here (caudal and rostroventral), and potentially in the remaining 90% of entire NAc (shell and core combined). Our results also mapped a separate suppressive coldspot in the caudal half of shell, approximately ~$1.3mm^3$ in volume, where each opioid stimulation oppositely reduced sucrose positive 'liking' reactions to approximately one-half normal levels. Confirming a unique reward-related role for the rostrodorsal quadrant, we further showed that mu or kappa stimulations produced positive

conditioned place preference at sites specifically within the hotspot (and a positive trend for delta), but not at outside sites in other subregions. Finally, we identified several anatomical differences between NAc opioid controls of 'liking' versus 'wanting' to eat. These results highlight NAc anatomical heterogeneity and localization of opioid reward-related functions within medial shell.

Neurobiological sources for endogenous opioid ligands in NAc include dynorphin (kappa ligand) released from medium spiny neurons (MSNs) that express D1 receptors, and enkephalin (delta and mu ligand) from D2-expressing MSNs (Ghazarossian, Chavkin, & Goldstein, 1980; Raynor et al., 1994). Dynorphin and enkephalin are also released by input projections from other structures, including ventral pallidum and lateral hypothalamus (Baldo et al., 2003; Groenewegen, Wright, Beijer, & Voorn, 1999; Haber, Groenewegen, Grove, & Nauta, 1985; Peyron et al., 1998). In addition, beta-endorphin (potent mu ligand) may reach NAc medial shell via projections from the arcuate nucleus of hypothalamus (Khachaturian, Lewis, Haber, Akil, & Watson, 1984; Raynor et al., 1994).

Post-synaptic receptors for mu, delta and kappa in NAc are reported on dendrites of most MSNs (Gracy, Svingos, & Pickel, 1997; Svingos, Chavkin, Colago, & Pickel, 2001; Svingos, Clarke, & Pickel, 1999; Svingos, Colago, & Pickel, 1999). Mu receptors likely predominate on D1 MSNs (e.g., direct path), but can also occur on D2 MSNs (indirect path) as well as on acetylcholine interneurons (Svingos, Colago, & Pickel, 2001; Svingos, Moriwaki, Wang, Uhl, & Pickel, 1996). In contrast, delta and kappa receptors may predominate on D2 MSNs (Svingos, Clarke, & Pickel, 1998; Svingos, Colago, et al., 1999; Svingos et al., 1996), and also exist on pre-synaptic terminals of glutamate and dopamine projections into NAc (Hjelmstad & Fields, 2001; Svingos, Clarke, et al., 1999; Svingos, Colago, et al., 1999). All three opioid receptors are G protein-coupled receptors that can recruit ERK 1/2 inside neurons in a Ras-dependent manner via the Gβγ subunit, though

through different intracellular pathways (Belcheva et al., 1998). For example, mu activation recruits ERK via PKCε, whereas kappa stimulation recruits PLC3 (Belcheva et al., 2005). These neurobiological distributions may be relevant to the functional pattern of effects described here.

Potential neurobiological basis for the rostrodorsal hotspot

Several distinctive neurobiological features exist for the hotspot's rostrodorsal quadrant of medial shell that may be relevant to its unique hedonic function (Figure 2.4) (Thompson & Swanson, 2010; Zahm et al., 2013). For example, Thompson and Swanson (2010) anatomically traced a unique closed-loop cortico-limbic-thalamo-cortical circuit through the NAc hotspot in the rostrodorsal quadrant of medial shell, potentially in parallel to other segregated loops involving different shell subregions. They reported that the rostrodorsal quadrant received corticolimbic inputs from one particular subregion of prefrontal infralimbic cortex (homologous to Brodmann's area 25 in humans), and sent unique output projections to distinct subregions of lateral hypothalamus and ventral pallidum. From there, pallido-thalamic and hypothalamo-thalamic projections were relayed via thalamus to the original infralimbic subregion, thereby completing a segregated corticolimbic – thalamocortical loop (Thompson & Swanson, 2010).

Separately, Zahm and colleagues (2013) reported additional distinguishing features of the rostral subregion containing the medial shell hotspot, suggesting that the rostral shell constitutes a distinct anatomical NAc-septal transition zone related to lateral septum. By contrast, they suggested that the caudal half of medial shell constitutes a different transition zone related to structures of extended amygdala. Further neurochemical and cellular specializations may additionally distinguish the rostrodorsal quadrant or rostral half from other subregions of medial shell (Britt & McGehee, 2008; Hanlon, Baldo, Sadeghian, & Kelley, 2004; Park, Aragona, Kile,

Carelli, & Wightman, 2010). Such features may eventually help explain the rostrodorsal hotspot's unique opioid ability to robustly generate hedonic enhancements.

Comparison to neuroimaging measures

Human neuroimaging studies also implicate NAc circuitry and opioid activation in both reward and pain modulation (Leknes, Lee, Berna, Andersson, & Tracey, 2011; Wanigasekera et al., 2012; Zubieta et al., 2005; Zubieta & Stohler, 2009). However, it is difficult to compare such neuroimaging results to our localization of a NAc hedonic hotspot until future refinements in anatomical resolution permit better contrast of the rostrodorsal quadrant to other subregions of medial shell. Even then, a potentially important difference may remain: our opioid hotspot localization applies particularly to opioid causation (i.e. magnification of sucrose 'liking'), whereas neuroimaging (and even anatomically precise electrophysiological and neurochemical measures of neural activation) assess regional coding that occurs in correlation with hedonic events. It remains for now an open question whether the same localization rules will apply to opioid causation and to coding of the same hedonic functions (especially since some coding sites may reflect hedonic-guided causation of other learning, cognition, etc. functions distinct from 'liking') (K. C. Berridge & Kringelbach, 2013).

Paradoxical kappa hedonic enhancement in rostral hotspot

Perhaps our most surprising finding was that kappa stimulation produced any positive enhancements at all for 'liking' and conditioned place preference – even though only at sites limited to the rostrodorsal hotspot. No other NAc study to our knowledge has previously reported positive reward effects for kappa stimulation. Instead kappa stimulation is commonly viewed to cause mostly negative effects, such as conditioned place avoidance, at least at sites in NAc core, ventral tegmentum or prefrontal cortex (Bals-Kubik et al., 1993; Bals-Kubik, Herz, &

Shippenberg, 1989; Kim, Pollak, Hjelmstad, & Fields, 2004; McLaughlin, Land, Li, Pintar, & Chavkin, 2006; Mucha & Herz, 1985). In accordance with such negative-valence dominance, we found kappa stimulation produced place avoidance effects at most sites in NAc medial shell: the entire caudal half of shell (which also produced suppression of 'liking' reactions to sucrose), as well as rostral sites that were ventral to the hotspot, or that were so far anterior as to be outside the NAc hotspot. We suggest that anatomical heterogeneity gates the valence of kappa effects in NAc, inducing positive reward within the rostrodorsal shell hotspot but mostly negative effects elsewhere.

Controlling motivation for food: 'wanting'

Opioid enhancements of 'wanting' to eat more food, expressed as increases in eating behavior and consumption, differed somewhat from 'liking' enhancements. Delta stimulation of intake (>140%) by DPDPE microinjection was anatomically limited to the rostrodorsal hotspot. At all other sites in medial shell, delta stimulation failed to stimulate intake at all. It seems plausible that previous positive reports of delta stimulation inducing eating increases in NAc may have predominantly targeted the rostrodorsal zone of shell (Majeed et al., 1986; Ragnauth, Moroz, et al., 2000; Richard, Castro, Difeliceantonio, Robinson, & Berridge, 2013; Zhang & Kelley, 1997).

By comparison, mu and kappa stimulations produced strong anatomical dissociations between 'wanting' versus 'liking' effects. For mu stimulation of eating (>140%), 'wanting' substrates anatomically extended additionally beyond the hotspot and throughout the entire medial shell (remaining strong even in caudal shell). This is consistent with previous reports that DAMGO stimulates eating at many NAc shell and core sites, as well as at sites of neostriatum, amygdala, etc. (DiFeliceantonio et al., 2012; Echo, Lamonte, Ackerman, & Bodnar, 2002;

Gosnell & Majchrzak, 1989; Mahler & Berridge, 2009; Majeed et al., 1986; Pecina & Berridge, 2005; Zhang & Kelley, 1997, 2000). It is also consistent with anatomically widespread mu enhancements for other aspects of 'wanting', as for example reflected in measures of learned seeking for rewards (Hanlon et al., 2004; Pecina & Berridge, 2013; Zhang et al., 2003).

Finally, kappa stimulation failed to consistently increase in food intake at any shell sites. Kappa stimulation doubled intake in some individuals but reduced intake by half in other individuals, producing variability with no discernible anatomical pattern. Our failure to find kappa-induced increases in intake seems consistent with previous reports of no change.

Clinical applications

Deficits of positive hedonic impact (anhedonia or dysphoria) may be involved in major depression or in bipolar disorder (Der-Avakian & Markou, 2012; Treadway & Zald, 2011), whereas excessive motivational 'wanting' to consume rewards characterizes compulsive consumption disorders, such as binge eating and addiction (Avena, Rada, & Hoebel, 2008a; Rubin, 2012; Shin, Pistell, Phifer, & Berthoud, 2010; Spijker, de Graaf, Ten Have, Nolen, & Speckens, 2010). Opioid reward circuitry in NAc is implicated in both types of dysfunction (Bruchas, Land, & Chavkin, 2010; Giuliano, Robbins, Nathan, Bullmore, & Everitt, 2012; Katsuura & Taha, 2014; Kupchik et al., 2014), suggesting that aberrant localizable mechanisms within NAc shell may be relevant to understanding such disorders. Better understanding of NAc heterogeneity, and localization of opioid functions in generating intense 'liking' and 'wanting', may prove useful in unraveling such psychopathologies, and eventually aid in creating more effective treatments.

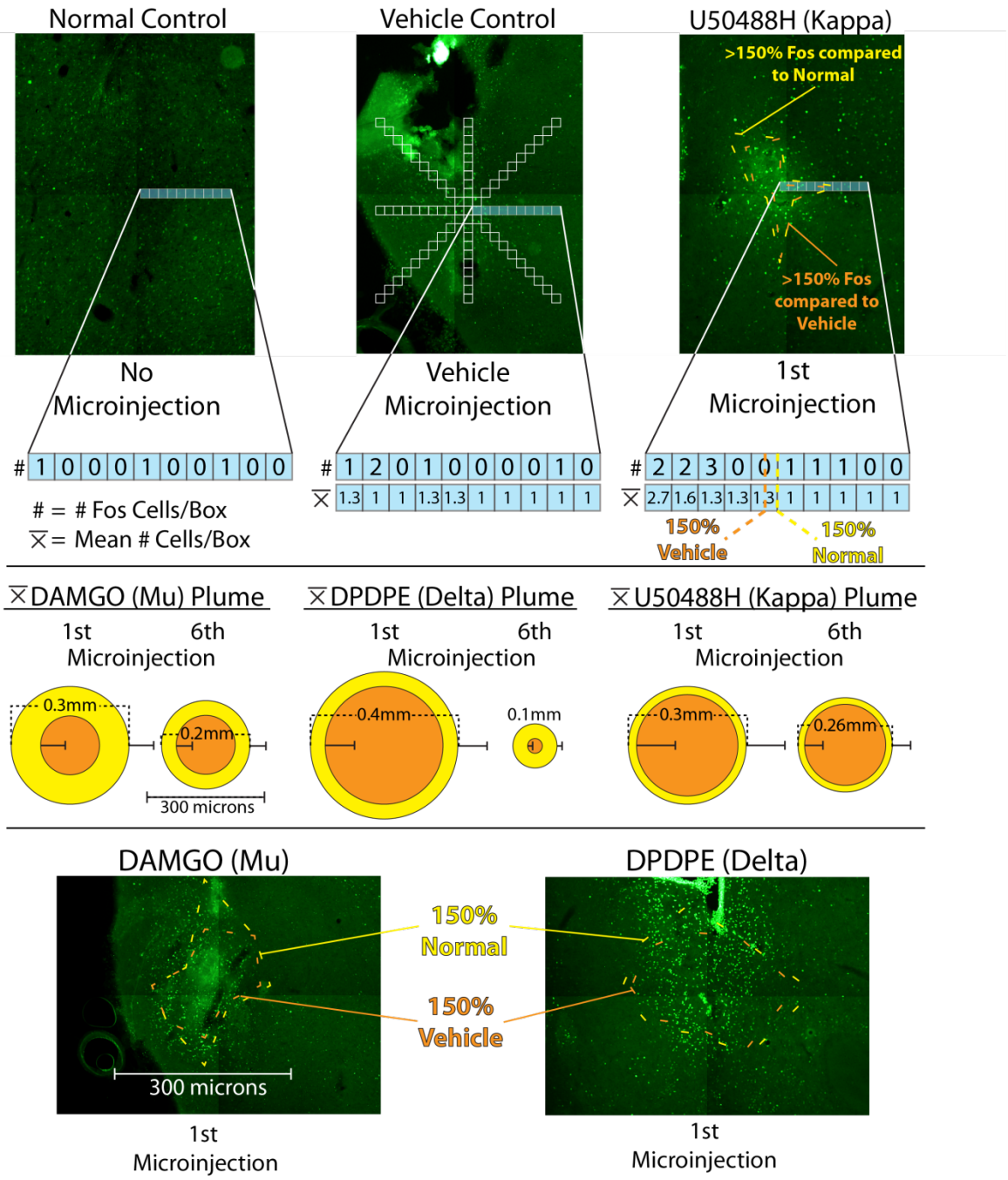

Normal Control
Vehicle Control
U50488H (Kappa)
>150% Fos compared to Normal
>150% Fos compared to Vehicle
No Microinjection
Vehicle Microinjection
1st Microinjection
1 0 0 0 1 0 0 1 0 0
1 2 0 1 0 0 0 0 1 0
2 2 3 0 0 1 1 1 0 0
X 1.3 1 1 1.3 1.3 1 1 1 1 1
X 2.7 1.6 1.3 1.3 1.3 1 1 1 1 1
# = # Fos Cells/Box
X = Mean # Cells/Box
150% Vehicle
150% Normal
X DAMGO (Mu) Plume
X DPDPE (Delta) Plume
X U50488H (Kappa) Plume
1st Microinjection
6th Microinjection
0.3mm
0.2mm
300 microns
0.4mm
0.1mm
0.3mm
0.26mm
DAMGO (Mu)
DPDPE (Delta)
150% Normal
150% Vehicle
300 microns
1st Microinjection
1st Microinjection

Figure 2.1. Fos plume maps for drug microinjections. Local Fos plumes reflect impact spread of a microinjected drug. Photomicrographs (top) show Fos expression in NAc shell of a normal (unoperated) control brain, a control brain that received a vehicle microinjection, and a brain that received a microinjection of U50488 (kappa κ). Each photo also depicts a sample bar showing Fos-expressing neuron counts at points along a radial axis emanating from center of microinjection (or equivalent bar for normal control brain). Numbers reflect the number of neurons in a box that expressed Fos. For microinjections, the average count for the corresponding box in the entire vehicle-microinjection group (*n = 4*) or U50488H (*n = 6*) group is also shown below. Such counts were used to compute elevations of drug-induced Fos compared with normal brains or vehicle brains for the corresponding location. The kappa plume shows the outer limits of where U50488H stimulated Fos expression >150% above vehicle microinjection levels (orange dashed line), and slightly larger region where Fos was stimulated >150% at least above normal control levels (yellow dashed line). Equivalent plumes are shown in photomicrographs at bottom for microinjections of DAMGO (*n* = 6; mu (μ) agonist) and of DPDPE (*n* = 6; delta (δ) agonist). The center row show illustrates the magnitude of shrinkage due to serial repetition, from maximal plumes measured after first-time microinjections in the dedicated Fos groups (shown in photomicrographs) to the sixth microinjection in rats that were tested 5 times behaviorally before a final Fos microinjection. Fos-positive labeled cells were individually counted in 50x50μm squares along radial arms extending from the center of the microinjection site at 10x magnification. Such shrinkage implies that dedicated Fos groups may provide more accurate measures of maximal plume diameter than groups previously tested for behavioral effects.

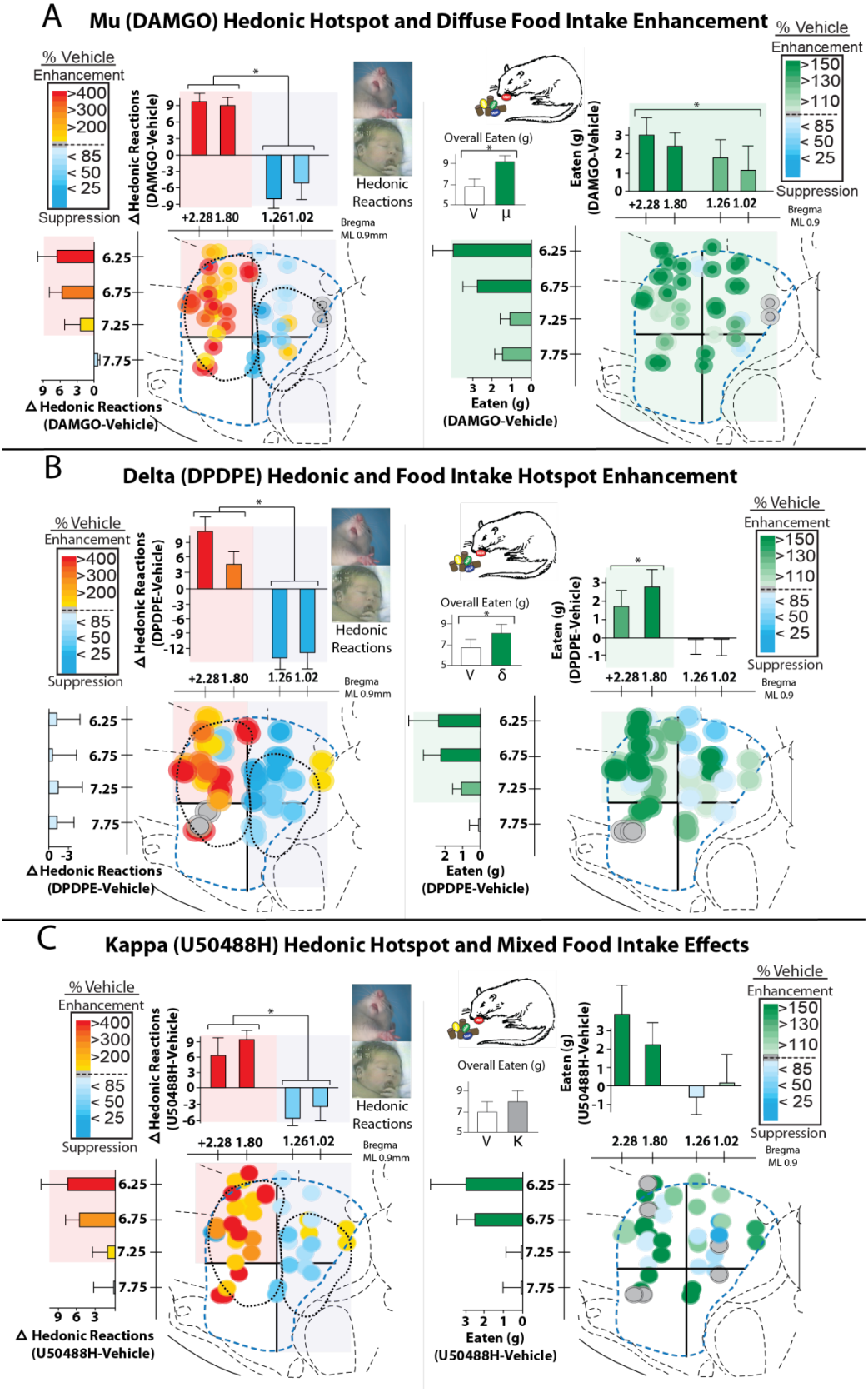

Hedonic Reactions
Food Intake
A
Mu (DAMGO) Hedonic Hotspot and Diffuse Food Intake Enhancement
% Vehicle Enhancement
>400
>300
>200
< 85
< 50
< 25
Suppression
Δ Hedonic Reactions (DAMGO-Vehicle)
Hedonic Reactions
+2.28 1.80
1.26 1.02
Bregma ML 0.9mm
6.25
6.75
7.25
7.75
Overall Eaten (g)
V
μ
Eaten (g) (DAMGO-Vehicle)
>150
>130
>110
Suppression
B
Delta (DPDPE) Hedonic and Food Intake Hotspot Enhancement
Δ Hedonic Reactions (DPDPE-Vehicle)
Eaten (g) (DPDPE-Vehicle)
V
δ
C
Kappa (U50488H) Hedonic Hotspot and Mixed Food Intake Effects
Δ Hedonic Reactions (U50488H-Vehicle)
Eaten (g) (U50488H-Vehicle)
V
K

Figure 2.2. Causation maps for localization of "liking" versus "wanting" enhancements. Sagittal maps of NAc medial shell show changes that each type of opioid agonist microinjection induced in hedonic reactions to sucrose taste (left column) and in food intake (right column) within the same individual rat (compared with taste reactions and food intake after vehicle microinjection; $n = 21$). Rows show mu (DAMGO; ***A***), delta (DPDPE; ***B***), or kappa (U50488H; ***C***) effects. Behavioral changes are displayed as percentage changes from vehicle control levels, and both bilateral microinjection sites are plotted on the single sagittal map. Bars show mean intensity of behavioral changes produced at each stereotaxic level (anterior–posterior and dorsal–ventral levels). Colors also show intensity of behavioral changes (percentage change from within-subject vehicle control levels) induced at each site. Enhancements of "liking" in the left column are depicted in yellow, orange, and red, whereas suppression of "liking" reactions is depicted by blue. In the right column, eating enhancement is displayed by green, and suppression of eating by blue. In both columns, gray indicates no change from vehicle control level. The size of sagittal map symbols is scaled to measured Fos plume diameters for each drug. The dashed circle in rostral accumbens shows the anatomical outline of the mu hotspot originally described by Pecina and Berridge (2005) for comparison to present data, and the caudal dashed circle represents their original mu coldspot.

Opioid Hotspot Induces Condition Place Preferences

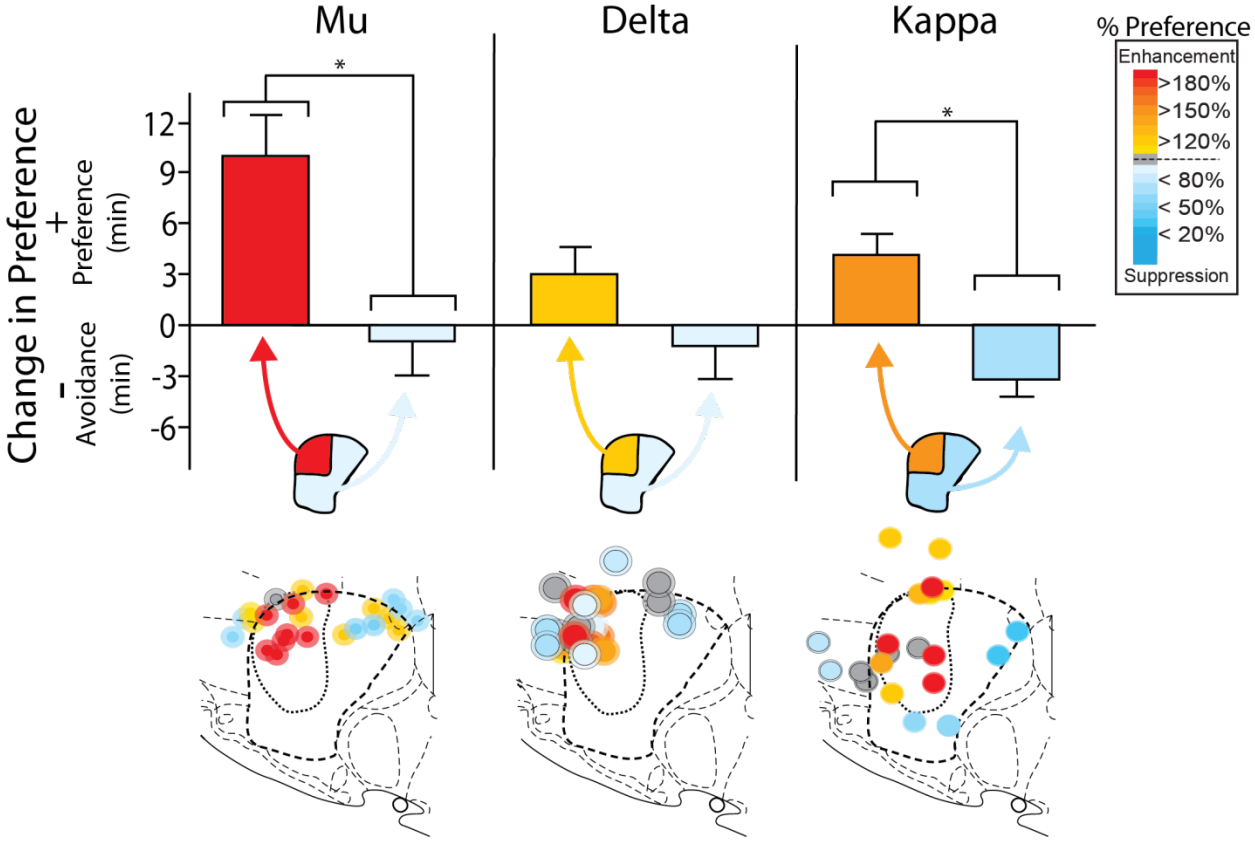
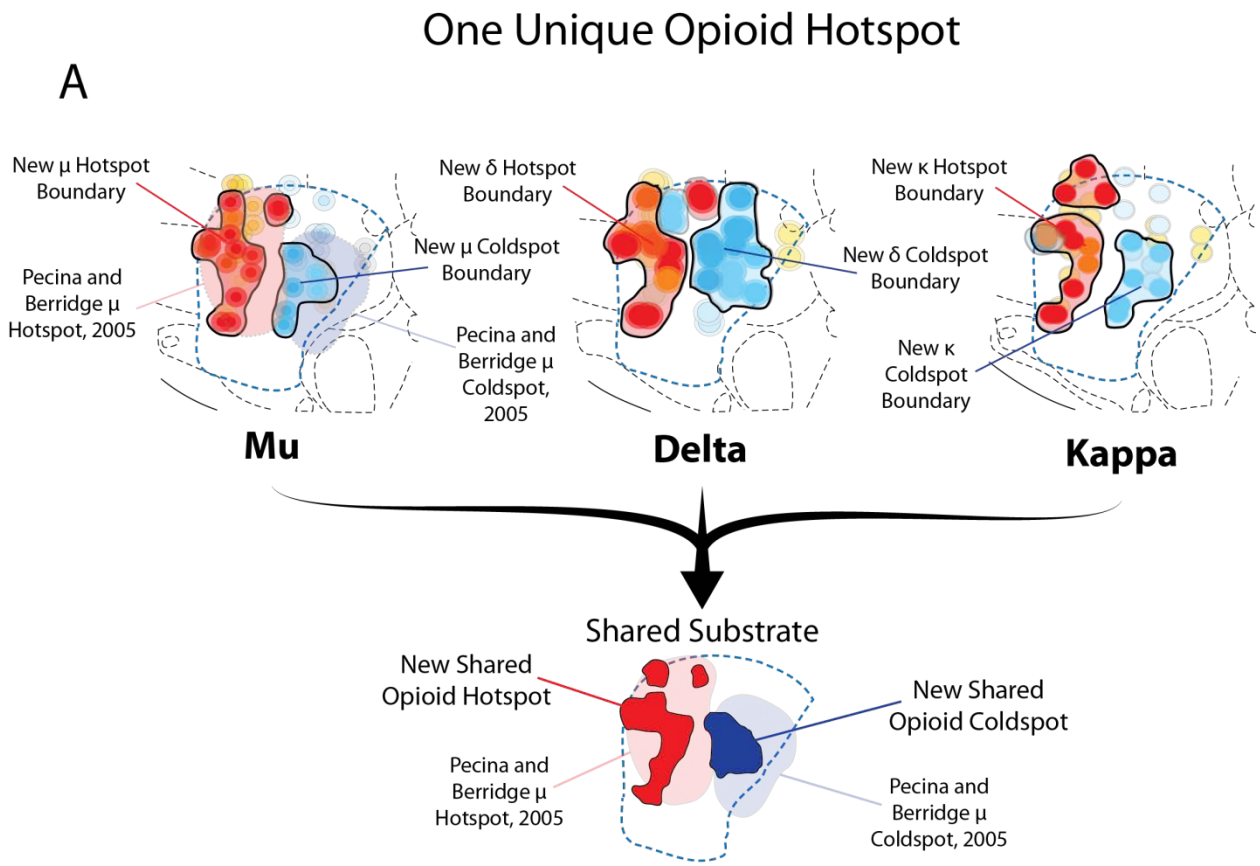


Figure 2.3. Causation maps for conditioned place preference. Sagittal maps and overall effects for establishment of conditioned place preference or avoidance is shown for mu (n = 13), delta (n = 13), and kappa (n = 13) agonist microinjections. Bars extending above the axis represent an overall conditioned place preference for a NAc region; bars below the axis represent a conditioned place avoidance. Regions are the rostrodorsal hotspot (left bar of each pair) versus the entire remaining three-quarters of medial shell outside the hotspot (rostroventral 1/3 of medial shell plus entire caudal half; right bar of each pair). Sagittal maps are similar to Figure 2.2. Red and orange indicates establishment of a positive place preference, and blue indicates a negative place avoidance. The dotted outline in the rostrodorsal portion of large slices indicate the original mu hotspot as originally defined by Pecina and Berridge (2005).


One Unique Opioid Hotspot
A
New μ Hotspot Boundary
Pecina and Berridge μ Hotspot, 2005
New μ Coldspot Boundary
Pecina and Berridge μ Coldspot, 2005
New δ Hotspot Boundary
New δ Coldspot Boundary
New κ Hotspot Boundary
New κ Coldspot Boundary
Mu
Delta
Kappa
Shared Substrate
New Shared Opioid Hotspot
Pecina and Berridge μ Hotspot, 2005
New Shared Opioid Coldspot
Pecina and Berridge μ Coldspot, 2005

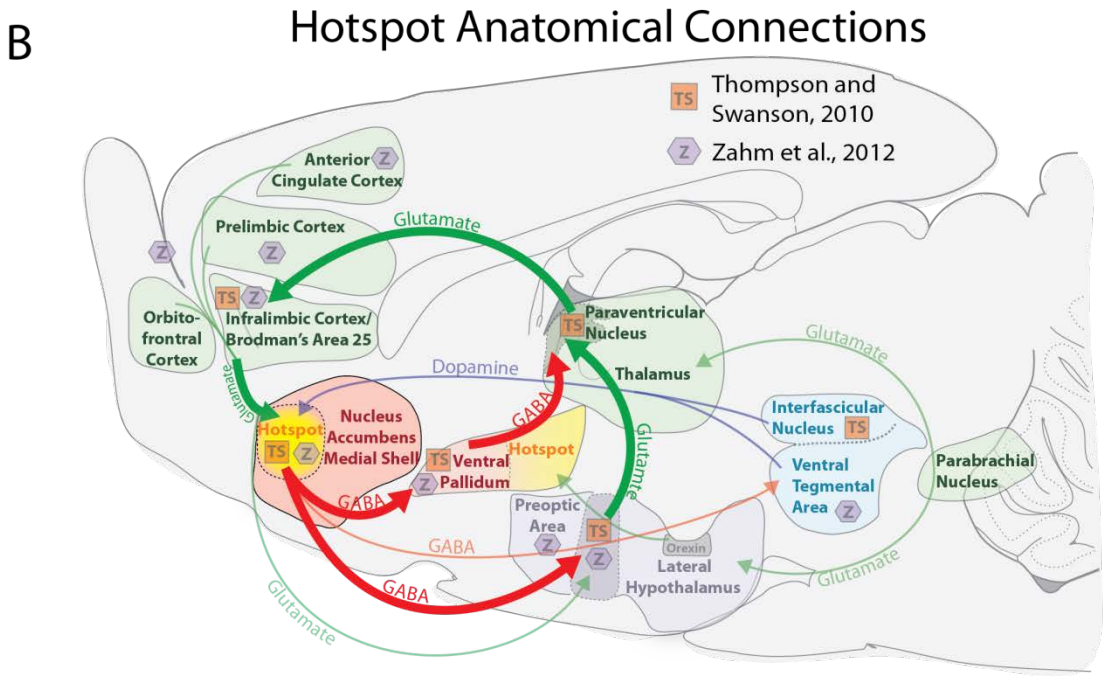

B
Hotspot Anatomical Connections
TS Thompson and Swanson, 2010
Z Zahm et al., 2012
Anterior Cingulate Cortex
Prelimbic Cortex
Orbito-frontral Cortex
Infralimbic Cortex/ Brodman's Area 25
Glutamate
Dopamine
GABA
Nucleus Accumbens Medial Shell
Hotspot
Ventral Pallidum
Paraventricular Nucleus
Thalamus
Interfascicular Nucleus
Ventral Tegmental Area
Parabrachial Nucleus
Preoptic Area
Orexin
Lateral Hypothalamus

Figure 2.4. Summary maps for NAc opioid hotspots and coldspots in medial shell and relevant anatomical circuitry. ***A***, Sagittal summary maps of NAc medial shell for mu, delta, or kappa hedonic enhancements (rostral hotspots) and suppressions (caudal coldspots) of sucrose "liking" observed here. Hotspot outlines are defined anatomically by contiguous groups of microinjection sites (sized to match Fos plumes) that caused >250% enhancements of positive orofacial hedonic reactions elicited by sucrose taste, and coldspot boundaries are defined by contiguous sites that caused suppressions to below one-half of control vehicle levels for sucrose "liking" reactions. The mu panel also shows for comparison the original hotspot and coldspot boundaries (Pecina and Berridge, 2005). The shared substrate map below is a subtraction map showing sites that produced equivalent hotspot enhancements for all three opioid stimulations, or equivalent coldspot suppressions for all three agonist microinjections. ***B***, Anatomical circuitry features relevant to the hotspot of rostrodorsal medial shell, as described in text (based on Thompson and Swanson (2010) and on Zahm et al., (2013); TS symbol in orange boxes depicts unique features of rostrocaudal quadrant of medial shell described by Thompson and Swanson; Z symbol in purple hexagons depicts features of rostral half of medial shell described by Zahm et al.). Hedonic hotspots are shown in yellow, GABAergic projections in red, glutamatergic projections in green, and dopaminergic projections in blue. Modified from (Richard et al., 2013).

CHAPTER 3:

OREXIN IN ROSTRAL HOTSPOT OF NUCLEUS ACCUMBENS ENHANCES SUCROSE 'LIKING' AND INTAKE BUT SCOPOLAMINE IN CAUDAL SHELL SHIFTS 'LIKING' TOWARD 'DISGUST' AND 'FEAR'

Introduction

Pleasure 'liking', or hedonic impact, is a fundamental aspect of sensory reward, and pathological dysfunction of hedonic brain circuitry may contribute to addiction, mood disorders, eating disorders and obesity. To better understand and map the neural mechanisms underlying hedonic impact, several affective neuroscience studies have used the taste reactivity test to measure orofacial 'liking' reactions to sweet tastes (K. C. Berridge & Kringelbach, 2015). These affective facial expressions to taste are homologous in human infants, non-human primates, and even rodents (K. C. Berridge, 2000; Steiner et al., 2001), and hedonic brain mechanisms can be mapped by their ability to cause changes in such 'liking' reactions.

One example of this hedonic localization involves the nucleus accumbens (NAc), which contains a roughly cubic millimeter sized "hedonic hotspot" in the rostral half of medial shell in rats. In that rostral hotspot of NAc shell opioid agonist microinjections can double or triple the number of positive hedonic orofacial reactions (i.e., 'liking' reactions) elicited by the taste of sucrose (Castro & Berridge, 2014b; Pecina & Berridge, 2005). Conversely, in caudal shell, agonist microinjections

reveal a “hedonic coldspot”, where opioid stimulation suppresses sucrose hedonic impact (Castro & Berridge, 2014b; Pecina & Berridge, 2005). By contrast to the localized hotspot for sweetness ‘liking’, mu opioid stimulations increase motivation to eat much more widely and homogeneously throughout the entire NAc shell (and in related structures), measured as increases in cue-triggered ‘wanting’ to obtain food rewards (e.g., in instrumental breakpoint and Pavlovian-Instrumental-Transfer tests), as well as in food consumption (Castro & Berridge, 2014b; Covelo, Patel, Luviano, Stratford, & Wirtshafter, 2014; Maldonado-Irizarry, Swanson, & Kelley, 1995; Pecina & Berridge, 2005, 2013; K. S. Smith & Berridge, 2005; K. S. Smith et al., 2011; Zhang & Kelley, 2000).

Other neurotransmitter systems in NAc also modulate food consumption, motivation, and hedonic impact of food rewards including, endocannabinoids and amino acids (Maldonado-Irizarry et al., 1995; Shinohara, Inui, Yamamoto, & Shimura, 2009; Soria-Gomez et al., 2007), some of which might interact with opioid signals in NAc shell (Faure, Richard, & Berridge, 2010; Mahler, Smith, & Berridge, 2007). Here we extended our analyses to orexin and acetylcholine systems in NAc, which modulate intake and food-motivated behaviors (Pratt & Kelley, 2004; Pratt, Spencer, & Kelley, 2007; Thorpe & Kotz, 2005).

Orexin-A (hypocretin) is a hypothalamic peptide implicated in reward (Barson, Ho, & Leibowitz, 2015; Berthoud & Munzberg, 2011; Harris et al., 2005; Sharf et al., 2010), as well as in arousal (Espana, Valentino, & Berridge, 2003; A. Rolls et al., 2011; Sutcliffe & de Lecea, 2002). Orexin can also amplify hedonic ‘liking’ reactions to sucrose taste, comparably to mu opioid stimulation, if microinjected into another opioid hedonic hotspot located in posterior ventral pallidum (Ho & Berridge, 2013; K. S. Smith & Berridge, 2005). Anatomically, hypothalamic orexin neurons send projections throughout the brain, including to NAc shell (but not core) (Baldo et al., 2003; Peyron et al., 1998). This projection pattern raises the question of

whether orexin might also enhance hedonic reactions within the NAc opioid hotspot, similarly to the ventral pallidum hotspot. Orexin-induced increases in food intake can be prevented by opioid blockade (2004) , and orexin in NAc can modulate phasic dopamine release, which may be related to incentive motivation to eat (Patyal, Woo, & Borgland, 2012; Thorpe & Kotz, 2005).

Acetylcholine (ACh) in NAc has also been implicated in food reward (Perry, Baldo, Andrzejewski, & Kelley, 2009; Perry, Pratt, & Baldo, 2014; Pratt et al., 2007), and this role has been suggested to involve interactions with NAc opioid and dopamine systems (Perry et al., 2014; Pratt & Kelley, 2005; Stouffer et al., 2015). Although early studies suggested that ACh might primarily suppress intake via satiety or aversion, since ACh levels rise gradually during food intake and after exposure to aversive tastes (Avena, Rada, & Hoebel, 2008b; Mark, Rada, Pothos, & Hoebel, 1992; Mark, Weinberg, Rada, & Hoebel, 1995), more recent studies have implicated endogenous ACh in NAc and striatum in the appetitive motivation for food rewards (Perry et al., 2009; Perry et al., 2014; Pratt et al., 2007). For example, blockade of endogenous ACh in NAc by microinjections of the muscarinic antagonist scopolamine suppresses food intake, and establishes learned taste or place avoidances. Further, the ability of mu opioid agonist microinjections in NAc to stimulate food intake is also blocked by simultaneous blockade of ACh muscarinic receptors (Perry et al., 2014), which alters striatal preproenkephalin mRNA levels (Pratt & Kelley, 2005), suggesting the possibility of an ACh-opioid interaction in NAc in food reward. Therefore it is of interest to examine the roles of endogenous ACh in NAc on the hedonic impact of palatable foods as well as on the motivation to eat.

Here we compared orexin-A microinjections and scopolamine microinjections at various sites in NAc medial shell for their effects on 1) intake of a palatable sweet food (chocolate candies), 2) positive ‘liking’ taste reactions elicited by oral infusions of sucrose

solution, and 3) negative 'disgust' reactions elicited by infusions of bitter quinine solution. Our results suggest the existence of a localized hedonic hotspot for orexin enhancement of hedonic impact in rostral NAc shell (similar to opioid enhancement). The results also suggest a more widespread anatomical substrate, distributed throughout entire NAc shell, for orexin stimulation of food intake. A similar distributed NAc network is suggested for endogenous ACh contributions to positive hedonic impact and to food intake, with an additional motivational role for the caudal half of NAc, where ACh blockade additionally releases a fear-related anti-predator reaction of defensive treading.

Methods & Materials

Subjects

Sprague Dawley rats (~3 months old) or male rats weighing at surgery (3-4 months old) (total *n* = 25 [female = 14, weight 250-300g; male = 11, weight 350-450g] were housed in same-sex pairs at ~21°C on a reverse 12h light/dark cycle and used in the microinjection-behavior tests. An additional, separate group of 4 rats were used solely for histological analysis of Fos plume diameters (to assess diameters of a 'first drug microinjection'). All rats had *ad libitum* access to food and water in their home cage. All experimental procedures were followed and approved by the University of Michigan Committee on the Use and Care of Animals.

Surgery

Rats were implanted with oral and cranial cannulas as described previously (Castro & Berridge, 2014b). Briefly, bilateral oral cannulas entered the mouth in the upper cheek pouch lateral to the first maxillary molar, ascended beneath the zygomatic arch, and then exited through the skin at the dorsal head cap (Grill & Norgren, 1978a). In the same surgery, permanent microinjection guide cannulas were bilaterally implanted. Bilateral coordinates were identical for

a given rat, but sites were staggered across rats to fill the entire NAc medial shell for the group as a whole. Rostral shell placements (n=15) averaged around +3.1 anteroposterior (AP) from Bregma, bilateral ±0.9mm mediolateral (ML), and -5.7mm dorsoventral (DV). Caudal placements (n=10) averaged between +2.6 to +2.8mm AP, ±0.9mm ML, and -5.7mm to -6.0mm DV. After surgery, each rat received subcutaneous injections of carprofen (5mg/kg) for pain relief, as well as topical antibiotic around the perimeter of the head cap. Rats received another dose of carprofen 24 h later, and reapplication of topical antibiotic, and were allowed to recover for one week before behavioral testing began.

Drug Microinjections

Rats were hand-held in the lap of the experimenter during NAc microinjections. Polyethylene PE-20 tubing was connected to a stainless steel microinjection cannula injector, which had a tip (16mm, 29 gauge) extending 2mm beyond the ventral end of guide cannulas to reach the NAc target site. On test days, solutions were brought to room temperature (~21ºC) prior to bilateral microinjection. Drugs were dissolved in a vehicle of artificial cerebrospinal fluid (ACSF). Microinjection solutions contained one of the following (drug order was counter-balanced across rats used for behavioral tests): 1) Orexin-A, a hypothalamic neuropeptide (500pmol/0.2 µl; also known as hypocretin-1); 2) Scopolamine, a muscarinic antagonist (10µg/0.5µl); or 3) ACSF vehicle alone in a volume of 0.2µl per side (vehicle control condition). Drug doses and volumes were chosen based on most behaviorally effective dose/volume from Thorpe and Kotz (2005) for orexin, and from Pratt et al. (2007) for scopolamine. Drugs were prepared fresh at the beginning of each test group, and then either frozen (ACSF, Orexin) or refrigerated (Scopolamine) in solution for testing later that week. Each 0.2µl microinjection was delivered during a 1min period at a speed of 0.2µl/min by syringe pump. After bilateral

microinjections, injectors were left in place for 1 minute to allow for drug diffusion, after which obturators were replaced and rats were immediately placed in the taste reactivity testing chamber. Each rat received bilateral microinjections of only one drug or vehicle solution per test day.

Taste Reactivity Testing

Before testing, rats were each extensively handled to familiarize them with experimenters. They were then habituated to the test chamber for 25minutes for 4 consecutive days, and received a mock injection of vehicle ACSF on the final day of habituation.

The taste reactivity test (Grill & Norgren, 1978a; Steiner, 1973; Steiner et al., 2001) was used to measure a rat's affective orofacial reactions to either a sucrose solution (1.0%, 0.029M) or a quinine solution ($3x10^{-3}$M). A 1ml volume of each solution was infused over a 1min period via syringe pump through plastic tubing connected to the rat's oral cannula (PE-50 connected to a PE-10 delivery nozzle). On each test day, the sucrose solution was infused 25min after a NAc microinjection of vehicle, orexin, or scopolamine. After a 5min delay, a 1min infusion of quinine followed for a second taste reactivity test. This order was used because if quinine were first, the bitterness disgust could easily carry over and suppress positive reactions to subsequent sucrose. However, sweet tastes do not appear to disrupt negative 'disgust' reactions to subsequent bitterness in our experience, and so a sucrose-quinine order of testing was used (Pecina & Berridge, 2005). Orofacial taste reactivity responses to both solutions were video recorded via close-up lens for subsequent slow-motion video-analysis as described previously (Castro & Berridge, 2014b).

Males and females were run in separate same-sex cohorts on different days to prevent any lingering opposite-sex odors from affecting behavior. Test chambers were cleaned with soap and

water at the end of each test day. Male and female Fos groups were also run separately for microinjections and perfusions to prevent any pheromone modulation of neuronal gene expression.

Food intake testing

A 1 hr free intake test was administered immediately following the taste reactivity test on each test day. Rats previously had been habituated to the food intake chamber during the 4 habituation days. Each intake chamber (23 x 20 x 45 cm) contained a large pile of palatable chocolate (M&Ms), and an ad lib water spout, and the floor was covered with 1 cm depth of corncob bedding. The amount of M&M candies (~20g) was weighed before and after testing to calculate amount of food intake, and water consumption was measured. All behavior was video recorded and later scored for eating behavior (duration in seconds), water drinking behavior (in seconds), grooming behavior (in seconds), treading (in seconds) and for number of bouts of food sniffs, food carrying or burying, cage crosses, and rears.

Histology and Fos-like protein immunohistochemistry

After the last day of behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital. Rats were decapitated and the brains were extracted and fixed in 10% paraformaldehyde solution for 1-2 days followed by a 25% sucrose solution in 0.1M NaPB for 2-3 days before slicing. 60 μm slices through the NAc were taken from each rat on a cryostat, mounted, dried, and stained with cresyl violet. Microinjection center was determined for each bilateral injection site and slides were compared with the stereotaxic atlas (Paxinos & Watson, 2007) to determine placement in the NAc.

Fos immunohistochemistry and plume analysis was performed on 4 naïve rats so that plume diameters would be maximal, and not shrunken due to gliosis/necrosis from any previous

microinjection. Fos analysis was also performed on 10 rats from the microinjection-behavior test groups for comparison (as previously described in Castro and Berridge (2014b)). In brief, rats received a microinjection of vehicle, orexin, or scopolamine 90 minutes before being euthanized and perfused. Brains were extracted, left in 4% paraformaldehyde for 24 hours, and switched to a 25% sucrose solution the following day. 40 μm slices through the NAc were taken on a cryostat and processed for Fos-like immunoreactivity using normal donkey serum, goat anti-c-fos (Santa Cruz Biotechnology), and donkey anti-goat AlexaFluor 488 (Invitrogen). Sections were mounted, air-dried, and coverslipped with Prolong Gold antifade reagent (Invitrogen).

The mapping of site effects for localization of function was constructed in a sagittal plane to allow representation of the entire rostrocaudal and dorsoventral extent of NAc medial shell. Symbols were color-coded to express the intensity of taste reactivity or food intake behaviors relative to vehicle. Symbols were sized to match the mean maximal diameter of measured Fos plumes. For statistical contrasts, sites between +2.4mm to +1.5mm anterior to Bregma were classified as in rostral half of medial shell (i.e., rostral to +1.5), and sites between +0.4mm to +1.5mm were classified as caudal placements (i.e., caudal to +1.5).

Statistical analysis

Statistical analyses were performed using non-parametric tests for within-subject (Friedman, Wilcoxon) and between-subject comparisons, and effect sizes and confidence intervals were included when appropriate.

Results

Fos plumes

Without drug, vehicle microinjections by themselves mildly increased local Fos by 125% over levels found in normal NAc tissue of intact brains, within a 0.38mm radius of the injector

tip (volume = 0.23mm^3). Orexin microinjections produced more intense local inner plumes of Fos elevation, which were >200% over those vehicle levels as well as over normal tissue levels (radius = 0.14mm for >200% over vehicle levels; volume = 0.011mm^3). This inner orexin-induced plume was surrounded by a larger plume of moderate >125% Fos elevation over vehicle levels (radius = 0.24mm; volume = 0.058mm^3), and that outer plume radius was similar to the orexin radius of 200% elevation over normal tissue levels (radius = 0.26mm; volume = 0.074mm^3). Thus the total diameter of a Fos plume induced by orexin was approximately 0.5mm, which was assigned to be the size of orexin site symbols in functional maps. Scopolamine microinjections produced a less intense inner excitatory Fos plume of >125% elevation over vehicle with a (radius = 0.20mm; volume = 0.034mm^3), surrounded by a larger inhibitory anti-plume where Fos expression was actually decreased by 25% below vehicle levels (radius = 0.23mm; volume = 0.051mm^3). This total diameter of 0.46mm was assigned to scopolamine symbols in functional maps. The scopolamine-induced inhibitory surround could reflect either lateral interactions between medium spiny neurons, or opposing drug effects at different drug concentrations as the drug diffuses away from the microinjection center. Collectively, these data indicate that even vehicle microinjections produce a local plateau of mild elevation in Fos immunoreactivity, while scopolamine adds a 125% greater inner peak plus an inhibitory outer surround, and orexin produces an even greater 200% inner peak with a broader base of 125% local elevation.

No sex differences in drug effects

Females and males were first compared for behavioral sex differences in taste reactivity or food intake measures after vehicle or drug microinjections. At baseline, female rats emitted more taste-elicited orofacial reactions than males after vehicle microinjections (vehicle, X^2 =

6.945, $p = 0.008$; sucrose: positive reactions, Z = 2.635, $p = 0.008$; sucrose: negative reactions, Z = 2.70, $p = 0.007$; quinine: negative reactions: Z = 2.097, $p = 0.035$). These results are consistent with earlier reports that females generally display higher orofacial reactivity to taste palatability than males (Clarke & Ossenkopp, 1998; Flynn, Schulkin, & Havens, 1993). Despite these baseline differences, males and females did not differ for orexin/scopolamine drug effects, expressed as percent change from vehicle baselines in taste reactivity (orexin, $X^2 = 2.865$, $p = 0.091$; scopolamine, $X^2 = 1.277$, $p = 0.258$), suggesting that the drugs similarly altered palatability in females and males. Since males and females did not differ in drug effects on hedonic impact, their data was pooled together for subsequent analyses of drug effects expressed as percent change from vehicle baselines. For food intake, no sex differences were observed in vehicle baseline, or in orexin or scopolamine conditions (vehicle, Z = 0.408, $p = 0.689$; orexin, Z = 4.633, $p = 0.110$; scopolamine, Z = 0.245, $p = 0.810$). Female and male data for food intake were therefore similarly pooled in subsequent analyses. All main effects described below applied to both sexes, unless noted.

Orexin in rostral shell enhances hedonic reactions to sucrose

Orexin-A microinjections altered orofacial reactions to sucrose, but only at particular rostrocaudal locations in NAc shell (Kruskal-Wallis, rostral vs caudal sites, $X^2 = 5.867$, $p = 0.015$) (Figure 3.1). At sites located in the rostral half of medial shell of NAc (i.e., overlapping with the previously identified opioid hotspot), orexin microinjection caused a 200% to 400% increase in the number of positive hedonic reactions elicited by sucrose taste, compared to vehicle control trials for the same rats (Friedman's ANOVA, $X^2 = 17.868$, $p = 0.000132$; Wilcoxon, Z = -2.559, $p = 0.010$; Rostral NAc, $X^2 = 21.571$, $p = 0.000021$; Z = -3.413, $p = 0.001$; $r = 0.88$; 96.5% CI [3, 6]). By contrast, orexin stimulation in caudal NAc shell produced

no change in hedonic reactions to sucrose (X^2 = 6.914, p = 0.032; Z = -1.193, p = 0.233). Thus, hedonic enhancements appeared to be restricted to a subregion in the rostral half of medial shell.

Within the rostral half of medial shell, the magnitude of enhancement of sucrose hedonic impact did not differ between dorsal and ventral portions of the rostral zone (Entire shell: X^2 = 0.153, p = 0.395; rostral, X^2 = 1.233, p = 0.267) (Figure 3.2). However, our ventral sites in this study did not extend into the most ventral 25% (~0.5mm) portion of rostral shell, making it difficult to know if the orexin hotspot filled the entire rostral half of medial shell or merely the dorsal two-thirds of the rostral half. The latter would be most similar to the original map of opioid hotspot (Pecina & Berridge, 2005). Microinjections of orexin in rostral shell did not alter aversive reactions to either sucrose (which always remained near zero (Z = -0.813, p = 0.416)) or to quinine (Z = -1.630, p = 0.103). Similarly, orexin microinjections even at rostral sites failed to alter positive hedonic reactions to quinine (which always remained near zero; Z = -1.414, p = 0.157).

Using the 0.5mm diameter outer Fos plume measurements to estimate extent of drug impact spread from effective sites, the rostral boundary of the orexin hedonic hotspot extended to where the corpus callosum joins hemispheres (AP +2.52). It is difficult to know if the boundary extends any more anteriorly because we did not have any sites further rostral than +2.28mm to Bregma. The caudal boundary was well mapped by posterior silent sites, which revealed the orexin hotspot extended caudally to the edge of the paralamboid septal nucleus (AP+1.44), beyond which orexin sites no longer had hedonic effects. The medial boundary reached approximately the lateral septum dorsally, the rostral ventral pallidum caudally, and the islands of Calleja at some mid AP sites ventrally (ML $\pm$0.51). The lateral boundary was always the border between NAc shell and core (ML $\pm$1.44). The hotspot extended dorsally to the lateral

septum and lateral ventricle (DV -6.06), and ventrally at least to the bottom 1/4th of medial shell (DV -8.34).

We calculated the volume of the orexin enhancement hotspot to be approximately ~1.34mm^3 using the inner 200% Fos plumes volumes. This volume is similar to the opioid hotspot previously reported in the same rostrodorsal region of medial shell (Castro & Berridge, 2014b; Pecina & Berridge, 2005), though slightly larger (112% compared to opioid hotspot). This similarity suggests that orexin and opioid signals share nearly the same anatomical hotspot within medial shell for hedonic enhancement of sucrose 'liking' reactions.

Orexin enhances food intake throughout entire shell

Orexin microinjection at virtually all sites throughout the entire medial shell of NAc increased palatable food intake by ~150% (chocolate M&M candies) compared to vehicle trials in the same rats (X^2 = 30.333, p = 0.0000001; Z = -2.001, p = 0.045; r = 0.28; 95.7% CI [2.8, 4.9]). Sites in the caudal half of shell were as effective as sites in the rostral half at supporting increases in eating (caudal mean = 7.41, S.E. = 1.09; rostral mean = 6.69, S.E. = 0.93; X^2 = 0.077, p = 0.781). Similarly, there was no difference between dorsal versus ventral sites in medial shell for orexin-induced increases in intake (X^2 = 0.013, p = 0.909). Thus, orexin increased eating equally throughout virtually the entire medial shell (Figure 3.4), consistent with previous reports by Thorpe and Kotz (2005). Widespread distribution of sites throughout NAc for orexin-induced increase in intake is also similar to mu opioid stimulation of eating throughout the entire NAc shell (despite the localization of hedonic hotspots for both in rostral shell, and not caudal shell) (Castro & Berridge, 2014b; Zhang & Kelley, 2000).

Scopolamine at all sites suppresses sucrose hedonic impact and elevates quinine 'disgust'

Microinjections of the muscarinic acetylcholine antagonist scopolamine in medial shell suppressed positive hedonic orofacial reactions elicited by the taste of sucrose by ~30% below vehicle control levels in the same rats (Figure 3.2) (X^2 = 17.868, p = 0.000132; Z = -2.585, p = 0.010; r = 0.37; 95.7% CI [-3, 0]). Essentially all sites throughout medial shell generated similar suppressions of 'liking' reactions to sucrose, with no difference in magnitude between rostral versus caudal sites (X^2 = 0.946, p = 0.397), or dorsal versus ventral sites (X^2 = 2.432, p = 0.119). However, despite suppressing hedonic reactions to sucrose, scopolamine microinjections never actually induced aversive gapes or other 'disgust' reactions to sucrose (X^2 = 4.617, p = 0.099; Z = -1.451, p = 0.147).

By contrast, aversive 'disgust' reactions to bitter quinine, which were already robust on control trials after vehicle microinjections, were nearly doubled in number after scopolamine microinjections at essentially all sites throughout medial shell (X^2 = 21.273, p = 0.000024; Z = -3.311, p = 0.001; r = 0.47; 95.7% CI [8, 23]). The elevation of quinine 'disgust' reactions was equally robust at sites, whether in rostral halves or caudal halves of medial shell (X^2 = 1.294, p = 0.255). Positive hedonic reactions to quinine, which were already nearly zero after vehicle microinjections, remained near zero and unchanged after scopolamine microinjections (X^2 = 2.8, p = 0.247).

Scopolamine suppresses food intake

Scopolamine microinjections throughout medial shell similarly caused a 50% suppression of intake of palatable M&M chocolate candies (X^2 = 30.333, p = 0.0000001; Z = 3.760, p = 0.00017; r = 0.53; 95.7% CI [-2.9, -2.5]). This intake suppression did not differ between rostral and caudal sites (Figure 3.3) (X^2 = 1.632, p = 0.201), again consistent with previous reports of intake suppression at various NAc sites by Pratt and Kelley (2005). ACh blockade also decreased

time spent drinking water (X^2 = 12.194, p = 0.002; Z = 2.898, p = 0.004) and time spent rearing (X^2 = 11.810, p = 0.003; Z = 3.00, p = 0.003).

Scopolamine increases fearful/defensive treading

Scopolamine microinjections, especially in the caudal half of medial shell, also caused a 5-fold increase in emission of defensive treading compared to vehicle days (X^2 = 12.194, p = 0.002; Z = 2.898, p = 0.004; r = 0.41; 95.7% CI [0, 20]) (Figure 3.4). Defensive treading was elicited more intensely at microinjection sites in the caudal half of shell than in the rostral half of shell (X^2 = 4.963, p = 0.026; Rostral: Z = 1.718, p = 0.086; Caudal: Z = 2.293, p = 0.022). Defensive treading or burying is a natural anti-predator response of rodents, which is used to throw debris forward toward a localized threat, sometimes actually burying the object (i.e. rattlesnake or shock prod) (Coss & Owings, 1978; Reynolds & Berridge, 2001, 2008; Treit, Pinel, & Fibiger, 1981). Defensive treading was not emitted randomly within the chamber (indicating it was not simply a motor reaction), but rather was directionally focused toward the four corners of the transparent plastic chambers, which may have reflected light in a slightly glittering fashion.

Potential independence of changes in taste reactivity, food intake, and defensive treading

Food intake and 'liking' reaction enhancement by orexin microinjections in the NAc rostral shell hotspot were not highly correlated (Spearman's R; ρ = -0.148, p = 0.598, R^2 = 0.0219). That appeared to be because orexin microinjections produced roughly 250% increases in sucrose-elicited 'liking' reactions, regardless of whether the intake increase was small (110-149% of vehicle levels) or large (>150%). Although scopolamine microinjections in NAc shell tended to suppress sucrose 'liking' reactions, increase bitterness 'disgust' reactions, suppress food intake, and in caudal shell increase defensive treading, there was not a close statistical

association among these effects. Similarly, the degree of scopolamine suppression of food intake was not correlated to the degree of suppression of hedonic reactions to sucrose or enhancement of quinine aversion (sucrose suppression: $\rho = -0.027$, $p = 0.90$, $R^2 = 0.05$; quinine enhancement: $\rho = 0.224$, $p = 0.293$, $R^2 = 0.014$). Finally, the increase in fearful/defensive treading caused by scopolamine microinjections was also statistically independent of the increase in 'disgust' reactions to quinine caused by those same microinjections ($\rho = 0.137$, $p = 0.0.672$, $R^2 = 0.019$). However, since taste reactivity and intake/treading behaviors were tested at different times after microinjections, their temporal separation might have promoted a degree of uncoupling. For sites outside the hotspot, orexin-induced increases in intake were not accompanied by any hedonic enhancement, making the stimulation of eating even more independent.

The hedonic suppression caused by scopolamine microinjections also was not tightly correlated to the elevation of quinine 'disgust' reactions ($\rho = -0.174$, $p = 0.417$, $R^2 = 0.03$). This result appears to be because scopolamine microinjections roughly doubled the number of quinine 'disgust' reactions regardless of whether it only slightly reduced sucrose 'liking' reactions (25% reduction; i.e., 75% of vehicle levels) or produced a greater hedonic suppression.

Discussion

Overview

Microinjections of orexin-A in the rostral half of NAc medial shell caused a 300% increase in the number of positive orofacial 'liking' reactions elicited by sucrose taste, whereas sites in the caudal half of shell failed to increase sucrose 'liking', revealing an anatomical orexin hotspot for hedonic enhancement. This hedonic hotspot was anatomically similar to the opioid hotspot in rostral shell previously mapped for mu, delta and kappa opioid stimulations (and overlapped with an endocannabinoid hedonic hotspot in dorsal shell previously mapped for

anandamide enhancements (Castro & Berridge, 2014b; Mahler et al., 2007). However, unlike opioid stimulation in NAc, orexin stimulation never suppressed 'liking' reactions at posterior sites in the caudal half of medial shell, which were merely hedonically silent. By contrast, the motivation to eat, expressed as higher food consumption, was increased by orexin at all sites throughout the entire medial shell. Widespread anatomical NAc stimulation of intake is also similar to mu opioid stimulation, which increases eating at all sites throughout shell and core, as well as in dorsal and ventrolateral regions of neostriatum, in central nucleus of amygdala, and in medial prefrontal cortex (Castro & Berridge, 2014b; DiFeliceantonio et al., 2012; Mahler & Berridge, 2009; Mena et al., 2011; Pecina & Berridge, 2005; Ragnauth, Moroz, et al., 2000; Richard et al., 2013; Thorpe & Kotz, 2005; Zhang & Kelley, 2000).

Regarding ACh in hedonic impact and motivation, scopolamine blockade of NAc acetylcholine muscarinic receptors typically suppressed both sucrose 'liking' reactions to below 50% normal levels and reduced palatable food intake to 50-70% of normal levels at most sites throughout medial shell. Scopolamine microinjections throughout nearly the entire NAc medial shell also doubled the number of 'disgust' reactions elicited by bitter quinine. However, scopolamine's induction of negative affect was never strong enough to actually create 'disgust' reactions to the sweet taste of sucrose (unlike GABA stimulations in caudal shell, which can reverse sucrose reactions from 'liking' to 'disgust' (Faure et al., 2010; Ho & Berridge, 2014; Reynolds & Berridge, 2002)). Finally, ACh blockade by scopolamine specifically in the caudal half of medial shell additionally elicited fear-related defensive treading behavior, which was directed towards locations in the chamber that may have been perceived as more threatening than others (e.g., light-reflecting corners). These results suggest that endogenous muscarinic ACh signals, when present, help maintain the overall positive hedonic impact of the taste of food, as

well as amplifying the motivation to eat, and in the caudal shell also potentially exerting an anxiolytic action.

Orexin rostral hotspot

Why the rostral half of NAc medial shell contains an anatomical hotspot for orexin and opioid hedonic enhancements needs further explanation, but it is known that rostral shell has several unique anatomical features that differentiate it from caudal shell, and which could be relevant. For example, the NAc rostrodorsal quadrant of medial shell has distinct inputs from a region in infralimbic cortex and outputs to ventral pallidum and hypothalamus that are different from other medial shell quadrants (Thompson and Swanson, 2010), resulting in a closed-circuit corticolimbic-thalamocortical loop that runs parallel to loops passing through other regions of NAc shell. In addition, the rostral half of shell also has septal-like anatomical features that distinguish it from the extended amygdala-like features of caudal shell (Thompson & Swanson, 2010; Zahm et al., 2013). Neurons in rostrodorsal medial shell also have distinct morphological features, such as fewer spiny dendrites and smaller medium spiny neuronal (MSN) cell bodies than other areas of NAc (Meredith et al., 2008; Zahm et al., 2013).

Neurochemically, the cellular mechanism for orexin enhancement of sucrose 'liking' also remains unclear. Orexin is typically thought to have excitatory depolarization effects on neurons (Korotkova, Sergeeva, Eriksson, Haas, & Brown, 2003; Marcus et al., 2001; Sakurai, Amemiya, Ishii, Matsuzaki, Chemelli, Tanaka, Williams, Richarson, et al., 1998; Trivedi, Yu, MacNeil, Van der Ploeg, & Guan, 1998; van den Pol et al., 2002; Zhu et al., 2003). However, neurons in NAc may exclusively contain orexin-2 receptors (OX_r2) (Ch'ng & Lawrence, 2015; Trivedi et al., 1998), which can be coupled to either Gi or Gq subunits, and may inhibit neurons via the augmentation of inhibitory GABA signals (Martin, Fabre, Siggins, & de Lecea, 2002; Zhu et al.,

2003). If so, neuronal inhibition by orexin could be more similar to GABAergic hyperpolarization or Gi coupled opioid or endocannabinoid signaling. Future work could clarify the role of NAc neuronal inhibition versus excitation for hedonic enhancement.

Scopolamine causes a shift towards negative affect and motivation

The ability of scopolamine microinjections throughout medial shell to suppress hedonic reactions to sucrose, increase aversive 'disgust' reactions to bitter quinine, and suppress intake of palatable food is consistent with the hypothesis by Kelley and colleagues (Kelley, Baldo, & Pratt, 2005) that endogenous ACh signals in NAc shell promote food intake (potentially by enhancing palatability). Their ACh-appetite hypothesis arose from the original demonstrations that scopolamine microinjections into NAc suppressed food intake, and that NAc scopolamine microinjections also could serve as an unconditioned stimulus to induce conditioned avoidance of either a paired taste or a paired place (Pratt & Kelley, 2004; Pratt et al., 2007). ACh also appears to interact with mu opioid signals in NAc shell, as indicated by reports that NAc scopolamine reduces preproenkephalin mRNA levels, and blocks the ability of mu opioid agonist microinjection in NAc to stimulate eating (Perry et al., 2014; Pratt & Kelley, 2005). A role for NAc ACh in incentive motivation and reward also seems consistent with reports that spontaneous firing in NAc neurons (including ACh interneurons) is evoked by reward events (Morris, Arkadir, Nevet, Vaadia, & Bergman, 2004), interactions between insulin and ACh interneurons can directly modulate dopamine release in response to food rewards (Stouffer et al., 2015), and that optogenetic inhibition of ACh interneurons in NAc prevents the establishment of a cocaine conditioned place preference (Witten et al., 2010).

Conclusion

These results reveal an anatomically localized hedonic hotspot in NAc rostral half of medial shell for orexin enhancement of sweetness 'liking', but more distributed orexin mechanisms in NAc shell for stimulating motivation or 'wanting' to eat palatable food. They also support a positive role for endogenous NAc acetylcholine signals in both hedonic impact and appetitive motivation. Collectively, these data help elucidate how orexin and ACh neurochemical signals in NAc contribute to sensory hedonic impact and the motivation to eat.

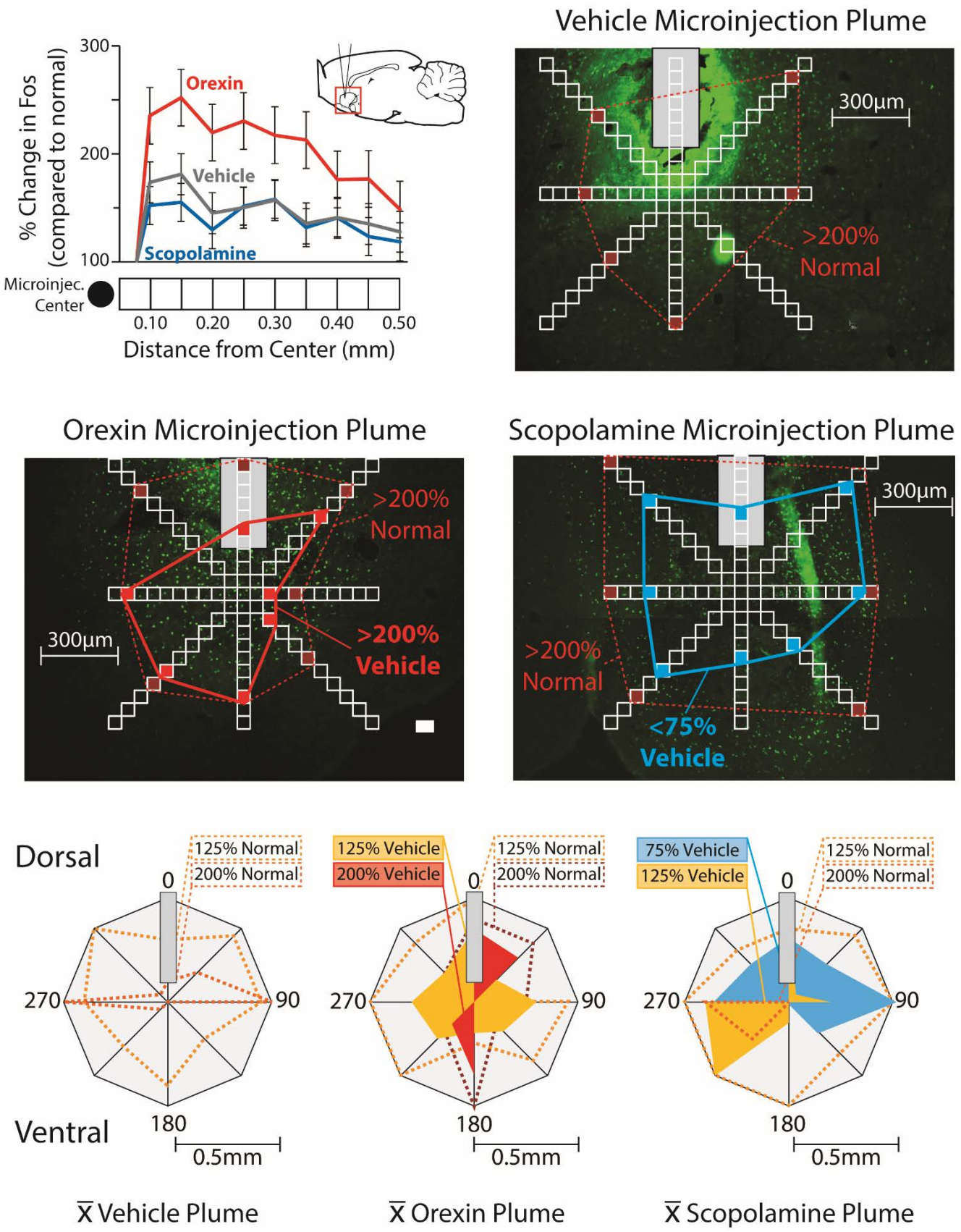
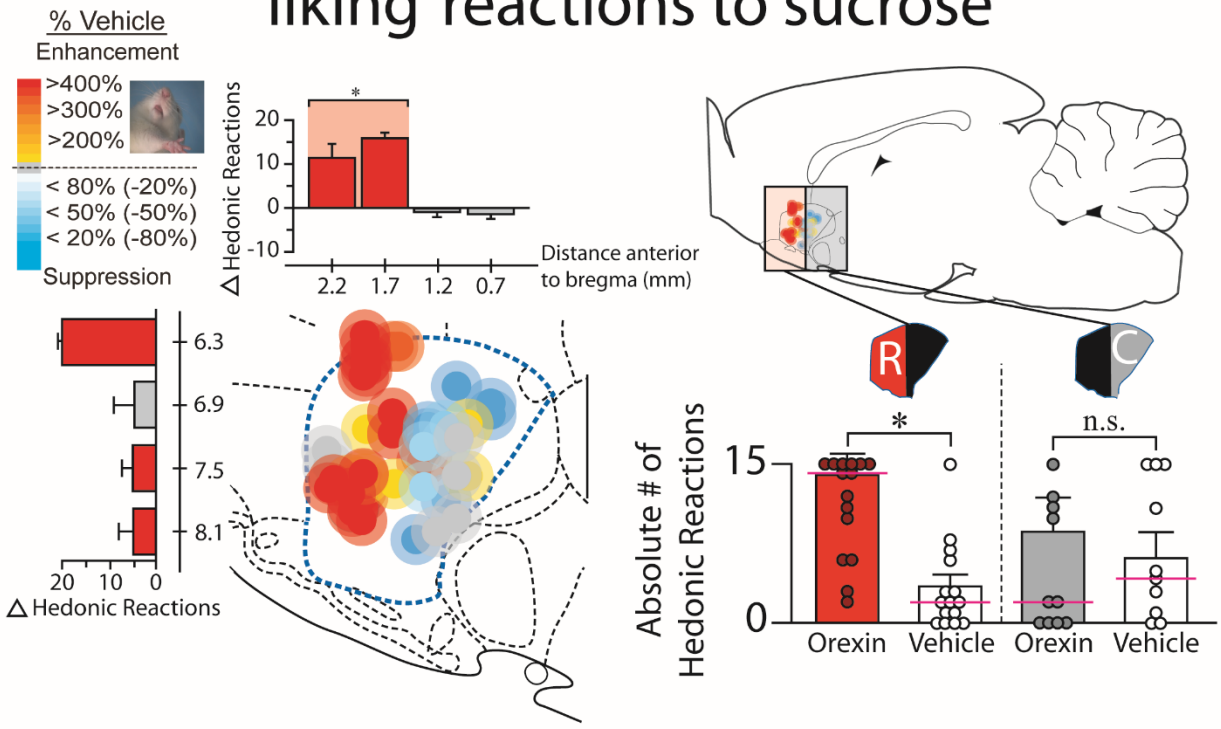

% Change in Fos (compared to normal)
300
200
100
Orexin
Vehicle
Scopolamine
Microinjec. Center
0.10
0.20
0.30
0.40
0.50
Distance from Center (mm)
Vehicle Microinjection Plume
300µm
>200% Normal
Orexin Microinjection Plume
>200% Normal
300µm
>200% Vehicle
Scopolamine Microinjection Plume
300µm
>200% Normal
<75% Vehicle
Dorsal
125% Normal
200% Normal
0
270
90
180
Ventral
0.5mm
X̄ Vehicle Plume
125% Vehicle
200% Vehicle
125% Normal
200% Normal
0
270
90
180
0.5mm
X̄ Orexin Plume
75% Vehicle
125% Vehicle
125% Normal
200% Normal
0
270
90
180
0.5mm
X̄ Scopolamine Plume

Figure 3.1. Orexin and scopolamine drug microinjection Fos plumes. Local Fos expression surrounding vehicle, orexin or scopolamine microinjections (top left). Photomicrographs show Fos expression after vehicle (top right), orexin (middle left) or scopolomaine (middle right) microinjections in NAc shell for individual rats, compared to levels in normal NAc tissue. Plumes 200% elevated over normal tissue levels are outlined with a dashed red line, whereas 200% elevations over slightly higher vehicle microinjection levels are outlined by red solid lines (blue solid lines = 25% decrease below vehicle-induced levels). Mean plume radius shown for vehicle (bottom left), orexin (bottom middle) and scopolamine (bottom right) microinjections relative to normal control brains (dashed lines) or vehicle brains (filled lines).

Rostral NAc orexin enhances hedonic 'liking' reactions to sucrose



NAc scopolamine suppresses hedonic 'liking' reactions to sucrose

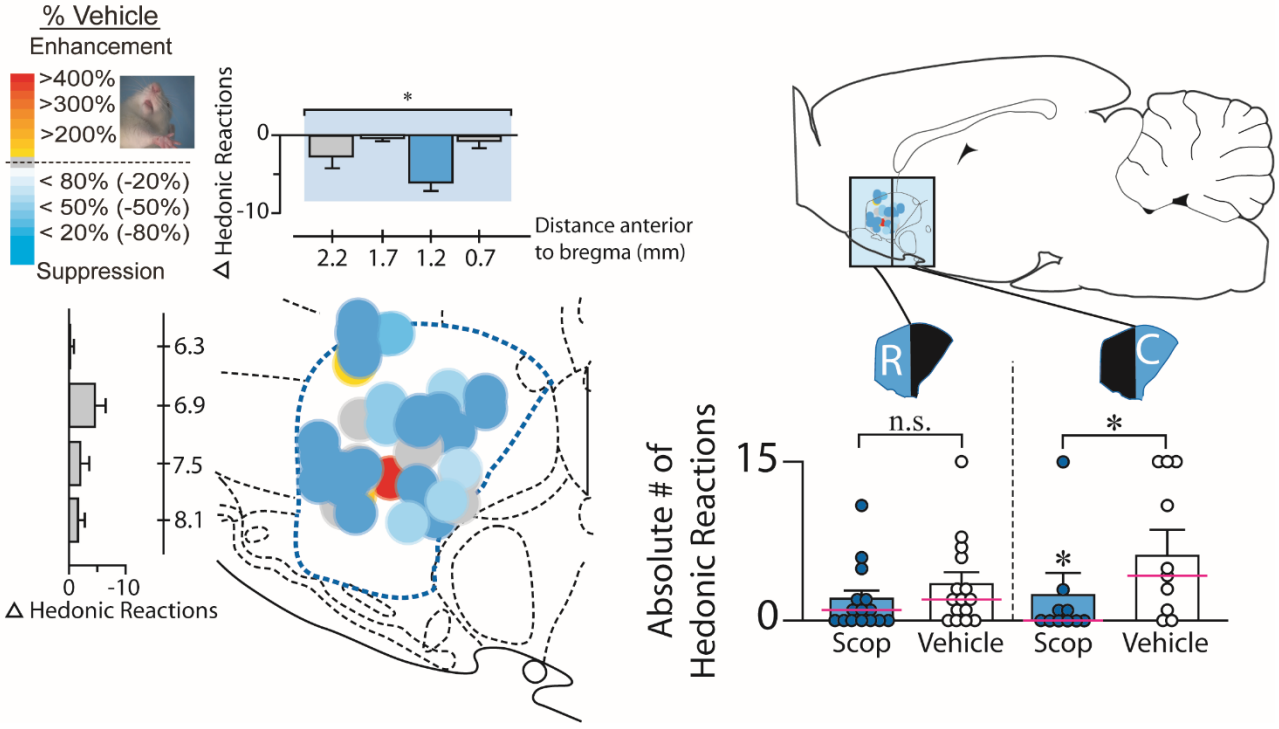
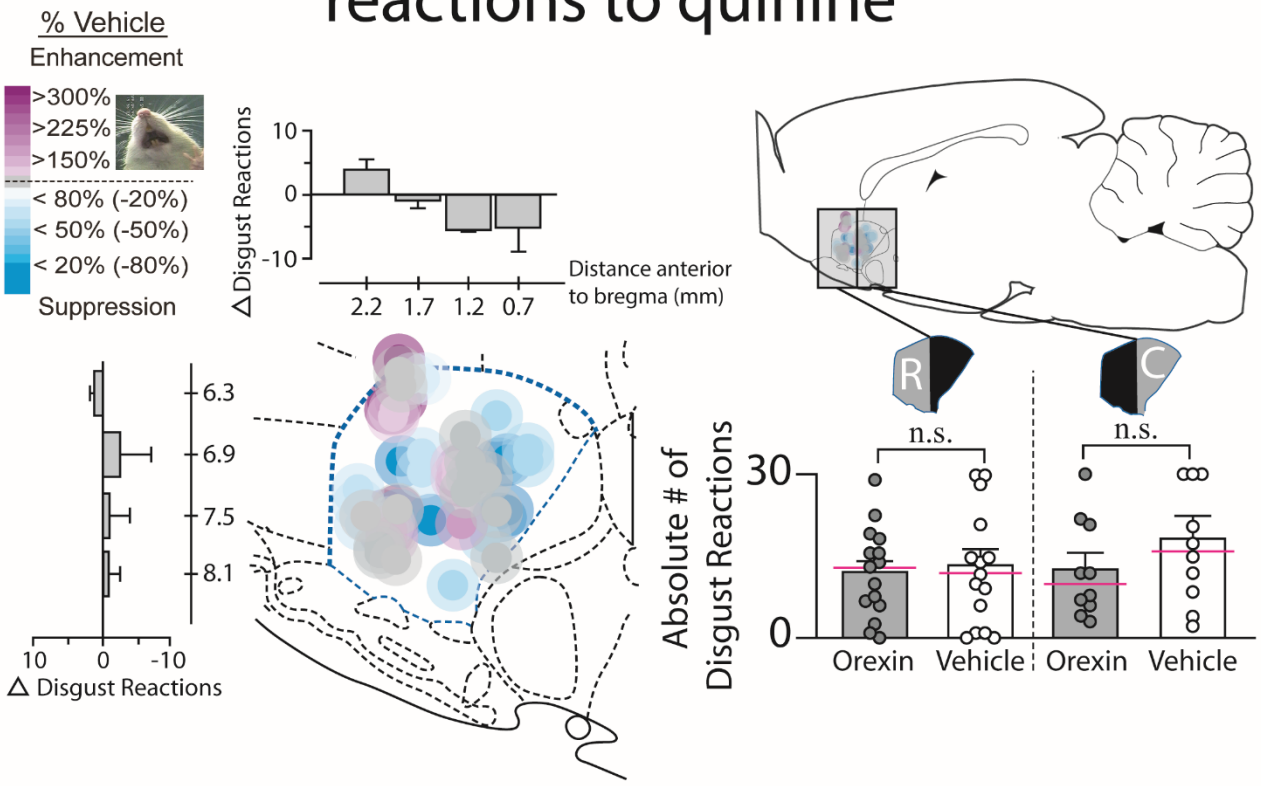


Figure 3.2. Orexin and scopolmaine oppositely modulate hedonic reactions to sucrose. Sagittal causation maps for localization of function in NAc medial shell, showing changes in orofacial hedonic ('liking') reactions elicited by sucrose taste after microinjections of either orexin or scopolamine (bottom; both compared to vehicle microinjection tests in the same rat). Each symbol placement indicates a microinjection site, the symbol size reflects the size of Fos plumes produced by that drug, and symbol color reflects the behavioral effects of the drug microinjection, shown as percentage change from vehicle control levels (enhancements: yellow-orange-red; suppressions: blue). Bars above and to the left of sagittal maps show mean absolute change in number of orofacial reactions induced by drug microinjection at that anterior–posterior or dorsal–ventral level. Total numbers of hedonic reactions are depicted in the bars graphs to the right of the sagittal maps, showing means with SEMs as bars (median as pink line), and individual data points as scatter-plot circles.

Orexin does not alter negative 'disgust' reactions to quinine



Scopolamine increases negative 'disgust' reactions to quinine

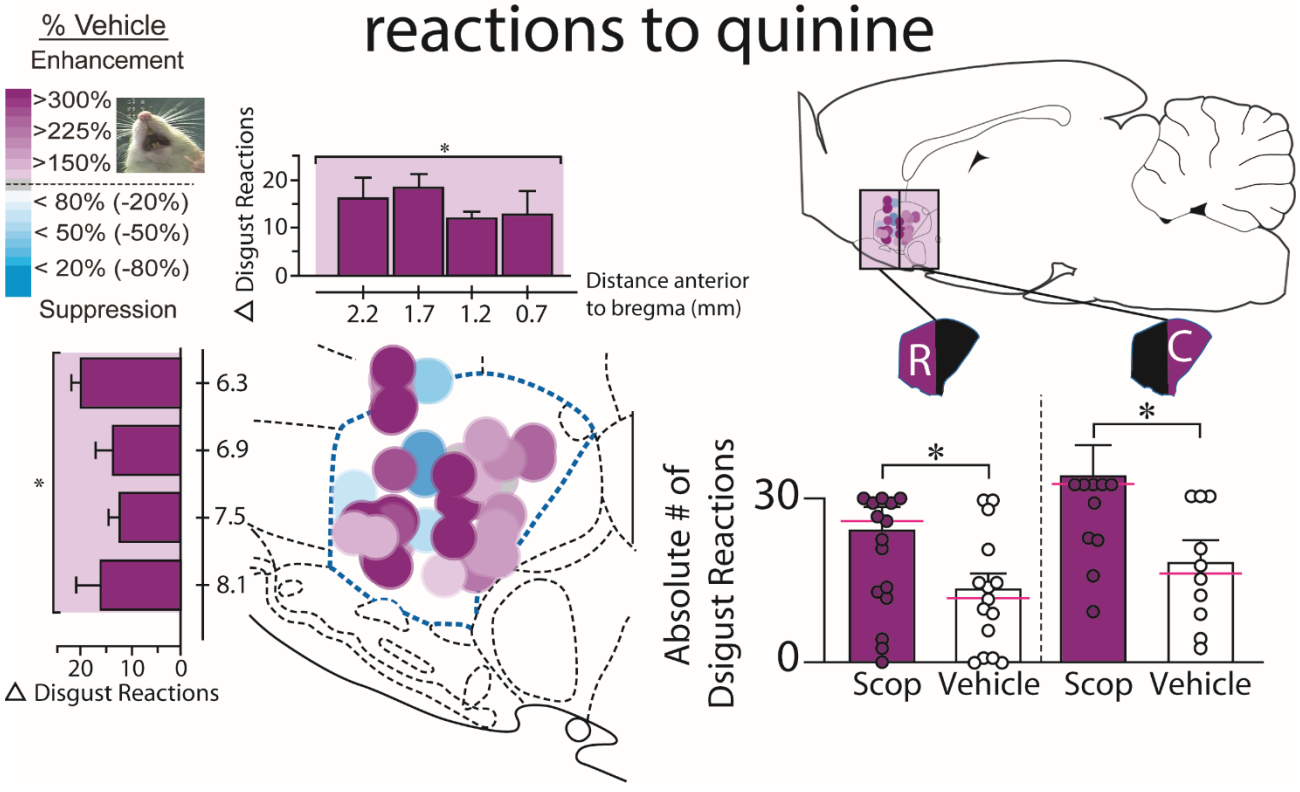


Figure 3.3. Scopolamine increases aversive 'disgust' reactions to bitter quinine. Sagittal maps showing changes in aversive ('disgust') orofacial reactions to bitter quinine taste after microinjections of orexin or scopolamine (bottom). Maps and symbols as in Figure 3.2, but with increases in 'disgust' reactions reflected by shades of purple.

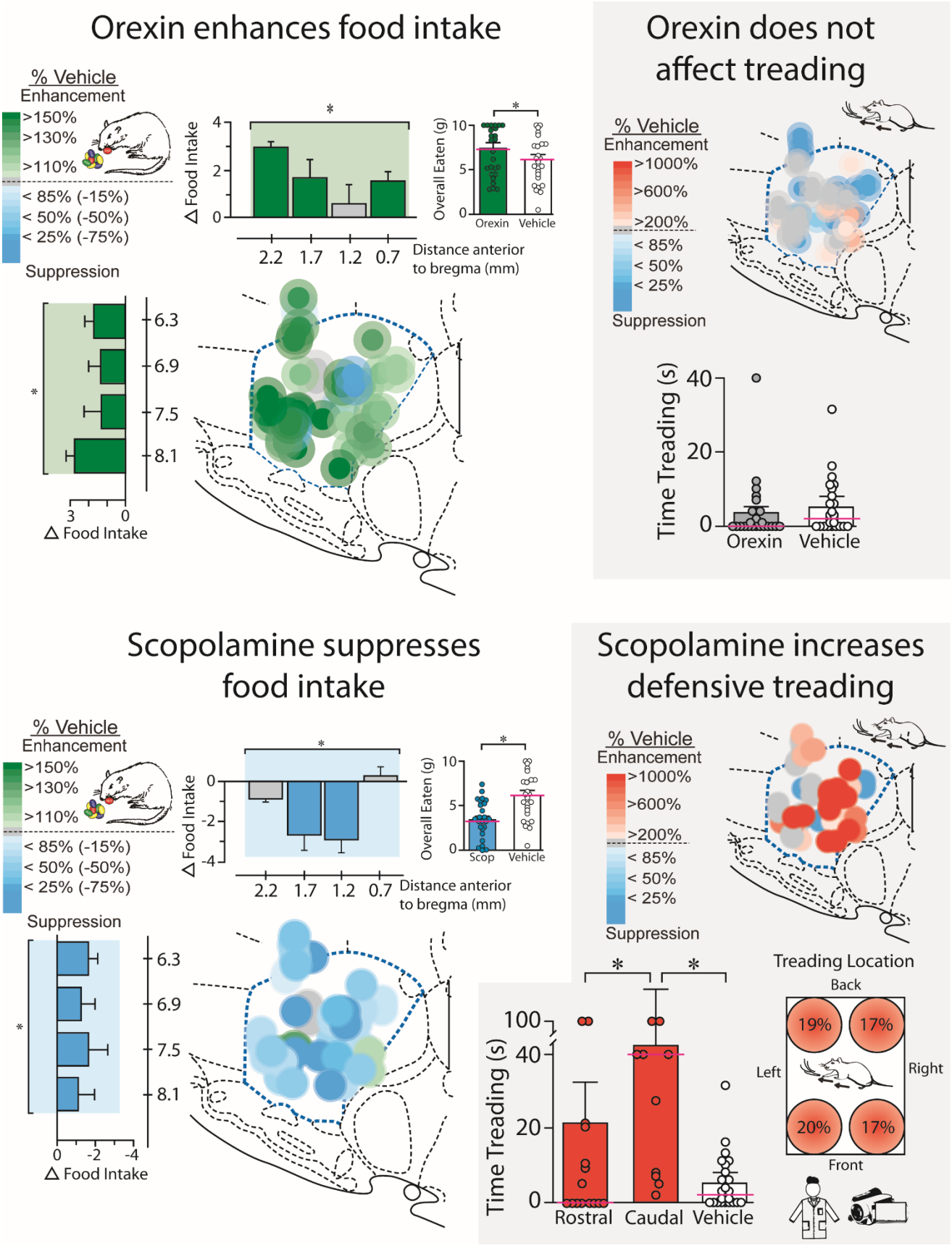

Orexin enhances food intake
% Vehicle Enhancement
>150%
>130%
>110%
< 85% (-15%)
< 50% (-50%)
< 25% (-75%)
Suppression
Δ Food Intake
Overall Eaten (g)
Orexin
Vehicle
2.2 1.7 1.2 0.7
Distance anterior to bregma (mm)
6.3
6.9
7.5
8.1
Δ Food Intake
Orexin does not affect treading
% Vehicle Enhancement
>1000%
>600%
>200%
< 85%
< 50%
< 25%
Suppression
Time Treading (s)
Orexin
Vehicle
Scopolamine suppresses food intake
Scop
Vehicle
Scopolamine increases defensive treading
Treading Location
Back
Left
Right
Front
19%
17%
20%
17%
Rostral
Caudal
Vehicle

Figure 3.4. Orexin and scopolamine oppositely affect food intake. Sagittal causation maps for changes in food intake (palatable chocolate M&M candies) and treading after either orexin (top) or scopolamine (bottom) microinjections. Intake effects are displayed as percentage changes from vehicle levels (enhancements: green; suppressions: blue) and treading effects are displayed as percentage changes from vehicle levels (enhancements: red; suppressions: blue). Maps and symbols otherwise as in Figure 3.2. The bottom right panel displays the amount of time spent treading at each corner of the chamber. Location of experimenter and video camera designate the front of the chamber.

CHAPTER 4:

CAUSAL MECHANISMS OF HEDONIC IMPACT AND MOTIVATION IN ORBITOFRONTAL CORTEX AND INSULA

Introduction

Positive hedonic reactions to pleasant events are needed for normal well-being, and pathological hedonic dysfunction contributes to depression, mood disorders, addiction binge eating, and related disorders. Affective neuroscience studies have identified brain mechanisms underlying hedonic reactions (K. C. Berridge & Kringelbach, 2015; Castro & Berridge, 2014a), but the role of cortex remains unclear.

On the one hand, human neuroimaging studies report that orbitofrontal cortex (OFC) and insula cortex encode the pleasantness of foods (Kringelbach & Rolls, 2004). For example, human fMRI studies have found increased BOLD signals in response to palatable food tastes/odors and decreased activity as participants reach satiety (Kringelbach, O'Doherty, Rolls, & Andrews, 2003; E. T. Rolls, Kringelbach, & de Araujo, 2003; Small, Zatorre, Dagher, Evans, & Jones-Gotman, 2001). These results suggest the possibility that limbic cortex activations may code the hedonic impact of a sensory pleasure. On the other hand, lesions of orbitofrontal cortex in humans do not reliably suppress positive hedonic or emotional reactions to pleasant stimuli (Beer, Heerey, Keltner, Scabini, & Knight, 2003; Bramham, Morris, Hornak, Bullock, & Polkey, 2009; Szczepanski & Knight, 2014), and even complete prefrontal lobotomy transection or

massive encephalitic lesions that damage medial orbitofrontal cortex, as well as insula and anterior cingulate cortex, can leave affective reactions and emotional exclamations remarkably intact despite causing cognitive impairments (Feinstein et al., 2010; Philippi et al., 2012). Similarly, in animal studies, neither cortical lesions nor complete decortication strongly suppress appetitive preference and seeking behavior or consummatory responses to palatable foods (Bales, Schier, Blonde, & Spector, 2015; Schier, Hashimoto, Bales, Blonde, & Spector, 2014; Wirsig & Grill, 1982). Thus cortical damage does not produce loss of positive hedonic reactions.

However, causal gains of hedonic function can be produced in some brain structures, even when lesions do not produce much loss of hedonic function (K. C. Berridge & Valenstein, 1991; Ho & Berridge, 2014). In subcortical structures, several "hedonic hotspots" have been identified, where neurochemical stimulations cause increases in positive 'liking' reactions elicited by sweetness. These subcortical hotspots are located in nucleus accumbens (NAc), ventral pallidum (VP), and in the brainstem parabrachial region of the pons in rats (Castro & Berridge, 2014a; Pecina, Smith, & Berridge, 2006; Soderpalm & Berridge, 2000). For example, microinjections of either a mu opioid agonist (DAMGO) or orexin-A into the rostrodorsal hotspot of medial shell in nucleus accumbens, or into the caudal hotspot of ventral pallidum, can double or triple the number of affective orofacial 'liking' reactions elicited by the taste of sucrose (Castro, Terry, & Berridge, 2016; Ho & Berridge, 2013; Pecina & Berridge, 2005; K. S. Smith & Berridge, 2005). However, only in the ventral pallidum do lesions induce loss of 'liking' reactions, and replace them with 'disgust' reactions to sweetness (Cromwell & Berridge, 1993; Ho & Berridge, 2014).

Recently, evidence for cortical causation of motivation has been reported. For example, optogenetic stimulation of 'sweet coding' site in insula gustatory cortex has been shown to

induce conditioned preference, whereas stimulation of a 'bitter coding' site induces conditioned avoidance and more directly causes mice to emit gapes when drinking water (Peng et al., 2015). Further, local mu opioid stimulation by DAMGO microinjections in OFC and medial PFC (infralimbic and prelimbic cortex) in rats causes increased food intake (Mena et al., 2011).

Opioid-induced stimulation of eating is a feature shared with hedonic hotspots of nucleus accumbens and ventral pallidum (Castro & Berridge, 2014b; K. S. Smith & Berridge, 2005). Although opioid-stimulation of eating also extends to many brain sites that lack hedonic enhancement capability (DiFeliceantonio et al., 2012; Mahler & Berridge, 2009; Noel & Wise, 1995; Zhang & Kelley, 2000), possession of that feature by limbic cortical regions raises the possibility that cortex might also contain an opioid hedonic hotspot(s) capable of hedonic enhancements. We aimed to assess that possibility here by making microinjections of the mu opioid agonist DAMGO at sites throughout the OFC and insula, and assessing hedonic effects on affective taste reactivity to sucrose or quinine. Further, since orexin-A stimulation in hedonic hotspots of nucleus accumbens and ventral pallidum is known to enhance 'liking' reactions to sweetness, we also aimed to compare hedonic effects of orexin-A microinjections in the same cortex regions.

Materials & Methods

Animals

116 Sprauge-Dawely rats (250-400g; male: n = 59, female: n = 47; behaviorally tested n = 95; cortical Fos plumes n = 21) were housed in a reverse 12h light/dark cycle at 21°C constant temperature. Chow and water were provided *ad libitum*. All procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Taste reactivity and cannulation surgery

Rats were anesthetized with a ketamine hydrochloride (80 mg/kg, i.p.) and xylazine (5mg/kg, i.p.) mixture, and then pretreated with atropine (0.05 mg/kg, i.p.) to prevent respiratory distress. Rats were then implanted with bilateral oral cannulae to permit oral infusions of sucrose and quinine solutions [polyethylene (Pecina & Berridge)-100 tubing]. Oral cannulae entered the mouth in the upper cheek pouch lateral to the first maxillary molar, ascended beneath the zygomatic arch, and then exited through the skin at the dorsal head cap (Grill & Norgren, 1978a). Rats were then placed in a stereotaxic apparatus (David Kopf Instruments), with the incisor bar set at -3.3mm below intraoral zero for flat skull measurements. Rats received bilateral NAc implantation of permanent microinjection guide cannulae (OFC: 12.5mm, 23-gauge, stainless steel; Insula: 14mm, 23-gauge, stainless steel). OFC placements (n = 19) ranged from +5.64mm to +2.76mm (AP) from bregma, ±0.2mm to ±3.4mm mediolateral, and -4.0mm to -6.8mm dorsoventral (DV). Insular cortex placements (n = 29) ranged from +4.2mm to -2.64mm (AP) from bregma, ±3.5mm to ±6.6mm mediolateral, and -5.6mm to -7.8mm dorsoventral (DV). Placements were scattered to fill in the majority of OFC or IC, but all placements were intended to be bilaterally symmetrical within each animal. Microinjection guide cannulae were anchored to the skull using surgical screws and dental acrylic, and stainless-steel obturators (28 gauge) were inserted to avoid any clogging, except for on behavioral testing days. After surgery, each rat received subcutaneous injections of carprofen (5mg/kg) for pain relief. Rats received another dose of carprofen 24 h later and were allowed to recover for one week before beginning behavioral testing.

Drug Microinjections

Rats were handled in the lap of the experimenter during NAc microinjections. PE-20 polyethylene tubing was connected to stainless steel microinjection cannula (OFC: 12.5mm, 29

gauge; IC: 14mm, 29 gauge) extending 1mm (OFC) or 2mm (IC) beyond the guide cannulae to reach the NAc target site. On test days, solutions were brought to room temperature (~21ºC) prior to microinjection. Microinjection solutions contained one of the following: 1) DAMGO, a selective mu receptor agonist at a dose of 0.05µg/0.2µl per side; 2) Orexin-A, an excitatory neuropeptide hormone (500pmol/0.2 µl per side); 3) ACSF vehicle alone in a volume of 0.2µl per side (vehicle control condition). Drugs were prepared at the beginning of each test group, and frozen in between test days. Drugs were dissolved in a vehicle of artificial cerebrospinal fluid (ACSF) and microinjected over a 1min period at a volume of 0.2µl per side at a speed of 0.2µl/min by syringe pump. After each microinjection, injectors were left in place for 1 minute to allow for drug diffusion, after which obturators were replaced. Rats were then immediately placed in the taste reactivity testing chamber. Each rat received only one drug or vehicle microinjection per test day.

Taste Reactivity Testing

Before testing, rats were each handled and habituated to the testing conditions for 25 minutes for 4 consecutive days, and they received a mock injection of vehicle, artificial cerebral spinal fluid (ACSF) on the final day of habituation. The taste reactivity test (Grill & Norgren, 1978a; Steiner, 1973; Steiner et al., 2001) is used to measure rat's affective orofacial reactions to either a 1ml volume sucrose solution or a 1ml volume quinine solution (sucrose: 1.0%, .029M; quinine: $3x10^{-3}$M, 1ml per test), which is infused via hollow tubing (PE-50 connected to a PE-10 delivery nozzle) connected to the rat's oral cannula and then attached to a syringe pump. At the peak pharmacological activation of microinjected drugs (25min for all three; vehicle, orexin, and scopolamine) testing occurred, where sucrose was infused evenly for 1min followed by a 1min infusion of quinine. Orofacial taste reactivity responses to both solutions were video recorded via

close-up lens and an angled mirror placed underneath the transparent floor for subsequent slow-motion video analysis.

Taste reactivity video scoring

Each rat's hedonic, aversive, and neutral taste reactivity responses were scored after testing in slow motion (1/30 s frame-by-frame to $1/10^{th}$ actual speed) using Observer software. Hedonic responses were classified as lateral tongue protrusions, rhythmic midline tongue protrusions, and paw licks (K. C. Berridge, 2000). Aversive reactions were classified as head shakes, gapes, forelimb flails, face washes, and chin rubs. Neutral responses were classified as ordinary grooming, passive dripping of solution out of the mouth, and rhythmic mouth movements. A time-bin scoring system was used to quantify positive and aversive reactions and to ensure that each component contributed equally to the overall calculations of taste reactivity responses (K. C. Berridge, 2000). Rhythmic mouth movements, paw licking, and passive dripping reactions were scored in 5 s time bins, while rhythmic midline tongue protrusions and chin rubs were scored in 2 s time bins. More discrete events, such as lateral tongue protrusions, gapes, forelimb flails, and head shakes, were scored individually every time they occurred. Total hedonic and aversive responses were then calculated. The sum of lateral tongue protrusions, rhythmic tongue protrusions, and paw lick scores represented total hedonic reactions. The sum of gapes, head shakes, face washes, forelimb flails, and chin rub scores represented total aversive reactions.

Food intake testing

For 1-hr immediately following the taste reactivity test on each day of microinjections rats were given free access to food and drink and subsequently video-recorded. Rats were habituated to the food intake conditions during the 4 habituation days. Each food intake chamber

(23 x 20 x 45 cm) was filled with 1 cm high of corncob bedding, a large pile of palatable chocolate (M&Ms, ~20g), and a water bottle. The amount of M&M candies was weighed before and after testing to calculate amount of food intake. Video was recorded and later scored for calculation of eating behavior (duration in seconds), water drinking behavior (in seconds), grooming behavior (in seconds), and for number of bouts of food sniffs, food carrying, cage crosses, and rears.

Histology and Fos-like protein immunohistochemistry

After the last day of behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital. Rats were decapitated and the brains were extracted and fixed in 10% paraformaldehyde solution for 1-2 days followed by a 25% sucrose solution in 0.1M NaPB for 2-3 days before slicing. 60 μm slices through the NAc were taken from each rat on a cryostat, mounted, dried, and stained with cresyl violet. Microinjection center was determined for each bilateral injection site and slides were compared with the stereotaxic atlas (Paxinos & Watson, 2007) to determine placement in the NAc.

To map the spread and impact of microinjections on local tissues, Fos immunohistochemistry was performed on a new set of rats (n = 21). Previous studies have shown that multiple microinjections and reduce the size of the Fos plume, thereby underestimating the functional spread of the drug. Rats were anesthetized and transcardially perfused 90 minutes after their last microinjection of vehicle, orexin, or DAMGO. Brains were sliced and processed for Fos-like immunoreactivity using normal donkey serum, goat anti-c-fos (Santa Cruz Biotechnology), and donkey anti-goat Alexa Fluor 594 (Invitrogen). Injection sites were scattered across OFC and IC to develop a single representative “cortical plume.” Sections were mounted, air-dried, and coverslipped with Prolong Gold antifade reagent (Invitrogen). Zones that

showed elevated expression of fluorescent Fos in the neurons surrounding microinjection sites were then assessed via microscope (Castro & Berridge, 2014a; Reynolds & Berridge, 2008).

The mapping of microinjection tips was done on two different sagittal planes to allow for the presentation of the rostrocaudal, dorsoventral and mediolateral components of OFC and IC. Color-coding was used to express the percent change of hedonic and aversive behaviors, as well as food intake, after drug microinjections compared to vehicle test days. Symbols were sized to match the maximal expansion of Fos plumes. The distinction between rostral and caudal placements in OFC was determined by placements around +3.72mm anterior to bregma, where sites falling between +5.16mm to +3.72mm were classified as rostral placements, and sites falling between +3.72mm to +2.52mm were classified as caudal placements. In IC, distinction between rostral and caudal placements was determined by placements around +0.0mm anterior to bregma, where sites falling between +3.72mm to +0.00mm were classified as rostral placements, and sites falling between +0.0mm to -3.0mm were classified as caudal placements. Sites falling outside of OFC or IC were also mapped and color coded on the same maps.

Results

DAMGO and orexin microinjections produced excitatory plumes surrounding the microinjection tip relative to vehicle microinjections ($F_{(2, 2885)} = 3.66$, $p = 0.026$; DAMGO: $p = 0.29$; orexin: $p = 0.010$). DAMGO produced a large outer plume within which Fos was elevated by 125% compared to vehicle injections (radius = 0.51mm; volume = 0.056mm^3). DAMGO injections also produced a more intense inner plume where Fos was elevated by 150% above vehicle levels within a radius of 0.25mm (volume = 0.065mm^3). Like DAMGO, orexin microinjections produced a large, mild outer plume where Fos was increased by 125% (radius = 0.55mm; volume = 0.7mm^3). A smaller, more intense inner plume with a radius of 0.16mm

showed 200% elevations (volume = 0.017mm^3). Both statistically and observationally, DAMGO and orexin plumes did not differ (p = 0.573), suggesting similarly sized effects of drug microinjections on local tissue. The plumes derived from DAMGO and orexin microinjections were assigned to be the size of drug site symbols in the functional maps.

No sex differences in drug effects

Females and males were first compared for behavioral sex differences in taste reactivity or food intake measures after vehicle or drug microinjections. Overall, there were no detectable differences between males and females on baseline reactions to sucrose (X^2 = -1.066, p = 0.286). There were also no differences in hedonic reactions between males and females after DAMGO or orexin microinjections (DAMGO: X^2 = -1.297, p = 0.195; Orexin: X^2 = -0.083, p = 0.934). Males and females also did not differ in the amount eaten at baseline (X^2 = 0.324, p = 0.973) or after DAMGO or orexin-A microinjections (DAMGO: X^2 = 0.243, p = 0.622; Orexin: X^2 = 0.506, p = 0.477).

To verify that there were no sex differences within brain regions, we also tested OFC or IC sites separately. We found that there were no baseline differences in hedonic reactions to sucrose (X^2 = 1.939, p = 0.164) but that males and females differed in baseline intake (X^2 = -2.064, p = 0.039) in OFC. We found a similar pattern after DAMGO (sucrose 'liking': X^2 = 0.913, p = 0.339; food intake: X^2 = -1.704, p = 0.088) or orexin microinjections (sucrose 'liking': X^2 = 0.078, p = 0.780; food intake: X^2 = -2.22, p = 0.026) microinjection. The difference in intake appears to be caused by females eating less at baseline compared to males (female mean intake = 4.45g, male mean intake = 7.71g). However, while this difference was maintained after DAMGO or orexin microinjection, the magnitude of the drug effect did not differ between males and females (change in food intake after DAMGO: X^2 = 0.002, p = 0.968; change in food intake

after orexin: $X^2 = 0.077$, $p = 0.781$), indicating that males and females are similarly affected by the drug.

No sex differences were found for baseline hedonic reactions to sucrose ($X^2 = 0.480$, $p = 0.489$) or food intake ($X^2 = 0.129$, $p = 0.720$) in insula, or after DAMGO (sucrose 'liking': $X^2 = 0.142$, $p = 0.707$; food intake: $X^2 = 0.353$, $p = 0.553$) or orexin (sucrose 'liking': $X^2 = 0.0001$, $p = 0.984$; food intake: $X^2 = 0.078$, $p = 0.780$) microinjection. Collectively, these results indicate that females and males may have differences in baseline food intake (at least in OFC), but that they are similarly affected by the drug microinjections. Therefore, data for both taste reactivity and food intake was pooled in subsequent analyses. All main effects described below applied to both sexes, unless noted.

OFC and insula each contain a "hedonic hotspot"

DAMGO or orexin microinjections in OFC altered affective taste reactivity elicited by infusions of sucrose into the mouth, but oppositely at different locations within OFC (rostromedial versus caudolateral OFC: $X^2 = 15.589$, $p = 0.000079$). A hedonic hotspot was revealed in the rostral and medial subregions of OFC, where either mu opioid or orexin stimulation doubled or even tripled the number of positive orofacial 'liking' reactions to sucrose (compared to control vehicle microinjection levels measured in the same rats) ($X^2 = 14.370$, $p = 0.001$; DAMGO: Z = -3.016, $p = 0.003$, $r = -0.81$, CI [2,12]; orexin: -2.835, $p = 0.005$, $r = -0.76$, CI [1,16] (Figure 4.2 and 4.3). By contrast, bitter quinine evoked robust numbers of negative 'disgust' reactions, and were left unchanged by either DAMGO or orexin microinjections within the OFC hotspot (quinine 'liking': $X^2 = 0.875$, $p = 0.646$; quinine 'disgust': $X^2 = 2.178$, $p = 0.337$). Thus opioid or orexin stimulation within the rostral OFC hotspot enhanced the positive hedonic impact of sweetness, but did not detectably alter the negative impact of bitterness.

The anatomical borders of the OFC hedonic hotspot were essentially identical for both DAMGO and orexin microinjections. This rostromedial hedonic hotspot in OFC began anteriorly and medially in orbital cortex extending medially along the midline of the brain into the medial orbital area at the front of the cingulate lobe (i.e., homologous to area 14, and immediately ventral to rostral prelimbic area/area 32v (Paxinos & Watson, 2007). The hotspot also extended laterally into ventral (area 13) and lateral (area12/47) orbital areas; in other words, the hotspot stretched throughout the entire ventral surface of rostral OFC. Our sites did not probe the dorsolateral orbital region of OFC, leaving undefined the hotspot's anterior dorsolateral edge. Traveling caudally, the dorsal edge of the hotspot comprised of prelimbic cortex medially and claustrum laterally, with the midline providing the medial border. Far rostral agranular insula provided the lateral border adjacent to lateral orbital area (more below). Along the A-P axis, the OFC hotspot extended from the OFC rostral pole through posterior portions of medial orbital area and the rostral half of ventral and lateral orbital areas (ending approximately at +3.5mm anterior to bregma A-P; ± 3.0mm lateral to midline M-L). The entire volume of this OFC hedonic hotspot was approximately 9.43mm^3, based on dimensions of 2.14mm A-P, 1.82mm D-V (based on average medial and lateral dorsoventral borders), and 2.42mm M-L (based on average dorsal and ventral mediolateral borders). M-L and D-V dimensions were calculated by averaging the dimensional borders at individual coronal sections located 0.5mm apart along the A-P axis of the hotspot. This sampling method accounted for the unusually shaped OFC at various points along the A-P, thereby reducing overestimations of the total volume. This method was applied to the coldspot and insula hotspot describe below. The OFC hotspot did not penetrate into the posterior one-third of ventral and lateral orbital region, which instead marked the rostromedial border of a suppressive hedonic coldspot, described below.

Suppressive coldspot: posterior OFC and Insula.

In the suppressive coldspot that filled posterolateral OFC and most of the insula, both DAMGO or orexin microinjections reduced the number of positive hedonic orofacial reactions elicited by sucrose to roughly one-half normal levels (33% - 50% suppression; $X^2 = 15.02$, $p < 0.001$; DAMGO: Z = -3.366, $p = 0.001$, $r = 0.65$, CI [-9, -2]; orexin: Z = -2.879, $p = 0.004$, $r = 0.55$, CI [-9, 0]) (Figure 4.2 and 4.3). Like the hotspot, the coldspot's affective modulation was specific to positive hedonic valence of sweetness, and did not alter the low number of negative 'disgust' reactions to sucrose ($X^2 = 2.239$, $p < 0.326$; DAMGO: Z = -0.086, $p = 0.931$; orexin: Z = -0.716, $p = 0.474$). Similarly, the robust levels of 'disgust' reactions elicited by bitter quinine were not altered by coldspot microinjections of either DAMGO or orexin ($X^2 = 3.314$, $p = 0.191$). Likewise, 'disgust' reactions to sweetness were never induced by DAMGO or orexin microinjections anywhere in the 4mm strip of OFC/insula coldspot ($X^2 = 2.1967$, $p = 0.338$), which merely reduced positive 'liking' reactions in a univalent fashion.

In a short A-P plane in posterior OFC (e.g., +3.72mm in front of bregma), the hedonic coldspot (lateral) and hedonic hotspot (medial) overlapped together (Figures 4.2 & 4.3). But apart from that plane, the suppressive OFC/insula coldspot was mostly posterior to the OFC hotspot, and the coldspot continued approximately 4mm caudally, through the whole anterior insula and even posterior insula (caudal to the middle cerebral artery). Throughout the extent from anterior insula to mid-posterior insula, coldspot sites were equally distributed across the agranular zone that is positioned ventromedially in the brain, the dysgranular zone lying immediately dorsal and lateral to it, and the granular zone that is most dorsolateral within insula. The hedonic coldspot therefore filled most of the insula: both anterior insula, which often has been assigned 'emotional' functions (Craig, 2011; Wager et al., 2013), and posterior insula that

has been traditionally assigned sensory functions, including all the gustatory insula region and mid-caudal portions of visceral cortex (~0.5mm from Bregma), just caudal to primary gustatory cortex.

The medial boundary of the rostral coldspot was ventral claustrum, infralimbic cortex (area 25) and dorsal peduncular cortex, and of the caudal coldspot also the dorsal endopiriform nucleus. The dorsal boundary for OFC portion of coldspot was claustrum (above OFC), and for the insula coldspot was frontal cortex (area 3), primary somatosensory cortex (e.g., jaw region), and posteriorly secondary somatosensory cortex. The ventral boundary was piriform cortex. The suppressive coldspot extended posteriorly past Bregma until reaching the caudal portions of visceral insula (~ -0.5mm behind Bregma). The volume of the entire coldspot of OFC/Insula was approximately 20.04mm^3, based on dimensions of 4.8mm A-P, 2.13mm D-V, and 1.96mm M-L. Posterior to the coldspot was a contiguous second cortical hotspot, which filled the remainder of posterior insula as described below.

Another hedonic hotspot in far posterior insula

The insula hedonic hotspot began anteriorly at approximately the same A-P level of the brain in coronal section as where the fornix diverges from a medial mass into bilateral columns, where the anterior edge of the third ventricle is located, and where the anterior commissure fades out posteriorly. Hotspot sites were uniformly distributed across ventral agranular, (mid) dysgranular and (dorsal) granular regions of far posterior (parietal) insula. This far posterior region of insula has been suggested to receive visceral and respiratory sensory inputs, on the basis of an electrophysiological mapping study (Cechetto & Saper, 1987), as well as somatosensory information (Shi & Cassell, 1998a).

Within the posterior insula hotspot, microinjections of either DAMGO or orexin again doubled to tripled the number of hedonic reactions elicited by sucrose (X^2 = 14.372, $p < 0.001$; DAMGO: Z = -2.937, p = 0.003, r = 0.63, CI [1, 11]; Z = -2.809, p = 0.005, r = 0.60, CI [1, 22]). These effects were selective to positive hedonic 'liking' reactions, and did not alter the robust 'disgust' gapes and related negative reactions elicited by quinine (X^2 = 4.769, p = 0.092). Anatomical comparisons confirmed that opioid/orexin stimulation produced significantly opposite effects in the rostral to mid posterior coldspot versus the far posterior hotspot in insula (X^2 = 34.320, $p < 0.0001$).

Anatomically, the insula hotspot extended posteriorly about 1.5mm, until ending at the border between insula and perirhinal/ectorhinal cortex. The rostral border is located just rostral to the A-P level that includes the caudal edge of ventral anteromedial thalamic nucleus (-2.0mm from Bregma) and of claustrum, and the rostral edge of dorsolateral amygdala. Medially, the insula hotspot extended to the claustrum through most of its length, and to the external capsule at caudal sites. Dorsally, the insula hotspot extended to the secondary somatosensory cortex, and ventrally to the piriform cortex. The total volume of the caudal insula hotspot was 4.83mm^3, based on dimensions 1.8mm A-P, 1.7mm D-V and 1.58mm M-L.

These results indicate that both OFC and IC contain hedonic hotspots where mu opioid or orexin stimulation can amplify the positive hedonic impact of sucrose taste. Further, those two hotspots are positioned like bookends, containing between them a continuous long 'coldspot' strip where the same neurochemical stimulations oppositely suppress the positive hedonic impact of sweetness. The functional hotspots also appear to be the same anatomical hotspots for DAMGO and orexin stimulations, as the degree of hedonic enhancement is highly correlated between these two drugs at individual microinjection sites. In other words, sites that produce

enhancements in hedonic 'liking' sucrose after DAMGO microinjections are also likely to be the same sites that enhance 'liking' after orexin stimulation ($r = 0.583$, $p = 0.002$.).

OFC and IC differently affect food intake

To compare the pharmacological manipulations of affective taste reactivity to the motivation to consume a sweet food, we also measured food intake of palatable M&M chocolate candies in 1-hr intake tests conducted immediately after each taste reactivity session. We found that throughout the entire OFC, comprising both the hedonic hotspot and coldspot regions, DAMGO microinjections increased food intake by about 130% over vehicle control days in the same rats over ($X^2 = 6.632$, $p = 0.036$; DAMGO, Z = -2.334, $p = 0.020$, $r = 0.54$, CI [-0.7, 4.3]). Within the OFC rostromedial hotspot, DAMGO increased intake by 169%, but did not significantly increase food intake within the posterolateral coldspot. Similarly, we found that chocolate intake was increased by 150% after orexin microinjections in the OFC hotspot, but that effects appeared to diminish as sites entered caudolateral OFC (Entire OFC: orexin, Z = -2.175, $p = 0.030$, $r = 0.50$, CI [0.7, 2.7]; hotspot: 150%) (Figure 4.5). Though somewhat in conflict with work by Mena et al. (2011) who showed that DAMGO stimulations could enhance food intake in caudolateral sites of OFC, it is likely that the difference can be explained by differences in dose. Mena et al. found that only doses that were 20 times more potent than the dose used in this study enhanced food intake. Therefore, we conclude that while the entire OFC can enhance food intake, sites in the rostral hotspot may be especially sensitive to mu-stimulated eating.

Unlike OFC, neither orexin nor DAMGO microinjections reliably stimulated intake when stimulated sites moved further lateral into insula ($X^2 = 2.78$, $p = 0.249$). Although few individual rats (15% of rats) with insula sites did show >150% increases in intake after either DAMGO or orexin microinjection, most insula sites produced no change (65%), and the remaining sites

(20%) showed modest increases (~115%). Thus overall, insula sites for orexin or DAMGO produced no change in intake, not even in the hedonic hotspot site of far posterior insula (X^2 = 2.516, p = 0.284). As a caveat, our food intake tests were always conducted serially after taste reactivity tests on the same day. Our serial procedure was chosen because we were primarily interested in hedonic impact mechanisms; serial testing on the same day allowed us to additionally sample intake effects as a secondary aim without either exceeding 4 microinjections per rat or doubling the total number of rats in the study. However, serial testing arguably made our intake test less sensitive than if intake were measured separately (e.g., in different rats or on a different day) immediately after a microinjection (Bakshi & Kelley, 1993; Mena et al., 2011) because serial testing imposed a 30min delay before food intake could begin, and interposed oral infusions of sucrose followed by quinine. We cannot rule out the possibility that this delay and intervening stimuli disrupted weaker intake stimulation effects that might otherwise have been detected within insula.

Thus regarding intake, we conclude that OFC sites support robust stimulation of eating behavior and consumption by DAMGO and orexin, both in OFC hedonic hotspot and OFC hedonic coldspot. By contrast, insula sites for DAMGO and orexin do not share the same robust intake stimulating capacity. Whether weaker intake stimulation sites might be found in future in insula, especially in the hedonic hotspot of far posterior insula, is a question that would require future studies to answer.

Surrounding cortical sites

Unlike the OFC and insula, microinjection sites into prelimbic, anterior cingulate, olfactory, piriform, primary/secondary sensory, or primary/secondary motor cortex did not demonstrate any anatomically dissociable pattern of hedonic capability. Stimulation of medial

PFC as a whole (cingulate, prelimbic, infralimbic) via DAMGO or orexin did appear to alter hedonic reactions to sucrose ($X^2 = 0.559$, $p = 0.756$), nor did more anatomically driven analysis (cingulate: $X^2 = 0.667$, $p = 0.717$; prelimbic: $X^2 = 0.744$, $p = 0.689$; infralimbic: $X^2 = 2.923$, $p = 0.232$). Interestingly, prelimbic stimulation via orexin (but not DAMGO) consistently decreased 'disgust' reactions to quinine ($X^2 = 9.33$, $p = 0.009$; DAMGO: Z = -0.312, $p = 0.755$; orexin Z = -2.319, $p = 0.020$) without altering aversive reactions to sucrose ($X^2 = 3.00$, $p = 0.223$). Other than this, mu opioid or orexin stimulation in mPFC never consistently altered hedonic reactions, with roughly 55% of sites being totally silent and the other 45% randomly enhancing (>150%) or suppressing (<50%) reactions. Like mPFC, neither olfactory nor piriform cortex showed any ability modulate hedonic reactions to sucrose (olfactory: $X^2 = 1.733$, $p = 0.42$; piriform: $X^2 = 1.067$, $p = 0.587$). While a few individual sites show increases/decreases, there is no localizable pattern that we could detect, and, like mPFC, ~55% of the tested sites were completely silent. Lastly, neither motor nor sensory cortices showed any consistent effects on affective reactions to sucrose ($X^2 = 0.364$, $p = 0.834$).

For food intake, we did not observe increased food intake after DAMGO or orexin stimulation in mPFC ($X^2 = 0.187$, $p = 0.911$). These results were somewhat surprising, as Mena et al. (2011) has previously reported that DAMGO stimulation in mPFC can robustly enhance food intake. However, as noted above, our dose of DAMGO was 20-fold lower than the most effective dose reported in Mena et al. (2011). These results suggest that mu stimulated eating in OFC may be easier to elicit than mPFC, or perhaps that more pure tests of food intake may yield more sensitive results (whereas our animals were tested after taste reactivity testing). Like mPFC, we do not see an increase in food intake after drug microinjection into olfactory cortex ($X^2 = 2.80$, $p = 0.247$) or into other frontal cortices including primary and secondary

somatosensory and motor cortex (X^2 = 1.652, p = 0.438). By contrast, we do appear to see a general increase in consumption in piriform cortex after DAMGO stimulation (Overall: X^2 = 6.222, p = 0.045; DAMGO: Z = -1.886, p = 0.059; Z = 0.471, p = 0.637). This is the first report that we know of that extends DAMGO stimulated food intake to also include piriform cortex. Interestingly, our sites appear to overlap with sites in piriform cortex where NPY, somatostatin or GABA stimulation reduces amino acid consumption in amino acid deprived rats, further implicating piriform cortex in food reward (Cummings, Truong, & Gietzen, 1998; Truong, Magrum, & Gietzen, 2002). This diffuse pattern of stimulated eating (relative to the anatomically confined hotspots) is consistent with mu receptor stimulation in subcortical structures like NAc, central amygdala, and dorsal striatum. Unlike DAMGO, orexin stimulated eating seemed somewhat more constricted, and did not appear to extend into piriform cortex.

Altogether, our results show that while anatomically discrete sites in OFC and insula are involved in affective generation, the mechanisms underlying motivated 'wanting' for food rewards is more broadly distributed throughout cortex.

Discussion

Code versus cause

In the results presented above, we examined the causal role of cortex in affect and motivation, focusing on brain sites that have extensive correlative evidence suggesting their involvement. One candidate area was OFC. Human and nonhuman studies (either through fMRI or electrophysiology) have found OFC to respond to and potentially track the valence of various sensory stimuli. In particular, increased BOLD activity has been shown to correlate with self-reported pleasantness ratings of tastes/odors/flavors. In a study by Rolls et al. (2003), it was shown that odor pleasantness ratings correlated with medial OFC activity, whereas odor

unpleasantness was associated with increased BOLD activity in lateral OFC. A similar localization was found with chocolate (milk) consumption, where initial positive ratings were correlated with increased BOLD activity in medial OFC (Kringelbach et al., 2003; Small et al., 2001). Like the human imaging studies, there are also a few non-human studies showing that OFC may code reward value. For example, neuronal activity in macaque OFC has been shown to code both rewarding and aversive outcomes, perhaps implicating OFC in monitoring incentive value of stimuli (Hosokawa, Kato, Inoue, & Mikami, 2007; Roesch & Olson, 2004; Tremblay & Schultz, 1999). OFC has also been shown to selectively monitor reward amount, whereas other regions, like the nearby dorsolateral PFC, appears to direct executive/motor output (Wallis & Miller, 2003).

BOLD activity in insula has also been associated with both positive and negative emotional/affective valence, especially at rostral sites. In the chocolate study by Small et al. (2001) mentioned above, decreased ratings of chocolate pleasantness as participants reached satiety were correlated with decreased activity in rostral insula, perhaps suggesting that rostral insula was sensitive to initial reward value or appraisal. By contrast, rostral insula has also been shown to have increased BOLD responses to disgusting images (Calder et al., 2007; Mataix-Cols et al., 2008), unpleasant visuo-tactile sensations (e.g., slimy substance with an image of a snail) (Lamm, Silani, & Singer, 2015), and intense, aversive shocks (Wager et al., 2004). Although less is known about mid/caudal insula, Simmons et al. (Simmons et al., 2013) found that lower peripheral glucose levels predicted greater increased BOLD activity after participants viewed pictures of food relative to non-food images. The enhanced signal could reflect the affective changes that occur during hunger alliesthesia. Caudal insula has also been associated with

pleasant touch, with greater BOLD responses correlating with the pleasantness of the tactile stimulation (Segerdahl, Mezue, Okell, Farrar, & Tracey, 2015).

Although fewer in number, there are several notable studies showing gain of function, or causal, roles for cortex in affect and motivation. In 1972, Routtenberg and Sloan showed that medial OFC and some rostral sites in IC supported electrical self-stimulation in rats (Routtenberg & Sloan, 1972). More recently, Mena et al. (2011) demonstrated that opioid stimulation in medial PFC or ventrolateral OFC was sufficient to drive spontaneous eating. In rostral insula, electrical stimulation can induce ecstatic auras ("…intense feeling of bliss, enhanced well-being, and heightened self-awareness…") in patients suffering from seizures seeded in the same area (Picard & Craig, 2009). Electrical stimulation in monkeys also produces positive emotional behaviors (though this time clustered in mid-caudal insula), increasing affiliative behaviors (Caruana, Jezzini, Sbriscia-Fioretti, Rizzolatti, & Gallese, 2011). By contrast, electrical stimulation studies in cat and monkey have shown that stimulation of rostral insula can elicit 'disgust' reactions, with cats displaying 'disgust'-like licking ("…as if to remove an irritant…") (Hess, Akert, & Mc, 1952), and monkeys actively spitting out normally preferred food or throwing away food in their hands (Caruana et al., 2011).

Here we show that OFC and insula each contain a hedonic hotspot that possesses the functional capacity to amplify the hedonic impact of sweetness. These hotspots are defined as anatomically distinct sites in which mu opioid or orexin-A stimulation cause 200%-300% increases in orofacial 'liking' reactions elicited by sucrose. OFC possessed a far anterior hotspot at the rostral tip of the cortex (caudal and dorsal to olfactory bulb/nerve). 'Liking' reaction enhancements were observed in all three OFC subregions, so long as they were in the rostral 4/5 of OFC, filling nearly 80% of the entire structure. Insula also contained a hedonic hotspot that

was roughly 5mm^3, located in the caudal 1/3 of the structure. These two hotspots are positioned like bookends, encasing a long suppressive hedonic coldspot where opioid or orexin stimulation oppositely suppressed 'liking' reactions to sucrose by 30%-50%. The coldspot stretched between the hotspots as a continuous 20mm^3 strip along the ventrolateral surface of the brain, running from posterolateral OFC to posterior insula. The position, shape and size of these hedonic hotspots and coldspots in cortex were virtually identical across mu opioid and orexin maps of cortical hedonic causation.

Cross-species homologues

How do our functionally and anatomically localized hotspots in the rat map onto human/primate cortex? Humans have three types of prefrontal tissue: rostral granular cortex (areas 10, 11), middle granular cortex (14, 13), and caudal agranular cortex (14, 13, 12/47) cortex. By contrast, rat OFC is exclusively agranular, making it unlikely that there are any sites homologous to human areas 10, 11, and some sites in areas 14 and 13. However, most anatomists agree that rostral OFC in rats is most similar to caudal OFC zones of human/primate cortex (which includes areas 14c and 13a) (Wallis, 2012). Extending out of caudolateral OFC appears to be a transition zone leading to rostral insula (Price, 2007). This portion of rostral insula is agranular and receives strong inputs from amygdala, and is likely comparable to Brodmann areas Iam, Iapm, and Iai. Although not widely considered insula in rats until the mid-1990's (Paxinos and Watson, 1998), the caudal most portions of rat insula do share features of connectivity similar to human insula, with direct efferents to amygdala and afferents from visceral S2 cortex (Shi & Cassell, 1998a, 1998b). Early parcellation of insula based on cytoarchitectonic features identified all three basic subregions of insula rodents, with the major difference between rodent and human brains attributable to a greater number of subregions in humans (Nieuwenhuys, 2012;

Rose, 1928). The other notable difference between rodent and primate insula is the orientation of the three subregions. In humans, there appears to be a rostrocaudal gradient created from three concentric belts of tissue, with rostroventral insula comprised of agranular tissue, followed by a large middle dysgranular zone, and culminating in a caudodorsal granular zone. By contrast, rodent insula is more or less organized as a flattened band along of the brain with the three subregions oriented dorsoventrally (ventral agranular, middle dysgranular, dorsal granular). In other words, human insula appears to reorient itself by 90 degrees (relative to rodent insula), as well as curl the tissue to account for the Sylvian fissure, abutting the surrounding operculum. These architectural deviations suggest two potential ways for our rat insula cold/hotspot to be overlaid onto human insula: 1) If the hot/coldspot is perfectly conserved in humans, it is possible that there may be horizontal columns of hot or cold sites along the rostrocaudal axis (since our hot and coldspot infiltrated all three subregions of insula), or 2) it is possible that individual hot and cold sites exist at comparable rostrocaudal locations within each subregion, e.g., a hotspot in the caudal half of each subregion and a coldspot in the rostral half of each subregion. Both hypotheses could account for the functional discrepancies described above for rostral or caudal localizations of function, though future work will be necessary before any strong claim can be made.

Since rodents and humans appear to share more or less common cortical anatomy (except perhaps far rostral portions of human OFC), it is worth considering whether the functional boundaries defined by our microinjection maps already adhere to previously defined functional zones in cortex. One example of a functionally defined zone in insula is primary gustatory cortex (GC). In rats, GC extends rostral to and somewhat caudal from the middle cerebral artery, occupying a ~2mm long strip of rostral agranular insula (Kosar, Grill, & Norgren, 1986). Human

and nonhuman primate imaging studies likewise show rostral insula/frontal operculum as containing primary gustatory cortex (Small, 2006). Further elaborating GC localization, Peng et al. (2015) have proposed distinct zones for sweet and bitter coding, and Shier et al. (2014) have mapped a special site in far caudal GC and visceral cortex important for the expression of conditioned taste avoidances. Does our OFC/insula coldspot map onto any of these functionally defined regions? Based on our microinjection map, the coldspot extends beyond traditional GC, both rostrally and caudally, infiltrating OFC at rostral sites and extending throughout caudal visceral cortex. Our coldspot also infiltrates all three subregions of insula (granular, dysgranular, and agranular), whereas GC and the specialized taste modality zones are localized to agranular insula. Further, the Shier site noted above sits on the border between the coldspot and IC hotspot, leaving unresolved its contribution to our affective maps. Altogether, it does not appear that the functionally defined hotspots and coldspot in our study can be explained through previously delineated zones of taste or visceral processing, indicating novel sites for affective generation within cortex, in addition to whatever other roles OFC and insula may have.

Necessity versus sufficiency

Though we have shown localized causal sites within cortex for affective generation, previous work suggests that cortex may not be necessary at all for affective processing. One extreme example involves Patient R, who is missing substantial portions of medial OFC and only has 10% of his insula remaining after a traumatic brain injury (with the remaining insula likely not functional) (Philippi et al., 2012). Despite these massive lesions, R does not appear to lack the ability to feel and/or express various emotions. For example, he

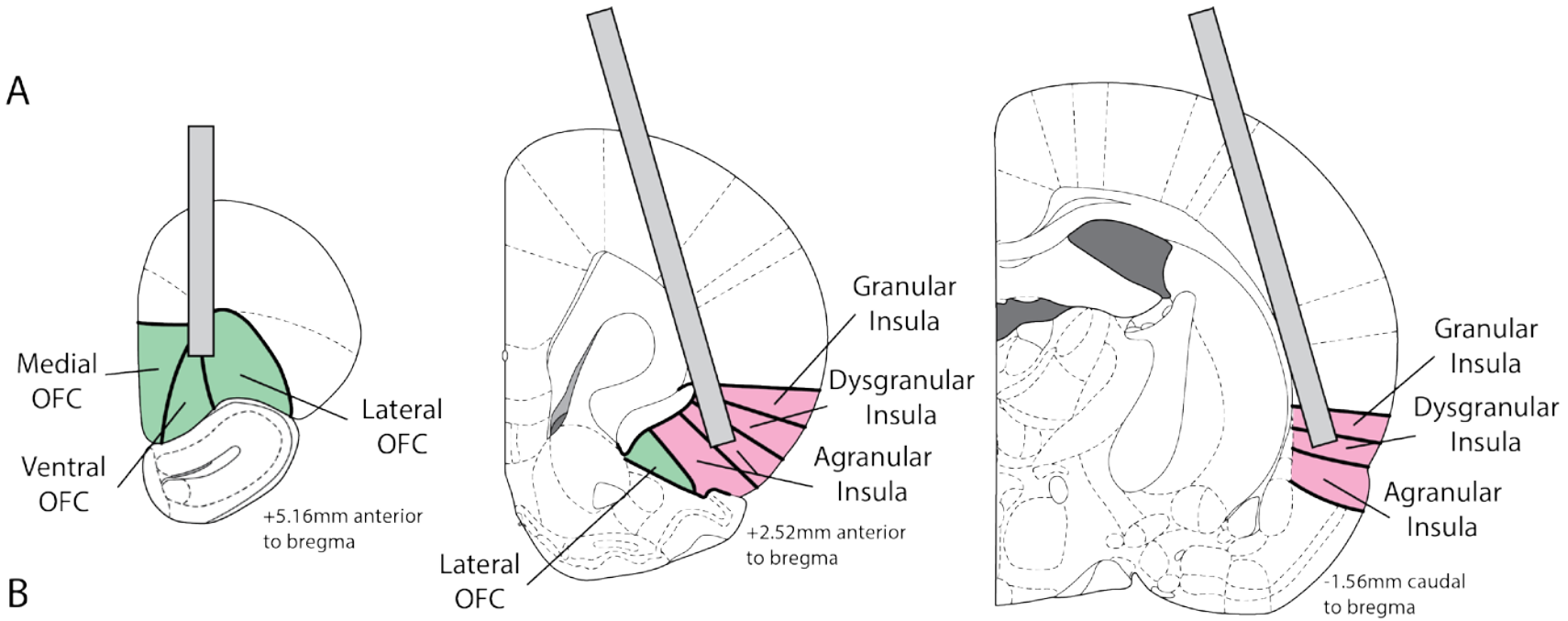
"…does not demonstrate significant impairments in recognizing other emotions…such as fear or disgust…He readily displays signs of positive emotion including happiness, amusement, interest, and excitement." (Feinstein et al., 2010).

Another example of selective lesions (this time in ventromedial PFC, including OFC) not disrupting the generation of affect looks at skin conductance responses during the Iowa Gambling Task. Here, participants with ventromedial lesions failed to show anticipatory skin responses before selecting a card (controls show robust responses after learning the task), but both controls and VM patients showed skin responses to the *result* of the card, suggesting that while VM lesions may affect prospective inference of a reward or punishment, the somatic reaction to the result itself remains intact (Bechara, Tranel, Damasio, & Damasio, 1996) (although see Dunn et al. (2006)). Lastly, damage to areas like OFC have been shown to disrupt social behavior regulation and anticipatory responses to startle cues, but again the emotions themselves are still intact and producible (Beer et al., 2003; Beer, Knight, & D'Esposito, 2006; Roberts et al., 2004).

Although not definitive, work from the late 1980's in rats also suggests that PFC is not necessary for the experience of 'liking', as decortication of PFC and insula, a technique that selectively aspirates and causes the degradation of cortical tissue, failed to disrupt responding for hypertonic sodium chloride during salt replete or depleted states (Wirsig & Grill, 1982). However, whether or not the cortical hotspots are necessary for the experience of hedonic 'liking' does not rule out their sufficiency to provide gains of function. For example, lesions to the nucleus accumbens hotspot does not disrupt normal sucrose 'liking', even though microinjections of opioid, orexin, GABA, or endocannabinoid agonist drugs robustly amplifies hedonic 'liking' (Castro & Berridge, 2014b; Faure et al., 2010; Mahler et al., 2007). While it is

possible to enhance negative ‘disgust’ in NAc via GABA stimulations, glutamate metabotropic blockade or acetylcholine muscarinic blockade (Castro et al., 2016; Faure et al., 2010; Richard & Berridge, 2011a), the inability for lesions to also enhance ‘disgust’ suggests that active neurochemical modulation within NAc is necessary for ‘disgust’ enhancement. It may be that the cortical hotspots found here are similar to the NAc hotspot, such that they are not necessary for raw generation of ‘liking’. Future studies may localize other neurochemical systems capable of modulating ‘disgust’ in cortex.

In sum, we show that sites in OFC and insula are causally capable of modulating the hedonic impact of a sweet taste in localized hotspots after mu opioid or orexin stimulation. Additionally, an anatomically distinct hedonic coldspot suppresses ‘liking’ throughout rostral and mid insula, and infiltrates caudolateral portions of OFC. By contrast, robust food intake was only generated in OFC, with weak and/or inconsistent stimulated eating elicited by insula microinjections. These results provide the first evidence that cortex can provide gains of function for affective processing, potentially indicating hierarchical cortical control over hedonic impact. Future work will hopefully explore this hypothesis, examining cortical-subcortical interactions, which may have important implications for affective processing.



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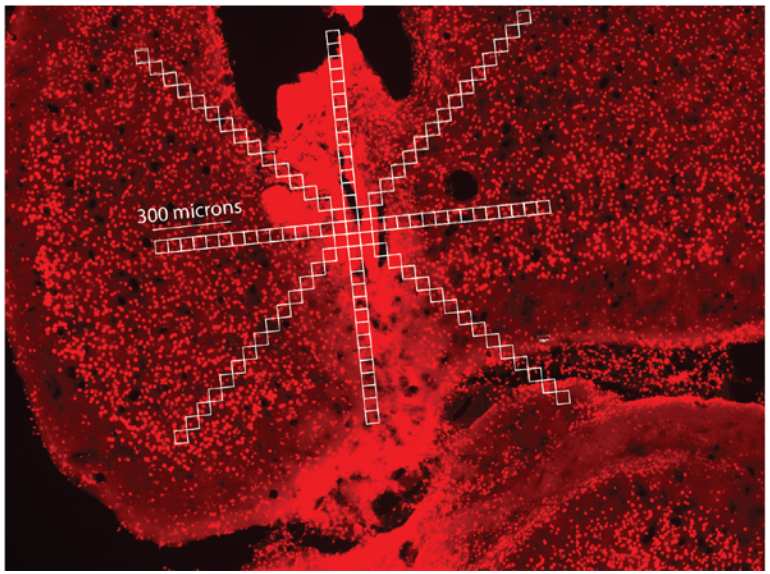

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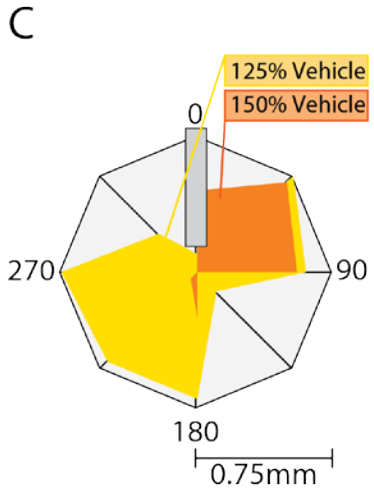
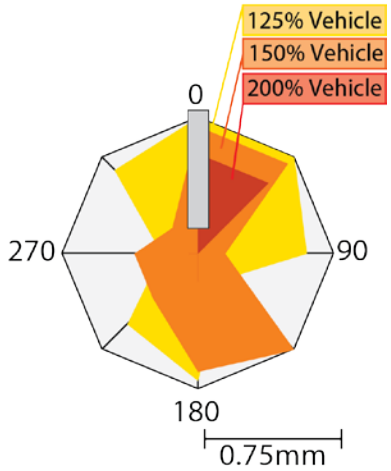


$\bar{x}$ DAMGO Plume



$\bar{x}$ Orexin Plume

Figure 4.1. Cortical Fos plumes. *A* Representative slices showing where microinjections were targeted throughout OFC (green) and insula (pink). *B* Representative image of a drug microinjection in OFC hotspot. Radial arm was overlaid onto microinjection site and the number of Fos positive cells within each square was counted to sample changes in Fos expression at increasing distances from microinjection site. *C* Average Fos plumes generated by DAMGO (left) or orexin (right) microinjections relative to vehicle microinjections throughout cortex (yellow = 125% increase, orange = 150% increase, red = 200% increase).

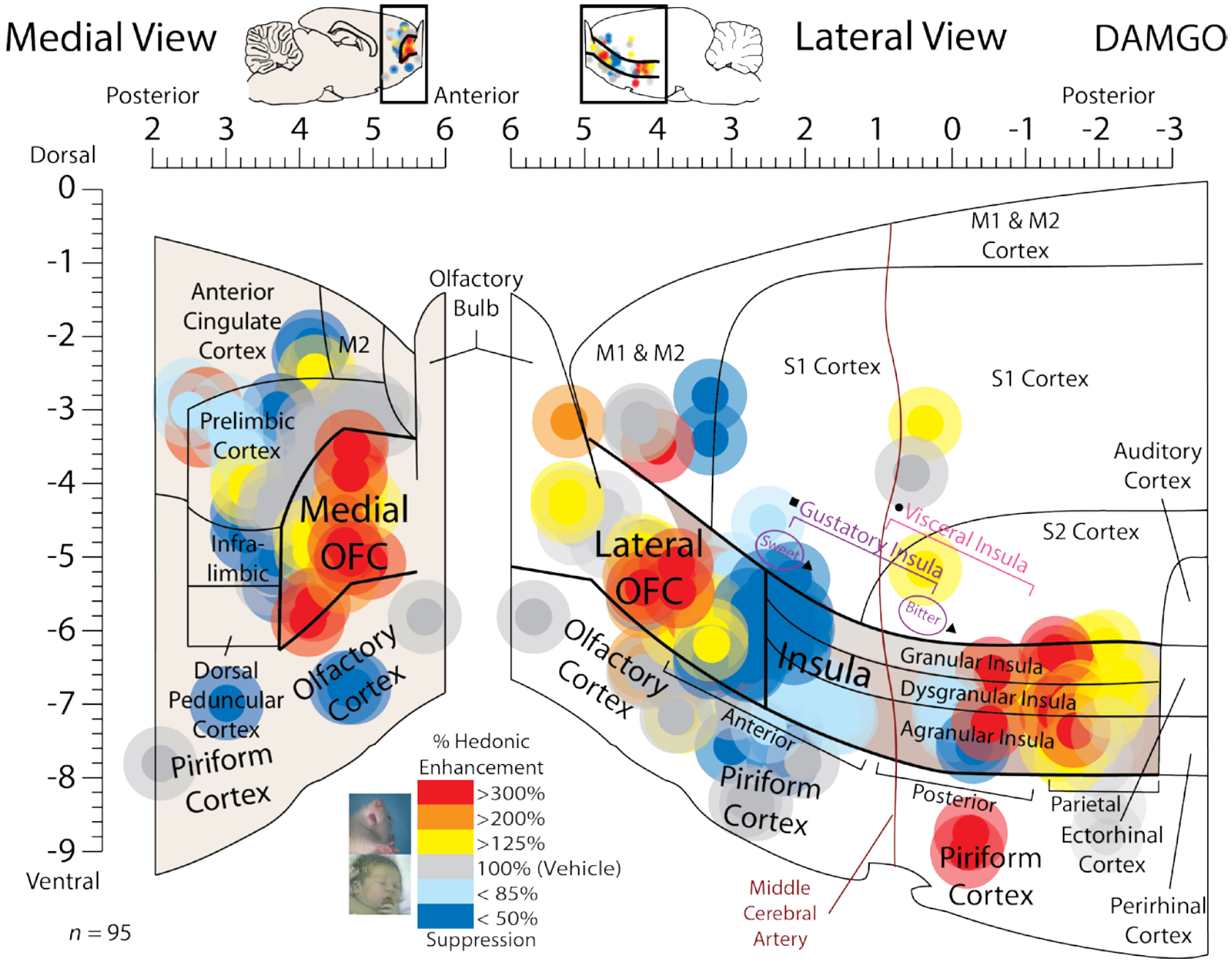
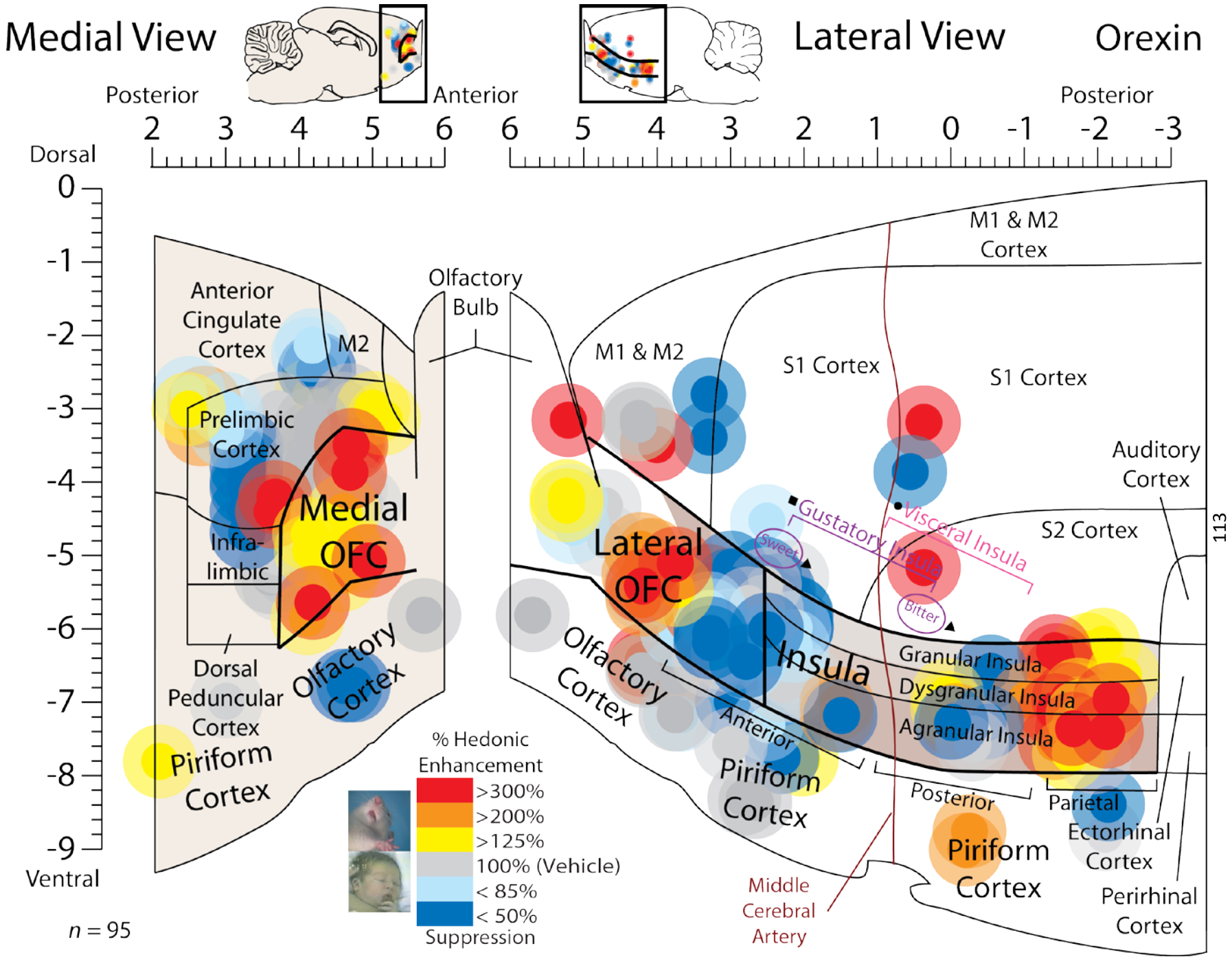

Medial View
Lateral View
DAMGO
Posterior
Anterior
Posterior
2 3 4 5 6
6 5 4 3 2 1 0 -1 -2 -3
Dorsal
0 -1 -2 -3 -4 -5 -6 -7 -8 -9
Ventral
n = 95
Anterior Cingulate Cortex
M2
Prelimbic Cortex
Infra-limbic
Medial OFC
Dorsal Peduncular Cortex
Olfactory Cortex
Piriform Cortex
Olfactory Bulb
M1 & M2
M1 & M2 Cortex
S1 Cortex
S1 Cortex
Auditory Cortex
S2 Cortex
Gustatory Insula
Visceral Insula
Sweet
Bitter
Lateral OFC
Olfactory Cortex
Insula
Granular Insula
Dysgranular Insula
Agranular Insula
Anterior
Posterior
Piriform Cortex
Parietal
Ectorhinal Cortex
Perirhinal Cortex
Middle Cerebral Artery
% Hedonic Enhancement
>300%
>200%
>125%
100% (Vehicle)
< 85%
< 50%
Suppression

Figure 4.2. OFC and insula contain mu opioid hedonic hotspots. Sagittal causation maps for localization of function throughout the cortex showing changes in orofacial hedonic reactions elicted by sucrose taste after microinjections of the mu opioid receptor agonist DAMGO. Each symbol placement indicates a microinjection site, the symbol size reflects the size of the Fos plumes produced by DAMGO, and symbol color reflects the behavioral effects of the microinjection, shown as percentage change from vehicle control levels (enhancements: yellow-orange-red; suppressions: blue). The sagittal map on the right provides a view of the lateral surface of the brain, and the left map provides a view of the medial surface. OFC and insula are thickly outlined in black. Primary gustatory cortex is bracketed in purple ■ (Kosar et al., 1986) and potential chemotopic locations for sweet and bitter taste modalities are also marked in purple ▲ (Peng et al., 2015). Primary visceral cortex is bracketed in pink ● (Cechetto & Saper, 1987). The middle cerebral artery runs along the lateral surface of the brain and is colored dark red.


Medial View
Lateral View
Orexin
Posterior
Anterior
Posterior
2 3 4 5 6
6 5 4 3 2 1 0 -1 -2 -3
Dorsal
0 -1 -2 -3 -4 -5 -6 -7 -8 -9
Ventral
n = 95
Anterior Cingulate Cortex
M2
Prelimbic Cortex
Infra-limbic
Medial OFC
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Olfactory Cortex
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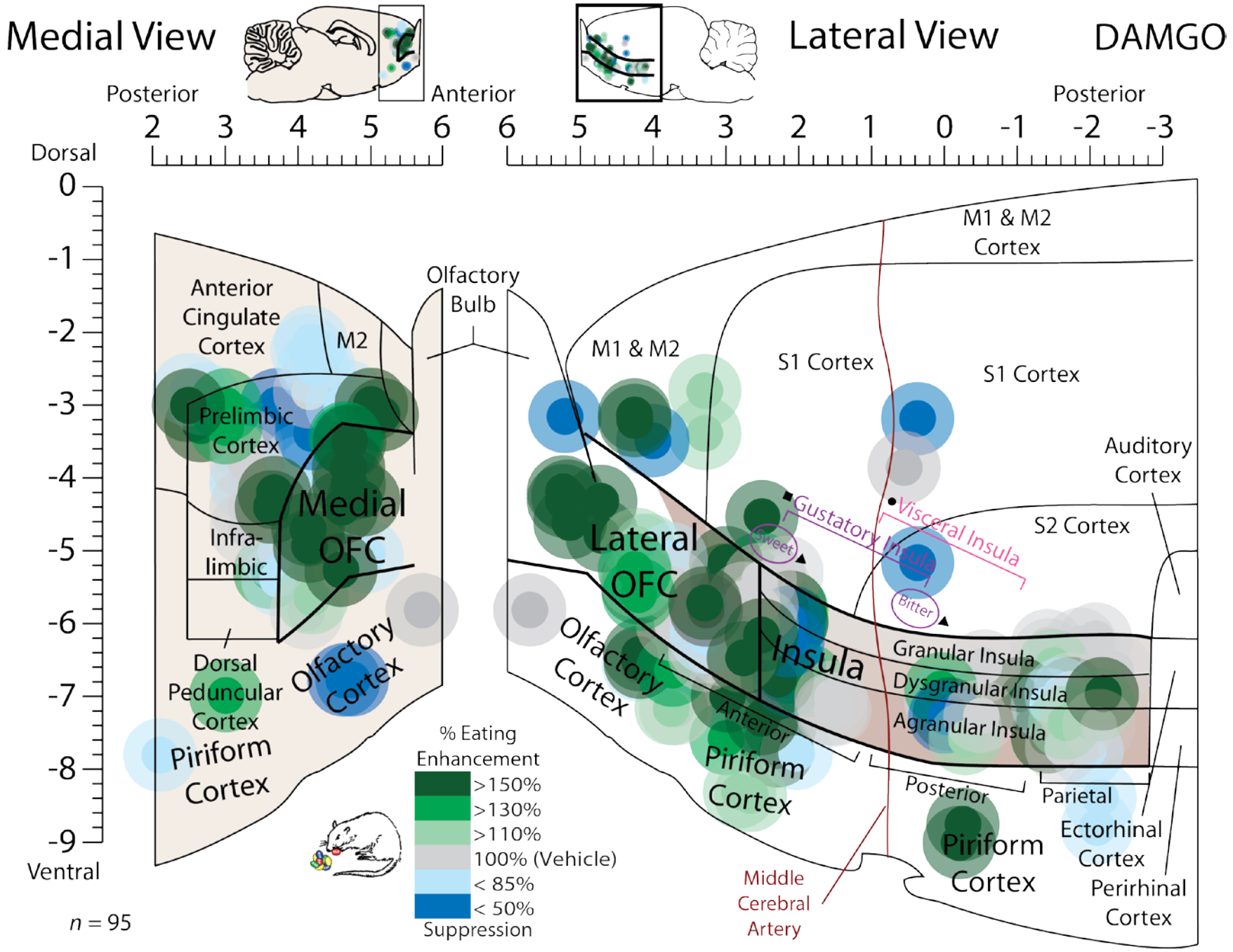
Olfactory Bulb
M1 & M2
M1 & M2 Cortex
S1 Cortex
S1 Cortex
Auditory Cortex
S2 Cortex
Gustatory Insula
Visceral Insula
Sweet
Bitter
Lateral OFC
Insula
Granular Insula
Dysgranular Insula
Agranular Insula
Olfactory Cortex
Anterior
Posterior
Piriform Cortex
Parietal Ectorhinal Cortex
Perirhinal Cortex
Middle Cerebral Artery
% Hedonic Enhancement
>300%
>200%
>125%
100% (Vehicle)
< 85%
< 50%
Suppression
113

Figure 4.3. OFC and insula contain orexin hedonic hotspots. Sagittal causation maps for localization of function throughout the cortex showing changes in orofacial hedonic reactions elicted by sucrose taste after microinjections of the orexin receptor agonist orexin-A. Each symbol placement indicates a microinjection site, the symbol size reflects the size of the Fos plumes produced by orexin, and symbol color reflects the behavioral effects of the microinjection, shown as percentage change from vehicle control levels (enhancements: yellow-orange-red; suppressions: blue). The sagittal map on the right provides a view of the lateral surface of the brain, and the left map provides a view of the medial surface. OFC and insula are thickly outlined in black. Primary gustatory cortex is bracketed in purple ■ (Kosar et al., 1986) and potential chemotopic locations for sweet and bitter taste modalities are also marked in purple ▲ (Peng et al., 2015). Primary visceral cortex is bracketed in pink ● (Cechetto & Saper, 1987). The middle cerebral artery runs along the lateral surface of the brain and is colored dark red.


Medial View
Lateral View
DAMGO
Posterior
Anterior
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2 3 4 5 6
6 5 4 3 2 1 0 -1 -2 -3
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-7
-8
-9
Ventral
Anterior Cingulate Cortex
M2
Prelimbic Cortex
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Olfactory Cortex
Piriform Cortex
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S2 Cortex
Gustatory Insula
Visceral Insula
Sweet
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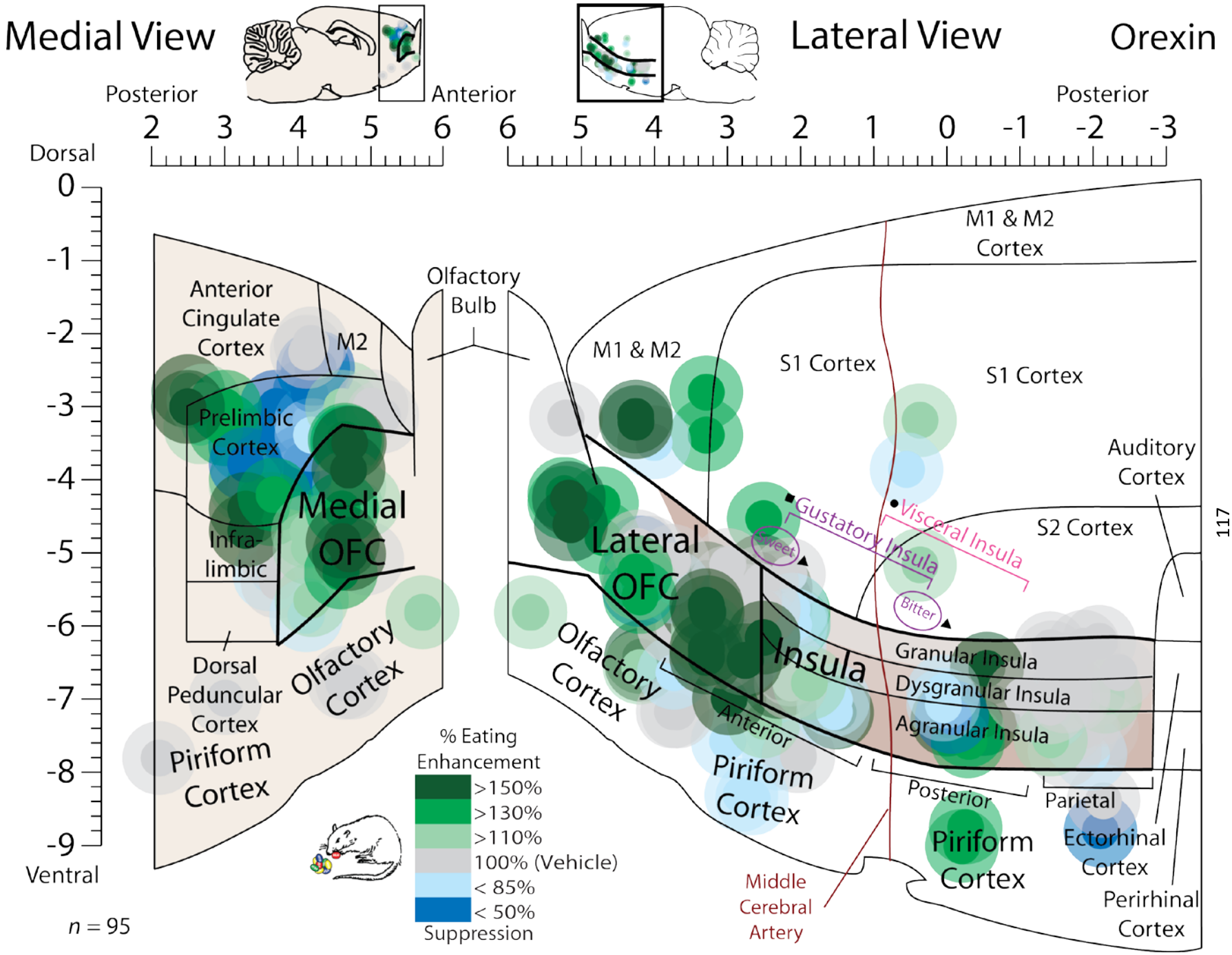
Insula
Granular Insula
Dysgranular Insula
Agranular Insula
Olfactory Cortex
Anterior
Posterior
Parietal
Piriform Cortex
Ectorhinal Cortex
Perirhinal Cortex
Middle Cerebral Artery
% Eating Enhancement
>150%
>130%
>110%
100% (Vehicle)
< 85%
< 50%
Suppression
n = 95

Figure 4.4. Mu opioid stimulation increases food intake in OFC and piriform cortex. Sagittal causation maps for localization of function throughout the cortex showing changes in food intake of palatable M&M candies after microinjections of DAMGO. Each symbol placement indicates a microinjection site, the symbol size reflects the size of the Fos plumes produced by DAMGO, and symbol color reflects the behavioral effects of the microinjection, shown as percentage change from vehicle control levels (enhancements: green; suppressions: blue). The sagittal map on the right provides a view of the lateral surface of the brain, and the left map provides a view of the medial surface. OFC and insula are thickly outlined in black. Primary gustatory cortex is bracketed in purple ■ (Kosar et al., 1986) and potential chemotopic locations for sweet and bitter taste modalities are also marked in purple ▲ (Peng et al., 2015). Primary visceral cortex is bracketed in pink ● (Cechetto & Saper, 1987). The middle cerebral artery runs along the lateral surface of the brain and is colored dark red.


Medial View
Lateral View
Orexin
Posterior
Anterior
Posterior
2 3 4 5 6
6 5 4 3 2 1 0 -1 -2 -3
Dorsal
0 -1 -2 -3 -4 -5 -6 -7 -8 -9
Ventral
Anterior Cingulate Cortex
M2
Prelimbic Cortex
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Medial OFC
Dorsal Peduncular Cortex
Olfactory Cortex
Piriform Cortex
Olfactory Bulb
M1 & M2
M1 & M2 Cortex
S1 Cortex
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Auditory Cortex
S2 Cortex
Gustatory Insula
Visceral Insula
Sweet
Bitter
Lateral OFC
Insula
Granular Insula
Dysgranular Insula
Agranular Insula
Olfactory Cortex
Anterior
Piriform Cortex
Posterior
Parietal
Piriform Cortex
Ectorhinal Cortex
Perirhinal Cortex
Middle Cerebral Artery
% Eating Enhancement
>150%
>130%
>110%
100% (Vehicle)
< 85%
< 50%
Suppression
n = 95
117

Figure 4.5. Orexin stimulation increases food intake in OFC. Sagittal causation maps for localization of function throughout the cortex showing changes in food intake of palatable M&M candies after microinjections of orexin-A. Each symbol placement indicates a microinjection site, the symbol size reflects the size of the Fos plumes produced by orexin, and symbol color reflects the behavioral effects of the microinjection, shown as percentage change from vehicle control levels (enhancements: green; suppressions: blue). The sagittal map on the right provides a view of the lateral surface of the brain, and the left map provides a view of the medial surface. OFC and insula are thickly outlined in black. Primary gustatory cortex is bracketed in purple ■ (Kosar et al., 1986) and potential chemotopic locations for sweet and bitter taste modalities are also marked in purple ▲ (Peng et al., 2015). Primary visceral cortex is bracketed in pink ● (Cechetto & Saper, 1987). The middle cerebral artery runs along the lateral surface of the brain and is colored dark red.

CHAPTER 5:

VENTRAL PALLIDUM HOTSPOT FOR SWEETNESS 'LIKING' IS MODULATED BY LATERAL HYPOTHALAMUS CIRCUITRY FOR 'WANTING' TO EAT

Introduction

Rewards evoke both hedonic impact ('liking') and incentive motivation ('wanting'), and dysfunction of either component can produce consequences that range from depression to addiction and eating disorders. Within mesocorticolimbic reward circuitry, the ventral pallidum (VP) is an especially crucial node (Mickiewicz, Dallimore, & Napier, 2009; Root, Melendez, Zaborszky, & Napier, 2015). VP neurochemical stimulation can produce gains in hedonic impact and/or motivation to enhance 'liking' and intake of food or drug rewards. Conversely, VP lesions or impairment can produce loss of hedonic/motivation functions, eliminating 'liking' and making normally palatable sweet tastes evoke 'disgust' reactions, and simultaneously produce aphagia (Cromwell & Berridge, 1993; Ho & Berridge, 2014; Mahler et al., 2014; K. S. Smith & Berridge, 2005; Tang, McFarland, Cagle, & Kalivas, 2005). Of particular interest for both gain and loss of hedonic functions is the posterior half of VP, which contains a cubic-millimeter 'hedonic hotspot' (Castro & Berridge, 2014a; Groenewegen, Berendse, & Haber, 1993; Kupchik & Kalivas, 2013).

Hypothalamic regulatory circuitry, especially involving the lateral hypothalamus (LH), can modulate mesocorticolimbic circuitry through homeostatic signals, thus allowing hunger and

satiety states to alter motivated and hedonic aspects of food rewards (Berthoud & Munzberg, 2011). One example of this involves orexin, also known as hypocretin, which is produced in LH and DMH, and is an important neurochemical signal in arousal and sleep/wake cycles (A. Rolls et al., 2011; Sakurai, 2014). Orexin additionally has roles in appetite, reward and addiction, especially in a mid-tuberal subregion of LH (Harris & Aston-Jones, 2006; Harris et al., 2005; Sakurai, Amemiya, Ishii, Matsuzaki, Chemelli, Tanaka, Williams, Richardson, et al., 1998). LH orexin neurons project directly to posterior VP, and microinjection of orexin into VP amplifies 'liking' reactions to sucrose taste (Baldo et al., 2003; Ho & Berridge, 2013; Peyron et al., 1998; Swanson et al., 2005), providing a potential route for homeostatic hunger signals to enhance 'liking' and 'wanting' for palatable foods.

Here, we compared the effects of optogenetic stimulation of 1) intrinsic VP neurons, 2) intrinsic LH neurons or 3) LH-VP projections on the hedonic impact of sweetness and the motivation to eat. First, bilateral microinjections were made of an adeno-associated viral vector serotype 5 ($AAV_{5)}$ coding channelrhodopsin (ChR2) and enhanced yellow fluorescent protein (eYFP), guided by either a neuron-specific promoter or general cellular promoter; other rats received a control virus with only eYFP but not ChR2. Microinjection sites were either in the posterior VP or in the mid-tuberal orexin field of LH. Optic fibers simultaneously were bilaterally implanted in either posterior VP or LH. In the same surgery, all rats were also implanted with bilateral oral cannulas for later taste reactivity testing. After a 5 week recovery, rats were tested in two behavioral tests: 1) affective taste reactivity test, measuring orofacial expressions elicited by infusions of sucrose or quinine solutions directly into the mouth, and 2) voluntary intake test, measuring consumption of a palatable sweet food (M&M chocolate candies).

Methods & Materials

Animals

Sixty-two (LH-LH *n* = 20; LH-VP *n* = 11; VP-VP *n* = 20; Control LH-LH/LH-VP *n* = 4, Control VP-VP *n* = 4; Fos *n* = 16) male Sprague-Dawley rats were housed at ~21°C on a reverse 12 h light/dark cycle. All rats had *ad libitum* access to both food and water. All experimental procedures were approved by the University of Committee on the Use and Care of Animals at the University of Michigan.

Surgery

Rats were anesthetized with intraperitoneal injections of ketamine hydrochloride (80mg/km and xylazine (5mg/kg), treated with atropine (0.05 mg/kg) to prevent respiratory distress, and placed in a stereotaxic apparatus (David Kopf Instruments), with the incisor bar set at -3.3 for flat skull measurements. For optogenetic virus infection in VP, rats received bilateral microinfusions (AAV5-CAG-ChR2-eYFP, *n* = 6; AAV5-hSyn-ChR2-eYFP, *n* = 14) into the hotspot of posterior VP (AP, -0.8; ML, ±3.0; DV, -8.1). Animals receiving the control virus (AAV5-hSyn-eYFP, *n* = 4) were also aimed at the posterior VP hotspot. In the same surgery, each rat also received bilateral implants of 220μm optic fibers above the same VP sites, positioned 0.3mm relative to virus infusion location (AP, -0.8; ML, ±3.0; DV, -7.8).

For optogenetic virus infection in LH, rats received bilateral microinfusions (AAV5-CAG-ChR2-eYFP, *n* = 9; AAV5-hSyn-ChR2-eYFP, *n* = 9; AAV5-CAMKIIα-ChR2-eYFP, *n* = 2) into the mid-tuberal portion of LH (AP, -2.9; ML, ±1.8; DV, -8.4). Animals receiving the control virus (AAV5-hSyn-eYFP, *n* = 4) were also aimed at the mid-tuberal LH. In the same surgery, each rat also received bilateral implants optic fibers above the same LH sites, positioned 0.3mm relative to virus infusion location (AP, -2.9; ML, ±1.8; DV, -8.1). For the LH-VP

condition, these same rats also received bilateral fiber implants into the posterior VP hotspot (AP, -0.8; ML, ±3.0; DV, -7.8).Virus microinjections were infused 1.0µl/side at a rate of 0.1µl/1 m for 10min, followed by a 10min wait period to allow for virus diffusion. Microinjectors were slowly removed from the infusion site after virus diffusion. Headcaps were secured to skull by skull screws and acrylic cement. Optic fibers had 230µm diameter cores and were inserted into 9mm long zirconia ferrules. Fibers were tested before and after experimentation to ensure fiber integrity.

For subsequent taste reactivity testing, all rats were also implanted in the same surgery with bilateral oral cannulae [polyethylene-100 tubing] to permit oral infusions of sucrose or quinine solutions. Oral cannulae entered the mouth in the upper cheek pouch lateral to the first maxillary molar, traveling beneath the zygomatic arch, and exited the skin at the dorsal headcap. Oral cannulae did not disrupt normal eating. After surgery, each rat received subcutaneous injections of chloramphenicol sodium succinate (60mg/kg) to prevent infection and carprofen (5mg/kg) for pain relief. Rats received carprofen again 24hr later and were allowed to recover for at least 5 weeks to allow optimal virus expression.

Optogenetic taste reactivity and food intake tests

Animals in behavioral experiments were given three days of habituation in the testing chamber where there was access to M&M candies and water. This was followed by two test days, one in which animals received laser stimulation, and second on which no photostimulation occurred. These tests days were counterbalanced across animals. Laser stimulation was administered at 1-3mW for at 25Hz (15msec ON/25msec OFF pulses). During taste reactivity tests and intake tests, laser was delivered in a 5-sec bin once every twenty sec (5sec On/15sec Off).

Taste reactivity testing

The taste reactivity test was used to measure affective orofacial reactions of rats to a 1ml volume of sucrose solution infused into the mouth via oral cannula. Tests occurred during 1min infusions administered during laser activation of either LH or the VP hotspot. To infuse sucrose solution into the mouth, a syringe containing sucrose or quinine in a syringe pump (Sucrose: 1.0%, 0.029M, 1ml per test; Quinine: $3x10^{-4}$M, 1ml per test) was attached via hollow tubing (PE-50 connected to a PE-10 delivery nozzle) to a rat's oral cannula. A 1ml volume of solution was infused evenly over a period of 1min duration. Orofacial taste reactivity responses were video recorded via close-up lens and an angled mirror placed underneath the transparent floor for subsequent slow-motion video analysis.

Taste reactivity video scoring

Hedonic, aversive, and neutral taste reactivity patters were scored off-line in slow motion (1/30 s frame-by-frame to 1/10th actual speed). Hedonic responses were classified as rhythmic midline tongue protrusions, lateral tongue protrusions, and paw licks. Aversive responses were classified as gapes, head shakes, face washes, forelimb flails, and chin rubs. Neutral responses were classified as passive dripping of solution out of the mouth, ordinary grooming, and rhythmic mouth movements. A time-bin scoring procedure was used to ensure that taste reactivity components of different relative frequencies still contribute equally to final affective hedonic/aversive totals, and that frequent components such as rhythmic tongue protrusions do not swamp rare but equally informative components, such as lateral tongue protrusions. Specifically, rhythmic mouth movements, passive dripping, and paw licking reactions, which occur in long bouts, were scored in 5sec time bins (e.g., 5sec continuous paw licking behavior equals one bout occurrence). Rhythmic midline tongue protrusions and chin rubs, which occur in

shorter bouts, were scored in 2s time bins. Lateral tongue protrusions, gapes, forelimb flails, and head shakes, which typically occur as discrete events, were scored as single occurrences each time they occurred (e.g., one gape equals one occurrence). Individual totals were calculated for hedonic versus aversive categories. A hedonic reaction total was quantified as the sum of scores for lateral tongue protrusion, rhythmic tongue protrusion, and paw lick scores. An aversive reaction total was quantified as the sum of gape, head shake, face wash, forelimb flail, and chin rub scores.

Food intake testing

Spontaneous eating behavior was video-recorded and food consumption measured in a 1hr free intake test that began 1min after the taste reactivity test on each test day. Each food intake chamber (12 x 18 x 12 cm) had a pile of pre-weighed amount of palatable chocolates (M&Ms, ~25g) or standard laboratory chow on separate test days, and *ad libitum* water was also available. Rats had already been habituated to the food intake testing chamber for 3 daily 1hr sessions prior to the first test day. Amount remaining of M&Ms or chow were weighed again at the end of the test to calculate amount eaten (after chamber inspected for spillage). All behavior in the chamber was also video recorded during the 60min test, and scored later offline for video analysis by a researcher blind to the drug microinjection condition. Videos were scored for eating behavior (duration in seconds), water drinking behavior (in seconds), grooming behavior (in seconds), and for number of bouts of food sniffs, food carrying (grasping and transport of food by 2 or more steps), cage crosses, and rears (each counted separately).

Conditioned taste aversion

A subset of animals (n = 7) received 3 pairings of a 2 minute intraoral infusion of either saccharin or glucose, and an injection of lithium chloride immediately afterwards. 48hr after the

final pairing, animals underwent taste reactivity testing with the conditioned flavor either with laser stimulation of the VP hotspot or not. 24hr later, they had the opposite condition to test whether laser stimulation could enhance the palatability of a conditioned taste aversion, or whether 'disgusting' stimuli would be unavailable to laser enhancements like quinine.

Laser self-stimulation

Med-Associates chambers were fitted with a metal grid floor as well as two empty liquid sippers on the back wall of the chamber. Contact with the sippers was recorded over a thirty minute session. When animals (n = 15) contacted one of the two sippers (assignment counterbalanced between animals), they received a 1sec 25Hz pulse of laser illumination. Animals were given free access to two empty sippers in the operant chamber for 30m for 3 days. Licking one sipper resulted in a 1sec laser pulse (25Hz, 15ms on/25ms off), whereas contact with the other sipper yielded no laser. After three days, animals that contacted the laser sipper >100 times were tested in a reversal task where the sipper producing laser stimulation was switched with the other sipper.

Histology

All rats were perfused and brains treated as described previously (M. J. Robinson, Warlow, & Berridge, 2014). Fos counts were assessed in a dedicated Fos group that were not behaviorally tested (n =16). Fos was assessed after a 60 minute stimulation session using the same 5s ON, 15s OFF pattern (25Hz, 1-3sW) used for food intake testing. Brain slices were processed for Fos-like immunoreactivity using normal donkey serum, goat anti-c-fos (Santa Cruz Biotechnology), and donkey anti-goat Alexa Fluor 594 (Invitrogen). Sections were mounted, air-dried, and coverslipped with Prolong Gold antifade reagent (Invitrogen). Zones where the expression of fluorescent Fos was elevated in neurons in targeted regions of interest were

assessed via microscope as described previously (Richard & Berridge, 2013).

Fos plume analysis

Fos plumes surrounding fiber optic tips were assessed in a separate dedicated Fos group (n = 16) that was included to detect the maximal spread of neuronal impact after laser photoexcitation. In the dedicated group, which could be expected to have maximum laser spread, a single laser or no laser photostimulation session LH was administered under conditions similar to the first day of food intake testing for behavioral rats. Rats were then anesthetized and transcardially perfused after high laser power stimulation of ChR2 infected neurons (7-10mW, n = 5, low laser power stimulation of ChR2 infected neurons (1-3mW, n = 5) or high laser power stimulation of control virus infected neurons (7-10mW, n = 4). Rats with no surgery were also included in a "normal" group (n = 2) to assess counts in naive brains. Brain slices were processed for Fos-like immunoreactivity using normal donkey serum, goat anti-c-fos (Santa Cruz Biotechnology), and donkey anti-goat AlexaFluor 594 (Invitrogen). Sections were mounted, air-dried, and coverslipped with Prolong Gold antifade reagent (Invitrogen).

Zones where the local expression of fluorescent Fos was elevated in neurons surrounding optic fiber tips ("Fos plumes") were assessed via microscope. Individual Fos plumes were constructed for each laser stimulated brain by comparing them to "normal" brains. These plumes were defined by whether an elevation in Fos occurred within 0.5mm of the fiber optic tip along individual arms, and if so, whether a subsequent square with increase Fos occurred within 0.15mm of the last Fos enhanced square within that arm. These rules were applied to avoid over-estimating the size of the plume. Once each individual plume had been constructed for a given conditioning (e.g., high power ChR2), the average furthest distance of Fos enhancement was calculated for each arm, allowing for an analysis of the shape of the plume.

Mapping behavioral effects of VP or LH sagittal, horizontal and coronal planes allows for the presentation of all sites on the same map of the entire rostrocaudal, mediolateral, and dorsoventral extent of VP and LH. Functional effects on hedonic and motivated behaviors were mapped using color-coding to express the intensity changes in affective and motivated behaviors for individual behaviorally tested rats. Map symbols were sized to match the Fos plume sizes described below.

Results

Hedonic reactions to sweet or bitter tastes

Taste reactivity results showed that low intensity (1-3mW) ChR2 photostimulation of intrinsic VP neurons caused the sweet taste of sucrose to elicit a 200%-400% increase in the number of positive hedonic orofacial ('liking') reactions, such as lateral tongue protrusions or paw licks, compared to the within-subject control number of reactions elicited by sucrose without laser (Figure 5.2, 5.3 and 5.7) (No laser: mean = 3.4, SEM = 0.9; Laser: mean = 8.4, SEM = 1.4; VP: $F_{(1,8)} = 27.441$, $p = 0.001$; $d = 1.484$). The VP ChR2 stimulation effect was selective to the positive hedonic impact of sucrose taste, and did not raise the near-zero number of negative ('disgust') reactions (e.g., gapes or chin rubs) (VP: $F_{(1, 8)} = 0.908$, $p = 0.369$), nor impair the ability of bitter quinine taste to elicit high numbers of negative reactions (VP: $F_{(1, 8)} = 1.0$, $p = 0.347$). VP photostimulation of inactive-virus control rats produced no change in positive or negative hedonic reactions (No laser: mean = 6.0, SEM = 2.4; Laser: mean = 3.8, SEM = 1.9; $F_{(1, 3)} = 2.670$, $p = 0.201$), indicating that ChR2 photoreceptor excitation was crucial to the VP enhancement of positive hedonic impact. For posterior VP virus microinjections, virus immunofluorescence extended medially to substantia innominata or sublenticular extended amygdala, laterally to the interstitial nucleus of the posterior limb of the anterior commissure,

dorsally toward globus pallidus and ventrally to the magnocellular preoptic nucleus. On average, each virus microinjection appeared to fill a volume of 0.5mm^3, or ~37% of posterior VP.

For rats that received ChR2 virus and optic fiber implants both in the LH, photostimulation of intrinsic LH neurons failed to alter positive hedonic reactions elicited by sucrose taste (Figure 5.2, 5.3, and 5.9) (No laser: mean = 7.9, SEM = 1.3; Laser: mean = 6.8, SEM = 1.9; LH-LH/LH-VP: $F_{(2,16)} = 188.481$, $p = 0.015$; LH-LH: $p = 0.723$). Similarly, LH neuronal photostimulation failed to alter aversive reactions to quinine (LH-LH/LH-VP: $F_{(2, 16)} = 0.873$, $p = 0.437$; LH-LH: $p = 0.664$). Like VP, photostimulation within LH affected a smaller volume of space than the portion of tissue infected with virus. Virus expression for LH microinjection conditions was consistently located in the mid-rostral tuberal region of LH, lateral to fornix but medial to internal capsule. Virus was densest in mid and dorsal portions of LH, with no expression in the ventral-most part of LH. Using laser induced Fos plumes, we can estimate the volume of space affected laser illumination (M. J. Robinson et al., 2014). Within LH, each laser plume was ~0.013mm^3 and was shaped like an elongated cone, within which Fos was elevated by ~200% compared to normal tissue. The plume extended ventrally away from the fiber tip (total dorsoventral height = 0.25mm; mediolateral width diameter = 0.32mm; radius = 0.16mm). These data indicate that laser photostimulation only affected ~3% of the region infected with ChR2 virus, only ~0.3% of the entire LH (Figure 5.1).

By contrast, photostimulation of ChR2 in the LH-VP projection condition, roughly doubled the number of positive hedonic reactions elicited by sucrose taste (Figure 5.4 and 5.8) (No Laser: mean = 7.9, SEM = 1.3; Laser: mean = 14.2, SEM = 3.4; $p = 0.018$; effect size = 0.994). Positive hedonic enhancement by laser excitation of LH-VP projections again occurred

without altering negative aversive reactions to quinine (LH-VP: $p = 0.471$) or sucrose (LH/LH-VP: $F_{(2, 16)} = 0.598$, $p = 0.562$; $p = 0.766$).

Ruling out VP modulation of sensory signal as mechanism of hedonic enhancement

Given that the VP and LH receive ascending gustatory inputs from the parabrachial nucleus of the pons (i.e., second gustatory relay site in hindbrain of rats) (Norgren & Leonard, 1973; Pfaffmann, Norgren, & Grill, 1977), it seemed possible that VP photostimulation might have distorted the sensory gustatory signal (e.g., making the sensation of sucrose more sweet) rather than directly modulating hedonic impact. To probe this possibility, we assessed if VP ChR2 excitation distorted gustatory perception in a way that would disrupt recognition of a previously learned sweet taste. This was approached using a taste aversion procedure that induced learned disgust to one CS+ taste (either 0.007M saccharin or 0.3M glucose, randomly assigned) which had been paired with illness (1.5 M LiCl, 1.33 ml/kg, i.p.). After conditioning, rats were tested on subsequent days with additional infusions of CS+ taste, but this time with either VP photostimulation or no laser (2 tests, randomized order).

Taste aversion results showed that LiCl pairings abolished positive orofacial reactions to the CS+, indicating a loss of hedonic impact for the sweet CS+ taste (Figure 5.5) ($F_{(2,12)} = 52.081$, $p = 0.000001$; Pre- versus Post-Conditioning with No Laser: $p = 0.001$; $d = 3.044$). VP photostimulation did not disrupt recognition of the CS+ taste needed to express the learned aversion or alter taste reactivity elicited by the CS+ (Pre- versus Post-Conditioning with Laser: $p = 0.001$; effect size = 2.24; Laser versus No Laser: $p = 0.733$). These results suggest that a conditioned aversion is impervious to laser hedonic enhancements because the CS+ has become affectively ‘disgusting’ like quinine. If laser stimulation was altering the taste quality, then there

should have been a restoration of hedonic reactions since the perceived taste would no longer be the CS+.

LH stimulation of food intake

Intrinsic LH photostimulation increased consumption of sweet M&Ms chocolates by ~130-200% compared to the no laser control condition in the same rats (Figure 5.6) (No Laser: mean = 9.1g, SEM = 1.2; Laser: mean = 12.7g, SEM = 1.2; $F_{(2,16)} = 38.766$, $p = 0.0001$; $d = 0.849$). Although there was an average increase of 130% across all stimulated animals, a closer inspection shows that some animals started at a lower baseline thereby allowing for a larger percent increase. LH stimulation of inactive virus control rats did not alter intake ($F_{(1, 3)} = 1.452$, $p = 0.315$). Similarly, stimulation of LH projections to the VP hotspot increased consumption by ~130% (Figure 5.4) (No Laser: mean = 9.1g, SEM = 1.2; Laser: mean = 12.1g, SEM = 1.4; $p = 0.0001$; $d = 0.828$), but there was no change for inactive virus control rats.

In contrast to intrinsic and projection stimulation of LH infected neurons, direct illumination of VP did not increase food intake detectably, even in VP rats that had shown ChR2 enhancement of sucrose hedonic impact on the same day (Figure 5.6) (No Laser: mean = 11.5g, SEM = 0.8; Laser: mean = 10.8g, SEM = 0.9; VP: $F_{(1,8)} = 0.705$, $p = 0.426$; eYFP-control: $F_{(1,3)} = 5.717$, $p = 0.097$). To verify that the lack of VP stimulated eating was not due to exposure to quinine before food intake testing, a separate group of rats that did not receive oral cannulas were run on a pure food intake test. Like the oral cannulated animals, these rats did not eat chow or M&Ms when VP was directly photostimulated (M&Ms No Laser: mean = 5.9g, SEM = 0.78; Laser: mean = 7.5g, SEM = 1.09; VP: $F_{(1,4)} = 0.174$, $p = 0.698$; Chow No Laser: mean = 0.11g, SEM = 0.27; Laser: mean = 0.30g, SEM = 0.14) even though a comparable LH group still showed robust eating for both chow (No Laser: mean = 0.03g, SEM = 0.02; Laser: mean =

5.74g, SEM = 1.79; $F_{(1,6)} = 10.375$, $p = 0.018$) and M&Ms (No Laser: mean = 3.2g, SEM = 0.64; Laser: mean = 10.1g, SEM = 2.27; $F_{(1,6)} = 9.456$, $p = 0.022$) after laser stimulation.

Self-stimulation

Although direct VP photostimulation did not increase food intake specifically, it is possible that VP stimulation may contribute to appetitive motivations more generally. Therefore, to determine whether caudal VP is capable of modulating motivated behaviors other than food intake, rats were placed in a chamber in which they could earn short pulses of laser photostimulation by contacting an empty sipper. Animals also had access to an inactive spout that never delivered laser photostimulation.

We found that laser photostimulation of VP did not support self-stimulation. Of the 7 animals tested, none contacted the laser spout >100 times (Laser spout mean = 21.1, SEM = 9.6; non-laser spout mean = 14.2, SEM = 5.9). These results are consistent with the inability for laser to stimulate motivated behaviors, including food intake as described above.

By contrast, we found that LH stimulation generated robust self-stimulation in about half of animals tested (Laser spout mean = 624.67, SEM = 487.53; non-laser spout mean = 27.78, SEM = 4.64) but not in the other half (Laser spout mean = 22.92, SEM = 11.36; non-laser spout mean = 11.92, SEM = 4.02). Animals that did self-stimulate were tested on a reversal task, where contacts with the previously unrewarded spout now delivered laser pulses and contact with the spout that previously administered laser was no longer rewarded. Animals that self-stimulated on the first spout tracked the laser and self-stimulated at the new laser spout (Laser spout mean = 1580.44, SEM = 862.18; non-laser spout mean = 18.56, SEM = 0.99). These results support the food intake results, namely that LH stimulation can enhance motivated and reward-related behaviors.

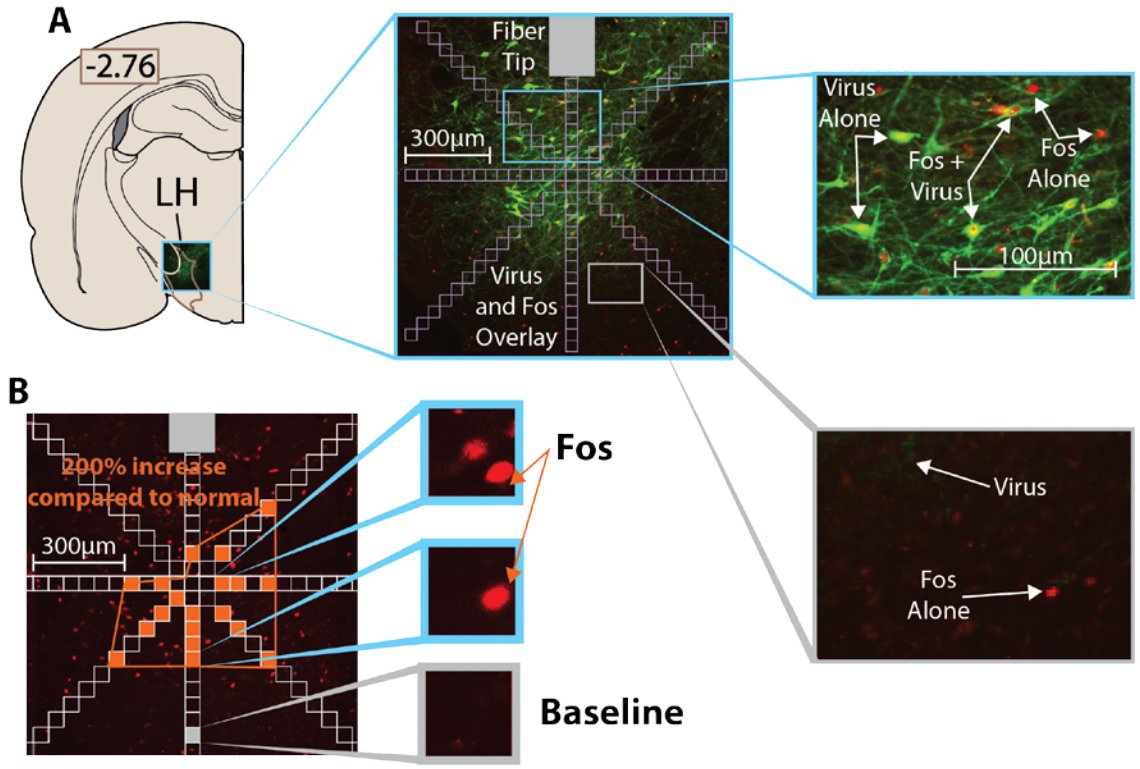
Discussion

Here we showed that optogenetic stimulation of posterior VP, but not mid-tuberal LH, selectively doubled the number of positive 'liking' reactions elicited by the taste of sucrose. VP enhancement of 'liking' is consistent with previous reports of a hedonic hotspot able to amplify and encode sensory pleasure located in the caudal half of VP (Castro & Berridge, 2014a; Richard et al., 2013; K. S. Smith & Berridge, 2005; K. S. Smith et al., 2011). A role for VP in sweetness hedonic enhancement is also consistent with our observation that ChR2 stimulation of LH-to-VP projections similarly increased 'liking' reactions. However, since many neurotransmitter systems project from LH to VP (Bittencourt et al., 1992; Elias et al., 2001; Koylu, Couceyro, Lambert, & Kuhar, 1998), it is not clear which neurochemical system is mediating the projection stimulated enhancements of 'liking' and wanting'. One potential causal mechanism involves the orexin neuron population in LH, which colocalizes with glutamate and dynorphin. Previous work has shown that direct orexin receptor stimulation in the VP and NAc hotspots can amplify 'liking' reactions to sucrose, and GABA blockade (which should increase tonic neural activity of VP) increases food intake (Castro et al., 2016; Covelo et al., 2014; Ho & Berridge, 2013; K. S. Smith & Berridge, 2005). Together, orexin/glutamate signals may be acting to enhance food hedonic impact. By contrast, the anatomically nearby but distinct MCH-containing neuronal population (Swanson et al., 2005) has been shown to colocalize with both GABA (shown to abolish 'liking' and food intake in caudal VP) and CART (shown to reduce food intake) (Broberger, 1999; Elias et al., 2001; Ho & Berridge, 2014; Kristensen et al., 1998). As far as is known, VP does not appear to contain MCH receptors (Saito, Cheng, Leslie, & Civelli, 2001), making the MCH/CART/GABA projection an unlikely mediator of the effects described above.

Unlike direct VP stimulation, LH stimulation increased consumption of food by ~130% (i.e., 'wanting' to eat more food). LH induction of 'wanting-without-liking' is consistent with previous reports that electrode-induced deep brain stimulation of LH neurons, which elicits eating, similarly failed to enhance 'liking' reactions to tastes (K. C. Berridge & Valenstein, 1991). However, our results are also slightly puzzling given that LH-to-VP projection illumination did enhance 'liking' reactions as well as 'wanting' to eat. One possible explanation might be that LH neuronal stimulation activated projections that synapse in many other targets besides VP, with some of those additional targets cancelling the hedonic enhancement effect that could otherwise be induced via projections to posterior VP. It is also interesting that LH-to-VP stimulation increased food intake, even though direct VP stimulation did not. These results may be related to back-propagation, such that LH terminal stimulation activated LH cell bodies, thereby making this stimulation more similar to direct LH stimulation. Another possibility is that stimulating LH terminals caused the release of neurotransmitters (e.g., orexin, glutamate, dynorphin) which provided information not given by direct optogenetic stimulation of VP. This would further develop a functional role for LH-VP circuitry, suggesting that LH is able to focus motivation as well as amplify food palatability at the same neuroanatomical site. Future studies on VP and may yield more effective stimulation parameters that generate food intake directly.

In sum, direct stimulation of posterior VP neurons, as well as indirect activation via LH terminal illumination, amplified the hedonic impact of a sweet solution. By contrast, direct illumination of LH neurons or LH-to-VP terminals enhanced intake of palatable M&Ms and chow. Altogether, these data support a functional, but regionally specific, role for LH and VP in motivated and hedonic behaviors.

Laser intensity and ChR2 determines amount and spread of Fos activity



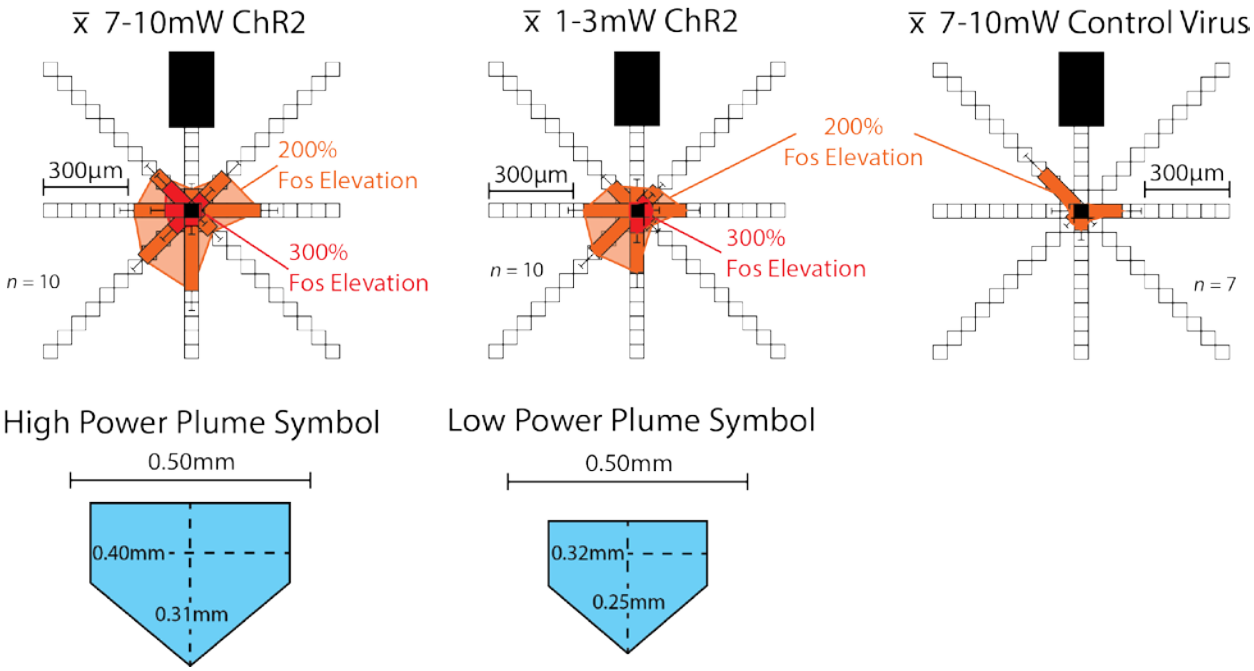
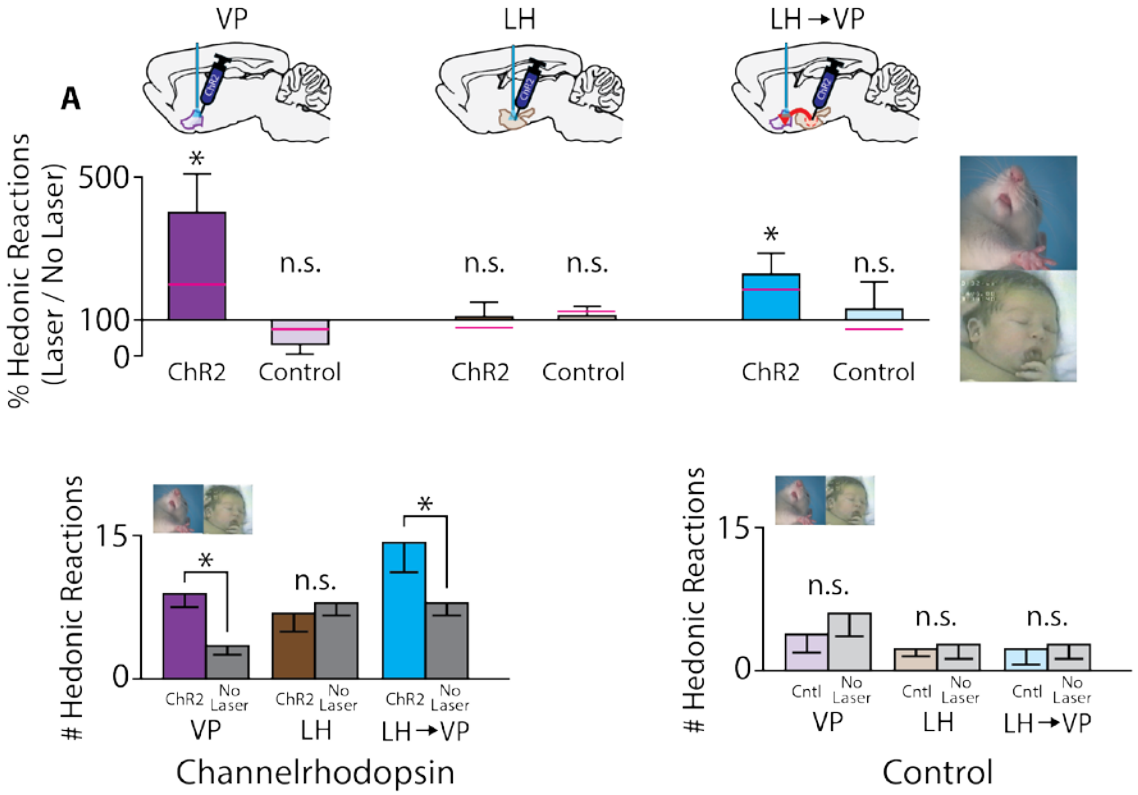


Figure 5.1. Functional spread of laser photostimulation. (A) Representative image of a ChR2 infected animal in LH with an overlaid grid (purple; each square 50µm x 50µm). The blue-outlined image shows overlap between Chr2 infected neurons (green) and Fos-positive neurons (Meredith et al.), whereas the gray outlined image displays no overlap. (B) Representative Fos plume for an individual animal. Orange indicates a 200% increase in Fos compared to tissue with no virus or photostimulation. Blue outlined squares display Fos-positive cells whereas the gray outlined box displays a Fos-negative square. (C) Average Fos plumes for High (7-10mW), Low (1-3mW), and control virus (7-10mW) conditions. Extrapolated plume shapes displayed below plumes.

VP laser stimulation enhances hedonic 'liking' for sucrose



LH laser stimulation enhances food 'wanting' for M&Ms

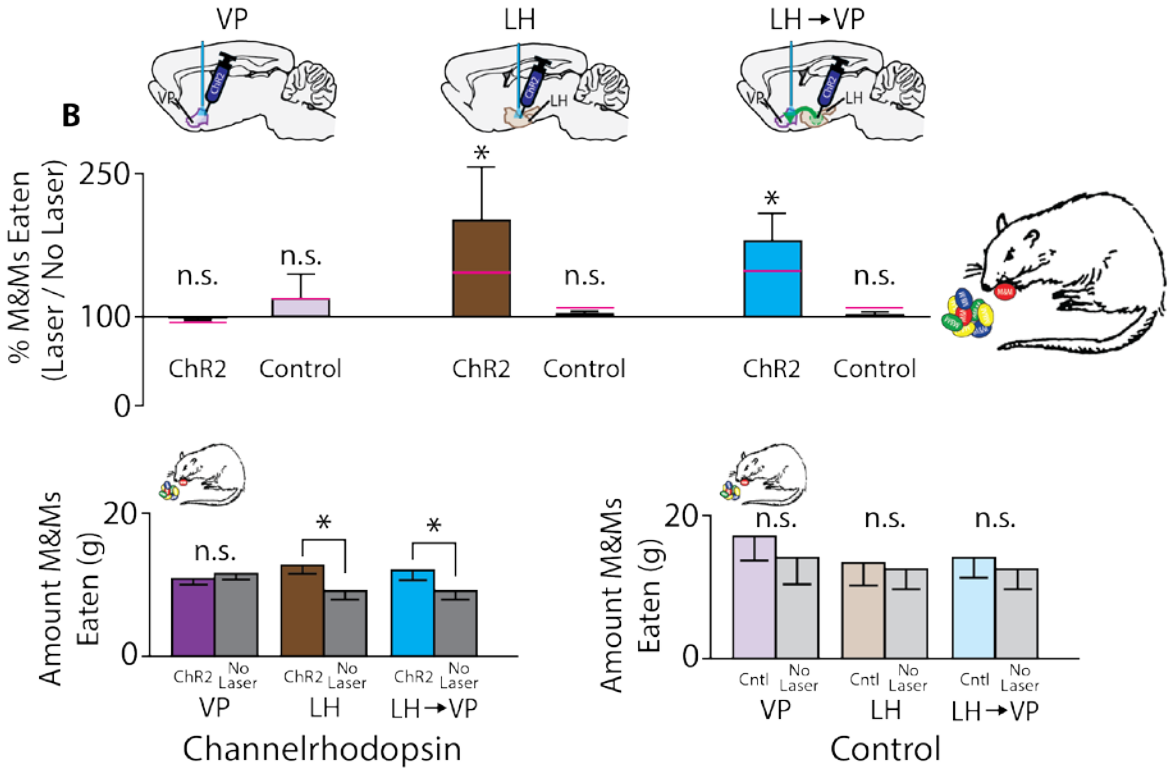
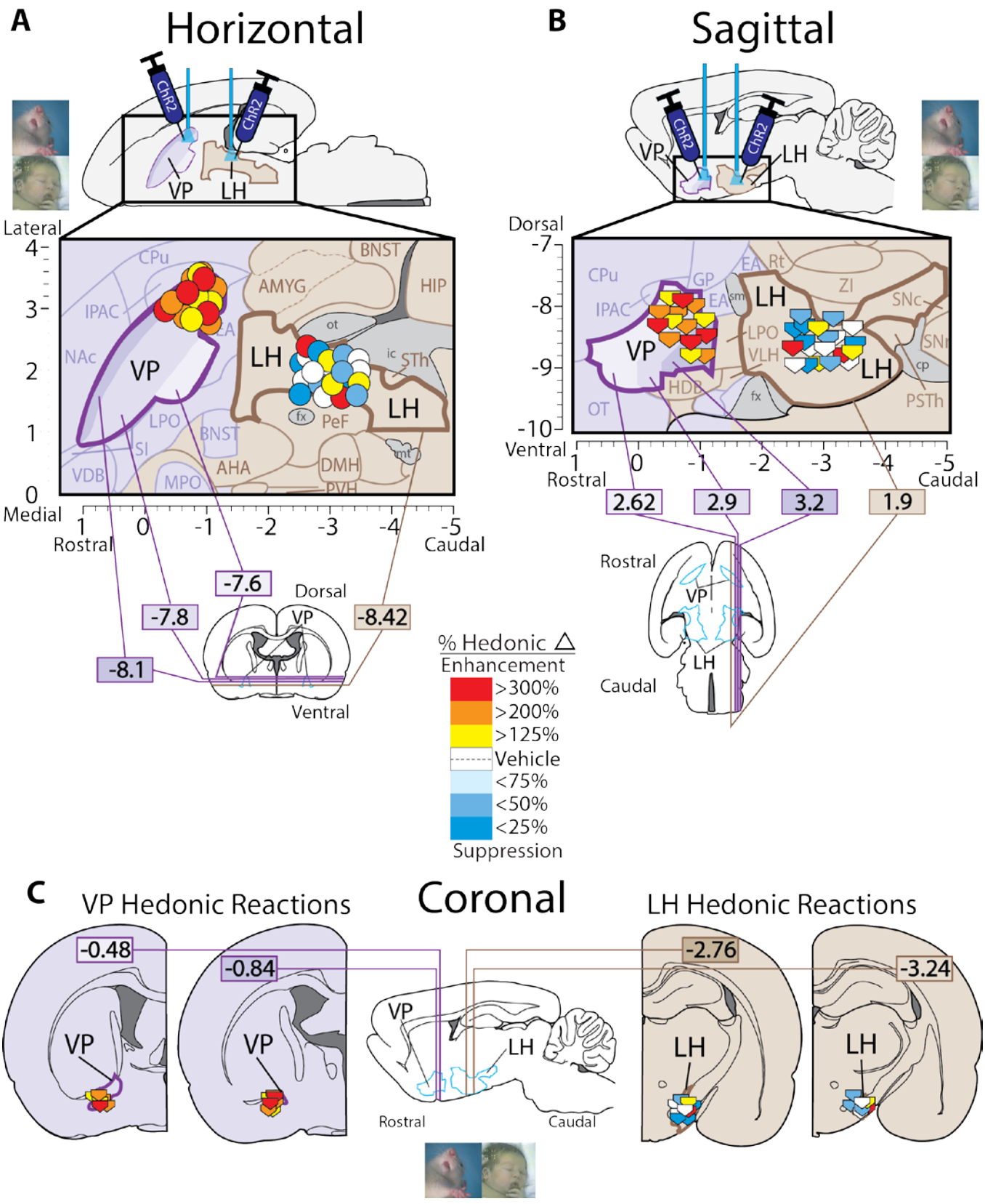


Figure 5.2. Summary of laser stimulated effects on hedonic impact and food intake. (A) Bar graphs in the top panel show percentage change in hedonic reactions after laser stimulation relative to a no laser baseline. Purple bars indicate virus and fibers implanted into VP, brown bars indicate virus and fibers in LH, and blue bars indicate virus in LH and fibers in VP. Pink lines indicate the median percent change for each group. Lower left and right panels shows raw hedonic scores for each condition with channelrhodopsin animals on the left, and control virus animals on the right. (B) Bar graphs in the top panel show percentage change in food intake after laser stimulation relative to a no laser baseline. Purple bars indicate virus and fibers implanted into VP, brown bars indicate virus and fibers in LH, and blue bars indicate virus in LH and fibers in VP. Pink lines indicate the median percent change for each group. Lower left and right panels shows raw food intake for each condition with channelrhodopsin animals on the left, and control virus animals on the right.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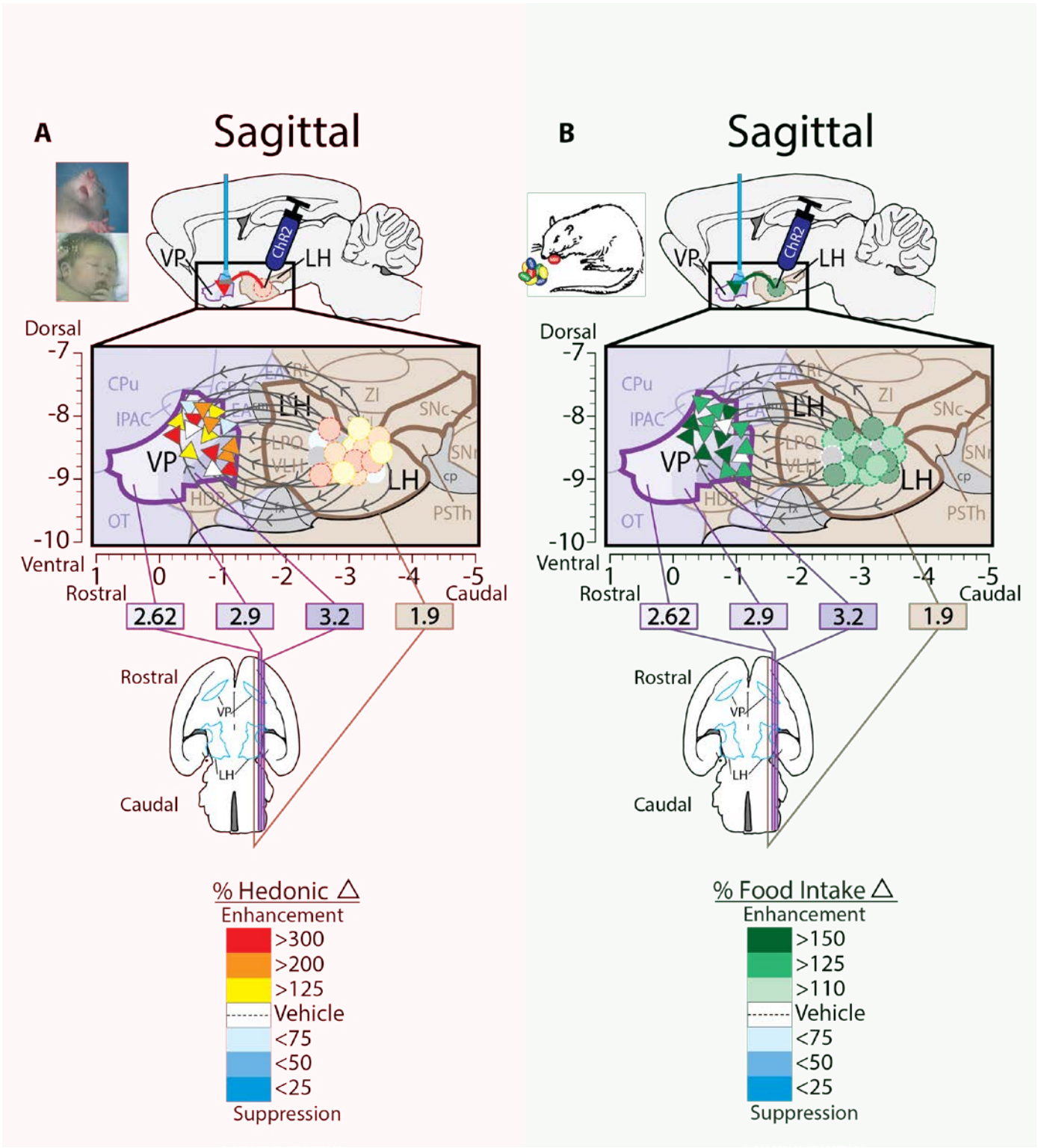
-2.76
-3.24
LH
LH

Figure 5.3. Functional maps of laser effects on hedonic impact in VP or LH. (A) Horizontal, (B) sagittal or (C) coronal placement maps showing spread of VP or LH virus infusions, with the behavioral effects of ChR2 illumination painted onto individual animals. Maps display several levels of the atlas onto one frame to more completely visualize the placements. Yellow to red indicates an increase in hedonic reactions, whereas light to dark blue indicates a suppression. EA, extended amygdala; LPO, lateral preoptic area; LH, lateral hypothalamus; VP, ventral pallidum.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Suppression

Figure 5.4. Functional maps of laser effects on taste reactivity and food intake in LH-VP projections. Sagittal placement maps showing spread of LH virus infusions and the fiber site in VP, with the behavioral effects of ChR2 illumination painted onto individual animals. Maps display several levels of the atlas onto one frame to more completely visualize the placements. (*A*) Percent changes in hedonic reactions to sucrose, with yellow to red indicating an increase in hedonic reactions, and light to dark blue indicates a suppression. (*B*) Percent changes in food intake for palatable M&Ms, with light to dark green indicates an increase in food intake, and light to dark blue indicates a suppression. EA, extended amygdala; LPO, lateral preoptic area; LH, lateral hypothalamus; VP, ventral pallidum.

VP hotspot stimulation does not alter taste reactivity to aversive tastes

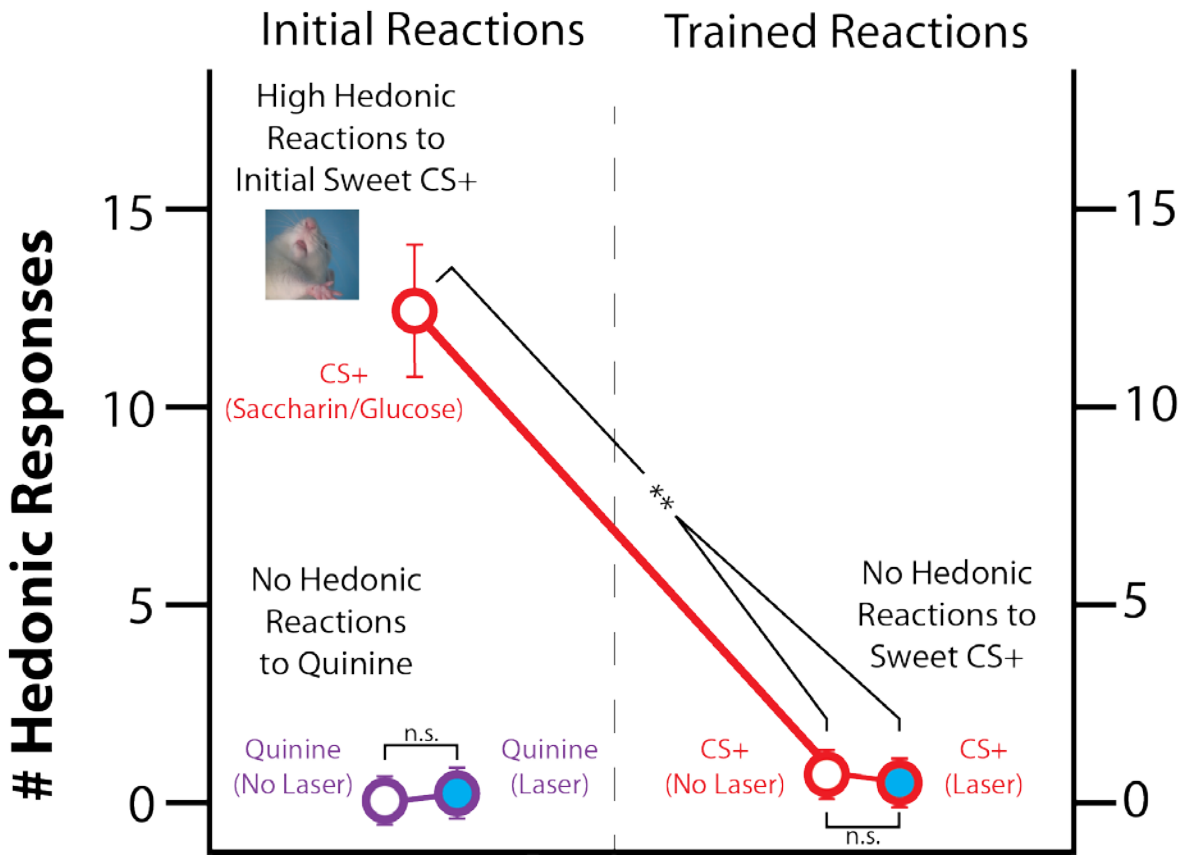
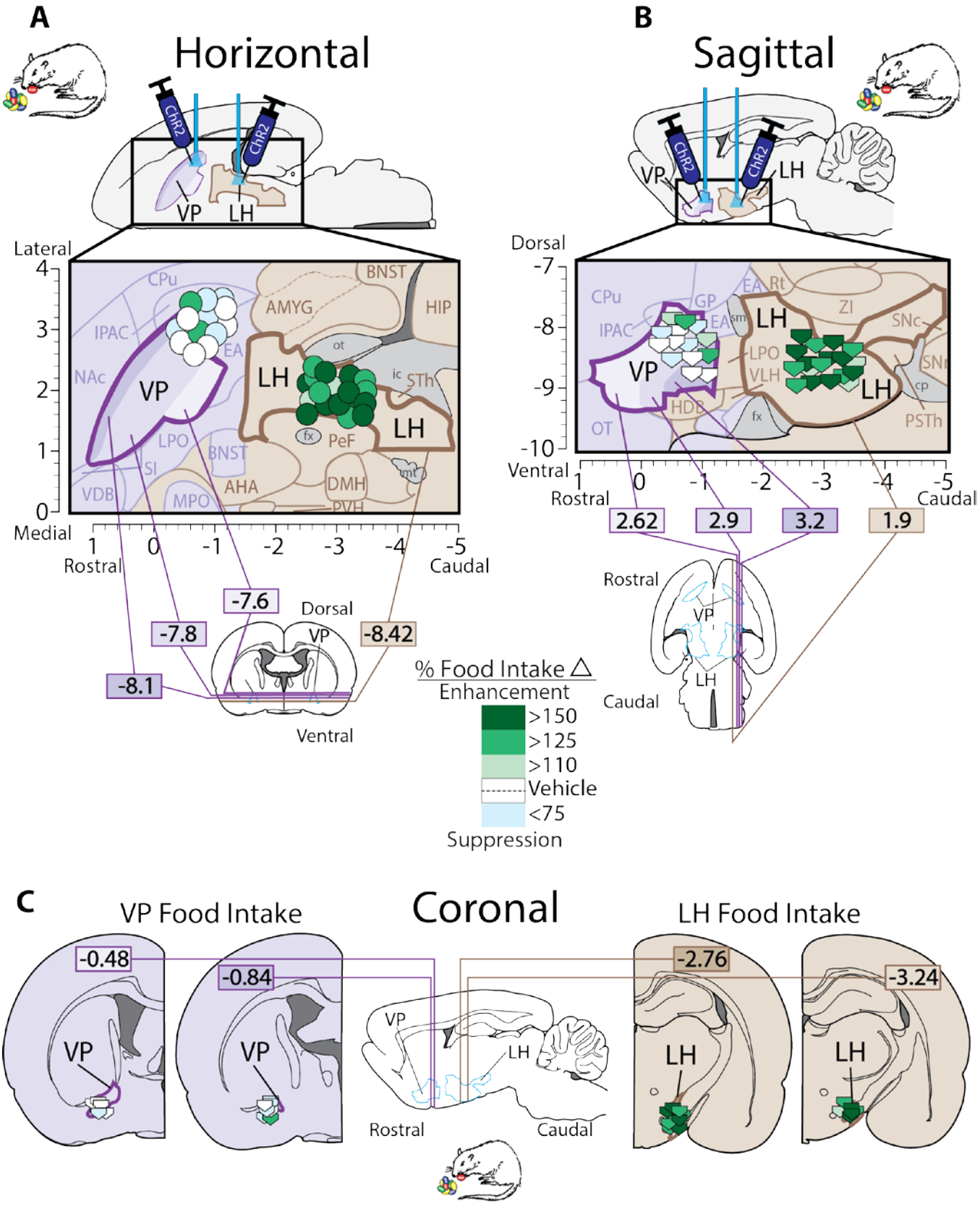


Figure 5.5. Laser stimulation is unable to reverse a conditioned taste aversion. The number of positive hedonic orofacial reactions to different taste stimuli are plotted before and after conditioned taste aversion pairings. Red circles indicate palatable sweet solutions (saccharin or glucose) and purple circles indicate bitter quinine. Open circles show baseline reactions without laser stimulation and blue filled circles show the number of positive reactions after laser stimulation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BNST
SI
VDB
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AHA
DMH
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-8.42
-8.1
Dorsal
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Ventral
B
Sagittal
ChR2
ChR2
VP
LH
Dorsal
Ventral
Rostral
Caudal
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OT
2.62
2.9
3.2
1.9
Rostral
VP
LH
Caudal
% Food Intake △
Enhancement
>150
>125
>110
Vehicle
<75
Suppression
C
VP Food Intake
Coronal
LH Food Intake
-0.48
-0.84
VP
VP
VP
LH
Rostral
Caudal
-2.76
-3.24
LH
LH

Figure 5.6. Functional maps of laser effects on food intake in VP or LH. (A) Bar graphs showing that LH-LH ChR2, but not VP-VP ChR2 or control eYFP, illumination enhances food intake of M&M candies. (B) Horizontal, (C) sagittal or (D) coronal placement maps showing spread of VP or LH virus infusions, with the behavioral effects of ChR2 illumination painted onto individual animals. Maps display several levels of the atlas onto one frame to more completely visualize the placements. Light to dark green indicates an increase in food intake, whereas light to dark blue indicates a suppression. EA, extended amygdala; LPO, lateral preoptic area; LH, lateral hypothalamus; VP, ventral pallidum.

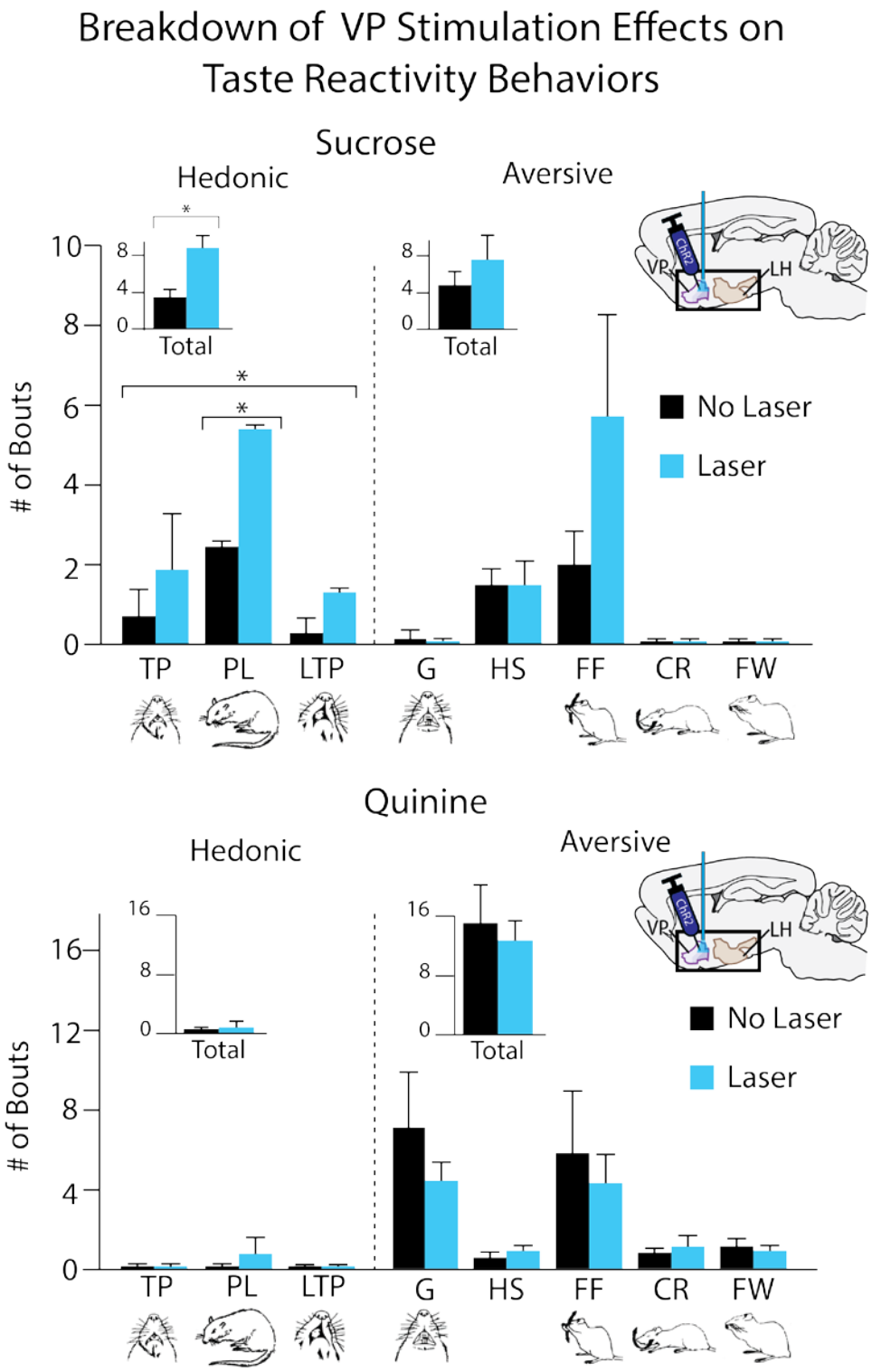
Breakdown of VP Stimulation Effects on Taste Reactivity Behaviors
Sucrose
Hedonic
Aversive
Total
Total
# of Bouts
No Laser
Laser
VP
LH
ChR2
TP
PL
LTP
G
HS
FF
CR
FW
Quinine
Hedonic
Aversive
Total
Total
# of Bouts
No Laser
Laser
VP
LH
ChR2
TP
PL
LTP
G
HS
FF
CR
FW

Figure 5.7. Distribution of orofacial reactions after VP stimulation. Individual positive 'liking' (TP: tongue protrusions; PL: paw licking; LTP: lateral tongue protrusions) and negative 'disgust' (G: gape; HS: head shake; FF: forelimb flail; CR: chin rub; FW: face wash) reactions to sucrose (top) or quinine (bottom). Laser stimulation broadly increased 'liking' reactions to sucrose, without altering aversive reactions to sucrose or orofacial reactions to quinine (black: no laser baseline; blue: laser stimulation).

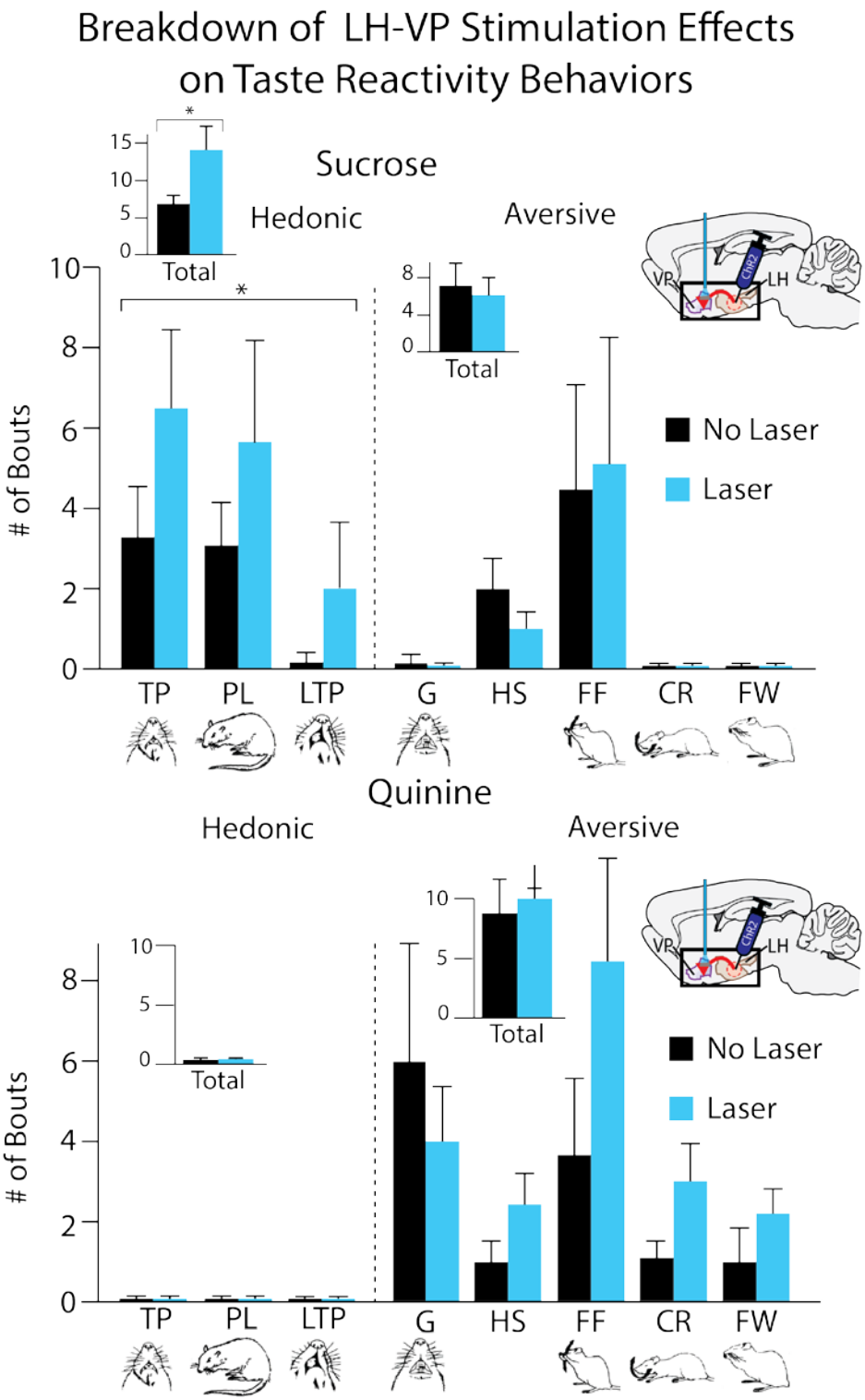
Breakdown of LH-VP Stimulation Effects
on Taste Reactivity Behaviors
Sucrose
Hedonic
Aversive
Total
# of Bouts
No Laser
Laser
VP
LH
ChR2
TP
PL
LTP
G
HS
FF
CR
FW
Quinine
Hedonic
Aversive
Total
# of Bouts
No Laser
Laser
TP
PL
LTP
G
HS
FF
CR
FW

Figure 5.8. Distribution of orofacial reactions after LH-VP stimulation. Individual positive 'liking' (TP: tongue protrusions; PL: paw licking; LTP: lateral tongue protrusions) and negative 'disgust' (G: gape; HS: head shake; FF: forelimb flail; CR: chin rub; FW: face wash) reactions to sucrose (top) or quinine (bottom). Laser stimulation broadly increased 'liking' reactions to sucrose, without altering aversive reactions to sucrose or orofacial reactions to quinine (black: no laser baseline; blue: laser stimulation).

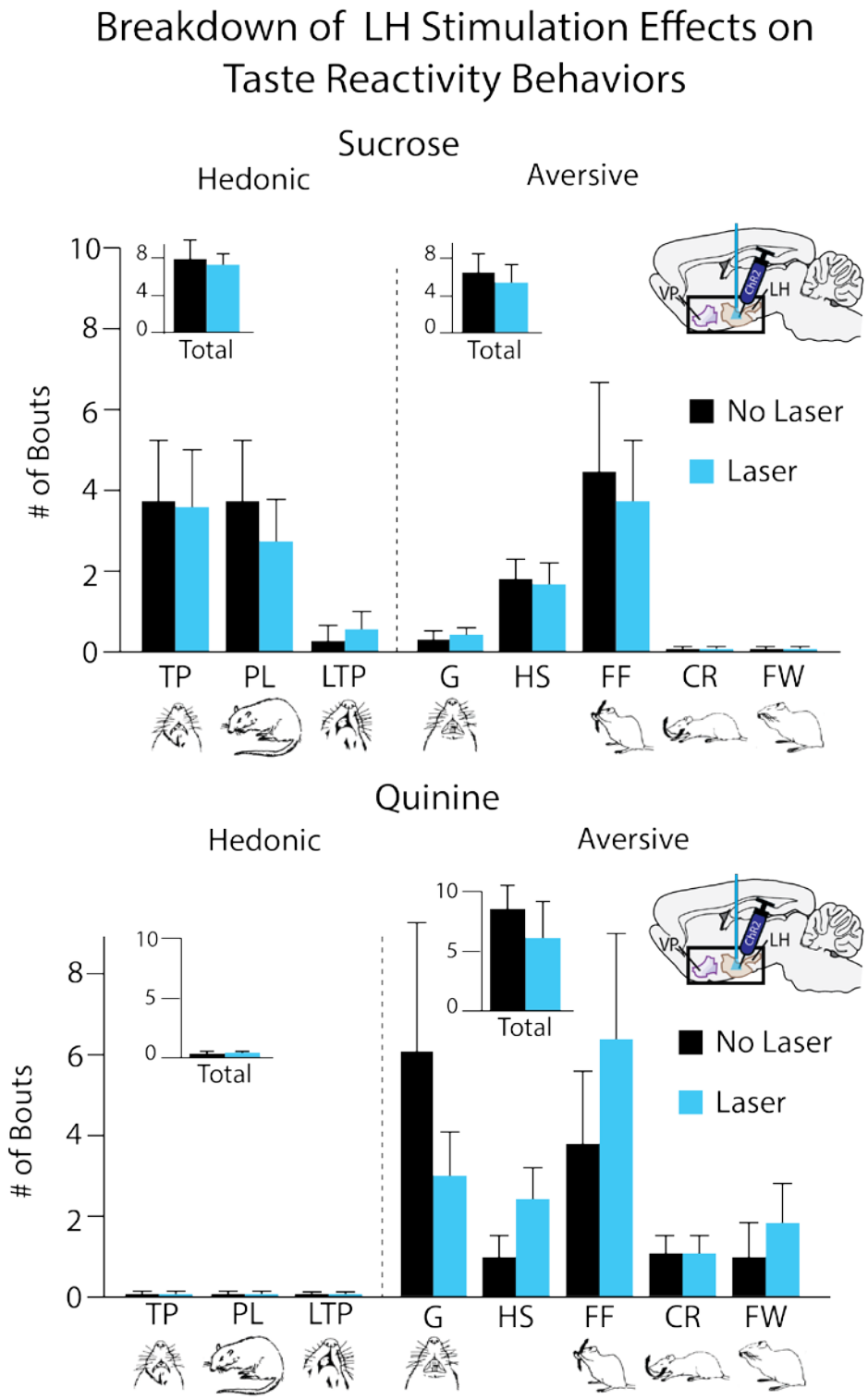
Breakdown of LH Stimulation Effects on Taste Reactivity Behaviors
Sucrose
Hedonic
Aversive
# of Bouts
Total
No Laser
Laser
VP
LH
TP
PL
LTP
G
HS
FF
CR
FW
Quinine
Hedonic
Aversive
# of Bouts
Total
No Laser
Laser
VP
LH
TP
PL
LTP
G
HS
FF
CR
FW

Figure 5.9. Distribution of orofacial reactions after LH stimulation. Individual positive 'liking' (TP: tongue protrusions; PL: paw licking; LTP: lateral tongue protrusions) and negative 'disgust' (G: gape; HS: head shake; FF: forelimb flail; CR: chin rub; FW: face wash) reactions to sucrose (top) or quinine (bottom). Laser stimulation never increased positive or negative reactions to sucrose or quinine (black: no laser baseline; blue: laser stimulation).

CHAPTER 6:

DISCUSSION

The studies presented in this dissertation explored the neuroanatomical substrates of hedonic 'liking' in subcortical and cortical nuclei, focusing on mu opioid and orexin neurochemical systems.

A unique site for hedonic enhancement in nucleus accumbens medial shell

One of the first hedonic hotspots was discovered in the rostrodorsomedial shell of NAc by mapping anatomical sites of hedonic 'liking' enhancement after mu opioid receptor stimulation (Pecina & Berridge, 2005). In chapter 2, I sought to evaluate the veracity of this surprising localization of hedonic enhancement, and to extend our knowledge of opioid function in NAc shell by also stimulating delta or kappa opioid receptors via drug microinjections. Work on food intake had indicated that delta receptors may be able to elicit motivated 'wanting' for food, but its inconsistency in the literature made it difficult to hypothesize its role (if any) in hedonic function. In some reports, it was shown that delta stimulation was sufficient to drive motivated intake, but in others it seemed to have no effect (Bakshi & Kelley, 1993; Katsuura & Taha, 2010). Kappa opioid receptors have long been associated with aversion and stress (especially in NAc), potentially indicating that instead of contributing to hedonic 'liking', they

may be involved in aversive 'disgust' (Bals-Kubik et al., 1993; Bals-Kubik et al., 1989; Land et al., 2008).

To determine how these three opioid receptor subtypes were involved in affective and motivated behaviors, I performed microinjections of receptor selective agonists (mu: DAMGO, delta: DPDPE, kappa: U50488H) throughout medial shell and tested subsequent behaviors on taste reactivity responses to sucrose, followed by an hour long food intake session. I found that stimulation of any of the three receptor subtypes was sufficient to enhance hedonic reactions by ~300%, but only if the injections were in the rostral half of NAc medial shell. Microinjection sites located at caudal levels oppositely suppressed hedonic reactions by ~33% in what we call a hedonic coldspot.

In contrast to the anatomically homogeneous results on hedonic reactions across drug conditions, drug effects on food intake differed greatly between receptor subtypes. Mu stimulation increased food intake throughout the entire rostrocaudal axis of NAc, even at coldspot sites in caudal shell. Delta stimulation also increased food intake, but only within the boundaries of the rostral hotspot. Kappa stimulation never consistently enhanced food intake at any localized site in NAc shell, which is consistent with previous studies (Bakshi & Kelley, 1993).

To further establish that rostromedial shell was uniquely capable of positively enhancing the affective qualities of a stimulus, I tested a separate group of animals in a conditioned place preference paradigm, in which stimulation of the hotspot was associated with a unique context. I found that animals developed a place preference for the drug-paired context, even animals that received kappa receptor stimulations. The CPP did not occur in sites surrounding the hotspot, and in fact the sites surrounding the hotspot that received kappa stimulation seemed to induce

what might be a place avoidance. These results are consistent with what Bals-Kubik et al. found in the mid 90's; that is, mu receptor stimulation in the nearby core did not generate a place preference, and kappa stimulation produced a place avoidance (Bals-Kubik et al., 1993).

In considering the results of kappa stimulation on food intake and conditioned place preference, it is puzzling that one behavioral task, but not the other, was sensitive to hotspot stimulations. One potentially relevant difference between the two tasks is the presence of the drug itself; kappa stimulation was occurring during the food intake task, and animals in the CPP paradigm were tested in a drug free state. Kappa receptors are predominantly found on dopamine and glutamatergic terminals (Svingos, Chavkin, et al., 2001; Svingos, Colago, et al., 1999), potentially allowing kappa stimulations to inhibit those inputs. By contrast, mu receptors are typically found on MSNs or local interneurons (Svingos, Colago, et al., 2001; Svingos et al., 1996), indicating that kappa stimulations may have the unique ability to modulate incoming glutamate or dopamine signals. I propose that the interaction of reduced glutamate and dopamine transmission would generate competing effects on motivated food intake, but leave hedonic functions unaltered. In 1995, Maldonado-Irizarry et al. (1995) reported that blockade of glutamate receptors in NAc medial shell generated an intense desire to eat. This effect has since been replicated (Reynolds & Berridge, 2003), and has been extended to show that the stimulated food intake requires local dopamine signals (although dopamine itself may not be necessary for general food intake) (Baldo, Sadeghian, Basso, & Kelley, 2002; Faure, Reynolds, Richard, & Berridge, 2008; Richard & Berridge, 2011b). However, while glutamate and dopamine signals are important for modulating motivated food intake, neither neurochemical system appears to be sufficient or necessary to enhance hedonic 'liking' reactions to sucrose in NAc shell (Faure et al., 2010; Wyvell & Berridge, 2000). Thus, the competing effects of blocking glutamate (which

should increase food intake) and dopamine transmission (which should prevent glutamate blockade induced eating) might account for the *inconsistent* ability for kappa stimulation to increase motivated food intake, as well as explain why the stimulations could still consistently alter hedonic orofacial reactions. However, this inconsistent disruption would only manifest during testing periods in which active kappa receptor stimulation occurred; animals tested in a drug free state would not be susceptible to the same neurochemical disruptions. In sum, kappa stimulation may disrupt the *expression* of motivation, but not the experience of pleasure 'liking', which may help explain why rats still showed a conditioned place preference (since they would have experienced the rewarding effects of kappa stimulation during conditioning, and would then be able to express that positive conditioning during the drug-free test day).

Orexin and acetylcholine systems modulate 'liking' and 'wanting' in NAc

In Chapter 3, I explored the potential roles of both incoming orexin signals and local acetylcholine signals in hedonic and motivated behaviors in NAc medial shell. I found that orexin stimulation in rostromedial NAc shell enhanced hedonic reactions to sucrose in a hotspot that nearly identically overlapped with the opioid hotspot described in Chapter 2. However, one difference between the two neurochemical systems was that orexin did not generate a hedonic coldspot in caudal shell. Orexin also did not modulate orofacial reactions to quinine, neither suppressing quinine 'disgust' nor increasing its palatability, even in the hotspot. For food intake, orexin, like mu stimulation, increased eating throughout NAc shell. Taken together, these results suggest that orexin may primarily be involved in enhancing already positively valenced stimuli, at least within NAc.

The reason for orexin's exclusivity for modulating positively valenced stimuli remain unresolved, but it is worth noting the similarities between the effects of orexin stimulation and

endocannabinoid stimulation in NAc on hedonic and motivated behaviors. In both cases, simulations only enhanced sucrose 'liking', without altering aversive reactions to sucrose or 'disgust' reactions to quinine (Mahler et al., 2007). In addition, stimulation of both systems was shown to increase food intake at all tested sites. Beyond taste reactivity and food intake, it has been shown that orexin and endocannabinoid systems can work synergistically to modify motivated behaviors, such as the formation of a conditioned place preference (Yazdi, Jahangirvand, Pirasteh, Moradi, & Haghparast, 2015). If these two neurochemical systems are working together to generate the same type of psychological signal, then it might be useful to consider where these receptors can be found within NAc, as this could indicate potential mechanisms of interaction. Endocannabinoid receptors are exclusively expressed on fast-spiking interneurons in NAc, and it is thought that they may exert their actions via a feed-forward inhibition signal onto MSNs (Winters et al., 2012). Further, the interneurons that contain the highest density of CB1 receptors are found at rostral sites of NAc; in other words the greatest number of CB1 receptors overlaps with our functionally defined hotspot. It is unknown whether such a concentration of OX-2 receptors in NAc also exists, but exploring the distribution and localization of OX-2 receptors may prove worthwhile for understanding its relationship to CB1 signaling and ultimately hedonic signaling.

In contrast to the effects of orexin, general muscarinic blockade via scopolamine promoted a broad shift toward aversive 'disgust'. Scopolamine microinjections decreased positive 'liking' reactions to sucrose at all sites tested in NAc medial shell (although it should be noted that it did not enhance aversive 'disgust' reactions to sucrose), and it similarly suppressed food intake of palatable M&Ms. These same microinjections also doubled the number of negative 'disgust' reactions to quinine, and generated fearful/defensive treading toward the light

refracting corners of the food intake chamber. Like the suppression of appetitive behaviors, these 'disgust' and 'fearful' responses were not anatomically localized. For the first time in the studies I have reported, I did not observe a localized effect of drug microinjections on affective taste reactions. These results might be related to how endogenous acetylcholine signals are transmitted, as well as the type of neurons that generate those signals. Within NAc, large acetylcholine interneurons account for only 1-2% of the total population of neurons, but their dendritic arborizations can infiltrate up to nearly a millimeter of tissue (Zhou, Wilson, & Dani, 2002). Since these neurons likely play a role in coordinating activity across NAc, it is not surprising that blocking ACh signaling anywhere in NAc would result in wide-spreading effects (i.e., why rostral muscarinic blockade still enhanced aversive 'disgust').

In relation to how acetylcholine signals themselves might be guiding the shift towards aversion, I suggest that the paradoxical pharmacological effects of scopolamine is important for explaining the simultaneous reduction of some behaviors (i.e., hedonic 'liking' and food intake) while increasing others (i.e., aversive 'disgust' and treading). Work by Pratt and Blackstone (2009) showed that the decreased food intake caused by scopolamine microinjections is unlikely to be mediated by the blockade of the M2 autoreceptors (which should increase ACh transmission). Instead, increased autoreceptor activity was shown to mimic the effects of scopolamine, suggesting that scopolamine's effects on food intake are likely due to blockade of post-synaptic muscarinic receptors. Direct inhibition of local ACh interneurons has also been shown to block cocaine CPP, which similarly suggests that decreased local ACh results in decreases reward-related behaviors (Witten et al., 2010). However, microdialysis assays have shown that scopolamine reliably and intensely increases local acetylcholine (Pfister, Boix, Huston, & Schwarting, 1994). If decreased appetitive motivation is mediated by post-synaptic

muscarinic blockade, then what effects might ambient increased acetylcholine (via autoreceptor blockade) have on behavior? Zhou et al. (2002) have shown that tonic acetylcholine acts on the nicotinic receptors found on incoming dopamine terminals, which results in increased dopamine release. Thus, if scopolamine is already generating a shift toward an aversive psychological state (via post-synaptic muscarinic blockade), then it follows that increased dopamine release in NAc, which would increase the incentive value of surrounding sensory stimuli, might selectively increase the salience of *aversive* stimuli. In the experiment described in this dissertation, this manifested as increased treading towards the brightly lit corners of the food intake chamber. Altogether, the simultaneous blockade of muscarinic signaling, coupled with increased nicotinic signaling, could result in the general shift toward aversive 'disgust' that I observed in this study.

In sum, the results from Chapters 2 and 3 suggest that the rostral NAc hotspot is a unique anatomical site capable of positively modulating hedonic impact. While the particular mechanism mediating each neurochemical enhancement of sucrose 'liking' remains unknown, it is likely that there is some synergistic interactions between them. Future work evaluating these local interactions, as well as a more thorough understanding of receptor location, will help to answer some of these lingering questions.

Orbitofrontal cortex and insula each contain a hedonic hotspot

In Chapter 4, I used the same microinjection mapping technique from Chapters 2 and 3 to functionally map the entire orbitofrontal and insular cortices, as well as nearby structures including medial PFC and piriform cortex. I found that both OFC and insula contain a mu/orexin hotspot, with the OFC hotspot localized to the rostral 2/3rd of OFC, and the insula hotspot restricted to its caudal 1/3rd. By contrast, one single, long coldspot was bookended by the two hotspots, stretching from rostral insula/caudolateral OFC and extending throughout mid-insula.

Like in NAc, the hedonic maps did not overlap with the maps showing changes in food intake. Food intake was increased at all sites in OFC, even in the caudolateral coldspot, but did not extend into insula. Mu opioid stimulation in piriform cortex was also sufficient to enhance food intake, even though the same sites did not alter hedonic reactions to sucrose. Whether insula is simply not capable of generating intense motivation, or whether purer tests of food intake would reveal 'wanting' enhancements remains unknown.

One surprising phenomenon that was uncovered in this experiment was the existence of one single cortical coldspot. Previous studies in NAc and VP have revealed discrete coldspots localized to the same structure as their corresponding hotspot, but the cortical coldspot bridged the transition zone between OFC and insula, functionally ignoring the anatomical boundaries. While initially surprising, I suggest that we may have actually misinterpreted the subcortical coldspots as individual functional zones; in other words, the NAc and VP coldspot may be one continuous strip comparable to the strip found in OFC and insula. Anatomically, opioid stimulation suppresses hedonic impact in NAc from where medial septal nucleus and ventral limb of the diagonal band bridge the hemispheres (~1.5mm anterior to bregma) to sites just caudal to the middle cerebral artery (~0.80mm anterior to bregma). Dorsoventrally, the coldspot fills the entire NAc (or at least at all sites tested; ~-6.25 to -7.75mm below bregma). The ventral pallidum coldspot begins at ~1.0mm anterior to bregma, and continues until roughly the genu of the anterior commissure (~0.0mm). Dorsoventrally, the VP coldspot extends the entire VP, from -7.75mm to almost -9.0mm below bregma. If both coldspots are plotted on the same map, it becomes clear that no true functional boundary exists to separate the two coldspots. In reality, what can observed is a ventral shift of the coldspot as NAc ends and VP begins, the same trend observed with the cortical coldspot. Further, unpublished work by Eric Jackson in our lab

showed that DAMGO microinjections into the bed nucleus of the stria terminals (BNST) suppressed hedonic reactions to sucrose. BNST is located directly caudal to NAc and dorsal to VP. If these results can be substantiated, it would seem that the subcortical coldspot is much bigger than previously thought, and may begin in NAc, but continues into BNST where it drops ventrally into VP. While there is not enough information to speculate how viewing the cortical and subcortical hedonic systems as having one large coldspot bookended by two hotspots is functionally relevant for affective processing, reshaping how we define the hot and coldspots may alter how we interpret potentially relevant inputs/outputs to these areas, as well as reveal new commonalities between these functionally defined zones that may not be obvious through investigations of discrete brain areas.

Optogenetic stimulation of LH inputs to the VP hotspot causally enhances 'liking' and 'wanting'

With the addition of the OFC and insula hotspots, it is becoming increasingly clear that affective processing is comprised of a set of distributed affective nodes. Work by Kyle Smith has already indicated that this may be the case, as functional interactions between NAc and VP have been shown. For example, mu opioid receptor stimulated enhancements of 'liking' in NAc or VP can be blocked by simultaneous blockade of opioid receptors in the other hotspot (K. S. Smith & Berridge, 2007). Further, increased neural firing in VP (which correlates with coding of hedonic value), can be boosted above normal levels by NAc opioid stimulations (K. S. Smith et al., 2011). If the hotspots do indeed work together to generate hedonic signals, then the hedonic signals generated at each hotspot must be somehow relayed across the brain.

One candidate system to relay their activity includes orexin, which is exclusively produced in hypothalamus, and has been shown to causally enhance hedonic 'liking' in all four hotspots (Baldo et al., 2003; Peyron et al., 1998). In particular, orexin neurons found in lateral

hypothalamus have been especially implicated in reward, whereas dorsomedial hypothalamic orexin neurons are associated with arousal (Adamantidis et al., 2010; Harris et al., 2005). Anatomically, LH orexin neurons are in a good position to modulate the hotspots as it shares reciprocal connections with all of them. Therefore, if LH neurons are involved in recruiting the hotspots, it follows that by stimulating direct lateral hypothalamic/orexinergic inputs into a hotspot, I should be able to replicate the effects observed with direct orexin microinjections.

In Chapter 5, I sought to determine if selective stimulation of lateral hypothalamic inputs in the VP hotspot could modulate hedonic 'liking' for sucrose or food 'wanting'. I found that selective stimulation did enhance both hedonic and motivated behaviors, without altering aversive 'disgust' behaviors to quinine. Further, I tested whether direct stimulations of either the VP hotspot or LH cell bodies specially contributed to the enhanced behaviors. VP stimulation was found to selectively enhance hedonic reactions to sucrose without altering food intake, whereas LH stimulation only increased motivated food intake without altering hedonic reactions. Collectively, these data suggest that an anatomically functional circuit between LH and VP exists whereby LH can tap into the hedonic VP hotspot to boost hedonic impact.

While the results of this study support a functional connection between LH and VP, it is somewhat perplexing that direct VP stimulation did not enhance food intake, but stimulation of the LH-VP projection did. Pharmacologically, disinhibition via GABA blockade can produce intense hyperphagia at all sites in VP (Covelo et al., 2014; K. S. Smith & Berridge, 2005), suggesting that increased excitation may be related to increased motivation. However, general disinhibition in VP likely alters the neurochemical profile of local microcircuits, altering the composition of ambient and incoming levels of neurotransmitters as well as how neurons ultimately fire. Direct optogenetic stimulation supersedes that neurochemical shift; by eliciting

action potentials directly, the surrounding changes in neurochemical microcircuitry may become irrelevant, or at the very least ignored. This artificial excitation may then be sufficient to enhance hedonic impact (likely through downstream recruitment of the other hotspots), but may not convey potentially relevant neurochemical signals required to enhance motivated food intake (e.g., orexin-A, dynorphin). Alternatively, the eating may have been a byproduct of back propagation, in which terminal stimulation produced an action potential back toward the cell bodies in LH. Increased LH activity through local circuits is likely to enhance food intake as this is more similar to direct LH stimulated eating. Future studies combining local pharmacologic blockade in VP with optogenetic terminal stimulation will be useful for assessing these types of interactions.

Although direct stimulation of VP or LH each resulted in no overall change in eating or hedonic impact, respectively, the manner in which they were ineffective was strikingly different. In VP, laser stimulation seemed to not affect behavior at all; animals ate exactly the same amount of M&Ms across test days. By contrast, LH stimulation sometimes had no effect, sometimes increased hedonic reactions, and at other times actually suppressed hedonic reactions. Based on the positive effects of projection stimulation in VP, coupled with orexin's hedonically suppressive role in the cortical coldspot, I hypothesize that this is indirect evidence for LH involvement with the other hotspots and coldspots to modulate hedonic impact. It is possible that laser stimulation in some rats may have somewhat preferentially projected to targets like the cortical or NAc coldspot in animals that showed a decrease in hedonic reactions, which would not prevent or contest those same regions' abilities to simultaneously enhance food intake. In the same vein, LH sites that increased hedonic reactions likely projected to one of the hotspots to recruit their hedonic abilities (as I saw with selective stimulation of LH-VP). While future work

will be needed to establish whether LH to hotspot/coldspot projections are functionally relevant as I observed with VP, it can at least be said that LH does appear to have the ability to recruit the VP hotspot to selectively augment hedonic impact.

A role for lateral hypothalamus in affective processing

The results of the optogenetic experiment indicate that although LH itself may not be a site of hedonic generation, it has the ability to recruit the hotspots to enhance the hedonic impact of a taste stimulus. Based on the orexin microinjection results in NAc, OFC, and insula, it would be of interest to test whether stimulation of LH terminals in these sites would likewise enhance hedonic impact. If LH does not house a hotspot (as far as we can tell), then the question becomes: what purpose does the LH orexin system serve in the hedonic circuit?

One potential explanation could be that its role in affect is merely one of several roles that LH subsumes in its generation of behavior. LH has long been associated with many types of motivated behaviors, being important for food and drug seeking (Anand & Brobeck, 1951; Jennings, Rizzi, Stamatakis, Ung, & Stuber, 2013; Richardson & Aston-Jones, 2012), affiliative and aggressive behaviors (Haller, 2013; Woodworth, 1971), and approach/avoidance elicited by reward/punishment cues (Cole, Hobin, & Petrovich, 2015; Harris et al., 2005; Petrovich, Holland, & Gallagher, 2005). As discussed in Chapter 1, motivation and affect can be teased apart as distinct psychological mechanisms, but in reality these two components often work together to generate behavior. LH may act as a site that integrates affect and motivation, directly modulating the hedonic impact of a stimulus (via its projections to the hotspots) that then redirects and guides the attribution of incentive salience and subsequent motivated behavior (via its projections to striatum, VTA, cortex, etc.). Although a relatively new hypothesis, there does seem to be evidence for LH, and even LH orexin neurons themselves, being involved in affective

coding. In one study, LH neural activity appears to track the palatability of sweet versus bitter tastes, independently of any sensory stimulus/quality coding that may be occurring (Li, Yoshida, Monk, & Katz, 2013). A more recent study has shown that orexin neurons may even track the positive or negative valence of a reward cue, potentially supporting the idea that LH may integrate affective and motivated signals (Hassani, Krause, Mainville, Cordova, & Jones, 2016).

In addition to being sufficient to modulate hedonic impact, LH (or at the very least the forebrain in general) may in fact be necessary for transformations of hedonic value, especially for the expression of sensory alliesthesia. With its diffuse connections with mesocorticolimbic motivated systems, and its particular connections with the known hedonic hotspots, it is well positioned to modulate affective responses (experience of alliesthesia) as well as act on those changes to best suit the individual (motivated response). Early decerebrate studies, in which rats received transections at the level of the midbrain (effectively cutting off forebrain control of brainstem reflex systems) showed that decerebrate rats do *not* demonstrate the ability to adapt to learned or homeostatic pressures that alter taste palatability. Explicitly, decerebrate rats are unable to learn conditioned taste aversions and do not display hunger alliesthesia (Grill & Norgren, 1978b). These studies provided the first clue that forebrain sites are required for flexible affective responses, even if the motor effector systems are contained within the brainstem. Future studies evaluating the role of LH-hotspot projections during various types of homeostatic challenges (e.g., salt depletion, hunger) would interesting and useful for determining whether it is playing an active role in this process.

Titrating affect: A specific role for LH in the VP hotspot

The ventral pallidum was the first hotspot to be mapped in 2005 using gain of function anatomical maps, but the localization of caudal VP as a site important for generating hedonic

impact had been established more than 10 years earlier through discrete lesions (Cromwell & Berridge, 1993; K. S. Smith & Berridge, 2005). The early lesion studies that led to the discovery of the VP hotspot were striking not only because its destruction led to the disappearance of hedonic 'liking' for palatable food, but because they flipped positive affective responses into aversive 'disgust' reactions. However, the manifested 'disgust' was transient, such that reactions to sucrose returned to normal even before the aphagia symptoms disappeared. If the VP hotspot was truly necessary for the generation of positive affect, then positive reactions to sucrose should never have been restored after lesions. But because hedonic reactions do return, this suggests that VP lesions (or inactivations) may actually cause an affective imbalance that initially allows negative affect to obscure or trump positive affect. This imbalance can be conceptualized as an overwhelming release of 'disgust', rather than a true loss of hedonic 'liking', which implies that not only is VP an important site for generating hedonic impact, but that it serves a second, equally important role of tempering the expression of 'disgust'.

What neural mechanisms might underlie this second role for VP in 'disgust' regulation? To begin answering this question, we can examine the results of a pilot study I conducted that are related to the results presented in Chapter 5. In that chapter, I showed that stimulation of LH terminals in the VP hotspot could selectively enhance sucrose 'liking' without altering quinine 'disgust'. However, while that study did provide evidence for LH sufficiency, it did not address whether this LH projection was necessary for affective processing. In an effort to address that, I performed a pilot study in which I optogenetically inhibited the LH-VP hotspot pathway during sucrose or quinine infusions and measured subsequent orofacial reactions. While I did not see a decrease in hedonic reactions to sucrose, I did observe a marked increase in aversive 'disgust' reactions to quinine. Although somewhat surprising, these results at least partially replicated the

effects of direct VP hotspot lesions, suggesting that perhaps the 'disgust' release observed in lesion studies might in part be related to a disruption of communication between LH and VP. More broadly, changes in LH activity in the VP might be important for titrating, or dialing up or down the degree of positive and negative affective valence that is ultimately experienced. In other words, when LH-VP hotspot activity is high, preferential augmentation of positively valenced stimuli occurs. In contrast, when LH-VP hotspot activity is low, preferential augmentation of negatively valenced stimuli occurs. If such an interaction is important for mediating how much of an affective gloss will be generated, then it would be interesting to test whether LH-VP *coldspot* stimulations or inhibitions might cause the opposite response: increased activity could result in decreased 'liking', whereas decreased activity might result in decreased 'disgust' reactions. This hypothesis could account for why selective disruption of LH-VP hotspot did not alter sucrose hedonic impact; although decreased LH-VP hotspot activity was sufficient to begin increase aversive 'disgust', the lack of a concurrent increase in LH-VP coldspot activity allowed normal hedonic impact to be preserved (thus preventing a full affective flip). While still speculative, the opposing effects of LH-VP hotspot stimulations versus inhibitions indicate that the LH-VP pathway may be especially important for titrating the expression of positive or aversive signals in VP.

Functional extrapolation for multiple hotspots

While the results of this dissertation provide evidence for four discretely localized hedonic hotspots (and potentially 2 large coldspots), it is somewhat perplexing to have so many hedonically vocal sites. Based on work by Kyle Smith, it seems unlikely that the four hotspots are redundant fail safes for generating hedonic impact, as interactions between the hotspots are necessary for hedonic amplifications (at least in the subcortical hotspots) (K. S. Smith &

Berridge, 2007). Future work will need to ascertain whether such interactions are necessary between the cortical hotspots, between the cortical and subcortical hotspots, and between the two coldspots. If interactions are required, then it seems likely that each hotspot is probably contributing some sort of unique signal important for the holistic experience of affect. What special contribution each hotspots makes remains unknown, but it may be useful to examine what sort of information is relayed through these hedonic areas, as unique connectivity may reveal potential functional roles.

One example of functional extrapolation has already been explored with the VP hotspot, namely, that activity in this area via LH may be important for determining the degree of affective valence that is assigned to a stimulus. Another case for functional extrapolation can be explored with the insula. As I discussed in Chapter 4, the insula has been shown to house both primary gustatory and visceral cortex. But in addition to these sensory modalities, insula has been shown to receive inputs from auditory, olfactory, vestibular, and visual cortex, as well as pain/temperature/somatosensory related information via SI, SII and thalamus (Cechetto & Saper, 1987; Kimura, Imbe, & Donishi, 2010; Nieuwenhuys, 2012; Shi & Cassell, 1998a, 1998b). Unlike the other known hedonic zones, insula appears to be specially innervated by all sensory modalities. Access to the pure sensory information puts insula in an excellent position to paste hedonic value directly onto sensory stimuli. While the afferents for each sensory modality are not represented across all insular sites (e.g., gustatory cortex lies completely within the coldspot), the extremely dense projections within insula still allow neurons in the functionally defined hot and coldspot to have access to the localized sensory information. While this is still speculative, having an affective zone designated to pasting hedonic value onto sensory stimuli (i.e., insula), in combination with an affective zone designated to assigning affective valence (i.e., VP), helps to

provide a logic for the existence of multiple hedonic zones. Future studies that deliberately disrupt particular inputs will hopefully reveal distinct roles for each hedonic area.

Where is 'disgust'?

Another remaining mystery is whether or not a similarly distributed 'disgust' hotspot circuit exists. While individual studies have localized potential sites of interest (e.g., caudal accumbens), no comprehensive system has been identified. Although speculative, I believe one potential iteration of a 'disgust' circuit could be the reverse of the hotspot circuit. In other words, from 'disgust's' point of view, each hotspot may be an aversive coldspot, and each hedonic coldspot may be an aversive hotspot. Evidence for a "reversed" 'disgust' circuit mainly stems from work in NAc. Within caudal NAc, GABA stimulations have been shown to robustly increase aversive reactions to both quinine and sucrose (Ho & Berridge, 2014). The scopolamine results described in Chapter 3 also suggest that caudal shell may be especially important for eliciting negatively valenced behaviors, since the treading observed was most robust at caudal sites.

One reason for the paucity of findings concerning sites that increase 'disgust' may be related to the neurochemical systems that have been tested. As the list of neurochemicals that can enhance 'liking' in the hotspots continues to grow, it is likely that our work so far has been biased toward testing neurochemicals that are already associated with reward (i.e., opioids, orexin, endocannabinoids). I hypothesize that a separate suite of neurochemicals such as melanocortin or CART, which are already associated with aversion (Aja, Robinson, Mills, Ladenheim, & Moran, 2002; Chaki, Kawashima, Suzuki, Shimazaki, & Okuyama, 2003; Lim, Huang, Grueter, Rothwell, & Malenka, 2012; Zheng et al., 2010), may be better candidate systems for exploring the neuroanatomical localization of aversive 'disgust'. Alternatively,

future studies could examine unique anatomical features to help choose novel neurochemical systems to test. For example, caudal shell of NAc receives a dense innervation of norepinephrine that is absent at rostral sites (BNST and VP also receives substantial inputs) (Baldo et al., 2003; Foote, Bloom, & Aston-Jones, 1983; Swanson & Hartman, 1975). Though surprisingly little research has explored the local role of norepinephrine on motivated behaviors, recent work has shown that glucocorticoid enhanced memories of a conditioned taste avoidance requires local norepinephrine, as does cannabinoid induced conditioned place avoidance (Carvalho & Van Bockstaele, 2011; Wichmann, Fornari, & Roozendaal, 2012). The unique projection pattern of norepinephrine, as well as some hints that it may be important for aversion, suggests that this may be a useful neurochemical system to employ in the search for the neural sites of 'disgust' generation.

Conclusion

In conclusion, the studies described in this dissertation introduced two novel affective cortical sites, and integrated LH into a broader hedonic circuit, providing a scaffold for how to interpret the hedonic system as a dispersed neural process. It also examined the role of three distinct neurotransmitter systems throughout a distributed hedonic network. Collectively, these results demonstrate the need for a more comprehensive investigation into the neural mechanisms of affect which may ultimately lead to a better understanding of normative affective processing in the brain.

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