Dissociable Mesocorticolimbic Contributions to Pleasure and Motivation

by

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Dedication

For my grandfather, Oscar. My first teacher.

Para mi abuelo Oscar. Mi primer maestro y la persona que despertó en mí el amor por el apredinzaje y la búsqueda del conocimento.

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Abstract

Mesocorticolimbic systems are heavily implicated in the control of reward. Reward contains multiple components that include 'liking', 'wanting', and learning processes (Berridge, 2004; Berridge & Robinson, 2003; Morales & Berridge, 2020). Over many decades, most attention has been paid to understanding 'wanting' and learning components, and 'liking' has remained the least understood. However, recent progress in understanding brain generators of hedonic impact has been made through the identification of brain hedonic hotspots, or small subregions of mesocorticolimbic systems that causally amplify affective 'liking' expressions to pleasant tastes in nucleus accumbens medial shell (NAc), caudolateral ventral pallidum (VP), rostromedial orbitofrontal cortex (OFC), and caudal insula in response to a few neurochemical signals including orexin and mu-opioid receptor agonists (Castro et al., 2016; Castro & Berridge, 2014c, 2017; Ho & Berridge, 2013; Mahler et al., 2007; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005; Söderpalm & Berridge, 2000).

Thus far, hedonic hotspot sites within mesocorticolimbic regions have primarily been studied using drug microinjection techniques, such as through the use of mu-opioid, orexin, and endocannabinoid agonists. This leaves open the possibility that hedonic hotspot amplification of 'liking' reactions is a mere artifact of the pharmacological approaches used, rather than a true neurobiological mechanism that exerts hedonic control. In order to provide triangulating evidence that hedonic hotspots are true neurofunctional entities capable of controlling affective responses, I use optogenetic techniques as an alternative method of controlling neuronal activity

within known hedonic hotspot sites. Chapter 2 of this dissertation investigates cortical control of 'liking' reactions to determine how neuronal excitation in rostromedial OFC and caudal insula hedonic hotspots increases positive affective responses to sweetness and other pleasant tastes.

The existence of cortical hedonic hotspots in OFC and insula raises the possibility that other corticolimbic regions heavily implicated in emotion and affective responses may exist in areas not yet identified. Thus, in Chapter 3 I use ChR2 activations to map a region of mid cingulate cortex in rats that has never been previously tested for hedonic function. I show that activating neurons within a mid-to-caudal region of cingulate cortex nearly doubles positive 'liking' reactions to pleasant tastes, indicating the existence of a new hedonic hotspot not previously characterized.

In Chapter 4, my efforts move subcortically to probe the *necessity* of the caudolateral ventral pallidum hedonic hotspot for normal 'liking'. First, I inhibited local neurons in subregions of VP to determine 'wanting' vs 'liking' contributions in rostral and caudal sites. Then, I further probe VP control of hedonic function by selectively manipulating the activity of VP^{GABA} neurons using ChR2 to excite and iC++ to inhibit GABA populations. This work shows that caudal VP^{GABA} neurons bidirectionally control 'liking' reactions. By comparison, rostral VP^{GABA} neuron activations, which oppositely suppress 'liking' still increase incentive motivation for palatable rewards, and even promote a maladaptive pursuit of pain in some rats.

Finally, in Chapter 5 I investigate amygdala control of incentive motivation for intravenous opioids. I pair central amygdala (CeA) neuron stimulation with receipt of a specific laser-paired intravenous reward, the synthetic opioid remifentanil, and show that rats exclusively pursue this laser-paired remifentanil and ignore an identical remifentanil infusion that is never laser paired. Further, in rats choosing between intravenous remifentanil and natural sucrose

rewards, pairing CeA with either reward caused CeA ChR2 rats to become ‘sucrose addicts’ or ‘remifentanil addicts’ so that only the laser-paired reward becomes pursued.

Altogether, this dissertation demonstrates that mesocorticolimbic systems in OFC, insula, cingulate cortex, ventral pallidum, and central amygdala are crucial sites for the control of ‘liking’ and/or ‘wanting’ for reward. Importantly however, ‘liking’ is restricted to small subregions of hedonic hotspots where optogenetic manipulations casually amplify hedonic impact for sweetness. Outside of these hotspots, optogenetic manipulations fail to increase ‘liking’ reactions, and sometimes even oppositely suppress affective reactions. In some cases, such in central amygdala, maladaptive ‘wanting’ can be generated for natural and drug rewards that is never matched in changes in ‘liking’. The neural mechanisms underlying these different motivational and hedonic processes provide important insights onto hedonic and motivational dysfunctions that may contribute to various affective and other psychological disorders.

Chapter 1 Introduction

A fundamental question of psychology and affective neuroscience is how the brain enables us to identify, seek, and obtain things in the world that are biologically relevant. This psychological process, known as reward, guides us in our everyday pursuits and needs, often without our conscious participation. Importantly, reward as a functional and psychological construct is anything but simple. It involves distinct 'liking', 'wanting', and learning process that must each occur and interact in order for true reward to occur (Berridge et al., 2009; Berridge & Robinson, 2003). Under normal conditions in the world, 'liking', 'wanting', and learning occur together (Berridge & Kringelbach, 2015), and this inherent entanglement can complicate our efforts to understand their unique functions.

Some insights into 'liking', 'wanting', and learning components of reward have come from extreme, but real-world examples in clinical populations of human patients who suffer from various affective and motivational disorders including addiction, schizophrenia, and depression (Olney et al., 2018; Salamone et al., 2016; Treadway & Zald, 2011, 2013; Whitton et al., 2015). But rodent models that study causal neural function can also greatly help us understand how the brain generates these distinct reward processes. In the chapters that follow, I will argue that reward subcomponents are reducible to distinct neurobiological underpinnings, and through paired brain manipulations with specifically designed behavioral tests, we can isolate each of these separate processes in order to better understand them and their unique functions. In particular, I focus my pursuits on better characterization of the unique brain systems that control 'liking' vs. those that mediate 'wanting'.

1.1 'Liking' and 'Wanting' as separate psychological processes.

The words 'liking' and 'wanting' are often used interchangeably in ordinary life when talking about rewards. For example, people may want a palatable piece of chocolate because they like the flavor and other sensations of consuming it. In ordinary use, liking means conscious pleasure and wanting means conscious desire, which typically involve cognitive appraisals and declarative goals mediated by cortically weighted circuitry. But here I use quotations for 'wanting' and 'liking' in order to distinguish specific psychological processes from ordinary use (Berridge & Kringelbach, 2015). 'Wanting' here refers to incentive salience, which can occur either consciously or unconsciously, generated by brain mesolimbic circuitry in the form of cue-triggered motivation. When rewards such as palatable foods and their predictive cues are imbued with incentive salience by mesocorticolimbic circuitry, those cues and foods become attractive, and in conscious form able to elicit subjective cravings. Whether conscious or not, incentive salience triggered by cues can also generate behavioral urges to seek and consume their associated rewards (Berridge, 2018; Winkielman & Berridge, 2004). In the laboratory, 'wanting' is typically measured in humans by subjective craving ratings, and in animals by how much food is pursued, consumed, or preferred over an alternative. 'Liking' refers to the hedonic impact of pleasant rewards, which when surfaced into consciousness can result in subjective pleasure ratings in adult humans, but in which in animals and human infants can be assessed via objective measures of hedonic orofacial expressions elicited to taste using the taste reactivity test (Berridge, 2000; Grill & Norgren, 1978c; Steiner, 1973, 1974; Steiner et al., 2001). 'Liking' and 'wanting' can become separated in some conditions, as discussed below.

1.2 The taste reactivity test: Objective measure of hedonic impact

The hedonic taste reactivity task measures affective orofacial reactions to tastes of sucrose, quinine, water, etc., and the reactions to any given taste can also be shifted by a variety of relevant physiological, learning, and brain manipulation factors that alter its palatability. Originally pioneered by Steiner for use in human infants (Steiner, 1973), the test was adapted for rodents by Grill and Norgren (Grill & Norgren, 1978c). Orofacial responses to taste are grouped into positive, neutral, and aversive categories. Positive hedonic 'liking' evaluations are reflected in tongue protrusions, paw licks, and lateral tongue protrusions, and are typically elicited by palatable tastes such as sucrose. By comparison, negative aversive or 'disgust' evaluations are reflected by gapes, forelimb flails, headshakes, paw treading and face washes, and typically elicited by bitter quinine. Many of these orofacial expressions to taste are evolutionarily conserved across mammalian species ranging from human infants to non-human primates, rodents, and horses (Berridge, 2000; Jankunis & Whishaw, 2013; Steiner et al., 2001). Our laboratory uses rodents who have been implanted with bilateral oral cannula, which allow taste solutions to be directly infused into their mouths without them having to engage in any appetitive activity to obtain them, allowing experimenter control of stimulus intensity and duration. Independence from appetitive or instrumental decisions to consume is important in allowing taste reactivity to provide a relatively pure measure of taste-elicited 'liking', without being altered by changes in 'wanting' that can influence most other behavioral measures of food reward (Berridge, 2000; Castro & Berridge, 2014a).

Tastants with very different sensory properties like sucrose, saccharin, salt, and fats can all evoke similar positive 'liking' responses, indicating that hedonic reactions are palatability-specific rather than sensory-specific (Davidson et al., 2011; Peciña & Berridge, 2005; M. J. F.

Robinson & Berridge, 2013; Shin et al., 2011; Steiner et al., 2001). Accordingly, taste reactivity behaviors are not simple inflexible reflexes to a particular sensation, but rather reflect a hedonic evaluation that also depends on the internal state of the organism, including physiological appetite and satiety states, neurobiological states, as well as learned associations carried from previous experiences with the taste.

Physiological states like hunger and satiety can shift subjective ratings of palatability for a particular taste in humans, in a phenomenon known as alliesthesia (Cabanac, 1971, 1979; Laeng et al., 1993). In rodents too, caloric hunger magnifies hedonic 'liking' reactions to palatable sweet taste, whereas satiety conversely reduces 'liking' (Berridge, 1991; Cabanac & Lafrance, 1990). Similarly, salt appetite modulates the hedonic impact of the intense saltiness taste of concentrated NaCl. For example, hypertonic concentrations of salt are normally aversive, in the sense that rats mostly display 'disgust' reactions when a seawater concentration of NaCl is placed into their mouths. However, when a hormonal state of sodium deficiency or salt depletion is induced, orofacial reactivity to the same intensely salty taste shifts to mostly positive 'liking' (Berridge & Grill, 1984; Berridge & Schulkin, 1989; Clark & Bernstein, 2006; M. J. F. Robinson & Berridge, 2013; Tindell et al., 2006, 2009). Conversely, modulation by learned associations can be induced by pairing a novel 'liked' sweet taste of saccharin as a Pavlovian conditioned stimulus (CS+) with an injection of lithium chloride, which induces malaise, as an unconditioned stimulus (UCS), to produce a conditioned taste aversion (CTA) so that subsequent exposures to saccharin taste instead elicit negative gapes and related 'disgust' reactions (Berridge et al., 1981; Grill & Norgren, 1978a; Parker, 2003; Spector et al., 1988, 1992; Wilkins & Bernstein, 2006).

1.3 Hedonic hotspots: Brain mechanisms of hedonic 'liking'

Affective neuroscience research has primarily focused its efforts on understanding brain generators of learning and incentive motivation. Over several decades these efforts have been fruitful and enabled us to partially understand the complexities of how the brain regulates behavior related to food and drug reward, hunger and thirst, and given us key insights into how dysfunctions in these systems may give rise to various affective disorders (Berridge, 2009; Castro et al., 2015; de Araujo et al., 2020; Morales & Berridge, 2020; Olney et al., 2018; Volkow, Wang, & Baler, 2011; Volkow, Wang, Fowler, et al., 2011) . However, normal hedonic function, or 'liking' reactions to positive affective tastes is also essential for mental health and wellbeing. Dysfunction in hedonic circuitry may contribute to mood disorders, addiction, and eating disorders (Morales & Berridge, 2020; Olney et al., 2018). To date, brain mechanisms of reward 'liking' remain less understood and studied than those that control incentive motivation and learning.

Our laboratory has studied brain generators of taste 'liking' by combining central neural manipulations of hedonic circuitry with the taste reactivity measure of 'liking' versus 'disgust'. Traditionally, we have relied on pharmacological microinjections or excitotoxin lesions to systematically turn on or turn off particular neural systems in various brain locations during the taste reactivity test. This is coupled with an analysis of local Fos protein expression that allows us to more directly determine the spread of neuronal changes induced by a manipulation that alters 'liking', to identify localization of function, and map subregional localization of hedonic mechanisms within a brain structure.

This approach has revealed a distributed network of limbic hotspots or small sites within subregions of cortical and subcortical structures in the rat that are capable of amplifying the

hedonic impact of sucrose taste (Castro & Berridge, 2017; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005; Söderpalm & Berridge, 2000). Brain hedonic hotspots appear to be restricted to particular subregions of limbic structures such as the rostrodorsal quadrant of the nucleus accumbens medial shell (NAc), caudolateral half of the ventral pallidum (VP), rostromedial orbitofrontal cortex (OFC), a far posterior zone of insula cortex (IC), and the parabrachial nucleus of the brainstem pons (PBN). Brain hedonic hotspots that generate 'liking' are embedded within larger mesocorticolimbic circuitry (spanning several entire structures) that is capable of generating incentive salience 'wanting', underlying the close interconnection between 'liking' and 'wanting' functions in reward (Cole et al., 2018; DiFeliceantonio et al., 2012; DiFeliceantonio & Berridge, 2012, 2016; Mahler & Berridge, 2009; Peciña et al., 2006; M. J. F. Robinson et al., 2014; K. S. Smith & Berridge, 2005; Warlow et al., 2017b). In the following sections, I will discuss roles of these hedonic hotspots and mesocorticolimbic circuitry involved in 'liking' and 'wanting' processes.

1.3.1 The hindbrain computes early hedonic evaluations

Rudimentary hedonic processing of tastes begins to occur in the brainstem early in pathway of ascending gustatory signals (Berridge, 2009; Grill & Norgren, 1978b, 1978d; Peciña & Berridge, 1996; Steiner, 1973). For example, brainstem (4^{th}-ventricle) microinjections of a benzodiazepine drug that promotes GABA signaling enhanced positive 'liking' reactions to sweet taste, as did microinjections limited to the parabrachial nucleus of the pons, revealing that site as a brainstem hedonic hotspot (Berridge & Peciña, 1995; Soderpalm & Berridge, 2000). Brainstem capacity for early hedonic-related processing was also revealed by classic studies of taste reactions in decerebrate rats and in anencephalic infants, both of which lack a functioning forebrain, yet are able to adequately respond to sucrose taste with positive affective reactions,

and to quinine with aversive reactions (Grill & Norgren, 1978d; Steiner, 1973). Similarly, decerebrate rats show increases in positive 'liking' reactions to intra-oral sucrose after systemic administration of a benzodiazepine drug (Berridge, 1988). For humans and other primates, the causal role of PBN in food hedonics has sometimes been questioned (Rolls, 2016; Scott & Small, 2009) on the basis that in primates, gustatory neuroanatomical projections may ascend directly from the hindbrain nucleus of the solitary tract to forebrain thalamus and limbic structures, rather than making an obligatory intermediary relay in PBN as in rodents (Norgren & Leonard, 1973; Pritchard et al., 2000). However, very little data actually exists yet on PBN roles in food reward functions in primates, including humans.

A crucial need for forebrain hierarchical contributions to normal 'liking' exists even in rats, evident from observations that many features of normal physiological and associative modulation of 'liking' reactions that occur in normal rats are missing in decerebrate rats. For example, decerebrate rats that are transected above the midbrain cannot learn or retain behavioral conditioned taste aversions to a nausea-paired sweet flavor that normally would switch 'liking' to 'disgust' reactions, suggesting that higher order affective processing involving experience and learning requires forebrain control and cannot be fully mediated by the brainstem on its own (Berridge et al., 1981; Grill & Norgren, 1978d; Spector et al., 1992; Wilkins & Bernstein, 2006). Caloric hunger similarly is reported to fail to enhance positive hedonic reactions to sweet tastes in decerebrate rats (Kaplan et al., 2000) unlike in normal rats (Berridge, 1991; Grill et al., 1996), and inducing a hormonal salt appetite state fails to not enhance positive orofacial reactions to the taste of salt (Grill et al., 1986) again unlike in normal rats (Berridge & Grill, 1984; Berridge & Schulkin, 1989; Clark & Bernstein, 2006; M. J. F. Robinson & Berridge, 2013; Tindell et al., 2006, 2009). Those decerebrate failures suggest that the brainstem by itself cannot integrate

physiological state or learned associations with tastes to modulate alliesthesia changes in hedonic orofacial reactions, even though some rudimentary processing of such modulating inputs has been reported in brainstem based on electrophysiological measures of neural activity (F. C. Chang & Scott, 1984; Giza et al., 1993; Giza & Scott, 1983, 1987; Glenn & Erickson, 1976).

1.3.2 The nucleus accumbens medial shell hedonic hotspot

Several decades of research have implicated the nucleus accumbens (NAc) as especially important in motivation, and the NAc also plays important roles in controlling 'liking' reactions. Relevant to 'wanting', opioid, dopamine, and GABA/glutamate drug microinjections in the nucleus accumbens, especially in medial shell, can robustly enhance motivation to pursue and eat palatable foods (Bakshi & Kelley, 1993b, 1993a, 1994; Basso & Kelley, 1999; Castro & Berridge, 2014c; Kelley et al., 2002; Kelley & Swanson, 1997; Maldonado-Irizarry et al., 1995; Peciña & Berridge, 2005; Reynolds & Berridge, 2002, 2003; Stratford et al., 1998; Stratford & Kelley, 1997; Stratford & Wirtshafter, 2004; Urstadt et al., 2013; M. Zhang & Kelley, 2000). Importantly however, the nucleus accumbens is a heterogenous structure with multiple anatomical subregions (Groenewegen et al., 1993; Humphries & Prescott, 2010; Z. Li et al., 2018; Reynolds & Berridge, 2001; Thompson & Swanson, 2010; West & Carelli, 2016; Zahm et al., 2013) that differentially mediate 'liking' and 'wanting', at least in response to particular manipulations (Castro & Berridge, 2014c; Peciña & Berridge, 2005; Reynolds & Berridge, 2001, 2002, 2003). Beyond the anatomical components of core and shell, there also are important subregional hedonic specializations within the shell, such as the hedonic hotspot within the rostrodorsal quadrant of medial shell. The rostrodorsal quadrant of NAc medial shell was first identified as an important hedonic hotspot for 'liking' enhancement by Peciña and Berridge (Peciña & Berridge, 2005). That hedonic mapping study used microinjections of the mu-opioid

receptor agonist (DAMGO) to show that, only in the 1 mm^3 rostrodorsal subregion of medial shell did mu opioid stimulation enhance 'liking' reactions to sucrose taste, even though opioid stimulation anywhere throughout the entire NAc shell generated robust 'wanting' to eat reflected in increased food intake. Opioid stimulations at NAc shell sites other than the rostrodorsal hotspot completely failed to enhance sweetness 'liking' reactions at all, even decreasing sucrose 'liking' at a hedonic 'coldspot' site in caudal shell, despite still increasing 'wanting' to eat (Peciña & Berridge, 2005). That and subsequent mapping studies revealed a clear NAc subregional dissociation between amplification of 'liking', which is limited to the rostral medial shell hotspot, versus of 'wanting', which can be generated by opioid and some other neurochemical manipulations throughout the entire medial shell as well as NAc core (Peciña & Berridge, 2005; M. Zhang & Kelley, 2000). Further illustrating the unique hedonic features of this NAc hotspot, delta opioid and even kappa opioid agonists can enhance sucrose 'liking' similarly to mu opioid stimulations when microinjected within the 1 mm^3 hotspot in rostrodorsal shell, although kappa opioid stimulation is known to produce negative aversive effects at many other brain sites (Castro & Berridge, 2014c).

Beyond opioid stimulation, orexin and endocannabinoid microinjections within the NAc rostrodorsal shell hotspot also can enhance sucrose 'liking' reactions (endocannabinoid enhancements might possibly also extend to caudodorsal shell) (Ho & Berridge, 2013; Mahler et al., 2007). Endocannabinoids bind to presynaptic receptors on axonal terminals of NAc neurons and influence the release of other postsynaptic neurotransmitters (Howlett et al., 2002). The ability for endocannabinoids in the NAc hotspot to enhance sucrose 'liking' appears to require local endogenous opioid mediation (Mitchell et al., 2018). For example, if opioid-blocking naloxone is mixed in the same microinjection into NAc hotspot that contains the

endocannabinoid anandamide, the simultaneous opioid blockade prevents the endocannabinoid stimulation from enhancing 'liking' reactions to sucrose at all. These findings seem in accordance with research showing that opioid and cannabinoid receptors often co-localize on the same neurons to form heterodimers, and that the two neurochemical signals can functionally interact together to influence motivation for food and drug rewards (Ferré et al., 2009; Robledo et al., 2008; Wenzel & Cheer, 2018).

While opioid, endocannabinoid, orexin, and a few other neurotransmitters act in the NAc hotspot to enhance 'liking'(Castro et al., 2016; Faure et al., 2010; Mahler et al., 2007; Mitchell et al., 2018; Reynolds & Berridge, 2002; K. S. Smith & Berridge, 2005, 2007), mesolimbic dopamine is notably missing from the list of hedonic neurochemical signals. Even in the NAc hotspot of rostrodorsal shell, synaptic dopamine stimulations, such as by amphetamine microinjection or genetic knockdown of the dopamine transporter that boosts dopamine levels in NAc synapses, completely fail to enhance 'liking' at all (although potently stimulating cue-triggered 'wanting' for sweet reward)(Peciña et al., 2003a; Wyvell & Berridge, 2000). Conversely, removing NAc dopamine signals via permanent 6-OHDA lesions or through pharmacological blockade can suppress 'wanting' during consuming and instrumental responding tasks (Berridge et al., 1989; Cousins et al., 1994; Galistu & D'Aquila, 2012; Higgs & Cooper, 2000; Hsiao & Smith, 1995; Muscat & Willner, 1989; Oltmans & Harvey, 1976; Rolls et al., 1974; Schneider et al., 1990a; G. Smith, 1995; Wise & Raptis, 1986; Zis & Fibiger, 1975), but fails to impair 'liking' reactions (Berridge et al., 1989; Peciña et al., 1997; Treit & Berridge, 1990).

1.3.3 The Ventral Pallidum Hedonic Hotspot

The ventral pallidum receives the densest output projections from nucleus accumbens (Groenewegen & Russchen, 1984; Mogenson et al., 1983; Zahm & Heimer, 1990, 1993), and ventral pallidum is important in both reward and aversion (Ahrens et al., 2016, 2018; Calder et al., 2007; S. E. Chang et al., 2017, 2018; Cromwell & Berridge, 1993; Faget et al., 2018; Farrell et al., 2019; Itoga et al., 2016; Knowland et al., 2017; Mahler et al., 2014; Ottenheimer et al., 2018; Reichard et al., 2019; Richard et al., 2016; Shimura et al., 2006; K. S. Smith & Berridge, 2005; Tindell et al., 2004, 2005, 2009; Wulff et al., 2019). The posterior half of the ventral pallidum of rats contains another 0.8 mm^3 hedonic hotspot where microinjections of the mu-opioid agonist DAMGO more than doubles hedonic 'liking' reactions to sucrose (K. S. Smith & Berridge, 2005, 2007). Similar to NAc, though reversed in front to back valence polarity, the VP appears organized along a bivalent anatomical gradient (K. S. Smith & Berridge, 2005). For example, local opioid stimulation by DAMGO microinjection in the posterior (the same subregion is also lateral and dorsal in VP) half of VP enhanced sucrose 'liking' reactions (and increased food intake), whereas the same opioid stimulation in anterior (which is also medial and ventral) VP oppositely suppressed positive 'liking' reactions (and suppressed food intake), revealing a rostral VP hedonic coldspot. It may be related that a human neuroimaging study found similar rostrocaudal bivalence, in that anterior VP was reported to activate in response to disgusting images, whereas posterior VP activated to images of palatable foods (Beaver et al., 2006; Calder et al., 2007). However, anterior VP still can participate in generating incentive motivation or 'wanting' for rewards. A different manipulation of anterior VP, namely local GABA blockade induced via bicuculine antagonist microinjections to disinhibit or excite anterior VP neurons, caused increases in food intake (K. S. Smith & Berridge, 2005). Similarly, anterior

VP has also been shown by others to be important in motivation to pursue drug and foods rewards (Farrell et al., 2019; Mahler et al., 2014).

Within the hedonic hotspot of posterior VP, orexin microinjections also have been found to enhance 'liking' reactions to sucrose, just as opioid microinjections do (Ho & Berridge, 2013). Similarly implicating these subregional differences for VP in reward, others have reported that frequency thresholds for electrical self-stimulation in VP are lower in posterior subregions of VP than anterior subregions supporting a special role for caudal ventral pallidum in some reward-related functions (Panagis et al., 1995). However, as mentioned, anterior VP neurons also contribute to motivation to seek reward, at least in some neurobiological modes and in some situations (Farrell et al., 2019; Mahler et al., 2014; Ottenheimer et al., 2018; K. S. Smith & Berridge, 2005, 2007). The functional flexibility and multiple roles of VP subregions is a topic that deserves further investigation.

1.3.3.1 VP Hotspot is crucial for normal 'liking.'

Although all hedonic hotspots can produce *gains* in hedonic 'liking' reactions when appropriately stimulated, damage to most hotspots does not produce *loss* of normal 'liking' reactions. The posterior VP hotspot is the only known brain region where excitotoxic or electrolytic neuron-destroying lesions can result in loss of normal 'liking' reactions and replacement by excessive 'disgust' reactions even to sweet taste. These effects can persist for weeks, underlining the special importance of VP hotspot to normal hedonic function (Cromwell & Berridge, 1993; Ho & Berridge, 2014). For example, after VP lesions, normally 'liked' sucrose taste instead elicits 'disgust' reactions such as gapes, headshakes, paw treading, etc., as though the sweet taste had become bitter or otherwise strongly unpalatable (Cromwell & Berridge, 1993; Ho & Berridge, 2014; Khan et al., 2019).

Classic studies in the 1960s using large electrolytic lesions originally attributed lesion-induced 'disgust' to damage to the LH (Schallert & Whishaw, 1978; Teitelbaum & Epstein, 1962). However, subsequent more precise mapping using smaller excitotoxin lesions indicated that the crucial 'disgust-induction' lesion site was not in LH but was actually the hedonic hotspot of posterior VP (Cromwell & Berridge, 1993). The large electrolytic lesions to LH of earlier studies typically also damaged posterior VP in addition to the LH, which may account for the negative affective reactions reported by early LH studies (Castro et al., 2015). In other words, only damage to the VP hotspot produces dramatic loss of hedonic function. Both LH lesions and VP lesions can cause loss of 'wanting' to eat or drink, producing severe adipsia and aphagia, so that lesioned rats require intragastric feeding and hydration to be kept alive. But if they receive that intense nursing for days to weeks, rats slowly begin to independently feed again on soft palatable food, eventually progressing to normal eating and then drinking behavior, although some subtle ingestive functions still remain impaired (Rodgers et al., 1965; Teitelbaum et al., 1969; Teitelbaum & Epstein, 1962; Teitelbaum & Stellar, 1954).

Beyond 'disgust' induction by posterior VP lesions, pharmacological inhibition of posterior VP hotspot neurons, such as by microinjections of GABA agonists, also can induce temporary excessive 'disgust' to sweetness that lasts at least for hours (Ho & Berridge, 2014; Khan et al., 2019). Excessive 'disgust' induced by pharmacological muscimol/baclofen microinjections in the VP hotspot, as well as by posterior VP lesions, has been interpreted as a 'release phenomenon' (Ho & Berridge, 2014; Khan et al., 2019), a century-old concept from the early neurologist Hughlings-Jackson for explaining how a neuronal dysfunction produces an active behavioral disorder (Hughlings Jackson, 1958). That is, the excessive disgust probably results from negative-affect generating circuitry in other brain structures outside the VP, which is

released or disinhibited by damage to the positively-valenced hedonic hotspot of posterior VP (Ho & Berridge, 2014; Khan et al., 2019).

1.3.4 Cortical Hedonic Hotspots: Orbitofrontal cortex and insula

Beyond subcortical hedonic hotspots, two hotspots in cortex were recently discovered by our lab: one in the anteromedial orbitofrontal cortex, and another in the far-posterior insula cortex of rats. Both of these cortical hedonic hotspots similarly caused hedonic gains of function in sucrose 'liking' reactions in response to drug microinjections that deliver mu opioid stimulation or orexin stimulation to local neurons (Castro & Berridge, 2017). By contrast, the same opioid/orexin microinjections in other limbic cortex sites outside these hotspots, even in other regions of OFC or insula, fail to enhance sucrose 'liking' (and some sites suppress 'liking'), even if they stimulate 'wanting' to eat (Castro & Berridge, 2017).

The finding that hedonic hotspots exist in the cortex was surprising in one sense, because lesions in cortical areas do not reliably reduce hedonic reactions in either rats or humans (Beer et al., 2003; Feinstein et al., 2010; Hashimoto & Spector, 2014; King et al., 2015a; Philippi et al., 2012a; Wirsig & Grill, 1982a). That is, damage to the orbitofrontal cortex or insula does not necessarily cause loss of 'liking' reactions to foods or other pleasant events. However, gain of hedonic function is different from loss of hedonic function, and in a neural hierarchy a superior structure such as cortex might plausibly cause hedonic gains by activating subcortical hedonic circuitry, without causing hedonic losses when damaged, if the subcortical circuitry is capable on its own of generating basic hedonic reactions. In any case, human neuroimaging data and animal electrophysiological studies have also reported that orbitofrontal cortex and insula at least encode hedonic values of food and other rewards (de Araujo et al., 2003, 2006; DiFeliceantonio et al., 2018; Jezzini et al., 2013; Kringelbach et al., 2003; Mena et al., 2011, 2013; Small, 2001).

In keeping with the hierarchical triggering and cross-hotspot recruitment notions, DAMGO or orexin into the OFC or insula hotspot that enhanced 'liking' caused distant increases in neural activation measured by Fos expression in the hedonic hotspots in NAc and VP. This supports the hypothesis that 'liking' enhancements caused by neurochemical stimulation of a particular hotspot are mediated by recruiting the entire hedonic circuitry across the brain to activate all hotspots together (Castro et al., 2015; Castro & Berridge, 2017; K. S. Smith et al., 2011; K. S. Smith & Berridge, 2007). The two cortical hedonic hotspots were also shown to bookend a long 'hedonic coldspot' strip between them where orexin and DAMGO microinjections oppositely suppressed sucrose hedonic reactions (i.e., stretching from lateral orbitofrontal cortex through insula). Orexin or opioid microinjections in the coldspot strip produced a pattern of Fos changes across the brain quite different from cortical hotspot microinjections, suggesting activation of a separate anti- 'liking' neural circuitry that dampens positive hedonic reactions (Castro & Berridge, 2017). It is interesting that an overlapping subregion of posterior insula (posterior to gustatory sensory cortex) also appears crucial to taste aversion learning (Schier et al., 2014). Increases in motivational 'wanting' to eat, measured as increased consumption of chocolate M&M candies were also produced by all OFC hotspot microinjections and some insula hotspot microinjections , and were also produced by a number of nonhedonic sites in infralimbic cortex, prelimbic cortex, or anterior cingulate cortex (ACC), and even by some sites in the intervening hedonic coldspot strip of posterior-lateral OFC and anterior insula (Castro & Berridge, 2017). However, more cortical mapping may be needed given that a recent report suggested that optogenetic stimulation in anterior insula of mice promotes positive affective reactions whereas posterior insula stimulation evoked 'disgust' reactions (Dolensek et al., 2020; Peng et al., 2015). We also note that some others have reported

optogenetic laser self-stimulation of glutamate neurons in insula regions, or of insula-to-amygdala projections (Peng et al., 2015; L. Wang et al., 2018), although others report avoidance of laser-stimulation at some insula sites (Gehrlach et al., 2019; Peng et al., 2015; L. Wang et al., 2018), suggesting the insula picture in particular may need further clarification.

1.4 Optogenetic probes of hedonic function in hedonic hotspot sites.

Thus far, characterization of hedonic hotspots that control 'liking' reactions has been limited to drug microinjection techniques and large excitotoxin brain lesions. This raises the question of whether hedonic hotspots are true neurobiological and functional entities capable of amplifying hedonic impact or mere artifacts of drug microinjection techniques. One way to help resolve this question is by using alternative techniques to manipulate the activity of hedonic hotspot neurons and determine if we can produce similar changes in affective 'liking' reactions to different tastes. In this dissertation I present a series of experiments that seek to extend our understanding of the neural mechanisms of hedonic 'liking' and aversive 'disgust' vs. motivational 'wanting' using optogenetic techniques to directly control the activity of hedonic hotspots across various mesocorticolimbic sites.

Chapter 2 uses optogenetics to study neural causation of 'liking' reactions within the cortical hotspots in rostromedial OFC and caudal insula. I replicate the original findings by Castro and Berridge and show that optogenetic activations within rostromedial OFC and caudal insula cortex double positive reactions to intra-oral sucrose (Castro & Berridge, 2017). My findings provide some of the first triangulating evidence that hedonic hotspots are true, robust neurofunctional entities capable of producing hedonic gains of function when optogenetically stimulated. I also map a site of hedonic suppression that spans caudolateral OFC and continues posteriorly through rostral and mid insula where the same ChR2 neuron excitations oppositely

reduce affective expressions to sweetness. The mapped functional boundaries of hotspots in OFC and insula had a high degree of overlap with the mapped orexin and mu-opioid hotspot boundaries (Castro & Berridge, 2017). Further, I show that that cortical sites that produce 'wanting', here measured via propensity of animals to self-stimulate for laser extend beyond the anatomical boundaries of rostromedial OFC and caudal insula to the entirety of those structures, so that even sites that decreased 'liking' robustly promote 'wanting' for laser photostimulation.

The finding that cortical OFC and insula regions can compute hedonic gains of function in rats raises the possibility that other corticolimbic areas implicated in emotion and motivation may contain hedonic hotspots not yet discovered. One cortical region implicated in negative and positive affective states is the cingulate cortex. Thus, Chapter 3 extends our knowledge of cortical contributions to hedonic function into the cingulate cortex (CC). I used ChR2 activations in CC to identify a novel hedonic hotspot located in a mid to posterior region where neural activations nearly doubled hedonic 'liking' reactions to sucrose. I then demonstrate that pairing optogenetic stimulation of these CC hotspot neurons with instrumental lever pressing for a laser-paired sucrose reward caused rats to focus and intensely pursue this laser-paired sucrose, suggesting CC neurons that increase 'liking' for sucrose also mediate 'wanting' for sucrose. Finally, CC hotspot neuron activations also cause rats to self-stimulate for laser alone in two tests of laser self-stimulation, similar to self-stimulation we observed in OFC and insula hotspot rats in Chapter 2, further providing evidence that ACC hotspot neurons generate both 'liking' and 'wanting'.

Chapter 4 then moves subcortically to probe hedonic function and incentive motivation in the ventral pallidum. The ventral pallidum is a critical mediator of hedonic function and unique even among the other hotspots. The caudolateral VP hotspot not only produces gains in hedonic

function, but it also appears to be necessary for normal 'liking' as loss of VP hotspot neurons abolishes positive reactions to sucrose and replaces them with aversive 'disgust' (Cromwell & Berridge, 1993; Ho & Berridge, 2014; Khan et al., 2019). In this chapter I aim to answer three questions about VP function: First I probe the bidirectional role of VP neurons on 'liking' and 'wanting' by asking 1) can general optogenetic inhibitions (using chloride conducting optogenetic inhibitory viruses) in ventral pallidum suppress motivation and control aversive 'disgust' in an anatomically specific manner. I show that posterior VP neuron inhibitions suppress positive reactions to sucrose and replace them with aversive 'disgust' expressions. This hedonic modulating function was restricted to caudal VP neurons, as the same neuronal inhibitions in rostral subregions failed to alter hedonic impact, despite still reducing incentive motivation to eat in a voluntary test of food intake. Finally, I implement a more cell specific approach to probe hedonic and motivational functions in VP. Specifically, I use Cre-dependent ChR2 activations in GAD1-cre rats (Sharpe et al., 2017), to target local populations of GABA neurons in VP. My results provide striking anatomical overlap with previous pharmacological studies and show that optogenetically stimulating VP^{GABA} neurons increases positive 'liking' reactions similar to DAMGO and orexin microinjections. These effects are dissociable from rostral VP^{GABA} neuron activations, which oppositely suppress 'liking' reactions yet still increase incentive motivation measured as instrumental responses for laser-paired sucrose rewards during an operant task and laser self-stimulation. Further, we show that activating hedonic suppressive sites in rostral VP^{GABA} neurons creates a powerful and maladaptive attraction to a painful electrified shock rod.

1.5 Distributed brain mechanisms of 'wanting': amygdala and beyond.

The mesocorticolimbic brain system that generates incentive salience or 'wanting' is anatomically larger than the hedonic hotspot network, including entire structures of NAc, central nucleus of amygdala and parts of neostriatum, etc. Neurochemically, it includes dopamine and glutamate, as well as opioid orexin, and endocannabinoid transmitters so that its functionally more robust than the 'liking' network. (Berridge et al., 2010; Campus et al., 2019; Ferrario et al., 2016; Flagel et al., 2007, 2011; Haight et al., 2017; Haight & Flagel, 2014; Kuhn et al., n.d.; Olney et al., 2018; Yager et al., 2015a). This robust network can generate intense incentive motivation, even without enhancing hedonic 'liking'. Particularly important node for incentive motivation is the amygdala, which is thought to assign motivational valence to stimuli in the environment through learned associations.

Historically, research into amygdala function has extensively highlighted its role in generating aversive and fear-related motivations, but the focus of positive incentive 'wanting' onto particular targets is a function in which amygdala also plays an important role in. The amygdala is composed of multiple nuclei, including the basolateral nucleus of amygdala (BLA), the medial nucleus of the amygdala (MeA), and the central nucleus of amygdala (CeA) (Alheid & Heimer, 1988; Baxter & Murray, 2002a; De Olmos & Heimer, 1999; Janak & Tye, 2015; J. Kim et al., 2016, 2017; Swanson, 2003; Swanson & Petrovich, 1998), and of these, the CeA is particularly important to generating intense incentive salience. The CeA has 'striatal-level' status within a cortico-striatal-pallidal macrosystem organization of forebrain structures (in which the BLA has cortical status, and the bed nucleus of stria terminalis (BNST) holds 'pallidal status' within the extended amygdala complex (Swanson, 2003)). The striatal-level status of the CeA may be relevant to its ability to amplify appetitive motivation. For example, the CeA contains

many GABAergic neurons that receive BLA glutamate inputs and mesolimbic dopamine inputs (glutamate-dopamine convergence similar to NAc and neostriatum), and project primarily to BNST as a pallidal-type target (McDonald, 1982a).

Eating palatable food causes increases in Fos expression in the central amygdala (Valdivia et al., 2014; Wu et al., 2014) and direct manipulations that alter opioid, glutamate, GABA, and several peptides within CeA can potentiate unconditioned food intake (Andrezjewski et al., 2004; Baldo et al., 2005; Corwin et al., 1993; Fekete et al., 2007; Giraudo, Billington, et al., 1998; Giraudo, Kotz, et al., 1998; Gosnell, 1988; Kask & Schiöth, 2000; E. M. Kim et al., 2004; Levine et al., 2004; Mahler & Berridge, 2009, 2012; Y. Y. Pang et al., 2015; Vígh et al., 1999; Will et al., 2004). Conversely, GABAergic inactivation of the CeA or dopamine blockade in CeA suppresses food intake (Anderberg et al., 2014; Miñano et al., 1992). Some recent optogenetic studies have similarly reported that ChR2 activation of various CeA neuronal types amplifies food intake and drinking of palatable sweet solutions (Douglass et al., 2017; W. Han et al., 2017; Hardaway et al., 2019; Torruella-Suárez et al., 2020).

The CeA may also play a special role in targeting enhanced 'wanting' on to particular learned cues for food rewards. For example, in a sign-tracking/goal-tracking situation, CeA mu-opioid stimulation by DAMGO microinjection selectively enhances the incentive salience of the sucrose-predicting lever CS+ in sign-trackers, but selectively enhances the incentive salience of the sucrose-contiguous dish CS+ in goal-trackers. In both cases it enhances approach towards, and consummatory bites and nibbles to the individual's preferred metal lever or dish cue (DiFeliceantonio & Berridge, 2012; Mahler & Berridge, 2009, 2012). That suggests the CeA can amplify incentive motivation and focus 'wanting' specifically on an already preferred CS+ stimulus (DiFeliceantonio & Berridge, 2012). Similarly, in a Pavlovian-to-instrumental transfer

situation (PIT), CeA opioid stimulation specifically enhances cue-triggered 'wanting' by increasing bouts of instrumental lever pressing for sucrose reward when the CS+ is presented, and not in its absence (Mahler & Berridge, 2012). In addition to its role in food motivation and appetite, CeA signaling has also been shown to be important for cue-induced motivation for drug rewards (Funk et al., 2016; X. Li et al., 2015; Y. Q. Li et al., 2008; Lu et al., 2005; Venniro et al., 2017, 2020). Conversely, lesion studies suggest that loss of CeA function impairs cue-induced 'wanting', suppressing PIT, and other forms of motivation (Corbit & Balleine, 2005; Gallagher et al., 1990; Hall et al., 2001; Holland & Gallagher, 2003).

Recently, optogenetic CeA stimulations have been used to amplify and control the direction of 'wanting' for a particular target, such as sucrose, cocaine, or even a noxious shock-rod stimulus that delivers electric shocks if touched (M. J. F. Robinson et al., 2014; Warlow et al., 2017b, 2020). Initial studies by Mike Robinson and Shelley Warlow in our lab showed that pairing such CeA optogenetic stimulation with a sucrose target could make the rat exclusively pursue that laser-paired sucrose target while ignoring an equally good sucrose alternative. CeA stimulation also amplified breakpoint incentive motivation to obtain sucrose in a progressive ratio task (M. J. F. Robinson et al., 2014). Another study by Robinson and colleagues showed that rats will withstand a painful foot shock in order to gain access to the laser-paired sucrose, and pursue it even when the alternative non-laser paired sucrose reward is 10 times larger (Tom et al., 2018).

When the pairing of CeA ChR2 stimulation with a target is applied to rats choosing between sucrose pellets versus intravenous cocaine infusions, motivation can be intensified and narrowed at the whim of the experimenter (Warlow et al., 2020). Rats that have CeA stimulation paired with earning sucrose become 'sucrose addicts' and pursue sucrose exclusively while

ignoring the opportunity to earn intravenous cocaine (Warlow et al., 2020). Other rats that have CeA ChR2 photostimulation paired with earning cocaine become 'cocaine addicts', exclusively pursuing the drug while ignoring the sucrose. The CeA role is powerful enough to make a rat 'want what hurts it' when laser stimulation is paired with voluntary encounters of the noxious shock-rod. Paradoxically, CeA ChR2 photostimulation caused rats to paradoxically become compulsively attracted to the shock-rod and subject themselves to shocks again and again (Warlow et al., 2020). This CeA-driven attraction is mediated in part via recruiting activation of distributed mesocorticolimbic circuitry for incentive motivation (Warlow et al., 2020).

Despite CeA neurons' ability to generate powerful attractions for external rewards, most CeA ChR2 rats find the laser by itself a relatively weak reward, and many will not self-stimulate CeA ChR2 laser at all, even if it makes them strongly attracted to their paired sucrose, cocaine, or shock-rod target (M. J. F. Robinson et al., 2014; Tom et al., 2018; Warlow et al., 2017b, 2020; Warlow & Berridge, 2021). [although c.f. (Douglass et al., 2017; Hardaway et al., 2019; J. Kim et al., 2017; Servonnet et al., 2020; Torruella-Suárez et al., 2020; L. Wang et al., 2018).

CeA ChR2 enhancement of incentive motivation does not necessarily need to be viewed in contradiction to the many demonstrations that CeA and related circuitry generate oppositely valenced aversive and fearful motivations (Fadok et al., 2018a; Keifer et al., 2015; LeDoux, 2007; H. Li et al., 2013; Moscarello & LeDoux, 2013; Zimmerman et al., 2007). In the same study from our lab I previously described, Shelley Warlow and colleagues demonstrated the same CeA ChR2 activations potentiated conditioned freezing and fearful responses to auditory cues paired with an inescapable Pavlovian conditioned footshock, even in the same rats that previously preferred laser paired sucrose, cocaine, or voluntary shock rod contacts (Warlow et al., 2020). The mechanism by which CeA neuron activations can both promote intense attraction

and potentiate fear responses remains somewhat unclear. One possibility is that aversive motivation vs. incentive motivation is controlled by distinct populations of CeA neurons. Although CeA neurons are primarily GABAergic, they express a rich variety of peptides including corticotropin-releasing factor (CRF), somatostatin (SOM), protein-kinase-c delta (PKC-d), and other signaling molecules including dopamine from the midbrain (Avegno et al., 2021; Cassell et al., 1986; Hu et al., 2020; J. Kim et al., 2017; McCullough, Daskalakis, et al., 2018; McCullough, Morrison, et al., 2018; Pomrenze et al., 2015; Swanson & Petrovich, 1998). Some evidence of this proposed cell specific CeA neuronal function comes from studies in mice that that selectively manipulate nonoverlapping populations of PKC-d neurons vs. somatostatin (SOM) neurons (J. Kim et al., 2017; J. Y. Kim et al., 2017; Wilson et al., 2019), or potentially by distinct populations of neurons that express dopamine D1 vs D2 neurons (J. Kim et al., 2017; McCullough, Morrison, et al., 2018; Venniro et al., 2017).

Work in our lab has also begun to use Cre-dependent manipulations in central amygdala to probe whether distinct neuronal populations control incentive motivation using D1-cre, A2A-cre, and CRF-cre rats (Pettibone et al., 2019; Pomrenze et al., 2015). Hannah Baumgartner showed that optogenetically activating CRF-expressing neurons in CeA, which have been traditionally implicated in aversive stress stated related to withdrawal and thought to promote consumption as a means of alleviating that unpleasantness (de Guglielmo et al., 2019; Heilig & Koob, 2007; Koob, 2013, 2020; Koob & Le Moal, 1997; Koob & Schulkin, 2019; Roberto et al., 2017), caused ChR2 rats to oppositely *prefer* a sucrose pellet that was paired with CeA CRF neuron activation, and even promoted self-stimulation for CRF neuron activation alone, suggesting that CeA CRF neurons also have incentive properties outside these traditionally proposed roles in stress and aversive motivation (Baumgartner et al., 2021). But allostatic models

of motivation and CRF neuron role in stress and aversion were primarily developed to explain motivation for drugs and addiction (Koob & Le Moal, 1997; Koob & Schulkin, 2019; Roberto et al., 2017). So perhaps even more importantly, Baumgartner and colleagues subsequently showed that pairing CeA CRF neuron activations could also direct and focus incentive motivation for CRF neuron activation paired with intravenous cocaine through a positive incentive mechanism, and that this recruits mesocorticolimbic circuitry, measured as increases Fos protein expression within distant sites (Baumgartner et al., 2022). An alternative hypothesis to the view that CeA functions may be entirely reducible to specific neuronal populations is that that CeA has several affective valence modes it can generate and which dynamically change across different environmental contexts (Berridge, 2019; Warlow & Berridge, 2021). This means that the environment may retune CeA neurons to generate either fearful, aversive, or incentive motivations depending on current situations. Clearly there is much still to be resolved about amygdala functions and roles in incentive motivation.

Can CeA-mediated powerful attractions be explained, at least in part by CeA's ability to modulate affective 'liking' reactions for reward? The currently available evidence suggests the answer may be no. Robinson and Warlow found that CeA ChR2 stimulation did not enhance orofacial 'liking' reactions in rats allowed to freely ingest sucrose pellets despite making rats 'want' to instrumentally respond for sucrose more (M. J. F. Robinson et al., 2014). These findings may serve as another powerful illustration of how 'liking' and 'wanting' dissociate within brain mesocorticolimbic systems, and raises the possibility that the CeA is a pure generator of incentive motivation and not hedonic function. Although I caution that Robinson and colleagues' experiments used voluntary measures of ingestion to probe hedonic impact in CeA ChR2 rats (M. J. F. Robinson et al., 2014), and that taste reactivity more faithfully tracks

hedonic impact since tastes are delivered directly into the mouth without any need for voluntary ingestion. Relying on voluntary intake requires animals to engage in appetitive approach that may likely engage 'wanting' brain systems. Appetitive approach and voluntary intake tests thus pose a challenge in measuring hedonic impact because incentive motivation and hedonic impact can change independently, such as within known hedonic hotspot sites I've discussed in previous sections (Castro et al., 2016; Castro & Berridge, 2014c, 2017; Ho & Berridge, 2013, 2014; Khan et al., 2019; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005), and through manipulations of dopamine systems that change incentive motivation via intake and instrumental measures (Berridge et al., 1989; Higgs & Cooper, 2000; Oltmans & Harvey, 1976; Rolls et al., 1974; Schneider et al., 1990a; G. Smith, 1995; Zis & Fibiger, 1975), without changing affective reactions to tastes during taste reactivity (Berridge et al., 1989; Peciña et al., 1997, 2003b; Wyvell & Berridge, 2000). Thus, a more direct probe of potential hedonic functions in CeA could help clarify this question.

Overall, CeA and its control over other mesocorticolimbic circuitry may be involved in sharpening the focus of amplified 'wanting' onto cues for a particular incentive target, like a high-caloric palatable food, drug rewards, and even noxious and painful stimuli that contributes to intense urges to indulge in those rewards. How this translates to other rewards, such as distinct classes of drugs like opioids, and whether CeA generated incentive motivation is matched by changes in hedonic impact are questions I tackle in the final chapter of this dissertation.

Psychostimulants and opioids often have distinct effects on the brain. For example, certain manipulations, such as those that abolish dopamine function decrease self-administration for cocaine but not heroin. Findings such as these raise the question of how motivation, and by extension, addiction may differ between psychostimulants and opioids (Badiani et al., 2011). In

Chapter 5 I ask whether central amygdala circuitry can control motivation for intravenous opioids as it has been shown to do for cocaine and sucrose rewards. I trained rats to instrumentally respond for intravenous infusions of the synthetic opioid remifentanil paired with optogenetic stimulation of CeA neurons or alternatively for intravenous remifentanil that was never laser paired. This caused CeA ChR2 rats to singly pursue the laser-paired remifentanil infusion and altogether ignore remifentanil alone that was never laser paired. I then trained separate rats to choose between a sucrose reward or an intravenous infusion of remifentanil. For half the rats, CeA neuron stimulation was paired with the sucrose, causing them to become 'sucrose addicts' that singly consume sucrose and ignore intravenous remifentanil. By comparison, rats who had remifentanil paired with CeA photostimulation became 'remifentanil addicts' and exclusively responded for remifentanil and altogether ignored sucrose. Finally, these intense CeA-generated attractions were not matched by changes to the hedonic impact of intra oral sucrose during taste reactivity, suggesting CeA circuitry mediates 'wanting' but never 'liking'.

Chapter 2 Optogenetic Hedonic Hotspots in Orbitofrontal Cortex and Insula: Enhancement of Sweetness 'Liking'

2.1 Abstract

Hedonic hotspots are brain subregions that causally amplify the hedonic impact of palatable tastes, measured as increases in affective orofacial 'liking' reactions to sweetness. Previously, two cortical hedonic hotspots were identified in orbitofrontal cortex and insula using neurochemical stimulation by opioid and orexin microinjections. Here we used optogenetic stimulations in rats as an independent neurobiological technique for activating cortical hedonic hotspots to identify hedonic functions and map boundaries. We report that channelrhodospsin stimulations within rostral orbitofrontal and caudal insula hotspots doubled the number of hedonic 'liking' reactions elicited by sucrose taste. This confirms their robust functional identity as causal amplifiers of hedonic 'liking' and confirms their anatomical boundaries. Additionally, we confirmed an intervening suppressive hedonic coldstrip, to stretching from caudal orbitofrontal cortex to rostral insula. By contrast to localized hedonic hotspots for 'liking' enhancement, motivational 'wanting' for reward appeared mediated by more widely distributed cortical sites, measured as laser self-stimulation.

2.2 Introduction

Reward contains multiple core components of 'liking', 'wanting', and learning. Among those, possibly the least understood remains the neural mechanisms of 'liking', able to enhance the actual hedonic impact of a pleasant stimulus. However, some progress has been gained by

identification of ‘hedonic hotspots’, or small subregions within mesocorticolimbic brain structures that are uniquely capable of enhancing ‘liking’ reactions to sweetness when neurochemically stimulated, based on affective taste reactivity studies (Castro et al., 2016; Ho & Berridge, 2013; Mahler et al., 2007; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005; Söderpalm & Berridge, 2000).

Hedonic hotspots were originally identified in subcortical structures, such as nucleus accumbens shell (NAc), ventral pallidum (VP), and brainstem pons (Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005; Söderpalm & Berridge, 2000). More recently two hedonic hotspots were also identified in cortex: an 8 mm^3 subregion of rostromedial orbitofrontal cortex (OFC), and a 6 mm^3 subregion of posterior insula cortex (Castro & Berridge, 2017). Each of those studies used local microinjections in rats of either opioid, orexin, or endocannabinoid agonists to double or triple affective orofacial expressions of positive ‘liking’ that are elicited by sweetness and other pleasant tastes, versus negative ‘disgust’ elicited by bitterness and other unpleasant tastes, in human infants, other primates, and rats (Berridge, 2000; Berridge & Kringelbach, 2015; Grill & Norgren, 1978c; Steiner, 1973).

Opioid/orexin stimulation of OFC hotspots that increased sucrose ‘liking’ also recruited Fos increases in the insula cortical hotspot, as well as vice versa, and further increased Fos in other subcortical hedonic hotspots in rostrodorsal NAc medial shell and posterolateral VP. That prompted the hypothesis that ‘liking’ enhancement induced by neurochemical stimulation of any one hotspot may recruit other hotspots into simultaneous neural co-activation as a unified hedonic network to increase hedonic impact (Castro & Berridge, 2017; K. S. Smith & Berridge, 2007). Between the rostral OFC and caudal insula hotspot, an 18 mm^3 suppressive hedonic ‘coldstrip’ was found to comprise posterolateral OFC and rostral insula, where the same orexin

or opioid microinjections oppositely reduced 'liking' reactions to sucrose taste and failed to recruit Fos activation in other hedonic hotspots.

However, the exclusive use of pharmacological stimulations in hedonic hotspots to enhance 'liking' reactions raises the question of whether hedonic hotspots are mere neurochemical artifacts, limited to the effects of local drug microinjections? Alternatively, hedonic hotspots may be robust neurofunctional entities that mediate 'liking' enhancements. If so, their hedonic capacities might also be revealed by independent nonpharmacological techniques of neural stimulation. To methodologically triangulate and potentially provide independent confirmation that hedonic hotspots have special capability for 'liking' enhancement, here we assessed whether optogenetic channelrhodospsin (ChR2) stimulations in cortical hotspots would alter 'liking' reactions to sucrose or quinine tastes. In support of this optogenetic effort, we noted that others have reported previous optogenetic studies indicating that that ChR2 stimulation in the anterior insula of mice elicited positive affective taste-elicited expressions (Dolensek et al., 2020), and that optogenetic stimulation in a gustatory region of insula promoted intake of palatable solutions and ingestive patterns of spout licking in mice (Peng et al., 2015; L. Wang et al., 2018).

Here we intended to assess further whether 1) optogenetic stimulation in previously identified orbitofrontal or insula hotspots enhanced positive 'liking' reactions to sweetness, 2) the anatomical locations and boundaries of cortical hedonic hotspots when mapped optogenetically were similar to those previously mapped by neurochemical stimulations, and 2) whether optogenetic mapping similarly revealed a suppressive hedonic coldstrip intervening between hotspots, where optogenetic stimulations reduced 'liking' reactions to sweetness. Finally, using laser self-stimulation tests, we assessed whether sites able to support motivational

'wanting' to obtain an incentive were more widely distributed across cortex, extending outside of hedonic hotspots. Our results suggest that neurochemically mapped OFC and insula hotspots are indeed able to enhance hedonic 'liking' reactions to sweetness when activated optogenetically, with similar anatomical boundaries, and that a cortical optogenetic hedonic coldstrip also exists between them.

2.3 Material and Methods

Animals

Female and male Sprague Dawley rats (n = 88; n = 44 female, n = 44 male; weighing 250-400 g at surgery), were group housed in separate same-sex rooms, maintained at 21º C constant temperature, on a reverse 12h dark/light cycle at the University of Michigan. *Ad libitum* access to both food and water was given throughout the experiments. Experimental procedures were approved by the Committee on the Use and Care of Animals at the University of Michigan.

Surgery

Optogenetic Virus Infusion. Rats were anesthetized with isoflurane gas (4-5% induction, 1-2% maintenance) and received atropine (0.04 mg/kg; i.p.; Henry Schein) before surgery, and then placed into a stereotaxic apparatus (David Kopf Instruments). Bilateral microinjections either of AAV channelrhodopsin virus (ChR2: AAV5-hSyn-ChR2-eYFP; UNC Vector Core, Chapel Hill; 0.5 µL in insula sites - 0.75 µL in OFC sites) or of control virus lacking the opsin gene (eYFP: AAV5-hSyn-eYFP; UNC Vector Core, Chapel Hill, NC) were targeted at cortical sites in OFC (ChR2 n = 41; eYFP n = 13) and insula (ChR2 n = 19, eYFP n = 8) as described below. A separate group of rats received an inhibitory optogenetic virus (AAV5-iC++-eYFP; Stanford Vector Core; n = 7) in the rostromedial orbitofrontal cortex to determine whether

neuronal inhibition in the OFC hedonic hotspot suppressed 'liking' reactions. At OFC sites a 0.75 µL volume of virus was infused per side over a 7.5-minute period at a constant rate of 0.1 µL/min. At insula sites, a lower 0.5 µL volume was infused per side, because pilot results indicated that 0.75 µL insula infusions may induce seizures during subsequent laser stimulation in ChR2 rats. Following virus infusion, the microinjector was subsequently left in place for an additional 10 min to allow for virus diffusion.

Sites were aimed to be as identical bilaterally as possible within each individual rat but were staggered across individuals so that the group's sites filled the entire rostral-caudal extent of the lateral cortex from midline tip of anterior OFC to posterior insula. OFC coordinates ranged from +5.16 mm to + 3.00 mm AP, ± 0.2 to ±2.5 mm ML and -4.00 mm to -6.00 mm DV (all relative to bregma). OFC sites included medial orbitofrontal (MO) and ventral orbitofrontal (VO) subregions of medial OFC (± 0.2 to ±1.0 mm ML), and lateral to cover lateral orbitofrontal (LO), and dorsolateral (DLO) subregions of lateral OFC (± 1.5 to ±2.5 mm). Insula coordinates ranged from +3.00 to –1.56 mm AP, ± 4.00 to ±6.00 mm ML, and -5.00mm to -6.00 mm DV. Insula sites included anterior insula, middle insula and posterior insula subregions. After surgery, cefazolin (100 mg/kg, s.c.; Henry Schein) was administered to prevent infection, and carprofen (5 mg/kg, s.c.; Henry Schein) given for post-operative pain relief. Carprofen and cefazolin were repeated at 24-h and 48-h post-operation.

Oral Cannula Surgery and Fiber Optic Implantation. Three weeks after the initial viral infusion surgery, rats were re-anesthetized with isoflurane as described above for implantation of intracranial optic fibers and of bilateral oral cannulas, which allowed for direct oral infusions of sucrose, quinine, and water solutions. Each oral cannula (polyethylene-100 tubing) entered the upper cheek just lateral to the secondary maxillary molar, ascended beneath the zygomatic arch,

and exited the skin at the dorsal head, where it was secured with skull screws and a dental acrylic headcap. In the same surgery, rats were implanted with bilateral optic fibers (200 μm), aimed to place each fiber tip 0.3 mm dorsal to the rat's bilateral virus microinjection sites, and anchored with the same acrylic headcap. Cefazolin and carprofen were again administered and repeated post-operatively as above. All rats were allowed to recover for 1 week prior to behavioral testing.

Stimulation Parameters and Order of Behavioral Tests

Laser stimulation was tested at 5 Hz, 10 Hz, 20 Hz, and 40 Hz frequencies (counterbalanced in order on a within-subject basis) at 1-3 mW intensity. Use of multiple frequencies assessed whether any effects of ChR2 stimulation were robust across a wide range, or instead limited to a particular frequency. Laser was always delivered bilaterally to OFC sites. Pilot insula results indicated that bilateral stimulation of insula sometimes produced seizures, and so unilateral laser stimulation was subsequently used at insula sites.

Behavioral Procedures

Taste Reactivity Testing. Each rat was habituated to the test chamber for 30 minutes on four consecutive days before any behavioral testing occurred. On the last two days of habituation, rats received oral infusions of a 0.03M sucrose solution to habituate them to infusion of fluids into the mouth. In subsequent taste reactivity tests, affective orofacial reactions (i.e., positive 'liking' versus negative 'disgust' patterns) elicited by oral infusions either of water or of three different taste solutions: two concentrations of sucrose solutions (0.03M and 0.10M), and one concentration of bitter quinine (3×10^{-4} M) (Berridge, 2000; Grill & Norgren, 1978c). Orofacial reactions were videorecorded through a close-up lens facing an angled mirror

underneath the transparent floor, positioned to capture a clear view of the mouth and face, and saved for subsequent offline analysis. Taste solutions (1 ml) were delivered into the mouth of rats through PE-50 tubing connected to a PE-10 delivery nozzle, at a constant 1ml/min rate during the 1 min infusion, via a syringe pump, connected to the oral cannula.

On each test day, a rat received two separate 1-ml/1-min infusions of the same solution (e.g., 0.1 M sucrose), one infusion accompanied by laser stimulation and the other infusion not accompanied by laser as a within-subject baseline (counterbalanced order across rats), spaced 8-10 min apart. Different tastants were tested on different days. During a laser-paired infusion, laser illumination (1-3 mW; 15 ms pulses) was cycled in 5-s ON, 5 Sec OFF bins throughout the 60-sec trial test. Several different frequencies of laser illumination within 5-s ON bins were tested on different days: Every laser parameter was tested on at least two days for each rat in separate daily tests.

Taste Reactivity Scoring/ Taste reactivity videos were scored subsequently for positive hedonic 'liking' reactions, aversive 'disgust' reactions, and neutral taste reactions in slow-motion at speeds ranging from frame-by-frame to $1/5^{th}$ normal speed, using The Observer Software (Noldus; Leesburg, VA). Positive hedonic or 'liking' responses were considered to be: lateral tongue protrusions, paw licks, and rhythmic midline tongue protrusions (Berridge, 2000; Castro et al., 2016; Castro & Berridge, 2014c, 2017). Aversive 'disgust' reactions were: gapes, forelimb flails, head shakes, face washes, chin rubs, and paw treading. Neutral responses (i.e., relatively uncoupled from hedonic impact) were: passive dripping of solution out of the mouth, rhythmic mouth movements, and grooming. A time-bin scoring system was used to ensure each type of affective reaction contributed equally to the overall affective score (Berridge, 2000; Castro et al., 2016; Castro & Berridge, 2014c, 2017). Rhythmic mouth movements, paw licks, passive

dripping, and grooming were all scored in 5-s time bins, because these behaviors typically are emitted in bouts of relatively long duration. Any continuous emission of these behaviors up to 5-sec was counted as a single occurrence; continuous emissions of 5-sec to 10-sec counted as two occurrences, etc. Midline tongue protrusions and paw-treading were scored similarly, but in 2-s bins, because they are typically emitted in shorter bouts. Lateral tongue protrusions, gapes, flails, headshakes, and chin rubs were counted as discrete events every time they occurred, because these can occur singly or in brief repetitions. A total positive hedonic (i.e., 'liking') score was then calculated by combining component scores of rhythmic tongue protrusions, paw licks, and lateral tongue protrusions. A total negative aversive (i.e., 'disgust') score was calculated by combining gapes, forelimb flails, head shakes, paw treading, face washes, and chin rubs (Berridge, 2000; Castro & Berridge, 2017).

Laser Self-Stimulation Tasks

To test whether laser ChR2 stimulation of cortical sites by itself would support incentive motivation for reward, in the absence of any taste stimulus, laser self-stimulation was assessed in two different tasks. A place-based self-stimulation task, similar to that used in early electrical brain-stimulation reward studies (Olds & Milner, 1954; Valenstein & Meyers, 1964), allowed rats to earn laser illuminations by entering a particular chamber in a 2-chamber apparatus and remaining there. Each side of the chamber was marked by a distinctive floor surface and different visual patterns on walls. Entry into the designated laser chamber triggered onset of laser stimulation (1-3 mW) at either 20 Hz or 40 Hz, depending on test day. Laser illumination continually cycled at 3-s ON, 8-s OFF as long as the rat remained within the designated laser

chamber. Exit from the laser chamber terminated the laser pulses. Entry into the other chamber produced nothing.

Separately, an active-response or 'spout-touch' laser self-stimulation task allowed rats to earn brief laser illuminations each time they touched a particular one of two empty metal drinking spouts, positioned 5 cm apart on the wall of a Med-Associates operant chamber (Fairfax, VT). One spout was arbitrarily designated as the active 'laser spout', and each touch on it earned either a 1-s or 5-s duration bin (depending on day) of 15 ms laser pulses (1-3 mW) at either 20 Hz or 40 Hz (depending on day). Touches on a second inactive spout produced nothing and contacts on it simply served to measure baseline levels of exploratory touching. Spout assignments were balanced across rats. Each combination of laser parameters was repeated on 3 consecutive days of self-stimulation (30-min sessions, order of combinations balanced across rats).

Immunohistochemistry and Histology

Beginning 75-min prior to euthanasia, a final controlled laser stimulation session was administered with one of the same parameters that produced hedonic modulation in the taste reactivity tests (40 Hz, 15 ms pulse, 5-sec ON/5-sec OFF; 30-min session). This final laser stimulation was given to a) induce local Fos plumes around optic fiber tips that would indicate the anatomical spread of local neuronal stimulation at that cortical site induced by ChR2 laser illumination, and b) potentially also recruit distant Fos activation in various limbic brain structures, to identify recruited circuitry that potentially might mediate optogenetic modulation of hedonic reactions (Baumgartner et al., 2021; Cole et al., 2018; Warlow et al., 2020).

Following the final laser stimulation, rats were deeply anesthetized with a lethal dose of sodium pentobarbital (150-200 mg/kg) and transcardially perfused with PBS followed by 4% PFA. Brains were removed and post-fixed in 4% PFA for 24-h and then transferred to a 25% sucrose solution for at least two days. Tissue was coronally sectioned at 40 micrometers using a cryostat (Leica), slices were processed for GFP and cFos immunohistochemistry, and imaged using a digital camera (Qimaging) and fluorescence microscope (Leica). For immunohistochemistry, coronal sections were rinsed for 10 min in 0.1 M sodium phosphate buffer three times, then blocked in 5% normal donkey serum / 0.2% triton-X PBS solution for 60 min and incubated overnight in a polyclonal rabbit anti-cfos igG primary antibody (1:2500; Synaptic Systems) and chicken polyclonal anti-GFP igY primary antibody (1:2000; Abcam). Tissue was again rinsed three times in 0.1M NaPb for 10 min followed by 2-h in biotin-SP-conjugated donkey anti-rabbit (1:300; Jackson ImmunoResearch) secondary antibody and AlexaFluor-488 donkey anti-chicken secondary antibody (1:300; Jackson ImmunoResearch). Tissue was rinsed three times in 0.1M NaPB for 10 min followed by1.5-h in streptavidin-conjugated Cy3 (1:300; Jackson ImmunoResearch). Brain sections were mounted, air-dried, and cover-slipped with anti-fade Pro-long gold (Invitrogen).

Local Fos Plume Analysis. Immunoreactivity for Fos-like protein was visualized using a fluorescent microscope filter with a band of excitation at 515-545 nm. Coronal sections were imaged (10x magnification) to localize fiber tips and surrounding Fos plumes, spread of virus expression, and to quantify Fos expression in distributed structures. Local Fos plumes, which are local Fos elevation induced by laser illumination that immediately surround an optic fiber tip, reflect how far local ChR2 neuronal excitation spreads (Baumgartner et al., 2020, 2021; Warlow et al., 2020). Fos plumes were mapped at 10x magnification by counting the number of Fos+

neurons within a 50 µm x 50 µm block sample of tissue, sampled consecutively along 8 radial arms emanating from the optic fiber tip. Counting continued outward along each arm until at least two consecutive blocks did not contain any Fos+ cells. This point determined the radius of the local Fos plume along that particular arm, and the same was done for all 8 arms. Percent increases in ChR2 Fos expression were calculated against a control baseline level measured at the same sites in eYFP rats with inactive virus control that also received laser illumination prior to euthanasia (to control for any Fos elevation merely due to local heat or light). Symbols matched to the size of observed Fos plumes were used to construct maps of ChR2 localization of function in OFC and insula figures (Baumgartner et al., 2020, 2021; Cole et al., 2018; Warlow et al., 2020). Stimulation sites were plotted onto corresponding maps using a brain atlas (Paxinos & Watson, 2013).

Recruitment of Fos changes in distant brain structures. Functional activation of circuitry recruited by laser stimulation of OFC or insula immediately prior to euthanasia was assessed by measuring change in Fos expression at distant sites in multiple structures: infralimbic cortex, prelimbic cortex, orbitofrontal cortex, insula, anterior and posterior ventral pallidum, anterior and posterior nucleus accumbens shell and core, anterior and posterior lateral hypothalamus, anterior and posterior anterior cingulate cortex, central amygdala, basolateral amygdala, medial amygdala rostral and caudal ventral tegmentum, dorsolateral striatum, dorsomedial striatum, and paraventricular thalamus. For brain structures known to contain hedonic hotspots or coldspots (OFC, NAc, VP, and insula) separate Fos counts were conducted in the hotspot and coldspot subregions of each structure. LASX software was used to capture tiled images of whole brain coronal sections at 10x magnification, using a filter with 515-545 excitation band. Within each subregion, Fos-expressing neurons were counted in two to three sample boxes, placed

equidistantly within the structure, and approximately at the same locations across rats, guided by a template on a corresponding brain atlas to facilitate consistent box placements. The size of the sample boxes was adjusted to each brain structure, so that each box contained approximately 10 Fos+ neurons in naïve rats. Fos+ neurons were counted in each sample box by someone blind to experimental conditions. Fos counts across the 3 sample boxes were added together to determine expression for each subregion or structure (Baumgartner et al., 2020).

Statistical Analyses

Taste reactivity tests were analyzed using repeated-measures ANOVAs, followed by *t*-tests for individual comparisons with a Bonferroni correction. Self-stimulation tasks were analyzed using mixed ANOVAs. Kruskal-Wallis tests and Friedman's two-way ANOVAs were used for nonparametric tests, followed by Wilcoxon sign-ranked tests. Significance was set at $p < 0.05$.

2.4 Results

Local Fos and Neuronal Spread of Activation

Fos expressing cells around optic fibers were counted to measure local 'Fos plumes' induced by ChR2 laser stimulation (Fig. 1). The averaged diameters of Fos plumes were used to set symbol sizes in maps showing localization of function of hedonic enhancement sites or hedonic suppression sites based on, and self-stimulation sites (Fig 2; Fig 10). Localization of function maps were used to calculate the anatomical boundaries and volumes of optogenetic 'liking' enhancement hotspots or suppressive coldspots in OFC and insula. Fos plumes typically had a 2-layer structure, with inner zones of intense 250% Fos elevation averaging 0.54 ± 0.05 mm in diameter (volume = 0.08 mm^3) surrounded by outer plumes of moderate 150%-250% Fos

elevation averaging 0.9± 0.04 mm in diameter (volume =0.39 mm^3), relative to control Fos expression measured at equivalent sites in laser-illuminated eYFP rats (Fig 1) and separately, relatively to completely naïve rats (Fig 8). The size of inner plumes ($F_{3,25} = 2.06$, $p < 0.13$) and outer plumes did not differ across cortical sites ($F_{3,25} = 0.98$, $p < 0.42$; $n = 11$ rostral OFC, $n = 9$ caudal OFC, $n = 4$ caudal insula, $n = 5$ rostral insula). The outer plume 0.90 mm diameter was used to set the maximum size of individual symbols in anatomical localization of function maps. The color of symbols in those maps represents intensity of functional effects induced by ChR2 stimulation at that site: optogenetic-induced changes in hedonic 'liking' reactions to sucrose, or of optogenetic self-stimulation, expressed as a within-subject ChR2-induced percent change compared to control no-laser baseline measured in the same rat.

Optogenetic ChR2/eYFP virus expression typically extended further than laser-induced Fos plumes (Fig. 1k), indicating there was a minimum threshold of laser stimulation required to induce neuronal Fos excitation. The mean ~1.4 mm diameter (1.3 mm^3 volume) of virus infection did not differ between OFC and insula sites ($F_{3,22} = 0.93$, $p < 0.44$; $n = 9$ rostral OFC hotspot, $n = 4$ caudal insula hotspot, $n = 9$ caudal OFC coldspot, $n = 4$ rostral to mid insula coldspot). Since ChR2 Fos plumes were smaller than zones of virus infection, plume diameter was taken as the best indicator of how far neuronal excitation spread from an optic fiber tip, and was used to set the size of individual map symbols in our localization of function maps (Fig. 2; Fig. 3; Fig. 4; Fig 10).

Hedonic taste reactivity

Baseline affective reactions elicited by tastes. Oral infusions of sucrose solution at both concentrations (0.03M; 0.1M) elicited positive 'liking' reactions in both female and male rats on control baseline trials without laser. Dilute 0.03 M sucrose elicited moderate numbers of positive

reactions (e.g., lateral tongue protrusions; rhythmic midline tongue protrusions; paw licking; *M* =12.24, *SEM* = 0.65; $F_{1,79}$ = 167.0, $p < 0.0001$, n = 81; Fig. 9a). Very few negative aversive reactions were elicited by 0.03M sucrose (e.g., gapes, headshakes, forelimb flails, etc; *M* = 3.06, *SEM* = 0.38). A baseline sex difference was found to the low concentration, in that females emitted about 20% more 'liking' reactions to dilute 0.03M sucrose than males in absence of laser (($F_{1,79}$ = 6.28, p =0.01, female 'liking': *M* = 13.56, *SEM* = 0.86; n = 40 females, male 'liking' score: *M* = 10.95, *SEM* = 0.98, n = 41; t_{158} =2.66, p = 0.02). However, more concentrated 0.10 M sucrose elicited higher numbers of positive 'liking' reactions similarly from both sexes *(M* = 17.19, *SEM* = 1.12; $F_{1,38}$ = 163.9, $p < 0.0001$, *n = 40;* Fig. 9*.),* and again very few negative 'disgust' reactions (*M* = 2.55, *SEM* = 0.37;); Sex: $F_{1,38}$= 0.78, p = 0.38, n = 20 males, n = 20 females).

Conversely, baseline oral infusions of bitter 3x 10^{-4} M quinine solution elicited predominantly negative 'disgust' reactions (e.g., gapes, headshakes, forelimb flails; face washing; 'disgust' score *M* = 33.03, *SEM* = 1.73), and almost no positive 'liking' expressions ('liking' *M* = 1.31, *SEM* = 0.24, $F_{1,66}$ = 308.4, $p < 0.0001$, n = 68; Fig 9c), with no sex differences detected ($F_{1,66}$= 1.24, p = 0.27, n =33 females, n = 35 males). Finally, oral infusions of water elicited low numbers of both positive and negative reactions, although water still elicited more positive 'liking' reactions than aversive 'disgust' expressions from both male and female rats ('liking' reactions, *M* = 9.83, *SEM* = 0.67; 'disgust' reactions *M* = 7.14, *SEM* = 0.70, $F_{1,68}$ = 7.33, p = 0.001, n = 70; Fig 9c). Again, females emitted slightly more positive 'liking' reactions to water than male rats in the absence of laser stimulation ($F_{1,68}$= 5.78, p = 0.02, n = 33 females, n = 35 males).

Rostromedial OFC hedonic hotspot: optogenetic ChR2 stimulation enhances 'liking' reactions.

No detectable motor effects of laser on spontaneous orofacial reactions. In the absence of any oral infusion, laser illuminations in OFC ChR2 rats failed to induce detectable orofacial movements at any cortical sites. Neither positive hedonic reactions (M = 0.50, SEM = 0.34) nor negative 'disgust' reactions (M = 1.5, SEM = 0.85; $F_{1,5}$ = 3.00, $p = 0.15$, n = 6; Fig. 12c) were elicited by ChR2 laser stimulations in the absence of oral infusions, indicating that OFC and insula cortical stimulations did not directly cause motor reactions.

OFC ChR2 enhancement of sucrose 'liking' reactions. At ChR2 sites within the anteromedial subregion of OFC, which was previously identified by opioid/orexin microinjections as a hedonic hotspot (Castro & Berridge, 2017), laser stimulation (5, 10, 20, 40 Hz; 5-s ON/ 5-s OFF) approximately doubled the overall number of positive hedonic reactions elicited by oral sucrose infusions of both 0.03M or 0.1M concentrations (rhythmic tongue protrusions, lateral tongue protrusions, and paw licks). Laser illumination increased positive 'liking' reactions in anteromedial OFC ChR2 rats by 235% ± 32% for 0.03M sucrose over measured control baseline levels in the same individuals without laser (Fig. 12a; 0.03M Sucrose: $F_{1,22}$ = 23.46, $p < 0.0001$; n = 11 females, n = 12 males; Sum of rhythmic tongue protrusions, lateral tongue protrusions, and paw licks: *Laser-ON*: M = 16.52, SEM = 1.22; *Laser-OFF*: M = 8.80, SEM = 0.87; paired comparison t_{22} = 7.79, $p < 0.0001$). The magnitude of laser enhancement of 'liking' reactions was similar in females and males (Fig 12k; ChR2: sex x valence x laser interaction: $F_{1,21}$ = 1.15 p = 0.30; n = 23 OFC ChR2 rats; n = 12 males n = 11 females). Anteromedial OFC ChR2 laser stimulation did not alter the few negative 'disgust'

reactions elicited by 0.03M sucrose (gapes, headshakes, forelimb flails; chin rubs; *Laser-ON*: *M* = 3.24, *SEM* = 0.62, *Laser-OFF*: *M* = 2.30, *SEM* = 0.42, t_{22} = 0.94, *p* = 0.72).

Similarly, for the higher sucrose0.1 M concentration, anteromedial OFC laser stimulation nearly doubled (183 ± 21%) the number of positive hedonic reactions compared to within-subject baseline levels (Figure 12b; *n* = 11, Laser-ON *M* = 19.36*, SEM* = 2.27*; Laser OFF M* = 11.45, *SEM* = 1.45; $F_{1,10}$*= 8.98*, *p* = 0.01). The percentage magnitude of laser hedonic enhancement of 'liking' reactions over baseline levels was comparable for both 0.03M and 0.10 M sucrose ($F_{1,32}$*= 0.004*, *p* = 0.93), and the magnitude of laser enhancement of hedonic 'liking' reactions to 0.1M sucrose was similar in male and female ChR2 rats ($F_{1,38}$ = 0.78 *p* = 0.38; *n* = 11 OFC ChR2 rats; *n* = 4 males *n* = 7 females)

By contrast, in control eYFP rats with optically inactive virus, laser illumination in anteromedial OFC sites failed to alter either positive hedonic reactions or negative reactions to sucrose from baselines measured in the same individuals, for either 0.03 or 0.1 M sucrose (Fig. 12a; $F_{1,12}$ = 2.88 *p* = 0.19, *n* = 13). Hedonic reactions of OFC eYFP rats in both conditions remained similar to baseline reactions of OFC ChR2 rats in the absence of laser (t_{34} = 1.79, *p* = 0.10). No sex differences in affective reactions were detected between this group of male and female rats (Supplementary Fig 5l; sex x valence x laser interaction: $F_{1,11}$ = 0.19 *p* = 0.67; *n* = 13 OFC ChR2 rats; *n* = 7 males *n* = 6 females).

Different laser frequencies produce similar enhancements: Hedonic enhancement effects were robust and similar across a range of different laser frequencies in the rostromedial OFC hotspot (5, 10, 20, 40 Hz; 1-3 mW intensity), and was not limited to any single parameter. All frequencies produced similar magnitudes of enhancements of positive 'liking' reactions to sucrose, ranging between ~150% - 300% above within-subject no-laser baselines, and did not

differ statistically from each other (Fig. 121e: $F_{3,67} = 1.01$, $p = 0.39$). Assessed separately, 5 Hz, 10 Hz, 20 Hz and 40 Hz frequencies each increased positive hedonic reactions by 150% - 250% above no-laser baselines measured in the same rats (20 Hz =211% ± 24% increase, $F_{1,19} = 40.54$, $p < 0.0001$, $n = 20$; 10 Hz =240% ± 59 % increase, $F_{1,13} = 12.01$, $p = 0.004$, $n = 14$; 5 Hz = 157% ± 16% increase, $F_{1,13} = 14.17$, $p = 0.0024$, $n = 14$).

Anatomical boundaries of optogenetic hedonic hotspot in anteromedial OFC:

Localization of function was mapped for optogenetic OFC hedonic enhancements caused across cortical sites (Figure 2). Hedonic hotspots were considered to be sites where ChR2 laser illumination caused 125% - 400%+ increases in 'liking reactions to sucrose, compared to no-laser baseline levels measured in the same individual. Hedonic enhancement sites clustered anatomically into two cortical hotspots: anteromedial OFC and far caudal insula.

The anterior border of the OFC hedonic hotspot began at the far rostral tip of OFC (~+5.64 mm AP), near the anterior edge of medioventral orbital cortex, and then extended caudally along both lateral and medial surfaces. The OFC hotspot was bordered dorsally on the medial surface by prelimbic cortex, and dorsally on the lateral surface by secondary motor cortex. Moving posteriorly along the medial surface, the OFC hedonic hotspot extended ~1.4 mm to the far caudal edge of medial orbital cortex (~ +4.28 mm AP). Along the lateral surface, the hotspot extended posteriorly ~2.1mm to a point approximately ~3.72 mm anterior to Bregma. There the hotspot was bordered dorsally by the claustrum, medially by the dorsal peduncular cortex, and laterally by the rostral insula. Overall, the OFC hedonic hotspot thus extended rostrocaudally (AP) in length ≈ 2.1mm, mediolaterally (ML) ≈ 2.4 mm, and dorsoventrally (DV) ≈ 2.2 mm, with a total volume of ≈ 11.1 mm^3. We note these optogenetic OFC hotspot boundaries corresponded closely to those originally mapped neurochemically using

opioid/orexin microinjections (Castro & Berridge, 2017), The only difference between our optogenetic map and the earlier opioid/orexin map is that our study probed further in a dorsolateral direction than the earlier microinjections , and we found that the optogenetic OFC hotspot additionally extended into the rostral tip of the dorsolateral orbital cortex, making our total volume slightly larger by ~25%. Thus, portions of medial orbital (MO), ventral orbital (VO), lateral orbital (LO), and dorsolateral orbital (DLO) were all included within the ChR2 hedonic hotspot of OFC.

Beyond these boundaries, laser ChR2 stimulations at sites more caudal or lateral in OFC failed to increase 'liking' reactions to sucrose taste, including sites in caudolateral orbitofrontal cortex and caudoventral orbitofrontal cortex. Similarly, medial sites in dorsally neighboring prelimbic cortex, or ventrally in neighboring olfactory bulb, failed to enhance 'liking' reactions to sucrose (Fig 12; $F_{1,6} = 0.04$, $p = 0.84$, $n = 7$).

Microstructure of taste reactivity components fits hedonic enhancement pattern. To confirm that laser ChR2 stimulation within the OFC hotspot induced hedonic enhancements, rather than a mere sensorimotor reaction, we assessed whether changes in individual taste reactivity components were grouped into larger affective categories of positive 'liking' versus negative 'disgust'. For example, a shared increase among multiple components within the positive hedonic category (rhythmic midline tongue protrusions [TP], lateral tongue protrusions [LTP] and paw licks [PL]), but no increase in any component of the negative 'disgust' category (gapes [G], headshakes [HS], face washes [FW], forelimb flails [FF], or chin rubs [CR] (Berridge, 2000)would be required to be categorized as a hedonic increase in positive 'liking' reactions.

For OFC hotspot sites, ChR2-induced enhancements fit this category-based pattern. Hedonic increases were not dominated by any single taste reactivity component, which if so, might have reflected a simpler motor effect. Rather, increases in 'liking' elicited by laser-accompanied sucrose taste were distributed across multiple reaction components within the positive hedonic category: (Fig 11a; TP: *Laser-OFF: M*= 4.95 *SEM= 0.66; Laser-ON: M*= 8.86, *SEM= 0.81,* laser main effect*: $F_{1,32} = 31.12$, $p < 0.0001$* ; LTP: *Laser-OFF: M*= 2.22 *SEM= 0.38; Laser-ON: M*= 5.28, *SEM= 0.68,* laser main effect*: $F_{1,32} = 27.02$, $p < 0.0001$;* PL: *Laser-OFF: M*= 2.72 *SEM= 0.40; Laser-ON: M*= 3.58, *SEM= 0.40,* laser main effect*: $F_{1,32} = 4.65$, $p = 0.04$.)*

OFC hotspot hedonic enhancement of water. Laser ChR2 excitation in the OFC hotspot similarly increased positive 'liking' reactions to water by >30% over within-subject baselines (Fig 12f; laser x valence interaction: $F_{1,20} = 6.06$, $p = 0.02$, paired comparison: $t_{20} = 2.79$, $p = 0.02$), with no change in the low number of aversive 'disgust' expressions to water ($t_{20} = 0.69$, $p = 0.99$). In absence of laser, oral infusions of tap water at room temperature elicited only a few positive 'liking' reactions ($M = 8.36$, $SEM = 1.08$) and a few aversive 'disgust' reactions on baseline tests ($M = 7.38$, $SEM = 1.35$). In eYFP control rats, adding laser illumination to OFC did not alter either positive or negative reactions to water compared to baseline (Fig 12f; $F_{1,11} = 2.58$, $p = 0.15$; $n = 12$).

OFC hotspot suppression of quinine 'disgust'. Oral infusions of bitter quinine solution (3×10^{-4} M) elicited predominately aversive 'disgust' reactions in the absence of laser ($M = 34.74$, $SEM = 2.98$; $F_{1,20} = 140.0$, $p < 0.0001$, $n = 21$). Within the OFC hotspot, adding laser stimulation in either ChR2 rats or eYFP rats (40 Hz; 5-s ON/ 5-s OFF) moderately suppressed the number of aversive reactions elicited by quinine by about 20%-30% below no-laser baselines

measured in the same rats (Fig. 12g; ChR2 rats: 35% ± 7% suppression, *M*= 20.90, *SEM* = 2.25, $F_{1,20} = 33.02$, $p < 0.0001$, $n = 21$; eYFP control rats: 18.4% ± 10% suppression, $F_{1,12} = 8.49$, $p < 0.01$, $n = 13$). The magnitude of quinine 'disgust' suppression was nearly twice as large in ChR2 rats as eYFP rats, although this magnitude difference was not significant ($F_{1,32} = 0.94$, $p = 0.34$), suggesting that light or heat from laser in OFC may partly contribute to reduce 'disgust' reactions, independently of ChR2-induced neuronal excitation (Owen et al., 2019; Stujenske et al., 2015). Multiple components of 'disgust' reactions elicited by quinine were suppressed together by OFC hotspot laser, supporting the interpretation that the aversive 'disgust' of bitterness was reduced: headshakes (Fig 11d. *Laser-OFF*: *M* = 6.31, *SEM* = 0.66; *Laser-ON*: *M* = 3.74, *SEM*= 0.66, $t_{20} = 5.18$, $p < 0.0001$), forelimb flails (*Laser-OFF*: *M* = 18.33, *SEM* = 2.34; *Laser-ON*: *M* = 9.10, *SEM*= 1.51, $t_{20} = 5.41$, $p < 0.0001$), and face washes (*Laser-OFF*: *M* = 2.12, *SEM* = 0.42; *Laser-ON*: *M* = 1.29, *SEM*= 0.30, $t_{20} = 2.05$, $p = 0.05$).

OFC hotspot neuron inhibition fails to alter affective reactions. A separate group of rats received the inhibitory virus iC++ in the rostromedial OFC hotspot. Laser delivery within the OFC hotspot failed to alter affective reactions to 0.03M sucrose (Fig 12h; 104% ± 13% laser enhancement; $F_{1,6} = 0.01$, $p = 0.92$; $n = 4$ females, $n = 3$ males; Sum of rhythmic tongue protrusions, lateral tongue protrusions, and paw licks: *Laser-ON*: *M* = 14.64, *SEM* = 3.14; *Laser-OFF*: *M* = 14.36, *SEM* = 2.83), or to water (Fig 12i; 162% ± 83% laser enhancement; $F_{1,6} = 1.83$, $p = 0.22$; $n = 4$ females, $n = 3$ males; Sum of rhythmic tongue protrusions, lateral tongue protrusions, and paw licks: *Laser-ON*: *M* = 6.50, *SEM* = 1.41; *Laser-OFF*: *M* = 7.36, *SEM* = 1.72), or quinine (Fig 12j; 216% ± 62% laser enhancement; $F_{1,6} = 0.26$, $p = 0.63$; $n = 4$ females, $n = 3$ males; Sum of face washes, forelimb flails, headshakes, gapes, and chin rubs: *Laser-ON*: *M* = 17.64, *SEM* = 5.63; *Laser-OFF*: *M* = 15.86, *SEM* = 6.16. This suggests that while neuronal

activations can generate gains of hedonic function that increase hedonic 'liking' reactions, this is not matched by loss of hedonic function when OFC hotspot neurons are optogenetically inhibited.

Optogenetic hedonic coldspot strip spans from caudal OFC through rostral insula: 'Liking' suppression

Anatomical boundaries of OFC/Insula optogenetic hedonic coldspot: Beginning at the caudal boundary of the anteromedial OFC hotspot on the lateral surface of cortex, ChR2 laser stimulation at posterior sites on the lateral surface of OFC oppositely suppressed positive 'liking' reactions to both 0.03M and 0.10M sucrose tastes in a hedonically suppressive 'coldstrip' (Fig 2a,c). This suppressive coldstrip extended ~ 3 mm posteriorly through entire posteriolateral OFC, anterior insula and a middle portion of insula, to end at a mid-posterior insula point just dorsal to where the anterior commissure crosses the midline (AP coordinates ~+3.00 mm to ~-0.12mm bregma). This coldstrip therefore included sites in ventral and lateral orbital subdivisions of caudal OFC, caudal dorsolateral OFC and caudal ventrolateral OFC as well as anterior and middle insula. At its rostral tip, the hedonic coldstrip was bordered dorsally by the claustrum, and medially by the dorsal peduncular cortex, and rostral strip spanned mediolaterally through the lateral orbitofrontal cortex and anterior insula. At its caudal end, the suppressive coldstrip was bordered dorsally by secondary somatosensory cortex, ventrally by piriform cortex, and medially by the claustrum. Within the insula, agranular, dysgranular, and granular horizontal layers were all included in the hedonic coldstrip.

Laser stimulation (40 Hz) at sites in this OFC-insula coldspot strip of ChR2 rats suppressed positive 'liking' reactions to 0.03M or 0.1M sucrose to approximately one-half the levels emitted by the same ChR2 rats in baseline tests when no laser was delivered (Fig 13a;

0.03M sucrose: *Laser-ON*: $M = 9.33$, $SEM = 0.86$; *Laser-OFF*: $M = 17.05$, $SEM = 1.08$; $F_{1,20} = 37.48$, $p < 0.0001$; paired comparison: $t_{21} = 5.78$, $p < 0.0001$; Fig 13b: 0.1M sucrose: *Laser-ON*: $M = 9.18$, $SEM = 1.25$; *Laser-OFF*: $M = 20.32$, $SEM = 1.69$; $F_{1,16} = 47.10$, $p < 0.0001$; paired comparison: $t_{17} = 6.53$, $p < 0.0001$). The percentage magnitude of suppression was similar for both 0.03M and 0.1M sucrose concentrations ($F_{3,69} = 0.60$, $p = 0.62$). Hedonic suppression was similarly robust across all laser frequencies tested here (Supplementary Fig 6; $F_{3,69} = 0.60$, $p = 0.62$), and when assessed separately, 40 Hz, 20 Hz, 10 Hz, and 5 Hz each suppressed positive 'liking' reactions to sucrose (40 Hz: 41%± 6% decrease, $F_{1,20} = 29.93$, $p < 0.0001$, $n = 21$; 20 Hz: 34%± 6% decrease, $F_{1,19} = 16.32$, $p = 0.0007$, $n = 20$; 10 Hz: 39%± 8% decrease, $F_{1,15} = 20.21$, $p = 0.0004$, $n = 16$; 5 Hz: 28%± 10% decrease, , $F_{1,14} = 12.16$, $p = 0.004$, $n = 15$). Similarly, both posterior OFC and anterior insula portions of the coldstrip suppressed sucrose 'liking' reactions to similar extents, (Fig 13c; brain site x laser interaction effect; $F_{1,19} = 1.81$, $p = 0.19$). Posterior OFC and insula sites of coldstrip were also similar in their magnitude of aversive 'disgust' induction ($F_{1,19} = 0.27$, $p = 0.61$). By comparison, in eYFP control rats, laser stimulation of sites in the OFC-insula coldstrip failed to alter positive or negative affective reactions to sucrose (Fig 13a, b; *Laser-ON*: $M = 12.92$, $SEM = 2.71$; *Laser-OFF*: $M = 11.83$, $SEM = 1.57$; $F_{1,5} = 2.74$, $p = 0.16$).

Coldstrip microstructure of taste reactivity: Hedonic suppression pattern. Multiple components of positive 'liking' reactions elicited by sucrose were suppressed together by ChR2 laser stimulation at sites in the OFC-insula coldstrip (e.g., midline tongue protrusions ($t_{21} = 4.91$, $p < 0.001$) and paw licks ($t_{21} = .45$, $p = 0.003$)). This suggests coldstrip ChR2 stimulations suppressed positive hedonic reactions as an entire affective category.

W*ater infusions: hedonic suppression*. In response to oral water infusions, photoexcitation (40 Hz) of ChR2 sites within the hedonic OFC-insula coldstrip again decreased positive 'liking' reactions to approximately one-half the number elicited on control trials without laser in the same rats (Fig 13f; *Laser-ON*: $M = 7.26$, $SEM = 1.00$; *Laser-OFF*: $M = 13.24$, $SEM = 1.24$; laser main effect: $F_{1,17} = 27.62$, $p = <0.0001$). By comparison, in eYFP control rats, laser stimulation did not alter affective reactions to water (*Laser-ON*: $M = 6.00$, $SEM = 0.35$; *Laser-OFF*: $M = 11.41$, $SEM = 2.39$; $F_{1,5} = 1.20$, $p = 0.09$).

Quinine infusions: Potential suppression of 'disgust' reactions. Laser stimulation of OFC-insula 'hedonic coldstrip' sites in ChR2 rats similarly suppressed aversive 'disgust' reactions to bitter quinine by approximately 30% *(Fig 13g; Laser-ON: $M = 25.08$, $SEM = 2.57$; Laser-OFF: $M = 32.68$, $SEM = 2.90$; laser main effect: $F_{1,18} = 4.86$, $p = 0.04$),* just as it suppressed positive hedonic reactions above. Global suppression of both negative aversive reactions to quinine and positive hedonic reactions to sucrose and water, suggests a general affective suppression of both positive 'liking' and negative 'disgust'. Alternatively, it could reflect a general sensorimotor disruption of orofacial reactions. However, in the absence of any taste infusion, laser illumination in OFC/insula coldstrip ChR2 rats failed to produce any detectable orofacial movements on its own (Fig 13d; $F_{1,13} = 4.48$, $p = 0.14$, $n = 14$). Posterolateral OFC sites and rostral-middle insula sites within the coldstrip similarly suppressed quinine 'disgust' reactions *(*Fig. 13h; *brain region x laser interaction: $F_{1,18} = 0.36$, $p = 0.55$*). Only a few positive 'liking' reactions were elicited by quinine, and these were not detectably altered by laser ChR2 excitations at coldstrip sites, perhaps because they were near zero to begin with *($F_{1,18} = 1.50$, $p = 0.24$).*

A second optogenetic hotspot in far caudal insula magnifies hedonic 'liking' to sucrose.

In the far-caudal subregion of insula, a second cortical hedonic hotspot was confirmed, where laser stimulation of ChR2 sites doubled-to-tripled the number of 'liking' reactions to sucrose tastes. The insula hedonic hotspot included agranular, dysgranular, and granular zones of the farthest caudal one-third of insula, spanning ~-2 mm from ~-0.84 mm AP from bregma to ~-2.92 mm AP (Fig. 2). The caudal insula hotspot was bordered medially by the claustrum, dorsally by secondary somatosensory cortex, ventrally by piriform cortex, and posteriorly by ectorhinal and perirhinal cortex (at its caudal end where it medially abutted external capsule).

Within this caudal insula hotspot, laser ChR2 stimulations increased hedonic 'liking' reactions to 0.03M sucrose taste by over 300% ± 116% over baseline levels elicited from the same rats on no-laser trials (Fig. 14a; *Laser OFF*= 8.2 ± 1.48; *Laser ON*: = 19.35 ± 2.47; $F_{1,9}$= 16.31, p =0.003; n = 10). All laser frequencies (40 Hz, 20 Hz, 10 Hz, and 5 Hz) produced similar magnitude hedonic enhancements at these sites (Fig. 14f; $F_{1.57,\ 9.45}$ = 2.64, p = 0.13), and male and female rats with ChR2 sites in the caudal insula hotspot also showed similar hedonic enhancements, without detectable sex differences (Fig. 14c*;* $F_{1,8}$= 0.28, p = 0.61).

As caveat, we tested 0.03 M sucrose in all insula hotspot rats but were able to test 0.1M sucrose in only a few rats. That was because we observed laser-induced seizures appear in 50-80% of rats at posterior insula sites after multiple optogenetic ChR2 stimulations, and so restricted most subsequent rats to as few laser stimulations as possible. In the two posterior insula ChR2 rats we were able to test with 0.10M sucrose, we observed 150% and 133% increases in hedonic 'liking' reactions.

Water infusions: Hedonic enhancement. In response to oral water infusions, laser ChR2 stimulation in the posterior insula hotspot increased positive 'liking' reactions by 500% over normally low baselines in the absence of laser (Fig. 14b; $F_{1,8}$= 10.99, p =0.01; n = 9).

Taste reactivity microstructure: Hedonic enhancement pattern. Multiple orofacial components within the positive 'liking' category for sucrose and water were increased together by ChR2 laser stimulations in the insula hotspot: rhythmic midline tongue protrusions (Fig 11c; *Laser-ON: M = 7.45, SEM = 3.50; Laser-OFF; M = 3.50, SEM 0.84;* t_{10} = 3.800, p = 0.00), and lateral tongue protrusions (*Laser-ON: M = 8.15, SEM = 1.36; Laser-OFF; M = 1.95, SEM 0.50*; t_{10} = 4.21, p = 0.002*).* However, in the absence of any taste infusion, ChR2 laser stimulations failed to elicit any detectable orofacial reactions (positive *'liking', M* = 0.5, *SEM* = 0.19; aversive 'disgust' *M* = 1.6, *SEM* = 0.42; $F_{1,7}$= 2.39, p = 0.17, n = 8). This pattern suggests that ChR2 excitation in the posterior insula hotspot specifically enhanced the hedonic impact of tastes that were initially pleasant or neutral.

Quinine infusions: no detectable change. For bitter quinine infusions, laser ChR2 stimulation in the insula hotspot failed to suppress the substantial level of 'disgust' reactions, or to increase positive 'liking' reactions above their low baselines to bitterness (Fig. 14d; $F_{1,7}$= .01, p =0.91; n = 8). This suggests that a strongly disgusting taste may resist hedonic enhancement by posterior insula stimulation.

Incentive value of laser by itself? Self-stimulation measures.

The incentive motivation value of laser ChR2 stimulation on its own, in the absence of any taste infusion, was measured in two laser self-stimulation tests: an active instrumental spout-touch task and a relative passive place-based self-stimulation task.

Spout-Based Self-Stimulation

OFC and insula hotspots support spout-touch self-stimulation. In the spout-touch self-stimulation task, each instrumental touch on a designated empty waterspout (laser-spout) earned a brief laser bin of either 1-sec or 5-sec duration (depending on test day). By contrast, touching an alternative control spout produced nothing, and served merely as a measure of baseline exploratory touches. Rats were considered to be 'high self-stimulators' if they earned >50 laser illuminations in a 30-min session and made at least twice as many contacts on their laser-spout than on control spout (Fig 3). Rats were considered to be 'low self-stimulators' if they earned >10 but <50 illuminations per session, and still made twice as many contacts on laser-spout than on control spout. Finally, rats were considered 'failures to self-stimulate' if they earned <10 illuminations or failed to touch the laser spout at least twice as frequently as the control spout. All rats were categorized on day 1 and retested for reliability on days 2 and 3.

When OFC or insula hedonic hotspot rats could earn brief 1-s 40 Hz laser pulses, ~77% of OFC hotspot rats and ~75% of insula hotspot rats met criteria for at least low levels of self-stimulation behavior, and 10% to 25% met criteria for high self-stimulation (OFC hotspot: high self-stimulation: 9.1%, low self-stimulation: 68.2%, no self-stimulation: 22.7%; Insula hotspot: high self-stimulation: 25.0%, low self-stimulation: 50.0%, no self-stimulation: 25%). When touches earned longer 5-s laser pulses (which had been used in taste reactivity tests to increase hedonic 'liking' reactions to sucrose) ~ 50% to 60% met criteria for at least at low-levels of self-stimulation, and 10% - 20% met criteria for high self-stimulation (5-sec OFC hotspot: high self-stimulation: 18.2%, low self-stimulation: 45.5%, no self-stimulation: 36.4%; Insula hotspot: (high self-stimulation: 12.5%, low self-stimulation: 37.5%, no self-stimulation: 50.0%). OFC hotspot sites were slightly more effective than insula hotspot sites at promoting self-stimulation

when longer 5-s pulses were delivered (Fig. 15; 20 Hz laser x brain region x pulse duration interaction: $F_{1,19}$= 8.54, p = 0.0009; 40 Hz laser x brain region x pulse duration interaction: $F_{1,28}$= 3.56, p = 0.07).

By contrast, eYFP rats with sites in OFC or insula hotspots failed to meet any criteria for laser self-stimulation in the spout-touch task. Control eYFP rats made similar numbers of contacts on the laser spout and non-laser spout, both for 1-s laser bins (Fig. 15; Spout contacts: Laser-ON: M =17.1, SEM = 4.6; Laser-OFF: M = 24.0, SEM = 8.1), and for 5s laser bins (Spout contacts: Laser-ON: M =7.9, SEM = 2.3; Laser-OFF: M = 8.3, SEM = 2.1; laser x pulse duration interaction: $F_{1,8}$= 1.2, p = 0.31, n = 11).

Hedonic coldstrip sites also support spout-touch laser-self-stimulation. Many sites in the hedonic-suppressive coldstrip from caudolateral OFC to mid insula also supported laser self-stimulation in the spout-touch task, despite having suppressed hedonic 'liking' reactions in taste-reactivity tests (Fig 15; 20 Hz laser main effect: $F_{1,18}$= 5.55, p = 0.03; n = 9 OFC, n = 11 insula; 40 Hz laser main effect: $F_{1,18}$= 11.87, p = 0.003; n = 9 OFC, n = 11 insula;). When earning 1-sec laser bins, virtually all posterior OFC coldstrip ChR2 rats met criteria for at least low levels of self-stimulation, and ~50% met criteria for high self-stimulation (caudolateral OFC: high self-stimulation: 55.6%; low self-stimulation: 44.4%; no self-stimulation: 0%). Similarly, ~70% of insula coldstrip ChR2 rats met criteria for at least low self-stimulation, and ~25% met criteria for high self-stimulation (anterior & middle insula: high self-stimulation 18.2%; low self-stimulation 54.5%; no self-stimulation 27.3%).

When laser duration was extended to longer 5-s bins, more similar to durations used in the taste reactivity test, coldstrip sites continued to support laser self-stimulation (Supplementary Fig 9 caudolateral OFC: high self-stimulation: 55.6%; low self-stimulation: 33.3%; no self-

stimulation 11.1%; Anterior & middle insula: high self-stimulation: 18.2%; low self-stimulation: 72.7%; no self-stimulation: 9.1%). Coldstrip sites in both caudolateral OFC and anterior insula supported laser self-stimulation equally at both 1-s and 5-s laser durations, and at both 20 Hz and 40 Hz frequencies (Fig. 15; 20 Hz laser x brain region x pulse duration interaction: $F_{1,18}$= 0.17, p = 0.68; 40 Hz laser x brain region x pulse duration interaction: $F_{1,18}$= 0.18, p = 0.68), suggesting robust incentive or 'wanting' value of ChR2 stimulations in the hedonic coldstrip when tested in the spout-touch task, despite lack of 'liking' enhancement and even suppression of 'liking' reactions in the taste reactivity test at these sites.

By contrast, eYFP control rats failed to reach self-stimulation criteria in the spout-touch task, and made equal numbers of touches on the laser-delivering spout and non-laser spout for 1-s bins (Fig. 15; Spout contacts: *Laser-ON: M* =10.5, *SEM* = 5.7; *Laser-OFF: M* = 31.9, *SEM* = 12.2) and 5s bins (Spout contacts: *Laser-ON: M* =26.8, *SEM* = 17.; *Laser-OFF: M* = 27.3, *SEM* = 13.8; laser x pulse duration interaction: $F_{1,4}$= 2.98, p = 0.16, n = 5).

Place-Based Self-Stimulation

OFC and Insula Hotspots Support Place-Based Self-Stimulation. In the place-based task, rats could earn laser illuminations by entering a designated chamber, or simply remaining in it while laser continued (3 sec ON, 8 sec OFF cycle). Hedonic hotspot sites in both anteromedial OFC and far-caudal insula supported place-based self-stimulation. ChR2 rats with OFC or insula hedonic hotspot sites spent 150% - 200% more time in the laser-delivering chamber than in the alternative chamber without laser (Figure 4; Fig 16;laser main effect; $F_{2,73}$= 4.80, p =0.01; n = 22 OFC, n = 10 insula), and OFC vs insula hedonic hotspots did not differ in levels of place-based self-stimulation at either 20Hz or 40 Hz frequencies (Figure 4, Supplementary Fig 10; 20 Hz

Difference Score: *M = 153.3*, *SEM* = 62.4; 40 Hz Difference Score: *M = 236.8*, *SEM* = 41.3; No Laser Score: *M = -111.2*, *SEM* = 55.5; laser x brain site interaction: $F_{2,73}$= 0.92, *p* =0.63). Male and female rats insula/OFC hotspot ChR2 rats also showed comparable levels of laser self-stimulation, with no detectable sex difference (laser x brain site x sex interaction: $F_{2,29}$ = 0.41 *p* = 0.67; *n* = 16 males *n* = 16 females).

By comparison, eYFP control rats failed to self-stimulate in the place-based task (Figure 4; Fig. 16; laser x virus interaction; $F_{2,73}$= 3.69, *p* =0.46), and spent equal amounts of time in both the non-laser and laser-delivering chambers (20 Hz Difference Score: *M = -119.28*, *SEM* = 91.3; 40 Hz Difference Score: *M = -102.2*, *SEM* = 97.9; No Laser Score: *M = -133.9*, *SEM* = 89.5; laser x brain site interaction: $F_{2,73}$= 0.92, *p* =0.63; laser x brain site interaction: $F_{1,16}$= 1.76, *p* = 0.20; *n = 12* OFC, *n* = 6 insula).

Coldstrip relatively fails to support place-based self-stimulation. Coldstrip sites in caudal OFC or rostral insula as an entire group did not reliably support place-based laser self-administration (Figure 4; Fig. 16; laser main effect: $F_{2,46}$= 0.46, *p* = 0.63), although there appeared non-significant trends toward place based self-stimulation at caudolateral OFC sites, and opposite place-based avoidance in rostral to mid-insula sites that did not reach p<.05 statistical significance. ChR2 rats with caudolateral OFC coldspot sites spent ~175% more time in the laser-delivering chamber than no-laser chamber (20 Hz Difference Score: *M = 58.8*, *SEM* = 57.4; 40 Hz Difference Score: *M = 246.7*, *SEM* = 39.2; No Laser Score: *M = -139.3*, *SEM* = 84.5; n= 9 caudal OFC; Laser x site interaction; $F_{2,46}$= 3.09, *p* = 0.06). By contrast, ChR2 rats with anterior insula coldspot sites oppositely spent only ~40% as much time in the laser-delivering chamber as the no-laser chamber (Figure 4, Fig. 16; 20 Hz Difference Score: *M = -204.0*, *SEM* = 49.8; 40 Hz Difference Score: *M = -234.1*, *SEM* = 90.1; No Laser Score: *M* = -

35.92, *SEM* = 61.53, *n* = 12 anterior insula; Laser x site interaction; $F_{2,46}$= 3.09, *p* = 0.06). Control eYFP virus rats failed to show either place based self-stimulation or laser avoidance (Figure 4, Fig. 16; 20 Hz Difference Score: *M* = *52.6*, *SEM* = 37.4; 40 Hz Difference Score: *M* = *29.1*, *SEM* = 59.8; No Laser Score: *M* = *-11.38*, *SEM* = 120.6; laser x brain site interaction: $F_{1,4}$= 0.0001, *p* = 0.99; *n* = *3* OFC, *n* = 3 insula). One potential reason for why results of the place based self-stimulation task might differ from active self-stimulation task is that the cumulative duration of laser per minute was approximately 2x to 4x higher in the place-based task than in the spout-touch task. It is possible that greater laser durations exceeded an optimal level for self-stimulation, and potentially became aversive especially for anterior insula and middle insula sites.

Fos protein expression in distant structures

We assessed distant changes in Fos expression in several mesocorticolimbic structures recruited by laser ChR2 excitation of neurons within cortical sites in OFC and insula. For all structures, Fos was measured after laser ChR2 illumination in a cortical site, and was compared with a) control eYFP Fos baseline levels measured in eYFP rats receiving laser illuminations and b) control naïve baseline levels in rats that were lightly handled, but received no surgery, virus microinjection, laser or behavioral testing.

Rostromedial OFC hotspot neuron stimulation. Laser ChR2 stimulation in the rostromedial OFC hotspot also recruited distant 150%-300% increases in Fos expression in the caudal insula hotspot (Fig. 5; Fig. 17). Similarly, OFC hotspot stimulation recruited ~175%-300% increases in Fos in previously identified subcortical hedonic hotspots, such as in rostrodorsal hotspot of NAc medial shell, and caudal hotspot of ventral pallidum (Castro et al.,

2016; Castro & Berridge, 2017; Mahler et al., 2007; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005). By contrast, we did not observe increases in Fos in previously identified subcortical suppressive coldspots, such as the caudal subregion of the NAc medial shell or the anterior ventral pallidum. Significant Fos increases were observed also in prelimbic cortex, infralimbic cortex, anterior cingulate cortex, nucleus accumbens core and medial shell, ventral pallidum, perifornical areas of the lateral hypothalamus, medial amygdala, and ventral tegmental area (Fig. 5; Fig. 17)

Caudal insula hotspot neuron stimulation. Laser ChR2 stimulation in the caudal insula hotspot recruited distant >175% Fos increases in the rostromedial OFC hotspot (Fig. 6; Fig. 18). Insula hotspot stimulation also recruited >175% Fos increases in the subcortical hedonic hotspot in rostrodorsal quadrant of NAc medial shell, although no Fos change was detected in the posterior hotspot of ventral pallidum (Fig. 6; Fig. 18). Other significant changes in Fos were detected in prelimbic cortex, infralimbic cortex, anterior cingulate cortex, paraventricular thalamus, nucleus accumbens core and shell, olfactory tubercle, lateral hypothalamus, arcuate nucleus, and ventral tegmental area (Fig. 6; Fig. 18)

Overall, our results suggest that optogenetic neuron activation of either the rostromedial OFC hotspot or of the far-posterior insula hotspots is sufficient to also recruit Fos activation in each other, and simultaneously in at least one subcortical hedonic hotspot. This seems consistent with the hypothesis that local neurobiological stimulation of any one hedonic hotspot may recruit co-activation in other hotspots, to activate an entire distributed hedonic enhancement circuit to cause increases in taste-elicited orofacial 'liking' reactions.

Caudal OFC to rostral-mid insula coldstrip. Laser ChR2 stimulation in the suppressive hedonic coldstrip, which stretched from caudolateral OFC through anterior and middle insula

failed to recruit distant Fos increases in either cortical hotspot of rostromedial OFC or caudal insula, nor in subcortical hedonic hotspots in rostrodorsal medial shell or caudal ventral pallidum. However, stimulation in the hedonic coldstrip did increase Fos in other cortical coldstrip sites, as well as in other previously identified subcortical suppressive coldspots. For example, within the cortical coldstrip, caudolateral OFC stimulation recruit >200% Fos increases in rostral insula, and conversely rostral insula stimulations recruited >200%Fos increases in caudolateral OFC sites (Fig 7; Fig. 19). Similarly, caudal OFC/rostral insula coldstrip stimulation also recruited subcortical >175% increases in the caudodorsal coldspot quadrant of NAc medial shell, and recruited >200% Fos expression in the rostral coldspot of ventral pallidum (Fig 7; Fig. 19), where opioid microinjections suppress hedonic 'liking' reactions to sucrose (Castro et al., 2016; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005). Other >175%-300% Fos increases were observed in o prelimbic cortex, infralimbic cortex, paraventricular thalamus, nucleus accumbens core, central amygdala, basolateral amygdala, hypothalamic arcuate nucleus, and ventral tegmental area (Fig 7; Fig. 19).

2.5 Discussion

Here we confirmed the existence and boundaries of localized hedonic hotspots in rostromedial OFC and far-caudal insula, and extended their definition by showing that optogenetic ChR2 stimulation within each approximately doubled the hedonic impact of sucrose taste, as reflected in the number of positive orofacial 'liking' expressions in rats. These OFC and insula hedonic hotspots were previously mapped only neurochemically, in studies which used microinjections of mu opioid agonist or of orexin to enhance 'liking' reactions (Castro & Berridge, 2017).

Our optogenetic results provide independent triangulating evidence that these hedonic hotspots are robust neurofunctional entities with special capacities to enhance the hedonic impact

or ‘liking’ reaction to a pleasant stimulus in response to local neurobiological stimulations. Our results show that ChR2-induced depolarization of neurons within the cortical hotspots is as effective as opioid/orexin receptor stimulation for enhancement of hedonic ‘liking’ reactions. Anatomically, the locations and boundaries of the rostromedial OFC and caudal insula hotspots mapped optogenetically here were nearly identical to the boundaries previously mapped neurochemically. Here, the optogenetic OFC hedonic hotspot began anteriorly at the rostral tip of the OFC and extended caudally ~2.5 mm along both the medial and lateral surfaces of OFC. Moving further posteriorly along the lateral surface of OFC, the hedonic hotspot was replaced by a 4.5 mm long (17.0 mm^3 volume) suppressive OFC-insula ‘hedonic coldstrip’, where ChR2 laser excitations reduced the number of ‘liking’ reactions elicited by sweet tastes to one-half control levels. The suppressive coldstrip included the most-caudal one-third of OFC, and both the anterior insula and middle one-third of insula. Finally, we confirmed that the most posterior one-third of insula cortex contained a second hedonic hotspot, approximately 5.3 mm^3 in volume where ChR2 laser excitation again doubled the number of ‘liking’ expressions elicited by sucrose or water tastes.

Motivational ‘wanting’ for laser anatomically more widespread than hedonic ‘liking’ enhancement. By contrast to the restricted localization of hedonic hotspots for ‘liking’ enhancement, cortical sites that supported incentive motivational ‘wanting’ effects of ChR2 excitation, measured as laser self-stimulation in an active spout-touch task, included both rostromedial OFC and caudal insula hotspots but also extended between them into the hedonically-suppressive coldstrip, including posteriolateral OFC and anterior-mid insula sites. This is consistent with the idea that cortical substrates for enhancement of motivational ‘wanting’ are anatomically more widespread than hedonic hotspots for ‘liking’, consistent with

studies that reported increased motivation measured as increases in food intake, instrumental responding during reward choice tasks, or self-stimulation of cortical electrical, or optogenetic, or drug microinjection stimulations (Baldo et al., 2016; Ballesta et al., 2020; Castro & Berridge, 2017; Giacomini et al., 2021, 2022; Jennings et al., 2019; Khani et al., 2015; Koolhaas et al., 1977; Mena et al., 2011, 2013; Münster et al., 2020; Münster & Hauber, 2018; Peng et al., 2015; Routtenberg, 1971; Routtenberg & Sloan, 1972; Selleck et al., 2015, 2018).

Comparison to other optogenetic studies of hedonic taste modulation. Previous optogenetic studies of cortical stimulation in mice reported that optogenetic stimulation in anterior insula enhanced voluntary licking of a drink spout, supported self-stimulation or elicited computer-scored positive facial expressions to tastes (Dolensek et al., 2020; Peng et al., 2015; L. Wang et al., 2018). In partial agreement with positive incentive motivation or 'wanting' effects, we similarly found that anterior insula sites supported optogenetic self-stimulation in the spout-touch task. However, regarding hedonic impact, anterior insula sites here fell within our hedonic coldstrip where ChR2 excitation suppressed facial 'liking' reactions to sucrose. One possible explanation of the discrepancy is that those other studies primarily employed measures of reward motivation rather than 'liking' or hedonic impact. The one exception was the AI-scored study facial reaction to tastes, as mice licked from a spout (Dolensek et al., 2020). However, voluntary licking is also a 'wanting' measure, as licking is instrumentally required to execute a decision to ingest, rather than a purely affective reaction to the hedonic impact of a taste delivered to the mouth, as measured here. For example, voluntary licking is reduced by systemic administration of dopamine antagonists e (D'Aquila et al., 2012; Schneider et al., 1990), but antagonists or dopamine lesions fail to reduce hedonic 'liking' facial expressions to sweetness measured in taste reactivity tests (Pecina et al. 1997; Berridge & Robinson, 1998), nor does dopamine

blockage or loss reduce subjective liking ratings in humans of the hedonic impact of sweet tastes, cocaine, or amphetamine even when it reduces wanting ratings of the same reward (Brauer & De Wit, 1997; Leyton et al., 2005; Sienkiewicz-Jarosz et al., 2005). The AI-scored study did not specify which particular movements were identified by its AI algorithm as 'positive facial expressions', leaving it unclear whether the 'positive facial expression' score reflected specific increases in lateral tongue protrusions, rhythmic tongue protrusions and paw licks elicited by the hedonic impact of sweetness, as measured here, or whether instead included increases in voluntary lick movements used to instrumentally ingest sucrose from its source (Dolensek et al., 2020). If voluntary licking of an external object to obtain liquid sucrose was included in the positive score, then the increases reported by that study could have reflected increased 'wanting' for sucrose, rather than increased 'liking' reaction to its hedonic impact once obtained. By comparison, direct delivery of taste solutions to the mouth via oral cannula, as used here, skips over voluntary decisions and actions to ingest, and more selectively filters purely affective reactions elicited by the hedonic impact of a taste. Conversely, other optogenetic studies have reported that aversive motivation and 'disgust' facial reactions in mice were evoked in caudal insula by optogenetic ChR2 stimulation (Peng et al., 2015; L. Wang et al., 2018), including specifically stimulation of glutamate neurons using a CamKII promoter (Gehrlach et al., 2019). Those aversive effects contrast to our observation of enhancement of sucrose 'liking' reactions and laser self-stimulation for ChR2 excitation in the far caudal hotspot of insula in rats. However, the insula sites in mice that produced aversive effects might actually have been in what we categorized as the mid-insula portion of our hedonic coldstrip, where we similarly promoted aversive 'disgust' reactions to sucrose, rather than far caudal insula. Further, Peng et al., used

laser intensities that were 10-20x higher (10 – 20 mW) than ours (1-3 mW), and it's conceivable that higher intensities in insula might promote aversive reactions (Peng et al., 2015).

Gains vs losses of hedonic function

Optogenetic iC++ inhibition in the rostromedial OFC hotspot failed to alter 'liking' reactions to tastes, although ChR2 excitation enhanced sweetness and water 'liking' at comparable OFC sites. Failure of local inhibitions to alter hedonic impact may be consistent with reports that even cortical insula lesions or complete decortication in rats similarly fails to impair taste 'liking' reactions or motivation for food reward (King et al., 2015b; Wirsig & Grill, 1982b).

We hypothesize this difference between cortical gain vs loss of hedonic function may reflect the hierarchical nature of cortical hedonic contributions to 'liking' reactions. That is, functional activation of cortical hedonic hotspot sites causes hierarchical facilitation of positive 'liking' reactions mediated by subcortical hedonic circuitry. Conversely, functional activation of cortical hedonic coldstrip sites hierarchically suppresses 'liking' reactions mediated by subcortical circuitry. Loss or inhibition of cortical hotspot sites may impair hierarchical facilitation but does not necessarily impair autonomous hedonic functions of subcortical circuitry, allowing baseline 'liking' reactions to remain intact.

Potential Neuronal Mechanisms within Hedonic Hotspots

Our findings indicate that optogenetic depolarization of neurons within previously identified hotspot subregions of OFC and insula, induced by ChR2-mediated influx of Na+ and Ca+ ions, enhances hedonic 'liking' reactions to palatable tastes. This raises the question of how optogenetic enhancement relates to neurochemical enhancements produced by microinjections of either a mu opioid agonist or orexin in the same hedonic hotspots (Castro & Berridge, 2017).

Orexin is reported to depolarize neurons in layer 6 of neocortex (Bayer et al., 2004; Combremont et al., 2016), as well as in central amygdala (Dustrude et al., 2018), nucleus basalis (Hoang et al., 2004), and hypothalamus (Y. Li et al., 2002). Thus, orexin-induced enhancement of 'liking' in OFC or insula hotspots might conceivably involve neuronal depolarization as a mechanism, similarly to optogenetic ChR2 stimulation.

However, DAMGO, a selective mu-opioid agonist, acts at Gi-protein coupled inhibitory receptors that suppress intra-neuronal adenylyl cyclase and are associated with IPSPs (CHILDERS et al., 1992; Connor & Christie, 1999; Finnegan et al., 2005; Koehl et al., 2018; Margolis & Fields, 2016; Matsui-Sakata et al., 2005; Nijssen et al., 1992; Stanford & Cooper, 1999). Opioid enhancement of 'liking' therefore presents a puzzle for understanding how both DAMGO microinjection and optogenetic stimulation in the same OFC or insula hedonic hotspots produce similar 'liking' enhancements. One possible resolution might be that mu-opioid microinjections inhibit local or afferent GABA inhibitory neurons, which if inhibited, disinhibit other neurons in the cortical site into depolarization, as has been proposed to occur in VTA, hippocampus, and periaqueductal gray (Dunwiddie et al., 1980; Gysling & Wang, 1983; Johnson & North, 1992; Lupica et al., 1992; Madison & Nicoll, 1988; Matsui & Williams, 2011; K. Pang & Rose, 1989; Vaughan & Christie, 1997; Zieglgänsberger et al., 1979). In support, DAMGO is reported to reduce inhibitory synaptic transmission in insula cortex and ventrolateral and medial subregions of OFC (Lau et al., 2020; Qu et al., 2015; Yokota et al., 2016). An alternative possibility might be that a U-shaped polarization curve characterizes hedonic enhancement mechanisms within cortical hotspots, similar to reports that both local inhibitory neuronal manipulations (e.g., GABA agonist microinjections; glutamate antagonist microinjections) (Baumgartner et al., 2020; Carlezon & Thomas, 2009; Cheer et al., 2005; Krause et al., 2010;

Meredith et al., 2008; Reynolds & Berridge, 2001, 2002, 2008; Richard et al., 2013; Richard & Berridge, 2011b; Roitman et al., 2008; Taha & Fields, 2006) and local excitatory neuronal manipulations (e.g. optogenetic excitation; electrical stimulation) nucleus accumbens shell similarly increase appetitive motivation for rewards (Cole et al., 2018; Lobo et al., 2010; Mogenson et al., 1979; Phillips, 1984; Rolls, 1971; Soares-Cunha et al., 2016; Van Ree & Otte, 1980). Clearly, future research is needed to solve this puzzle.

Recruitment of Distant Hedonic Circuitry

Optogenetic stimulation in the rostromedial OFC hotspot, which enhanced 'liking' recruited neurobiological activation of Fos-expressing neurons in the caudal insula hedonic hotspot, as well as increasing Fos expression in subcortical hedonic hotspots including the NAc rostrodorsal medial shell and the caudolateral ventral pallidum (Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005). Similarly, optogenetic stimulation of the caudal insula hotspot recruited distant Fos activation in the rostromedial OFC hotspot and in the NAc rostrodorsal medial shell hotspot. The observation that stimulation of one hedonic hotspot recruits distant neural activation in multiple other hedonic hotspots is consistent with previous pharmacological studies (Castro & Berridge, 2017; K. S. Smith & Berridge, 2007), and supports the hypothesis that the mechanism underlying 'liking' enhancements may involve recruiting other hotspots into unanimous co-activation, as an entire integrated hedonic network. Although subcortical hedonic hotspots may not be directly connected anatomically (Thompson & Swanson, 2010; Zahm et al., 2013), cortical and subcortical hedonic hotspots do appear to be functionally interact, likely via intermediary sites, and to activate together as a cooperative circuit in enhancing 'liking' reactions.

Do hedonic hotspots exist in humans? We define hedonic hotspots here as causal entities, or special subregions able to amplify 'liking' reactions to the hedonic impact of a pleasant stimulus in response to local neurobiological stimulations. Whether humans possess equivalent hedonic hotspots remains an open question, given that it is not ethical to employ similar brain manipulations in people. It is not logically necessary that sites with special causal functions should also encode higher endogenous neural activations when reacting to pleasant stimuli, but human neuroimaging evidence may still be of interest. A recent meta-analysis and fMRI study of encoding of human pleasure ratings for beverages or humorous cartoons concluded "The spatial layout of the pleasure signature is consistent with… observations of hedonic hotspots identified in rodent studies" as well as being correlated with opioid binding (Kragel et al., 2023). Many other fMRI neuroimaging studies, as well as electrophysiological studies of nonhuman primates have implicated OFC and insula sites more generally in various aspects of reward (Berridge & Kringelbach, 2015; de Araujo et al., 2003, 2006; Hosokawa et al., 2007; Kringelbach et al., 2003; Lamm et al., 2015; Rolls, Kringelbach, et al., 2003; Small et al., 2001; Tremblay & Schultz, 1999). For example, mid-anterior OFC activity is reported to track subjective ratings of taste, odor, or tactile pleasantness, including changes in subjective taste pleasure ratings induced by sensory-specific satiety (de Araujo et al., 2003; Kringelbach et al., 2003; Lamm et al., 2015; Rolls, Kringelbach, et al., 2003; Rolls, O'Doherty, et al., 2003). Similarly, insula is activated by pleasant food images (Simmons et al., 2013), and insula activation is reported to track decreases in chocolate taste pleasure ratings as people eat chocolate to satiety (Small et al., 2001).

Clinical Implications

A better scientific identification of brain hedonic mechanisms that amplify 'liking' reactions to pleasant events may be relevant to understand hedonic dysfunctions that may occur in depression and other affective disorders, and to efforts to improve clinical therapies (Devoto et al., 2018; Ferrario, 2017; Morales & Berridge, 2020; Nguyen et al., 2021; Olney et al., 2018; M. J. F. Robinson et al., 2016; T. E. Robinson & Berridge, 1993). Some individuals with major depression or schizophrenia are reported to have symptoms of anhedonia, or inability to experience pleasure, while others may have a more selective avolition or loss of motivational 'wanting' for life rewards even if hedonic reactivity remains intact (McCarthy et al., 2016; Thomsen et al., 2015; Treadway et al., 2012, 2015; Treadway & Zald, 2011, 2013; J. Wang et al., 2015; Whitton et al., 2015). A recent study in humans found that patients with major depressive disorder with anhedonia had blunted fMRI BOLD responses in OFC and insula during a monetary gain/ loss task, suggesting that in humans too, these regions may be important mediators of reward impact (Steinmann et al., 2022). Whether the cortical suppressive 'hedonic coldstrip' described here may be relevant to reduced pleasure in anhedonia, or whether promoting activity in hedonic hotspots could reverse hedonic deficits remain open questions, but such possibilities would be in line with RDoC criteria, which breaks down psychological functioning into a subset of domains with underlying neurobiological determinants (Insel et al., 2010; National Institutes of Mental Health, 2018; Sanislow et al., 2010).

Our results show that optogenetic stimulation of neurons within previously identified opioid/orexin hedonic hotspots of orbitofrontal cortex and insula can enhance hedonic impact, similarly to previous reports of pharmacologically induced enhancements in the same cortical hotspots. Hedonic enhancements were expressed as increases in affective orofacial 'liking' reactions elicited by sweet taste of sucrose. Here we mapped the boundaries of a 11 mm^3 hotspot

in rostromedial OFC, and a 6 mm^3hotspot in far-caudal insula. By contrast, throughout an intervening suppressive coldstrip that stretched from caudal OFC to anterior-mid insula, optogenetic stimulations oppositely reduced ‘liking’ reactions. Finally, we find that sites able to support incentive motivation or ‘wanting’ to self-stimulate laser excitations, extended both throughout the two hedonic hotspots and beyond into much of the suppressive hedonic coldstrip, indicating a partial dissociation between cortical mechanisms of ‘wanting’ versus ‘liking’. Understanding how hedonic hotspot mechanisms generate ‘liking’ enhancements for rewards may lead to improved understanding of hedonic dysfunctions in various affective disorders.

2.6 Figures

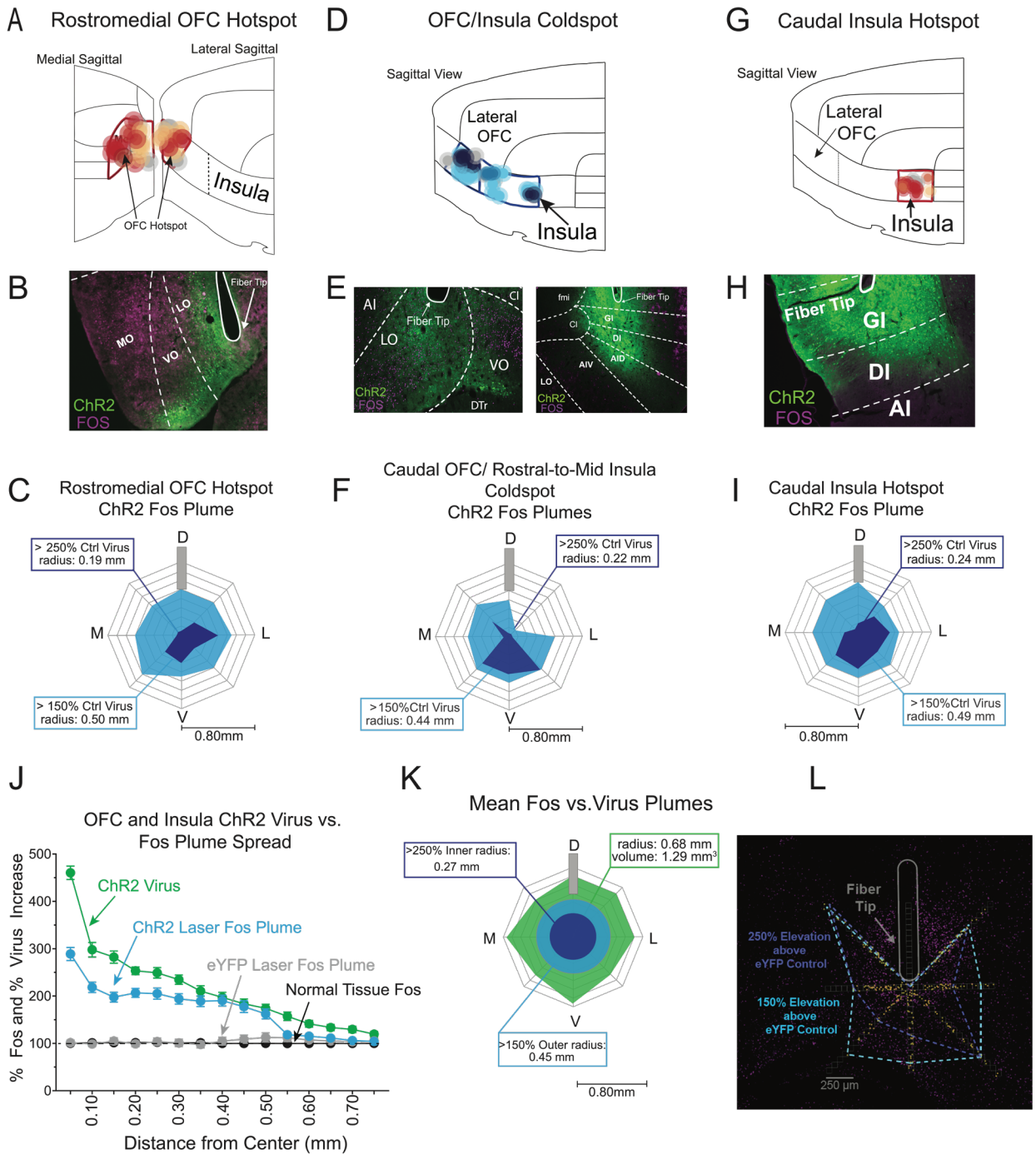

A
Rostromedial OFC Hotspot
Medial Sagittal
Lateral Sagittal
Insula
OFC Hotspot
D
OFC/Insula Coldspot
Sagittal View
Lateral
OFC
Insula
G
Caudal Insula Hotspot
Sagittal View
Lateral
OFC
Insula
B
Fiber Tip
LO
MO
VO
ChR2
FOS
E
AI
Fiber Tip
LO
VO
DTr
Cl
ChR2
FOS
fmi
Fiber Tip
Cl
GI
DI
AID
AIV
LO
ChR2
FOS
H
Fiber Tip
GI
DI
AI
ChR2
FOS
C
Rostromedial OFC Hotspot
ChR2 Fos Plume
> 250% Ctrl Virus
radius: 0.19 mm
> 150% Ctrl Virus
radius: 0.50 mm
D
M
L
V
0.80mm
F
Caudal OFC/ Rostral-to-Mid Insula
Coldspot
ChR2 Fos Plumes
>250% Ctrl Virus
radius: 0.22 mm
>150%Ctrl Virus
radius: 0.44 mm
D
M
L
V
0.80mm
I
Caudal Insula Hotspot
ChR2 Fos Plume
>250% Ctrl Virus
radius: 0.24 mm
>150%Ctrl Virus
radius: 0.49 mm
D
M
L
V
0.80mm
J
OFC and Insula ChR2 Virus vs.
Fos Plume Spread
% Fos and % Virus Increase
500
400
300
200
100
0
ChR2 Virus
ChR2 Laser Fos Plume
eYFP Laser Fos Plume
Normal Tissue Fos
0.10
0.20
0.30
0.40
0.50
0.60
0.70
Distance from Center (mm)
K
Mean Fos vs.Virus Plumes
>250% Inner radius:
0.27 mm
radius: 0.68 mm
volume: 1.29 mm3
>150% Outer radius:
0.45 mm
D
M
L
V
0.80mm
L
Fiber
Tip
250% Elevation
above
eYFP Control
150% Elevation
above
eYFP Control
250 µm

Figure 1. Cortical hotspot ChR2 virus and Fos plumes.

(A) Rostromedial OFC sites of hedonic enhancement (medial and lateral sagittal views). (B) Rostromedial OFC photomicrograph (10x magnification) shows green channelrhodopsin (ChR2) virus infection (AAV5-ChR2-eYFP) and magenta Fos protein. (C) Average ChR2 laser-induced Fos plume in rostromedial OFC (>250% above eYFP: light solid blue, > 150% above eYFP rats: dark solid blue (D) Caudolateral OFC and rostral insula sites of hedonic suppression in OFC/insula coldstrip (lateral sagittal view). (E) caudolateral OFC and rostral insula photomicrographs showing green channelrhodopsin (ChR2) virus infection (AAV5-ChR2-eYFP) and magenta Fos protein. (F) Average ChR2 laser-induced Fos plume in caudolateral OFC and anterior insula- (>250% above eYFP: light solid blue, > 150% above eYFP rats: dark solid blue. (G) Caudal insula sites of hedonic enhancement (lateral sagittal view). (H) caudal insula photomicrograph showing green channelrhodopsin (ChR2) virus infection (AAV5-ChR2-eYFP) and magenta Fos protein. (I) Average ChR2 laser-induced Fos plume in caudal insula (>250% above eYFP: light solid blue, > 150% above eYFP rats: dark solid blue. (J) Graph shows how quantitative increases in virus and in Fos protein decline as a function of distance from the fiber tip (Combined rostromedial OFC virus, caudolateral OFC and anterior insula virus, and caudal insula virus, n =26, Combined rostromedial OFC laser Fos, caudal OFC/ anterior insula laser Fos, and caudal insula laser Fos, n =21; Ctrl eYFP Fos, n = 19; Naïve tissue Fos, n = 6). All data represented as mean and standard error (SEM). (K) shows average ChR2 virus spread away from fiber optic tip relative to average size of Fos plume. (L) Fos expression showing example local plume surrounding fiber in the OFC hotspot D: dorsal, M: medial, V: ventral, L: lateral

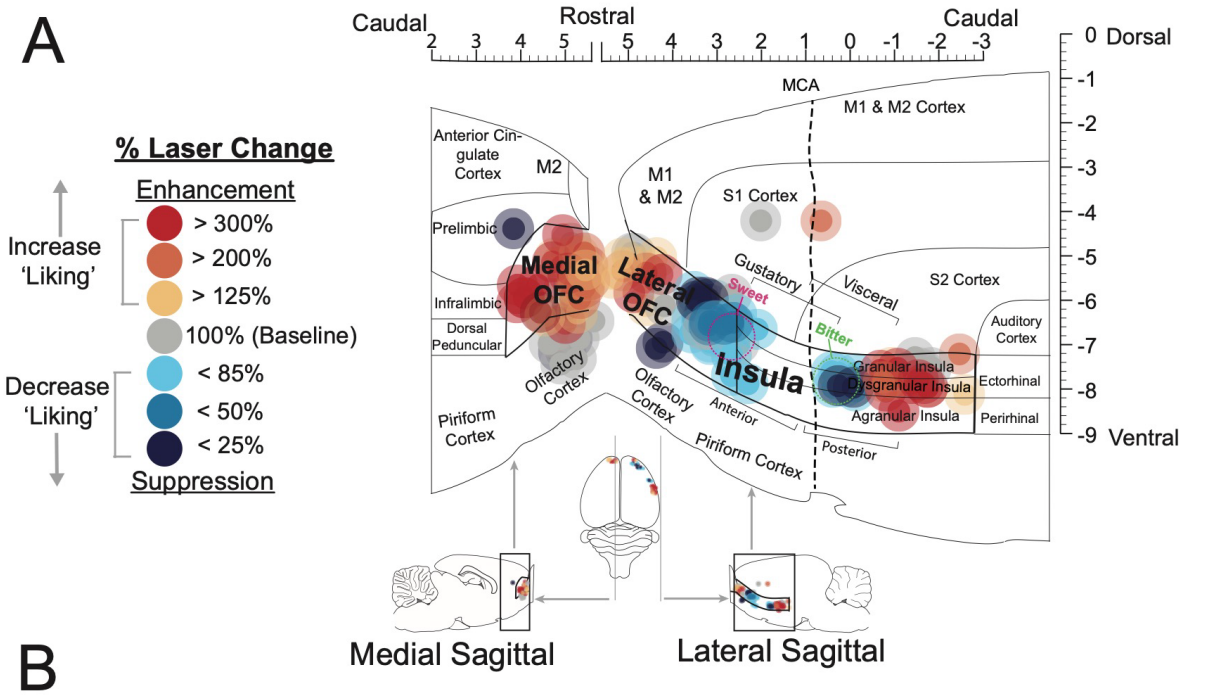

A
% Laser Change
Enhancement
Increase 'Liking'
> 300%
> 200%
> 125%
100% (Baseline)
Decrease 'Liking'
< 85%
< 50%
< 25%
Suppression
Caudal
Rostral
Caudal
2 3 4 5 5 4 3 2 1 0 -1 -2 -3
0 Dorsal
-1
-2
-3
-4
-5
-6
-7
-8
-9 Ventral
MCA
M1 & M2 Cortex
Anterior Cingulate Cortex
M2
M1 & M2
S1 Cortex
Prelimbic
Medial OFC
Lateral OFC
Gustatory
Sweet
Visceral
S2 Cortex
Bitter
Infralimbic
Dorsal Peduncular
Auditory Cortex
Granular Insula
Dysgranular Insula
Agranular Insula
Ectorhinal
Perirhinal
Olfactory Cortex
Insula
Piriform Cortex
Anterior
Posterior
Medial Sagittal
Lateral Sagittal
B
OFC Hotspot 'Liking' Enhancement
%Δ Laser / Non Laser
800
400
300
200
100
****
eYFP
ChR2
Sagittal
Insula
Medial OFC
Lateral OFC
OFC/ Insula Coldspot 'Liking' Suppression
200
150
100
50
0
****
Lateral Sagittal
Lateral OFC
Insula
Insula Hotspot 'Liking' Enhancement
1500
1000
600
500
400
300
200
100
**
Lateral Sagittal
Lateral OFC
Insula

Figure 2. Cortical sites in orbitofrontal cortex and insula that support hedonic enhancement or suppression.

(A) Localization of hedonic function map shows how optogenetic ChR2 stimulation altered the hedonic impact of sucrose at each individual's cortical site. Colors reveal hedonic enhancement/suppression effects of ChR2 laser stimulation at each cortical site, measured as laser-induced changes in hedonic taste reactivity (positive 'liking' reactions) elicited by intraoral sucrose infusions. Each symbol placement indicates an individual rat's site size of symbol reflects average size of Fos plumes). Color of symbol represents the within-subject behavioral change in hedonic reactions induced by ChR2 laser stimulation reflected as percent change from no laser control conditions measured in the same rats ('Liking' enhancements: red-yellow; 'Liking' suppression: Blue). (B) Laser ChR2 stimulations differentially alter hedonic 'liking' reactions depending on the anatomical subregion of OFC and insula. At rostromedial OFC hotspot and caudal insula hotspot sites, laser stimulation enhanced hedonic 'liking' reactions 200% - 300% in ChR2 rats, but not in eYFP controls (rostromedial OFC: $U = 18.00$, $^{****}p < 0.0001$; caudal insula: $U = 4.00$, $^{**}p < 0.01$. In the intervening coldstrip, spanning caudolateral OFC to mid insula, laser ChR2 stimulations oppositely suppressed sucrose 'liking' reactions to approximately 50% in ChR2 rats, but not in eYFP controls (OFC/insula coldstrip ($U = 12.00$, $^{****}p < 0.0001$)). Data presented as means and standard error (SEM). Anatomical abbreviations: M1: primary motor cortex, M2: secondary motor cortex, S1: primary somatosensory cortex. S2: secondary somatosensory cortex. Gustatory insula zones adapted from (Cechetto & Saper, 1987), visceral insula functional zone adapted from (Peng et al., 2015).

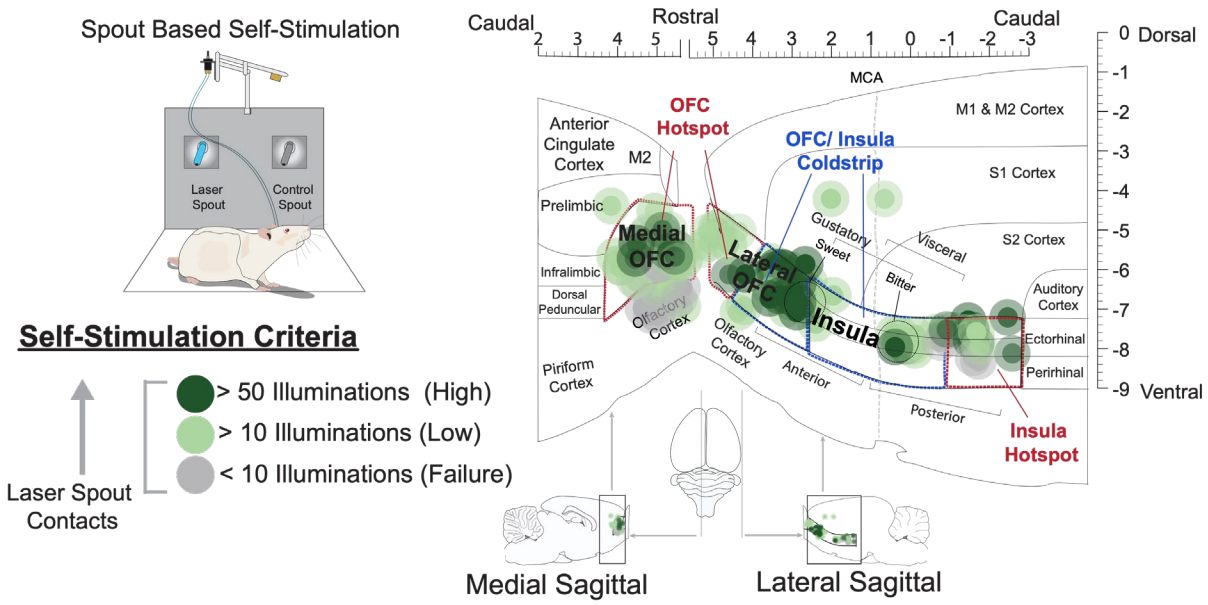


Figure 3. Cortical sites in orbitofrontal cortex and insula that support incentive motivation for reward: laser self-stimulation on spout-touch task.

Optogenetic ChR2 stimulation at various cortical sites, both in and outside of hedonic hotspots, support laser self-stimulation. Functional maps show instrumental performance to earn ChR2 laser stimulations at each cortical site on a spout-touch laser self-stimulation task (map based on 40 Hz, 1-s pulse data). Each symbol placement indicates an individual rat's channelrhodopsin expression (size of symbol reflects size of Fos plumes). Color of symbols represents the level of self-stimulation criteria met by each rat (high self-stimulation (>50 illuminations earned): dark green; low self-stimulation (10 to 49 illuminations earned: light green; Failures to self-stimulate (<10 illuminations earned): grey). For comparison purposes to hedonic 'liking' effects, red and blue outlines indicate the anatomical boundaries of the cortical hedonic hotspots and coldstrip mapped based on taste reactivity results in the same rats. Anatomical abbreviations: M1: primary motor cortex, M2: secondary motor cortex, S1: primary somatosensory cortex. S2: secondary somatosensory cortex. Gustatory insula zones adapted from (122), visceral insula functional zone adapted from (Cechetto & Saper, 1987) and sweet/bitter coding regions adapted from (Peng et al., 2015).

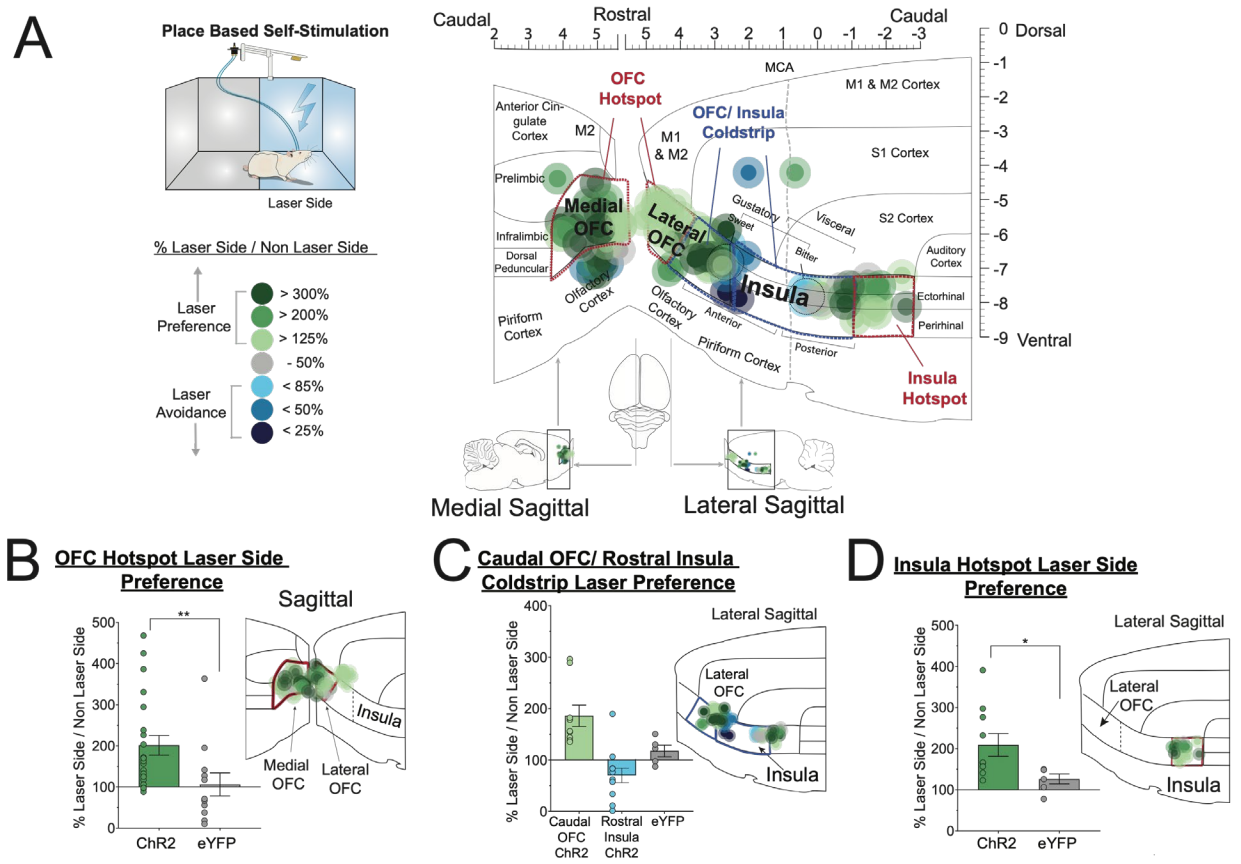


Figure 4. Cortical sites in orbitofrontal cortex and insula that support laser self-stimulation in place-based task.

Caudal OFC and rostral to mid insula sites did not reliably promote place-based self-stimulation, and at some sites laser stimulation was avoided. (A) Functional maps show preferences for laser-paired side (green) or avoidance of laser-paired side (blue) during the place-based self-stimulation task. The color reflects the percent preference or avoidance for the laser-delivering vs. non-laser- side in the same rats. (B, D) Bar graphs show quantified % laser side preference in rostromedial OFC and caudal insula ChR2 rats who showed evidence of self-stimulation relative to eYFP control rats (n = 22 rostromedial OFC ChR2, n = 12 rostromedial OFC eYFP; n =10 caudal insula ChR2; n = 6 caudal insula eYFP). (C) No reliable place-based self-stimulation was observed in ChR2 rats with caudal OFC or rostral insula sites (n = 9 caudal OFC ChR2, n = 12 rostral OFC ChR2; n = 5 eYFP). All data presented as means and SEM, *p< 0.05; **p< 0.01.

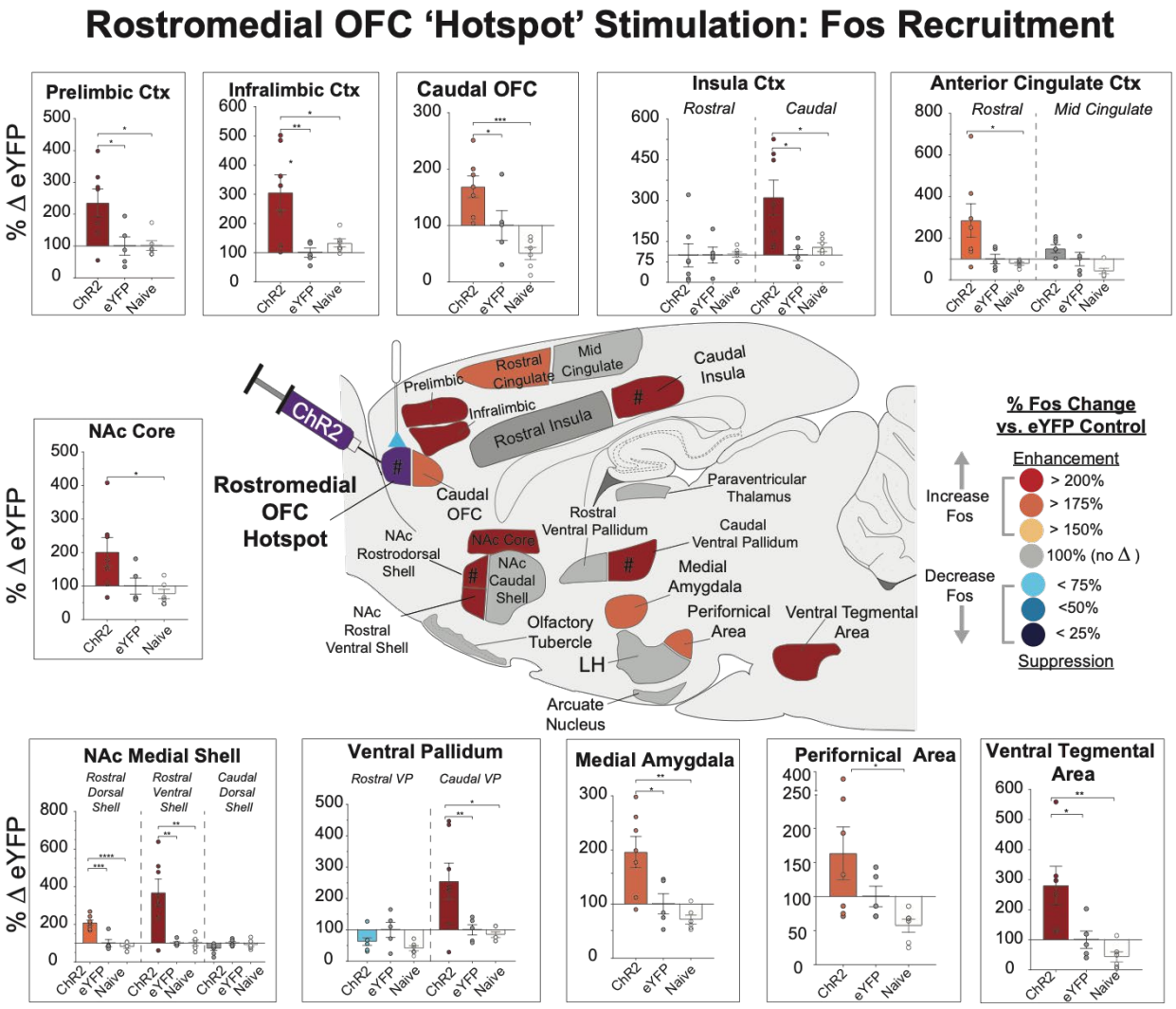


Figure 5. Distant Fos recruitment induced by rostromedial OFC hotspot.

OFC hotspot stimulation recruits limbic brain circuitry for hedonic enhancement. Brain map shows elevated Fos expression in recruited mesocorticolimbic structures after laser stimulation in rostromedial OFC hotspot of ChR2 rats (N = 7; colors denote % Fos elevation compared to illuminated eYFP control rats (N = 5), and to naïve control baseline rats (N = 6). Significant Fos elevation was recruited in other hedonic hotspots, including far caudal insula cortex, nucleus accumbens rostrodorsal medial shell, and caudal ventral pallidum. Fos elevation was also recruited in other limbic cortical regions, such as prelimbic cortex, infralimbic cortex, caudal orbitofrontal cortex, and rostral anterior cingulate cortex. Fos elevation was also recruited in subcortical limbic structures, such as ventral tegmental area, nucleus accumbens core, nucleus accumbens rostroventral medial shell, medial amygdala, and perifornical area of the lateral hypothalamus. Also see supplementary table 1. Bar graph data shown as mean and SEM of % Fos enhancements in that structure relative to eYFP controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. # symbol

denotes sites of previously identified hedonic hotspots (Castro & Berridge, 2017; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005).

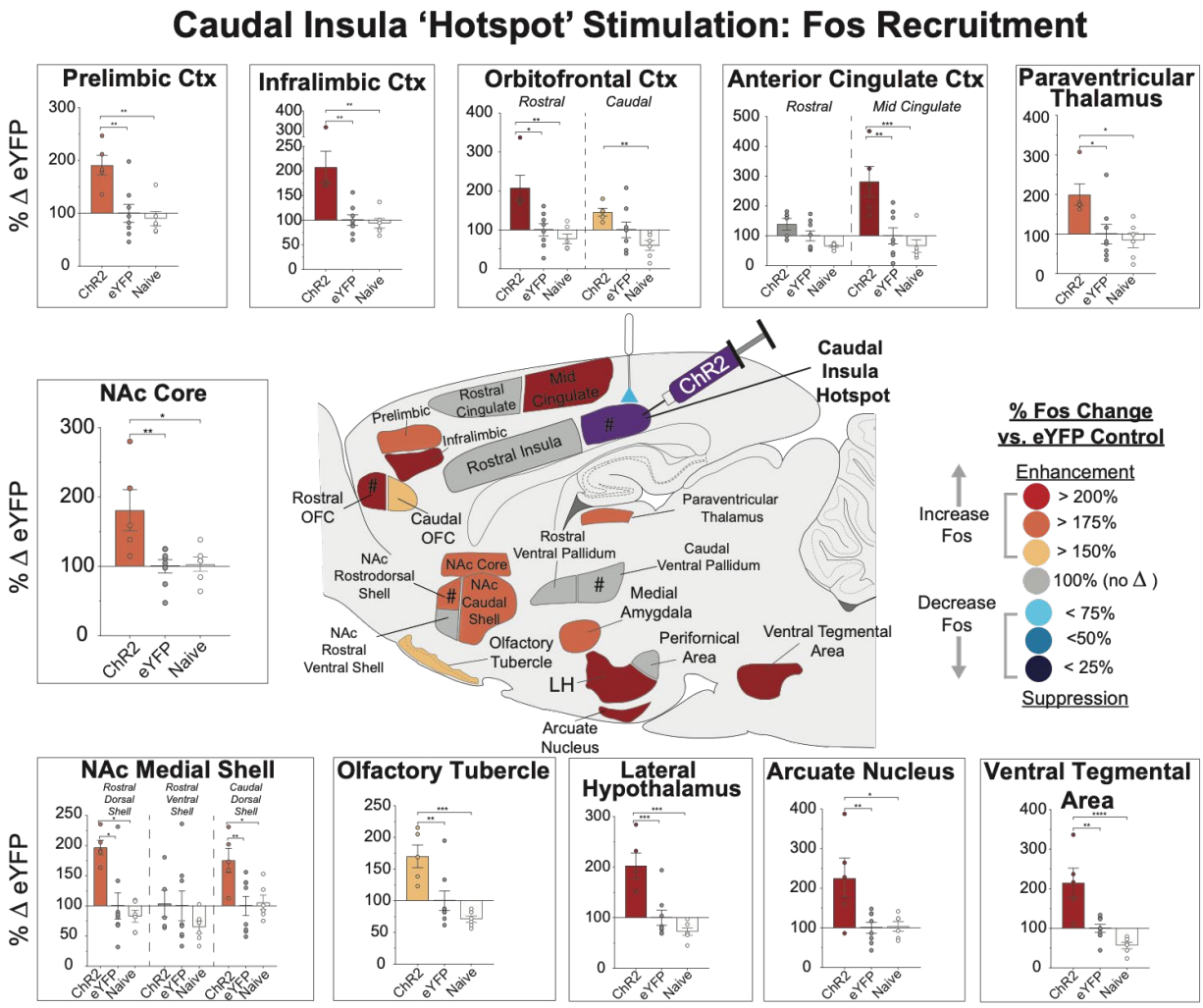


Figure 6. Distant fos recruitment induced by caudal insula hotspot.

Optogenetic stimulation in caudal Insula 'Hotspot' recruited limbic brain circuitry for hedonic enhancement. Brain map shows elevated Fos expression in recruited mesocorticolimbic structures after laser ChR2 stimulation in far-caudal insula hotspot (N = 5; colors denote % Fos elevation compared to eYFP control rats (N = 8), and to naïve control baseline rats (N = 6). Significant Fos elevation was recruited in other hedonic hotspots, including rostromedial orbitofrontal cortex, and nucleus accumbens rostrodorsal medial shell. Fos elevation was also recruited in other limbic cortical regions, such as prelimbic cortex, infralimbic cortex, caudolateral orbitofrontal cortex, and mid anterior cingulate cortex. Fos elevation was also recruited in other subcortical limbic structures, including ventral tegmental area, nucleus accumbens core, nucleus accumbens rostrocaudal medial shell, olfactory tubercle, paraventricular thalamus, lateral hypothalamus, and arcuate nucleus of ventromedial hypothalamus. Also see supplementary table 2. Bar graph data shown as mean and SEM of % Fos enhancements in that structure relative to

eYFP controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. # symbol denotes sites of previously identified hedonic hotspots (Castro & Berridge, 2017; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005).

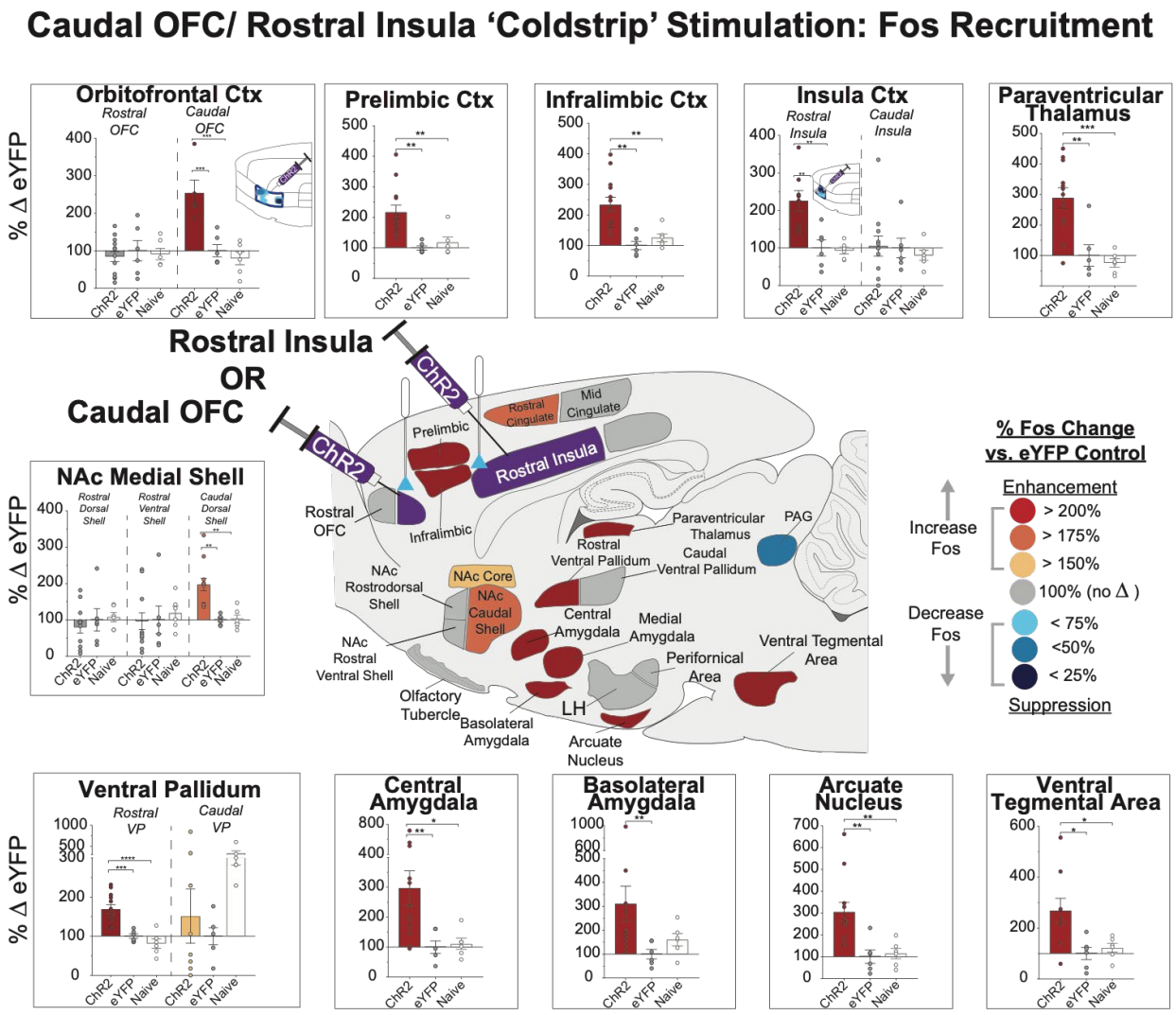


Figure 7. Caudal OFC/ rostral insula 'coldstrip' distant Fos recruitment.

Brain map shows elevated Fos expression recruited in mesocorticolimbic structures after laser ChR2 stimulation in OFC/insula coldstrip ChR2 rats (N = 7 caudal OFC, N = 5 rostral insula; colors denote %Fos elevation compared to eYFP control rats (N = 6), and to naïve control baseline rats (N = 6). Cortical regions included caudal orbitofrontal cortex (Fos counts based on rostral insula ChR2 rats only), prelimbic cortex, infralimbic cortex, rostral insula cortex (Fos counts based on caudal OFC ChR2 rats only). Subcortical structures included nucleus accumbens core, caudodorsal nucleus accumbens medial shell, rostral VP, paraventricular thalamus, central amygdala, basolateral amygdala, arcuate nucleus, and ventral tegmental area. Also see supplementary table 3. Bar graph data shown as

mean and SEM of % Fos Enhancements in that structure relative to eYFP controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

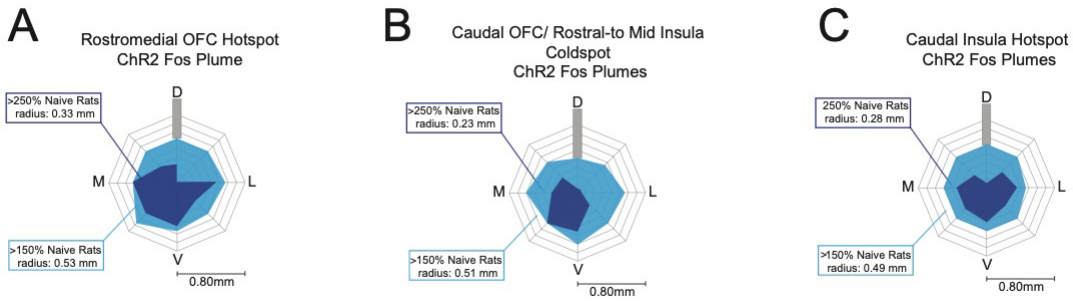


Figure 8. Cortical hotspots local fos plumes.

Local average Fos plumes around fiber tip measured after ChR2 laser stimulation at sites in in **A)** rostromedial OFC hotspot, **B)** Caudal OFC and rostral to mid insula suppressive coldstrip, and **C)** Far-caudal insula hedonic hotspot (Colors: >250% above naïve controls: light solid blue, > 150% above naïve control rats: dark solid blue).

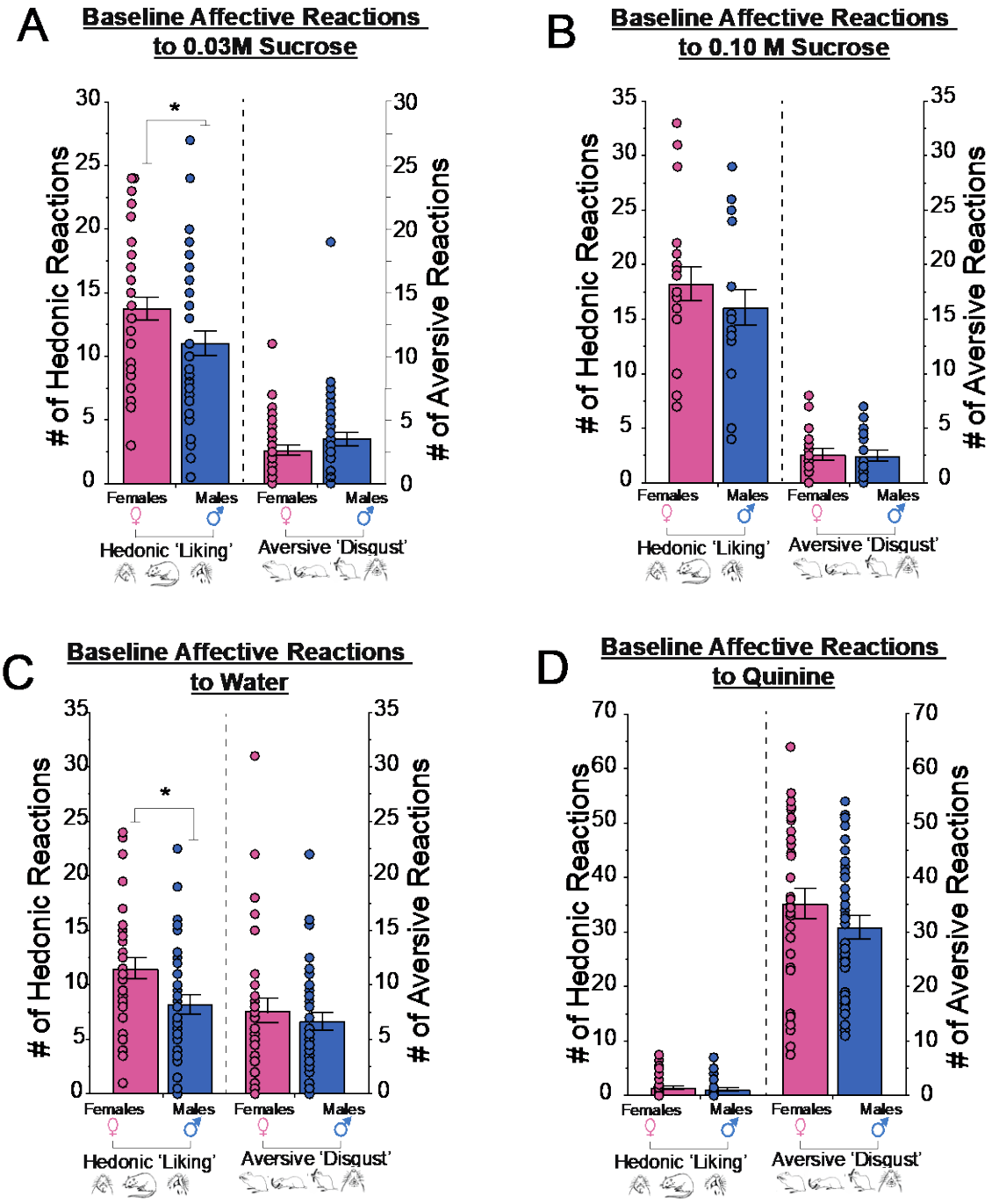

A
Baseline Affective Reactions to 0.03M Sucrose
# of Hedonic Reactions
# of Aversive Reactions
*
Females
Males
Females
Males
Hedonic 'Liking'
Aversive 'Disgust'
B
Baseline Affective Reactions to 0.10 M Sucrose
# of Hedonic Reactions
# of Aversive Reactions
Females
Males
Females
Males
Hedonic 'Liking'
Aversive 'Disgust'
C
Baseline Affective Reactions to Water
# of Hedonic Reactions
# of Aversive Reactions
*
Females
Males
Females
Males
Hedonic 'Liking'
Aversive 'Disgust'
D
Baseline Affective Reactions to Quinine
# of Hedonic Reactions
# of Aversive Reactions
Females
Males
Females
Males
Hedonic 'Liking'
Aversive 'Disgust'

Figure 9. Sex Differences in taste reactivity at baseline.

Sex differences and similarities in baseline affective taste reactivity elicited in male versus female rats in control condition without laser stimulation. **A)** Females emitted higher positive 'liking' reactions to a dilute 0.03M sucrose solution (n = 40 females, n = 41 males). **B)** No sex difference in positive hedonic reactions elicited by more concentrated 0.1M sucrose;(n = 20 females, n = 20 males). **C)** Females emitted higher positive 'liking' reactions to tap water infusions *(*n =33 females, n = 35 males). **D)** No sex differences in negative 'disgust' reactions elicited by bitter quinine (n = 33 females, n = 35 males). All data presented as mean and SEM. *$p < 0.05$.

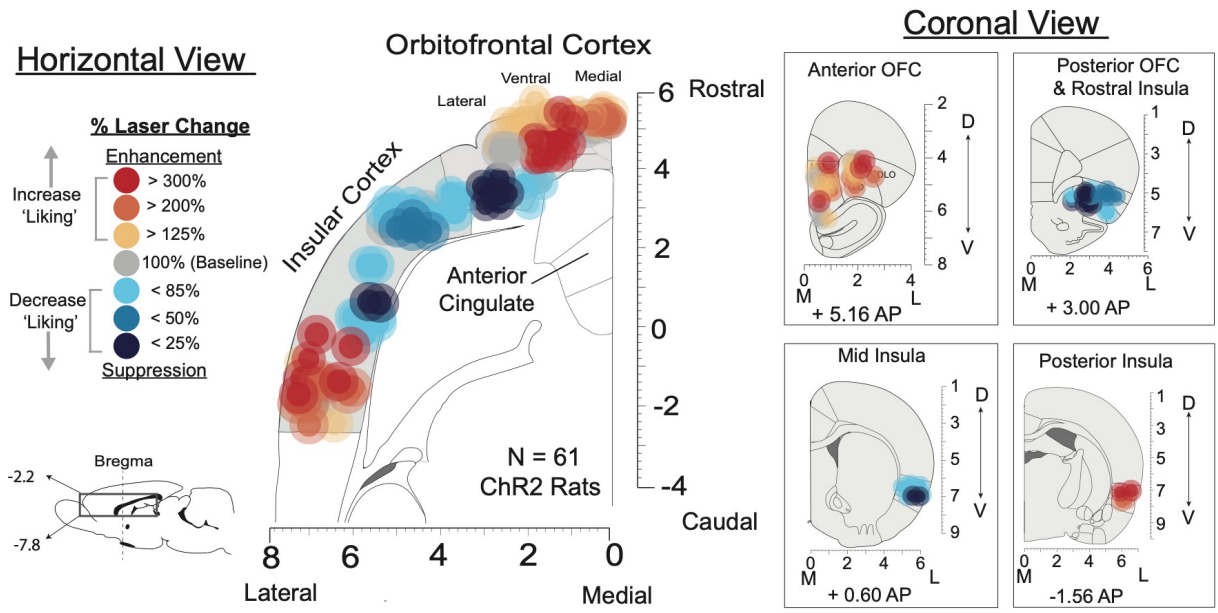


Figure 10. Coronal and horizontal hedonic localization of function maps.

Horizontal view (left) and coronal views (right) of hedonic hotspot localization of function. Each site shows laser ChR2 stimulation effects on positive 'liking' taste reactivity to sucrose. Each symbol placement reflects an individual rat. Size of symbols based on average size of Fos plumes. Color of each symbol represents the individual's within-subject change in hedonic reactions induced by ChR2 laser stimulation reflected as percent change from baseline hedonic reactions in no laser control condition in the same rats ('Liking' enhancements: red-yellow; 'Liking' suppression: Blue).

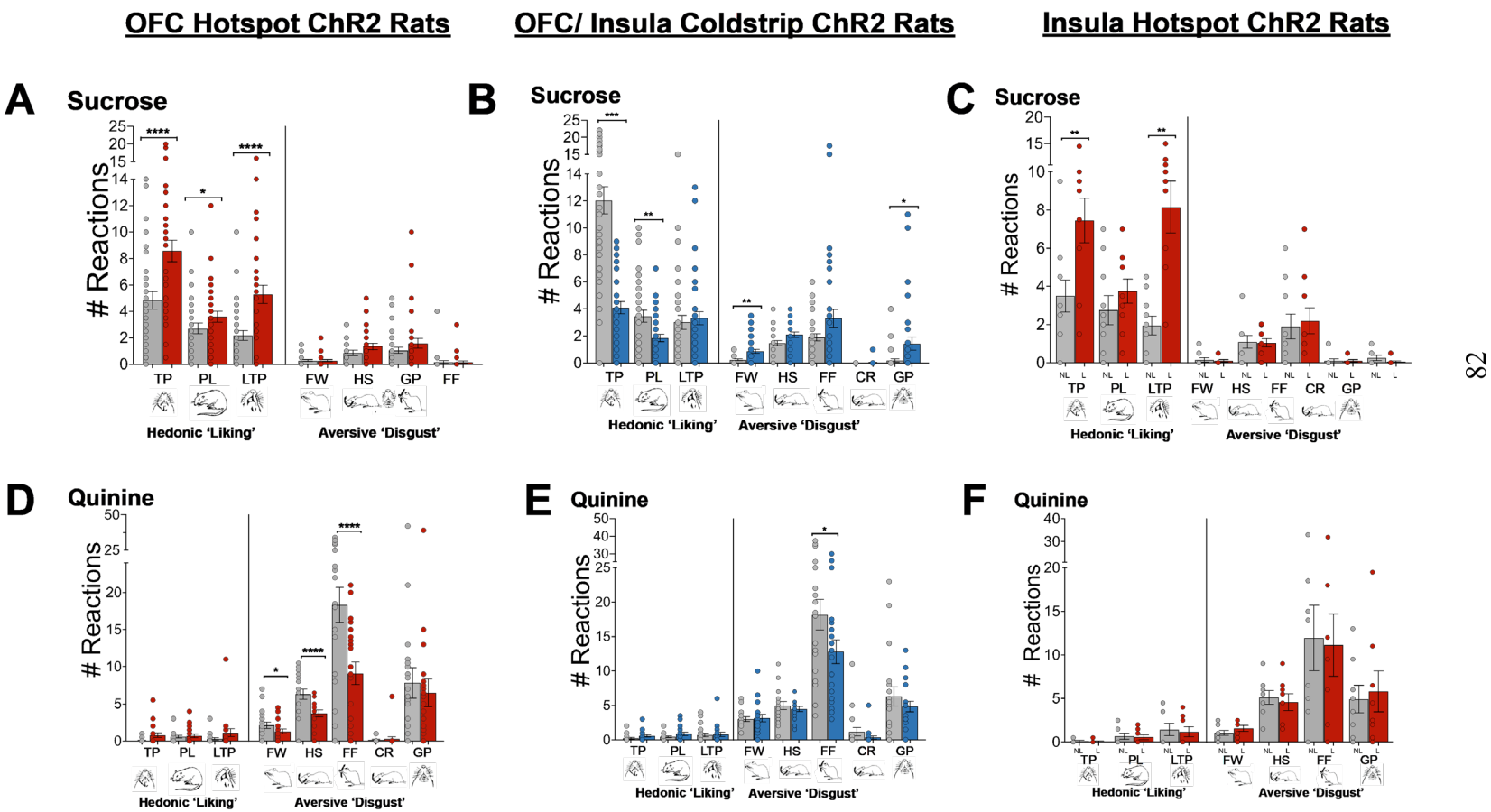

OFC Hotspot ChR2 Rats
OFC/ Insula Coldstrip ChR2 Rats
Insula Hotspot ChR2 Rats
A
Sucrose
B
Sucrose
C
Sucrose
D
Quinine
E
Quinine
F
Quinine
Reactions
TP
PL
LTP
FW
HS
FF
CR
GP
NL
L
Hedonic 'Liking'
Aversive 'Disgust'

Figure 11. Microstructure of taste reactivity components.

Positive hedonic taste reactivity components include: paw licks (PL), lateral tongue protrusions (LTP), and rhythmic tongue protrusions (TP). Negative 'disgust' components are: gapes (GP), face washes (FW), head shakes (HS), forelimb flails (FF), and chin rubs (CR). Relative neutral components are: rhythmic mouth movements and passive dripping (not shown). Scoring: each occurrence was counted for LTP, GP, HS, FF, and CR. TP was scored in 2-s bins, and PL was scored in 5-s bins. Bar graphs show absolute scores as mean and SEM for no laser baseline and ChR2 laser stimulation trials in the same ChR2 rats (grey bars: no laser trials; red and blue bars: laser trials). **A)** Laser ChR2 stimulation (40 Hz) in rostromedial OFC hotspot sites significantly increased TP, PL, and LTP hedonic reactions to 0.03M sucrose. **B)** Laser ChR2 stimulation in caudal OFC - rostral insula coldstrip sites oppositely suppressed TP and PL hedonic 'liking' reactions to sucrose and increased aversive 'disgust' FW and GPs. **C)** Laser ChR2 stimulation in far-caudal insula hotspot increased both TP and LTP positive 'liking' reactions elicited by 0.03M sucrose. **D)** Laser ChR2 stimulation in rostromedial OFC hotspot decreased aversive 'disgust' reactions of FW, HS, and FF elicited by bitter quinine. **E)** Laser ChR2 stimulation in caudal OFC - rostral insula coldstrip sites decrease FF 'disgust' reactions to bitter quinine **F)** Laser ChR2 stimulation in far caudal insula hotspot sites do not alter affective reactions to quinine. All data presented as Means ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

OFC Hotspot

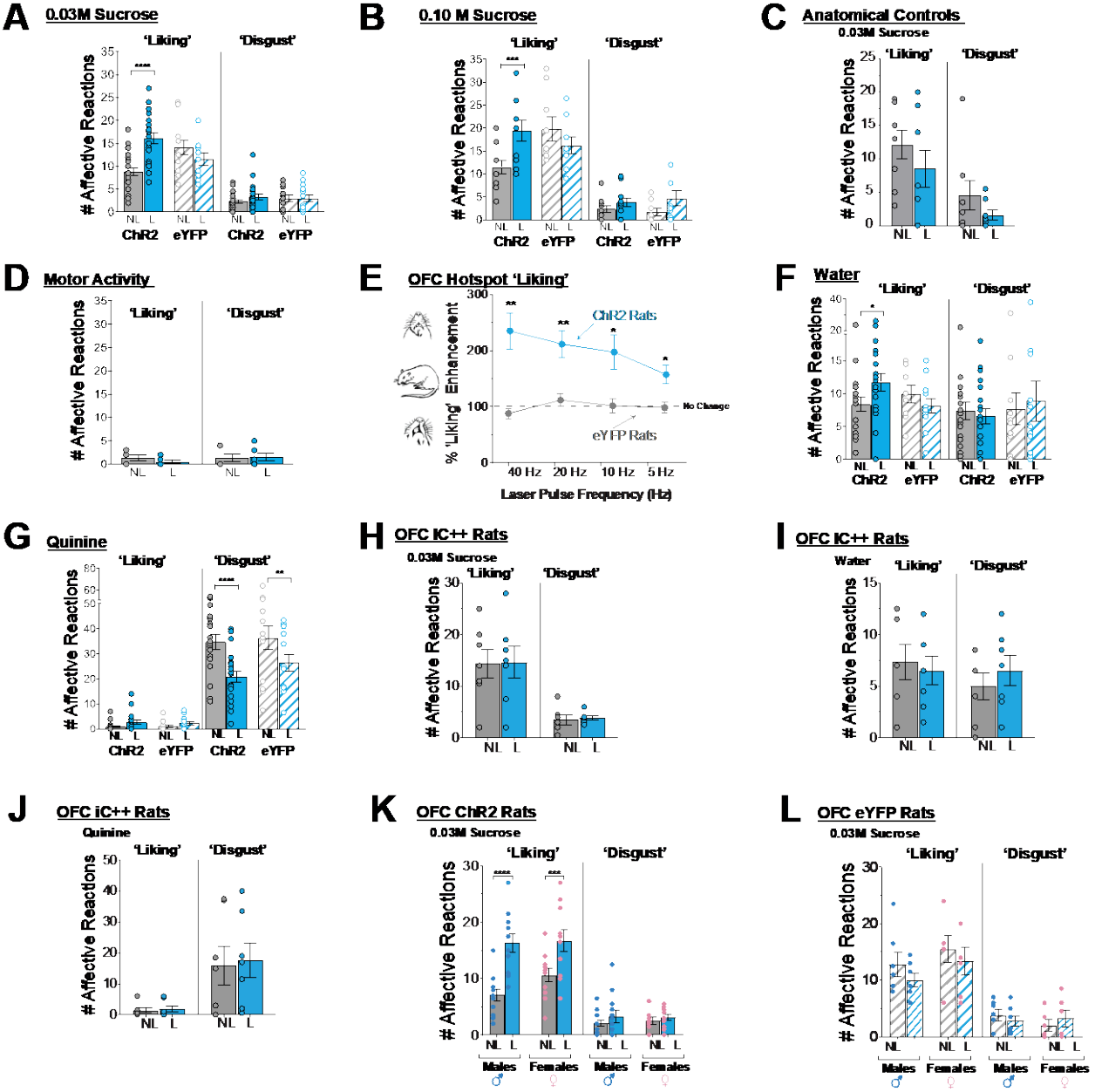
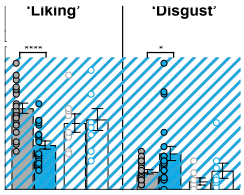

A
0.03M Sucrose
'Liking'
'Disgust'
Affective Reactions
NL L
ChR2 eYFP ChR2 eYFP
B
0.10 M Sucrose
C
Anatomical Controls
0.03M Sucrose
D
Motor Activity
E
OFC Hotspot 'Liking'
% 'Liking' Enhancement
ChR2 Rats
No Change
eYFP Rats
40 Hz 20 Hz 10 Hz 5 Hz
Laser Pulse Frequency (Hz)
F
Water
G
Quinine
H
OFC IC++ Rats
I
OFC IC++ Rats
J
OFC iC++ Rats
K
OFC ChR2 Rats
Males Females Males Females
L
OFC eYFP Rats

Figure 12. Rostromedial OFC hotspot taste reactivity.

Raw affective components counts elicited by various tastants following rostromedial OFC hotspot activations. **A)** Optogenetic laser activations increase hedonic 'liking' reactions to 0.03M sucrose in ChR2 rats, but not eYFP controls. **B)** Laser activations increase hedonic 'liking' reactions to 0.1M sucrose in ChR2 rats, but not eYFP controls. **C)** Laser ChR2 activations in anatomical control rats (prelimbic cortex and olfactory cortex) fail to increase affective reactions to 0.03M sucrose. **D)** No oromotor reactions observed from OFC hotspot ChR2 laser activations in the absence of taste infusions. **E)** Multiple laser frequencies (5, 10, 20, 40 Hz; all 1 mW) increase positive 'liking' reactions to 0.03M sucrose in chR2 rats. Data shown as within-subjects percent change from no laser baseline conditions. **F)** Rostromedial OFC ChR2 activations increase hedonic 'liking 'reactions to tap water. **G)** Aversive 'disgust' reactions to quinine are reduced by rostromedial OFC laser stimulation in both rostromedial ChR2 and eYFP rats. **H-J)** Rostromedial OFC hotspot optogenetic iC++ inhibitions fail to alter affective reactions elicited by 0.03M sucrose, water, or quinine. **K)** Male and female rostromedial OFC hotspot ChR2 rats show similar laser-induced increases in positive 'liking' reactions to 0.03M sucrose **L**) No sex differences in affective expressions to 0.03M sucrose in eYFP control rats. All data shown as means ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



H ne

Figure 13. Caudal OFC and rostral-to-mid insula hedonic 'coldstrip' taste reactivity.

Raw affective component counts elicited by various tastants following optogenetic activations of neurons in the caudal OFC/ rostral-to-mid insula hedonic 'coldstrip'. (A) Optogenetic laser activations decrease hedonic 'liking' reactions and increase aversive 'disgust' reactions to 0.03M sucrose in ChR2 rats, but not in eYFP controls (B) Laser ChR2 activations suppress hedonic 'liking' reactions to 0.1M sucrose (C) Laser ChR2 activations similarly suppress hedonic 'liking' reactions to 0.03M sucrose. (D) No oromotor reactions observed from caudal OFC and rostral insula coldstrip activations in the absence of taste. (E) Multiple laser frequencies (5, 10, 20, 40 Hz; all 1 mW) suppress positive 'liking' reactions to 0.03M sucrose in ChR2 rats. Data shown as within-subjects percent change from no laser conditions. (F) Laser ChR2 activations in caudal OFC and rostral insula coldstrip sites decrease positive 'liking' reactions to water. (G) Laser ChR2 activations in caudal OFC and rostral insula coldstrip sites decrease aversive 'disgust' reactions to quinine. (H) Laser ChR2 activations in either caudal OFC segment or rostral insula segment of intervening coldstrip similarly suppress aversive 'disgust' reactions to quinine. All data shown as means ± SEM. *p<0.05, **p<0.01, ****p<0.0001

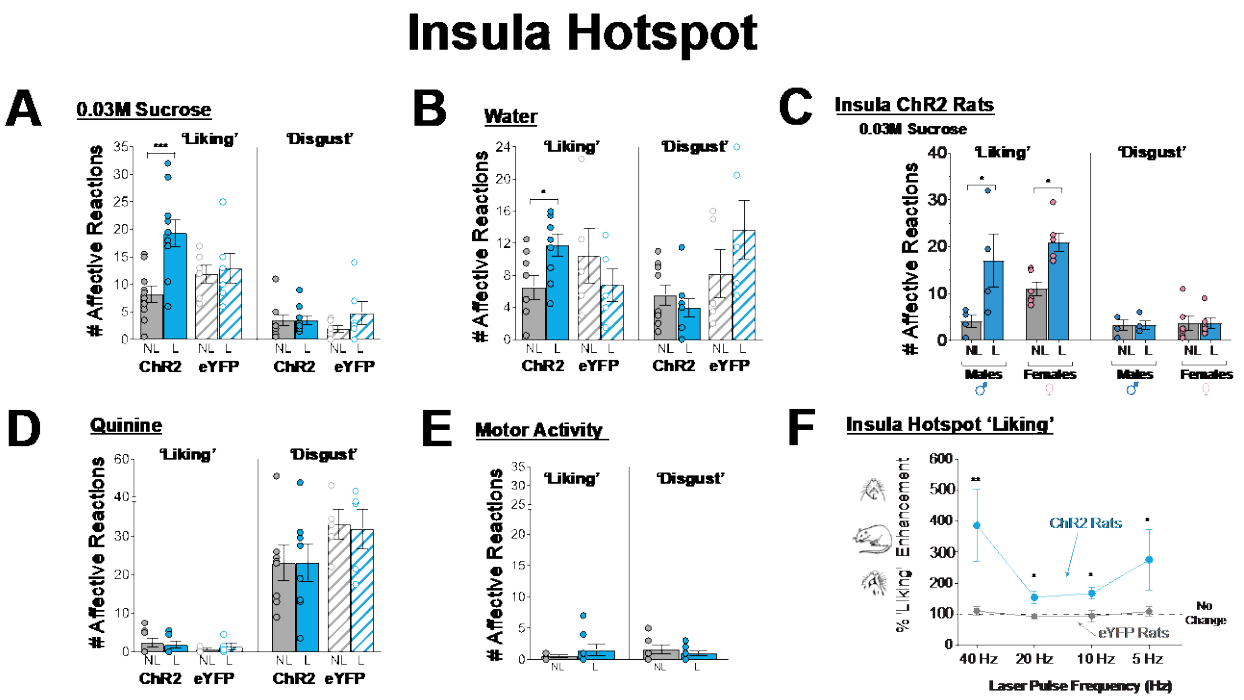


Figure 14. Caudal insula hedonic 'hotspot' taste reactivity.

Raw affective component counts elicited by various tastants following optogenetic activations in the far-caudal insula hedonic 'hotspot'. **A)** Optogenetic laser activations increase hedonic 'liking' reactions to 0.03M sucrose in ChR2 rats, but not eYFP controls. **B)** Caudal insula hotspot ChR2 laser activations increase positive 'liking' reactions to water. **C)** Male and female rats with sites in caudal insula hotspot show equal laser-induced ChR2 increases in hedonic reactions to sucrose D**)** No change in aversive 'disgust' reactions following caudal insula ChR2 activations **E)** No oromotor reactions observed during caudal insula ChR2 activations in the absence of taste. **F)** All laser frequencies (40 Hz, 20 Hz, 10 Hz, and 5Hz; all 1 mW) increased positive 'liking' reactions to 0.03M sucrose in insula ChR2 rats. All data shown as means ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001.

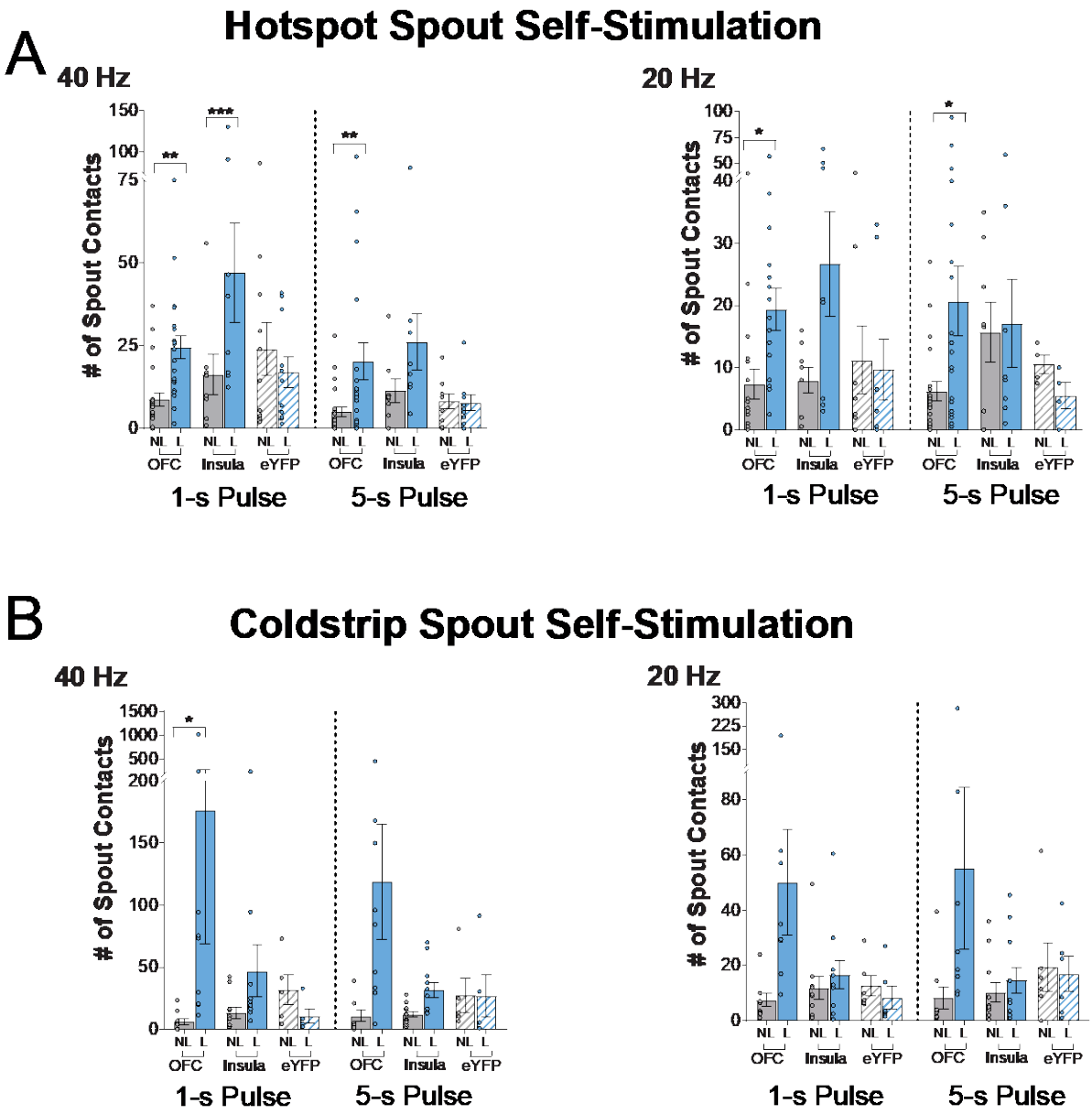


Figure 15. OFC and insula spout self-stimulation.

A) Total laser self-stimulations earned on spout-touch task by ChR2 rats with rostromedial and caudal insula hedonic hotspot sites (combined) at both 40 Hz (left) and 20 Hz (right) laser frequencies (5 sec and 1 sec pulse durations; 1 mW). **B)** Total laser self-stimulations earned by rats from caudal OFC and rostral-to-mid insula coldstrip sites at both 40 Hz (left) and 20 Hz (right) laser frequencies (5 sec and 1 sec pulse durations; 1 mW). All data presented as means and SEM; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

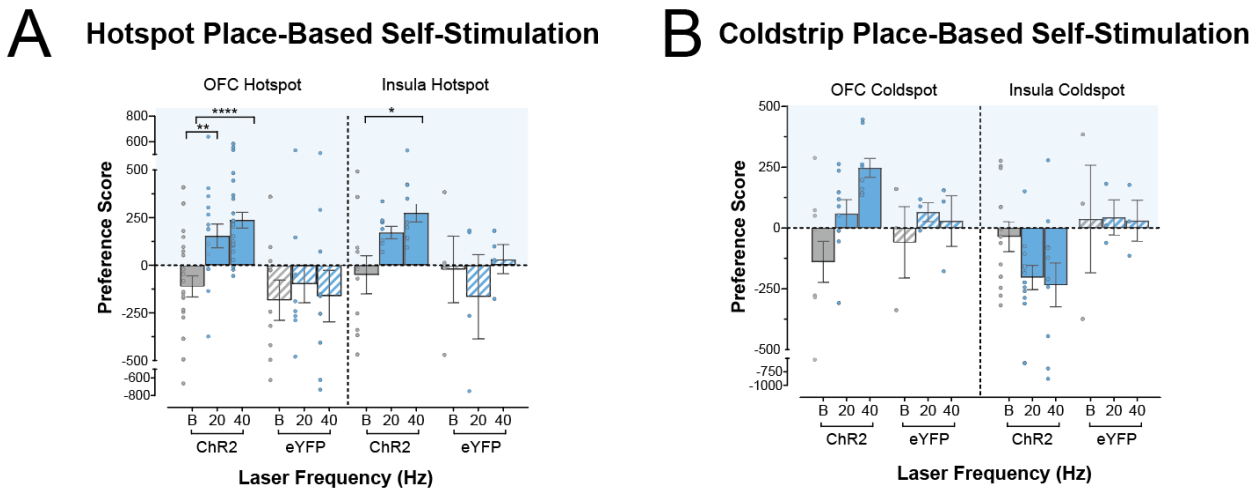


Figure 16. OFC and insula place-based self-stimulation.

A) Raw preference scores during place-based self-stimulation tests for ChR2 rats with sites in rostromedial and caudal insula hedonic hotspots (combined) (n = 22 rostromedial OFC ChR2; n = 9 rostromedial OFC eYFP; n =10 caudal insula ChR2; n = 4 caudal insula eYFP). B) Raw preference scores during place-based self-stimulation tests for ChR2 rats with sites in caudal OFC and rostral to mid insula coldstrip (n = 9 caudal OFC ChR2; n = 3 caudal OFC eYFP; n = 12 rostral to mid insula ChR2; n = 3 rostral insula eYFP). All data presented as means and SEM; Preference score reflects time (s) spent on the laser side – time (s) spent on non-laser side in the same rats; B: baseline habituation day; 20: 20 Hz stimulation tests; 40: 40 Hz stimulation tests; *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

OFC Hotspot Fos + Neurons	Fos+ Counts (MEAN ±SEM)			Statistic		Bonferroni Adjusted vs eYFP	95% CI	Effect Size
	ChR2	eYFP	Naïve					
Brain Region	n = 7	n = 5	n = 6	*F (ANOVA) or H[#] (Kruskal- Wallis)*	*p*	*p*		*d or η [2#]*
Prelimbic Cortex	136.9 ± 25.9	58.2 ± 16.8	59.0 ±8.9	5.43	0.017*	0.02*	(17.4, 139.9)	1.4
Infralimbic Cortex	122.4 ± 24.2	40.0 ± 6.2	53.3 ± 5.6	7.03	0.001**	0.004**	(30.2, 134.7)	1.78
Posterior OFC	143.1 ± 16.4	84.8 ± 22.4	42.7 ± 9.1	10.43	0.001**	0.02*	(8.6, 108.1)	1.2
Anterior Insula	45.0 ± 19.3	45.4 ±13.2	46.5 ± 4.5	1.12[#]	0.59			
Posterior Insula	122.1 ± 25.0	39.2 ± 8.1	50.7 ± 6.4	6.64	0.009*	0.006**	(28.2, 137.7)	1.71
Rostral Anterior Cingulate	111.3 ± 31.4	39.0 ± 8.9	30.3 ± 6.7	5.86[#]	0.04*	0.06		
Mid Anterior Cingulate	59.7 ± 7.8	40.0 ± 13.0	16.8 ± 5.4	6.58	0.009**	0.13		
NAc Core	70.1 ± 15.0	34.8 ± 8.3	26.5 ± 4.9	4.6	0.03*	0.04*	(0.75, 69.9)	1.1
NAc Dorsal Medial Shell	106.9 ± 7.1	51.2 ± 9.8	41.5 ± 4.8	25.5	<0.0001***	<0.0001****	(33.5, 77.85)	2.7
NAc Ventral Medial Shell	84.7 ± 16.7	23.0 ± 1.8	23.3 ± 3.6	9.96	0.002**	0.002**	(26.1,97.3)	2.0
NAc Caudal Dorsal Medial Shell	28.4 ± 4.0	40.0 ± 3.4	36.7 ± 4.1	2.3	0.13			
Dorsolateral Striatum	24.6 ± 11.8	22.0 ± 5.0	12.7 ± 1.6	0.57	0.58			
Olfactory Tubercle	32.4 ± 10.5	20.2 ± 7.3	13.8 ± 0.9	1.5	0.25			
Anterior VP Hotspot	26.1 ± 5.0	41.8 ± 10.1	17.2 ± 3.4	3.7	0.05			
Posterior VP Hotspot	46.1 ± 8.3	17.0 ± 2.8	14.3 ± 1.5	9.6	0.002**	0.004**	(11.1, 47.22)	2.0
Bed Nucleus Stria Terminalis	20.7 ± 3.6	27.4 ± 7.9	26.5 ± 3.0	0.61	0.56			
Lateral Hypothalamus	36.7 ± 9.4	29.0 ± 6.9	20.8 ± 2.1	3.32[#]	0.2			
Perifornical Area	45.7 ± 10.8	28.0 ± 4.2	16.0 ± 2.7	3.98	0.04*			
Central Amygdala	40.1 ± 15.8	20.6 ± 4.9	15.2 ± 2.5	2.30[#]	0.33			
Basolateral Amygdala	32.0 ±5.7	29.0 ±8.5	27.8 ± 4.5	0.13	0.87			
Medial Amygdala	63.1 ± 9.3	32.2± 6.1	23.0 ± 2.6	3.48	0.002**	0.008**	(9.2,52.7)	1.5
Paraventricular Thalamus	23.7 ± 2.8	17.2 ± 4.0	16.17 ± 3.0	1.76	0.21			
Arcuate Nucleus	21.0 ± 6.0	15.4 ± 2.1	20.2 ± 4.1	0.35	0.71			
Substantia Nigra	20.4± 6.1	17.6 ± 2.4	7.3 ± 3.2	5.56[#]	0.06			
Ventral Tegmental Area	58.6 ± 11.2	20.2 ± 5.9	8.7 ± 3.4	12.24[#]	0.0001***	0.04*		0.68[#]
Periacqueductal Gray	18.2 ± 3.7	18.6 ± 2.5	11.8 ± 0.8	2.1	0.16			

Figure 17. Raw Fos counts after rostromedial OFC hotspot stimulation.

Table shows counts of neurons expressing Fos+ protein in various mesocorticolimbic structures and subregions after final exposure to rostromedial OFC hotspot laser stimulation in ChR2 rats (N = 7), eYFP controls (N = 5), and naïve rats (N = 6). Fos+ counts reflect mean of each group at each site ± standard error (SEM). One-way ANOVA's or Kruskal-Wallis was performed followed by corrected, two sided-post hoc tests between ChR2 and eYFP or ChR2 and naïve rats. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$, ****$p<0.0001$.

Insula Hotspot Fos + Neurons	Fos+ Counts (MEAN ±SEM)			Statistic		Adjusted p vs eYFP	95% CI	Effect Size
	ChR2	eYFP	Naïve					
Brain Region	n = 5	n = 8	n = 6	*F (ANOVA) or H[#] (Kruskal- Wallis)*	*p*	*p*		*d or η²[#]*
Rostromedial OFC	273.6 ± 42.7	131.5 ± 20.7	101.0 ± 16.7	11.42[#]	0.0006***	0.02*		0.59[#]
Caudal OFC	103.6 ± 7.2	71.1 ± 14.3	42.7 ± 9.1	5.4	0.02*	0.16		
Prelimbic Cortex	125.4 ± 12.14	65.63 ± 11.25	59.0 ±8.9	9.43	0.002**	0.003**	(20.6, 99.0)	2.0
Infralimbic Cortex	81.00 ± 6.4	56.9 ± 6.1	53.3 ± 5.6	5.12	0.02*	0.03*	(2.3, 46.0)	1.5
Rostral Insula	36.2 ± 11.9	45.6 ± 6.5	46.5 ± 4.5	0.49	0.62			
Rostral Anterior Cingulate	68.2 ± 9.5	49.0 ± 8.4	31.8 ± 1.9	4.96	0.02*	0.2		
Mid Anterior Cingulate	71.8 ± 12.9	25.5 ± 6.8	16.8 ± 5.4	11.23	0.0009***	0.002**	(17.4, 75.3)	2.1
NAc Core	53.6 ± 8.8	29.6 ± 2.8	30.5 ± 3.1	7.23	0.006**	0.006**	(7.2, 40.8)	1.6
NAc Dorsal Medial Shell	98.8 ± 5.9	50.1 ± 10.9	41.5 ± 4.8	8.39[#]	0.009**	0.03*		0.40[#]
NAc Ventral Medial Shell	37.4 ± 8.0	36.0 ± 8.9	23.3 ± 3.6	0.95	0.41			
NAc Caudal Dorsal Medial Shell	60.8 ± 6.9	34.6 ± 5.5	36.7 ± 4.1	6.06	0.01*	0.009**	(6.5, 45.9)	1.7
Dorsolateral Striatum	12.8 ± 1.8	19.4 ± 3.8	12.7 ± 1.6	1.71	0.21			
Olfactory Tubercle	33.2 ± 3.5	19.5 ± 3.0	13.8 ± 0.9	10.94	0.001*	0.007*	(3.8, 23.6)	1.6
Anterior VP Hotspot	16.2 ± 3.1	22.9 ± 3.5	17.2 ± 3.4	1.2	0.34			
Posterior VP Hotspot	26.2 ± 9.2	17.8 ± 3.1	14.3 ± 1.5	0.75	0.49			
Bed Nucleus Stria Terminalis	26.6 ± 3.1	28.3 ± 4.0	26.5 ± 3.0	0.08	0.92			
Lateral Hypothalamus	58.6 ± 7.3	28.9 ± 4.3	20.8 ± 2.1	15.29	0.0002***	0.0009***	(13.1, 46.4)	2.1
Perifornical Area	25.2 ± 7.6	29.4 ± 4.4	16.0 ± 2.7	2.03	0.16			
Central Amygdala	24.2 ± 5.2	15.3 ± 3.3	15.2 ± 2.5	1.77	0.2			
Basolateral Amygdala	32.0 ± 8.6	18.4 ± 3.2	27.8 ± 4.5	1.92	0.19			
Medial Amygdala	58.8± 4.7	23.0 ± 3.6	23.0 ± 2.6	27.68	<0.0001****	<0.0001****	(22.8, 48.8)	3.5
Paraventricular Thalamus	34.4 ± 4.7	17.3 ± 4.2	14.5 ± 3.2	8.33[#]	0.009**	0.02*		0.40
Arcuate Nucleus	36.6 ± 8.3	16.3 ± 2.2	16.8 ± 2.0	6.77	0.007**	0.007**	(5.6, 35.1)	1.5
Substantia Nigra	21.6 ± 10.3	9.3 ± 2.0	7.3 ± 3.2	1.98	0.17			
Ventral Tegmental Area	62.6 ± 10.9	29.1 ± 3.0	16.5 ± 2.3	16.12	0.0005***	0.001**	(14.1, 52.9)	1.78
Periacqueductal Gray	20.2 ± 4.3	15.3 ± 2.7	11.8 ± 0.8	1.93	0.18			

Figure 18. Raw fos counts after caudal insula hotspot stimulation.

Table shows counts of neurons expressing Fos+ protein in various mesocorticolimbic structures and subregions final exposure to caudal insula hotspot laser stimulation in ChR2 rats (N = 5), eYFP controls (N = 8), and naïve rats (N = 6). Fos+ counts reflect mean of each group at each site ± standard error (SEM). One-way ANOVA's or Kruskal-Wallis was performed followed by corrected, two sided-post hoc tests between ChR2 and eYFP or ChR2 and naïve rats. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$, ****$p<0.0001$.

OFC/Insula Coldstrip Fos+ Neurons	Fos+ Counts (MEAN ±SEM)			Statistic		Adjusted *p* vs eYFP	95% CI	Effect Size
	ChR2	eYFP	Naïve					
Brain Region	n = 5 Insula, n = 7 OFC	n = 6	n = 6	*F (ANOVA) or H# (Kruskal- Wallis)*	*p*	*p*		*d or η 2#*
Rostromedial OFC	93.9 ± 14.8	110.8 ± 30.3	101.0 ± 16.7	0.19	0.83			
Caudal OFC *Counts from Insula ChR2 rats only	136.6 ± 18.4	53.5 ± 8.7	42.7 ± 9.1	16.79	0.0002***	0.0006***	(38.9, 126.1)	2.5
Prelimbic Cortex	108.5 ± 11.3	50.0 ± 3.4	59.0 ±8.9	15.25#	0.0005***	0.0011		0.63#
Infralimbic Cortex	99.4 ± 10.1	42.5 ± 5.8	53.3 ± 5.6	10.47	0.0006***	0.001**	(23.2, 90.6)	2.1
Rostral Insula **Counts from OFC ChR2 rats only	87.6 ± 8.9	50.0 ± 10.6	46.5 ± 4.5	7.56	0.005**	0.01*	(8.1, 67.2)	1.5
Caudal Insula	66.2 ± 14.3	52.5 ± 13.8	42.2 ± 7.2	2.50#	0.3			
Rostral Anterior Cingulate	64.3 ± 8.5	37.2 ± 8.3	34.5 ± 5.2	4.14	0.04*	0.07		
Mid Anterior Cingulate	18.4 ± 4.1	18.8 ± 10.9	24.2 ± 6.7	0.21	0.81			
NAc Core	62.8 ± 7.3	40.3 ± 3.7	30.5 ± 3.1	6.54	0.006**	0.06		
NAc Rostrodorsal Medial Shell	30.8 ± 6.2	38.5 ± 11.8	41.5 ± 4.8	0.56	0.58			
NAc Rostroventral Medial Shell	19.0 ± 4.5	19.7 ± 7.4	23.3 ± 3.6	1.53#	0.47			
NAc Caudal Dorsal Medial Shell	71.1 ± 6.1	36.0 ± 2.0	36.7 ± 4.1	15.45#	0.0004***	0.002**		0.64#
Dorsolateral Striatum	7.6 ± 1.5	15.33 ± 6.7	12.7 ± 1.6	3.51#	0.17			
Olfactory Tubercle	15.5 ± 2.8	14.0 ± 1.9	13.8 ± 0.9	0.12#	0.94			
Anterior VP Hotspot	51.8 ± 3.6	30.7 ± 1.8	17.2 ± 3.4	17.48	<0.0001****	0.0008***	(8.9, 33.4)	2.32
Posterior VP Hotspot	8.6 ± 3.9	5.7 ± 1.2	14.3 ± 1.5	8.1#	0.02*	>0.9999		
Bed Nucleus Stria Terminalis	18.4 ± 4.1	18.8 ± 10.9	26.5 ± 3.0	3.98#	0.14			
Lateral Hypothalamus	38.9 ± 9.0	15.5 ± 4.7	20.8 ± 2.1	3.24#	0.20			
Perifornical Area	23.0 ± 4.9	28.3 ± 8.42	16.0 ± 2.7	0.87	0.43			
Central Amygdala	40.7 ± 7.8	13.7 ± 2.8	15.2 ± 2.5	10.52#	0.005**	0.008**		0.41#
Basolateral Amygdala	53.8 ± 12.8	17.3 ± 3.4	27.8 ± 4.5	8.91#	0.012*	0.006**		0.33#
Medial Amygdala	113.3 ± 16.8	52.0 ± 11.0	30.7 ± 3.7	14.52#	0.0007***	0.06		
Paraventricular Thalamus	61.6 ± 7.1	21.3 ± 7.5	16.2 ± 3.0	13.46	0.0002***	0.001**	(15.8, 64.7)	1.90
Arcuate Nucleus	53.9 ± 7.8	17.7 ± 5.4	20.2 ± 4.1	13.75#	0.001**	0.003**		0.56#
Substantia Nigra	7.2 ± 1.4	8.0 ± 2.1	7.3 ± 3.2	0.03	0.97			
Ventral Tegmental Area	36.2 ± 6.6	13.5 ± 3.3	16.5 ± 2.3	5.85	0.01*	0.01**	(4.5, 41.0)	1.51
Periacqueductal Gray	17.3 ± 2.8	28.7 ± 3.8	11.8 ± 0.8	7.9	0.003**	0.02*	(-21.0, -1.6)	-1.28

Figure 19. Raw fos counts after caudal OFC/ rostral insula coldstrip stimulation.

Table shows counts of neurons expressing Fos+ protein in various mesocorticolimbic structures and subregions after final exposure to caudal insula hotspot laser stimulation in ChR2 rats (N = 7 caudal OFC; N = 5 rostral insula), eYFP controls (N = 6), and naïve rats (N = 6). Fos+ counts reflect mean of each group at each site ± standard error (SEM). One-way ANOVA's or Kruskal-Wallis was performed followed by corrected, two sided-post hoc tests between ChR2 and eYFP or ChR2 and naïve rats. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$, ****$p<0.0001$.

Chapter 3 Identification of a Novel Hedonic Hotspot in Cingulate Cortex: Enhancement of Sweetness 'Liking'.

3.1 Abstract.

Brain hedonic hotspots are small mesocorticolimbic systems with the capacity to causally amplify the hedonic impact or 'liking' reactions of palatable tastes in response to a few neurochemical signals. Hedonic hotspots have thus far been identified within the rostrodorsal quadrant of the nucleus accumbens medial shell, caudal ventral pallidum, and more recently in rostromedial orbitofrontal cortex, and insula cortex. The identification of hedonic hotspots in cortical areas raises the question of whether additional hotspots exist in other corticolimbic systems that have yet to be explored. Recently, an anatomically restricted site in the anterior cingulate cortex of human was discovered where deep brain stimulations in patients undergoing treatment for epilepsy produced intense happiness and joyful laughter. Here we use optogenetic stimulations paired with the taste reactivity test, which categorizes affective taste responses into positive 'liking' and aversive 'disgust' reactions in rats to probe hedonic gains of function in Cingulate neurons. We report that channelrhodopsin stimulations in a mid-to-caudal region of cingulate cortex in rats doubled the number of affective 'liking' reactions elicited by intra-oral sucrose and water infusions. Optogenetic manipulations at this same site also generated incentive motivation, here measured as focused and directed instrumental responding for a laser-paired sucrose reward relative to an identical sucrose reward that was never laser-paired. Finally, we show that activating cingulate hotspot neurons in the absence of an external sucrose reward also

promotes incentive motivation, resulting in robust levels of laser self-stimulation. Our results suggest a never previously identified site of hedonic enhancement exists in cingulate cortex of rats, capable of causally amplifying the hedonic impact of pleasant tastes.

3.2 Introduction.

The ability to produce normal affective reactions in response to pleasant events is crucial for wellbeing. Affective disorders may arise from dysfunctions to brain systems that regulate the hedonic impact, or 'liking' for reward. Some progress has been made in understanding neural control of hedonic responses via identification of brain hedonic hotspots. These are small subregions of mesocorticolimbic brain systems that increase positive 'liking' reactions to sweet taste in response to a few neurochemical signals during the taste reactivity test (Castro et al., 2016; Castro & Berridge, 2014c, 2017; Ho & Berridge, 2013; Mahler et al., 2007; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005). This test categorizes orofacial expressions elicited by various tastes into positive 'liking' or aversive 'disgust' that are shared across rodents, human infants, and other mammals (Berridge, 2000; Berridge & Kringelbach, 2015; Grill & Norgren, 1978c; Steiner, 1973).

Hedonic hotspots have thus far been identified in subcortical structures including the rostrodorsal nucleus accumbens medial shell (NAc), caudolateral ventral pallidum (VP), and the parabrachial nucleus in the brainstem (Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005; Söderpalm & Berridge, 2000). Cortical hotspots were also recently identified in rostromedial orbitofrontal cortex (OFC) and caudal insula using drug microinjections of orexin and the mu-opioid agonist DAMGO (Castro & Berridge, 2017). In Chapter 2 of this dissertation, I further confirmed the existence of these cortical hedonic hotspots using optogenetic techniques to depolarize neurons within the rostromedial OFC and caudal insula to show that alternative neural

manipulations within hedonic hotspot boundaries can also increase positive affective responses to sweetness. This positive verification of brain hedonic hotspots raises the question of whether additional hedonic hotspots might exist in other corticolimbic regions that have yet to be identified.

One potential corticolimbic contributor to affective responses is the cingulate cortex (CC) (Berridge, 2003; Berridge & Kringelbach, 2015; Kringelbach & Berridge, 2010a). The cingulate cortex comprises a strip of cortical tissue that runs along the medial wall of the dorsal edge of the brain, and is part of the original limbic systems developed by Broca, Papez, and Maclean (Broca, 1878; MacLean, 1949; Maclean, 1955; Papez, 1937). The cingulate cortex has been implicated in human emotion, including various affective disorders, and is thought to code both positive and unpleasant stimuli. Aversive stimuli including pain and unpleasant tastes are linked to activity in the cingulate cortex (K. D. Davis et al., 1997; Porro et al., 1998; Rainville et al., 1997; Tölle et al., 1999; Zald et al., 1998). Further, positive stimuli such as pleasant touch, palatable tastes, drug-related cues, and drugs including cocaine, fentanyl, and marijuana elicit neural activity in the cingulate cortex, (Childress et al., 1999; de Araujo & Rolls, 2004; Firestone et al., 1996; Grabenhorst et al., 2008; Grabenhorst & Rolls, 2008; Huang et al., 2024; Maas et al., 1998; Mathew et al., 1997; Rauch et al., 1999; Rolls, 2008; Rolls, O'Doherty, et al., 2003), suggesting that the cingulate cortex is a correlational neural marker for both positive and negative affective states.

Evidence against cortical necessity for hedonic function comes from studies in humans showing that lesions to cortical areas including anterior cingulate cortex do not abolish emotional behavior in humans (Damasio, 1994, 1996; Damasio et al., 2013). While certain emotional behaviors may seem odd or socially misplaced, the core processes that enable the generation of

affective responses appear to remain intact. For example, human patients with massive cortical lesions can still react to unpleasant stimuli, get angry, become fearful, seek palatable foods and other pleasant rewards, display aspects of intact emotional learning, and even report subjective feelings of happiness (Damasio et al., 2013).

However, brain capacity to produce gains of function may not necessarily always be matched by opposing loss of function. Some evidence of this comes from studies in rats that show increases in affective expressions or 'liking' reactions within brain hedonic hotspots that are not always matched by loss or abolished 'liking' (Castro & Berridge, 2017; Ho & Berridge, 2014; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005). Further a recent study in human patients undergoing treatment for epilepsy showed that deep brain stimulation within a highly localized site near a dorsal and mid-caudal region of anterior cingulate cortex produced intense happiness, joy, and, and laughter (Bijanki et al., 2019). This raises the possibility that a yet unidentified hedonic hotspot that exerts control over 'liking' reactions may exist in limbic cingulate cortex of rats, similar to what has been described in OFC and insula.

The original microinjection study that identified hedonic hotspots in OFC and insula failed to show amplification of hedonic 'liking' responses to sucrose when the mu-opioid agonist DAMGO or orexin was administered into prefrontal regions in prelimbic, infralimbic, and the most rostral tip of anterior CC, but this study did not explore more caudal regions of cingulate cortex as potential sites of hedonic enhancement (Castro & Berridge, 2017). Therefore, we aimed to investigate any potential cingulate cortex contributions to affective 'liking' reactions in rats by using optogenetics to stimulate neurons along a larger rostro-caudal gradient of cingulate cortex. We also compared the ability of the same photostimulation within CC neurons to promote

'wanting' for a laser-paired sucrose reward rats were instrumentally trained to respond to, and finally, to measures of laser self-stimulation.

3.3 Materials and Methods

Animals

Adult Sprague Dawley rats (n = 35, n = 17 females, n = 14 male) were group housed by sex and kept on a reversed 12-h dark/light cycle at constant temperature of 21°C. Rats had *ad libitum* access to food and water throughout all experiments. All experiments were approved by The Committee on the Use and Care of Animals at the University of Michigan (IACUC).

Surgery

Optogenetic virus infusion.

Rats were anesthetized with isoflurane gas (5% induction; 1-2% maintenance) and given atropine to precent respiratory distress. Rats were placed into a stereotaxic frame (David Kopf Instruments; Tujunga, CA) and received bilateral virus injections of an AAV5 channelrhodopsin virus (AAV5-hSYN-ChR2-eYFP, n = 11; or AAV5-CamK11-ChR2-eYFP, n = 10; UNC Vector Core) or an inactive control virus that lacked the ChR2 gene (AAV5-hSYN-eYFP, n = 4; AAV5-CamKII-eYFP, n = 4) targeted at cortical sites in anterior cingulate cortex. A 0.30 µL volume of virus was infused per side over a 3-minute period at a constant rate of 0.1 µL/min.

Virus microinjection sites were aimed to be bilaterally identical within an individual rat, but staggered across individuals so that the group's sites filled a large portion of the rostral-caudal extent of the mid anterior cingulate cortex. CC coordinates ranged from +2.16 mm to -1.20 mm AP, ± 0.1 to ±2.0 mm ML and -1.00 mm to -3.00 mm DV (all relative to bregma) and

included Cg1 and Cg2 subregions of CC (Paxinos & Watson, 2013). After surgery, cefazolin (100 mg/kg, s.c.; Henry Schein) was administered to prevent infection, and carprofen (5 mg/kg, s.c.; Henry Schein) given for post-operative pain relief. Carprofen and cefazolin were repeated at 24-h and 48-h post-operation.

Oral cannula and fiber optic implantation.

Three weeks after the initial viral infusion surgery, rats were re-anesthetized with isoflurane as described above for implantation of intracranial optic fibers and of bilateral oral cannulas, which allowed for direct oral infusions of sucrose, quinine, and water solutions. Each oral cannula (polyethylene-100 tubing) entered the upper cheek just lateral to the secondary maxillary molar, ascended beneath the zygomatic arch, and exited the skin at the dorsal head, where it was secured with skull screws and a dental acrylic headcap. In the same surgery, rats were implanted with bilateral optic fibers (200 μm), aimed to place each fiber tip 0.3 mm dorsal to the rat's bilateral virus microinjection sites, and anchored with the same acrylic headcap. Cefazolin and carprofen were again administered and repeated post-operatively as above. All rats were allowed to recover for 1 week prior to behavioral testing.

Behavioral Procedures

Taste Reactivity Test.

Each rat was habituated to the test chamber for 30 minutes on four consecutive days before any behavioral testing occurred. On the last two days of habituation, rats received oral infusions of a 0.03M sucrose solution to habituate them to infusion of fluids into the mouth. In subsequent taste reactivity tests, affective orofacial reactions (i.e., positive 'liking' versus negative 'disgust' patterns) elicited by oral infusions either of water or of three different taste solutions: two concentrations of sucrose solutions (0.03M and 0.10M), and one concentration of

bitter quinine (3 x 10^{-4} M) (Berridge, 2000; Grill & Norgren, 1978c). Orofacial reactions were videorecorded through a close-up lens facing an angled mirror underneath the transparent floor, positioned to capture a clear view of the mouth and face, and saved for subsequent offline analysis. Taste solutions (1 ml) were delivered into the mouth of rats through PE-50 tubing connected to a PE-10 delivery nozzle, at a constant 1ml/min rate during the 1 min infusion, via a syringe pump, connected to the oral cannula.

On each test day, a rat received two separate 1-ml/1-min infusions of the same solution (e.g., .01 M sucrose). One infusion accompanied by laser stimulation and the other infusion not accompanied by laser as a within-subject baseline (counterbalanced order across rats), spaced 8-10 apart. Different tastants were tested on different days. During a laser-paired infusion, laser illumination (1-3 mW; 15 ms pulses; 5 Hz, 10 Hz, 20 Hz, 40 Hz) was cycled in 5-s ON, 5 Sec OFF bins throughout the 60-sec trial test. Several different frequencies of laser illumination within 5-s ON bins were tested on different days. Every parameter was tested on at least two days for each rat in separate daily tests.

Taste Reactivity Scoring.

Taste reactivity videos were scored subsequently for positive hedonic 'liking' reactions, aversive 'disgust' reactions, and neutral taste reactions in slow-motion at speeds ranging from frame-by-frame to 1/5th normal speed, using The Observer Software (Noldus; Leesburg, VA). Positive hedonic or 'liking' responses were considered to be: lateral tongue protrusions, paw licks, and rhythmic midline tongue protrusions (Berridge, 2000; Castro et al., 2016; Castro & Berridge, 2014c, 2017). Aversive 'disgust' reactions were: gapes, forelimb flails, head shakes, face washes, chin rubs, and paw treading. Neutral responses (i.e., relatively uncoupled from hedonic impact) were: passive dripping of solution out of the mouth, rhythmic mouth

movements, and grooming. A time-bin scoring system was used to ensure each type of affective reaction contributed equally to the overall affective score (Berridge, 2000; Castro et al., 2016; Castro & Berridge, 2014c, 2017). Rhythmic mouth movements, paw licks, passive dripping, and grooming were all scored in 5-s time bins, because these behaviors typically are emitted in bouts of relatively long duration. Any continuous emission of these behaviors up to 5-sec was counted as a single occurrence; continuous emissions of 5-sec to 10-sec counted as two occurrences, etc. Midline tongue protrusions and paw-treading were scored similarly, but in 2-s bins, because they are typically emitted in shorter bouts. Lateral tongue protrusions, gapes, flails, headshakes, and chin rubs were counted as discrete events every time they occurred, because these can occur singly or in brief repetitions. A total positive hedonic (i.e., 'liking') score was then calculated by combining component scores of rhythmic tongue protrusions, paw licks, and lateral tongue protrusions. A total negative aversive (i.e., 'disgust') score was calculated by combining gapes, forelimb flails, head shakes, paw treading, face washes, and chin rubs (Berridge, 2000; Castro & Berridge, 2017).

Instrumental sucrose 2-choice test.

This test was used to determine whether CC photostimulation could narrow motivation onto a particular laser-paired sucrose target (Baumgartner et al., 2021; M. J. F. Robinson et al., 2014). Rats could choose between pressing on two levers mounted on the front wall of an operant chamber (Med Associates; Fairfax, VT). Presses on one lever delivered a sucrose pellet (Sucrose Alone) paired with its own auditory cue (CS+1; either tone or white noise; order counterbalanced across rats). Presses to the other lever delivered an identical sucrose pellet paired with ACC photostimulation (473 nm; 1-3 mW; 40 Hz; 8-s duration) also paired with its own unique auditory cue (CS+2). The assigned laser-paired lever for a rat remained constant

throughout the experiment but was counterbalanced across rats.

At the start of the session, four forced-choice trials were conducted to ensure that each rat was reminded of the two reward options. Briefly, a single lever was presented, and rats had to press that lever to earn its associated reward (either Sucrose Alone or Sucrose + Laser). Next, the alternate lever was presented, and the other reward was delivered if pressed. This cycle was repeated one more time, after which both levers were extended and rats could freely choose between the two options for the remainder of the session. Two-choice sessions were continued over a 9-day period with increasing reinforcement schedules (FR1, FR1, FR1, FR1, FR4, RR4, RR6, RR6, RR6).

Progressive ratio test of breakpoint effort

A progressive ratio test was conducted to determine whether paired CC photostimulation enhanced the intensity of incentive motivation for laser-paired sucrose. Testing was conducted across two separate days (1 day for Sucrose Alone; 1 day for Sucrose + Laser). On a single day, only one lever was made available (order counterbalanced across rats). During each test session, increased effort was required to earned the next sucrose pellet derived from the formula PR = $[5e^{\text{reward number x } 0.2}]$-5 rounded to the nearest whole number. The breakpoint was defined as the maximal ratio effort reached by a given rat, and was compared across Sucrose Alone and Sucrose + Laser days.

Place-Based Self-Stimulation

A passive place-based task was used to assess whether CC ChR2 rats would self-stimulate brief laser pulses by spending time within a laser-paired chamber. This task was adapted from original brain self-stimulation reward studies conducted using electrical stimulation

(Olds & Milner, 1954; Valenstein & Meyers, 1964). Each rat was placed into a two-chambered Plexiglas enclosure (76 cm x 38 cm x 38 cm). Entries into the control side produced nothing. Entries into the laser-paired side triggered brief laser pulses that continued to cycle (1-3 mW; 40 Hz; 3-s ON/4-s OFF) as long as the rat remained within the laser-paired chamber. Rats were given a no laser habituation day to assess side preference, then tested for self-stimulation across 3 consecutive days (15-min sessions).

Spout-Based Self-Stimulation

A more active spout-touching based task was used to determine whether ChR2 stimulation of CC neurons alone was rewarding. Rats were placed into Med-Associated operant chambers equipped with two empty metal water spouts. Licks or contacts to the control spout produced nothing. Licks or contacts to a laser-paired spout delivered brief laser pulses (1-s or 5-s duration; 1-3 mW; 40 Hz). Each pulse duration was tested across 3 consecutive days for a total of 6 x 30-min test sessions.

Immunohistochemistry and Histology

Beginning 75-min prior to euthanasia, a final controlled laser stimulation session was administered with one of the same parameters that produced hedonic modulation in the taste reactivity tests (40 Hz, 15 ms pulse, 5-sec ON/5-sec OFF; 30-min session). This final laser stimulation was given to a) induce local Fos plumes around optic fiber tips that would indicate the anatomical spread of local neuronal stimulation at that cortical site induced by ChR2 laser illumination, and b) potentially also recruit distant Fos activation in various limbic brain structures, to identify recruited circuitry that potentially might mediate optogenetic modulation of hedonic reactions (Baumgartner et al., 2021; Cole et al., 2018; Warlow et al., 2020).

Following the final laser stimulation, rats were deeply anesthetized with a lethal dose of sodium pentobarbital (150-200 mg/kg) and transcardially perfused with PBS followed by 4% PFA. Brains were removed and post-fixed in 4% PFA for 24-h and then transferred to a 25% sucrose solution for at least two days. Tissue was coronally sectioned at 40 micrometers using a cryostat (Leica), slices were processed for GFP and cFos immunohistochemistry, and imaged using a digital camera (Qimaging) and fluorescence microscope (Leica). For immunohistochemistry, coronal sections were rinsed for 10 min in 0.1 M sodium phosphate buffer three times, then blocked in 5% normal donkey serum / 0.2% triton-X PBS solution for 60 min and incubated overnight in a polyclonal rabbit anti-cfos igG primary antibody (1:2500; Synaptic Systems) and chicken polyclonal anti-GFP igY primary antibody (1:2000; Abcam). Tissue was again rinsed three times in 0.1M NaPb for 10 min followed by 2-h in biotin-SP-conjugated donkey anti-rabbit (1:300; Jackson ImmunoResearch) secondary antibody and AlexaFluor-488 donkey anti-chicken secondary antibody (1:300; Jackson ImmunoResearch). Tissue was rinsed three times in 0.1M NaPB for 10 min followed by1.5-h in streptavidin-conjugated Cy3 (1:300; Jackson ImmunoResearch). Brain sections were mounted, air-dried, and cover-slipped with anti-fade Pro-long gold (Invitrogen).

Immunoreactivity for Fos-like protein was visualized using a fluorescent microscope filter with a band of excitation at 515-545 nm. Coronal sections were imaged (10x magnification) to localize eYFP protein expression and fiber tips surrounding Fos plumes, spread of virus expression, and to quantify Fos expression in distributed structures. Stimulation sites were plotted onto corresponding maps using a brain atlas (Paxinos & Watson, 2013).

Statistical Analysis

Results were analyzed using repeated measures ANOVA followed by t-tests

with a Bonferroni for individual comparisons. Effect sizes were calculated using Cohen's *d.* When necessary, Wilcoxon sign-ranked tests were used for nonparametric tests. Significance level was set at $p < 0.05$.

3.4 Results

No spontaneous orofacial reactions are generated by ACC neuron stimulation alone. In the absence of intra-oral infusions of any tastant, optogenetic stimulation of ACC ChR2 rats failed to induce any orofacial movements ($M = 0.88$, $SEM = 0.31$; $F_{1,15} = 0.32$, $p = 0.58$, $n = 16$; Fig. 21a), indicating that ACC neuron stimulations did not directly cause spontaneous motor reactions.

Identification of a mid-caudal anterior cingulate cortex hedonic hotspot: optogenetic ChR2 stimulation enhances 'liking' reactions.

Rostral to mid ACC neuron ChR2 enhancement of positive 'liking' reactions to sucrose. At ChR2 sites within a mid to posterior region of cingulate cortex, laser stimulations approximately doubled hedonic 'liking' reactions, made up of rhythmic tongue protrusions, paw licks, and lateral tongue protrusions, elicited by 0.03M and 0.10M concentrations of sucrose (Fig 1). Laser illumination (40 Hz) increased positive 'liking' reactions in mid-to-caudal ACC ChR2 rats by 174% ± 29% (eYFP % Enhancement: 84.6% ± 3.9%; U = 1, CI [-116.0, -31.2, $p <$ 0.0001; Fig 1b), over measured control baseline levels in the same individuals without laser (0.03M Sucrose: Laser x virus x valence interaction: $F_{1,20} = 6.40$, $p = 0.01$; $n = 14$ ChR2; $n = 8$ eYFP; ChR2: Sum of rhythmic tongue protrusions, lateral tongue protrusions, and paw licks: *Laser-ON*: $M = 20.3$, $SEM = 2.9$; *Laser-OFF*: $M = 12.3$, $SEM = 0.9$; paired comparison $t_{20} = 5.27$, $p = 0.001$, $d = 3.80$, CI [-13.4, -4.2]; Fig. 20b, Fig 21). The magnitude of laser

enhancement of 'liking' reactions was similar in females and males (ChR2: sex x valence x laser interaction: $F_{1,12} = 0.66$ $p = 0.43$; $n = 6$ males $n = 8$ females; Fig. 21c). ACC ChR2 neuron stimulation did not alter the few negative 'disgust' reactions elicited by 0.03M sucrose (gapes, headshakes, forelimb flails; chin rubs; *Laser-ON*: $M = 4.3$, $SEM = 1.2$, *Laser-OFF*: $M = 4.0$, $SEM = 1.1$, $t_{20} = 0.17$, $p = 0.99$; Fig. 21b).

Both cingulate hSYN ChR2 neuron stimulations (40 Hz; 1-3 mW) and CAMKII ChR2 stimulations increased positive 'liking' reactions to sucrose in ChR2 rats; laser x valence interaction: $F_{1,12} = 6.65$, $p = 0.02$). The magnitude of hedonic enhancement was similar in hSYN ChR2 rats (203% ± 41.5%) relative to CaMKII ChR2 rats (121% ± 19%; laser x virus x valence interaction: $F_{1,12} = 2.1$, $p = 0.18$), but overall, hSYN ChR2 rats elicited about 2x as many total positive 'liking' reactions relative to CaMKII rats (hSYN sum of 'liking' reactions: *Laser-ON*: $M = 25.3$, $SEM = 3.3$; *Laser-OFF*: $M = 13.8$, $SEM = 1.0$; CaMKII sum of 'liking reactions: *Laser-ON*: $M = 11.3$, $SEM = 1.7$; *Laser-OFF*: $M = 9.5$, $SEM = 0.34$; virus x valence interaction: $F_{1,12} = 13.47$, $p = 0.003$; $F_{1,12} = 13.47$, $p = 0.003$). These differences in overall baseline 'liking' reactions may reflect individual differences in baseline hedonic reactivity across distinct cohorts of rats, as laser activation of ACC neurons promoted similar within-subject increases in positive 'liking' reactions across groups.

Similarly, for the higher 0.10M sucrose concentration, cingulate cortex neuron laser stimulation doubled (200 ± 32.5%) the number of positive hedonic reactions compared to within-subject baseline levels in ACC ChR2 rats (Laser-ON $M = 25.4$, $SEM = 3.0$; *Laser OFF* $M = 14.9$, $SEM = 1.7$; laser x valence x virus $F_{1,20} = 5.74$, $p = 0.03$, $n = 14$ ChR2 rats; paired comparison: $t_{20} = 5.5$, $p < 0.0001$, $d = 4.30$, CI [-15.7, -5.3]; Fig. 20c; Fig 21e). The percentage

magnitude of laser hedonic enhancement of 'liking' reactions over baseline levels in ChR2 ats was comparable for both 0.03M and 0.10M sucrose ($F_{1,26}$= 0.42, p = 0.52).

By contrast, in control eYFP rats with an optically inactive virus, laser illuminations in cingulate cortex sites failed to alter either positive hedonic reactions, or the few elicited negative reactions from baselines measured in the same individuals, for either 0.03M or 0.10 sucrose (0.03M sucrose: $F_{1,7}$ = 2.00 p = 0.20, n = 8; 0.10M sucrose: $F_{1,7}$ = 0.12 p = 0.74, n = 8; Fig. 20; Fig 21b, 21e). Thus, ACC ChR2 rats and ACC eYFP controls differed from each other (0.03M sucrose: laser x virus x valence interaction: $F_{1,20}$ = 6.75 p = 0.02; 0.10M sucrose: laser x virus x valence interaction: $F_{1,20}$ = 5.74 p = 0.03; Fig 20; Fig 21b; Fig 21e). No sex differences in affective reactions were detected between eYFP male and female rats (0.03M sex x valence x laser interaction: $F_{1,6}$ = 0.27 p = 0.63; n = 4 males n = 4 females; 0.10M sex x valence x laser interaction: $F_{1,6}$ = 2.04 p = 0.20; n = 4 males n = 4 females; Fig. 21f; Fig 21g). Further hedonic reactions of ACC eYFP rats remained similar to baseline reactions of ACC ChR2 rats in the absence of laser (t_{20} = 1.10, p = 0.28).

Similar hedonic enhancements across different laser frequencies. Hedonic enhancement effects were robust and similar across a range of different laser frequencies within the mid-to-caudal cingulate hotspot (20 Hz, 40 Hz; 1-3 mW intensity), and was not limited to a single parameter. All frequencies produced similar magnitudes of enhancements of positive 'liking' reactions to sucrose, ranging between ~160% - 250% above within-subject no-laser baselines, and did not differ statistically from each other (H (4, n = 50) = 0.30, p = 0.95). Assessed separately, 40 Hz, 20 Hz, 10 Hz, and 5 Hz each increased positive hedonic 'liking' reactions above no-laser baselines measured in the same rats (20 Hz = 163.8% ± 17% increase, W = 86.50,

Z = -2.87, p = 0.004, n = 14; 10 Hz = 246.6% ± 88.2% increase, W = 62.5, Z = -2.63, p = 0.009, n = 11; 5 Hz = 183.9% ± 43.5% increase W = 51.00, Z = -2.40, p = 0.02, n = 11; Fig. 21h).

Microstructure of taste reactivity components fits hedonic enhancement pattern. To confirm that laser ChR2 stimulation within the cingulate cortex hotspot generated increases in hedonic impact rather than nonspecific sensorimotor reactions, we assessed whether changes in individual taste reactivity components were grouped into larger affective categories of positive 'liking' versus negative 'disgust'. For example, a shared increase among multiple components within the positive hedonic category (rhythmic midline tongue protrusions [TP], lateral tongue protrusions [LTP] and paw licks [PL]), but no increase in any component of the negative 'disgust' category (gapes [G], headshakes [HS], face washes [FW], forelimb flails [FF], or chin rubs [CR] (Berridge, 2000)would be required to be categorized as a hedonic increase in positive 'liking' reactions.

ChR2-induced enhancements fit this category-based pattern in hotspot sites. Increases in hedonic impact were not dominated by a single taste reactivity component, which if so, might have reflected motor and not hedonic effects. Rather, increases in 'liking' elicited by laser-paired sucrose infusions were distributed across multiple reactions in the affective category (TP *Laser OFF:* TP: 0.03M Sucrose: *Laser-OFF: M*= 5.8, *SEM= 0.99; Laser-ON: M*= 8.5, *SEM= 1.6;* 0.10 M Sucrose: *Laser-OFF: M*= 7.6 *SEM= 1.4; Laser-ON: M*= 9.9, *SEM= 1.80,* $F_{1,42} = 5.76$, p = 0.02; PL: 0.03 M Sucrose: *Laser-OFF: M*= 2.8 *SEM= 0.7; Laser-ON: M= 3.5*, *SEM= 0.8,* ; 0.10M Sucrose: *Laser-OFF: M*= 2.9, *SEM= 0.9; Laser-ON: M*= 5.0, *SEM= 1.4;* $F_{1,42} = 2.93$, p = 0.09; LTP: 0.03 M Sucrose: *Laser-OFF: M*= 3.4 *SEM= 0.8; Laser-ON: M= 8.8*, *SEM= 2.3*; 0.10M Sucrose: *Laser-OFF: M*= 4.2, *SEM= 1.1; Laser-ON: M*= 10.6, *SEM= 2.9;* $F_{1,42} = 15.4$, p = 0.0003; n= 14; Fig. 21k).

Cingulate hotspot hedonic enhancement of water. Laser ChR2 excitation in the cingulate cortex hotspot similarly increased positive 'liking' reactions to water by 230% over within-subject baselines (Fig. 20d; laser x virus x valence interaction: $F_{1,18} = 5.32$, $p = 0.03$; paired comparison $t_{18} = 4.76$, $d = 5.36$, CI [-13.3, -3.5], $p = 0.006$) with no change in the number of aversive 'disgust' expressions to water ($t_{18} = 0.55$, $p = 0.99$). In absence of laser, oral infusions of tap water at room temperature elicited only a few positive 'liking' reactions ($M = 8.79$, $SEM = 1.20$) and a few aversive 'disgust' reactions on baseline tests ($M = 9.11$, $SEM = 2.99$). In eYFP control rats, adding laser illumination to ACC did not alter either positive or negative reactions to water compared to baseline ('Liking' paired comparison: $t_{18} = 0.54$, $p = 0.99$; 'disgust' paired comparison: $t_{18} = 0.34$, $p = 0.99$; $n = 8$), and thus eYFP controls differed from ChR2 rats (laser x virus x valence interaction: $F_{1,18} = 5.32$, $p = 0.03$; Fig. 21i).

No change in quinine 'disgust'. Oral infusions of bitter quinine solution ($3x10^{-4}$ M) elicited predominately aversive 'disgust' reactions in the absence of laser ($M = 31.3$, $SEM = 3.9$; $F_{1,20} = 87.09$, $p < 0.0001$, $n = 12$). Within the cingulate hotspot, adding laser stimulation in either ChR2 rats or eYFP rats (40 Hz; 5-s ON/ 5-s OFF) failed to alter the number of aversive reactions elicited by quinine compared to no-laser baseline in the same rats (ChR2: 103% ± 19%, $n = 14$; eYFP control rats:109% ± 5%, $n = 8$; laser x virus x valence interaction: $F_{1,18} = 3.36$, $p = 0.09$; Fig. 21j)

Anatomical boundaries of optogenetic hedonic hotspot in mid cingulate cortex. Localization of function was mapped for optogenetic cingulate hedonic enhancements caused across all cortical sites (Fig 20). Hedonic hotspots were considered to be brain regions where ChR2 laser illuminations caused 125% or greater increases in positive 'liking' reactions to sucrose, compared to elicited affective reactions under no laser conditions in the same rats.

Hedonic enhancement sites clustered anatomically along the caudal end of the mid cingulate cortex of rats (Area 24).

The center of the cingulate hedonic hotspot was highly concentrated in areas close to bregma. Beginning ~+0.60 mm AP, within the rostrocaudal axis that that coincides with the most posterior tip of the nucleus accumbens. At this rostral edge, the cingulate hedonic hotspot was bordered rostrally by cingulate cortex, ventrally by the corpus callosum, and dorsolaterally by secondary motor cortex. While the majority of virus expression in rats tended to concentrate in more dorsal areas, both A24a (Cg1) and A24b/A33 (Cg2) subregions effectively increased hedonic 'liking' reactions in rats. The hedonic hotspot extended ~0.96mm caudally along the medial wall of cingulate cortex to its far caudal tip (-0.36mm AP). There the hedonic hotspot was bordered ventrally by corpus callosum, dorsolaterally by secondary motor cortex, and caudally by the A24' subregions of cingulate cortex. Overall, the hedonic hotspot extended at least rostrocaudally (AP) in length ~0.96 mm, ~2.2 mm dorsoventrally, and ~2.4mm mediolaterally, for a total volume of 5mm^3. Within this concentrated zone, optogenetic stimulations of cingulate neurons nearly doubled hedonic 'liking' reactions.

We note the possibility that the boundaries of this identified cingulate hotspot might extend further rostrally than reported here, as our probe did not extend to more anterior regions of cingulate cortex. Mild (~130% increases) hedonic enhancements were also observed in a single rat with virus placement near the most anterior edge of Area 24 (+2.28 mm AP), raising the possibility that anatomical boundaries could extend further rostrally than reported here. Future studies could map into these more anterior areas to help resolve this question.

<u>Mid-caudal cingulate cortex neuronal stimulation enhances paired sucrose value</u>

We tested whether pairing CC neuron stimulation with earning sucrose rewards in the two-choice task could cause rats to pursue that laser-paired sucrose reward over the sucrose alone option never paired with laser stimulation (Fig. 22a). In this task, pairing ACC hotspot neuron stimulations biased CaMKII ChR2 rats for the Sucrose + laser option nearly exclusively over the otherwise identical sucrose Alone option without laser ($F_{8,40} = 7.85$, $p < 0.0001$, $n = 6$; Fig 22b). Rats reached a 6:1 ratio preference by final day 9 ($t_{40} = 7.1$, $p = 0.0001$, 95% CI [412, 987], $d = 1.7$). Both male and female CaMKII ChR2 showed equally strong preferences for Laser + Sucrose lever over the Sucrose Alone lever, (females: 5:1 ratio; males 6:1 ratio; $F_{8,32} = 0.60$, $p = 0.79$, $n = 3$ males, $n = 3$ females; Fig 22c.) By contrast hSYN ChR2 rats and ACC eYFP controls with inactive virus chose randomly between Laser + Sucrose and Sucrose Alone options (hSYN ChR2: $F_{8,64} = 0.37$, $p = 0.93$, $n = 9$; eYFP controls: $F_{8,56} = 0.30$, $p = 0.96$, $n = 8$; Fig 22c,d).

Overall, CaMKII ChR2, hSYN ChR2, and eYFP controls consumed equal total amount of sucrose pellets during the final instrumental session suggesting that while CC CamKII neuron excitation was able to direct and focus choice onto the laser-paired sucrose rewards, overall motivation to lever press and consume sucrose pellets remained the same across groups (CaMKII ChR2: 90.5 ± 18.0 pellets; hSYN ChR2: 101.4 ± 89.2 pellets; eYFP: 111.3 ± 6.3 pellets; χ^2 (3) = 1.47, $p = 0.48$; Fig. 22e).

<u>No change in intensity of motivation by ACC neuron stimulation during progressive ratio test</u>

A progressive ratio (PR) breakpoint test assessed whether stimulating CC neurons changed the intensity of incentive motivation to obtain sucrose. Pairing CC neuron stimulation with the laser-paired sucrose reward failed to change the intensity of motivation for one reward

over another in any of the groups tested, even in CamKII ChR2 rats that previously biased responding for laser-paired sucrose reached similar breakpoints (CaMKII ChR2: *Laser-OFF:* 67.8 ± 10.0 breakpoint, *Laser-ON:* 81.7 ± 19.4 breakpoint, W= 7.00, Z = -0.73, p= 0.47; hSYN ChR2: *Laser-OFF:* 104.1 ± 14.6 breakpoint, *Laser-ON:* 141.1 ± 26.8 breakpoint, W= 15.00, Z = 0.95, p= 0.34; eYFP control: *Laser-OFF:* 143.1 ± 15.0 breakpoint, *Laser-ON:* 129.5 ± 20.9 breakpoint, W= 15.00, Z = -0.42, p= 0.67; Fig. 23a). Similarly, all groups tested made similar lever presses between Sucrose Alone and Sucrose + Laser options during progressive ratio tests (CaMKII ChR2: *Laser-OFF:* 330.8 ± 55.3 lever presses, *Laser-ON:* 384.7 ± 102.1 lever presses, W= 13.00, Z = -0.52, p= 0.60; hSYN ChR2: *Laser-OFF:* 534.8 ± 97.2 lever presses, *Laser-ON:* 685.0 ± 137.9 lever presses, W= 23.00, Z= -0.70, p= 0.48; eYFP control: *Laser-OFF:* 659.8 ± 106.9 lever presses, *Laser-ON:* 738.4 ± 87.6 lever presses, W= 15.00, Z = -0.42, p= 0.67; Fig 22b). This suggests that while CC neuron stimulations may be able to direct motivation onto particular rewards, the intensity or magnitude of motivation may not be directly controlled by CC neurons.

Cingulate hotspot neurons promote ‘wanting’ for laser.

We measured the incentive motivation value of laser ChR2 stimulation on its own, in the absence of any taste infusion or external reward using two laser self-stimulation tests: an active instrumental spout-touch task and a relative passive place-based self-stimulation task.

ACC hotspot neurons support spout-self-stimulation. In the spout-touch task, each instrumental touch on a designated empty waterspout earned a brief laser pulse of either 1-sec or 5-sec duration (depending on test day). Contacts to an alternative control spout produced nothing and served as a measure of baseline exploratory touches. Rats were considered to be ‘high self-

stimulators' if they earned greater than 50 laser illuminations and made at least twice as many contacts to the laser spout than the control spout during a 30-min session (Fig. 23). Rats were considered to be 'low self-stimulators' if they earned more than 10 but less than 50 illuminations per session, and made twice as many contacts on the laser spout relative to the control spout. Rats were considered to be 'failures to self-stimulate' if they made less than 10 laser spout contact and did not meet any of the above criteria. All rats were categorized on day 1 and retested for reliability on days 2 and 3.

Cingulate hotspot ChR2 rats self-stimulated in the spout-touch task (laser x virus interaction: $F_{1,22}$= 16.54, p = 0.0005). When CC hedonic hotspot rats could earn brief 1-s laser pulses (either 40 Hz or 20 Hz, depending on the day tested), between 56% and 75% of CC ChR2 rats met criteria for at least low levels of self-stimulation (40 Hz: high self-stimulation: 5.6%, low self-stimulation: 50.0%, no self-stimulation: 43.8%; 20 Hz: high self-stimulation: 18.8%, low self-stimulation: 56.3%, no self-stimulation: 25.0%). CC hotspot ChR2 rats made approximately 2x as many contacts to the laser spout relative to the control spout for 40 Hz laser illuminations (40 Hz: Control Contact: 17.6 ± 3.4, Laser Contacts: 38.4 ± 5.2; t_{22} = 6.72, d = 4.7, CI [-28.2, -13.3], p < 0.0001) and ~3x as many laser spout contacts compared to control spout contacts for 20 Hz laser illuminations (20 Hz: Control Contacts: 8.0 ± 1.7, Laser Contacts: 26.8 ± 5.6; t_{22} = 4.78, d = 4.5, CI [9.4, 28.2], p = 0.0002, n = 16 ChR2 rats).

When touches earned longer 5-s 40 Hz pulses similar to those used in taste reactivity tests to increase hedonic 'liking' reactions to sucrose ~56% of rats met criteria for at least low levels of self-stimulation (40 Hz 5-s: high self-stimulation: 5.6%, low self-stimulation: 50.0%, no self-stimulation: 43.8%; 20 Hz: high self-stimulation: 18.8%, low self-stimulation: 56.3%, no self-stimulation: 25.0%). CC hotspot ChR2 rats made ~4x as many contacts to the laser spout

compared to the control spout when they could earn 5-s 40 Hz laser pulses that previously increases hedonic 'liking' reactions to sucrose in the same rats (40 Hz 5-s: Control Contact: 4.5 ± 1.2, Laser Contacts: 19.3 ± 3.4; virus x laser interaction: $F_{1,21}$= 13.97, p = 0.0012, paired comparison: t_{21} = 5.05, d = 5.8, CI [7.7, 21.9], p = 0.0001, n = 16 ChR2 rats).

No differences in self-stimulation by laser frequency, sex, or virus type in ACC hotspot rats. Both 40 Hz and 20 Hz promoted equal levels of self-stimulation in CC hotspot ChR2 rats (laser x virus x frequency interaction: $F_{1,22}$ = 0.007, p = 0.94). CC ChR2 excitations also promoted similar levels of self-stimulation in male and female rats (40 Hz 1-s: $F_{1,14}$= 0.44, p = 0.520, n = 7 males, n = 9 females; 20 Hz 1-s: $F_{1,14}$= 0.08, p = 0.78, n = 7 males, n = 9 females). Finally, hSYN and CaMKII ChR2 rats self-stimulated at similar magnitudes (40 Hz 1-s: $F_{1,14}$= 3.39, p = 0.09, n = 10 hSYN rats, n = 6 CaMKII rats; 20 Hz 1-s: $F_{1,14}$= 1.78, p = 0.20, n = 10 hSYN rats, n = 6 CaMKII rats).

By contrast, ACC eYFP control rats never self-stimulated, making equal contacts to the laser and non-laser spouts regardless of the laser frequency or duration that could be earned (40 Hz 1-s: t_{22} = 0.019, p > 0.99; 20 Hz 1-s: t_{22} = 1.14, p = 0.53), and thus differed from ChR2 rats (40 Hz 1-s: $F_{1,22}$ = 15.16, p = 0.0008, n = 8 eYFP; 20 Hz 1-s: $F_{1,22}$ = 8.96, p = 0.0067, n = 8 eYFP; 40 Hz 5-s: $F_{1,21}$ = 13.97, p = 0.0012, n = 8 eYFP).

Cingulate hotspot neurons promote self-stimulation in the place-based task. In the place-based task, rats could earn laser illuminations by entering a designated chamber, or simply remaining in it while laser continued (40 Hz, 3-sec ON, 4-sec OFF). CC hotspot sites supported place-based self-stimulation. Hotspot ChR2 rats spent ~150% more time in the laser-paired chamber than in the alternative chamber without laser (Fig. 24; $F_{2,37}$ = 8.92, p = 0.0002). CC ChR2 rats also spent ~ 480% time longer in the laser delivering chamber than they had during

previous baseline tests without laser stimulation (t_{37}= 3.47, p=0.003, CI [77.8, 3983.9], d = 4.0*),* and ~170% more time in their laser delivering chamber than inactive eYFP control rats (t_{37}= 3.62, p=0.002, CI [107.9, 501.2], d = 5.6*)*. Both male (n =7) and female (n =9) CC ChR2 rats spent comparable time in the laser-delivering chamber (male: 210% ± 7%; female: 150% ± 2%; $F_{1,14}$ = 1.23, p = 0.29). Further, hSYN (n =10) and CamKII (n =6) had equal preferences for the laser-delivering chamber (hSYN: 160% ± 2%; CaMKII: 200% ± 8%; $F_{1,14}$ = 2.91, p = 0.11).

3.5 Discussion

Here we investigated the existence of a new hedonic hotspot within the mid cingulate cortex of rats. We provide evidence that sites within cingulate cortex have the capacity to enhance the hedonic impact of sucrose ('liking') in rats. The hedonic hotspot was a discrete region of an approximately 5 mm^3 strip of mid-to-caudal region of the cingulate cortex in rats. Our results show that ChR2 depolarization of neurons within this novel cortical hedonic hotspot effectively doubled affective 'liking' reactions elicited by pleasant tastes including sucrose and water.

The cingulate hedonic hotspot began within the same rostro-caudal axis of the caudal boundary of NAc transition into stria terminalis, with its rostral boundary beginning near areas slightly anterior to bregma and extended posteriorly to the caudal edge of the anterior region of mid cingulate cortex, near the rostral border of A24' (van Heukelum et al., 2020; Vogt & Paxinos, 2014). It remains an open question whether this reflects the true boundaries of the hedonic hotspot, especially in regard to the rostral boundary noted here. The possibility remains that more rostral areas we did not map might also be a part of the cingulate hedonic hotspot. Effectively almost all sites tested in the present study were concentrated along a 5 mm^3 stretch of cortical tissue, and very few sites rostral to this region were mapped. A single rat's virus

expression was located in more rostral portions of cingulate, near the caudal boundary of PFC. For this rat, optogenetic stimulation of ACC neurons generated mild (~130%) hedonic enhancements to sucrose 'liking' reactions, but this was only observed across a single laser frequency, and not replicated across multiple tests as in many other rats. Relatedly, our current results did not find evidence of a site of hedonic suppression that diminishes positive 'liking' reactions to sweetness in cingulate cortex. The hedonic hotspots in nucleus accumbens, ventral pallidum, OFC, and insula sit adjacent to oppositely valenced regions of the same larger structures where the same microinjections or optogenetic manipulations that enhance 'liking' within hotspot boundaries oppositely suppress 'liking' in these hedonic coldspot areas (Castro & Berridge, 2017; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005). Does this pattern of functional dissociation between 'liking' enhancements and hedonic suppressions not exist in the cingulate cortex? This remains an open possibility and future studies could map larger portions of ACC and help clarify this question.

Motivational 'wanting' anatomically overlaps with hedonic 'liking' enhancement regions. The same cortical sites that enhanced sucrose 'liking' reactions in hedonic hotspot ChR2 rats also supported incentive motivational 'wanting' effects in those same rats. This was evidenced by a directed and focused pursuit of instrumental responding for a laser-paired sucrose reward that ChR2 rats actively pursued over an identical sucrose reward that was never laser paired. Further, cingulate hotspot sites also supported laser self-stimulation in the spout-touch and place-based tasks, suggesting that even in the absence of an external reward, activations of CC neurons in the hedonic hotspot are rewarding. This finding keeps in line with previous hedonic hotspot studies suggesting mesocorticolimbic sites that effectively increase positive 'liking' reactions also produce increases in incentive motivation.

One question raised is whether regions in cingulate cortex that produce incentive motivational effects extend beyond the boundaries of the cingulate hedonic hotspot, as has been found in most other studies of hedonic hotspots (Castro et al., 2016; Castro & Berridge, 2014c, 2017; Ho & Berridge, 2013; Mahler et al., 2007; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005). Our current investigation did not fully map all cingulate regions. Thus, it remains possible that more rostral areas that were not explored here, including prefrontal cortical regions of ACC or other areas rostral to our concentrated placements would also modulate 'wanting'. Previous pharmacological, optogenetic, and chemogenetic studies in rodents implicate a larger span of cingulate cortex than tested here, including prefrontal regions, and more rostral sites in Area 24 of rodents in the control of motivated behaviors, although the directionality of motivational effects may conflict across studies (Hart et al., 2020; Khani et al., 2015; Koolhaas et al., 1977; Schweimer et al., 2005; Schweimer & Hauber, 2005, 2006a, 2006b). Further, in the other cortical hedonic hotspots, localized microinjections restricted to rostromedial OFC and caudal insula cortex doubled positive 'liking' reactions to sucrose while effectively all tested cortical sites, including caudal OFC, rostral insula, and prefrontal robustly enhance food voluntary food intake, well as laser self-stimulation shown in my studies in Chapter 2 (Baldo et al., 2016; Castro & Berridge, 2017; Giacomini et al., 2021, 2022; Mena et al., 2011, 2013; Selleck et al., 2015, 2018; Selleck & Baldo, 2017). Future studies could expand our understanding of the cingulate hedonic hotspot to better determine anatomical boundaries and better understand the motivational roles of subregions of cingulate cortex.

Are 'wanting' and 'liking' neurons in cortex dissociable by cell type subclassification? We observed individual differences in affective 'liking' enhancements and incentive motivational effects across the two distinct ChR2 groups tested. Laser stimulations successfully

enhanced positive 'liking' reactions to sucrose in both hSYN and CaMKII groups, but the magnitude of hedonic enhancement was always slightly larger in hSYN relative to CaMKII groups. These overall differences did not reach a statistically significant threshold, suggesting that both virus types were able to recruit increases in positive 'liking' reactions. The more striking difference we found was in the instrumental sucrose choice task, where CaMKII ChR2 rats preferred to earn sucrose paired with CC neuron activations relative to the lever that delivered non-laser-paired sucrose. By comparison, hSYN ChR2 rats showed no such bias for laser-paired sucrose and behaved similarly to control rats. Could it be that hSYN driven expression of ChR2 protein more preferentially expressed on 'hedonic controlling' neurons while CaMKII driven expression was found in different neuronal populations that more aptly control motivation? This question is difficult to answer, as neither viral strategy truly delivers much cellular specificity. Channelrhodopsin or DREADD expression driven by the CaMKII promoter is often used in cortical areas to more directly target excitatory neuron populations (Benson et al., 1992; X. Han et al., 2009; Hart et al., 2020; Jones et al., 1994; Liu & Murray, 2012; Nathanson et al., 2009; Peng et al., 2015; L. Wang et al., 2018), but mounting evidence suggests that CaMKII driven promoters may not be as selective as previously thought. For example, CaMKII is robustly expressed in striatal medium spiny neurons of rodents and birds (Chuhma et al., 2011; Hein et al., 2007; Klug et al., 2012), and has also been recently detected within inhibitory interneurons in mouse cortical areas (Keaveney et al., 2020). There may be indeed important differences in neuronal expression of ChR2 across our two groups that contributed to slight individual differences in our manipulations, and this question needs further clarification.

Cortical gains vs. loss of function. Studies of affective expressions including the present experiment support the hypothesis that cortical activity is sufficient to produce gains in hedonic function, by increasing positive 'liking' reactions to sweetness and other pleasant stimuli. This contrasts with the growing evidence that loss of function in corticolimbic areas does not fully abolish affective responses. Patients with massive cortical lesions are still drawn to pleasant foods, can adequately react to painful and fearful stimuli, and even self-report feelings of fulfillment and happiness (Damasio et al., 2013). Similarly, cortical lesions or complete decortication in rodents does not abolish affective 'liking' reactions nor impair motivation for food (King et al., 2015b; Wirsig & Grill, 1982b). Although we did not directly probe cingulate hotspot neuron inhibitions, it is conceivable that cingulate hedonic hotspot neuron control of 'liking' reactions keeps with the previous studies mentioned here, and our findings in Chapter 2, where OFC hedonic hotspot neuron inhibitions did not alter sucrose 'liking' reactions in rats. Thus, we propose that cortical control of hedonic impact likely follows a hierarchical organization, with cortical areas being able to perform gains of function that turn on each other and also the subcortical hedonic hotspots in rostrodorsal NAc medial shell and caudal ventral pallidum, supported by increases in Fos expression following hedonic hotspot activations (Castro & Berridge, 2017; Chapter 2). If cortical regions are lost, then this hierarchical control may be lost, but not the actual hedonic generators that are found subcortically.

Clinical implications. Better understanding of brain hedonic systems that control 'liking' reactions may carry important implications for how we understand affective dysfunctions that contribute to depression, schizophrenia, and other affective disorders (Morales & Berridge, 2020; Olney et al., 2018; T. E. Robinson & Berridge, 1993; Zald & Treadway, 2017). For example, it has been proposed that some individuals with depression have symptoms that more closely

match anhedonia, while others experience avolition, or loss of motivation to pursue rewards even if their hedonic capacity is not altered (McCarthy et al., 2016; Thomsen et al., 2015; Treadway et al., 2012, 2015; Treadway & Zald, 2011, 2013; J. Wang et al., 2015; Whitton et al., 2015). In recent years, the cingulate cortex has emerged as a target of interest in the treatment of affective disorders. The mechanism is still unclear, but deep brain stimulation therapies have been shown to alleviate certain negative affective states, such as chronic pain and other symptoms associated with pain disorders like depression (Boccard et al., 2014, 2016, 2017; Kringelbach et al., 2007). Cingulate cortex activity may also serve as a neural predictor of treatment outcomes in patients with depression (Arns et al., 2015; Hunter et al., 2013; Korb et al., 2011; Tian et al., 2020; Whitton et al., 2019), suggesting cingulate as an important role with the potential to alleviate negative affective states. It is still an open question whether hedonic hotspots may be potential targets for treatments of hedonic dysfunction, but a better understanding of brain control of hedonic systems could help clarify such questions.

Conclusions. Investigations of affective responses using taste reactivity studies have now identified brain hedonic hotspots that control 'liking' reactions across every major mesocorticolimbic lobe. These include subregions of rostrodorsal nucleus accumbens medial shell, caudolateral ventral pallidum, rostromedial orbitofrontal cortex, caudal insula, brainstem pons (Castro et al., 2016; Castro & Berridge, 2014c, 2017; Mahler et al., 2007; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005; Söderpalm & Berridge, 2000). Our current findings now add the cingulate cortex to this list of brain hedonic systems and help cement the idea that cortical driven contributions to hedonic processes are distributed throughout a network of emotional, motivational, and affective zones that have now been described in both humans and rodents.

3.6 Figures

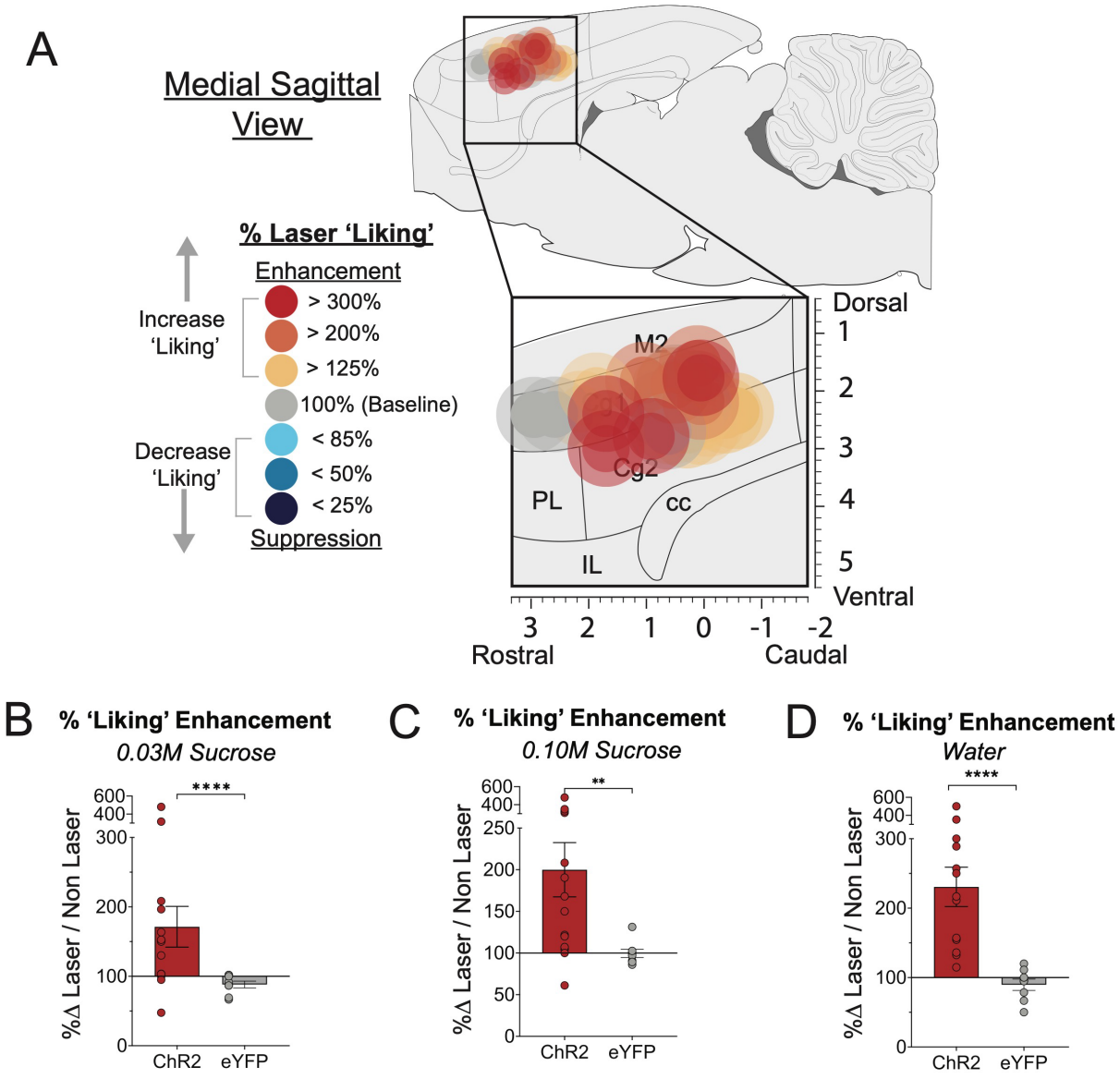

A
Medial Sagittal View
% Laser 'Liking'
Enhancement
Increase 'Liking'
> 300%
> 200%
> 125%
100% (Baseline)
Decrease 'Liking'
< 85%
< 50%
< 25%
Suppression
M2
Cg1
Cg2
PL
cc
IL
Dorsal
1
2
3
4
5
Ventral
3 2 1 0 -1 -2
Rostral
Caudal
B
% 'Liking' Enhancement
0.03M Sucrose
%Δ Laser / Non Laser
****
ChR2
eYFP
C
% 'Liking' Enhancement
0.10M Sucrose
%Δ Laser / Non Laser
**
ChR2
eYFP
D
% 'Liking' Enhancement
Water
%Δ Laser / Non Laser
****
ChR2
eYFP

Figure 20. Cingulate Cortex Sites that support hedonic enhancement for sucrose taste.

A) Localization of hedonic function map shows how optogenetic ChR2 neuron stimulation altered the hedonic impact of sucrose at reach individual rat's cortical site. Colors reveal magnitude of hedonic enhancement effects of ChR2 laser stimulation for each rat, measured laser induced changes in hedonic taste reactivity (positive 'liking' reactions) elicited by intraoral sucrose infusions. Each symbol placement indicates an individual rat's site. Color of symbol represents the within-subject behavioral change in hedonic reactions induces by ChR2 laser stimulation reflected as percent change from no laser control conditions measured in the same rats. (**B**) Laser ChR2 stimulations double hedonic 'liking' reactions for 0.03M sucrose, (**C**) 3x more concentrated 0.10M sucrose, and (**D**) room temperature tap water. Data presented as means and SEM. Anatomical abbreviations: M2: secondary motor cortex; PL: prelimbic cortex; IL: infralimbic cortex; cc: corpus callosum; CG1: cingulate cortex; CG2: cingulate cortex. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

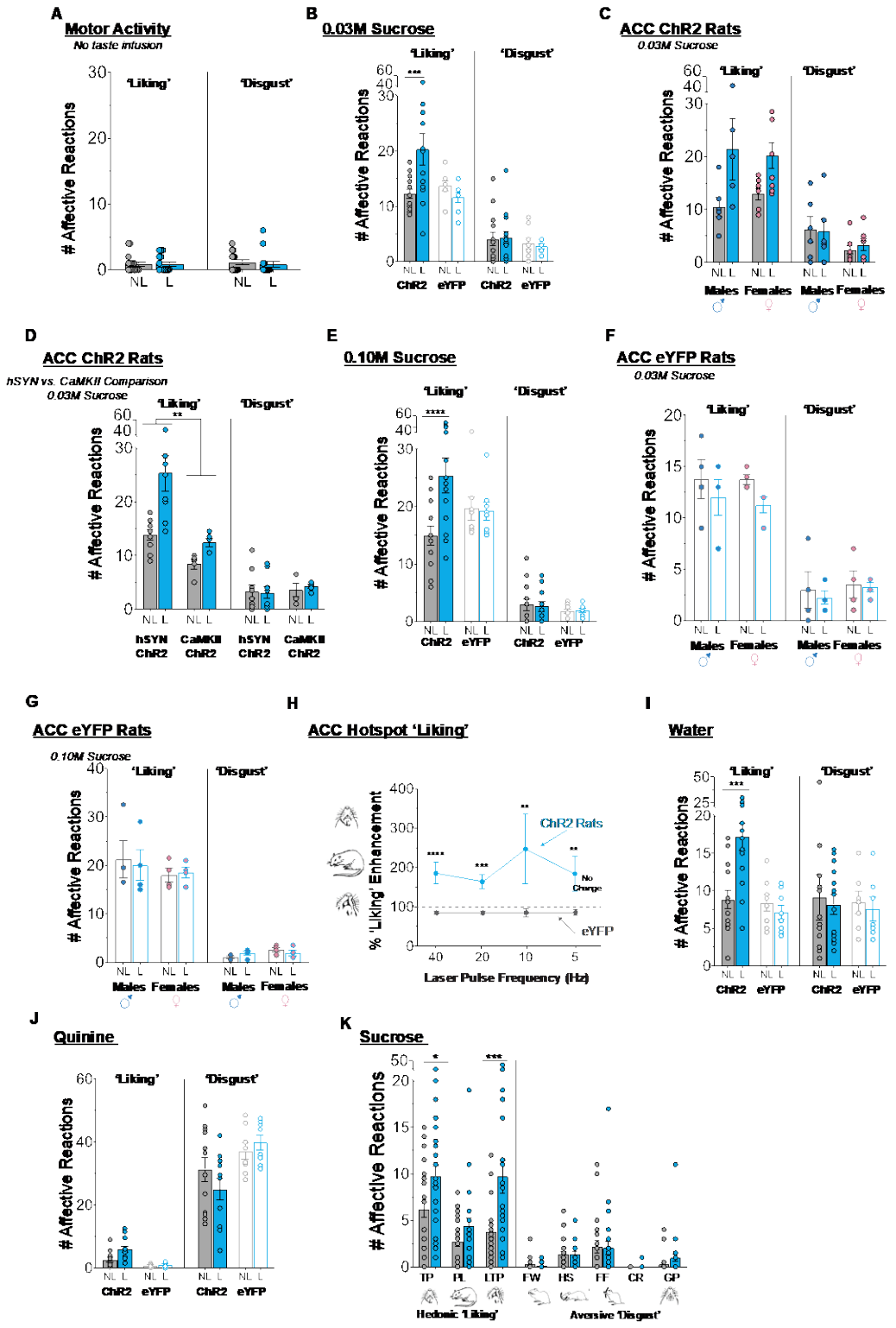

A
Motor Activity
No taste infusion
Affective Reactions
'Liking'
'Disgust'
NL L
B
0.03M Sucrose
ChR2 eYFP ChR2 eYFP
C
ACC ChR2 Rats
0.03M Sucrose
Males Females Males Females
D
ACC ChR2 Rats
hSYN vs. CaMKII Comparison
0.03M Sucrose
hSYN ChR2 CaMKII ChR2 hSYN ChR2 CaMKII ChR2
E
0.10M Sucrose
F
ACC eYFP Rats
0.03M Sucrose
G
ACC eYFP Rats
0.10M Sucrose
H
ACC Hotspot 'Liking'
% 'Liking' Enhancement
ChR2 Rats
No Change
eYFP
Laser Pulse Frequency (Hz)
I
Water
J
Quinine
K
Sucrose
TP PL LTP FW HS FF CR GP
Hedonic 'Liking'
Aversive 'Disgust'

Figure 21. Cingulate hotspot taste reactivity hedonic enhancement patterns.

Raw affective component counts elicited by various tastants following ACC hotspot neuron activations. **A)** No oromotor reactions observed from ACC hotspot ChR2 laser activations in the absence of taste infusions. **B)** Optogenetic laser activations increase hedonic 'liking' reactions to 0.03M sucrose in ChR2 rats, but not eYFP controls. **C)** Male and female ACC hotspot neuron activations produce similar laser-induced increase in positive 'liking' reactions to 0.03M sucrose. **D)** Similar hedonic enhancements in positive 'liking 'reactions between hSYN and CaMKII ChR2 rats. **E)** Laser activations increase hedonic 'liking' reactions to 0.1M sucrose in ChR2 rats, but not eYFP controls. **F)** No sex differences in affective expressions to 0.03M sucrose in eYFP control rats or in **G)** eYFP controls receiving 0.10M sucrose infusions. **H)** Multiple laser frequencies (5, 10, 20, 40 Hz; all 1 mW) increase positive 'liking' reactions to 0.03M sucrose in ChR2 rats. Data shown as within-subjects percent change from no laser baseline conditions. **I)** ACC neuron ChR2 activations increase hedonic 'liking 'reactions to tap water **J)** Aversive 'disgust' reactions to quinine are not altered by ACC hotspot neuron activations in ChR2 or eYFP rats. **K)** Positive hedonic taste reactivity components include: paw licks (PL), lateral tongue protrusions (LTP), and rhythmic tongue protrusions (TP). Negative 'disgust' components are: gapes (GP), face washes (FW), head shakes (HS), forelimb flails (FF), and chin rubs (CR). Relative neutral components are: rhythmic mouth movements and passive dripping (not shown). Scoring: each occurrence was counted for LTP, GP, HS, FF, and CR. TP was scored in 2-s bins, and PL was scored in 5-s bins. Bar graphs show absolute scores as mean and SEM for no laser baseline and ChR2 laser stimulation trials in the same ChR2 rats (grey bars: no laser trials; red and blue bars: laser trials) Laser ChR2 stimulation (40 Hz) in ACC hotspot sites significantly increased TP, LTP hedonic reactions to sucrose. All data shown as means ± SEM. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

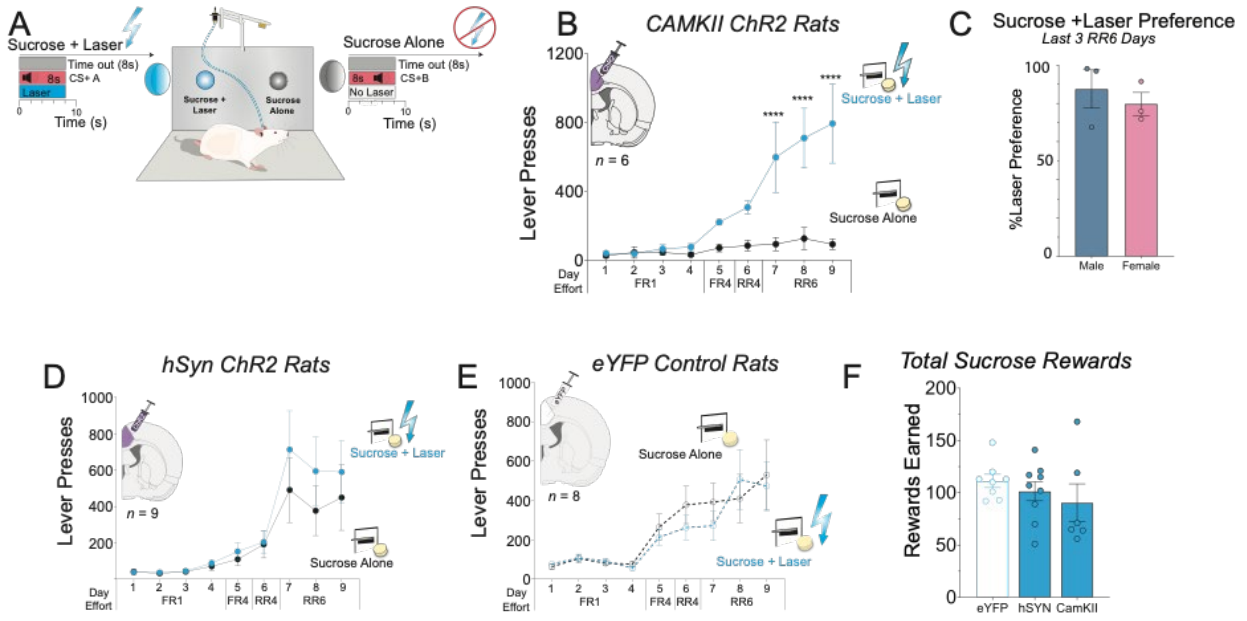


Figure 22. Cingulate hotspot CamKII ChR2 neuron stimulation biases sucrose motivation.

(A) Procedure for enhancement of laser-paired sucrose value in two-sucrose choice test. Two levers or nose ports protruded on either sucrose of a sucrose dish in center. Responses on either lever earned an equivalent sucrose pellet and a distinctive 8s sound that marked each lever/port's identity. One lever or port (Sucrose + Laser) was also paired with laser stimulation (40 Hz; 473 nm; 1-3 mW; 8-s duration). **(B)** Pairing ACC hotspot neuron stimulation in CaMKII ChR2 rats (n = 6) caused preference for laser-paired sucrose over sucrose alone, and reached a 6:1 ratio by Day 9. **(C)** Male (n = 3) and female (n = 3) CaMKII ChR2 rats equally prefer laser-paired sucrose. **(D)** ACC hSYN ChR2 rats (n = 9) and **(E)** eYFP control rats choose equally between sucrose options (n = 8). **(F)** Overall, all rats consume equal amounts of sucrose rewards. ****$p < 0.0001$. All data presented as means and SEM.

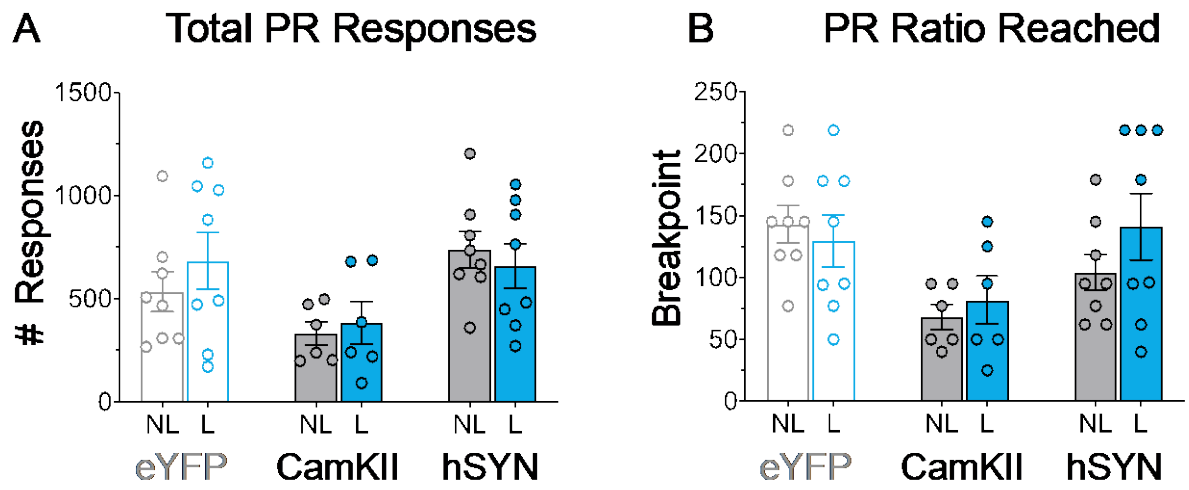
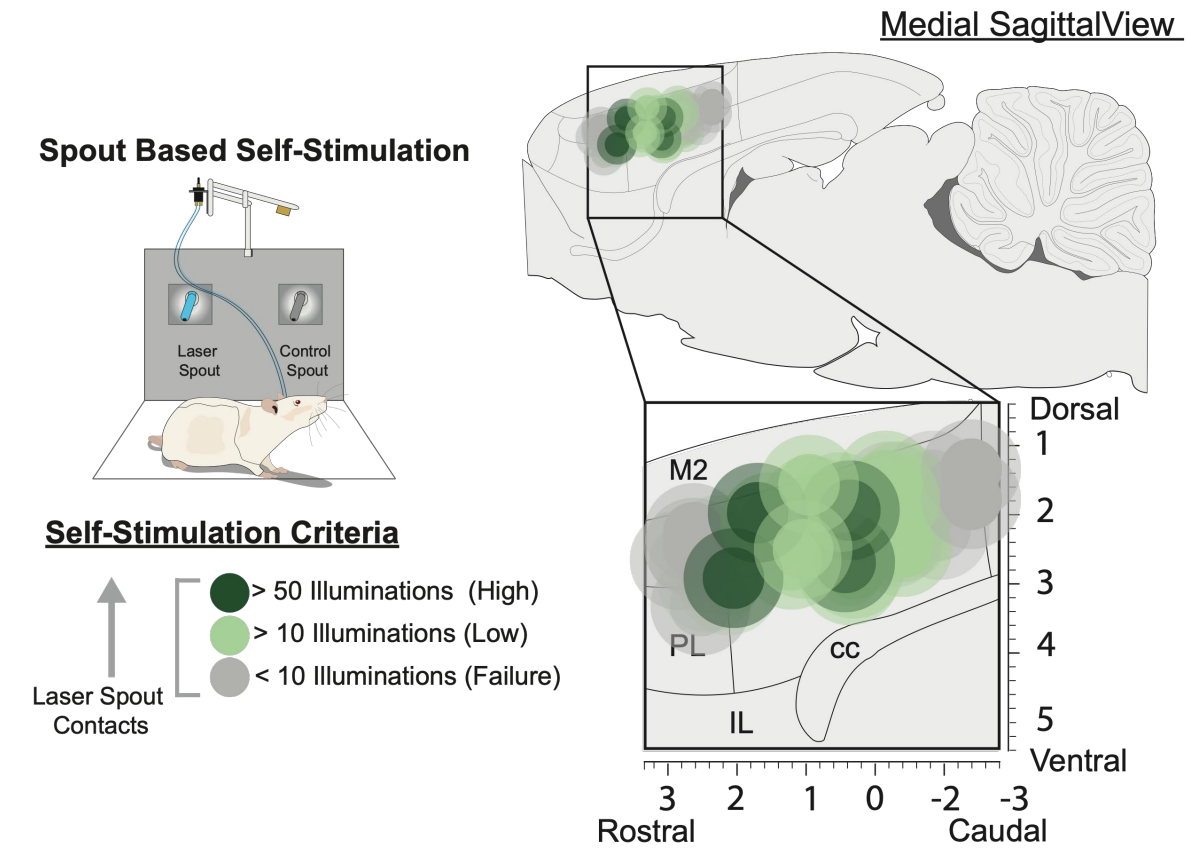


Figure 23. Cingulate cortex neurons fail to alter intensity of sucrose motivation.

(**A**) ACC hotspot ChR2 (CamKII: *n* = 6; hSYN: *n* = 8) and eYFP controls (*n* = 8) made similar responses, and **(B)** reached similar breakpoints for Sucrose + Laser and Sucrose Alone reward options during progressive ratio tests of motivation. All data presented as mean and SEM.


Medial SagittalView
Spout Based Self-Stimulation
Laser Spout
Control Spout
Self-Stimulation Criteria
> 50 Illuminations (High)
> 10 Illuminations (Low)
< 10 Illuminations (Failure)
Laser Spout Contacts
M2
PL
cc
IL
Dorsal
1
2
3
4
5
Ventral
3 2 1 0 -2 -3
Rostral
Caudal

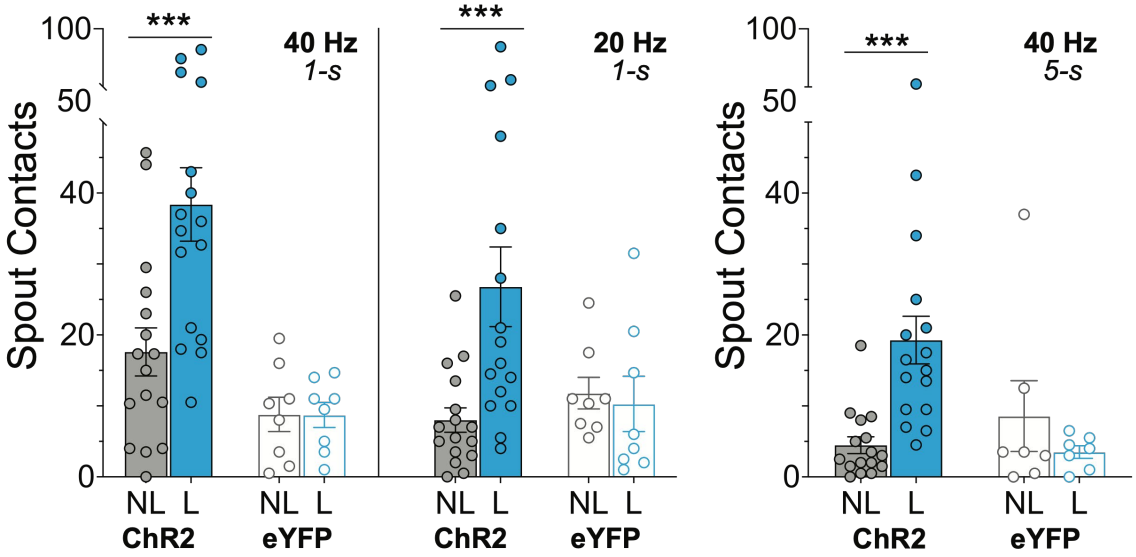
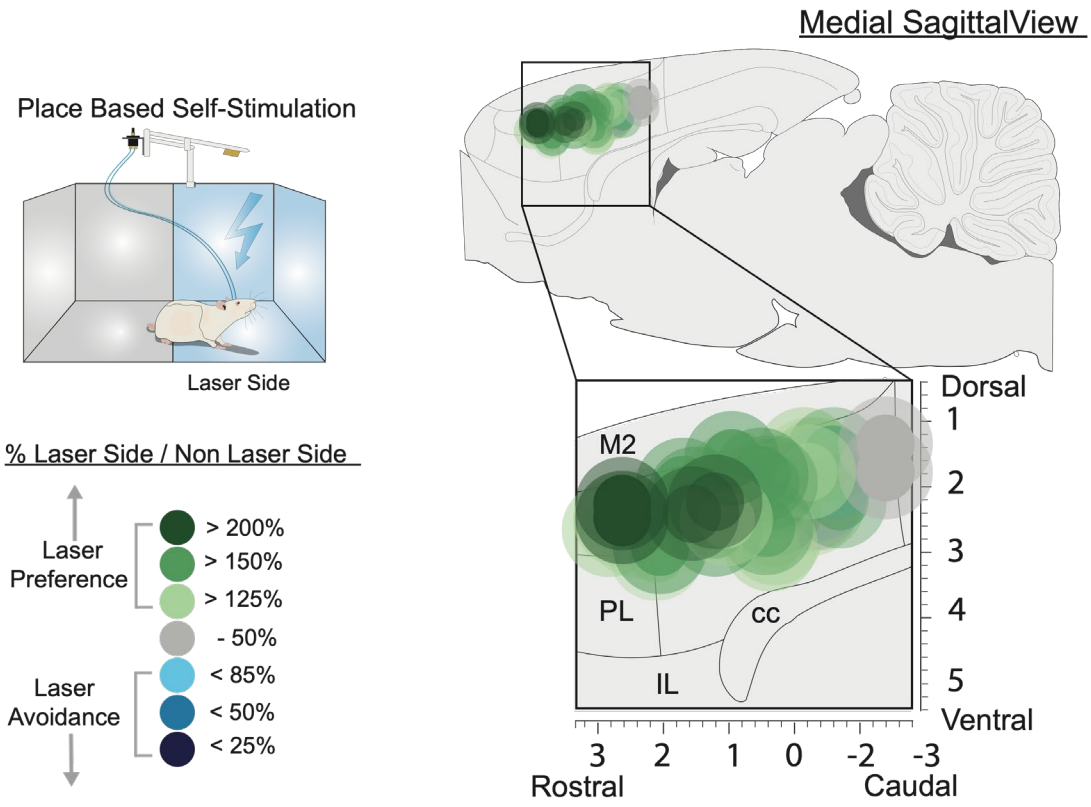

Spout Contacts
100
50
40
20
0
***
40 Hz
1-s
20 Hz
1-s
40 Hz
5-s
NL L
ChR2
eYFP

Figure 24. Cingulate hotspot sites that support laser self-stimulation in spout-touch task.

Optogenetic ChR2 stimulation at ACC hotspot sites support laser self-stimulation. Functional maps show instrumental performance to earn ChR2 laser stimulations at each cortical site on a spout-touch laser self-stimulation task (map based on 40 Hz, 1-s pulse data). Each symbol placement indicates an individual rat's channelrhodopsin expression. Color of symbols represents the level of self-stimulation criteria met by each rat (high self-stimulation (>50 illuminations earned): dark green; low self-stimulation (10 to 49 illuminations earned: light green; Failures to self-stimulate (<10 illuminations earned): grey). Anatomical abbreviations: M2: secondary motor cortex; PL: prelimbic cortex; IL: infralimbic cortex; cc: corpus callosum; CG1: cingulate cortex; CG2: cingulate cortex. Bottom shows total laser self-stimulations earned on spout-touch task by ChR2 rats with ACC hotspot sites (combined) at both 40 Hz (left) and 20 Hz (middle) 1-s laser frequencies, and 40 Hz 5-s laser frequencies (right). All data presented as mean and SEM.***$p < 0.001$.


Medial SagittalView
Place Based Self-Stimulation
Laser Side
% Laser Side / Non Laser Side
Laser Preference
> 200%
> 150%
> 125%
- 50%
Laser Avoidance
< 85%
< 50%
< 25%
M2
PL
cc
IL
Dorsal
1
2
3
4
5
Ventral
3 2 1 0 -2 -3
Rostral
Caudal

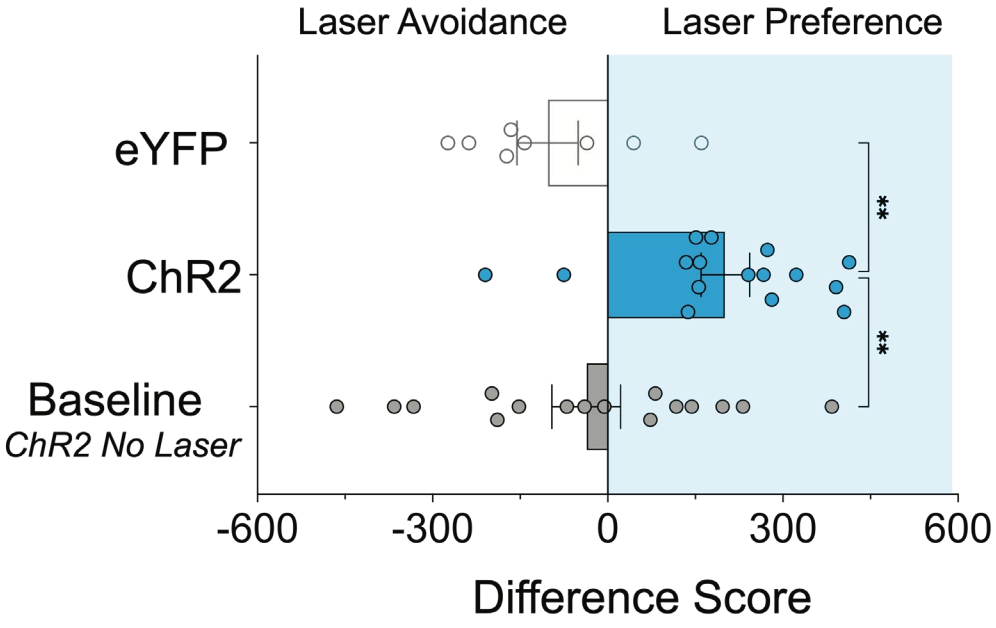

Laser Avoidance
Laser Preference
eYFP
ChR2
Baseline
ChR2 No Laser
**
**
-600
-300
0
300
600
Difference Score

Figure 25. Cingulate sites that support laser self-stimulation in place-based task.

Sites in mid-caudal ACC hotspot also supported place-based self-stimulation. Functional maps show preference for laser-paired side (green) or avoidance of laser-paired side (blue) during the place-based self-stimulation task. The color reflects the percent preference or avoidance for the laser-delivering side vs. non-laser-side in the same rats. Graph shows raw preference score during place-based self-stimulation tests for ACC ChR2 rats with sites in ACC hotspot (n = 16 ACC ChR2; n = 8 eYFP). All data presented as means and SEM; Preference score reflects time (s) spent on the laser side- time (s) spent on non-laser side in the same rats. Anatomical abbreviations: M2: secondary motor cortex; PL: prelimbic cortex; IL: infralimbic cortex; cc: corpus callosum; CG1: cingulate cortex; CG2: cingulate cortex. $^{**}p < 0.01$

Chapter 4 Bidirectional Control of Hedonic Impact and Incentive Motivation by Ventral Pallidum Neurons

4.1 Abstract

The ventral pallidum is a key node within mesocorticolimbic circuitry that controls both 'liking' and 'wanting' components of reward. It contains a hedonic hotspot 0.8mm^3 in volume where mu-opioid and orexin receptor agonists increase the number of positive 'liking' reactions rats elicit to sweetness during taste reactivity studies. The hedonic hotspot contained in VP is unique even among other hotspots that have been identified in the nucleus accumbens rostrodorsal medial shell, rostromedial orbitofrontal cortex, caudal insula, and brainstem pons. Unlike the other hotspots which only compute gains of hedonic function when stimulated, VP hotspot activity is necessary for normal affective reactions to pleasant stimuli. Lesions to VP hotspot neurons abolish normal 'liking' reactions to palatable tastes, and replace them with intense aversive 'disgust'. Previous loss of function studies in VP used large excitotoxin lesions or drug pharmacological manipulations to study loss of neuronal function in VP on 'liking' and 'wanting' for reward. Here we use optogenetic tools to deliver global neuronal inhibitions in either rostral or caudal VP, and demonstrate that only inhibitions in the most caudolateral portion of VP suppress sucrose 'liking' reactions and also increase the aversive impact of bitter tastes. Importantly however, neurons in VP are not heterogenous, and can be further subclassified into GABA, glutamate, and acetylcholine populations that are largely non-overlapping and thought to distinctly contribute to incentive motivation. Thus, we further probe VP neuronal contributions to 'liking' and 'wanting' by using GAD1-cre rats to either directly stimulate or inhibit VP^{GABA}

neurons across the rostrocaudal axis while testing rats on measures of 'liking' and 'wanting' for reward. We report that selective stimulation of VP^{GABA} neurons in posterior regions doubled hedonic 'liking' reactions to sucrose. By comparison, rostral VP^{GABA} neuron activations oppositely suppressed sucrose 'liking' reactions. Surprisingly, selective inhibition of caudal VP^{GABA} neurons suppressed sucrose 'liking' reactions, but did not enhance the aversive impact of sweetness as our previous general neuronal inhibitions in VP did. Finally, we report that while 'liking' control is dissociated between rostral and caudal VP^{GABA} neuron subregions, incentive motivational effects were observed uniformly across rostral and caudal sites. Optogenetic excitations of both rostral and caudal VP^{GABA} neurons increased incentive motivation while inhibitions oppositely suppress motivation. Our results support a critical role for ventral pallidum neurons in the control of 'liking' and 'wanting' and suggest GABA neuronal populations as bidirectional key mediators of these effects. Dysfunctions in mesolimbic circuitry that controls affective reactions may have important implications for the development of effective treatments for various affective disorders.

4.2 Introduction

Brain systems that control hedonic impact, or 'liking' have been identified in mesocorticolimbic systems using the taste reactivity test, which categorizes affective expressions elicited by various tastes into positive 'liking' and aversive 'disgust' (Berridge, 2000; Grill & Norgren, 1978c; Steiner, 1973). These 'hedonic 'hotspots' are small pleasure generating subregions located in the rostrodorsal quadrant of the nucleus accumbens medial shell (NAc), rostromedial orbitofrontal cortex (OFC), caudal insula cortex, parabrachial nucleus of the pons (PBN), and in caudolateral ventral pallidum (VP)(Castro et al., 2016; Castro & Berridge, 2014c; Ho & Berridge, 2013; Mahler et al., 2007; Peciña & Berridge, 2005; K. S. Smith & Berridge,

2005; Söderpalm & Berridge, 2000). Drug microinjections of the mu-opioid receptor agonist DAMGO, or orexin agonists into hedonic hotspot areas potently increase the number of positive 'liking' reactions rats emit in response to sweet tastes such as sucrose.

Like the other hotspots in NAc, PBN, OFC, and insula, posterior VP neuron stimulations can produce hedonic gains of function by increasing positive 'liking' reactions. For example, DAMGO and orexin microinjections into posterior VP regions nearly double the number of positive 'liking' reactions (Ho & Berridge, 2013; K. S. Smith & Berridge, 2005). These hedonic enhancing effects are localized to caudal VP, as the same drug microinjections into medial and anterior regions oppositely suppress 'liking', denoting the location of an oppositely valenced hedonic 'coldspot' (K. S. Smith & Berridge, 2005). The functionally specialized hedonic node contained in posterior VP is unique, even among the rest of the hedonic hotspots. Unlike the other hotspots, the posterior VP is also necessary for the normal production of affective responses. Excitotoxin lesions and GABA agonist pharmacological inactivations in caudal, but not rostral VP (or in NAc hotspot), not only suppress normal 'liking' to sweetness, but they also result in active 'disliking' with rats emitting primarily aversive 'disgust' reactions as if a bitter, sour, or otherwise unpleasant taste had been introduced into their mouth (Cromwell & Berridge, 1993; Ho & Berridge, 2014; Khan et al., 2019).

In addition to its role in 'liking', VP also plays crucial roles in incentive motivation or 'wanting'. Studies using microinjection techniques have shown that unlike hedonic localization, which is restricted to posterior VP, the entire rostro-caudal extent of VP neurons can exert some incentive motivational effects. For example, drug microinjections of glutamate agonists can potentiate consumption of palatable foods and solutions, while GABA agonists suppress intake (Ho & Berridge, 2014; Khan et al., 2019; Shimura et al., 2006; K. S. Smith & Berridge, 2005;

Stratford et al., 1999). However, some functional segregation may also exist between rostral and caudal VP control of motivation. For example, inhibition of rostral VP neurons that project to VTA can reduce cue-induced reinstatement of cocaine seeking, while inhibition of caudal VP neurons suppresses cocaine-primed reinstatement (Mahler et al., 2014). Importantly, however, VP neurons are not homogenous, and can be subclassified based on their cholinergic, glutamatergic, and GABAergic identities, which are largely nonoverlapping (Faget et al., 2018; Fallon et al., 1983; Tooley et al., 2018). Studies in mice suggest that VP control of motivation may differ based on the underlying cell populations. For example, optogenetic photostimulation of glutamatergic, VP^{Glu}, which tend to be most robustly expressed in rostral and mid VP regions, caused mice to avoid a laser-paired place (Faget et al., 2018; Tooley et al., 2018). By comparison, VP^{GABA} neurons promote self-stimulation and motivation for intravenous opioids (Faget et al., 2018; Farrell et al., 2022). Whether VP functional specialization, both in its control of 'liking' and 'wanting' can be explained solely by VP^{GABA} vs. VP^{Glu} neuron distinctions, or also potentially due to differences in VP^{GABA} neuron function along its rostro-caudal gradient remains an open question.

The use of pharmacological stimulations in hedonic hotspots to control 'liking' reactions in VP raises the question of whether hedonic hotspot effects are simply artifacts and limited to neurochemical stimulation rather than robust neurofunctional processes controlled by ventral pallidum neurons. Here we aimed to 1) provide independent confirmation of VP necessity in exerting hedonic control by using an optogenetic approach to inhibit (iC++ and SwiChR++) general VP neuronal populations to determine whether this reversible loss of function in VP would alter 'liking' reactions to sweet sucrose and bitter quinine solutions, and 2) to determine the amount of neuroanatomical overlap between these effects and VP control of incentive

motivation, here measured as voluntary intake of palatable chocolate candies. Given the proposed positive incentive role of VP^{GABA} neurons in mice, we further sought to probe VP control of 'liking' vs 'wanting' by using GAD1-cre rats (Sharpe et al., 2017) to 3) selectively activate (via ChR2) or inhibit (via iC++) VP^{GABA} neurons along rostral and caudal subregions determine effects on 'liking' vs 'wanting'. In support of this optogenetic effort, we note that others have reported optogenetic ChR2 effects in VP elicited positive affective expressions during voluntary licks and bouts of ingestion in mice (Dolensek et al., 2020; Vachez et al., 2021). Our results suggest that neurochemically mapped VP sites of hedonic control are indeed able exert hedonic control when manipulated via optogenetic methods. General caudal VP inhibitions suppressed sucrose 'liking' reactions and replaced them with aversive 'disgust' whole inhibiting neuronal populations across rostral and caudal subregions decreased food intake in rats. Finally, report evidence that VP^{GABA} neurons in rats may a bidirectional role in 'liking' and 'wanting' for sweetness. Site specific activations in posterior VP^{GABA} neurons doubled positive affective expressions while inhibitions decreased 'liking'. By comparison, both rostral and caudal VP^{GABA} neuron subregions bidirectionally controlled incentive motivations. Our results provide triangulating evidence that VP is a crucial node in the control of hedonic impact and incentive motivation, and extend our knowledge of brain mechanisms of 'wanting' and 'liking' by providing evidence that VP GABA neuron populations are crucial in these processes.

4.3 Materials and Methods

Animals

Male and female Sprague Dawley rats (*n = 64; n = 38 female, n = 26 male)* were used for all experiments testing general VP neuron manipulations. For experiments testing VP^{GABA} neuron function, male and female GAD1:Cre rats (*n = 85; n = 43 female, n = 42 male)* bred on a

Long Evans background were used (Sharpe et al., 2017). All rats weighed 250- 450g at surgery and were group housed in same-sex rooms maintained at constant temperature (21 º C) on a reverse 12-h dark/light cycle at the University of Michigan. Rats were given *Ad libitum* access to food and water throughout the duration of experiments. All experimental procedures were approved by the Committee on the Use and Care of Animals at the University of Michigan (IACUC).

Surgery

Optogenetic Virus Infusion

Rats were anesthetized with isoflurane gas (4-5% induction, 1-2% maintenance) and received atropine (0.04 mg/kg; i.p.; Henry Schein) before surgery. Animals were placed into a stereotaxic apparatus (David Kopf Instruments) and given bilateral virus microinjections (0.75 µL) of an optogenetic virus.

Some rats received a nonselective chloride conducting inhibitory virus (either AAV5-SwiChR++-eYFP, or AAV5-hSYN-iC++-eYFP; Stanford Vector Core) (Berndt et al., 2016) or inactive control virus that lacked either of the inhibitory virus genes (AAV5-hSYN-eYFP; UNC Vector Core, Chapel Hill, NC) targeted at distributed sites of the ventral pallidum (SwiChR+; *n = 20;* iC++: *n* = 26; eYFP: *n* = 18). For cre-dependent studies of GABA neuron function in VP, GAD1-cre rats received a cre-dependent excitatory ChR2 virus (AAV5-Ef1a-DIO-ChR2-eYFP; UNC Vector Core) or inactive control virus (AAV5-Ef1a-DIO-eYFP; UNC Vector Core) targeted at GABA neurons in the ventral pallidum (ChR2*: n* =42; eYFP: *n* = 14). A separate group of GAD1-cre rats received a cre-dependent inhibitory iC++ virus (iC++*: n* = 13; AAV5-Ef1a-DIO-iC++-eYFP; Stanford Vector Core) or inactive control virus (eYFP*: n* = 16; AAV5-Ef1a-DIO-eYFP; UNC Vector Core).

At all sites, the 0.75 µL volume of virus was infused per side over a 7.5-minute period at a constant rate of 0.1 µL/min. Following virus infusion, the microinjector was subsequently left in place for an additional 10 min to allow for virus diffusion. Virus microinjections were aimed to be bilaterally identical within a single rat and staggered across individuals so that the group's sites filled the entire rostral-caudal extent of the ventral pallidum. VP coordinates ranges from +0.96 mm AP to -0.96 mm AP from bregma. After surgery, cefazolin (100 mg/kg, s.c.; Henry Schein) was administered to prevent infection, and carprofen (5 mg/kg, s.c.; Henry Schein) given for post-operative pain relief. Carprofen and cefazolin were repeated at 24-h and 48-h post-operation.

Oral Cannula Surgery and Fiber Optic Implantation

At least three weeks after the initial virus infusion surgery, rats were re-anesthetized with isoflurane as described above for implantation of intracranial optic fibers and of bilateral oral cannulas, which allowed for direct oral infusions of sucrose or quinine. Each oral cannula (polyethylene-100 tubing) entered the upper cheek just lateral to the secondary maxillary molar, ascended beneath the zygomatic arch, and exited the skin at the dorsal head, where it was secured with skull screws and a dental acrylic headcap. In the same surgery, rats were implanted with bilateral optic fibers (200 µm), aimed to place each fiber tip 0.3 mm dorsal to the rat's bilateral virus microinjection sites, and anchored with the same acrylic headcap. Cefazolin and carprofen were again administered and repeated post-operatively as above. All rats were allowed to recover for 1 week prior to behavioral testing.

Behavioral Procedures

Taste Reactivity Testing

Taste Reactivity testing was used to measure affective 'liking' reactions to various tastes (Berridge, 2000; Grill & Norgren, 1978c). Each rat was habituated to the test chamber for 30 minutes and given oral infusions of sucrose before behavioral testing occurred. Sprague Dawley

rats were habituated with 1% sucrose, while GAD1-cre rats were habituated with 3% sucrose, as pilot studies indicated higher sucrose concentrations were needed to reach a baseline positive 'liking' score during taste reactivity studies. During subsequent taste reactivity tests, animals were placed in a clear round chamber set above an angled mirror to visualize affective orofacial reactions (positive 'liking' vs. aversive 'disgust' patterns) elicited by oral infusions of various solutions (hSYN Inhibitory Experiments: 0.03M and 0.10M sucrose and $3x10^{-}$M quinine; GAD1-cre experiments: 0.10M sucrose and $3x10^{-}$M quinine). Orofacial reactions were videorecorded through a close-up lens facing an angled mirror underneath the transparent floor, positioned to capture a clear view of the mouth and face. Solutions were infused via surgically implanted oral cannulas directly into the mouth of the animal at a rate of 1 mL/min for a total of 1-min through infusion lines made of PE-50 tubing connected to a PE-10 delivery nozzle. The rate of infusion was controlled by an automated infusion pump. Only one type of taste (sucrose, water, or quinine) was tested per day for a total of 2x 1mL infusions, either with laser stimulation or without laser stimulation as a within-subject baseline (Berridge, 1991; Cabanac & Lafrance, 1990). For ChR2 rats receiving optogenetic stimulation, we tested rats at low laser intensity (1-2 mw) and mid-high laser intensity (5-7 mW) at the same laser frequencies (40 Hz, 5-s ON/5-s OFF). For iC++ inhibition experiments, optogenetic laser inhibitions were delivered by 2-4 mW constant illumination cycled 8-s ON/ 8-s OFF). For inhibition experiments using the step function opsin SwiChR++, brief 1-s, 2-4 mW constant illumination laser pulses were cycled 1-s ON/ 15s-OFF). Each laser parameter was administered at least twice for each rat in separate daily tests.

Taste Reactivity Scoring

Videos were scored subsequently for positive hedonic 'liking' reactions, aversive 'disgust' reactions, and neutral taste reactivity components in slow-motion at speeds ranging

from frame-by-frame to 1/5th normal speed using The Observer Software (Noldus; Leesburg, VA). Positive hedonic or 'liking' responses were considered to be: lateral tongue protrusions, paw licks, and rhythmic midline tongue protrusions. Aversive 'disgust' reactions were: gapes, forelimb flails, head shakes, face washes, chin rubs, and paw treading. Neutral responses (i.e., relatively uncoupled from hedonic impact) were: passive dripping of solution out of the mouth, rhythmic mouth movements, and grooming. A time-bin scoring system was used to ensure each type of affective reaction contributed equally to the overall affective score (Berridge, 2000; Castro et al., 2016; Castro & Berridge, 2014c, 2017). Rhythmic mouth movements, paw licks, passive dripping, and grooming were all scored in 5-s time bins, because these behaviors typically are emitted in bouts of relatively long duration. Any emission of these behaviors up to 5-sec was counted as a single occurrence; emissions of 5-sec to 10-sec counted as two occurrences, etc. Midline tongue protrusions and paw-treading were scored similarly, but in 2-s bins, as typically emitted in shorter bouts. Lateral tongue protrusions, gapes, flails, headshakes, and chin rubs were counted as discrete events every time they occurred, because these can occur singly or in several brief repetitions. A total positive hedonic (i.e., 'liking') score was then calculated by combining component scores of rhythmic tongue protrusions, paw licks, and lateral tongue protrusions. A total negative aversive (i.e., 'disgust') score was calculated by combining gapes, forelimb flails, head shakes, paw treading, face washes, and chin rubs (Berridge, 2000; Castro & Berridge, 2017).

Laser Self-Stimulation Tasks

Place-based self-stimulation/aversion. To test whether laser stimulation by itself, in the absence of any taste infusion, had positive incentive value, laser self-stimulation was assessed in two different tasks. First, a place-based self-stimulation task, similar to that used in early electrical brain-stimulation reward studies (Olds & Milner, 1954; Valenstein & Meyers, 1964)

allowed rats to earn laser illuminations by entering a particular chamber in a 2-chamber apparatus. Each side of the chamber was marked by a distinctive floor surface and visual patterns on walls. Entry into the designated laser chamber triggered onset of laser stimulation or inhibition (For ChR2 experiments: 1-2 mW or 5-7 mW in separate rats at 40 Hz cycled 5-s ON/ 5-s OFF) Laser illumination continually cycled as long as the rat remained within the designated laser chamber. Exit from the chamber terminated laser pulses. Entry into the other chamber produced nothing. One habituation day was conducted during which no laser was administered in either compartment as served as a within-subject baseline. This was followed by 3-days of testing (15-min sessions). The side on which the laser-paired chamber was located was counterbalanced across animals, but remained constant for each rat across all testing days.

Spout-based self-stimulation. A second active-response 'spout-touch' laser self-stimulation task allowed rats to earn brief laser illuminations each time they touched a particular one of two empty metal drinking spouts, positioned 5 cm apart on the wall of a Med-Associates operant chamber (Fairfax, VT). One spout was arbitrarily designated as the active 'laser spout', and each touch on it earned either a 1-s or 5-s duration bin (depending on trial) (ChR2 rats: 1-2 mW or 5-7 mW at 40 Hz). Touches on a second inactive spout produced nothing and contacts on it simply served to measure baseline levels of exploratory touching. Spout assignments were balanced across rats. Each combination of laser parameters was repeated on 3 consecutive days of self-stimulation (30-min sessions, laser spout assignment counterbalanced across rats).

Unconditioned Food Intake Test

Rats were habituated to the food intake testing chamber for 4-days before undergoing 1-hr tests of voluntary food intake. Prior to the start of each intake test, a pre-weighed 20-g quantity of palatable milk chocolate candies (M&Ms) were placed in a Plexiglas chamber (23 cm x 20 cm x 45 cm). The floor was covered with approximately 1-cm of corncob bedding. On laser

tests, pulses of blue light were delivered (iC++ rats: 2-4 mW constant illumination cycled at 8-s ON 8-s OFF; SwiChR++ rats: 2-4 mW constant illumination cycled 1-s on / 30-s OFF) during the hour of testing. Chocolates were re-weighed after the test, and the amount consumed by each rat was calculated. Rats were tested a total of 4 days (2 days with laser delivery and 2 Days no laser).

Instrumental Sucrose 2-Choice Task

Using an instrumental two-choice task, we assessed whether pairing ChR2 stimulation or iC++ inhibition with VP neurons with one option for earning sucrose pellets (Laser + Sucrose) made that reward option either more or less preferred than an alternative sucrose option that was not accompanied by laser (Sucrose Alone) (Baumgartner et al., 2021; M. J. F. Robinson et al., 2014). The chamber contained two retractable illuminated levers mounted in the wall. Pressing one lever (Sucrose + Laser) delivered a sucrose pellet plus 8-sec illumination of blue laser (473 nm) stimulation (ChR2: 40 Hz/15 ms pulse; iC++: Constant illumination; 8-s duration) accompanied by a distinct auditory cue (white noise or tone). Pressing on the alternative lever (Sucrose Alone) delivered a single sucrose pellet accompanied by a different 8-sec auditory cue, but no laser illumination. Assignment of levers and sound cues was counterbalanced across rats but remained constant for each rat. At the beginning of each instrumental session, rats received a single-choice trial in which one lever was presented alone, and pressing it delivered its customary reward (either Sucrose Alone or Sucrose + Laser). Then that lever was retracted, and the alternative lever was presented, which delivered its other customary reward. These two single-choice trials were then repeated another time and served to remind the rat every day of each lever's associated reward. Then both levers were presented simultaneously, and remained available for the remainder of the 30-min sessions, allowing rats to repeatedly chose freely between the two options. A total of 9 daily 2-choice sessions were conducted at as schedules of

reinforcement became moderately more demanding over days (FR1, FR1, FR1, FR1, FR4, RR4, RR6, RR6, RR6).

Progressive ratio test of breakpoint effort

A progressive ratio or breakpoint test was subsequently used to assess the effect of VP^{GABA} neuron laser stimulation or inhibition on the intensity of incentive motivation to obtain sucrose reward. On one day, rats were tested with only the Sucrose + Laser lever available, and laser illuminations were paired with each earned sucrose pellet (ChR2: 40 Hz, 15 ms pulse, 8-s duration; iC++: Constant illumination, 8-s). On another day, only the Sucrose Alone lever was available, which earned its customary sucrose pellet without laser illumination. On both days, the effort requirement to earn a next sucrose pellet increased after each reward, following an exponential progression [$PR = 5e^{(reward\ number \times 0.2)}] - 5$ rounded to the nearest integer]. The maximal effort (breakpoint) and total number of presses by each rat were compared across Sucrose + Laser vs Sucrose Alone days (order counterbalanced across rats).

Laser-paired Aversive Shock Rod

We paired voluntary encounters with an aversive 'shock rod' (0.55 mA shock measured via ammeter) with VP^{GABA} neuron stimulation (only in GAD1-cre rats expressing ChR2) to determine the effect of VP^{GABA} neuron manipulations with a negatively valenced outcome (Treit et al., 1981; Warlow et al., 2020). In this situation, all touches to the shock rod were voluntary, as animals could move freely within the chamber and it could choose to avoid shocks. In that sense, instrumental shock pursuit would be similar to instrumental pursuit of laser-paired sucrose or laser stimulation, but with a negatively valenced outcome. The shock rod (1.5 x 1.5 x 9 cm core, wrapped with electrified wire) protruded 9 cm into the left side of a Plexiglas chamber containing approximately 4cm of corn cob bedding (chamber: 38-cm width × 38-cm length × 48-cm height; bedding: Bed'O'Cobs, Andersons Inc., Maumee).

Touching the shock rod delivered a shock that was maintained as long as contact was maintained. Touches to the shock rod were never forced for any rats, but each rat touched the rod at least once while exploring the chamber. A video camera recorded behavior throughout each session for subsequent off-line analysis. Laser stimulation was given when the rat was within 2 cm of the shock rod (ChR2: 40Hz; triggered with MATLAB program). Animals were tested once per day, with experiments lasting 20 minutes or until the rat received 20 shocks. A total of four test days were conducted. The first two days (Days 1-2), the shock rod was paired with laser stimulation. On Day 3 the laser-pairing was extinguished so that all contacts with the shock rod resulted in shock alone. This was followed by a single day (Day 4) where the laser was reinstated, and all contacts resulted in shock + laser stimulation.

Immunohistochemistry and Histology

Beginning 75-min prior to euthanasia, a final laser delivery session was given to all rats. For inhibition experiments, laser was administered with same parameters used in the taste reactivity tests (iC++: 2-4 mW; constant illumination 8-sON/8s-OFF; SwiChR2++: 2-4 mW; constant illumination cycled 1-s ON/ 30-s OFF; 30-min session). For VP^{GABA} neuron ChR2 experiments, GAD1-cre rats received a final sucrose 2-choice instrumental session. This final laser stimulation was given to a) induce local Fos plumes around optic fiber tips that would indicate the anatomical spread of local neuronal stimulation/inhibition of general neurons or GABA neurons at the tested VP site and b) potentially recruit activation of distant brain circuitry that mediated optogenetic effects on hedonic reactions (Baumgartner et al., 2021; Cole et al., 2018; Warlow et al., 2020).

Following the final laser session, rats were deeply anesthetized with a lethal dose of sodium pentobarbital (150-200 mg/kg) and transcardially perfused with PBS followed by 4% PFA. Brains were removed and post-fixed in 4% PFA for 24-h and then transferred to a 25%

sucrose solution for at least two days. Tissue was coronally sectioned at 40 micrometers using a cryostat (Leica), slices were processed for GFP and cFos immunohistochemistry, and imaged using a digital camera (Qimaging) and fluorescence microscope (Leica). For immunohistochemistry, coronal sections were rinsed for 10 min in 0.1 M sodium phosphate buffer three times, then blocked in 5% normal donkey serum / 0.2% triton-X PBS solution for 60 min and incubated overnight in a polyclonal rabbit anti-cfos igG primary antibody (1:2500; Synaptic Systems) and chicken polyclonal anti-GFP igY primary antibody (1:2000; Abcam). Tissue was again rinsed three times in 0.1M NaPb for 10 min followed by 2-h in biotin-SP-conjugated donkey anti-rabbit (1:300; Jackson ImmunoResearch) secondary antibody and AlexaFluor-488 donkey anti-chicken secondary antibody (1:300; Jackson ImmunoResearch). Tissue was rinsed three times in 0.1M NaPB for 10 min followed by1.5-h in streptavidin-conjugated Cy3 (1:300; Jackson ImmunoResearch). Brain sections were mounted, air-dried, and cover-slipped with anti-fade Pro-long gold (Invitrogen).

Fos-stained brains will eventually be used for analysis of Local Fos plumes surrounding the fiber optic and expression of Fos in mesocorticolimbic structures in the eventually published manuscripts that arise from these chapters. Virus localization was used to construct maps of localization of function for all experiments (Baumgartner et al., 2020, 2021; Castro & Berridge, 2017; Cole et al., 2018; Ho & Berridge, 2013; K. S. Smith & Berridge, 2005; Warlow et al., 2020). Virus localization sites were plotted onto corresponding maps using a rat brain atlas (Paxinos & Watson, 2013).

Statistical Analyses

Taste reactivity tests, self-stimulation, instrumental 2-choice tests, and food intake tests were analyzed using mixed ANOVAs followed by t-tests for individual comparisons with a

Bonferroni correction. Friedman's two-way ANOVAs were used for nonparametric tests, followed by Wilcoxon sign-ranked tests. Significance was set at $p < 0.05$.

4.4 Results

Dissociable rostral and caudal VP neuron contributions to affective expressions: general neuronal inhibitions

No detectable motor effects of laser on spontaneous orofacial reactions. In the absence of any oral infusion, laser inhibition in VP hSYN IC++ rats failed to induce detectable orofacial movements any VP site. Neither positive hedonic reactions (Rostral VP: $M = 0.9$, $SEM = 0.3$; Caudal VP: $M = 0.2$, $SEM = 0.1$) nor negative 'disgust' reactions (Rostral VP: $M = 0.4$, $SEM = 0.2$; Caudal VP: $M = 1.1$, $SEM = 0.5$, Fig. 26; Rostral VP: $F_{1,13} = 2.14$, $p = 0.16$, $n = 14$; Caudal VP: $F_{1,12} = 0.65$, $p = 0.44$, $n = 13$; Fig. 27) were elicited by iC++ laser inhibitions of oral infusions indicating that VP neuron inhibitions did not directly cause motor reactions.

A site of intense sucrose 'disgust' generated by posterior ventral pallidum inhibitions. At iC++ sites within the caudolateral subregion of VP, which has been previously identified as a hedonic hotspot where mu-opioid receptor agonists and orexin agonists increase positive 'liking' reactions to sucrose (Ho & Berridge, 2013; K. S. Smith & Berridge, 2005), and where excitotoxin lesions and pharmacological inactivations oppositely suppress hedonic impact (Cromwell & Berridge, 1993; Ho & Berridge, 2014; Khan et al., 2019), optogenetic inhibitions reduced the overall number of positive hedonic reactions by oral sucrose infusions of 0.03M sucrose (sum of rhythmic tongue protrusions, paw licks, and lateral tongue protrusions). Neuronal inhibitions induced by laser illuminations decreased positive 'liking' reactions for 0.03M sucrose in posterior VP iC++ rats by 64.3% ± 6.8% (Fig. 25) relative to measured control baseline levels in the same individuals without laser (0.03M Sucrose laser x virus x valence

interaction: $F_{1,31}$ = 11.83, p = 0.002; n = 14 females, n = 5 males; Sum of rhythmic tongue protrusions, lateral tongue protrusions, and paw licks: *Laser-ON*: M = 10.3, *SEM* = 1.3; *Laser-OFF*: M = 15.7, *SEM* = 1.2; paired comparison t_{31} = 4.85, p = 0.001, 95% CI[2.5, 8.4], d = -4.3; Fig. 26). Posterior VP neuronal inhibitions also increased aversive 'disgust' reactions elicited by sucrose infusions by 199% ± 25% relative to within-subjects no laser baseline reactions in the same rats (Sum of headshakes, forelimb flails, chin rubs, gapes, facewashes, paw treads: *Laser-ON*: M = 7.9, *SEM* = 2.0; *Laser-OFF*: M = 4.1, *SEM* = 0.9; paired comparison t_{31} = 2.74, p = 0.04, 95% CI[-6.1, -0.1], d = 2.5; Fig. 26). The magnitude of laser suppression of 'liking' and enhancement of aversive 'disgust' reactions was similar in female and male rat (iC++: sex x valence x laser interaction: $F_{1,17}$ = 0.04 p = 0.85; n = 19 iC++ rats; n = 5 males n = 14 females; Fig. 26)

Similarly, for the higher sucrose 0.1 M concentration, posterior VP neuron inhibitions reduced (63.7% ± 6.3%) the number of positive hedonic reactions compared to within-subject baseline levels (Laser-ON M = 9.9*, SEM* = 1.5*; Laser OFF M* = 15.1, *SEM* = 1.6; laser x virus x valence interaction: $F_{1,20}$= *13.8*, p = 0.001; paired comparison: t_{40} = 4.59, p = 0.0002, 95% CI[2.2, 8.2], d = -3.4, n = 14; Fig. 26) and also increased (236% ± 38%) the number of aversive responses elicited by the stronger sucrose concentration (Laser-ON M = 9.1*, SEM* = 2.2*; Laser OFF M* = 5.0, *SEM* = 1.2; t_{40} = 3.62, p = 0.0003, 95% CI[-7.1, -1.1], d = 2.3, n = 14; Fig. 26). The magnitude of hedonic suppression and 'disgust' enhancement over baseline levels was comparable for both 0.03M and 0.10M sucrose ($F_{1,31}$= *0.000*, p = 0.98), and the magnitude of hedonic suppression/ aversion enhancement was similar in male and female ChR2 rats ($F_{1,12}$ = 0.45 p = 0.52; n = 14 iC++ rats; n = 4 males n = 10 females)

By contrast, in control eYFP rats with optically inactive virus, laser illumination in caudal VP sites failed to alter either positive hedonic reactions or negative reactions to sucrose from baselines measured in the same individuals, for either 0.03M or 0.10M sucrose (0.03M sucrose: 'liking' paired comparison: $t_{31} = 0.22$, $p > 0.99$; 'disgust' paired comparison: $t_{31} = 0.25$, $p > 0.99$, $n = 14$; Fig. 26; 0.10M sucrose: 'liking' paired comparison: $t_{40} = 0.33$, $p > 0.99$; 'disgust' paired comparison: $t_{31} = 0.09$, $p > 0.99$, $n = 9$; Fig. 26)

Enhancement of aversive 'disgust' reactions to a bitter quinine. Oral infusions of bitter quinine solution ($3x10^{-4}$ M) elicited predominately aversive 'disgust' reactions in the absence of laser ($M = 26.9$, $SEM = 2.7$; valence main effect: $F_{1,30} = 169.7$, $p < 0.0001$, $n = 18$). Within posterior VP sies, adding laser illuminations increased the number of aversive reactions elicited by quinine by 159.2% ± 9.3% compared to no laser baselines measured in the same rats (M= 41.6, $SEM = 3.9$, laser x virus x valence interaction: $F_{1,20} = 33.02$, $p < 0.0001$, $n = 21$; eYFP control rats: 18.4% ± 10% suppression, $F_{1,30} = 21.3$, $p < 0.0001$; $t_{30} = 8.60$, $p < 0.0001$, 95% CI[-19.3, 10.2], $d = 4.4$) and did not change the few number of positive 'liking' reactions elicited by quinine infusions (Laser-ON $M = 1.9$, $SEM = 0.8$; *Laser OFF* $M = 4.0$, $SEM = 0.9$; $t_{30} = 1.22$, $p = 0.93$; Fig. 26). By comparison, eYFP controls emitted similar numbers of aversive 'disgust' reactions during laser and non-laser conditions (Laser-ON $M = 29.1$, $SEM = 3.0$; *Laser OFF* $M = 30.6$, $SEM = 3.2$; $t_{30} = 0.75$, $p > 0.99$; $n = 14$), and very few positive 'liking' reactions that remained unchanged following laser illuminations (Laser-ON $M = 0.6$, $SEM = 0.4$; *Laser OFF* $M = 0.8$, $SEM = 0.2$; $t_{30} = 0.07$, $p > 0.99$; $n = 14$)

Anatomical boundaries of optogenetic hedonic suppression in VP: Localization of function was mapped for optogenetic VP hedonic suppression across all rostral and caudal sites. These were considered to be sites where laser illumination caused >20% reductions in 'liking'

reactions to sucrose, compared to no-laser baseline levels measured in the same individual. Sites of hedonic suppression clustered anatomically along the caudal 1/3 of the most posterior and lateral VP regions.

The anterior border of VP-mediated hedonic suppression began near the caudal 1/3 of the ventral pallidum, just posterior to bregma and where the anterior commissure crosses the midline (-0.24 mm AP) and extended caudolaterally until the caudal edge of the ventral pallidum. Rostrally, the VP hedonic suppression site was bordered medially by the lateral preoptic area, ventrally by the magnocellular preoptic nucleus and the olfactory tubercle, dorsolaterally by IPAC, and dorsally by the anterior commissure. Moving caudolaterally, the VP 'hotspot' extended ~0.8mm to the far caudal and lateral edge of VP (-1.08mm AP). There, effective sites of hedonic suppression in VP were bordered medially by the magnocellular preoptic nucleus, dorsally by globus pallidus, laterally by IPAC, and ventrally by the anterior amygdaloid area. Overall, the VP hotspot extended rostrocaudally (AP) in length ~0.84mm, mediolaterally at its widest point (ML) ~1.6 mm, and dorsoventrally (DV) ~1.0 mm for a total volume of ~1.3 mm^3. We note that these optogenetic boundaries correspond closely to those originally mapped using lesions and pharmacological inactivations, although these studies report sites of hedonic suppression and 'disgust' induction into areas more rostral than our current study (Cromwell & Berridge, 1993; Ho & Berridge, 2014; Khan et al., 2019). One possibility is that our optogenetic approach, which may induce less robust levels of inhibitions compared to large electrolytic lesions and drug microinjections accounts for these small differences across studies.

Rostral VP inhibitions fail to alter affective expressions. The same optogenetic manipulations at rostral VP sites failed to alter affective reactions to any taste. In anterior VP iC++ rats, laser illuminations did not alter positive 'liking' or aversive 'disgust' elicited to 0.03M

(0.03M sucrose: 'liking' reactions: Laser-ON M = 12.1, SEM = 1.6; *Laser OFF* M = 11.1, SEM = 1.8; 'disgust' reactions: Laser-ON M = 8.0, SEM = 3.3; *Laser OFF* M = 6.8, SEM = 2.5; n= 10) or 0.10M sucrose (0.10M sucrose: 'liking' reactions: Laser-ON M = 14.3, SEM = 4.4; *Laser OFF* M = 12.9, SEM = 2.8; 'disgust' reactions: Laser-ON M = 10.2, SEM = 3.5; *Laser OFF* M = 11.6, SEM = 3.6; n= 6) and thus rostral VP iC++ rats did not differ from eYFP controls (0.03M sucrose: laser x virus x valence interaction: $F_{1,22}$= 0.76, p = 0.39, n = 14 eYFP; Fig. 27; 0.10M sucrose: laser x virus x valence interaction: $F_{1,12}$= 0.55, p = 0.47, n = *8*; Fig. 27).

Finally, laser illuminations also produced no change in affective expressions to quinine in either rostral VP iC++ rats or eYFP controls; laser x virus x valence interaction: $F_{1,21}$= 0.01, p = 0.92, n = 10 iC++; n = 13 eYFP), suggesting that loss of function in anterior VP neurons do not alter affective reactions to sweet or bitter tastes.

Rostral and Caudal VP control of Incentive Motivation: general neuronal inhibitions.

We compared the effects of rostral and caudal VP neuron inhibitions for their ability to influence incentive motivation, here measured as unconditioned intake of palatable chocolate M&M candies during a 1-h test of voluntary food intake. General optogenetic inhibitions throughout all of VP reduced total intake in all rats compared to no laser inhibition sessions conducted in the same rats (Fig. 28) Rostral VP inhibitions decreased intake to 66% ± 12% relative to within subjects baseline levels (Intake (g): Laser-ON M = 3.8, SEM = 0.5; *Laser OFF* M = 3.9, SEM = 0.7; laser x virus interaction: $F_{1,25}$= *6.51*, p = 0.02; paired comparison: t_{25} = 3.71, p = 0.002, 95% CI[1.1, 5.0], d = -5.1, n = 16; Fig. 28). The magnitude of feeding suppression was similar in male and female rostral VP iC++/ SwiChR++ rats ($F_{1,14}$= *0.52*, p = 0.48, n =*8* males, n = 8 females).

Similarly, posterior VP neuron inhibitions also reduced (61.1% ± 6.6%) the amount of M&M candies consumed (Intake (g): Laser-ON M = 6.1*, SEM* = 0.6*; Laser OFF M* = 3.6, *SEM* = 0.4; laser x virus interaction: $F_{1,30}$= *6.81*, p = 0.01; paired comparison: t_{30} = 4.48, p = 0.0002, 95% CI[1.2, 3.9], d = -4.9, n = 24; Fig. 28). The magnitude of feeding suppression was similar in male and female rostral VP iC++/ SwiChR++ rats ($F_{1,22}$= *0.31*, p = 0.58, n =*9* males, n = 15 females), and between rostral and caudal VP inhibition rats ($F_{1,38}$= *0.27*, p = 0.60). By comparison, both rostral and caudal VP eYFP controls consumed similar amounts of M&Ms between laser and non-laser conditions (Rostral eYFP: Intake (g): Laser-ON M = 6.7*, SEM* = 1.1*; Laser OFF M* = 6.5, *SEM* = 1.1; t_{25} = 0.24, p > 0.99; n = 11, Fig. 28; Caudal eYFP: Intake (g): Laser-ON M = 7.3*, SEM* = 0.7*; Laser OFF M* = 6.6, *SEM* = 0.9; t_{30} = 0.68, p = 0.99; n = 8; Fig. 28).

Bidirectional control of hedonic impact by posterior ventral pallidum GABA neurons

VP^{GABA} ChR2 neuron excitations increase sucrose 'liking' reactions. At ChR2 sites within the caudolateral subregion of VP, which has been previously identified as a hedonic hotspot where mu-opioid receptor agonists, and orexin agonists increase positive 'liking' reactions to sucrose (Ho & Berridge, 2013; K. S. Smith & Berridge, 2005), optogenetic ChR2 stimulations in VP^{GABA} neurons increases the overall number of positive hedonic reactions by oral infusions of 0.10M sucrose (sum of rhythmic tongue protrusions, paw licks, lateral tongue protrusions. We did not test lower concentrations of sucrose, as pilot studies indicated that transgenic GAD1-cre rats needed higher baseline sucrose concentrations to reach a base positive affective score during taste reactivity testing.

Laser ChR2 excitations increased positive 'liking' reactions for 0.10M sucrose in posterior VP^{GABA} ChR2 rats by 222.2%± 56% (Fig. 29) relative to measured control baseline levels in the same individuals without laser (0.10M Sucrose laser x virus x valence interaction: $F_{1,21} = 7.81$, $p = 0.02$; $n = 15$; Sum of rhythmic tongue protrusions, lateral tongue protrusions, and paw licks: *Laser-ON*: $M = 21.6$, $SEM = 2.8$; *Laser-OFF*: $M = 12.5$, $SEM = 1.7$; paired comparison $t_{42} = 6.22$, $p = 0.0001$, 95% CI[-12.9, -5.3], $d = 3.9$; Fig. 30). Posterior VP^{GABA} neuron excitations did not alter the few aversive 'disgust' reactions elicited to sucrose (Sum of headshakes, forelimb flails, chin rubs, gapes, facewashes, paw treads: *Laser-ON*: $M = 5.2$, $SEM = 1.3$; *Laser-OFF*: $M = 7.3$, $SEM = 1.7$; paired comparison $t_{42} = 1.48$, $p = 0.59$, Fig. 30). The magnitude of laser enhancement of 'liking' reactions was similar in male and female ChR2 rats (sex x valence x laser interaction: $F_{1,13} = 2.36$ $p = 0.15$; $n = 6$ males $n = 9$ females; Fig. 30). By contrast, in control eYFP rats with optically inactive virus, laser illumination in caudal VP sites failed to alter either positive hedonic reactions or negative reactions to sucrose from baselines measured in the same individuals (0.10M sucrose: 'liking' paired comparison: $t_{42} = 0.56$, $p > 0.99$; 'disgust' paired comparison: $t_{42} = 1.40$, $p = 0.67$, $n = 8$; Fig. 30)

VP^{GABA} iC++ neuron inhibitions decrease sucrose 'liking' reactions. Laser inhibitions in posterior VP^{GABA} neurons decreased positive 'liking' reactions for 0.10M sucrose by 48.5%± 6.8% (Fig. 28) relative to measured control baseline levels in the same individuals without laser (0.10M Sucrose laser x valence interaction: $F_{1,9} = 8.58$, $p = 0.02$; $n = 10$; Sum of rhythmic tongue protrusions, lateral tongue protrusions, and paw licks: *Laser-ON*: $M = 8.9$, $SEM = 1.7$; *Laser-OFF*: $M = 18.3$, $SEM = 2.0$; paired comparison $t_9 = 2.85$, $p = 0.04$, 95% CI[-18.3, -0.55], $d = -4.9$; Fig. 30). Caudal VP^{GABA} neuron excitations did not alter aversive 'disgust' reactions elicited to sucrose (Sum of headshakes, forelimb flails, chin rubs, gapes, facewashes, paw treads:

Laser-ON: *M* = 9.3, *SEM* = 3.0; *Laser-OFF*: *M* = 5.1, *SEM* = 1.6; paired comparison $t_9 = 1.29$, $p = 0.46$, Fig. 30). The magnitude of laser suppression of 'liking' reactions was similar in male and female iC++ rats (sex x valence x laser interaction: $F_{1,8} = 1.06$ $p = 0.33$; $n = 4$ males $n = 6$ females; Fig. 30). We were underpowered to analyze eYFP control rats ($n = 2$), but in the two animals tested, we observed no marked change in affective 'liking' reactions (81.9%± 13%) or aversive 'disgust' (139%± 43%) reactions to 0.10M sucrose.

Oppositely valenced hedonic coldspot in rostral VP^{GABA} neurons: ChR2 induced 'Liking' suppression.

Rostral VP^{GABA} ChR2 neuron excitations decrease sucrose 'liking' reactions. At rostral and medial sites within VP, laser ChR2 excitations oppositely suppressed positive 'liking' reactions for 0.10 sucrose in VP^{GABA} ChR2 rats by 6.2%± 6.7% (Fig. 29) relative to measured control baseline levels in the same individuals without laser (0.10M Sucrose laser x virus interaction: $F_{1,13} = 11.36$, $p = 0.005$; $n = 11$; Sum of rhythmic tongue protrusions, lateral tongue protrusions, and paw licks: *Laser-ON*: *M* = 10.1, *SEM* = 1.3; *Laser-OFF*: *M* = 17.8, *SEM* = 2.2; paired comparison $t_{13} = 5.51$, $p = 0.004$, 95% CI[3.6, 11.6], $d = -4.2$; Fig. 30). Rostral VP^{GABA} neuron excitations did not alter the few aversive 'disgust' reactions elicited to sucrose (Sum of headshakes, forelimb flails, chin rubs, gapes, facewashes, paw treads: *Laser-ON*: *M* = 3.8, *SEM* = 0.7; *Laser-OFF*: *M* = 5.5, *SEM* = 1.6; paired comparison $t_{13} = 1.21$, $p = 0.68$, Fig. 30). The magnitude of laser enhancement of 'liking' reactions was similar in male and female ChR2 rats (sex x valence x laser interaction: $F_{1,9} = 0.876$ $p = 0.38$; $n = 6$ males $n = 5$ females; Fig. 30). By contrast, in control eYFP rats with optically inactive virus, laser illumination in rostral VP sites failed to alter either positive hedonic reactions or negative reactions to sucrose from baselines

measured in the same individuals (0.10M sucrose: 'liking' paired comparison: $t_{13} = 0.00$, $p > 0.99$; 'disgust' paired comparison: $t_{13} = 0.11$, $p > 0.99$, $n = 4$; Fig. 30)

No change in sucrose affective reactions by rostral VP^{GABA} neuron inhibitions. Laser inhibitions in rostral VP^{GABA} iC++ rats failed to alter affective reactions emitted to intra oral infusions of 0.10M sucrose (valence x laser interaction: $F_{1,6} = 0.43$ $p = 0.54$; $n = 3$ males $n = 4$ females). We evaluated a single eYFP control rat for taste reactivity to sucrose, and thus were not powered to perform a statistical analysis. In this rat, intra-oral infusions of 0.10M sucrose did not alter affective expression to sucrose (96% laser change; 'Liking' reactions: *Laser-ON*: 21, *Laser-OFF*:22 ; 'Disgust 'reactions: *Laser-ON: 10; Laser-OF: 5.5).*

<u>Incentive value of laser-paired sucrose modulated by optogenetic VP^{GABA} neuron manipulations.</u>

We tested whether pairing VP^{GABA} neuron manipulations with earning sucrose reward in the choice task could cause rats to either pursue (via ChR2 excitations; 1-3 mW; 40 Hz) or avoid (via IC++ inhibitions; 2-4 mW, constant illuminations) that laser-paired sucrose reward over the sucrose reward never paired with laser stimulation.

Focused pursuit of paired sucrose by rostral and caudal VP^{GABA} neurons. Pairing VP^{GABA} neuron excitations in posterior hotspot ChR2 rats biased rats for Sucrose + Laser nearly exclusively over the otherwise identical sucrose alone option without laser (effort x virus x laser interaction $F_{8,200} = 5.52$, $p < 0.0001$, $n = 19$ ChR2 rats; Fig. 31). Posterior VP^{GABA} ChR2 rats reached a 5:1 laser preference ratio by final day 9 ($t_{144} = 9.00$, $p = 0.0001$, 95% CI [-440.1, -230.4], $d = 6.9$). Both male and female VP^{GABA} ChR2 rats showed equally strong preferences for Laser + Sucrose over the sucrose alone lever (females: 6:1 ratio; males 3:1 ratio; effort x laser x sex interaction: $F_{8,136} = 0.76$, $p = 0.64$, $n = 6$ males, $n = 13$ females; Fig. 31).

Rostral VP^{GABA} neuron activations also biased rats for Sucrose + laser relative to sucrose alone in the 2-choice task (effort x virus x laser interaction $F_{8,200} = 3.19$, $p = 0.002$, $n = 19$ ChR2 rats; Fig. 31). Rostral VP^{GABA} ChR2 rats reached an 8:1 laser preference ratio by final day 9 ($t_{144} = 10.41$, $p < 0.001$, 95% CI [-329.2, -189.1], $d = 9.0$). Both male and female VP^{GABA} ChR2 rats showed equally strong preferences for Laser + Sucrose over the sucrose alone lever (females: 9:1 ratio; males 7:1 ratio; effort x laser x sex interaction: $F_{8,136} = 0.16$, $p = 0.99$, $n = 10$ males, $n = 9$ females; Fig. 31). By contrast, both caudal VP^{GABA} eYFP rats and caudal VP^{GABA} control rats chose randomly between Laser + Sucrose and Sucrose Alone (Caudal VP eYFP: $F_{8,56} = 0.98$, $p = 0.46$, $n = 8$; Rostral VP eYFP: $F_{8,56} = 0.20$, $p = 0.99$, $n = 8$), and thus differed from ChR2 rats (Caudal VP: $F_{1,25} = 9.85$, $p = 0.004$, Rostral VP: $F_{1,25} = 9.87$, $p = 0.004$).

VP GABA containing neuron inhibition paired avoidance. Separate inhibition rats, with iC++ expressed in either rostral or caudal VP^{GABA} neurons developed a strong avoidance of the paired Laser + Sucrose option. Both posterior VP^{GABA} inhibition rats (Caudal VP Sucrose Alone Preference: 20:1; laser x effort x virus interaction: $F_{8,88} = 8.84$, $p < 0.0001$; paired comparison: $t_{40} = 10.62$, $p < 0.0001$, 95% CI [354.1, 624.2], $d = 7.7$, $n = 6$ iC++, $n = 7$ eYFP; Fig. 32) and rostral VP^{GABA} neuron inhibition rats avoided laser paired sucrose (Rostral VP Sucrose Alone Preference: 9:1; laser x effort x virus interaction: $F_{8,72} = 4.13$, $p = 0.004$; paired comparison: $t_{32} = 6.60$, $p < 0.0001$, 95% CI [148.1, 391.1], $d = 3.8$, $n = 5$ iC++, $n = 6$ eYFP; Fig. 32). By comparison, rostral and caudal VP eYFP control rats responded equally for sucrose alone and sucrose + laser reward options.

Intensity of incentive motivation bidirectionally modulated by VP^{GABA} neurons

VP^{GABA} neuron activations intensify incentive motivation for sucrose. Progressive ratio tests of breakpoint confirmed that VP^{GABA} neuron excitation throughout rostral and caudal

subregions increased the intensity of incentive motivation to work for laser-paired sucrose. Rostral VP^{GABA} ChR2 rats reached breakpoints that were 230% higher for Laser + sucrose (67.5. ± 9.3) than for sucrose Alone (29.6. ± 4.8; laser x virus interaction: $F_{1,24} = 9.70$, $p = 0.005$, $n = 18$; paired comparison $t_{24} = 4.49$, $p < 0.0001$, 95% CI [-54.0, -21.9], $d = 5.1$; Fig. 33). Overall rostral VP ChR2 rats made ~2.5x as many responses for laser-paired sucrose (289.4. ± 47.8) relative to the sucrose alone option (119.3. ± 25.2; $F_{1,24} = 4.36$, $p = 0.05$, $n = 18$; paired comparison $t_{24} = 4.63$, $p = 0.002$, 95% CI [-257.8, -82.3], $d = 4.5$; Fig. 33). Caudal VP^{GABA} ChR2 rats reached breakpoints that were 200% higher for Laser + sucrose (86.6. ± 7.7) than for sucrose Alone (43.4. ± 10.2; laser x virus interaction: $F_{1,23} = 4.36$, $p = 0.05$, $n = 17$; paired comparison $t_{23} = 3.87$, $p = 0.002$, 95% CI [-70.0, -16.5], $d = 4.8$; Fig. 33). Overall rostral VP ChR2 rats made ~2x as many responses for laser-paired sucrose (374.8. ± 36.4) relative to the sucrose alone option (190.4. ± 46.8; $F_{1,23} = 8.03$, $p = 0.009$, $n = 17$; paired comparison $t_{23} = 3.48$, $p = 0.004$, 95% CI [-311.4, -57.5], $d = 4.4$; Fig. 33). By contrast, rostral and caudal eYFP control groups reached equal breakpoints (Rostral eYFP Breakpoint: Sucrose + laser: 62.9 ± 8.7, Sucrose Alone: 68.5 ± 10.8; $t_{24} = 0.48$, $p = 0.63$; Caudal eYFP Breakpoint: Sucrose + laser: 54.1 ± 9.0, Sucrose Alone: 52.1 ± 12.8; $t_{23} = 0.12$, $p = 0.99$; Fig. 33)

VP^{GABA} neuron inhibitions suppress incentive motivation for sucrose. Inhibition of VP^{GABA} neurons tended to have the opposite effect on motivation, generally suppressing willingness to work for laser + sucrose in iC++ rats. In rostral VP, iC++ inhibition of GABA neurons suppressed PR breakpoints (Laser Breakpoint:35.2. ± 8.0; NL Breakpoint: 64.3± 12.4) and overall responses (Laser Responses: 146.5. ± 35.3; NL Responses: 294.8. ± 65.2) for sucrose by nearly 50% although this effect did not reach threshold for significance (Breakpoint: laser

main effect: $F_{1,9} = 6.01$, $p = 0.04$; laser x virus interaction: $F_{1,9} = 4.44$, $p = 0.06$; Responses: laser x virus interaction: $F_{1,9} = 4.33$, $p = 0.07$; $n = 6$).

Caudal VP^{GABA} ChR2 rats reached breakpoints that were 50% lower for Laser + sucrose (39.5. ± 8.3) than for sucrose Alone (85.7. ± 15.3; laser x virus interaction: $F_{1,11} = 15.70$, $p = 0.002$, $n = 6$; paired comparison $t_{11} = 4.63$, $p = 0.002$, 95% CI [20.3, 72.0], $d = -3.8$; Fig. 33). Overall rostral VP ChR2 rats made 60% less responses for laser-paired sucrose (152.5. ± 37.6) relative to the sucrose alone option (396.3 ± 79.8; $F_{1,11} = 17.1$, $p = 0.0002$, $n = 6$; paired comparison $t_{11} = 4.69$, $p = 0.001$, 95% CI [109.1, 378.5], $d = -3.9$; Fig. 33). By contrast, caudal eYFP controls reached equal breakpoints (Caudal eYFP Breakpoint: Sucrose + laser: 50.0 ± 10.7, Sucrose Alone: 42.3 ± 10.7; $t_{11} = 0.84$, $p = 0.84$, $n = 7$) and made similar responses for sucrose alone and sucrose + laser (Caudal eYFP responses: Sucrose + laser: 221.3 ± 57.0, Sucrose Alone: 172.7 ± 37.6; $t_{11} = 1.01$, $p = 0.67$, $n = 7$; Fig. 33).

Self-Stimulation Measures

Self-stimulation in place-based task. In the place-based task, rats could earn laser illuminations by entering a designated chamber, or simply remaining in it while laser continued (8-s ON/ 8-s OFF). Rostral and caudal VP^{GABA} neuron sites supported place-based self-stimulation. ChR2 rats in anterior VP coldstrip sites spent ~300% more time in the laser-delivering chamber than in the alternative chamber without laser (40 Hz Difference Score: 411.9 ± 47.8; $F_{2,47} = 19.74$, $p < 0.0001$; $n = 21$ ChR2 rats), and thus differed from eYFP controls who spent equal times in both chambers (eYFP difference score: 37.3 ± 62.9; 40 Hz vs. eYFP paired comparison: $t_{47} = 3.69$, $p = 0.001$, 95% CI [139.5, 609.7], $d = 6.7$; Fig. 34). The laser-induced preference by ChR2 rats also differed from the time they spent on the laser-delivering side

during a pre-laser habituation session when no laser photostimulations could be earned in either side (ChR2 habituation difference score: -47.7 ± 62.2; Laser vs. Habituation paired comparison: t_{47}= 6.09, p < 0.001, 95% CI [285.0, 634.3], d= 8.3; Fig. 34).

Posterior VP^{GABA} ChR2 rats also self-stimulated in the place-based task, spending ~250% more time in the laser-delivering chamber than in the alternative chamber without laser (40 Hz Difference Score: 376.6± 49.8; $F_{2,38}$ = 21.28, p < 0.0001; n = 17 ChR2 rats), and thus differed from eYFP controls who spent equal times in both chambers (eYFP difference score: 9.0± 76.0; 40 Hz vs. eYFP paired comparison: t_{38}= 3.62, p = 0.002, 95% CI [130.7, 604.6], d= 5.7; Fig. 34). The laser-induced preference by posterior VP^{GABA} ChR2 rats also differed from the time they spent on the laser-delivering side during a pre-laser habituation session when no laser photostimulations could be earned in either side (ChR2 habituation difference score: -119.5 ± 61.4; Laser vs. Habituation paired comparison: t_{38}= 6.40, p < 0.001, 95% CI [315.2, 677.1], d= 8.9; Fig. 34).

No reliable self-stimulation in the spout-task. We asked if activation VP^{GABA} neurons had motivational value on its own, in the absence of an external reward. In the spout-touch task, contacts to an empty metal spout earned brief laser illuminations (40 Hz; 1-s), whereas touching a different spout earned nothing, and served as baseline measure of exploratory contacts. Rostral VP^{GABA} ChR2 rats did not consistently self-stimulate in the spout task. In rostral VP, ChR2 rats made 22x as many contacts to the laser-paired spout (376.0. ± 127.4) relative to the control spout (16.7. ± 4.5) when they could earn 1-s laser illuminations, but this magnitude of difference did not reach a statistically significant threshold, and thus rostral ChR2 rats did not differ from eYFP controls (laser x virus interaction: $F_{1,21}$ = 1.81, p = 0.19, n =21 ChR2; n = 8 eYFP; Fig. 34) who made similar contacts to the laser-paired (10.1 ± 2.9) and control spout (11.8 ± 2.3).

Similarly, caudal VP^{GABA} ChR2 rats did not consistently self-stimulate in the spout task. Despite making 60x as many contacts to the laser-paired spout (672.3. ± 319.5) relative to the control spout (11.3 ± 3.5) when they could earn 1-s laser illuminations, this was not statistically significant from eYFP controls (laser x virus interaction: $F_{1,26} = 3.15$, $p = 0.09$, n =15 ChR2; n = 7 eYFP; Fig. 34) who made similar contacts to the laser-paired (8.3 ± 3.2) and control spout (11.0 ± 2.9).

Paired Aversive Shock Rod

Desire for noxious shock rod created by rostral VP^{GABA} neuron excitations. Our results thus far suggest that VP^{GABA} neurons play a bidirectional role in controlling incentive motivation for pleasant rewards including sucrose and optogenetic brain stimulations. In a different situation with a noxious shock rod, the same group of VP^{GABA} ChR2 rats and eYFP controls received pairings of VP^{GABA} neuron stimulations each time they voluntary approached within 2 cm of an electrified shock rod (laser 40 Hz; 1-3 mW; bin duration depending on how long the rat remained within 2-cm proximity of the shock rod).

Rostral VP^{GABA} ChR2 rats, in sites where laser activations during taste reactivity testing previously suppressed hedonic 'liking' reactions, became attracted to the shock rod unlike eYFP controls (laser x virus interaction: $F_{3,54} = 9.73$, $p < 0.0001$, n =10 ChR2). ChR2 rats approached and touched the rod 12 times on average the first day (Day 1 vs Day 3 paired comparison t_{54}= 7.40, $p < 0.0001$, 95% CI [-14.7, -7.3], d= 6.7; Fig. 35), and touched and received 12 shocks on the second day (Day 2 vs Day 3 paired comparison t_{54}= 7.13, $p < 0.0001$, 95% CI [-14.3, -6.9], d= 5.5). The number of contacts rats made with the shock rod decreased to near zero on Day 3 when shock rod contacts were no longer paired with laser stimulation (0.9 ± 0.4), then

again increased to 10 contacts on Day 4 when photostimulation was re-paired with the shock rod (Day 4 vs Day 3 paired comparison t_{54}= 6.12, $p < 0.0001$, 95% CI [-12.7, -5.4], d= 4.6). Male and female ChR2 rats developed a similar magnitude of attraction to the shock rod ($F_{3,24} = 0.31$, $p = 0.82$, n =6 females, n =4 females).

By comparison, control eYFP rats quickly learned to avoid the shock rod after touching it a few times on the first day. Control rats made more contacts with the rod during the initial session (Day1: 4.4 ± 0.3; paired comparison: t_{54}= 2.55, $p = 0.04$, 95% CI [-7.5, -0.2], d= 14.9; n = 10 eYFP), but these quickly fell to near zero contacts in subsequent sessions (Day 2: 1.1 ± 0.5; Day 3: 0.6 ± 0.2; Day 4: 0.2 ± 0.1; Fig. 35).

Both caudal VP^{GABA} ChR2 and eYFP control rats made some exploratory contacts with the shock rod when laser-stimulations were paired on the first day (ChR2 Day 1: 5.1 ± 0.9; eYFP Day 1: 4.0 ± 0.6; laser main effect: $F_{3,48} = 13.36$, $p < 0.0001$), but these exploratory investigations dropped to near zero levels by Day 2 (ChR2 Day 2: 3.1 ± 1.5; eYFP Day 2: 0.9 ± 0.3) and remained near zero for the rest of days rats were tested. Unlike rostral VP ChR2 rats, posterior VP^{GABA} ChR2 rats did not develop attraction to the shock rod, and thus did not differ from eYFP control rats (laser x virus interaction: $F_{3,48} = 1.50$, $p = 0.23$, n =9 ChR2; n =9 eYFP; Fig. 35).

4.5 Discussion

The ventral pallidum plays a key role generating incentive motivation and affective responses (Ahrens et al., 2018; S. E. Chang et al., 2017; Cromwell & Berridge, 1993; Ho & Berridge, 2013, 2014; Mahler et al., 2014; Ottenheimer et al., 2018; K. S. Smith & Berridge, 2005, 2007). Previous pharmacological studies have shown that VP bidirectionally controls hedonic impact of pleasant tastes. Its posterior half contains a hedonic hotspot where mu-opioid

and orexin microinjections enhance positive 'liking' reactions to sucrose (Ho & Berridge, 2013; K. S. Smith & Berridge, 2005). At rostral sites in VP, the same neurochemical manipulations oppositely suppress 'liking' reactions, despite still generating 'wanting' to eat (K. S. Smith & Berridge, 2005). Unlike other hedonic hotspots in NAc, OFC, insula, or PBN, lesions to the posterior VP hotspot also result in intense 'disgust' to normally pleasant tastes, suggesting the necessity of this subregion for normal affective reactions (Cromwell & Berridge, 1993; Ho & Berridge, 2014; Khan et al., 2019). Here we confirm the existence and boundaries of this localized site of hedonic suppression in VP, and extended its definition by showing that optogenetic inhibitions suppressed positive 'liking' reactions to sucrose by nearly 50% in rats.

Our results provide triangulating evidence that the VP hedonic hotspot is *necessary* for hedonic control and a robust neurofunctional entity using an alternative technique to locally inhibit neurons. Our results show that SwiChR++ and iC++ induced hyperpolarization of neurons within the caudolateral tip of VP is an effective means of suppressing hedonic impact similar to drug microinjections and excitotoxin lesions. The suppressive site in caudal VP began posteriorly to bregma and extended ~0.88 mm posteriorly to the caudal edge of VP. We further probe neuronal control of 'liking' reactions in VP by taking a cell-specific approach to investigate the role of inhibitory GABA neurons within VP. Using GAD1-Cre rats, we show that VP^{GABA} neuron activations within a similar site in caudal VP GABA neurons increases positive 'liking' reactions to sucrose in rats, and oppositely inhibiting VP^{GABA} neurons in this same caudolateral region suppress hedonic impact. Further, we identified a hedonic 'coldspot' region located rostrally to the posterior VP^{GABA} hedonic hotspot where ChR2 activation of GABA neurons reduced the number of positive 'liking' reactions to sweetness. The suppressive coldstrip

began at the rostral tip of VP, moving posteriorly and laterally through central VP regions just caudally to bregma.

Motivational 'wanting' modulation more widespread than hedonic 'liking'. In stark contrast to the highly localized sites in caudal VP that altered hedonic 'liking' reactions, most VP manipulations, including rostral and caudal subregions effectively produced changes in motivational 'wanting'. In our general inhibition experiments, iC++/SwiChR++ driven laser inhibitions in both rostral and caudal VP subregions effectively suppressed consumption during a 1-hr test of voluntary food intake. Relatedly, GABAergic manipulations in VP showed a similar pattern of 'wanting' control. ChR2 activations in rostral and caudal VP GABA neurons caused rats to selectively lever press for laser-paired sucrose in the instrumental choice-task, ignoring the otherwise identical sucrose alone option. Conversely, inhibitions throughout VP^{GABA} neuron sites caused iC++ rats to avoid the sucrose + laser option and primarily respond for sucrose alone. When tested using a progressive ratio schedule of reinforcement, rostral and caudal VP^{GABA} neuron photostimulations also caused ChR2 rats to work ~2x as hard for laser-paired sucrose, suggesting VP^{GABA} neurons not only direct incentive motivation onto particular targets, but can also effectively alter the intensity of this motivation and willingness to exert effort. Our findings are consistent with the idea that VP substrates that control motivational 'wanting' extend beyond the hedonic hotspots that control 'liking' (Cromwell & Berridge, 1993; Ho & Berridge, 2013; Khan et al., 2019; Shimura et al., 2006; K. S. Smith & Berridge, 2005; Stratford & Wirtshafter, 2012). For example, previous studies have shown that drug microinjections of $GABA_a$ and $GABA_b$ agonists throughout VP can suppress feeding in rats, as well as decrease appetitive social interactions (Ho & Berridge, 2014; Khan et al., 2019; Shimura et al., 2006) and pharmacological, electrical, and DREADD activations oppositely increase feeding and incentive

motivation (Farrell et al., 2019, 2022; Panagis et al., 1995; Shimura et al., 2006; Stratford & Wirtshafter, 2012)

Laser-generated attraction to a painful shock: 'wanting' what is never 'liked' in rostral VP^{GABA} neurons. In perhaps a striking display of 'wanting' completely divorcing from 'liking', we observed in a number of rostral VP^{GABA} sites where paired laser stimulations with an electrified shock rod produced maladaptive attraction in ChR2 rats, causing them to repeatedly approach, bite, and touch the rod despite receiving multiple shocks. Our findings suggest that incentive motivational 'wanting' induced by anterior VP^{GABA} neurons can transform an otherwise painful and aversive stimulus into an incentive target, causing rats to repeatedly shock themselves in a compulsive manner.

What psychological mechanisms is responsible for producing 'wanting' what hurts in VP? One possibility is that activating rostral VP^{GABA} neurons produces an analgesic effect that reduces the pain associated with electric shocks. Although limited, some studies have reported a role of VP neurons, including cholinergic neurons, in mediating pain and analgesia (Anagnostakis et al., 1992; Asgharieh-Ahari et al., 2023; Ji et al., 2023). We hypothesize that analgesia may not likely have played a large role in creating the shock rod attraction described in ChR2 rats. Upon contacting the rod, ChR2 rats still displayed pain-associated behaviors including jerks, jumps, and flinches that did not diminish over the course of testing. Rather, rats would flinch in pain, retreat from the rod's vicinity, and return to continue to interact with the rod. Alternatively, could paired rostral VP^{GABA} neuron activations have caused rats to 'like' the shocks? This explanation seems improbable as rostral VP sites that generated shock rod attraction failed to increase 'liking' during taste reactivity, and instead often suppressed 'liking'. Only ChR2 activation at caudal sites produces gains in hedonic impact, yet these rats failed to

develop shock rod attraction, indicating enhances 'liking' may not be a potential explanatory mechanism.

We hypothesize that ChR2 induced shock rod attractions generated by VP GABA neurons may reflect an incentive salience mechanism. In some pilot studies not presented here we introduced a barrier between the rat and shock rod, so that rats had to overcome this obstacle in order to interact with the rod. Our pilot studies indicated that shock rod attracted rats repeatedly climbed over the barrier and continued to shock themselves over the course of a session. Thus, our results suggest that VP ChR2 induction of 'wanting' what hurts may serve as a powerful proof of principle that strong motivational 'wanting' can be produced completely devoid of 'liking'.

Comparison with other optogenetic studies of hedonic taste modulation. Recent studies in mice have reported that optogenetic stimulation of VP^{GABA} and arkypallidal VP neurons that project back to NAc enhances voluntary 'liking' of a laser-paired spout, promotes laser self-stimulation, and elicited AI-scored positive facial expression in mice (Dolensek et al., 2020; Vachez et al., 2021). Our results are in agreement with these findings, and show that stimulation of VP^{GABA} neurons in rats also supported laser-stimulation and directed instrumental responses for laser-paired sucrose. Dolensek and colleagues do not report the anatomical site of their VP manipulations, and further do not state which facial responses were scored by machine learning algorithm and grouped into the 'pleasure' category, making it difficult to determine whether the overall affective score may have included more traditional measures of 'wanting' (Dolensek et al., 2020). Further, Vachez and colleagues used voluntary licking as a measure of affective 'liking' in mice, but this can be more aptly described as a measure of 'wanting', as it requires an appetitive response to execute a decision to ingest. Measures of motivation are sensitive to

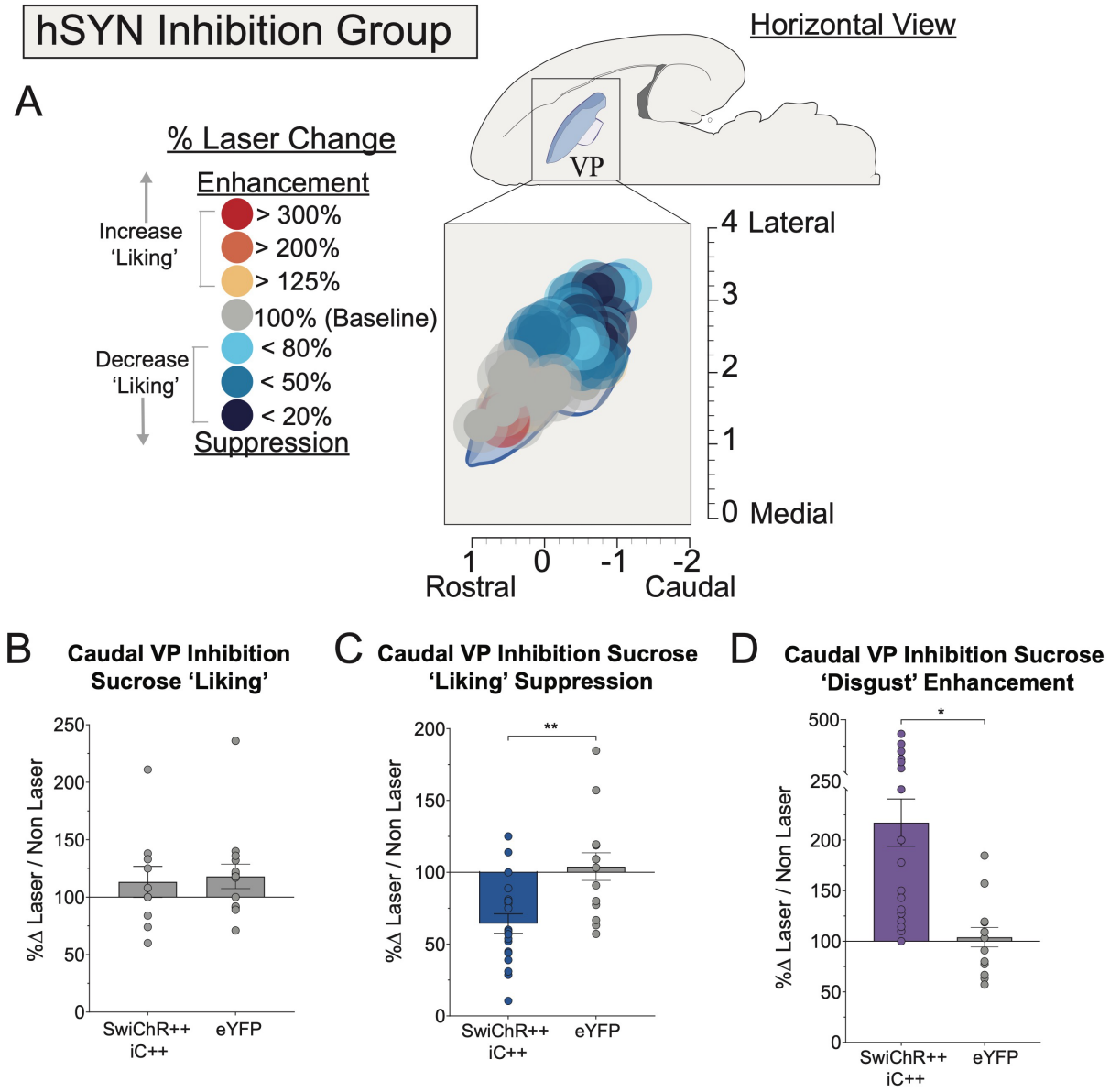
manipulations that do not alter affective 'liking' reactions, including dopamine manipulations (Berridge & Robinson, 1998; Brauer & De Wit, 1997; D'Aquila, 2010; Leyton et al., 2005; Peciña et al., 1997; Schneider et al., 1990b; Sienkiewicz-Jarosz et al., 2005). Our current study infused taste infusions directly into the mouth of rats in order to skip voluntary decisions and actions to ingest and served as a more direct measurement of affective state.

Human studies. Human studies support the hypothesis that VP contributes to affective and motivational processes. Neuroimaging studies have shown that images of palatable foods are associated with increased activity in posterior ventral pallidum (Beaver et al., 2006; Calder et al., 2007). Conversely, higher indices of 'disgust' in humans correlated with activity within rostral VP, the site of hedonic suppression identified in the current investigation (Calder et al., 2007). A recent fMRI study also reported that VP activity is suppressed in people shown pictures or odors who do not find cheese pleasant compared to participants that show no such aversion to cheese, further suggesting that VP activity may serve as a neural marker of hedonic value in humans too (Royet et al., 2016).

Conclusion. Our results show that optogenetic manipulations within the previously identified opioid and orexin hedonic hotspot in caudolateral ventral pallidum can control hedonic impact, similar to previous reports of excitoxic lesions or pharmacological drug microinjections within the same sites in VP (Cromwell & Berridge, 1993; Ho & Berridge, 2013, 2014; Khan et al., 2019; K. S. Smith & Berridge, 2005). Here we mapped sites that induced intense 'disgust' to otherwise palatable sucrose generated by general VP neuron inhibitions were restricted to posterior VP. Further, we show that directly stimulating VP^{GABA} neurons within this posterior region of VP oppositely produced hedonic gains of function by increasing positive 'liking' reactions to sweetness. Finally, we find that sites able to modulate incentive motivation, or

'wanting' food or laser self-stimulation extended beyond the VP hedonic hotspot into rostral VP areas, indicating a partial dissociation between VP mechanisms of 'liking' versus 'wanting'. Finally, we provide further evidence that 'wanting' dissociates from 'liking' in rostral VP^{GABA} neurons via generation of maladaptive attraction for pain in a subset of rats. Understanding how hedonic hotspot mechanisms generate 'liking' vs. 'wanting' for rewards may lead to improved understand of hedonic dysfunctions associated with affective disorders.

4.6 Figures.


hSYN Inhibition Group
A
Horizontal View
VP
% Laser Change
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Increase 'Liking'
> 300%
> 200%
> 125%
100% (Baseline)
Decrease 'Liking'
< 80%
< 50%
< 20%
Suppression
4 Lateral
3
2
1
0 Medial
1 0 -1 -2
Rostral
Caudal
B
Caudal VP Inhibition Sucrose 'Liking'
%Δ Laser / Non Laser
250
200
150
100
50
0
SwiChR++ iC++
eYFP
C
Caudal VP Inhibition Sucrose 'Liking' Suppression
**
%Δ Laser / Non Laser
200
150
100
50
0
SwiChR++ iC++
eYFP
D
Caudal VP Inhibition Sucrose 'Disgust' Enhancement
*
%Δ Laser / Non Laser
500
250
200
150
100
50
0
SwiChR++ iC++
eYFP

Figure 26. VP sites where neuronal inhibitions suppressed sucrose hedonic impact.

A) Localization of hedonic function map shows how optogenetic iC++/SwiChR++ inhibitions decreased affective 'liking' reactions at each individual's VP site. Colors reveal hedonic suppression/ enhancement effects of laser inhibition at each VP site, measured as laser-induced changes in hedonic taste reactivity (positive 'liking' reactions) elicited by intra-oral sucrose infusions. Each symbol placement indicates an individual rat's size site of virus expression. Color of symbols represents the within-subjects behavioral change in hedonic reactions induced by iC++/SwiChR++ laser inhibition reflected as percent change in hedonic reactions from no laser control conditions measured in the same rats ('Liking' enhancements: Red-yellow; 'Liking' suppression: blue). **B)** Laser iC++/SwiChR++ inhibitions differentially alter hedonic 'liking' reactions depending on the anatomical subregion of VP. At rostromedial VP, laser inhibitions did not later hedonic 'liking' reactions (U = 75.00, Z= 0.293, p = 0.80; n = 10 iC++/SwiChR++, n = 14 eYFP) to sucrose. **C)** At caudolateral VP sites, laser inhibitions suppressed sucrose 'liking' reactions in iC++/SwiChR++ rats but not eYFP controls (U = 215.00, Z= 3.06, **p < 0.002, n = 18 iC++/SwiChR++, n = 14 eYFP) and also D) increased aversive 'disgust' reactions elicited by sucrose infusions (U = 65.00, Z= -2.48, *p < 0.05). Data presented as means and SEM.

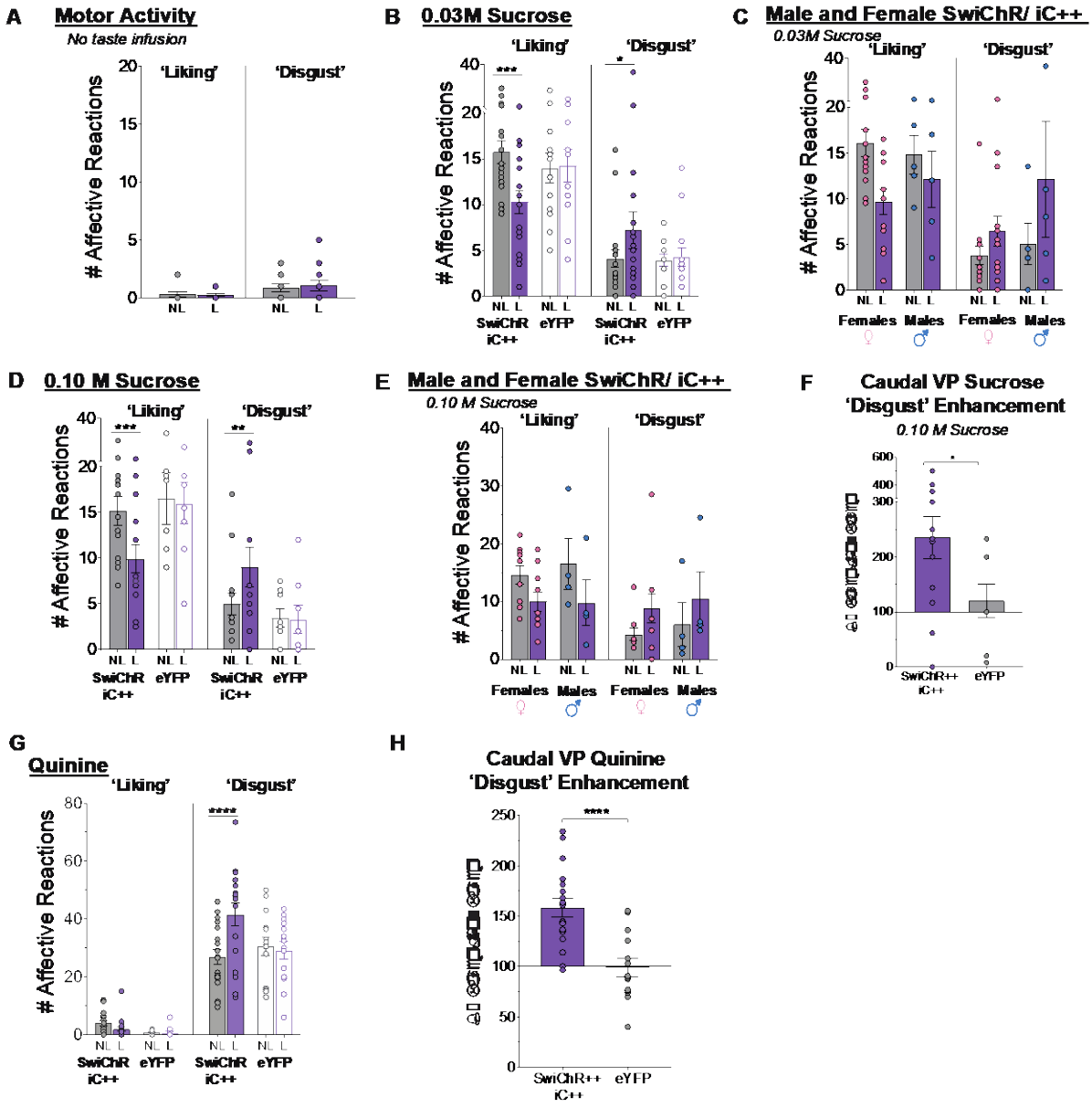

A
Motor Activity
No taste infusion
Affective Reactions
'Liking'
'Disgust'
NL
L
B
0.03M Sucrose
'Liking'
'Disgust'
***
*
NL L NL L NL L NL L
SwiChR iC++
eYFP
SwiChR iC++
C
Male and Female SwiChR/ iC++
0.03M Sucrose
'Liking'
'Disgust'
Females
Males
D
0.10 M Sucrose
'Liking'
'Disgust'
***
**
E
Male and Female SwiChR/ iC++
0.10 M Sucrose
'Liking'
'Disgust'
F
Caudal VP Sucrose
'Disgust' Enhancement
0.10 M Sucrose
SwiChR++ iC++
eYFP
G
Quinine
'Liking'
'Disgust'
****
H
Caudal VP Quinine
'Disgust' Enhancement
SwiChR++ iC++
eYFP

Figure 27. Caudal VP taste reactivity.

Raw affective component counts elicited by various tastants following caudolateral VP neuronal inhibitions **A)** No oromotor reactions observed from caudal VP inhibitions (n = 13) in the absence of taste infusions **B)** Optogenetic laser inhibitions suppress hedonic 'liking' reactions and also increase aversive 'disgust' reactions to 0.03M sucrose in iC++/SwiChR++ rats but not eYFP controls. **C)** Male (n = 5) and female (n = 14) caudal VP iC++/SwiChR++ rats show similar laser-induced decreases in positive 'liking' and increases in aversive 'disgust' reactions to 0.03M sucrose. **D)** Optogenetic VP laser inhibitions suppress hedonic 'liking' reactions and increase aversive 'disgust' reactions to 0.10M sucrose in iC++/ SwiChR++ (n = 14) but not eYFP (n = 8) controls. **E)** Caudal VP inhibitions suppress positive 'liking' reactions and increase aversive 'disgust' expressions by similar magnitudes in male (n = 4) and female (n = 10) iC++/SwiChR++ rats. **F)** Enhancement of 0.10M sucrose 'disgust' reactions reflected as a percent change from no laser baseline in iC++/ SwiChR++ rats and eYFP controls (U = 26.00, *p < 0.05). **G)** Aversive 'disgust' reactions to quinine are magnified by caudal VP neuron inhibitions in iC++/SwiChR++ rats (n = 18) but not eYFP controls (n = 14). **H)** Percent enhancement of aversive 'disgust' reactions elicited by quinine infusions are magnified in iC++/SwiChR++ rats but not eYFP controls (U = 30.00, ****p < 0.0001). All data presented as means and SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

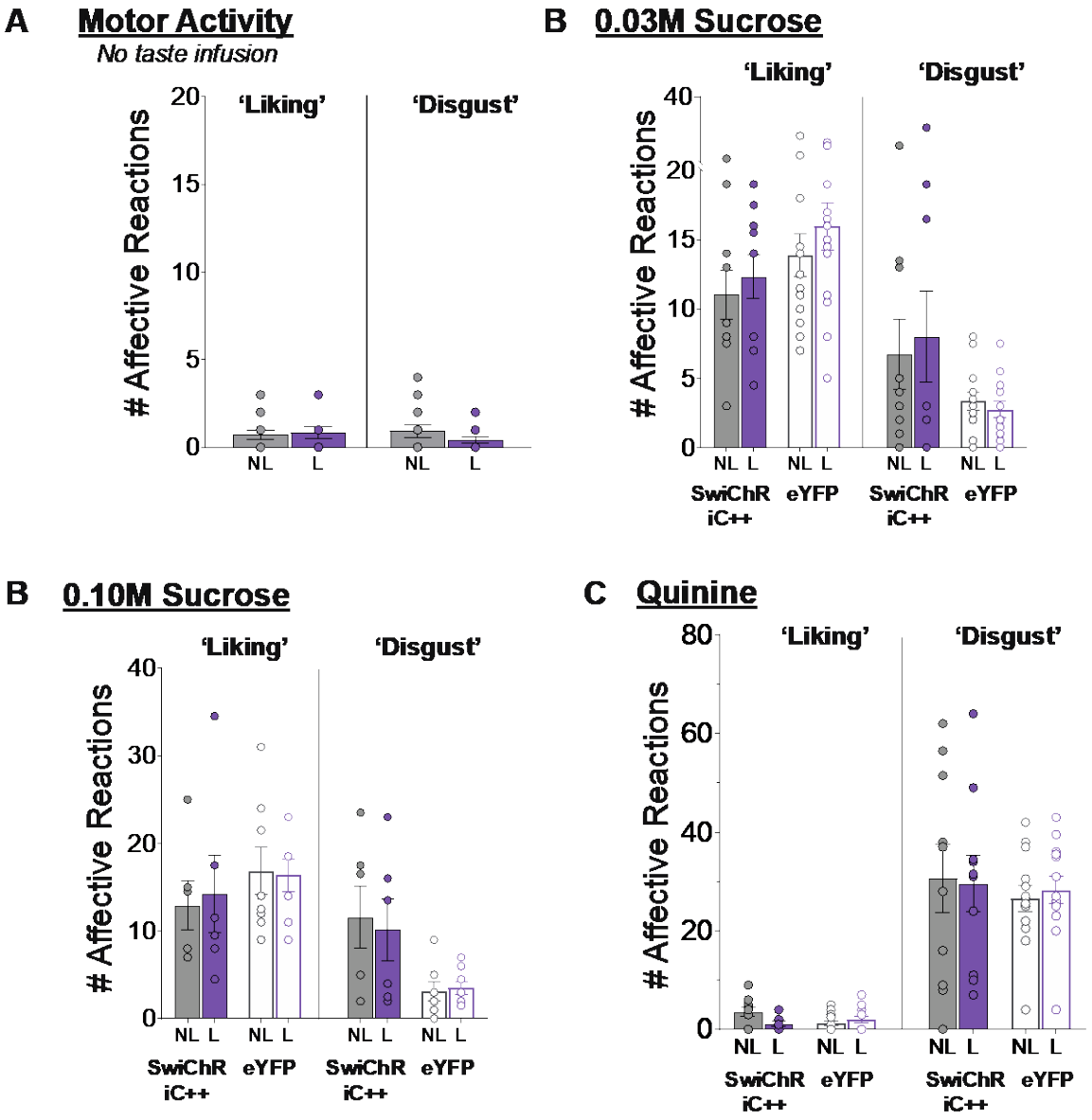

A
Motor Activity
No taste infusion
Affective Reactions
'Liking'
'Disgust'
NL L NL L
B
0.03M Sucrose
'Liking'
'Disgust'
NL L NL L NL L NL L
SwiChR iC++
eYFP
SwiChR iC++
eYFP
B
0.10M Sucrose
C
Quinine

Figure 28. Rostral VP taste reactivity.

Raw affective component counts elicited by various tastants following rostromedial VP neuronal inhibitions **A)** No oromotor reactions observed from rostral VP inhibitions in the absence of taste infusions (n = 14) **B)** Optogenetic laser inhibitions do not later affective reactions to 0.03M sucrose in iC++/SwiChR++ rats (n = 10) or eYFP controls (n = 14). **C)** No change in affective reactions emitted to 0.10M sucrose following rostral VP laser inhibitions in iC++/SwiChR++ rats (n = 6) or eYFP controls (n = 8). **D)** No change in quinine elicited aversive 'disgust' reactions rostral VP iC++/SwiChR++ rats or eYFP controls after optogenetic laser inhibitions. All data presented as means and SEM.

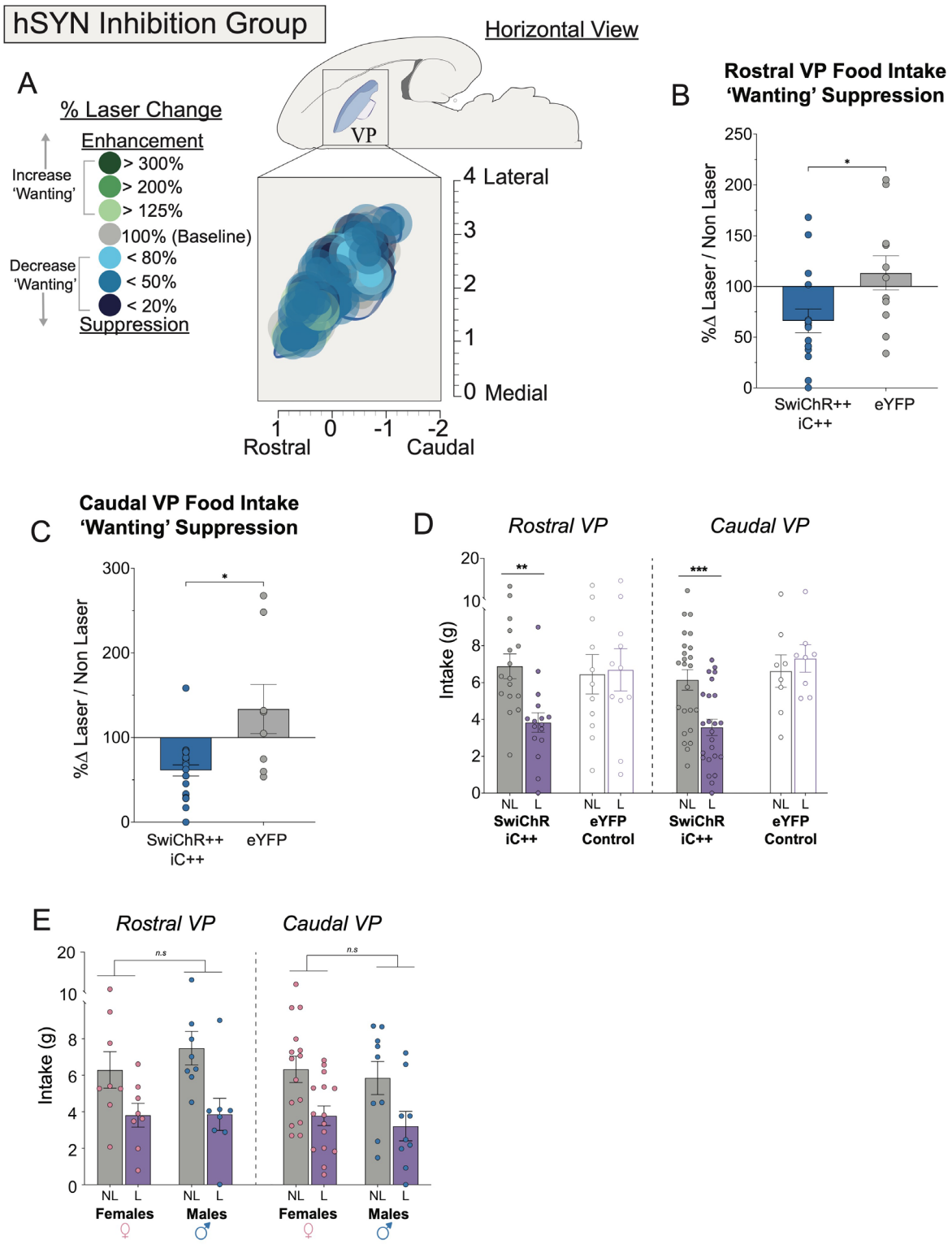

hSYN Inhibition Group
Horizontal View
A
% Laser Change
Enhancement
Increase 'Wanting'
> 300%
> 200%
> 125%
100% (Baseline)
Decrease 'Wanting'
< 80%
< 50%
< 20%
Suppression
VP
4 Lateral
3
2
1
0 Medial
1 0 -1 -2
Rostral
Caudal
B
Rostral VP Food Intake 'Wanting' Suppression
%Δ Laser / Non Laser
250
200
150
100
50
0
*
SwiChR++ iC++
eYFP
C
Caudal VP Food Intake 'Wanting' Suppression
%Δ Laser / Non Laser
300
200
100
0
*
SwiChR++ iC++
eYFP
D
Rostral VP
Caudal VP
Intake (g)
20
10
8
6
4
2
0
**
***
NL L
SwiChR iC++
eYFP Control
SwiChR iC++
eYFP Control
E
Rostral VP
Caudal VP
Intake (g)
20
10
8
6
4
2
0
n.s
n.s
NL L
Females
Males
Females
Males

Figure 29. VP neuron inhibitions suppress food 'wanting'.

Shows site in VP neurons where iC++/SwiChR++ inhibitions suppressed feeding during 1-h test of voluntary intake. Each symbol placement indicates an individual rat's iC++/SwiChR++ expression. Colors of symbols represent the magnitude of change in feeding following VP laser inhibitions reflected as a within subject change from no laser conditions (green: increases in intake; grey: no change; blue: decreased intake). **B)** Percent change in feeding induced by laser inhibitions in rostral VP rats. iC++/SwiChR++ rats but not eYFP controls show suppressed feeding after optogenetic VP neuron inhibitions (U = 43.00, *p = 0.03). **C)** Percent change in feeding induced by laser inhibitions in caudal VP rats. iC++/SwiChR++ rats but not eYFP controls show suppressed feeding after optogenetic VP neuron inhibitions (U = 43.00, *p = 0.02). **D)** Raw intake amounts (g) during 1-h voluntary food intake tests. iC++/SwiChR++ neuron inhibitions decrease feeding in rostral (n = 16) and caudal VP rats (n = 24), but not eYFP controls (rostral n = 11; caudal n = 8). **E**) The magnitude of feeding suppression is similar in male and female VP IC++/SwiChR++ rats (rostral male n = 8, rostral female n = 8; caudal male n = 9, caudal female n = 15). Data presented as mean and SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

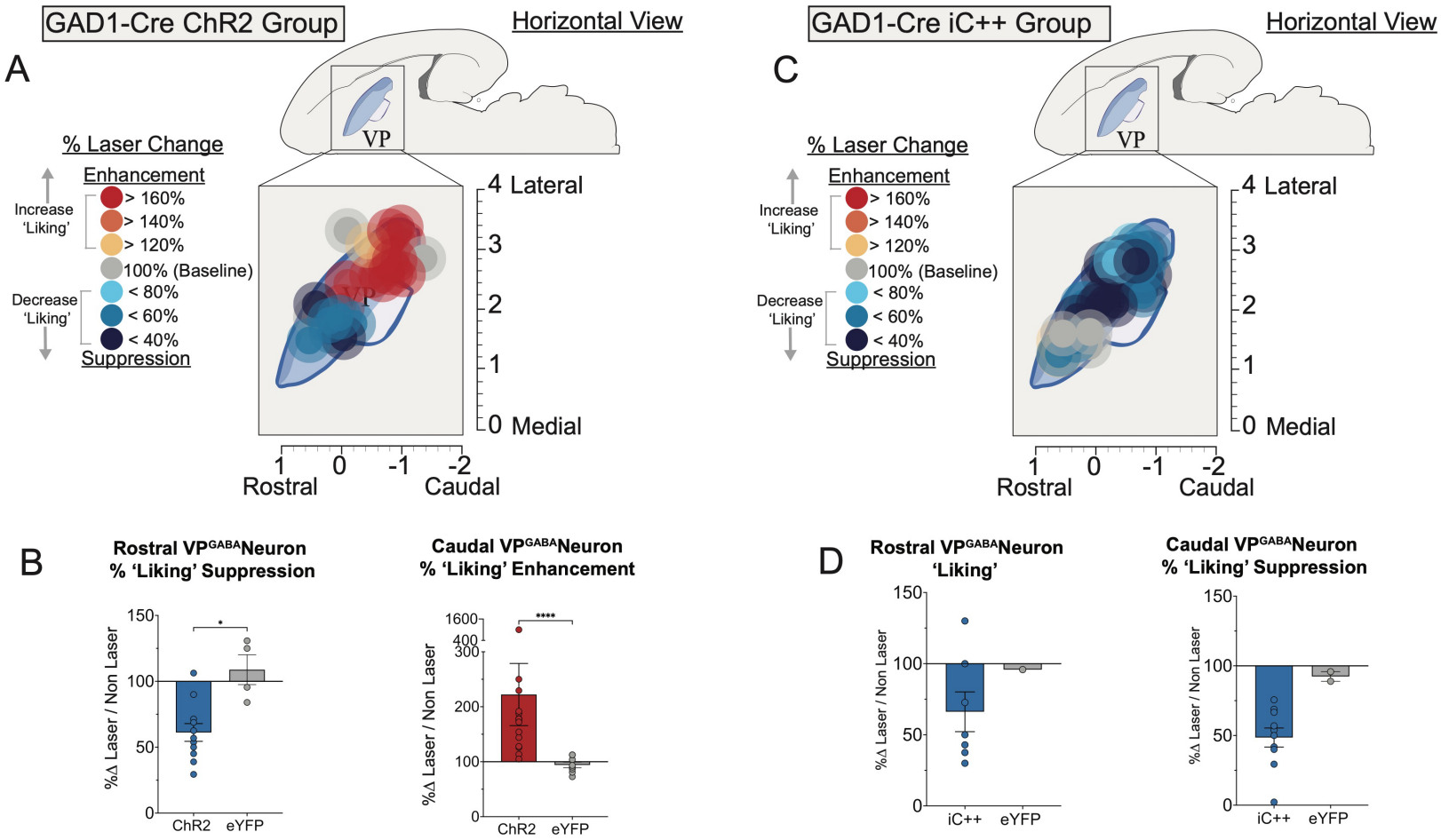

A
GAD1-Cre ChR2 Group
Horizontal View
VP
% Laser Change
Enhancement
Increase 'Liking'
> 160%
> 140%
> 120%
100% (Baseline)
Decrease 'Liking'
< 80%
< 60%
< 40%
Suppression
4 Lateral
3
2
1
0 Medial
1 0 -1 -2
Rostral
Caudal
C
GAD1-Cre iC++ Group
Horizontal View
VP
% Laser Change
Enhancement
Increase 'Liking'
> 160%
> 140%
> 120%
100% (Baseline)
Decrease 'Liking'
< 80%
< 60%
< 40%
Suppression
4 Lateral
3
2
1
0 Medial
1 0 -1 -2
Rostral
Caudal
B
Rostral VP^GABA Neuron
% 'Liking' Suppression
%Δ Laser / Non Laser
150
100
50
0
*
ChR2
eYFP
Caudal VP^GABA Neuron
% 'Liking' Enhancement
%Δ Laser / Non Laser
1600
400
300
200
100
0
****
ChR2
eYFP
D
Rostral VP^GABA Neuron
'Liking'
%Δ Laser / Non Laser
150
100
50
0
iC++
eYFP
Caudal VP^GABA Neuron
% 'Liking' Suppression
%Δ Laser / Non Laser
150
100
50
0
iC++
eYFP

Figure 30. Sites on VP GABA neurons that affect hedonic impact.

Localization of hedonic function maps shows how optogenetic ChR2 stimulation of VP^{GABA} neurons altered affective 'liking' reactions at each individual VP site. Colors reveal hedonic enhancement/suppressive effects of laser stimulation at each VP^{GABA} neuron site, measured as laser-induced changes in hedonic taste reactivity (positive 'liking' reactions) elicited by intra-oral sucrose infusions. Each symbol placement represents the within-subjects behavioral change in hedonic reactions induced by ChR2 laser activations reflected as percent change in hedonic reactions from no laser conditions in the same rats ('Liking' enhancements: red-yellow; no change: grey; 'liking' suppression: blue). **B)** ChR2 laser stimulations differentially alter hedonic 'liking' reactions depending on the anatomical subregion of VP. At rostromedial VP^{GABA} sites, ChR2 activations suppressed hedonic 'liking' reactions to sucrose (U = 3.00, p = 0.01; *n* = 11 ChR2; *n* = 4 eYFP). At caudolateral VP^{GABA} sites, photostimulation doubled hedonic 'liking' reactions in ChR2 rats but not eYFP controls (U = 2.00, p = 0.0001; *n* = 15 ChR2; *n* = 8 eYFP). **C)** Localization of hedonic function map shows how optogenetic iC++ GABA neuron inhibitions in VP altered affective 'liking' reactions at each individual VP site. **D)** Shows effects of iC++ inhibitions based on anatomical subregion of VP reflected as a percent change from a no laser condition in the same rats. We were not powered enough to analyze percent change data. Data presented as mean and SEM. **p* < 0.05, *****p* < 0.0001.

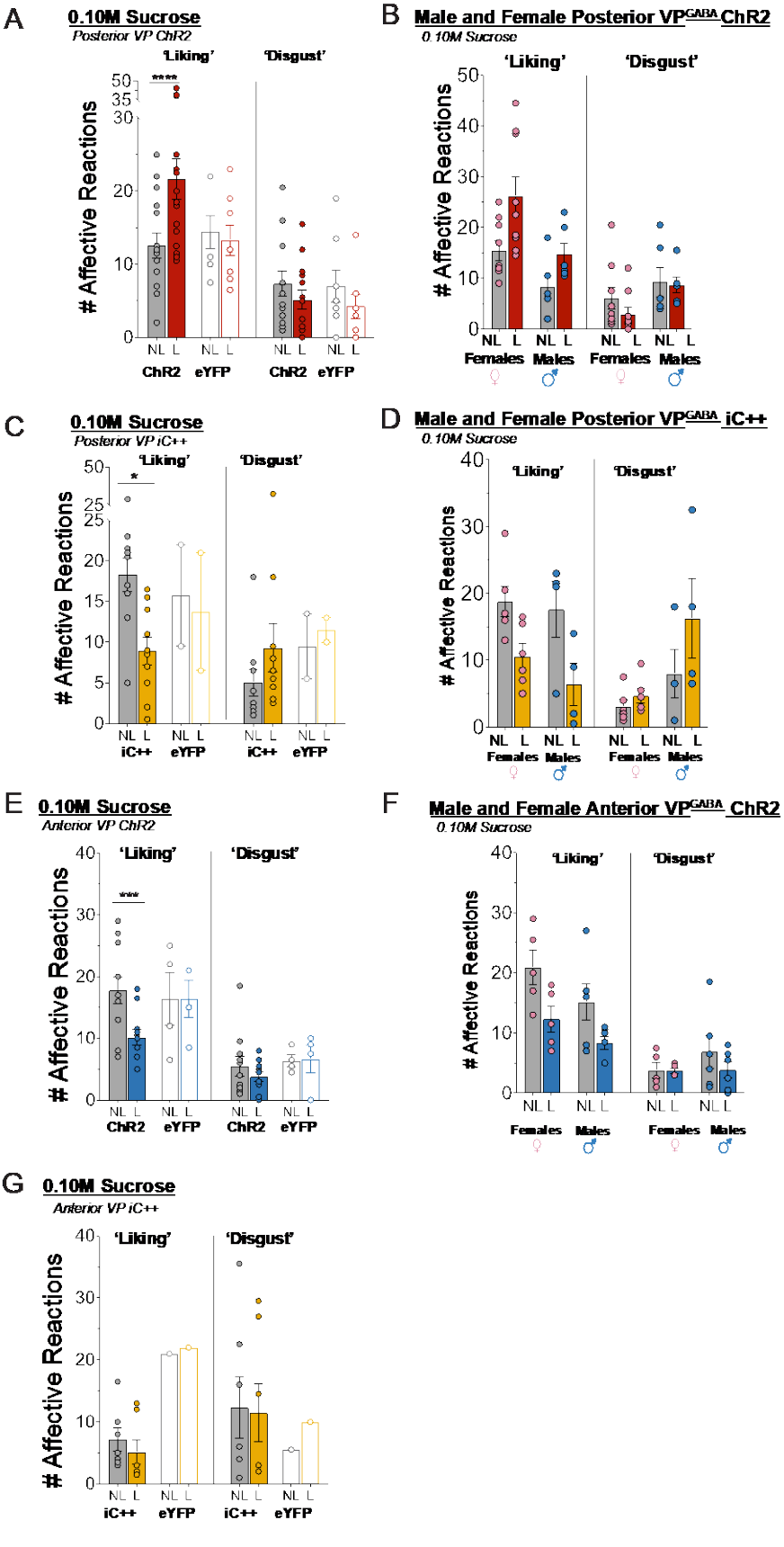

A
0.10M Sucrose
Posterior VP ChR2
'Liking'
'Disgust'
Affective Reactions
NL L NL L NL L NL L
ChR2 eYFP ChR2 eYFP
B
Male and Female Posterior VP GABA ChR2
0.10M Sucrose
'Liking'
'Disgust'
Affective Reactions
NL L NL L NL L NL L
Females Males Females Males
C
0.10M Sucrose
Posterior VP iC++
'Liking'
'Disgust'
Affective Reactions
NL L NL L NL L NL L
iC++ eYFP iC++ eYFP
D
Male and Female Posterior VP GABA iC++
0.10M Sucrose
'Liking'
'Disgust'
Affective Reactions
NL L NL L NL L NL L
Females Males Females Males
E
0.10M Sucrose
Anterior VP ChR2
'Liking'
'Disgust'
Affective Reactions
NL L NL L NL L NL L
ChR2 eYFP ChR2 eYFP
F
Male and Female Anterior VP GABA ChR2
0.10M Sucrose
'Liking'
'Disgust'
Affective Reactions
NL L NL L NL L NL L
Females Males Females Males
G
0.10M Sucrose
Anterior VP iC++
'Liking'
'Disgust'
Affective Reactions
NL L NL L NL L NL L
iC++ eYFP iC++ eYFP

Figure 31. VP GABA neuron taste reactivity.

Raw affective component counts elicited by various tastants following VP^{GABA} neuron manipulations. **A)** Optogenetic laser excitations increase positive 'liking' reactions to 0.10M sucrose in caudal VP^{GABA} ChR2 (n = 15) rats but not eYFP controls (n =8). **B)** Male (n = 6) and female (n = 9) caudal VP ChR2 rats have similar laser-induced enhancement of positive affective reactions to 0.10M sucrose. **C)** Optogenetic laser inhibitions in caudal VP^{GABA} neurons (n = 10) suppress 'liking' reactions to 0.10M sucrose in iC++ but not eYFP controls (n = 2). **D)** Similar suppression of positive 'liking' reactions in male (n = 4) and female (n = 6) caudal VP iC++ rats. **E)** Rostral VP^{GABA} neuron excitations suppress positive 'liking' 0.10M sucrose reactions in ChR2 rats (n = 11) but not eYFP controls (n =8). **F)** Male (n = 6) and female (n = 5) rostral VP^{GABA} ChR2 rats show similar suppression of positive 'liking' reactions to 0.10M sucrose. **G)** No change in affective expressions to 0.10M sucrose after laser inhibitions in rostral VP^{GABA} iC++ rats (n = 7) or eYFP controls (n =1). All data presented as means and SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

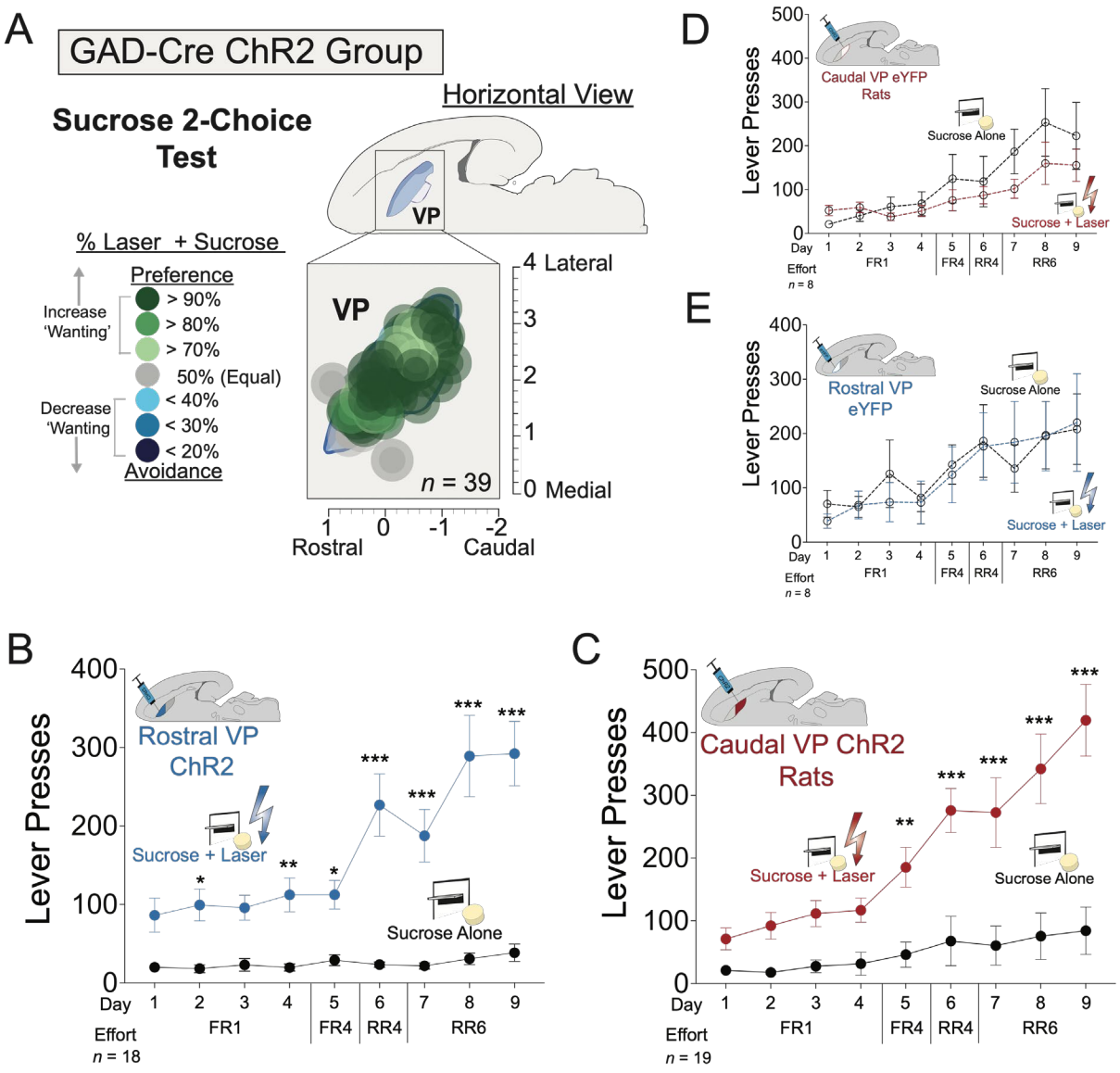

A
GAD-Cre ChR2 Group
Sucrose 2-Choice Test
Horizontal View
VP
% Laser + Sucrose
Preference
Increase 'Wanting'
> 90%
> 80%
> 70%
50% (Equal)
Decrease 'Wanting'
< 40%
< 30%
< 20%
Avoidance
VP
n = 39
4 Lateral
3
2
1
0 Medial
1 0 -1 -2
Rostral
Caudal
D
Caudal VP eYFP Rats
Sucrose Alone
Sucrose + Laser
Lever Presses
Day 1 2 3 4 5 6 7 8 9
Effort
FR1 FR4 RR4 RR6
n = 8
E
Rostral VP eYFP
Sucrose Alone
Sucrose + Laser
Lever Presses
Day 1 2 3 4 5 6 7 8 9
Effort
FR1 FR4 RR4 RR6
n = 8
B
Rostral VP ChR2
Sucrose + Laser
Sucrose Alone
Lever Presses
Day 1 2 3 4 5 6 7 8 9
Effort
FR1 FR4 RR4 RR6
n = 18
C
Caudal VP ChR2 Rats
Sucrose + Laser
Sucrose Alone
Lever Presses
Day 1 2 3 4 5 6 7 8 9
Effort
FR1 FR4 RR4 RR6
n = 19

Figure 32. VP GABA neuron stimulation biases sucrose motivation.

Shows sites on VP^{GABA} neurons where ChR2 activations directed motivation for laser-paired sucrose in the two-choice test. Each symbol placement indicates an individual rat's channelrhodopsin expression. Colors of symbols represents the level of preference for laser-paired sucrose (green: laser-sucrose preference; grey: no preference; blue: avoidance of laser-paired sucrose). **B)** Rostral VP^{GABA} ChR2 rats (n=19) reached 8:1 preference ratio by day 9. **C)** Caudal VP^{GABA} neuron ChR2 (n=19) rats reached a 5:1 Laser + sucrose preference by day 9. **D**) Rostral VP^{GABA} eYFP controls (n=8) and Caudal VP^{GABA} eYFP controls (n=8) choose equally between sucrose and sucrose + laser options. Data presented as mean and SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

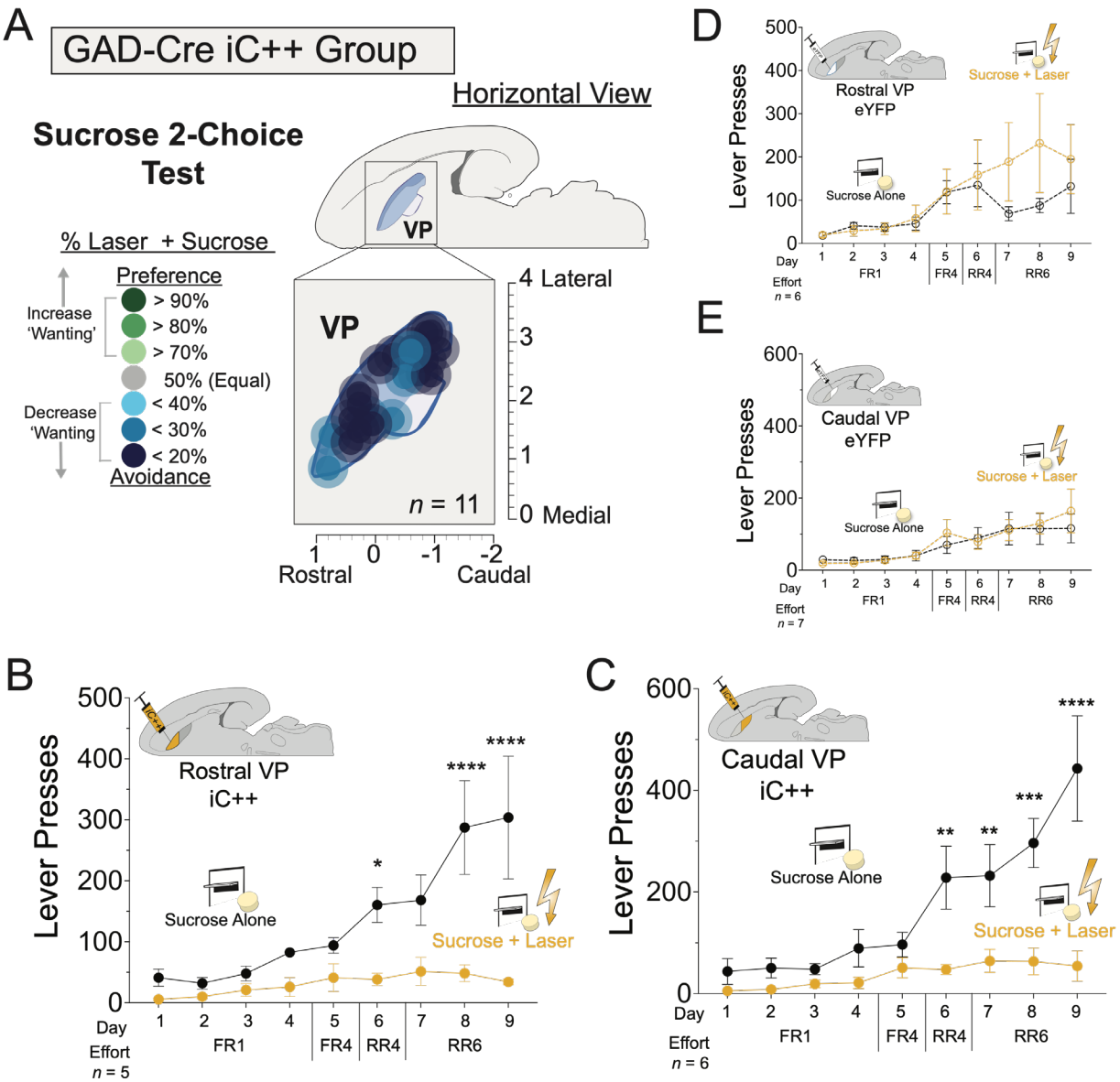
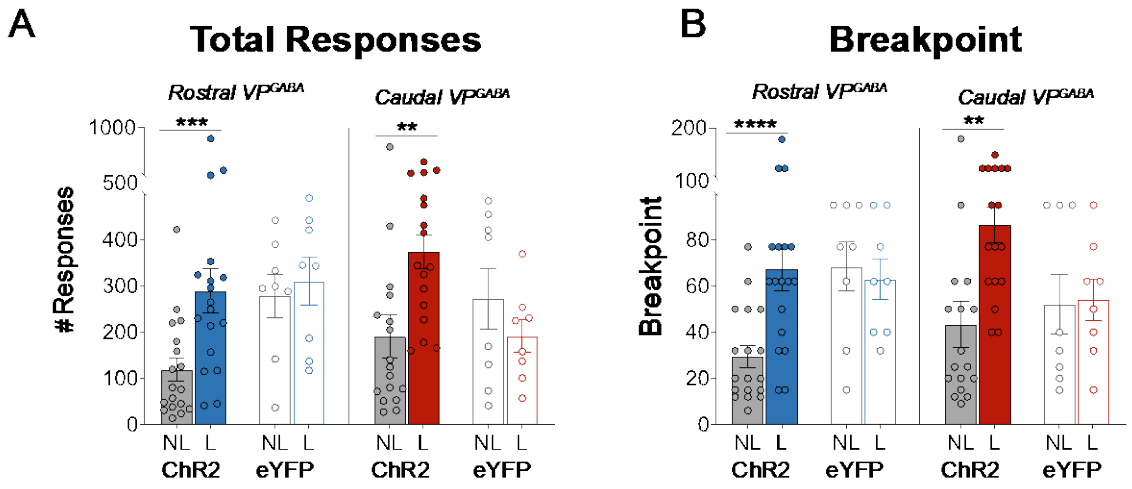

A
GAD-Cre iC++ Group
Horizontal View
Sucrose 2-Choice Test
% Laser + Sucrose
Preference
Increase 'Wanting'
> 90%
> 80%
> 70%
50% (Equal)
Decrease 'Wanting'
< 40%
< 30%
< 20%
Avoidance
VP
VP
n = 11
4 Lateral
3
2
1
0 Medial
1 0 -1 -2
Rostral
Caudal
D
Rostral VP eYFP
Sucrose + Laser
Sucrose Alone
Lever Presses
Day
Effort
n = 6
FR1
FR4
RR4
RR6
E
Caudal VP eYFP
Sucrose + Laser
Sucrose Alone
Lever Presses
Day
Effort
n = 7
FR1
FR4
RR4
RR6
B
Rostral VP iC++
Sucrose Alone
Sucrose + Laser
Lever Presses
Day
Effort
n = 5
FR1
FR4
RR4
RR6
C
Caudal VP iC++
Sucrose Alone
Sucrose + Laser
Lever Presses
Day
Effort
n = 6
FR1
FR4
RR4
RR6

Figure 33. VP GABA neuron inhibition causes laser-paired sucrose avoidance.

Shows sites on VP^{GABA} neurons where iC++ inhibitions generated avoidance of laser-paired sucrose in two-choice test. Each symbol placement indicates an individual rat's iC++ expression. Colors of symbols represents the level of avoidance for laser-paired sucrose (green: laser-sucrose preference; grey: no preference; blue: avoidance of laser-paired sucrose). **B)** Rostral VP^{GABA} iC++ rats (n=5) reached 20:1 avoidance ratio by day 9. **C)** Caudal VP^{GABA} neuron ChR2 (n=6) rats reached a 9:1 Laser + sucrose avoidance by day 9. **D**) Rostral VP^{GABA} eYFP controls (n=6) and Caudal VP^{GABA} eYFP controls (n=7) choose equally between sucrose and sucrose + laser options. Data presented as mean and SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

GAD-Cre ChR2 Group



GAD-Cre iC++ Group

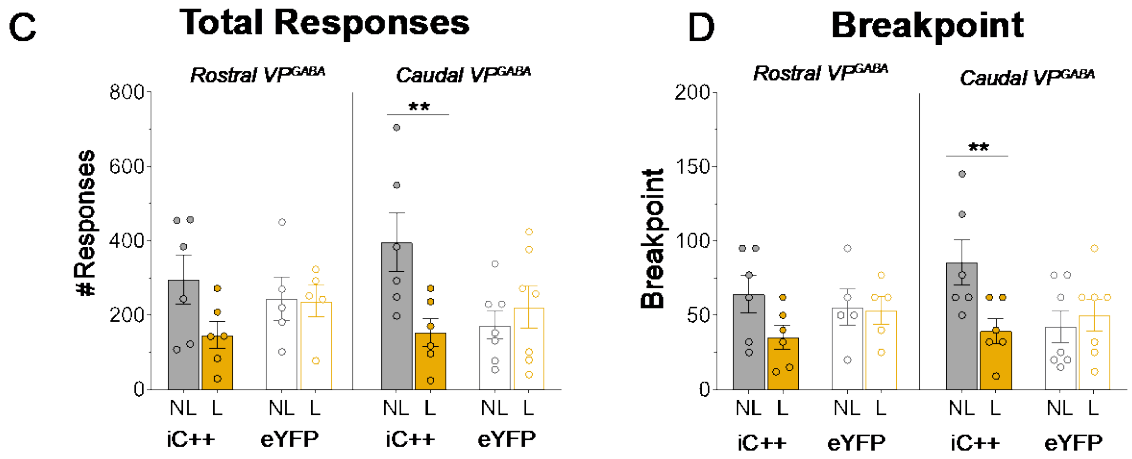
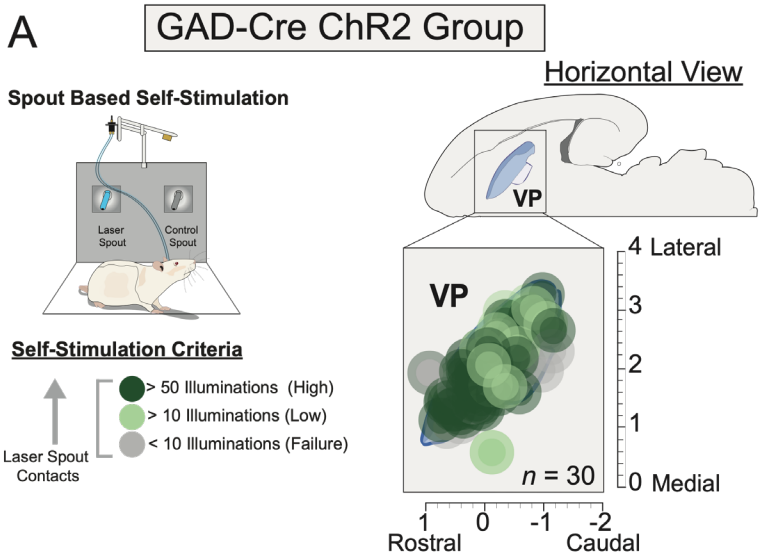
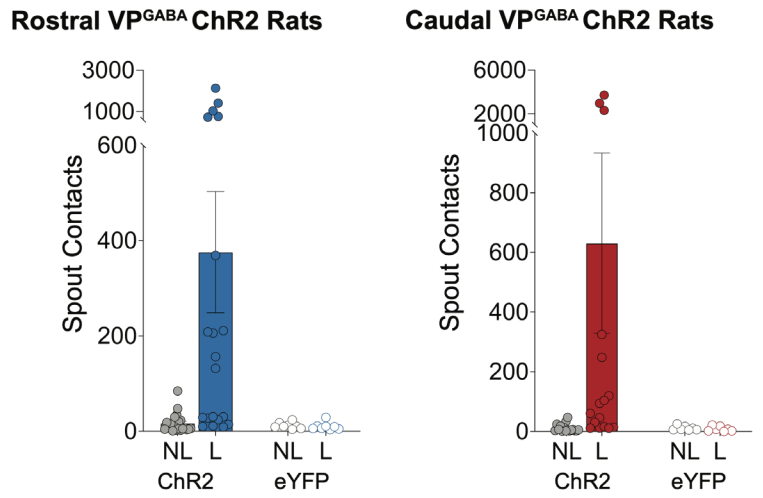
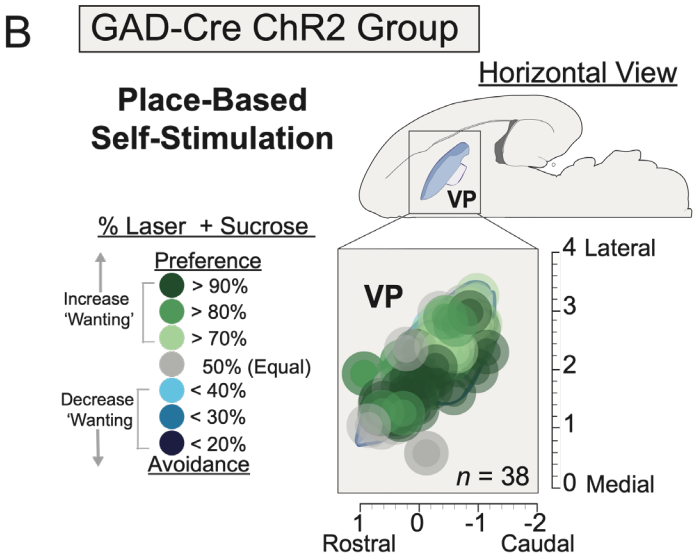


Figure 34. VP GABA neurons bidirectionally control intensity of incentive motivation for laser-paired sucrose in progressive ratio tests.

We used a progressive ratio test of motivation on two consecutive days. On one day, rats responded for sucrose + laser and on the other day, rats responded for sucrose alone on a progressive ratio schedule of reinforcement so that the effort required to obtain the next remifentanil infusion increases exponentially. **A)** Rostral (n = 18) and caudal (n = 17) VP^{GABA} ChR2 rats made greater overall responses for sucrose + laser than sucrose alone. eYFP controls made equal overall responses (rostral n = 8; caudal n = 9). **B)** VP^{GABA} ChR2 rats reached higher breakpoints for sucrose+ laser compared to sucrose alone during PR testing (rostral ChR2 n = 18; Caudal ChR2 n = 17). **C)** Caudal VP^{GABA} neuron iC++ (n = 6) inhibitions suppress total responses for sucrose + laser compared to sucrose alone. Non-significant trend observed in rostral VP iC++ rats (n = 6). No differences in total responses in eYFP controls (rostral n = 5; caudal n = 7). **D)** Caudal VP^{GABA} iC++ rats reached lower breakpoints for sucrose + laser relative to sucrose alone. Non-significant trend observed in rostral VP iC++ rats; similar breakpoints reached by eYFP controls. Data shown as mean and SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$.


A
GAD-Cre ChR2 Group
Spout Based Self-Stimulation
Laser Spout
Control Spout
Self-Stimulation Criteria
Laser Spout Contacts
> 50 Illuminations (High)
> 10 Illuminations (Low)
< 10 Illuminations (Failure)
Horizontal View
VP
VP
n = 30
4 Lateral
3
2
1
0 Medial
1 0 -1 -2
Rostral
Caudal


Rostral VP^GABA ChR2 Rats
Caudal VP^GABA ChR2 Rats
Spout Contacts
3000
1000
600
400
200
0
6000
2000
1000
800
600
400
200
0
NL L
ChR2
NL L
eYFP


B
GAD-Cre ChR2 Group
Place-Based Self-Stimulation
Horizontal View
% Laser + Sucrose
Preference
Increase 'Wanting'
> 90%
> 80%
> 70%
50% (Equal)
Decrease 'Wanting'
< 40%
< 30%
< 20%
Avoidance
VP
VP
n = 38
4 Lateral
3
2
1
0 Medial
1 0 -1 -2
Rostral
Caudal

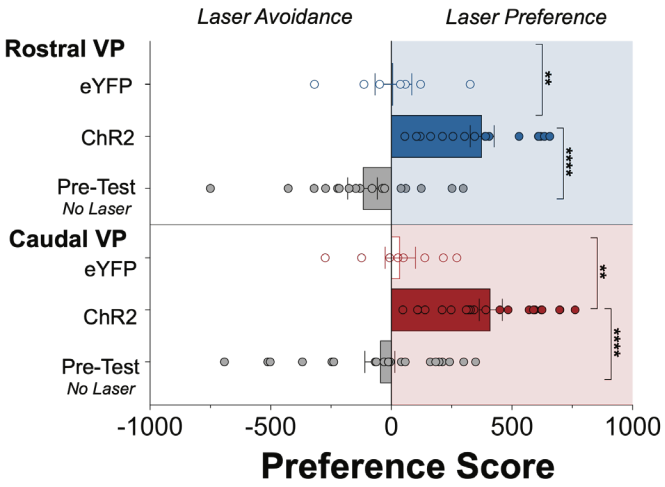

Laser Avoidance
Laser Preference
Rostral VP
eYFP
ChR2
Pre-Test
No Laser
Caudal VP
eYFP
ChR2
Pre-Test
No Laser
**
****
**
****
-1000
-500
0
500
1000
Preference Score

Figure 35. VP GABA sites that support incentive motivation for laser.

Functional maps show instrumental performance to earn ChR2 laser stimulations at each VP site on a spout-touch laser self-stimulation test (map based on 40 Hz, 1-s pulse data). Each symbol placement indicates an individual rat's channelrhodopsin expression. Color of symbols represents levels of self-stimulation criteria met by each rat (high self-stimulation (>50 illuminations earned): dark green; low self-stimulation (10 to 49 illuminations earned: light green; Failures to self-stimulate (<10 illuminations earned): grey). **B)** Although many rats met criteria for self-stimulation in the spout-task, the overall rostral and caudal ChR2 groups did not reliably self-stimulate in the spout task. **C)** Functional map shows preferences for laser-paired side (green) or avoidance of laser-paired side (blue) during the place-based self-stimulation task. The color reflects the percent preference or avoidance for the laser-delivering chamber in the same rats. **D)** Bar graphs show quantified % laser side preference in rostral VP^{GABA} (n = 21) and caudal VP^{GABA} ChR2 rats (n = 17), who showed evidence of self-stimulation relative to eYFP controls (rostral n = 8; caudal n =8) and the same ChR2 rats during a pre-habituation test with no laser. All data presented as means and SEM. ** $p < 0.01$, **** $p < 0.0001$.

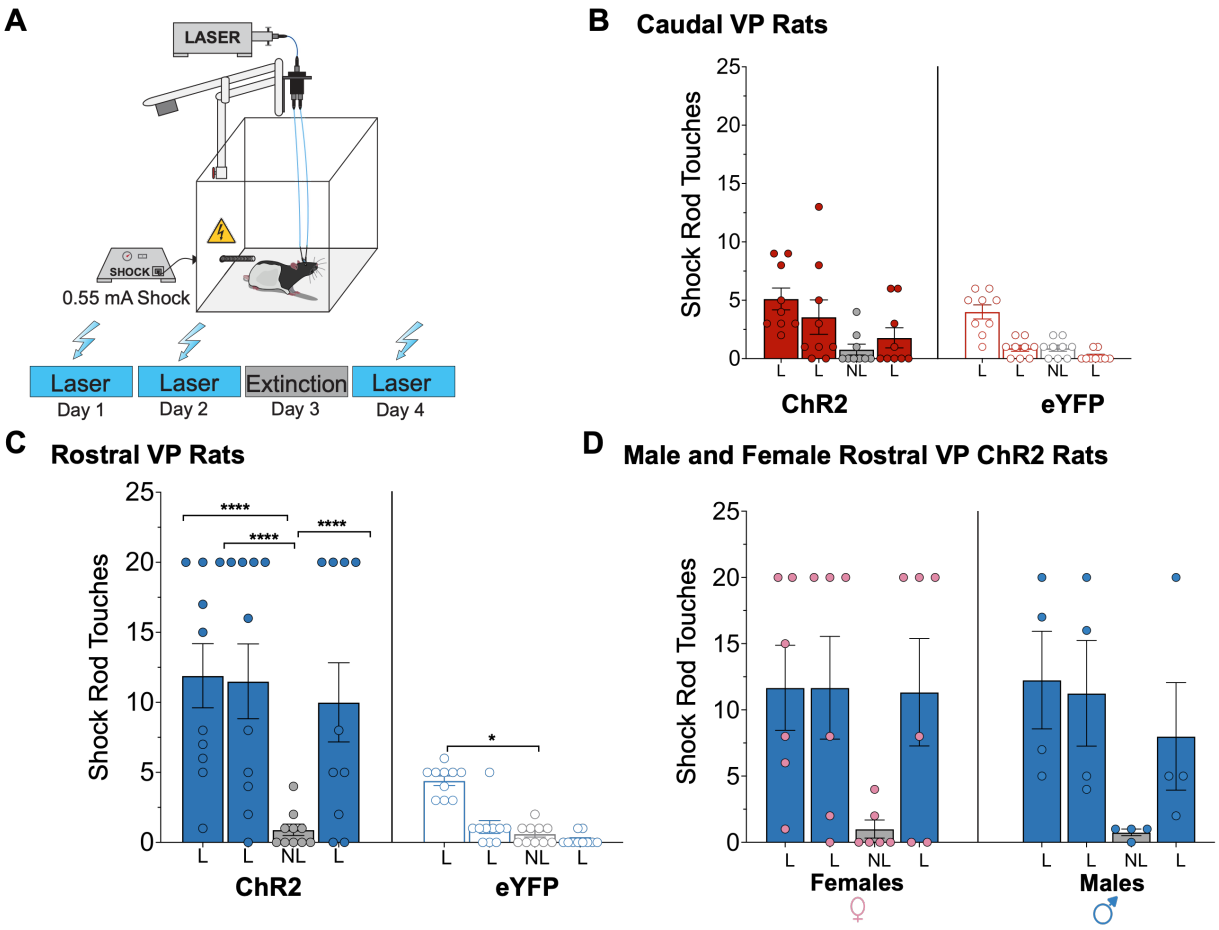


Figure 36. Rostral VP GABA neurons promote attraction to noxious shock rod.

Rostral VP^{GABA} neuron ChR2 rats (n = 10) voluntarily and willingly shock themselves to interact with an electrified shock rod paired with laser stimulation of VP^{GABA} neurons (Day 1, 2, and 4), but do not interact with rod when VP laser stimulation is placed on extinction (Day 3). No shock rod attraction in eYFP controls (n = 10). **B)** Male (n=4) and female (n = 6) VP^{GABA} ChR2 rats show equal levels of attraction to shock rod **C)** No shock rod attraction in caudal VP ChR2 or EYFP controls. Data presented as mean and SEM. * $p < 0.05$, **** $p < 0.0001$.

Chapter 5 'Opioid Addict' vs. 'Sucrose Addict': Paired Central Amygdala Excitation Controls, Amplifies, and Narrows Focus of Desire in Choice between I.V. Remifentanil and Natural Sucrose.

5.1 Abstract

Addiction is characterized by intense and narrowly focused motivation to pursue the addictive target at the expense of other rewards. Previous studies showed that pairing optogenetic channelrhodopsin (ChR2) stimulation of central amygdala (CeA) in rats could selectively recruit mesolimbic circuitry to arbitrarily direct, amplify, and narrow incentive motivation onto a laser-paired target for rats choosing among intravenous cocaine or sucrose rewards. However, opioid drugs have additional effects that differ from cocaine or other psychostimulants, and so opioid self-administration might be expected to resist such CeA ChR2 control. We tested this first in a paradigm that allowed CeA ChR2 rats to freely choose to earn either intravenous remifentanil infusions (2 μg/kg) paired with CeA ChR2 laser excitations or alternatively earn identical remifentanil alone without laser. CeA ChR2 rats intensely and exclusively pursued their ChR2-paired remifentanil option, while ignoring their alternative remifentanil-alone option, and elevated breakpoint for laser-paired remifentanil in a separate progressive ratio test of motivation intensity. Yet CeA ChR2 laser on its own had only modest incentive value, and some CeA ChR2 rats completely failed to self-stimulate laser despite being strongly motivated for laser-paired remifentanil. Further, in a second test, separate groups of CeA ChR2 were allowed to choose between intravenous remifentanil or sucrose pellet rewards, one group of CeA ChR2 rats had laser paired with sucrose but not remifentanil, whereas another group had laser paired with

remifentanil but not sucrose. CeA ChR2 rats that had laser paired with sucrose, exclusively chose sucrose and ignored the opportunity to earn intravenous remifentanil. By contrast, CeA ChR2 rats that had laser paired with remifentanil exclusively chose remifentanil and ignored sucrose. Yet again, CeA ChR2 laser self-stimulation by itself was relatively weak and failed to explain how laser pairing produced 'addictive-like' narrowed motivations for sucrose vs. remifentanil. We conclude that amygdala-related circuitry can transform incentive motivation to produce narrowly focused intense pursuit of a single target in an addictive-like fashion.

5.2 Introduction

Amygdala-related circuitry helps assign motivational significance to stimuli in the environment, involved in motivating both fear-related and reward-related behavior (Balleine & Killcross, 2006; Baxter & Murray, 2002b; Belova et al., 2007, 2008; DiFeliceantonio & Berridge, 2012; LeDoux, 2007; Mahler & Berridge, 2009, 2012; Morrison & Salzman, 2010; Sah et al., 2003; Warlow & Berridge, 2021). In particular, the central nucleus of the amygdala (CeA) has striatal-like status in macrosystem frameworks, with a rich population of largely GABAergic output neurons (Swanson, 2000, 2005; Swanson & Petrovich, 1998), and receives largely glutamate inputs from the 'cortical-like' basolateral amygdala (BLA) (Cassell et al., 1986; McDonald, 1982c, 1982b). Accordingly, local optogenetic or neurochemical stimulations in CeA can generate intense appetitive motivated behavior, similar to local stimulations in nucleus accumbens and parts of neostriatum (Baumgartner et al., 2020; DiFeliceantonio et al., 2012; DiFeliceantonio & Berridge, 2012, 2016; Mahler & Berridge, 2009).

Previous work has shown that pairing optogenetic channelrhodopsin (ChR2) stimulation of CeA either with sucrose reward or with cocaine reward can generate intense incentive motivation to obtain and consume that particular laser-paired reward (M. J. F. Robinson et al.,

2014; Warlow et al., 2017b, 2020). For example, when CeA ChR2 rats could choose between earning either of two sugar options or two intravenous cocaine options, with only one of the two options paired with CeA laser, they exclusively chose their laser-paired option and not the identical alternative without laser (Robinson et al., 2014; Warlow et al., 2017). Further, when rats could choose freely between earning either intravenous cocaine infusions or sucrose pellets, rats that had CeA ChR2 stimulations paired with cocaine developed a narrowly focused 'cocaine addiction' phenotype, avidly pursuing only laser-paired cocaine while ignoring alternative sucrose (Warlow et al., 2020). By contrast, other rats that had CeA ChR2 stimulations paired with sucrose developed a narrowly focused 'sucrose addiction' phenotype, pursuing only laser-paired sucrose while ignoring alternative cocaine (Warlow et al., 2020). In a third condition, CeA ChR2 pairing even produced 'wanting what hurts' as maladaptive attraction to a laser-paired shock rod (Warlow et al., 2020). Similarly showing perseverance despite adverse consequences, CeA ChR2 rats have been reported to be willing to pay a footshock price to pursue laser-paired sucrose rewards (Tom et al., 2018). Yet in all those studies, CeA ChR2 laser stimulation by itself was only moderately self-stimulated, and some rats completely failed to self-stimulate laser despite being strongly attracted to their laser-paired cocaine, sucrose, or shock rod.

Opioids are potent rewards capable of producing uniquely intense euphorigenic and withdrawal effects, and are often taken in ways that differ from psychostimulants (Badiani et al., 2011, 2019; De Pirro et al., 2018; Ettenberg et al., 1982; Pettit et al., 1984; Vassilev et al., 2020). Thus, it may be one thing to control addictive-like motivation for cocaine and sucrose rewards, but a different thing to control motivation for opioid drugs. Consequently, some might expect motivation for an i.v opioid drug to be more intense and resistant to optogenetic control by CeA ChR2 stimulation. Remifentanil is a fast-acting mu-opioid agonist that is approximately 100

times more potent than morphine (Glass et al., 1999; Hoffman et al., 1993), and is readily self-administered by rats (Farrell et al., 2022; Yager et al., 2015b). Here we asked whether paired CeA ChR2 activation controls opioid motivation. We report that the answer appears to be yes: optogenetic CeA ChR2 pairing can control the target of motivation and narrowly focus intense pursuit solely on laser-paired remifentanil or laser-paired sucrose, when rats freely choose either between two remifentanil options or between remifentanil and sucrose. Further, CeA ChR2 pairing increases the intensity of incentive motivation for remifentanil, measured as increased breakpoint in a progressive ratio test. Overall, our results confirm that CeA ChR2 stimulation of amygdala-related circuitry can control motivation for i.v. opioid drug similarly to its control of motivation for i.v. cocaine or for natural sucrose.

5.3 Materials and Methods

Animals

Male and female Sprague Dawley Rats (*n* = 69 total*; n = 30* males*, n = 39* females*)*, weighing 250-400 g at surgery were housed in separate-sex rooms at 21°C constant temperature on a reverse 12 h light/dark cycle. All rats had *ad libitum* access to both food and water through the duration of the experiments. All experimental procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Surgery

Optogenetic Virus Infusion and Fiber Implant

Rats were anesthetized with isoflurane gas (4-5% at induction and maintenance at 1-2%) and received atropine (0.04 mg/kg; IP; Henry Schein) before surgery. During surgery, each rat also received subcutaneous injections of cefazolin (100 mg/kg, Henry Schein) to prevent

infection and carprofen (5 mg/kg, Henry Schein) for pain relief. Cefazolin and carprofen were re-administered 24-h and 48-h post-operation. Rats were placed into a stereotaxic apparatus (David Kopf Instruments) and received bilateral 0.75 µL infusions into CeA or BLA of either an AAV ChR2 (AAV5-Hsyn-ChR2-eYFP; CeA *n* = 30; BLA *n* = 8 UNC Vector Core, Chapel Hill, NC) or inactive control virus lacking the ChR2 gene (AAV5-Hsyn-eYFP; *n* = 31; UNC Vector Core, Chapel Hill, NC). A separate group of rats received the inhibitory opsin IC++ into the CeA (AAV5-hsyn-iC++-eYFP; *n = 8;* Stanford Vector Core). Within an individual, CeA placements were bilaterally identical, but coordinates were slightly varied from individual to individual so that sites for the group spanned the entire CeA, ranging from 1.56 – 2.5 (AP), 4.00 – 4.50 (ML), and 7.60-8.00 (DV) all from bregma. The 0.75 µL volume was infused per side over a period of 7.5 minutes, at a constant rate of 0.1 µL per min, and the injector was left in place for an additional 10 minutes to allow for viral diffusion. For optogenetic stimulation, bilateral optic fibers (200 µm) were also implanted 0.3 mm dorsal to the virus infusion site for each rat.

Intravenous Jugular Catheter Implantation

A chronic intravenous jugular catheter for intravenous delivery of remifentanil solutions was implanted during a second surgery 3 weeks after the CeA procedure (Warlow et al., 2017b, 2020). Rats were anesthetized using isoflurane gas similarly to the method described above and administered perioperative treatment for infection, pain, and inflammation. The intravenous (i.v.) jugular catheter was Silastic tubing (id: 0.28 mm; od: 0.61 mm; Plastics1) threaded into the right jugular vein of each rat and secured proximally and distally using non-absorbable polyamide thread (Braum). The outer end of the catheter was passed under the skin of the shoulder, along the dorsal back, and exited from a secured subcutaneous anchor near the midscapular region.

Catheter patency was maintained during the initial 10-day post-operative period via daily flushes with 0.2 mL of saline solutions containing 5 mg/mL gentamicin sulfate (Henry Schein) to prevent infection, followed by 0.1 mL of 3.5 mg/mL heparinized saline (Sigma-Aldrich). Subsequently, catheters were flushed daily with either 0.1 mL heparinized saline or 0.2 mL of sterile saline on alternating days. Catheter patency was assessed via intravenous infusion of 0.2 mL methohexital sodium (20 mg/mL; JHP Pharmaceuticals) prior to the start of behavioral testing, roughly midway during the series of behavioral test days, and again at the conclusion of behavioral experiments. Catheters were considered patent if rats became ataxic within 10s of the intravenous injection, and only data from rats with patent catheters were included in the behavioral analysis.

Oral Cannula and Optic Fiber Implantation

To allow an affective taste reactivity test of whether CeA ChR2 stimulation enhanced hedonic impact or 'liking' of its paired stimulus, a separate group of rats first received an AAV5 ChR2 virus in CeA as above (AAV5-hsyn-ChR2-eYFP, n = 6 or AAV5-hsyn-eYFP control, n =6, UNC Vector Core), except that optic fibers were not implanted during the initial surgery. Following infusion of the optogenetic virus, the head wound was sutured, and rats were allowed to recover for at least three weeks and to allow time for virus expression. A second surgery was then performed to implant oral cannula and CeA optic fiber. Rats were re-anesthetized with isoflurane as described above for implantation of intracranial optic fibers and of bilateral oral cannulas, which allowed for direct oral infusions of sucrose, and quinine solutions. Each oral cannula (polyethylene-100 tubing) entered the upper cheek just lateral to the secondary maxillary molar, ascended beneath the zygomatic arch, and exited the skin at the dorsal head, where it was

secured with skull screws and a dental acrylic headcap. In the same surgery, rats were implanted with bilateral optic fibers (200 µm), aimed to place each fiber tip 0.3 mm dorsal to the rat's bilateral virus microinjection sites, and anchored with the same acrylic headcap. Cefazolin and carprofen were again administered and repeated post-operatively as above. All rats were allowed to recover for 1 week prior to behavioral testing.

Behavioral Tests

Experiment 1: Two-Choice Task: Remifentanil with CeA ChR2 stimulation versus Remifentanil without laser

First, we assessed whether CeA ChR2 neuron stimulation alters the incentive value of i.v. remifentanil, using an instrumental two-choice task similar to one used previously for i.v. cocaine to assess if CeA ChR2 pairing altered the motivational value of remifentanil (Warlow et al., 2017). In this task, rats could choose between two instrumental nosepoke options that delivered identical doses of i.v. remifentanil: one delivered remifentanil accompanied by 8-sec CeA ChR2 laser stimulation, and the other delivered remifentanil alone without laser. Behavioral sessions were conducted in operant chambers (Med-Associates) with clear Plexiglas floors and an auditory speaker housed within an individual sound attenuated chamber. Infusion pumps were located outside each chamber to allow for intravenous delivery of remifentanil.

Rats were initially trained to earn intravenous infusions of remifentanil by making nose pokes into one of two retractable portholes on the front wall of the chamber. Nose pokes into one porthole delivered an intravenous infusion of remifentanil by itself, without laser stimulation (Remifentanil Alone; 2 µg/kg in 50 µL of 0.9% isotonic saline; delivered over 2.8-s). Nose pokes into the alternative porthole delivered an identical remifentanil infusion paired with laser

excitation (Laser + Remifentanil) into the CeA (ChR2: n = 9 females, n = 8 males; eYFP: n = 6 females, n = 6 males; 1-2 mW; 25 Hz, 15-ms pulse; 8-s pulse train). Laser illumination was triggered by the nosepoke response and continued for 8-sec during and after the i.v. infusion. Laser parameters were based on previous published studies with cocaine (Baumgartner et al., 2022; Warlow et al., 2017). Remifentanil Alone or Laser + Remifentanil infusions were each accompanied by their own distinctive 8-sec auditory cue to provide an additional signal of which reward was earned (either tone or white noise; assignment counterbalanced across rats). Once an i.v. infusion was delivered, both nose ports retracted into the back wall for a 20-s time out intertrial period. During this time out, no responses could be made, and no reward was available. The very first session was 2-h in duration, and all subsequent sessions were 1-h. Besides automated beam-break measures of nose-poke responses, two video cameras, one placed below the transparent floor and the other mounted on a chamber wall recorded behavior for subsequent off-line video analysis.

Each 2-choice session began with a series of 4 single choice trials, in which only one porthole was presented to earn its customary outcome: either Remifentanil Alone or Laser + Remifentanil. Rats had to successfully poke into the available nose port until intravenous delivery of the drug was earned together with its customary laser or no-laser condition. After a 20-s time out period, the alternative nose port was presented until the rat earned the alternative reward, either Laser + Remifentanil or Remifentanil Alone. Then the sequence of first porthole followed by second porthole was repeated once more, so that each outcome was earned twice. These single-choice trials were conducted at the beginning of every session to ensure that rats experienced both types of remifentanil outcomes each day before having to choose between them.

After the 4 single-choice trials, both portholes were presented simultaneously for the rest of each session to allow choice between them. After each outcome was chosen and received, a 20-sec time out followed with portholes retracted, and then simultaneous presentation of both portholes was repeated. This continued for 1-hour or until the rat earned a maximum ceiling of 40 opioid infusions. Sessions were repeated daily for 10 days on a Fixed Ratio 1 (FR1) schedule, where each nose poke resulted in an intravenous remifentanil infusion, and subsequently the fixed ratio effort requirement was increased over days 11-16 to assess the robustness of any preference: FR2, FR4, RR6, RR6, RR6.

Is Laser the Goal? 2-Choice Remifentanil Extinction Tests

To assess whether any preference for Laser + Remifentanil over Remifentanil Alone was due to laser ChR2 stimulation adding a separate reward value that rats pursued, the same rats were tested in 7 daily drug-extinction sessions, in which remifentanil infusions were discontinued but laser could still be earned. Nose pokes into the former Laser + Remifentanil port still delivered a customary 8-s laser pulse (1-3 mW; 25 Hz) but no remifentanil infusion, and nosepokes into the former Remifentanil Alone porthole now delivered nothing. Rats could freely choose between the two portholes, essentially to assess whether laser by itself would maintain instrumental responding and preference when intravenous remifentanil was no longer delivered. Each session ended after 1 hr or when 40 total responses were made.

Progressive Ratio Tests of Remifentanil Incentive Motivation Intensity

To confirm whether laser excitation of CeA ChR2 neurons increases the magnitude of incentive motivation for remifentanil, after the 2-choice task was finished the same rats subsequently underwent two days of progressive ratio (PR) testing to assess laser effect on effort

breakpoint to obtain i.v. remifentanil rewards (i.e. also assessed the ability of optogenetic CeA stimulation to *amplify* incentive salience using a progressive ratio test of motivation that was conducted across two days. On one day (order balanced across rats) only the Remifentanil Alone port was available, to earn its customary opioid reward without laser. On the other day, only the Laser + Remifentanil porthole was available, which earned its customary opioid reward together with 8-sec CeA ChR2 laser stimulation. Within each session, the number of nose pokes needed to earn the next remifentanil infusion increased exponentially according to a progressive ratio schedule of reinforcement with the following formula [PR= [$5e^{(\text{reward number x } 0.2)}$]-5 rounded to the nearest integer] (M. J. F. Robinson et al., 2014; Warlow et al., 2017b). Work requirements for this schedule were 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, etc. The effort breakpoint reached when responding stopped was compared between laser vs no-laser conditions (i.e., the maximum effort price paid in terms of responses to earn remifentanil within each 1-hr session).

Experiment 2: Remifentanil vs. Sucrose 2-Choice Task:

A separate group of rats (CeA ChR2 n = 17; n = 11 females, n = 6 males; CeA eYFP: n = 13*;* n = 7 females n = 5 males*)* was used to assess whether pairing CeA ChR2 excitation could control choices between intravenous remifentanil versus natural sucrose pellets, similar to as it previously controlled choice between cocaine versus sucrose (Warlow et al., 2020). One group of CeA ChR2 rats (*Sucrose + Laser*) had 8-sec laser selectively paired with choosing sucrose when they made nosepokes into the designated porthole (Laser + Sucrose). Each sucrose pellet was delivered into a recessed dish located in the chamber wall between the two ports. By contrast, for these rats, remifentanil was earned alone without laser (2 µg/kg; *Remifentanil Alone*). A different

group of CeA ChR2 rats (Laser + Remifentanil rats) oppositely received laser pairings only with earning remifentanil but not with earning sucrose.

Training and choice sessions for Remifentanil v. Sucrose were conducted in MedAssociates operant chambers with clear Plexiglas floors and two retractable portholes or levers as in Experiment 1. Prior to 2-choice sessions, rats were initially pre-trained on alternating offered to earn either sucrose alone or remifentanil alone in 60-min sessions (laser paired with one reward, continuing daily until each had rat cumulatively earned a total of 50 sucrose pellets and 50 remifentanil infusions. During a pretraining session, only one of the two portholes were inserted into the box per day, each porthole permanently assigned to its own particular reward type, so that rat could only nosepoke onto that porthole to earn its particular associated reward (either sucrose or remifentanil). On the next day, the other porthole was solely available, and nosepokes in it earned the alternative reward, and so on. Half the group of CeA ChR2 rats were arbitrarily assigned to be Laser + Sucrose rats, and the remaining CeA ChR2 rats designated to be Laser + Remifentanil rats. Each rat's laser assignment was permanent for the duration of the experiment. Nose pokes into the porthole that earned laser-paired reward delivered photoexcitation (1-3 mW, 25 Hz, 8-s pulse train) that began with final nosepoke and continued to overlap with receipt of that reward. Successful completion of initial training was followed by 9 days of two-choice test sessions in which both portholes were simultaneously presented so that rats could choose freely between intravenous remifentanil or sucrose.

Each 2-choice session began with a single choice trial in which only one porthole was randomly inserted until a nose poke was made and its customary reward earned (either sucrose or remifentanil; accompanied by laser or not depending on individual assignment). Receipt of the first reward resulted in porthole retraction and a 20-s time out. The alternative porthole was then

presented until the rat earned its alternative reward. This single-choice cycle was repeated once more until each rat earned two remifentanil rewards and two sucrose rewards, with one reward type accompanied by laser and the other not depending on individual assignment, to ensure each rat sampled each reward condition twice prior to choosing between them. Then both portholes were simultaneously presented, allowing rats to freely choose between the two rewards. Upon nosepoke into a chosen porthole, its customary reward was delivered accompanied by an assigned auditory cue that identified choice of sucrose ore remifentanil (either tone or white noise; assignment counterbalanced across rats), plus CeA neuron laser stimulation if the rat chose its assigned laser-paired reward, followed by a 10-min time out This 2-choice opportunity was repeatedly presented again for the remainder of the 2-hr session, allowing up to 12 independent choices per session. These 2-choice daily sessions continued for 9 days.

Remifentanil vs. Sucrose Laser Extinction Task

On test days 10 -14 we assessed whether any narrowing of the focus of motivated pursuit on the laser-paired reward (either sucrose or remifentanil) would persist if laser were discontinued for several laser extinction trials. In each laser extinction trial, 2-choice sessions were offered similarly to above, but responses for the previously laser-paired reward no longer triggered CeA laser illumination, and so both sucrose and remifentanil were earned without laser for all rats. All sessions were similar to remifentanil vs. sucrose instrumental sessions described above, but a total of 5 laser extinction sessions were conducted on consecutive days, and each session was 2-h in length.

Laser Self-Stimulation: Place-Based and Spout-Based Self-Stimulation

To assess whether ChR2 CeA stimulation had incentive value on its own, in the absence of remifentanil or sucrose, each rat was tested for laser self-stimulation in two self-stimulation tasks. In a passive place-based self-stimulation task, rats could earn laser excitation (25 Hz; 3-s pulse trains) by entering one of two chambers of a two-chamber Plexiglas apparatus (76 cm x 38 cm x 38 cm). Each chamber was distinguished by its own set of visual cues (stripped vs dotted walls) and tactile cues (small grid vs. large grid floor). A pre-test was conducted to assess chamber preference, in which rats were allowed to move freely between the two compartments without any laser stimulation. After this pre-test one chamber was randomly assigned as the laser-delivering chamber (assignment balanced across rats). Rats could earn laser illumination by entering their laser-paired compartment (1-3 mw; 25 Hz; 3-s pulse trains). Laser continued to cycle (3-s ON/ 8-s OFF) as long as the rat remained in that chamber. Exiting the chamber terminated laser delivery. Entry into the alternative no-laser chamber ensured absence of laser stimulation. For each rat, the same chamber was assigned as its laser-delivering chamber for three daily and consecutive 15-min sessions.

In the active spout-touch task, rats could earn laser illuminations by instrumentally touching one of two empty metal drinking spouts, or one of two metal rods that resembled the drinking spouts in length but had no sipper ball bearing in 30 min sessions. Two sipper spouts placed ~10 cm apart along the back wall of a Med Associates chamber (a different chamber from that used in 2-choice tests). The floor of the chamber was a metal grid floor that enables a circuit to detect contacts onto each respective spout. Touching the designated laser spout (permanently assigned for each rat; assignments balanced across rats) delivered CeA laser pulses (1-3 mW; 25 Hz) of 8 sec duration (i.e., identical to laser bin duration in 2-choice task; 3 daily sessions; 8-sec tests balanced across rats). By comparison, touches on the alternative spout earned nothing

(control spout) and served simply as a baseline measure of exploratory touching. Rats were tested for both place-based and spout-based laser self-stimulation before 2-choice initial sessions began, and again after the conclusion of all 2-choice tests.

Taste Reactivity Testing

The taste reactivity test was used to determine whether CeA neuronal excitations that generated incentive motivation also increased the hedonic impact of the laser-paired rewards. Each rat was habituated to the test chamber for 30 minutes on four consecutive days before any behavioral testing occurred. On the last two days of habituation, rats received oral infusions of a 0.03M sucrose solution to habituate them to infusion of fluid into the mouth. In subsequent taste reactivity tests, affective orofacial reactions (i.e., positive 'liking' versus negative 'disgust' patterns) elicited by oral infusions of sucrose solutions (0.03M), or quinine (3×10^{-4} M), were videorecorded for offline analysis (Berridge, 2000; Grill & Norgren, 1978c). Orofacial reactions were videorecorded through a close-up lens facing an angled mirror underneath the transparent floor, positioned to capture a clear view of the mouth and face. Taste solutions (1 ml) were delivered into the mouth of rats through PE-50 tubing connected to a PE-10 delivery nozzle, at a constant 1ml/min rate during the 1 min infusion, via a syringe pump, connected to the oral cannula. Only one type of taste was tested per day (sucrose, water, or quinine) for a total of 2 x 1mL infusions per day, either with laser stimulation, or without laser as a within-subject baseline. During laser trials, laser illumination (1-3 mW; 25 Hz, 15 ms pulse/ cycled 5-s ON/ 5-s OFF) throughout the 1-min test. Each laser parameter was tested at least twice for each rat in separate daily tests.

Taste Reactivity Scoring

Videos were scored subsequently for positive hedonic 'liking' reactions, aversive 'disgust' reactions, and neutral taste reactions in slow-motion at speeds ranging from frame-by-frame to 1/5th normal speed, using The Observer Software (Noldus; Leesburg, VA). Positive hedonic or 'liking' responses were considered to be: lateral tongue protrusions, paw licks, and rhythmic midline tongue protrusions. Aversive 'disgust' reactions were: gapes, forelimb flails, head shakes, face washes, chin rubs, and paw treading. Neutral responses (i.e., relatively uncoupled from hedonic impact) were: passive dripping of solution out of the mouth, rhythmic mouth movements, and grooming. A time-bin scoring system was used to ensure each type of affective reaction contributed equally to the overall affective score (Berridge, 2000; Castro et al., 2016; Castro & Berridge, 2014c, 2017). Rhythmic mouth movements, paw licks, passive dripping, and grooming were all scored in 5-s time bins, because these behaviors typically are emitted in bouts of relatively long duration. Any emission of these behaviors up to 5-sec was counted as a single occurrence; emissions of 5-sec to 10-sec counted as two occurrences, etc. Midline tongue protrusions and paw-treading were scored similarly, but in 2-s bins, as typically emitted in shorter bouts. Lateral tongue protrusions, gapes, flails, headshakes, and chin rubs were counted as discrete events every time they occurred, because these can occur singly or in several brief repetitions. A total positive hedonic (i.e., 'liking') score was then calculated by combining component scores of rhythmic tongue protrusions, paw licks, and lateral tongue protrusions. A total negative aversive (i.e., 'disgust') score was calculated by combining gapes, forelimb flails, head shakes, paw treading, face washes, and chin rubs (Berridge, 2000; Castro & Berridge, 2017).

Anatomical Localization: ChR2 placement verification, virus expression, local and distant Fos analysis

Subsets of rats were assigned to have a final 2-choice session with laser, or a final laser-only stimulation session (25 Hz, 8-s ON/22-s OFF) 75-min prior to euthanasia. These were done to recruit distant circuitry into Fos activation that potentially mediated CeA ChR2 2-choice effects or laser self-stimulation effects, as well as to assess the size and spread of local neuronal modulations indicated by Fos plumes surrounding the tips of illuminated CeA optic fibers (Baumgartner et al., 2021; Baumgartner et al., 2022; Cole et al., 2018; Warlow et al., 2020). All rats were then anesthetized with a lethal dose of sodium pentobarbital (150-200 mg/kg) and perfused transcardially with PBS followed by 4% Paraformaldehyde. Brains were extracted and post-fixed in 4% PFA for 24-h to 48-h then transferred to a 25% sucrose solution for a minimum of two days. Coronal sections of brain tissue were collected at 40 micrometers using a cryostat (Leica), and slices were processed for GFP and Fos immunohistochemistry. Images were taken using a digital camera (Qimaging) and fluorescence microscope (Leica). Virus localization sites were mapped onto their corresponding plates using a brain atlas (Paxinos & Watson, 2013).

Coronal sections were rinsed for 10 min in 0.1 M sodium phosphate buffer three times to prep tissue for immunohistochemistry. Sections were then blocked in 5% normal donkey serum / 0.2% triton-X PBS solution for 60 min and incubated overnight in a polyclonal rabbit anti-cfos igG primary antibody (1:2500; Synaptic Systems) and polyclonal anti-GFP primary antibody (1:3000; Abcam). Tissue was rinsed three times in 0.1M NaPb for 10 min followed by 2-h in biotin-SP conjugated anti-rabbit (1:300; Jackson ImmunoResearch) and donkey anti-chicken (1:300; Jackson ImmunoResearch) secondary antibodies, and 1.5-h in streptavidin-conjugated Cy3 (1:300; Jackson ImmunoResearch). Brain sections were mounted, air-dried, and cover-

slipped with anti-fade Pro-long gold (Invitrogen). Coronal images were taken at 10x magnification to localize the fiber tip and surrounding Fos plumes and determine the spread of virus expression. Fos data was not analyzed for this dissertation but will be used to determine induced changes in Fos expression in distant structures against eYFP control levels and naïve control rats for published manuscript (Baumgartner et al., 2020; Cole et al., 2018; Warlow et al., 2020).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9.0 and SPSS. Remifentanil 2-choice sessions, sucrose vs. remifentanil, spout self-stimulation, and place-base self-stimulation tasks were analyzed by mixed ANOVA, followed by *t*-tests for individual comparisons with a Bonferroni correction. Friedman's two-way ANOVAs were used for non-parametric tests, following Wilcoxon sign-ranked tests. Significance was set at $p < 0.05$.

5.4 Results

Experiment 1: 2-Choice Laser + Remifentanil vs Remifentanil Alone

CeA ChR2 rats choosing between an i.v. remifentanil infusion accompanied by CeA neuron photoexcitation (Fig2a; 1-3 mW; 25 Hz; 8s) and an identical remifentanil infusion by itself without laser excitation, overwhelmingly came to pursue the laser + remifentanil option (Fig. 43a.; $F_{9,117} = 4.92$, $p < 0.0001$, $n = 14$). By the third day of testing, a 2: 1 preference for Laser + Remifentanil over Remifentanil Alone was detectable ($t_{117} = 3.25$, $p = 0.015$, $d = 0.92$, CI = 1.3 to 20.0). That preference continued and rose to a 4:1 preference by day 10 of FR1 testing ($t_{117} = 7.91$, $p < 0.0001$, $d = 2.04$, CI = 16.6, 35.3)

As response ratios became more difficult over days 11 to 15 (FR1, FR2, FR4, RR4, RR6), CeA ChR2 rats continued to prefer Laser + Remifentanil (Fig 37b.; $F_{9,94} = 11.61$, $p <$

0.0001, n = 13) by a 4:1 ratio on FR2 schedule (t_{106} = 3.40, p < 0.01, d = 1.62 , CI = 6.8, 80.0) and remained at 3:1 until the last day of RR6 testing (t_{106} = 6.27, d = 1.64 , CI = 45.6, 122.4, p < 0.0001). Female and male CeAChR2 rats showed similar preferences for Laser + Remifentanil over Remifentanil Alone both on FR 1 schedule (Fig 43b.; Day10; female ratio: 4:1; male ratio: 4.5:1; laser x sex x day interaction: $F_{9,108}$ = 0.56, p = 0.82, n = 9 females, n = 8 males) and on higher schedules ranging to RR6 (RR6 female preference: 3:1; RR6 male preference: 3:1; $F_{9,85}$ = 0.98, p = 0.46, n = 6 females, n = 7 males).

By contrast, control CeA eYFP rats with optically-inactive virus chose equally between Laser + Remifentanil and Remifentanil Alone options (Fig 37d., Fig S2b.; FR1; $F_{9,99}$ = 1.22, p = 0.29, n = 12; 1:1 FR2-RR6: $F_{9,72}$ = 0.42, p = 0.92, n = 9), without showing detectable preference or avoidance (1:1 FR1 (Day 10) Preference Ratio; 1:1 RR6 (Day 16) Preference Ratio) and thus the control eYFP group differed from the CeA ChR2 group on both FR1 schedule (laser x session day x virus; $F_{9,216}$ = 3.64, p = 0.0003) and higher RR6 schedules ($F_{9,166}$ = 4.16, p < 0.0001).

BLA ChR2 group

Interestingly, BLA ChR2 rats also showed preference for the Laser + Remifentanil option (Fig 43d., $F_{9,63}$ = 2.74, p = 0.009, n = 8). This preference reached statistical significance on Day 6 (Fig S1c; 3:1 preference; t_{63} = 3.42, p = 0.01) and continued to grow to a 5:1 preference by Day 10 of FR1 testing (t_{63} = 5.10, p < 0.0001). We observed a sex difference between male and female BLA ChR2 rats, so that females, on average, had more total nose pokes for i.v remifentanil (session day x sex interaction: $F_{9,54}$ = 3.86, p = 0.0008, n = 5 females; n = 3 males), but male and female and BLA ChR2 similarly preferred the Laser + Remifentanil option (laser x

session day x brain region interaction; $F_{9,54} = 1.72$, $p = 0.11$; Day 10; female ratio: 5:1; male ratio: 3:1).

Similar to CeA ChR2 rats, BLA ChR2 rats increased their responses as response ratios became more difficult (Fig 37c; $F_{9,45} = 7.24$, $p < 0.0001$, $n = 6$), and also showed a bias for the laser + remifentanil option ($F_{9,45} = 2.65$, $p = 0.015$). BLA ChR2 rats had a 4:1 preference for the laser + remifentanil option, which emerged as statistically significant during FR4 testing ($t_{45} = 4.99$, $p < 0.0001$, $d = 1.75$, CI = 29.7, 115.7), and persisted through the second day of RR6 testing ($t_{45} = 3.97$, $p = 0.0026$, $d = 1.39$, CI = 14.8, 100.8). However, the laser preference went away for the last day of RR6 testing ($t_{45} = 0.68$, $p = 0.99$. Despite that CeA and BLA ChR2 rats both preferred the laser + Remi, they also differed from each other ($F_{9,139} = 2.07$, $p = 0.04$).

Amount and rate of total opioid intake (combined Laser + Remifentanil and Remifentanil Alone)

By the end of RR6 testing days, CeA ChR2 rats took the maximum 40 infusions per day that were possible for each 1-hr session, with virtually all rats reaching a maximum of 80 µg/kg remifentanil each day (Fig 37e; CeA ChR2: 39.6 ± 0.3; CeA eYFP: 27.2 ± 3.6; BLA ChR2: 30.3 ± 5.3; $F_{2,23} = 5.4$, $p = 0.012$), and took more total remifentanil than eYFP controls ($t_{23} = 3.16$, $p = 01$, $d = 1.63$, CI = 2.3, 22.6). Further, CeA ChR2 rats also responded more rapidly, and reached their maximum 40 infusion ceiling within approximately 37 min, or 20 minutes earlier than eYFP and BLA ChR2 rats (Fig 37f; RR6 Day 16 Session Times; CeA ChR2: 37.0± 4.2; CeA eYFP: 57.3± 1.9; BLA ChR2: 60.0± 0.0; $F_{2,23} = 15.45$, $p < 0.0001$; CeA ChR2 vs eYFP comparison: $t_{23} = 4.65$, $p = 0.0003$, $d = -1.92$, CI = -31.6, - 9.0; CeA ChR2 vs BLA comparison: $t_{23} = 4.65$, $p = 0.0003$, $d = -2.34$, CI = -37.8, - 10.24). By comparison, BLA and eYFP rats' sessions were similar in length ($t_{23} = 0.52$, $p = 0.99$).

Central amygdala stimulation enhances laser-paired intravenous remifentanil breakpoints during progressive ratio test of motivation.

Progressive ratio tests of breakpoint confirmed that CeA ChR2 neuron excitation increased the intensity of incentive motivation to consume remifentanil. CeA ChR2 rats reached >160% higher breakpoints for Laser + Remifentanil (41. ± 5.0) than for Remifentanil Alone (Fig38a; 25.8 ± 4.6; $W = 41.5$, $Z = 2.25$, $p = 0.02$, $n = 9$). Overall, CeA Rats made twice as many nose pokes for opioid infusions on their Laser + Remifentanil Day (158.0 ± 223.9) than on the Remifentanil Alone day (Fig 38b; 83.9 ± 20.8; $W = 42.0$, $Z = 2.31$, $p = 0.02$, $n = 9$). This difference became significant within the first 10 min of the sessions, a time point when CeA ChR2 rats had already pressed 300% more on the Laser + Remifentanil Day than on the Remifentanil Alone day ($W = 33.0$, $Z = 2.1$, $p = 0.036$, $n = 9$). By contrast, CeA eYFP rats reached similar breakpoints on the Laser + Remifentanil day and Remifentanil Alone day (Fig3a; NL: 17.0 ± 4.2; L: 23.1± 3.9; $W = 17.0$, $Z = 1.4$, $p = 0.17$, $n = 8$), and made similar total numbers of nose pokes on both days (Fig 38b; NL: 50.1 ± 15.1; L: 74.4± 17.0; $W = 20.0$, $Z = 1.01$, $p = 0.31$, $n = 8$), and also differed from CeA ChR2 rats ($F_{1,15} = 8.06$, $p = 0.01$).

Male and female CeA ChR2 rats showed similar >160% elevations in breakpoint induced by laser stimulation, and so there was no detectable sex difference in CeA ChR2 increase in intensity of incentive motivation to obtain remifentanil (Fig 44b; Male: Laser + Remifentanil: 34.8 ± 5.3, Remifentanil Alone: 19.0 ± 7.3; Female: Laser + Remifentanil: 46.2 ± 7.6, Remifentanil Alone: 31.2 ± 5.1, $F_{1,7} = 0.003$ $p = 0.96$). Similar to eYFP controls, BLA ChR2 rats did not differ in breakpoints achieved during progressive ratio tests (Fig 38a; NL: 16.5

± 8.3; L: 26.0± 6.2; $W = 6.0$, $Z = 1.60$, $p = 0.11$, $n = 4$) or in the total number of nose pokes made (Fig 38c; NL: 45.8 ± 27.8; L: 87.3± 29.3; $W = 10.0$, $Z = 1.83$, $p = 0.07$, $n = 4$).

Experiment 2: Remifentanil vs. Sucrose

A separate group of rats were tested for their choice of remifentanil paired with CeA ChR2 neuron activation against sucrose alone, or sucrose paired with CeA ChR2 laser activation against remifentanil alone (Fig. 39a). Control rats with optically inactive virus in CeA (eYFP rats) chose equally (remifentanil + laser group: 0.5:1 preference ratio; sucrose + laser group: 1:1 laser preference ratio) between sucrose and remifentanil regardless of which reward was laser-paired (Fig. 4b; remifentanil + laser group: $F_{8,48} = 1.51$, $p = 0.18$, $n = 7$; sucrose + laser group: $F_{8,40} = 1.03$, $p = 0.18$, $n = 6$).

By contrast, CeA ChR2 rats with central amygdala neuron laser stimulation paired with sucrose almost exclusively pursued sucrose and ignored remifentanil (Fig. 39d; sucrose + laser group: $F_{8,32} = 4.95$, $p = 0.005$, $n = 5$). The laser -paired preference emerged by the second-choice session (Fig 45a; 2:1 laser reward preference; $t_{32} = 3.12$, $p < 0.03$) continued until the last day of testing, growing to a 6:1 laser-reward preference by day 9 ($t_{32} = 8.33$, $p < 0.0001$). CeA ChR2 rats that had remifentanil paired with CeA neuron excitation oppositely responded for intravenous remifentanil (1.5:1 preference) and ignored sucrose from the initial day of testing (Fig. 39d; remifentanil + laser group: $F_{1,4} = 50.99$, $p = 0.002$, $n = 5$), and this preference grew to 4:1 by the last choice session (Fig. 45b). Male and female CeA ChR2 rats equally preferred the laser-paired reward (4:1 female; 5:1 males), regardless of which reward was paired with CeA neuron activations (Fig 45c; $F_{8,64} = 1.00$, $p = 0.44$, $n = 6$ females, $n = 4$ males).

Does CeA-induced preference between remifentanil and sucrose persist?

In order to determine whether the established preference for remifentanil vs. sucrose would persist in the absence of continued paired CeA neuron excitation, we tested CeA ChR2 rats for 5 additional days of remifentanil vs. sucrose sessions under conditions of laser extinction. Under these conditions, the previously established reward choice (either sucrose for sucrose + laser ChR2 rats or remifentanil for remifentanil + laser ChR2 rats) persisted for the 5 laser extinction test sessions. CeA ChR2 rats that had sucrose paired with laser continued to selectively nose poke for sucrose (4:1 sucrose preference), ignoring the intravenous remifentanil option (Fig. 39e; laser main effect; $F_{1,3} = 34.41$, $p < 0.001$; $n = 4$). Similarly, CeA ChR2 rats who previously had remifentanil paired with laser continued to prefer intravenous remifentanil to sucrose by a ratio of 3:1 by Day 5 (Fig. 39f; laser main effect; $F_{1,4} = 83.65$, $p < 0.001$; $n = 5$).

Is the laser the goal? No reliable self-stimulation in CeA ChR2 rats

Our results from Experiments 1 and 2 suggest that optogenetic activations of CeA neurons can focus intense pursuit for a laser-paired reward, whether that reward is intravenous remifentanil or sucrose. This raises the question of whether CeA neuron stimulation has motivational value in the absence of a reward and may account for CeA ChR2 rats continued pursuit of the laser-paired reward options. We therefore assessed CeA ChR2 neuron valence using two self-stimulation tasks: an active instrumental spout-touch task, and a relative passive place-based self-stimulation task.

Spout Self-Stimulation – Remifentanil 2-Choice Rats

In the spout-task, each lick or contact to a designated empty laser-paired waterspout resulted in a brief 1-s or 5-s laser pulse duration (473 nm; 1-2 mW; 25 Hz). Contacts to an alternative empty waterspout produced nothing. We considered rats to be high self-stimulators if they made > than 50 contacts to the laser-paired spout and made at least 2x as many laser-spout contacts compared to the control spout. Rats were considered low self-stimulators if they made >10 contacts to the laser-paired spout and made at least 2x as many laser-spout vs. control spout contacts. Rats that did not meet either of these criteria were categorized as failures to self-stimulate. All rats were categorized on Day 1 and retested for reliability of self-stimulation on Days 2 and 3.

Most CeA ChR2 rats that were tested in the remifentanil 2-choice task met some criteria for self-stimulation when they could earn 8-s laser pulses of similar intensity and duration used during instrumental choice sessions (Fig. 40a; high self-stimulation: 35.7%, low self-stimulation: 21.4%; failure to self-stimulate: 42.9%; laser main effect; $F_{1,13} = 6.34$, $p < 0.03$, $n = 14$). CeA ChR2 rats made ~4x as many contacts to the laser-paired spout relative to the control spout when tested before remifentanil exposure (Drug Naive: *Control Spout:* 25.1 ± 5.6, *Laser Spout:* 98.6 ± 45.5). When re-tested for self-stimulation after drug exposure, CeA ChR2 rats made 11x as many laser vs. non laser spout contacts, although this difference in magnitude of self-stimulation between drug naïve and after drug experience sessions was not statistically significant (After remifentanil experience: *Control Spout:* 17.0 ± 2.9, *Laser Spout: 1*97.4 ± 82.3; laser x drug experience interaction; $F_{1,13} = 1.78$, $p = 0.21$, $n = 14$).

BLA ChR2 rats also self-stimulated for laser pulses 8-s in length (high self-stimulation: 25.0%, low self-stimulation: 12.5%; failure to self-stimulate: 62.5%; $F_{1,13} = 8.79$, $p = 0.01$, $n = 8$), and self-stimulated at similar intensities when they were drug naïve and after remifentanil

experience ($F_{1,13}$ = 2.09, p = 0.17, n = 8). Overall, BLA ChR2 rats made ~4.5x as many contacts to the laser spout compared to the control spout when drug naïve (Drug Naïve: *Control Spout:* 16.5 ± 4.7, *Laser Spout:* 75.9 ± 24.8), and ~3x as many laser spout contacts after remifentanil experience (Drug Experience: *Control Spout:* 10.64 ± 5.8, *Laser Spout:* 31.1 ± 14.2).

By comparison, eYFP control rats failed to self-stimulate in the spout-task (Fig 40a; high self-stimulation: 0%, low self-stimulation: 15.4%; failure to self-stimulate: 84.6%; $F_{1,12}$ = 1.80, p = 0.20, n = 13), and thus made equal contacts to the laser spout and control spout at any point tested (Drug Naïve: *Control Spout:* 10.2 ± 3.9, *Laser Spout:* 15.9 ± 5.8; Drug Experience: *Control Spout:* 14.7 ± 4.4, *Laser Spout:* 15.1 ± 3.8).

Spout Self-Stimulation – Remifentanil vs. sucrose rats.

CeA ChR2 rats that were trained to choose between intravenous remifentanil and sucrose self-stimulated for 8-s laser pulses in the spout task (Fig40b; laser main effect: $F_{1,15}$ = 4.92, p = 0.04, n =9). 33% of CeA ChR2 rats self-stimulated at low levels and 22% of ChR2 rats met the criteria for high self-stimulation. The remaining 44% of CeA ChR2 rats failed to self-stimulate. Prior to any sucrose or drug exposure, CeA ChR2 rats made approximately 5x as many laser spout contacts relative to the control spout (*Control Spout*: 32.1 ± 21.0, *Laser Spout*: 174.3 ± 139.5). When tested again for self-stimulation after remifentanil vs. sucrose sessions, CeA ChR2 rats made ~17x as many contacts to the laser-paired vs. control spout (*Control Spout:* 19.4 ± 6.5, *Laser Spout:* 312.4 ± 130.1). However, this difference in laser contacts after drug exposure was not statistically significant relative to drug naïve self-stimulation sessions (laser x drug exposure interaction: $F_{1,15}$ = 0.59, p = 0.45).

Place-based self-stimulation: remifentanil 2-choice rats

In the place-based task, rats could freely explore a designated laser-paired chamber that triggered laser illuminations which continued to cycle as long as the rats remained in that compartment (25 Hz; 3 sec ON, 8 sec OFF cycle). Rats were first assessed for a side preference during a no laser-habituation day, then tested for place-based self-stimulation when drug naïve, and again after drug experience. CeA ChR2 rats assessed in the remifentanil 2-choice test failed to self-stimulate in the place-based task, and spent equal amounts of time in the non-laser chamber and laser-paired chamber at all times tested (Fig 40c; Habituation Difference Score: *M = -50.4*, *SEM* = 73.6; Drug naïve difference Score: *M = 38.92*, *SEM* = 90.6; Drug experience difference score: *M = 107.5*, *SEM* = 89.9; $F_{2,25}$= 0.84, p =0.44, n = 11). Similarly, neither BLA ChR2 rats (Fig 40c; BLA ChR2: Habituation difference Score: *M = 104.4*, *SEM* = 163.8; Drug naïve difference Score: *M = 94.80*, *SEM* = 60.5; Drug experience difference score: *M = 57.9*, *SEM* = 92.4) or eYFP control rats (Fig. 40c; eYFP controls: Habituation difference Score: *M = 184.9*, *SEM* = 128.7; Drug naïve difference Score: *M = 139.3*, *SEM* = 119.1; Drug experience difference score: *M = -61.1*, *SEM* = 75.5) self-stimulated in the place-based task (BLA ChR2: $F_{2,8}$= 0.04, p =0.96, n = 5; eYFP; $F_{2,20}$= 1.3, p =0.30, n = 8).

Place-based self-stimulation: Remifentanil vs. sucrose rats

Both CeA and eYFP control rats tested in remifentanil vs. sucrose failed to self-stimulate in the place-based task. CeA ChR2 spent equal amounts of time in both the non-laser and laser-delivering chambers, regardless of whether they were tested during a no-laser habituation day (Fig. 40d; Difference Score: *M = 69.0*, *SEM* = 70.7), when drug naïve (Difference Score: *M = 144.7*, *SEM* = 60.3), or after drug experience (Difference Score: *M = 108.5*, *SEM* = 67.0; ($F_{2,25}$=

3.12, p =0.06, n = 10). eYFP also spent equal amounts of time in both chambers at any of the points tested (Fig. 5d; Habituation difference Score: M = *43.2*, SEM = 48.2; Before choice sessions difference Score: M = *-115.4*, SEM = 83.65; After choice sessions difference score: M = *-16.53*, SEM = 67.4; $F_{3,13}$= 1.96, p =0.18, n = 13), and did not differ from ChR2 rats ($F_{2,28}$= 0.91, p =0.42, n = 13). Overall, all of these results suggest that while CeA neuron activations can powerfully control pursuit of laser-paired rewards, CeA ChR2 stimulation is an unreliable reinforcer on its own.

Remifentanil 2-Choice Drug Extinction Sessions

To further rule out that CeA neuron self-stimulation was responsible for the laser-paired reward preference during instrumental choice sessions, we re-trained a subset of rats from Experiment 1 to respond on the remifentanil 2-choice task for 2 days under an FR1 schedule of reinforcement, then subsequently placed on drug extinction for a total of 7 days. Responses on the previous Laser + Remifentanil porthole now resulted in Laser alone, and nose pokes into the previous Remifentanil Alone port now produced nothing. This was done to assess whether CeA ChR2 laser by itself could maintain preference and high level of pursuit that were previously established by the simultaneous combination of CeA ChR2 stimulation plus remifentanil.

The previous laser-paired remifentanil preference was re-established on Day 1 and continued into Day 2 in CeA ChR2 rats, reaching 5:1 (Fig 41a; t_{80} = 5.12, p < 0.0001). During the subsequent remifentanil extinction condition, CeA ChR2 rats made fewer overall nose pokes as the drug extinction sessions progressed over a total of 7 days ($F_{8,80}$ = 7.94, p < 0.0001, n = 11). Remifentanil extinction reduced pursuit from 32.4 ± 2.9 nose pokes on the Laser + Remifentanil option on the first day of re-training to 8.9 ± 3.3 on the now Laser Alone by the last

day of extinction ($F_{8,72} = 9.27$, $p < 0.0001$, $n = 10$). Drug extinction also eliminated the preference for the previously Laser + Remifentanil porthole ($F_{8,72} = 4.58$, $p = 0.06$, $n = 10$). The preference ratio sunk from previous 5:1 ratio to ~1.6:1 on the first day of drug extinction ($t_{80} = 0.22$, $p > 0.99$) and to 2.9:1 by Day 7 of drug extinction ($t_{80} = 1.43$, $p > 0.99$).

Although BLA ChR2 rats showed preference for the laser-paired remifentanil nose port upon initial testing, they did not re-establish their preference for the laser-paired remifentanil nose port during re-training sessions and continued to choose equally for the remainder of extinction sessions (Fig 41c; $F_{8,32} = 1.37$, $p = 0.25$, $n = 5$). By comparison eYFP rats made fewer overall responses as extinction sessions progressed (Fig 41b; $F_{8,56} = 10.89$, $p < 0.0001$, $n = 8$), but they had no preference for either remifentanil port during any of the sessions tested ($F_{8,56} = 0.41$, $p = 0.92$). Overall, these studies suggest that CeA ChR2 laser stimulation is insufficient by itself to maintain a 2-choice preference over Remifentanil Alone that was previously established by the combination of that same CeA stimulation with an identical opioid reward. When CeA rats are given the same 2 choices under remifentanil extinction conditions, Laser Alone versus Nothing does not maintain pursuit or preference previously induced for Laser + Remifentanil over Remifentanil Alone.

<u>Optogenetic CeA neuron stimulation does not alter hedonic impact of sweet or bitter taste.</u>

Positive 'liking' reactions to sucrose. We tested whether CeA neurons' ability to focus incentive motivation onto a specific laser-paired reward might also enhance the hedonic impact of those rewards. In a taste reactivity test with sucrose (Fig 42a), we measured orofacial movements in response to 1 mL volume of 0.03M sucrose that was delivered intraorally at a constant rate during a 1-min period. Intraoral sucrose typically elicits a suite of primarily positive

'liking' reactions such as rhythmic tongue protrusions, paw licks, and lateral tongue protrusions. Consistent with this, in both CeA ChR2 rats and eYFP controls, intraoral sucrose elicited primarily affective 'liking' expressions (Fig. 42c; ChR2 'liking' Laser OFF: 14.9 ± 1.9; eYFP 'liking' Laser OFF: 12.0 ± 2.0) and little to no aversive 'disgust' reactions (ChR2 'disgust' Laser OFF: 1.3 ± 0.5; eYFP 'disgust' Laser OFF: 3.1 ± 1.0) under baseline conditions of no laser stimulation (CeA ChR2: $F_{1,4}$ = 51.36, p = 0.002, CI = 8.4, 19.1, n = 5; CeA eYFP: $F_{1,5}$ = 12.58, p = 0.02, CI = 2.7, 17.0, n = 6). Optogenetic laser stimulation failed to alter the total number of positive 'liking' reactions or the few aversive 'disgust' expressions elicited by sucrose infusion in CeA ChR2 rats compared to no laser conditions in the same rats (Fig 42c; ChR2 'liking' Laser On: 15.5 ± 1.8; ChR2 'disgust' Laser On: 1.6 ± 0.8; $F_{1,4}$ = 0.29, p = 0.62). Similarly, laser stimulation in eYFP control rats failed to alter affective reactions to sucrose (Fig 42c; eYFP 'liking' Laser On: 13.7 ± 20.5; eYFP 'disgust' Laser On: 2.8 ± 0.6; $F_{1,5}$ = 5.2, p = 0.08) and positive 'liking' reactions were similar between CeA ChR2 and eYFP controls ($F_{1,9}$ = 0.66, p = 0.44). Similarly, laser stimulation in CeA ChR2 rats failed to alter any individual component reactions that make up the total positive 'liking' score including: rhythmic midline tongue protrusions (Fig 42d; Laser-OFF: 6.2 ± 2.0, Laser-ON: 6.7 ± 2.3; t_4 = 0.77, p = 0.49), paw licks (Laser-OFF: 5.1 ± 1.6, Laser-ON: 4.7 ± 1.6; t_4 = 0.39, p = 0.71), or lateral tongue protrusions (Laser-OFF: 3.6 ± 0.9, Laser-ON: 4.1 ± 0.5; t_4 = 0.57, p = 0.60). Failure to alter overall 'liking' reactions or individual components suggests that CeA ChR2 neuron excitation does not alter the hedonic impact of sucrose, nor induces any motoric shift in component distribution.

Negative 'disgust' reactions to quinine. Bitter quinine infusions elicited primarily negative 'disgust' reactions including gapes, forelimb flails, headshakes, and chin rubs in both CeA ChR2 rats and eYFP controls (Fig 42e; ChR2 'disgust'' Laser OFF: 28.4 ± 6.4; eYFP

'disgust' Laser OFF: 21.0 ± 6.4) and almost no 'liking' reactions (ChR2 'liking' Laser OFF: 0.2 ± 0.1; eYFP 'liking' Laser OFF: 1.2 ± 0.2) under baseline conditions of no laser stimulation (CeA ChR2: $F_{1,4}$ = 20.91, p = 0.01, CI = -39.5, -9.6, n = 5; CeA eYFP: $F_{1,5}$ = 11.92, p = 0.02, CI = -32.3, -5.2, n = 6). Optogenetic laser stimulation failed to elicit a within subject change in affective reactions to quinine in both CeA ChR2 and eYFP control rats (Fig 42e; CeA ChR2: 'disgust'' Laser ON: 25.3 ± 4.5, 'liking' Laser ON: 0.7 ± 0.4; $F_{1,4}$ = 0, p = 0.99; CeA eYFP 'disgust' Laser ON: 21.3 ± 5.4, 'liking' Laser ON: 0.8 ± 0.3, $F_{1,5}$ = 0.06, p = 0.81). The number of aversive 'disgust' reactions was similar between CeA ChR2 and eYFP controls ($F_{1,9}$ = 0.001, p = 0.97). Overall, these studies suggest that while CeA neurons can direct and control incentive motivation onto a specific reward, they do not alter the hedonic impact of sucrose taste.

5.5 Discussion

Pairing CeA neuron activation with intravenous remifentanil or sucrose produced a strong incentive motivation that became intensely focused on the laser-paired target. In the remifentanil 2-choice test, CeA ChR2 and surprisingly, BLA ChR2 neuron stimulation narrowly focused motivation exclusively to its paired remifentanil option at the expense of nearly ignoring the alternative remifentanil option, and also intensified overall motivation for remifentanil by increasing the total intravenous remifentanil self-administration. In separate progressive ratio tests of motivation, only CeA but not BLA neuron excitation nearly doubled the breakpoint effort price that rats were willing to pay for laser-paired remifentanil. This pattern of focused pursuit resembles CeA ChR2 amplification and narrowing of motivation that has been previously reported for sucrose, cocaine, and alcohol (Fraser et al., 2024; M. J. F. Robinson et al., 2014; Warlow et al., 2017b, 2020). CeA neuron stimulation also controlled motivation in rats choosing between different rewards. Rats who received sucrose paired with CeA neuron excitation

displayed a ‘sucrose addiction’ phenotype, exclusively responding for sucrose and ignoring remifentanil. Conversely, rats with paired CeA neuron excitation + intravenous remifentanil exclusively responded for remifentanil and consumed little to no sucrose. These results indicate that CeA-related circuitry can also powerfully control the focus and intensity of motivation for a separate class of drugs: opioids.

Focused and targeted incentive salience -- not action reinforcement. The intense motivations induced by optogenetic CeA stimulations described above may involve various psychological components that each contribute to the focused incentive motivation we report. One such possibility is that CeA neuron excitation is highly reinforcing on its own, causing rats to choose laser-paired remifentanil or sucrose but they are actually seeking CeA stimulation alone. However, in our current experiments CeA neuron excitations alone was not a reliably reinforcer. While some rats robustly self-stimulated in the spout task, many CeA ChR2 rats failed to self-stimulate altogether. Yet, the same rats that railed to self-stimulate exhibited intensely focused pursuit of laser-paired remifentanil or sucrose comparably in intensity to CeA rats that robustly self-stimulated.

In the place-based task, CeA ChR2 rats neither preferred nor avoided the laser-paired chamber further indicating that CeA neuron stimulations alone are at best only mildly reinforcing. Finally, when rats were retested in the same context that previously established nose-pokes for laser-paired remifentanil, CeA neuron excitation alone failed to maintain instrumental nose-pokes during conditions of drug extinction. Others too have reported mixed results of CeA neuron self-stimulation in recent studies (Baumgartner et al., 2021, 2022; Douglass et al., 2017; J. Kim et al., 2017; M. J. F. Robinson et al., 2014; Seo et al., 2016; Servonnet et al., 2020; Warlow et al., 2017b, 2020). While most CeA neurons release GABA,

neuronal subtypes can be further classified based on expression of other signaling molecules including somatostatin, protein kinase C delta, corticotrophin releasing factor (CRF), and dopamine D1 vs. D2 receptors (Baumgartner et al., 2021, 2022; Cai et al., 2014; Haubensak et al., 2010; J. Kim et al., 2017). In the present study, the human synapsin (hSYN) promoter that controls ChR2 expression in CeA infects most CeA neurons. Thus, future studies could investigate differential contributions of CeA neuronal subtype roles in motivated behaviors (Baumgartner et al., 2021, 2022; Cai et al., 2014; Douglass et al., 2017; Fadok et al., 2018b; J. Kim et al., 2017; M. J. F. Robinson et al., 2014; Torruella-Suárez et al., 2020; Warlow & Berridge, 2021). Cell-type dependent self-stimulation may be reported in future situations, but within our testing parameters, CeA neuron activations alone does not appear to be a powerful reinforcer. The powerful incentive effects of CeA neuron mediated pursuit may require an external target stimulus. Thus, we propose that CeA ChR2 enhancement of opioid motivation transforms the value of the paired reward, whether it be sucrose, cocaine, or intravenous opioids to make those targeted rewards become more 'wanted'.

CeA focused motivation is not matched by changes in 'liking'. Reward is not a unitary phenomenon, but rather consists of distinct 'liking', 'wanting', and learning components (Berridge et al., 2009; Berridge & Robinson, 2003; Morales & Berridge, 2020). Hedonic 'hotspots' that causally amplify affective 'liking' reactions to sucrose have been described in other mesocorticolimbic sites including the rostrodorsal quadrant of the nucleus accumbens medial shell, posteriolateral ventral pallidum, rostromedial orbitofrontal cortex, and caudal insula (Castro et al., 2016; Castro & Berridge, 2014c, 2017; Mahler et al., 2007; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005). Within hedonic hotspot regions, amplified 'liking' is usually matched by enhancement of 'wanting' typically measured as increased motivation to eat.

CeA neuron appear to respond to various tastes (Azuma et al., 1984). So, could CeA neuron generation of 'wanting' be explained by CeA-mediated increases in 'liking'? Our results suggest the answer is no. Despite being interconnected with brainstem gustatory regions such as nucleus of the solitary tract (NTS), parabrachial nucleus of the pons (PBN) (Norgren, 1976, 1983; Schiff et al., 2018), and cortical taste regions including insula cortex (Allen et al., 1991; Schiff et al., 2018; L. Wang et al., 2018), a site containing a hedonic hotspot (Castro & Berridge, 2017), CeA neuron stimulation altogether failed to increase affective 'liking' reactions elicited to intra-oral sucrose infusions during a taste reactivity test.

A preliminary examination of whether CeA ChR2 neuron stimulation enhanced 'liking' reactions to sweetness did not find changes in orofacial expressions elicited to voluntary eating of sucrose pellets (M. J. F. Robinson et al., 2014). But voluntary consumption may have resulted in unequal amounts of sucrose intake across test rials, and physical pellets may have obstructed the view of the rats' mouth, causing some orofacial expressions to be missed. Most importantly however, voluntary intake is a 'wanting' measure, which requires motivational brain systems. Thus, intra-oral delivery during a taste reactivity test allows for a more controlled sucrose delivery and serves as a better objective measure of affective 'liking' that does not rely on voluntary consumption and motivation, as measured here.

Earlier studies using electrolytic lesions or drug microinjections of CeA neurons have also failed to report changes in affective 'liking' reactions in rats (Galaverna et al., 1993; Mahler & Berridge, 2012). For example, electrolytic lesions of CeA neurons that suppress intake of NaCl during hormonally induced sodium appetite states leave positive 'liking' reactions to intraoral NaCl intact (Galaverna et al., 1993). The mu-opioid agonist DAMGO, which increases 'liking' reactions when microinjected into the hedonic hotspots in NAc, VP, OFC, or insula

(Castro & Berridge, 2017; Peciña & Berridge, 2005; K. S. Smith & Berridge, 2005), similarly fails to alter sucrose 'liking' reactions despite increasing sign-tracking conditioned responses to a sucrose cue (Mahler & Berridge, 2012). Further proof of principle that CeA neurons control 'wanting' without 'liking' comes from recent studies that show pairing CeA neuron stimulation with contacts to an electrified shock rod caused rats to voluntarily and repeatedly shock themselves (Warlow et al., 2020). Auditory tones paired with CeA shock rod attraction also caused those associated cues to become conditioned reinforcers, suggesting CeA-shock paired cues had become imbued with incentive salience, despite never being 'liked' by rats. Thus, we suggest dissociable roles of CeA neurons in control of 'wanting' that is not matched by changes in 'liking'.

CeA neurons have also been implicated in analgesia and responses to pain (Ding et al., 2024; Paretkar & Dimitrov, 2019) as well as to behavioral responses to nausea (Ding et al., 2024). Despite being potent reinforcers, a common side effect of opioid administration is nausea and malaise, especially during initial stages of opioid treatment. An alternative hypothesis for CeA neuron function is the CeA ChR2 stimulation in the present studies reduced the perceived unpleasantness of intravenous opioids. However, we believe a more likely explanation is that CeA ChR2 pairing induced strong incentive motivation in ChR2 individuals that promoted greater overall intake that was not primarily mediated by analgesia or antiemetic mechanisms. While CeA ChR2 rats overall consumed more total remifentanil than control rats, this was primarily observed after several days of instrumental choice testing, and as schedules of reinforcement became more difficult. Any potential unpleasantness/malaise may have been more prominent during early days of self-administration, rates of intake during these periods were similar between ChR2 rats and eYFP controls. Opponent process theories of addiction have

proposed that aversive states such as drug withdrawal can result in drug-seeking behaviors as a means to hedonic self-medicate and reduce any experienced unpleasantness (Koob, 2013, 2020; R. Solomon, 1977; R. L. Solomon & Corbit, 1974). Presently, CeA neuron activation did appear to produce an aversive or painful state, as evidenced by ambivalence for laser-paired chamber stimulation in the place-based task, suggesting that CeA excitation enhances incentive motivations not necessarily mediated by a negatively valenced affective state such as nausea or pain.

Potential roles for amygdala-related circuitry in addiction. It has been proposed that addiction to psychostimulants is behaviorally and neurobiologically distinct from opioid addiction (Badiani et al., 2011). For example, loss of dopamine function potentially suppresses cocaine reward, but leaves heroin and morphine reward intact (Ettenberg et al., 1982; Pettit et al., 1984). That CeA neurons can intensely control motivation for natural and drug rewards including cocaine, opioids, and alcohol also suggests that brain generators of incentive motivation are shared across distinct classes of drugs. Important features of addiction are escalated intake, focused pursuit of drugs, often at the expense or neglect of other rewards, and continued use despite repeated and adverse consequences. Here we show escalated opioid intake and focused pursuit mediated by CeA neurons that has also been previously shown for alcohol, sucrose, and cocaine (M. J. F. Robinson et al., 2014; Warlow et al., 2017b, 2020). Further, our results show that irrational pursuit can be generated by CeA neurons at experimenter whim to create an ‘opioid addict’ that ignores sucrose or a ‘sucrose ‘addict’ that ignores remifentanil. This irrational feature of amygdala-generated desire may be shared with addiction phenotypes seen in humans.

5.6 Figures.

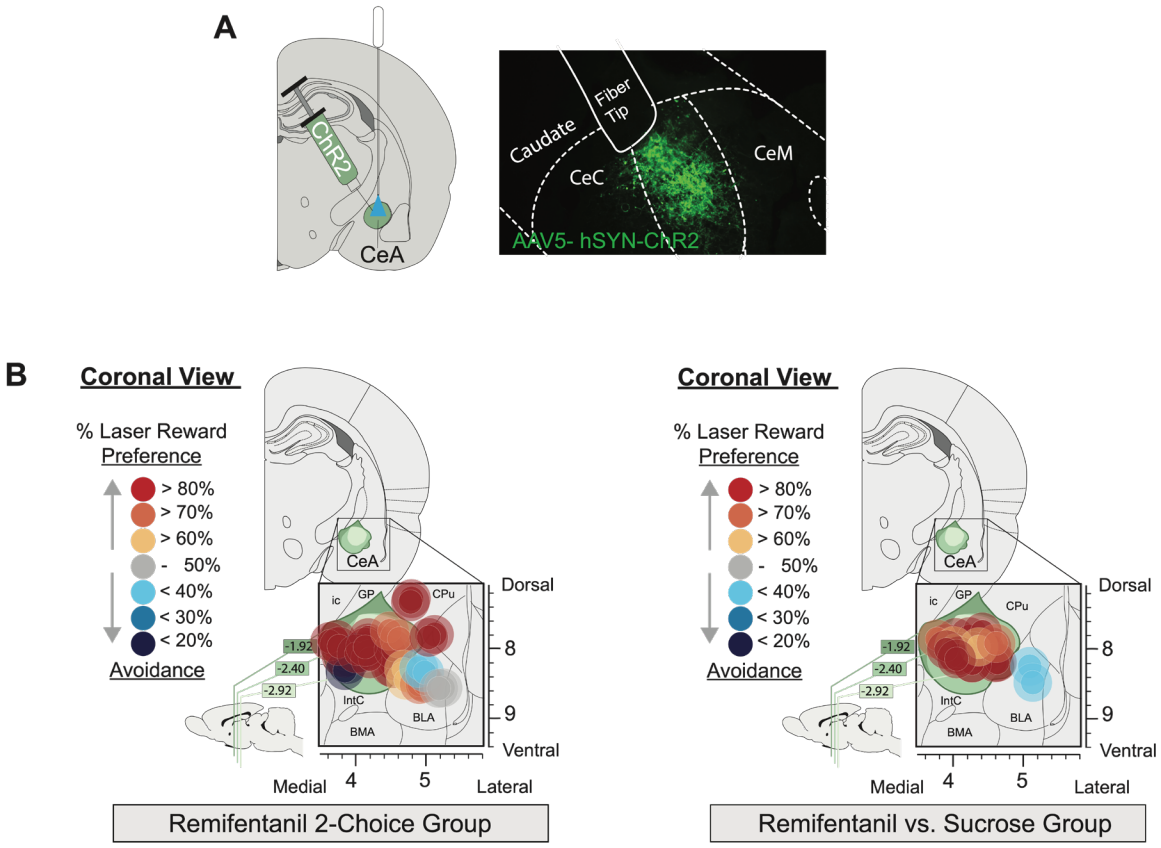


Figure 37. CeA ChR2 virus and localization of function placement map.

CeA photomicrograph shows green channelrhodopsin (ChR2; AAV5-hSYN-ChR2-eYFP) expression in CeA. **B)** Mapped sites of optic fiber implants for each ChR2 rat in remifentanil 2-choice group (left) and remifentanil vs. sucrose group (right). Symbol colors in both groups represent the percent preference for an individual rat's laser-paired reward over the non-laser paired reward calculated as the average of the last two test sessions. Ic, internal capsule; GP, globus pallidus; CPu, caudate putamen; BLA basolateral amygdala, CeA, central amygdala; IntC, intercalated amygdala; BMA, basomedial amygdala.

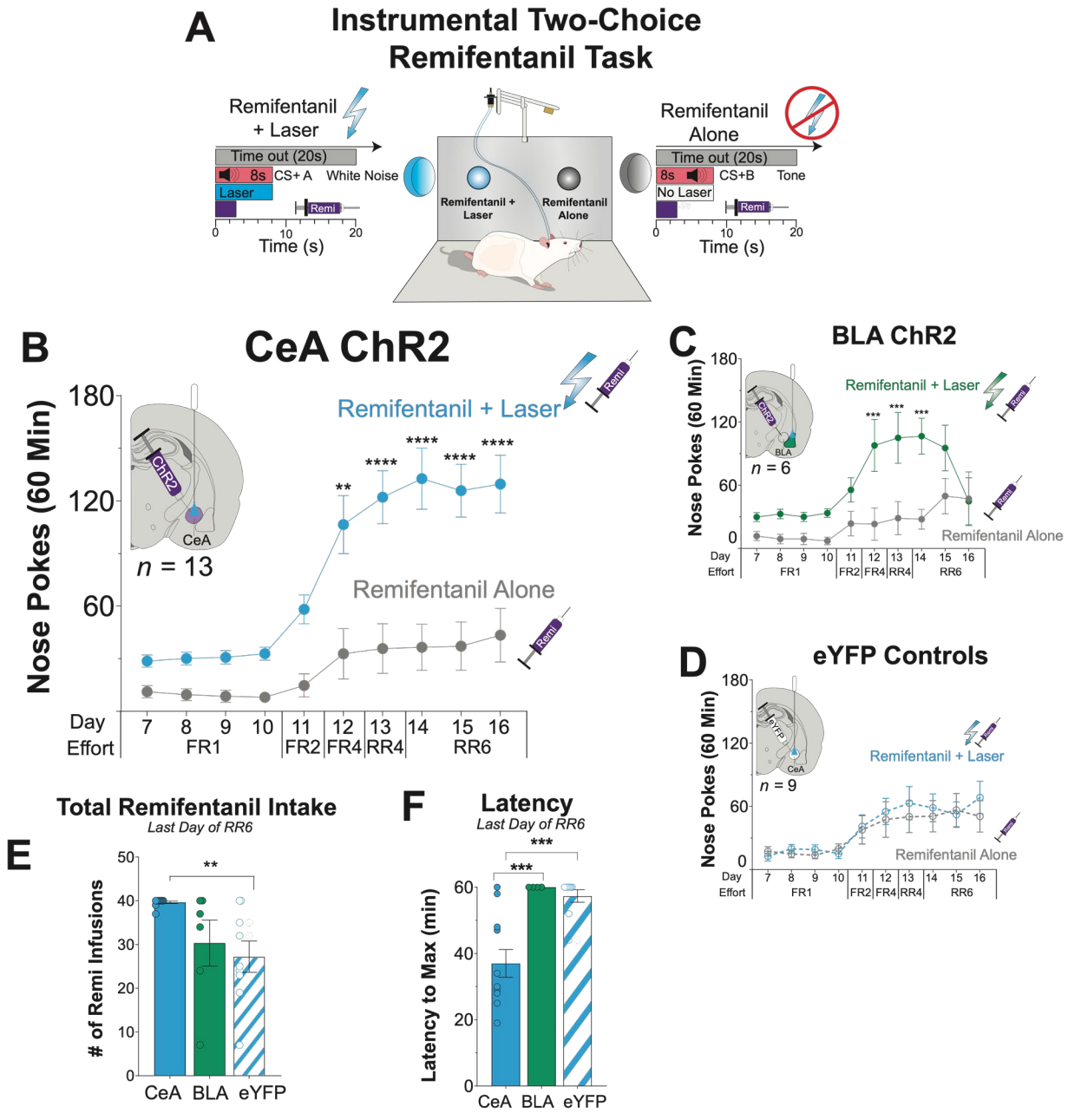

A
Instrumental Two-Choice Remifentanil Task
Remifentanil + Laser
Time out (20s)
8s CS+ A White Noise
Laser
Remi
Time (s)
Remifentanil + Laser
Remifentanil Alone
Remifentanil Alone
Time out (20s)
8s CS+B Tone
No Laser
Remi
Time (s)
B
CeA ChR2
Remifentanil + Laser
Remifentanil Alone
Nose Pokes (60 Min)
ChR2
CeA
n = 13
Day
Effort
FR1
FR2
FR4
RR4
RR6
C
BLA ChR2
Remifentanil + Laser
Remifentanil Alone
n = 6
D
eYFP Controls
Remifentanil + Laser
Remifentanil Alone
n = 9
E
Total Remifentanil Intake
Last Day of RR6
# of Remi Infusions
CeA BLA eYFP
F
Latency
Last Day of RR6
Latency to Max (min)
CeA BLA eYFP

Figure 38. Optogenetic CeA neuron excitations focus motivation for intravenous opioids.

Shows instrumental 2-choice task where rats nose poke into two different portholes. Responses to one port earn an intravenous remifentanil infusion (Remifentanil Alone: 2µg/kg; 2.8s duration; FR1-RR6 Schedule) accompanied by a discrete 8s CS+ tone. Nose poking into a second port located on the opposite side of the same wall earned an identical intravenous remifentanil infusion (Remifentanil + Laser: 2µg/kg; 2.8s duration; FR1-RR6 Schedule) paired with a different 8s CS+ tone and additional blue laser stimulation (25 Hz, 1-3 mW, 8s). **B)** ChR2 CeA neuron stimulation captures choice for intravenous remifentanil. CeA ChR2 rats (n = 13) singly pursue the laser-paired remifentanil reward (solid blue symbols) and leave the remifentanil alone option (solid grey symbols) relatively ignored. **C)** Basolateral amygdala ChR2 rats (n = 6) also show laser-paired remifentanil preference (solid green symbols), although this preference disappeared on the last day of testing. **D)** By contrast, control inactive virus rats (n = 9) lacking the ChR2 gene respond equally between remifentanil alone (dotted grey line and symbols) and remifentanil + laser (dotted blue line and symbols). **E)** By the end of 2-choice testing, CeA ChR2 rats self-administer more total remifentanil relative to eYFP controls. **F)** CeA ChR2 also consume their maximum much quicker than BLA ChR2 rats and eYFP controls, terminating their 60-min sessions ~20 min early. Data shown as mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

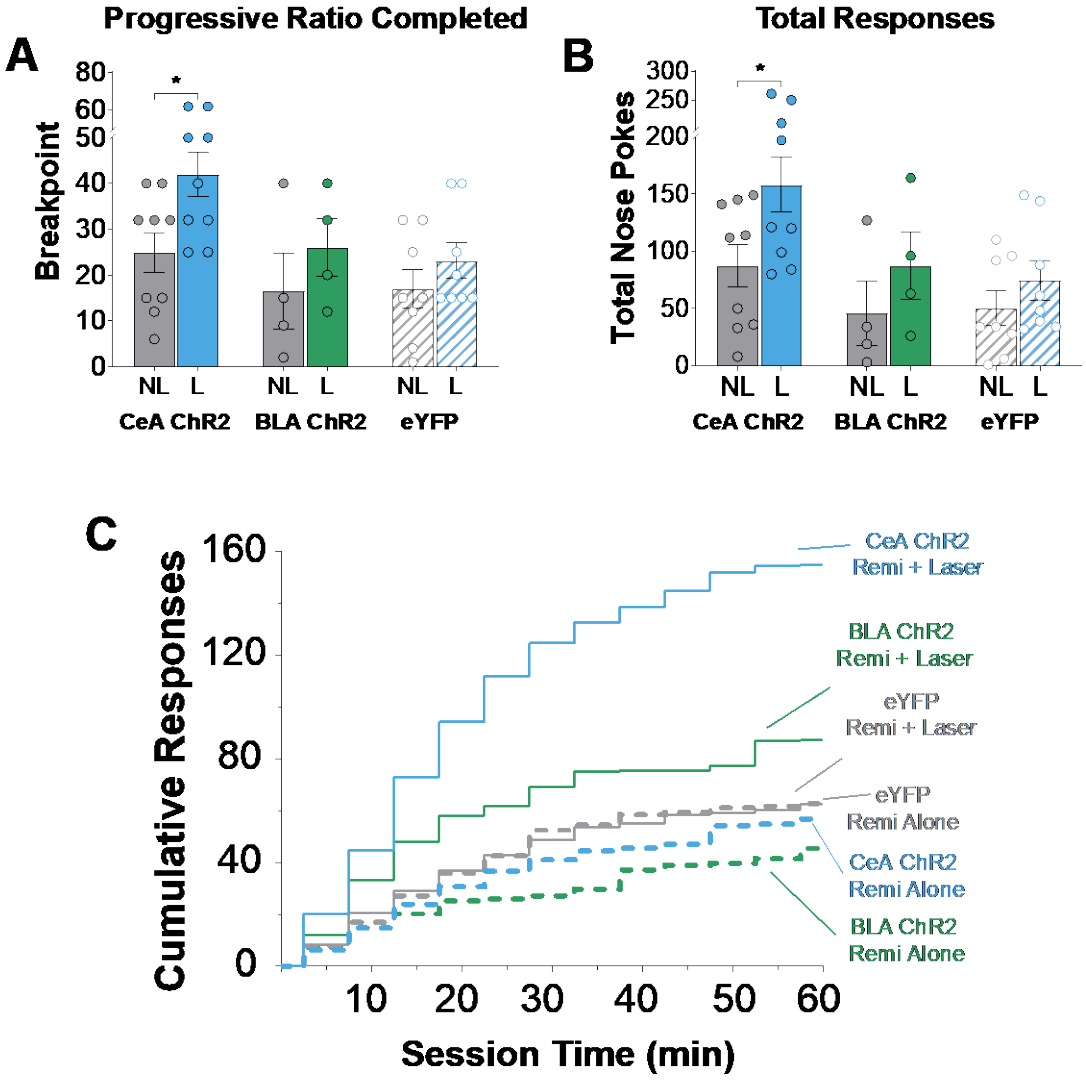

A
Progressive Ratio Completed
Breakpoint
80
60
50
40
30
20
10
0
*
NL L
CeA ChR2
NL L
BLA ChR2
NL L
eYFP
B
Total Responses
Total Nose Pokes
300
250
200
150
100
50
0
*
NL L
CeA ChR2
NL L
BLA ChR2
NL L
eYFP
C
Cumulative Responses
160
120
80
40
0
10
20
30
40
50
60
Session Time (min)
CeA ChR2
Remi + Laser
BLA ChR2
Remi + Laser
eYFP
Remi + Laser
eYFP
Remi Alone
CeA ChR2
Remi Alone
BLA ChR2
Remi Alone

Figure 39. CeA neuron stimulation amplifies breakpoint motivation.

We used a progressive ratio test of motivation on two consecutive days. On one day, rats responded for Remifentanil + Laser and on the other day, rats responded for Remifentanil Alone on a progressive ratio schedule of reinforcement so that effort required to obtain the next remifentanil infusion increased exponentially. A) CeA ChR2 rats (n = 9) reached higher breakpoints for Remifentanil + Laser and made more total responses B) and C). By contrast, BLA ChR2 rats (n = 4) and eYFP controls (n = 8) worked equally for remifentanil regardless of laser condition. Data are shown as mean ± SEM. * $p < 0.05$.

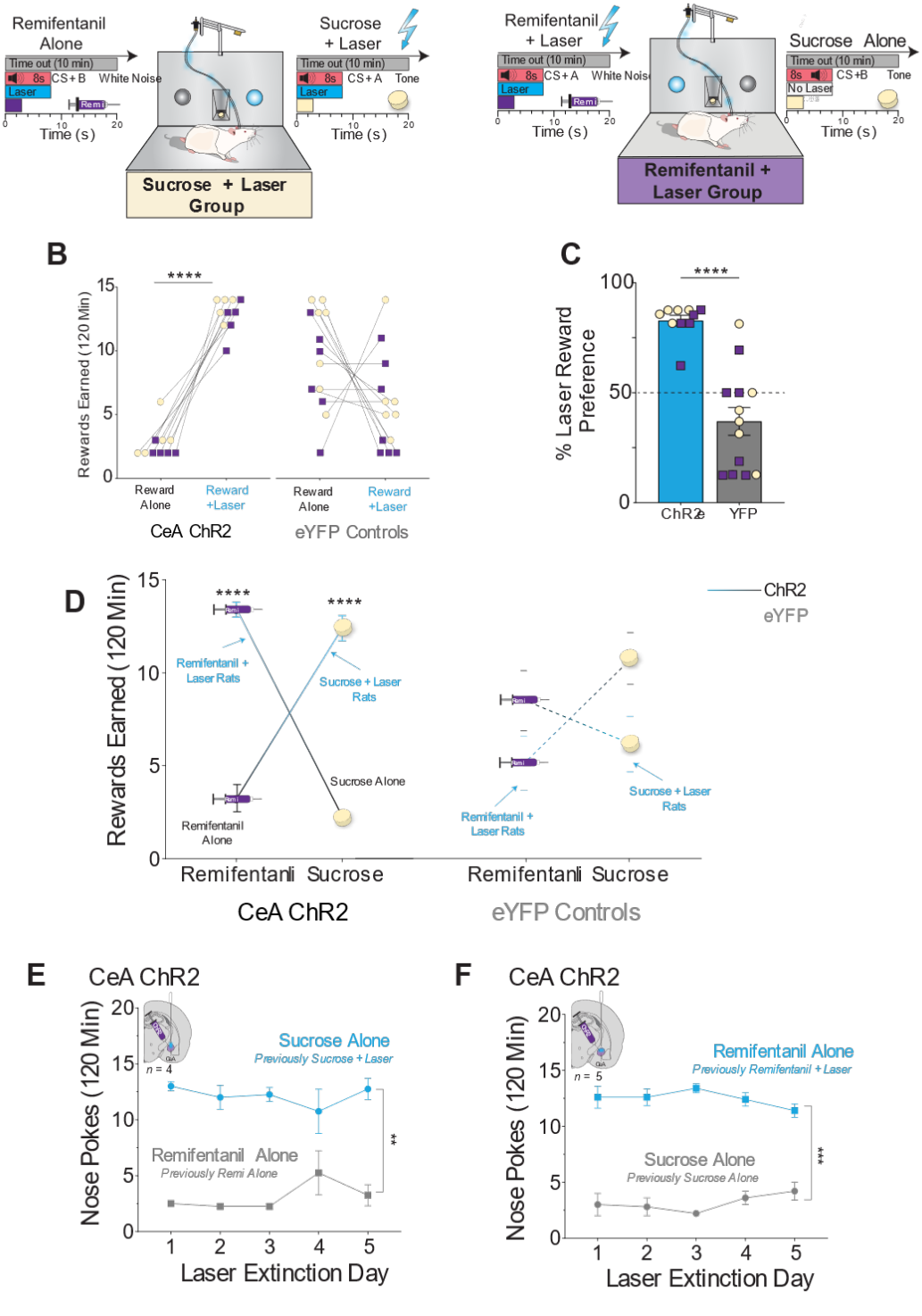

Remifentanil Alone
Time out (10 min)
8s CS+B White Noise
Laser
Remi
Time (s)
Sucrose + Laser
Time out (10 min)
8s CS+A Tone
Laser
Time (s)
Sucrose + Laser Group
Remifentanil + Laser
Time out (10 min)
8s CS+A White Noise
Laser
Time (s)
Sucrose Alone
Time out (10 min)
8s CS+B Tone
No Laser
Time (s)
Remifentanil + Laser Group
B
Rewards Earned (120 Min)
****
Reward Alone
Reward +Laser
CeA ChR2
eYFP Controls
C
% Laser Reward Preference
****
ChR2
YFP
D
Rewards Earned (120 Min)
ChR2
eYFP
****
****
Remifentanil + Laser Rats
Sucrose + Laser Rats
Sucrose Alone
Remifentanil Alone
Remifentanil
Sucrose
CeA ChR2
eYFP Controls
E
CeA ChR2
Nose Pokes (120 Min)
n = 4
Sucrose Alone
Previously Sucrose + Laser
Remifentanil Alone
Previously Remi Alone
**
Laser Extinction Day
F
CeA ChR2
Nose Pokes (120 Min)
n = 5
Remifentanil Alone
Previously Remifentanil + Laser
Sucrose Alone
Previously Sucrose Alone
***
Laser Extinction Day

Figure 40. CeA neurons control choice between remifentanil and sucrose.

Describes remifentanil vs sucrose choice paradigm. **B)** Earned rewards (nose pokes; FR1 schedule) during 120-min remifentanil vs. sucrose choice sessions for individual rats (ChR2: $n = 10$; eYFP: $n = 13$). **C)** Percent preference for laser-paired reward on day 9 (ChR2: $n = 10$; eYFP: $n = 13$). Individuals with laser paired with sucrose in yellow circles, and remifentanil in purple squares. Data presented as mean ± SEM **D)** Total number of rewards sucrose and remifentanil rewards earned on day, separated by reward type, and virus group. ChR2 rats in solid lines; eYFP rats in dotted lines**.** ChR2: remifentanil + laser $n = 5$, sucrose + laser $n = 5$; eYFP: remifentanil + laser $n = 7$; sucrose + laser $n = 6$. **E, F)** Reward preference between remifentanil and sucrose persists after CeA neuron excitation pairing is placed in extinction. Data presented as mean ± SEM**;** $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

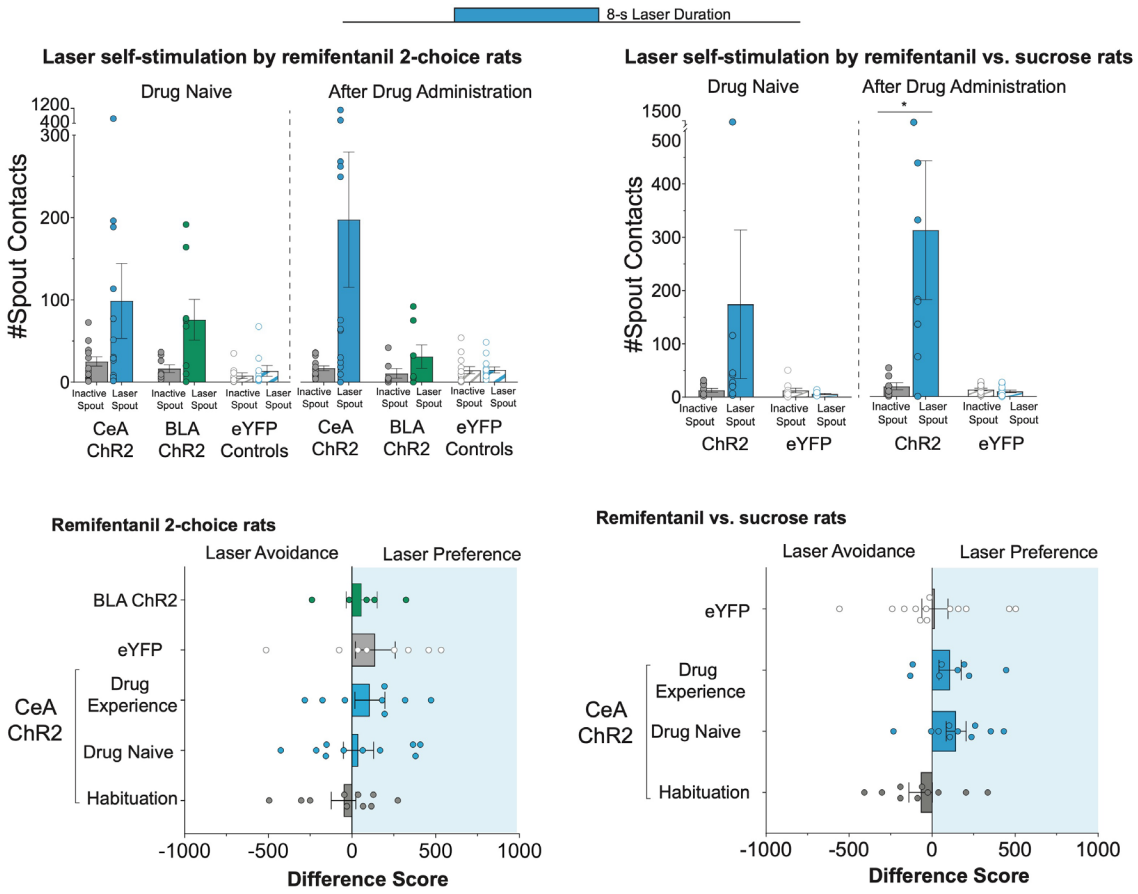

8-s Laser Duration
Laser self-stimulation by remifentanil 2-choice rats
Drug Naive
After Drug Administration
#Spout Contacts
Inactive Spout
Laser Spout
CeA ChR2
BLA ChR2
eYFP Controls
Laser self-stimulation by remifentanil vs. sucrose rats
ChR2
eYFP
Remifentanil 2-choice rats
Laser Avoidance
Laser Preference
BLA ChR2
eYFP
CeA ChR2
Drug Experience
Drug Naive
Habituation
Difference Score
Remifentanil vs. sucrose rats

Figure 41. CeA neurons do not reliably promote self-stimulation.

A, B) active spout-based laser self-administration task where touching a laser spout earned laser stimulation (25 Hz; 8-s duration) and touching a separate inactive spout earned nothing. **A)** total laser self-administrations earned by rats from remifentanil 2-choice experiments when drug naïve (left) and after drug self-administration experiments (right) (ChR2 rats, $n = 14$; BLA ChR2, $n = 8$, eYFP controls, $n = 13$). On average, CeA ChR2 and BLA ChR2 rats self-stimulate in the spout task equal amounts when drug naïve and after drug self-administration experiments. **B)** Total laser self-administration earned by rats from remifentanil vs. sucrose experiments when drug naïve (left) and after drug self-administration experiments (right). CeA ChR2 rats only self-stimulate in the spout task after drug experience. (ChR2 rats, $n = 8$; eYFP controls, $n = 10$). **C, D)** No self-stimulation in the passive place-based laser self-administration task where rats could earn laser stimulation (25 Hz; 3s ON/ 8s OFF) by spending time in the laser-paired chamber while spending time in another chamber earned nothing. Rats neither preferred nor avoided the laser-paired chamber where CeA ChR2 stimulation was delivered compared with the other chamber that lacked laser. Difference scores calculated as time (s) spent in laser-paired chamber – time (s) spent in non-laser chamber. **C)** Difference scores for CeA ChR2 rats ($n = 10$), BLA ChR2 rats ($n = 5$), and eYFP controls ($n = 8$) rats from remifentanil 2-choice experiments. **D)** Difference scores for CeA rats (ChR2 rats, $n = 8$; eYFP controls, $n = 13$). All data presented as means and SEM. $^{*}p < 0.05$.

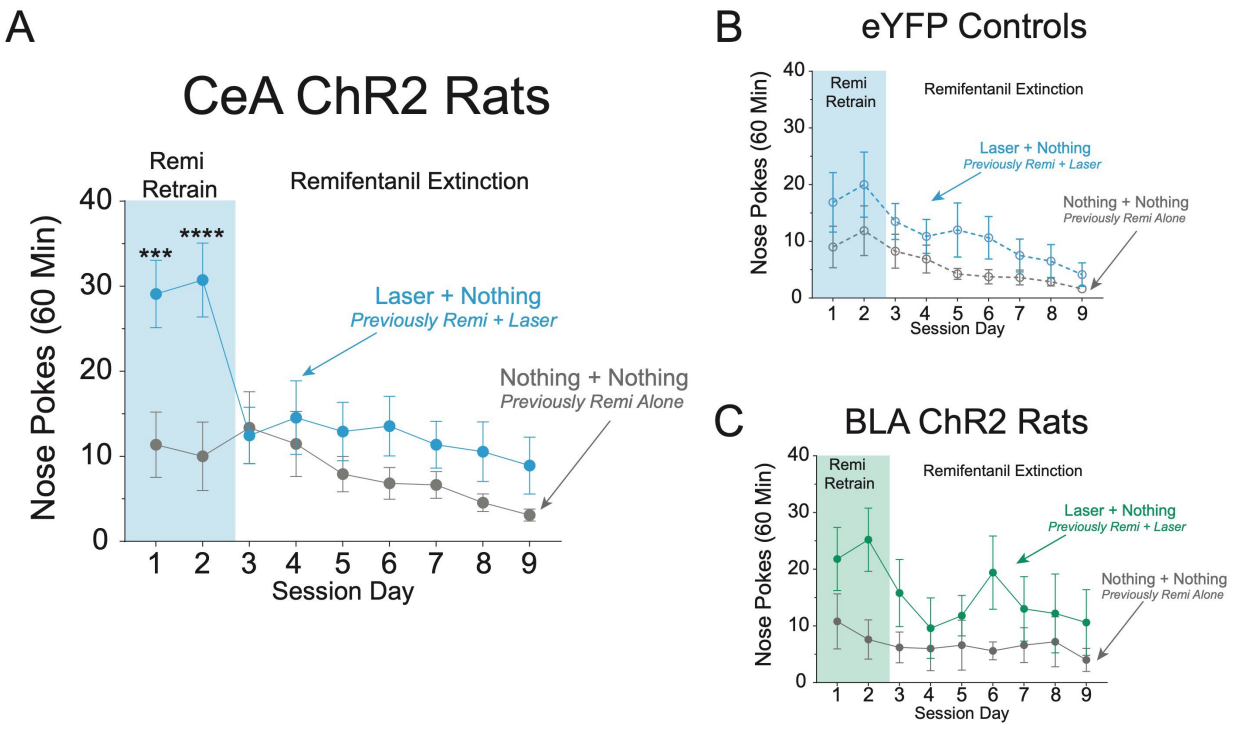
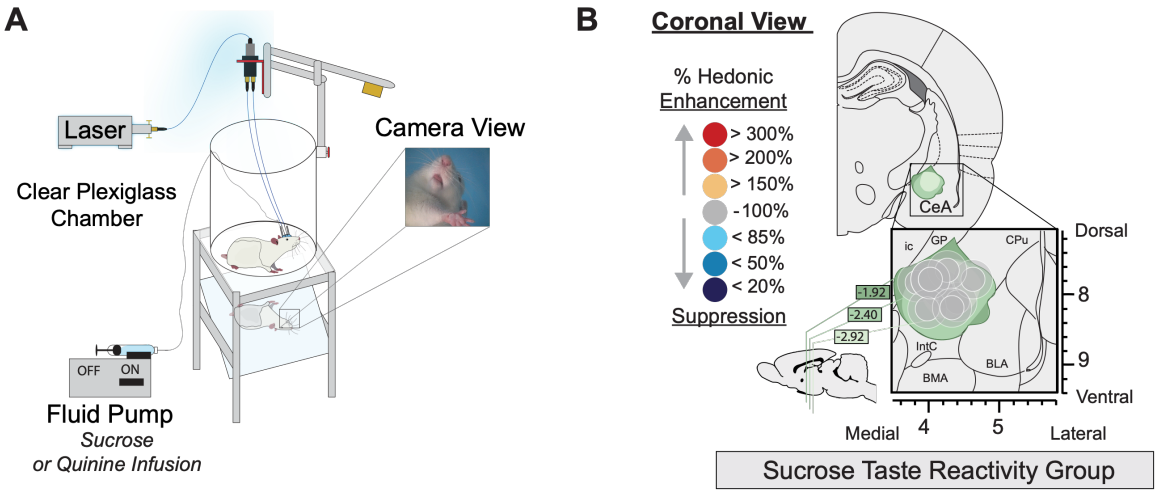
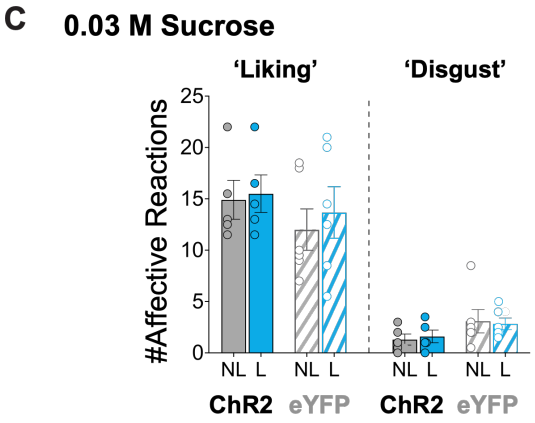
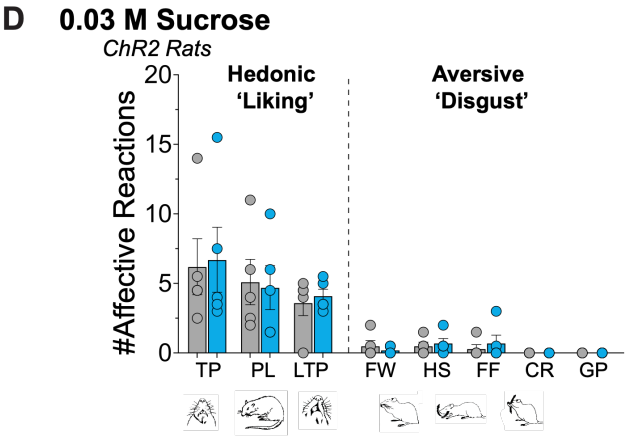


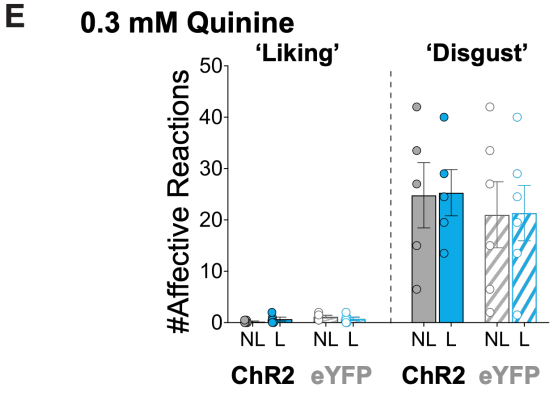
Figure 42. Remifentanil extinction does not maintain responding for CeA neuron activation.

Rats were retrained on the remifentanil two-choice task for two days. Starting on Day 3, intravenous remifentanil was removed, and rats could nose poke to earn laser stimulation by poking into the previous laser + remifentanil port (CeA ChR2 and eYFP: blue lines; BLA ChR2: green lines). Poked into the previous remifentanil alone port now earned nothing (grey lines). **A)** CeA ChR2 rats reestablished their preference for the laser-paired remifentanil option on Days 1 and 2. When laser stimulation as offered alone, responding declined and rats no longer preferred the laser-delivering port. **B)** eYFP control rats, and **C)** BLA ChR2 rats did not establish a preference for the laser-paired reward and responding declined over the course of 7 extinction sessions. Data are shown as mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$.


A
Laser
Camera View
Clear Plexiglass Chamber
OFF
ON
Fluid Pump
Sucrose
or Quinine Infusion
B
Coronal View
% Hedonic Enhancement
> 300%
> 200%
> 150%
-100%
< 85%
< 50%
< 20%
Suppression
CeA
ic
GP
CPu
Dorsal
8
9
Ventral
-1.92
-2.40
-2.92
IntC
BLA
BMA
Medial
4
5
Lateral
Sucrose Taste Reactivity Group


C
0.03 M Sucrose
'Liking'
'Disgust'
#Affective Reactions
25
20
15
10
5
0
NL L NL L NL L NL L
ChR2 eYFP ChR2 eYFP


D
0.03 M Sucrose
ChR2 Rats
Hedonic 'Liking'
Aversive 'Disgust'
#Affective Reactions
20
15
10
5
0
TP PL LTP FW HS FF CR GP


E
0.3 mM Quinine
'Liking'
'Disgust'
#Affective Reactions
50
40
30
20
10
0
NL L NL L NL L NL L
ChR2 eYFP ChR2 eYFP

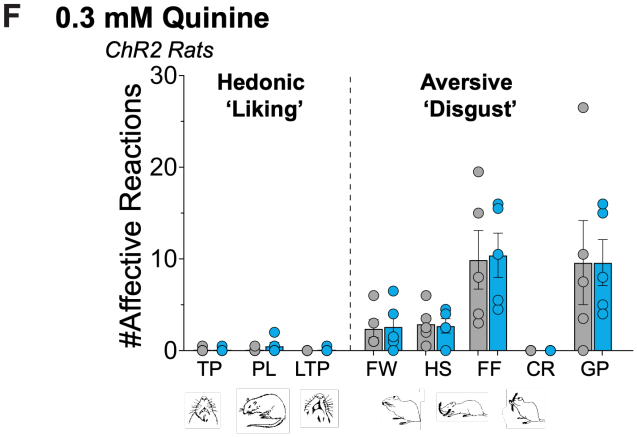

F
0.3 mM Quinine
ChR2 Rats
Hedonic 'Liking'
Aversive 'Disgust'
#Affective Reactions
30
20
10
0
TP PL LTP FW HS FF CR GP

Figure 43. CeA neuron stimulation does not alter hedonic impact for sweet or bitter reward.

A) Depicts taste reactivity testing situation. Rats with stimulus delivery tube (PE-10) tubing connected to oral cannula are placed in Plexiglass chamber. An angled mirror below the clear floor of chamber tilts the ventral view of rat's face to a video camera recording orofacial expressions. Sucrose (0.03M) or quinine (0.3 mM) infusions (1 mL) are infused directly into rat's mouth via intraoral cannula at a rate of 1 mL/ min via microinfusion pump. **B)** Coronal map depicts anatomical placements and behavioral effects of CeA ChR2 optic fibers in CeA (circles). Color of symbols represents functional hedonic effect on taste reactivity to sucrose of laser illumination at each side: within subjects % increase in sucrose-elicited 'liking' reactions by laser illumination over baseline measured to sucrose infusion without laser. **C)** Sucrose CeA ChR2 (solid bars; $n = 5$) and eYFP (striped bars; $n = 6$) affective reactions. No change in total number of positive 'liking' reactions (left) and aversive 'disgust' expression during no laser conditions (grey bars) and laser stimulation (blue bars). **D)** Separate component 'liking' and 'disgust' reactions to intraoral infusions of 0.03M sucrose are unchanged in CeA ChR2 rats ($n = 5$) at baseline (grey bars) vs. laser stimulation (blue bars). **E)** No change in total number of affective reactions to quinine of CeA ChR2 rats (solid bars; $n = 5$) and eYFP controls (striped bars; $n = 6$). Blue shows reactions during laser on vs. no laser (grey bars) conditions of the same rats. **F)** Separate component reactions to quinine within hedonic 'liking' and aversive 'disgust' categories emitted by CeA ChR2 rats ($n = 5$) in laser on (blue) vs. no laser (grey) conditions. Separate component reactions in positive hedonic category are unpacked as: tongue protrusions, TP; paw licks, PL; lateral tongue protrusions, LTP. Separate component reactions in negative aversive category are unpacked as: facewashes, FW; head shakes, HS; forelimb flails, FF; chin rubs, CR; gapes, GP.

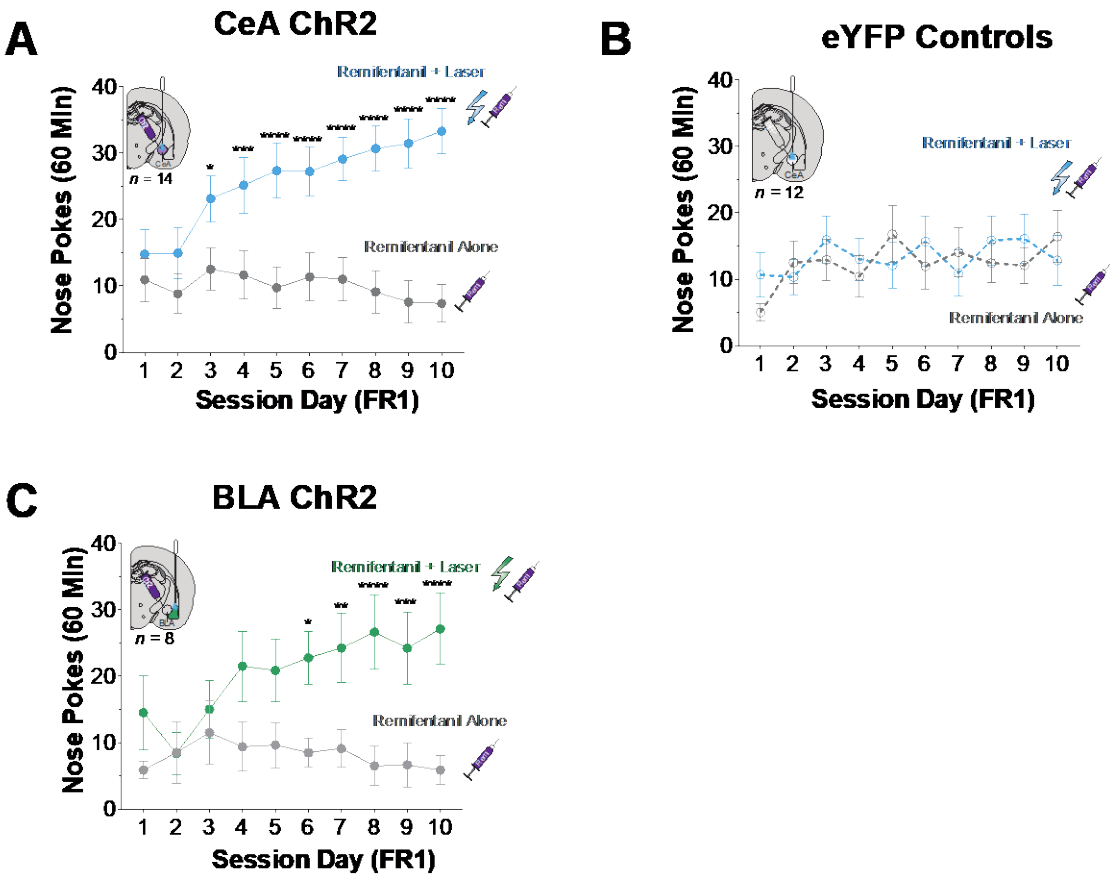


Figure 44. CeA ChR2 neuron stimulation captures choice for intravenous remifentanil.

A) CeA ChR2 rats (n = 14) almost exclusively pursue intravenous remifentanil reward paired with optogenetic CeA neuron excitation (solid blue lines) compared to intravenous remifentanil alone (solid grey lines) during initial 10 days of FR1 testing. **B)** eYFP control rats (n = 12) chose equally between intravenous remifentanil alone (dotted gray lines) and laser-paired remifentanil (dotted blue lines). **C** BLA ChR2 rats also showed preference for laser-paired remifentanil (solid green lines) compared to remifentanil alone (solid grey lines). Data are shown as mean and SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

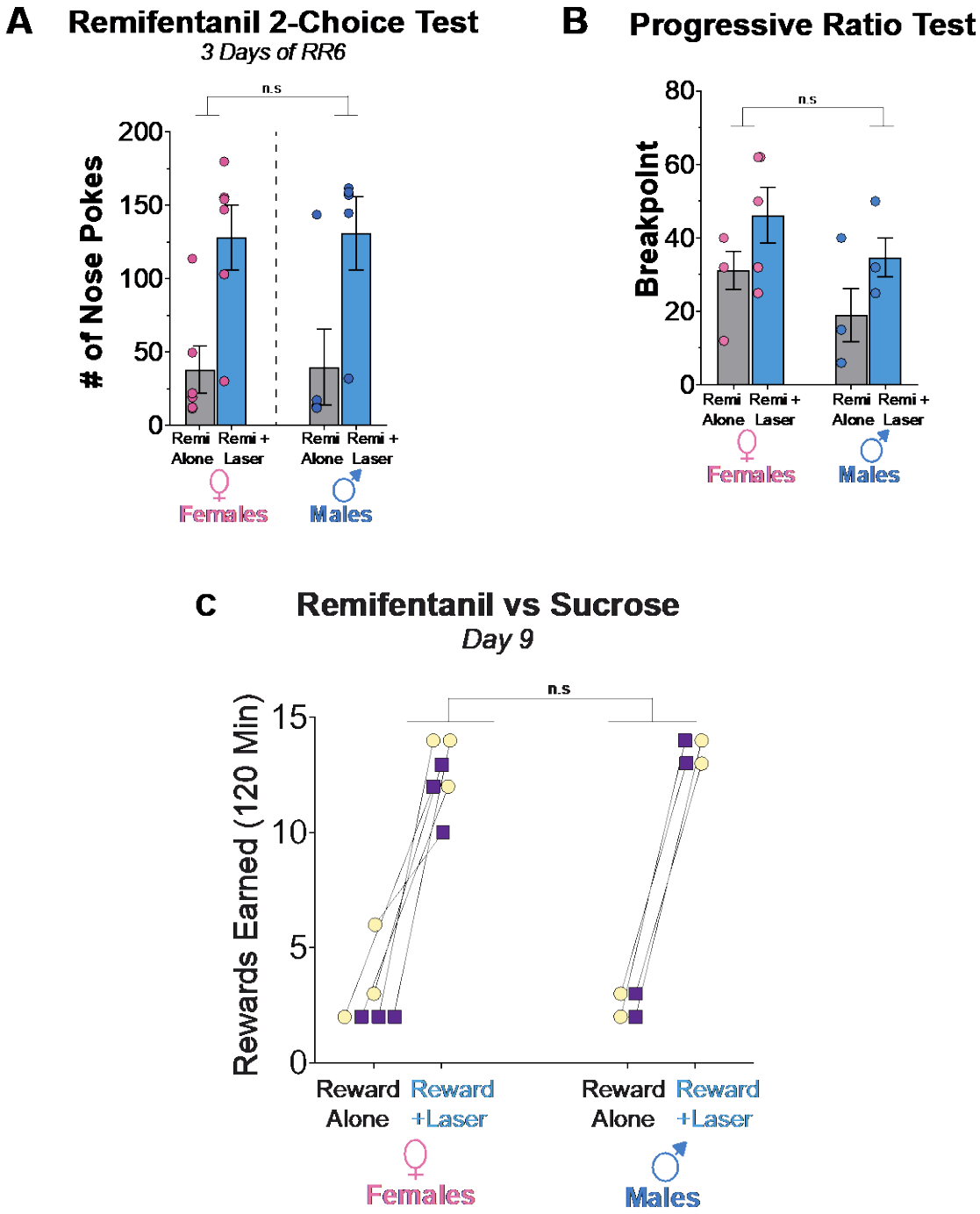

A
Remifentanil 2-Choice Test
3 Days of RR6
n.s
# of Nose Pokes
200
150
100
50
0
Remi Alone
Remi + Laser
Remi Alone
Remi + Laser
Females
Males
B
Progressive Ratio Test
n.s
Breakpoint
80
60
40
20
0
Remi Alone
Remi + Laser
Remi Alone
Remi + Laser
Females
Males
C
Remifentanil vs Sucrose
Day 9
n.s
Rewards Earned (120 Min)
15
10
5
0
Reward Alone
Reward +Laser
Reward Alone
Reward +Laser
Females
Males

Figure 45. Male and female groups across behavioral tests.

No sex differences detected between male and female CeA ChR2 rats during instrumental choice sessions. **A)** Male (laser preference ratio 3:1; *n* = 3) and female (laser preference ratio 3:1; *n* = 4) CeA ChR2 rats equally prefer laser-paired remifentanil over remifentanil alone during 2-choice test sessions. Data presented by averaging last three RR6 sessions. **B)** On average, male (*n* = 3) and female (*n* = 4) CeA ChR2 rats worked ~160% greater for laser-paired remifentanil vs. remifentanil alone during progressive ratio tests. **A, B)** Data presented as mean and SEM. **C)** Rewards earned by individual male (right) and female (left) ChR2 rats during the last remifentanil vs. sucrose choice session. Male (laser preference ratio 5:1; *n* = 4) and female (laser preference ratio 4:1; *n* = 3) ChR2 rats equally prefer the laser-paired reward. Sucrose responses in yellow circles and remifentanil responses in purple squares.

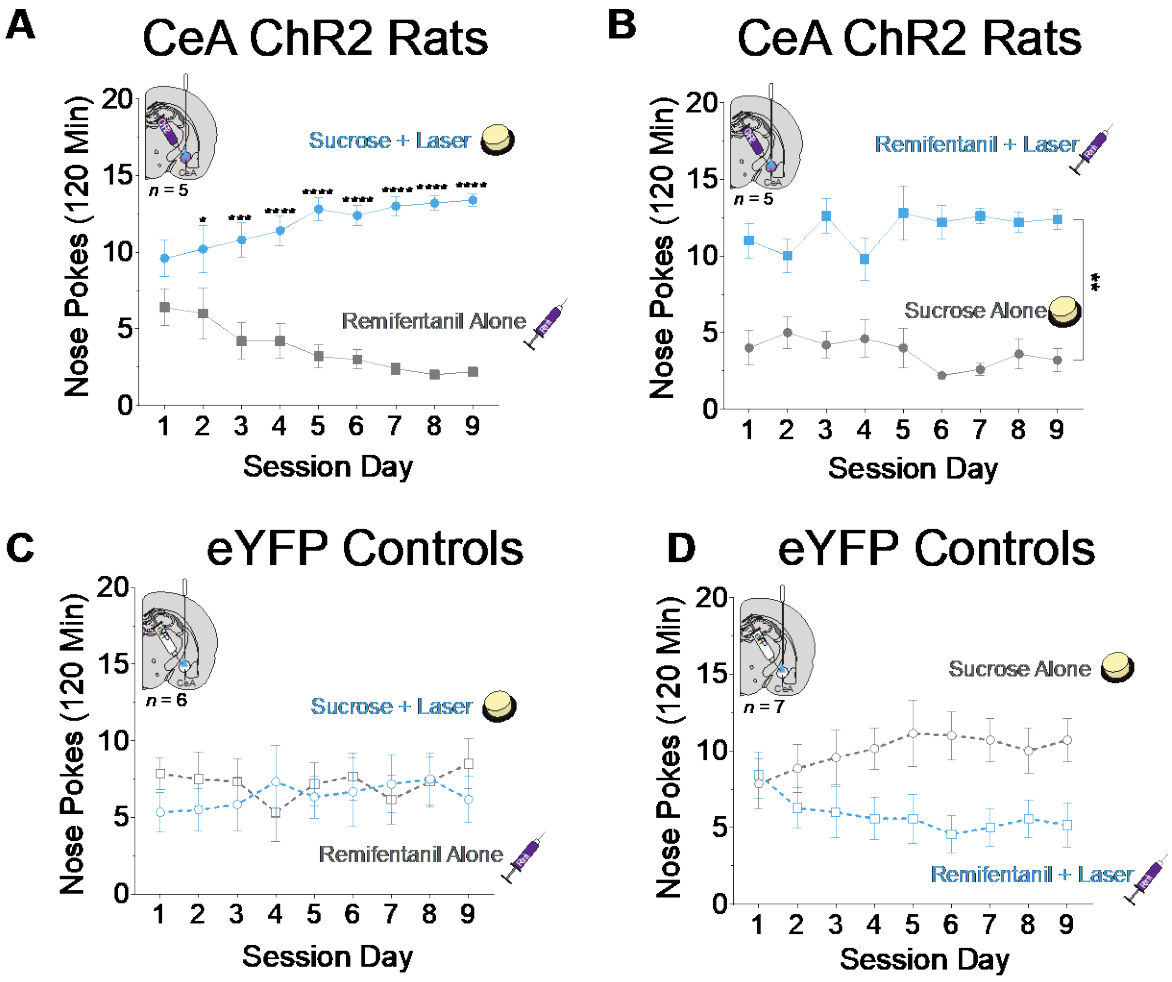

A
CeA ChR2 Rats
Nose Pokes (120 Min)
Session Day
n = 5
Sucrose + Laser
Remifentanil Alone
B
CeA ChR2 Rats
Remifentanil + Laser
Sucrose Alone
**
C
eYFP Controls
n = 6
Sucrose + Laser
Remifentanil Alone
D
eYFP Controls
n = 7
Sucrose Alone
Remifentanil + Laser

Figure 46. CeA ChR2 pairing controls pursuit of remifentanil vs. sucrose.

A-D) Nose pokes by rats in each group during each remifentanil vs. sucrose choice session. **A)** Sucrose + Laser CeA ChR2 rats (n = 5) exclusively poke for sucrose + laser and ignore intravenous remifentanil. **B)** Remifentanil + Laser CeA ChR2 rats (n = 5) singly pursue intravenous remifentanil and ignore sucrose. **C,D)** Neither group of eYFP controls (Sucrose + laser: n = 6; remifentanil + laser: n = 7) shows preference for either sucrose or remifentanil. All data presented as mean and SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Chapter 6 Discussion

Reward components of 'liking' and 'wanting' are controlled through interconnected mesocorticolimbic systems that allow us to seek out stimuli in the environment that are biologically relevant. Brain systems that control 'liking' or hedonic impact remain the least understood. Better characterization these brain circuits and their ability to exert hedonic control is critical to understanding hedonic dysfunction that may contribute to neuropsychiatric disorders such as depression, schizophrenia, obesity, and addiction. This dissertation investigated neuronal causation of 'liking' reactions by employing optogenetic techniques to directly control the activity of neurons in mesocorticolimbic sites known to contain hedonic hotspots.

Independent verification of hedonic hotspots in rostromedial OFC and caudal insula

The precise role of corticolimbic systems in generating affective responses has been topic of debate. Loss of function studies in rodents and case studies of cortical lesions in humans suggest that cortical regions are not *necessary* for the generation of affective responses. For example, insula lesions or complete decortication in rodents does not abolish affective 'liking' responses to pleasant tastes (Hashimoto & Spector, 2014; King et al., 2015b; Schier et al., 2014; Wirsig & Grill, 1982a). In humans, cortical lesions to prefrontal, OFC, insula, cingulate cortex, and beyond result in severe learning and memory impairments and emotional behaviors that appear odd or misplaced, but the capacity to produce emotions including fear, anger, and self-

reported happiness remains (Beer et al., 2003; Bramham et al., 2009; Damasio, 1994; Damasio et al., 2013; Feinstein et al., 2010; Philippi et al., 2012b; Szczepanski & Knight, 2014). Importantly however, human imaging studies suggest that corticolimbic systems may play important roles in affective behaviors. For example, OFC and insula activity is thought to encode the pleasantness of palatable stimuli and other rewards (Berridge & Kringelbach, 2015; de Araujo et al., 2003; Hosokawa et al., 2007; Kragel et al., 2023; Kringelbach et al., 2003; Lamm et al., 2015; Rolls, Kringelbach, et al., 2003; Rolls, O'Doherty, et al., 2003; Simmons et al., 2013; Small et al., 2001; Tremblay & Schultz, 1999), suggesting that cortical sites may serve as neural markers of affective states or even potentially compute gain in hedonic functions in humans.

In Chapter 2 I directly investigated cortical contributions to affective responses by stimulating sites in OFC and insula where orexin and opioid microinjections were previously shown to increase 'liking' reactions. The hypothesis that cortical sites can compute gains of hedonic function was indeed supported in the current work. My results show that neuronal depolarization via ChR2 activations within rostromedial OFC and caudal insula sites doubled hedonic 'liking' reactions to sucrose and other palatable tastes similar to drug microinjection studies (Castro & Berridge, 2017). The hedonic hotspot regions in OFC and insula bookended a larger hedonic coldstrip that spanned caudolateral OFC and continued laterally through anterior and mid insula. At these sites, ChR2 activations did not increase hedonic 'liking' reactions and even oppositely suppressed hedonic impact. In comparison to the localized sites of hedonic enhancement, nearly all cortical areas we tested effectively generated incentive motivation via their ability to promote laser self-stimulation. These sites of incentive motivational 'wanting' extended beyond the OFC and caudal insula hotspots, including sites that previously suppressed 'liking' during taste reactivity tests. Overall, there was striking overlap between the reported

anatomical boundaries of my optogenetic hedonic hotspots and those mapped using opioid and orexin (Castro & Berridge, 2017). Our OFC hedonic hotspot was slightly larger in volume, but this was primarily because we mapped into rostrodorsal areas OFC the original drug study did not investigate.

Our results also provide support for the proposal that cortical regions are not necessary for generating affective responses *de novo.* We directly silenced the OFC hedonic hotspot using iC++ mediated neuronal inhibitions and found that this failed to alter affective 'liking' reactions to palatable sucrose. The current results provide support for the hypothesis that cortical sites may exert hierarchical control over the hedonic generators that are likely found subcortically in ventral pallidum or nucleus accumbens (Cromwell & Berridge, 1993; Ho & Berridge, 2014; Khan et al., 2019). If cortical regions are lesioned or otherwise damaged, then this level of hierarchical control is lost, but the capacity for generating affective responses remains intact.

Next, I examined whether activating hedonic hotspots aligned with the hypothesis that hedonic hotspots communicate with one another and form a unified circuit for hedonic control (Castro & Berridge, 2014b; Morales & Berridge, 2020). For example, Kyle Smith's studies in VP showed that activating one hedonic hotspot (e.g in NAc) via DAMGO microinjections recruited Fos in the NAc hedonic hotspot (K. S. Smith & Berridge, 2007). Further, simultaneously inhibiting one hotspot (e.g., in VP) while stimulating the other (in NAc) with DAMGO prevented increases in 'liking' reactions otherwise generated by DAMGO alone (K. S. Smith & Berridge, 2007). Relatedly, DAMGO and orexin microinjections into the OFC and insula hedonic hotspots increase Fos protein expression in each other, and in the other subcortical hotspots, suggesting that 'liking' enhancements may require activity of multiple hotspots to produce gains in hedonic impact (Castro & Berridge, 2017). My current work found a

similar pattern of Fos recruitment when the OFC and caudal insula hedonic hotspots are optogenetically stimulated. ChR2 activations in the OFC hotspot recruited increases in Fos protein expression in caudal insula, rostrodorsal NAc medial shell, and caudal ventral pallidum hedonic hotspots. Overall, this provides additional support that hedonic hotspots recruit each other into action to increase positive affective responses.

Identification of a novel hedonic hotspot in mid cingulate cortex

Rodent and human neuroimaging studies have implicated additional corticolimbic regions in emotion and motivation (Berridge, 2003; Berridge & Kringelbach, 2013; Castro et al., 2015; Kringelbach & Berridge, 2010b; Morales & Berridge, 2020). One potential contributor is the anterior cingulate cortex, which has been shown to code pleasant and aversive affective states in humans, non-human primates, and rodents (Childress et al., 1999; K. D. Davis et al., 1997; de Araujo & Rolls, 2004; Firestone et al., 1996; Grabenhorst et al., 2008; Grabenhorst & Rolls, 2008; Huang et al., 2024; Maas et al., 1998; Mathew et al., 1997; Porro et al., 1998; Rainville et al., 1997; Rauch et al., 1999; Rolls, 2008; Rolls, O'Doherty, et al., 2003; Tölle et al., 1999; Zald et al., 1998).

A recent study in human patients raised the possibility that sites within cingulate cortex could potentially produce causal gains in hedonic function (Bijanki et al., 2019). Human patients undergoing deep brain stimulations as a treatment for epilepsy developed intense positive affect, laughter, and happiness when a localized region in dorsal and caudal anterior cingulate cortex was stimulated, and similar stimulations in other areas of ACC failed produce the same responses in other patients (Bijanki et al., 2019). In Chapter 3 I directly tested whether cingulate cortex in rats may contain a hedonic hotspot that can increase 'liking' reactions to intra-oral

infusions of pleasant tastes. I found that ChR2 activations within a mid-caudal region of mid cingulate cortex nearly doubled positive 'liking' reactions to different concentrations of sucrose as well as room temperature tap water, suggesting that this cingulate hedonic hotspot could robustly and repeatedly generate increases in hedonic 'liking' reactions.

In the same rats that ChR2 activations increased positive expressions to pleasant tastes, optogenetic stimulations also enhanced the incentive value of a laser-paired sucrose reward. I trained rats to choose between instrumentally responding for a palatable sucrose pellet (sucrose alone) or alternatively lever pressing for an identical sucrose pellet paired with cingulate neuron photostimulation. In this task, activating hedonic hotspot neurons caused rats to almost exclusively respond for sucrose + cingulate neuron photostimulation and altogether ignore sucrose alone. Further, cingulate neuron excitations promoted laser self-stimulation in cingulate hotspot ChR2 rats, indicating that even in the absence of an external sweet reward, ACC neuron excitation is rewarding.

Does the cingulate hotspot follow some of the same 'rules' (e.g., hierarchical control) and patterns of neuronal activity that are shared between the other hotspots? In regard to hierarchical control, this remains an open question. In the current study I did not perform loss of function experiments in ACC. However, it is conceivable that inhibition or lesions to cingulate neurons may not completely eliminate normal 'liking' reactions, which could certainly keep in line with evidence that ACC lesions do not abolish the capacity for affective responses in humans (Damasio et al., 2013). Future studies could directly test this hypothesis to compare neuronal activations vs. inhibitions influence over 'liking'.

Some evidence that cortical hotspots communicate with the cingulate hotspot comes from my Fos experiments in Chapter 2. There I found that optogenetic stimulations of caudal insula

sites, where the hedonic hotspot resides in insula, increased the number of Fos+ neurons in the cingulate hotspot region, suggesting that there may be indeed cross-communication between cortical hotspots. I plan to more directly test this in the future by looking at patterns of Fos expression following cingulate hotspot activations. The tissue was stained and imaged, and will be included in the final published manuscript that arises from my dissertation efforts.

Perhaps a question raised by the current work is whether the identified site of hedonic enhancement in cingulate should be viewed as a direct rodent homolog to the pleasure-generating site previously reported in humans? This is a reasonable question to ask, although cross-species homologies are difficult to directly assess, and may be especially challenging for cortical areas, which are so hugely differentiated in humans compared to rats. Regarding cingulate cortex and prefrontal regions of ACC, great disparities have been reported in terminology used to refer to prefrontal and cingulate regions across rodent and human researchers, which makes translational applications difficult to assess (Laubach et al., 2018; van Heukelum et al., 2020; Vogt & Paxinos, 2014). Older rodent atlases used prelimbic and infralimbic designations to refer to prefrontal regions of ACC, and split the rest of cingulate cortex along dorsal/ventral lines into Cg1 and Cg2 regions that extended caudally, and are not part of human and non-human primate terminology (Paxinos & Watson, 2007). It has been proposed that Cg1 an Cg2 terminology does not really match up with current knowledge regarding functional specialization across cingulate subregions, perhaps designated anterior, mid, and posterior cingulate designations may be more apt (van Heukelum et al., 2020). More recently, some atlases have dropped this terminology and instead use Broadman terminology to refer to various cortical sites (Paxinos & Watson, 2013). For example, infralimbic and prelimbic regions are now referred to as Area 32, and these may closely match pregenual areas of the

anterior cingulate cortex in humans. Areas 24 and 24' may instead now more closely reflect human and nonhuman primate functional areas that include mid cingulate and posterior cingulate subregions. Although entirely speculative, based on potential cross species views, it seems probable that if a human homologous site for hedonic enhancement exists, it may reside in regions that might constitute dorsocaudal anterior cingulate cortex and/or mid cingulate cortex areas.

Ventral Pallidum GABA neurons bidirectionally control 'liking' and 'wanting' for sweet reward.

The ventral pallidum is a crucial site that controls both 'liking' and 'wanting' for rewards. Lesion and pharmacological studies have indicated that the VP contains a hedonic hotspot in caudolateral regions that is both *necessary* and *sufficient* for normal affective responses. Drug microinjections in VP hotspot sites increase positive 'liking' reactions to sucrose, and lesions or drug inactivations completely abolish normal 'liking' and produce intense 'disgust' to otherwise palatable tastes (Cromwell & Berridge, 1993; Ho & Berridge, 2013, 2014; Khan et al., 2019; K. S. Smith & Berridge, 2005). Our current work supports this hypothesis, and provides additional triangulating evidence using optogenetic techniques and show that 1) VP is crucial for normal 'liking' and 2) 'liking' and 'wanting' functionally segregate across the VP rostro-caudal gradient.

Using optogenetic inhibitions in general neuronal populations in VP, I localized a posterior VP site that generates intense aversive 'disgust'. Within this region, iC++ and SwiChR++ inhibitions replaced normal 'liking' reactions with aversive expressions in rats, and further increased aversion to an already bitter and unpleasant quinine taste. I note that the intensity of my effects differs from those reported by previous drug microinjection and lesion

studies. While rats overall showed decreased positive responses and the overall constellation of behaviors did shift toward the 'disgust' category, perhaps a more appropriate assessment is that normally palatable sucrose had become more neutral, similar to water taste reactivity where somewhat equal numbers of positive 'liking' and aversive 'disgust' reactions are emitted by rats. Do these findings contradict previous studies? No, not necessarily as the directionality of effects is consistent between my optogenetic investigations and previous studies. A more likely explanation for our more moderate results is that this reflects the inherent technological limitations associated with current optogenetic inhibition techniques, which produce lower levels of inhibition compared to permanent lesions or drug microinjections (Guru et al., 2015; Mahn et al., 2016). Further, there are more specific challenges associated with the use of chloride conducting opsins such as iC++ and SwiChR++ that I employed in this current work (Berndt et al., 2016). These two variants of ChR2 become robustly expressed across the cell, which can lead to opposing neuronal depolarization when light is delivered to axon terminals given that unequal concentrations of chloride ions are found between cell body and terminal regions of neurons (Mahn et al., 2016).

My experiments in Chapter 5 also investigated VP roles in 'liking' vs 'wanting' beyond general neuronal manipulations to determine more specific mechanisms related to cell identity. I used GAD1-Cre + transgenic rats to exclusively manipulate the activity of GABA neurons in VP and determine any rostro-caudal differentiation between 'liking' in 'wanting'. In those experiments, I replicated original hedonic hotspot studies in VP using microinjections and showed that VP^{GABA} neuron activity bidirectionally controls 'liking' vs 'wanting' for sweetness. While rostral activations suppressed 'liking', indicating a hedonic coldspot site, caudal VP^{GABA} stimulations doubled affective expressions to sucrose. One potential question that arises from

these studies is whether additional neuronal populations that exist in VP and do not overlap with GABA neurons play any roles in modulating hedonic impact. One possible contributor could be the relatively small number of glutamate neurons that are most robustly found in rostral regions of VP (Faget et al., 2018; Tooley et al., 2018). In mice, VP glutamate neurons have been implicated in aversive motivation (Faget et al., 2018; Prasad et al., 2019; Saga et al., 2016; Tooley et al., 2018; Wulff et al., 2019). Could they play a similar role in aversive motivation or 'disgust' in rats? This remains and open question, but is a conceivable hypothesis based on the current findings of this dissertation. One striking difference between our hSYN inhibition experiments and VP^{GABA} studies is that general inhibitions of caudal VP induced 'disgust' while VP^{GABA} neuron iC++ inhibitions only suppressed 'liking' but did not increase aversive responses. These disparities may reflect differences in levels of neuronal inhibitions produced by the viruses we used. For example, hSYN inhibition experiments primarily used SwiChR++ rather than iC++ (although we also included some iC++ rats) but pilot studies suggested more consistent increases in aversive reactions with the SwiChR++ virus. By comparison, all VP^{GABA} experiments used iC++ to inhibit neurons. However, another conceivable explanation is that VP^{GABA} and VP glutamate neurons are somehow both necessary for the generation of 'disgust', and future studies could help clarify this question by targeting those neuronal populations.

One particularly striking result from these studies is the strong motivational effect of rostral VP^{GABA} neuron excitations that produced 'wanting' what hurts in a subset of hedonic coldspot ChR2 rats. To my knowledge, this is the first time that VP has been shown to completely transform the incentive value of a noxious and painful target and turn it into an objective of desire that elicits appetitive approach. In most studies, the incentive and hedonic qualities of stimuli that are pleasant at baseline can be enhanced by certain VP manipulations.

Here we show that VP^{GABA} neuron activations can flip the valence of a painful shock from aversive to become an incentive. Maladaptive attractions for painful shocks were initially shown by Shelley Warlow and colleagues in the lab following paired central amygdala ChR2 activations (Warlow et al., 2020). What psychological mechanism is responsible for VP^{GABA} neuron mediated attractions? In the original CeA experiments, CeA ChR2 rats that developed shock rod attractions also developed attraction to cues that had been paired with CeA activations and shocks, suggesting that the cues themselves had been imbued with incentive salience (Warlow et al., 2020; Warlow & Berridge, 2021). Could a similar incentive mechanism be at play in VP? The picture is incomplete, but some of my pilot evidence suggest the answer may be yes. For example, VP rats that show shock rod attraction will voluntarily jump a barrier to interact with interact with the shock rod, and do not interact much with an object that resembles the rod in shape and size that doesn't deliver shocks. Our initial pilot studies showing included very few rats but these initial results are promising.

Second, VP-generated shock rod attraction is not static. When laser-shock pairings are placed on laser extinction, VP^{GABA} ChR2 rats no longer willingly subject themselves to shocks. This mirrors incentive salience processes, which are dynamic and can change based on environmental contexts, internal states, and other factors (Baumgartner et al., 2020; Faure et al., 2010; Reynolds & Berridge, 2008; Richard et al., 2013; Richard & Berridge, 2011a, 2011b; Warlow et al., 2020). Another key feature of incentive salience attribution is that reward related cues imbued with incentive salience become conditioned reinforcers and elicit approach in their own right (Flagel et al., 2009). Future studies could pair auditory tones or olfactory cues with laser-paired shocks to determine if these CS+ cues become conditioned reinforcers such as in the original CeA studies (Warlow et al., 2020). Overall, these experiments suggest that 'wanting'

and ‘liking’ dissociate from one another even in VP, which is crucial node for the control hedonic impact, and shock rod experiments serve as a proof of principle that VP neurons can generate incentive motivation for stimuli in the environment that were never ‘liked’.

Central amygdala mechanisms of incentive motivation

Previous studies in our lab have shown that central amygdala ChR2 activations cause rats to pursue sucrose, cocaine, and even painful shocks in an addictive -like manner (M. J. F. Robinson et al., 2014; Tom et al., 2018; Warlow et al., 2017a, 2020). Research in humans and rodents suggests that classes of drugs have distinct effects on brain reward systems, and are self-administered differently by individuals (Badiani et al., 2011, 2019; De Pirro et al., 2018; Ettenberg et al., 1982; Vassilev et al., 2020). Thus, in my final chapter I extended our knowledge of CeA mechanisms of incentive motivation by showing it can also focus motivation for intravenous opioids. In these experiments I showed that pairing CeA ChR2 activation with receipt of an intravenous infusion of the synthetic opioid remifentanil caused rats to selectively respond for the laser-paired remifentanil option and ignore an otherwise identical remifentanil reward that was never laser paired. Further, I trained a separate group of rats to choose between intravenous remifentanil or a natural sucrose reward. In these rats, pairing CeA ChR2 activations with remifentanil caused CeA rats to become ‘remifentanil-addicts’ who ignored sucrose, and conversely, pairing CeA photostimulation with sucrose caused different rats to become ‘sucrose addicts’ who ignored intravenous opioids.

One characteristic hallmark of addiction is that individuals become focused on the pursuit of drugs at the expense of other life rewards. Pairing CeA ChR2 excitations mirrored this phenomenon in rats. In ChR2 rats, subsequently activating CeA neurons did not significantly increase hedonic ‘liking’ reactions to sucrose during taste reactivity, suggesting that reward

'wanting', even to irrational levels produced by CeA activations were not matched by changes in 'liking'. Thus, CeA and related circuitry may play crucial role in focused and irrational pursuit of drugs rewards that occurs in addiction.

Clinical Implications- Incentive sensitization, addiction, and obesity.

The discussion of brain mechanisms of 'wanting' versus 'liking' may carry potential implications for psychiatric disorders including addiction, eating disorders, and obesity. In the past decade, a number of obesity investigators have applied the brain-based 'wanting/liking' distinction to suggest that in some vulnerable individuals, 'wanting' for foods might dissociate and exceed 'liking' to cause excessive cue-trigged 'wants' to overeat (Ahmed et al., 2016; Berthoud et al., 2011; Devoto et al., 2018; Ferrario, 2017; Gearhardt et al., 2014; M. J. F. Robinson et al., 2015, 2016; Stice & Yokum, 2016). The idea that some cases of extreme over-eating or binge-eating disorders can reflect excessive 'wanting', without excessive 'liking' invokes the incentive-sensitization theory of addiction, which was originally proposed for drug addiction but recently has been extended to behavioral addictions and to over-eating (Berridge & Robinson, 2016; T. E. Robinson & Berridge, 1993, 2003). Incentive-sensitization applied to eating disorders suggests that some individuals may be especially vulnerable to developing neural sensitization of dopamine-related mesocorticolimbic systems of 'wanting', and consequently assign the exaggerated incentive salience that results specifically to palatable foods and the act of eating them. The result would be excessive 'wanting' to eat, typically triggered by palatable food cues or by vivid imagery about such foods, which could become especially exacerbated in moments of stress or emotional arousal that heighten mesolimbic reactivity. Evidence supporting this incentive-sensitization interpretation of overeating comes particularly from neuroimaging studies of obese or binge-eating individuals that have reported a

sensitization-type brain activation signature to food cues that is remarkably similar to the signature of people who suffer from drug addiction to drug cues (Ahmed et al., 2016; Devoto et al., 2018; Gearhardt et al., 2014; Stice & Yokum, 2016).

A potential incentive-sensitization brain explanation for eating disorders is also relevant to debates about the concept of food addiction (Ahmed et al., 2016; Avena & Hoebel, 2003b, 2003a; Cameron et al., 2017; Carlier et al., 2015; Carter et al., 2016; C. Davis & Loxton, 2014; Ferrario, 2017; Fletcher & Kenny, 2018; Gearhardt et al., 2014; Rogers, 2017; Schulte et al., 2015; Volkow et al., 2017; Westwater et al., 2016; Wiss et al., 2018). That is, a legitimate 'food addiction' might exist to the degree that some over-eaters truly show incentive-sensitization signatures of brain activation to foods, in the sense that those food-sensitized individuals may experience more intense cue-triggered food cravings than other people do. The ideal brain signature for an eating addiction in the sense of incentive-sensitization would be mesocorticolimbic hyper-reactivity in nucleus accumbens or striatum, ventral tegmentum, amygdala or limbic cortical regions in over-eaters that is triggered by food cues. An incentive-sensitization signature would be hyper-reactive in both of two ways: 1) more intense brain activations triggered by food cues than by money or other reward cues in the same over-eating individual, and 2) more intense brain activations triggered by food cues in sensitized over-eaters than triggered by the same food cues in nonsensitized normal eaters.

Extreme incentive salience attributed to foods is in one sense a natural phenomenon that nearly anyone could experience – at least, under extreme conditions of prolonged starvation, but which most people in the modern world fortunately never experience. For example, during World War 2 a controlled Minnesota study of starvation was carried out using conscientious objectors as volunteers of starvation to better understand starvation consequences and treatments

(Keys et al., 1950). Gradually the volunteers began to be gripped by intense food cravings as they became extremely underweight: “Some of them (volunteers) obsessively read cookbooks, staring at pictures of food with almost pornographic interest” (Keys et al., 1950). Despite being highly motivated, a number of volunteers could not resist succumbing to temptations to eat, and left the study. Thus, anyone can feel strong urges to eat during extreme physiological starvation that become nearly compulsive. What may be different in sensitized over-eaters is that similarly intense incentive salience is attributed to food cues, due to sensitized hyper-reactivity of mesocorticolimbic ‘wanting’ systems in some vulnerable individuals, even without ever being starved and despite developing obesity.

Some evidence for incentive sensitization in over-eating has come from reports that obesity and binge eating disorder is associated with heightened BOLD signals in ventral striatum, prefrontal cortex, and OFC in response to visual cues of palatable foods compared to individuals without obesity (Geliebter et al., 2006; Karhunen et al., 2000; Schienle et al., 2009). Similarly, individuals with obesity have been reported to have elevated brain responses in striatum, amygdala and orbitofrontal cortex to images of high calorie foods compared to foods low in calories or control images (Bruce et al., 2010; Dimitropoulos et al., 2012; Frankort et al., 2012; Holsen et al., 2012; Martin et al., 2010; Rothemund et al., 2007; Stice et al., 2010; Stoeckel et al., 2008). Using PET, one study reported elevation in striatal dopamine release in binge-eating individuals (compared to non-binge eating individuals) when they were given oral methylphenidate, which may pharmacologically prime mesolimbic dopamine reactivity, and their higher dopamine response was positively correlated with binge eating scores (G. J. Wang et al., 2011). Heightened brain activity to palatable foods also positively correlates with self-reported subjective cravings or ‘wanting’ to eat (Simon et al., 2016), and individuals with binge

eating are reported to have greater EEG reactivity in response to palatable chocolate pictures and increased craving ratings compared to healthy controls (Wolz et al., 2017). Elevated brain responses to food in individuals with obesity may also be associated with poorer outcomes to behavioral weight loss treatments (Murdaugh et al., 2012). Evidence suggests that enhanced brain limbic activity is selective to food rewards in over-eaters, as some studies have not observed increased brain activity to monetary rewards in individuals with binge-eating disorder (Balodis et al., 2013; Simon et al., 2016).

Neural patterns of brain activity that are predicted by incentive sensitization theories have also been recently shown in humans who suffer from heroin use disorder (Huang et al., 2024). Huang and colleagues used fMRI to directly compare brain responses to drug vs. neutral cues in heroin users relative to drug-free individuals. They report within subjects and between subjects differences in corticolimbic reactivity to drug cues in heroin users. Relative to drug-free controls, heroin users had higher limbic activations to presentations of drug-related cues, which supports the hypothesis that for certain individuals who suffer from addiction, incentive salience of drug and drug related cues become magnified. Further, limbic reactivity in heroin users was also higher to presentations for drug-related cues versus food cues or neutral cues in the same individuals (Huang et al., 2024). Overall, these studies suggest that individuals who suffer from eating disorders, addiction, and obesity may show incentive sensitization-like features in mesolimbic brain structures to food and food-associated cues, which could produce more intense cue-triggered ‘wanting’ to eat, even if not be matched by more intense ‘liking’ (Ahmed et al., 2016; Berthoud et al., 2011; Devoto et al., 2018; Ferrario, 2017; Gearhardt et al., 2014; M. J. F. Robinson et al., 2015, 2016; Stice & Yokum, 2016).

The evidence seems to point that sensitized and maladaptive 'wanting' is key feature of psychiatric disorders such as obesity, addiction, and eating disorders (Berridge, 2009; Berridge & Robinson, 2011, 2016; Morales & Berridge, 2020; T. E. Robinson & Berridge, 1993, 2000). Anecdotal evidence from drug users also supports this hypothesis. As individuals transition to addiction, they experience irresistible cravings for drugs that often become impossible to ignore, yet when they take drugs, the pleasure experienced seems diminished. So, does that mean that hedonic processes are not involved at all? This idea has been proposed by Ivan de Araujo, Mark, Schatzker and Dana Small (de Araujo et al., 2020). De Araujo et al. argue that less reliant are the hedonic properties of foods like flavor, taste, and aroma in their ability to generate excessive overeating (de Araujo et al., 2020). They note that vagal sensory projections from the viscera to the hindbrain sensory nucleus of the solitary tract carry signals about caloric content arising from food digestion, and show vagal signals may trigger dopamine release from substantia nigra axons in the dorsal neostriatum (de Araujo et al., 2020). Strikingly, direct optogenetic stimulation of vagal-to-medulla projections supports laser self-stimulation, which they suggest reveals a response-reinforcing signal (W. Han et al., 2018). Nutrient conditioning of flavor preferences similarly relies on intact dopamine signaling in the dorsal striatum (Tellez et al., 2016; L. Zhang et al., 2018). The vagal-neostriatal dopamine reinforcement signal, De Araujo et al. suggest, does not enhance food hedonic palatability but rather strengthens behavior more directly, similar to traditional stimulus-response (S-R) habit stamping-in theories. As de Araujo et al. put it "In other words, reinforcement and habit acquisition can occur seamlessly in the absence of any consciousness-borne flavor appreciation." (p. 153, [288]).

The hypothesis of de Araujo et al. that vagal nutrient signals act in neostriatum without any "consciousness-borne flavor appreciation" is consistent with our view that neostriatal

dopamine fails to enhance 'liking'. The hypothesis that vagal signals promote learned attraction to foods is also consistent, as de Araujo et al. point out, with many earlier demonstrations by Anthony Sclafani, Kevin Myers and colleagues that intra-gastric calories are able to act as a UCS to establish a conditioned preference for a paired CS flavor in rats, increasing 'wanting' to eat that food whether or not it also increases 'liking' for the more 'wanted' CS flavor (Myers & Sclafani, 2001, 2003; Sclafani, 2001, 2018; Tellez et al., 2016). For example, nutrient conditioning can enhance 'wanting' without enhancing 'liking' reactions for a bitter/sour CS+ flavor (Myers & Sclafani, 2003), although it can enhance both 'wanting' and 'liking' together if the CS+ flavor was initially sweet or palatable (Myers & Sclafani, 2001). Thus, enhanced 'liking' is a possible accompaniment but not an obligatory component of nutrient conditioned taste preferences.

Based on all this, we would suggest a possible alternative interpretation to S-R habit reinforcement for the role of vagal-evoked dopamine in neostriatum. That is, given that dopamine in dorsal neostriatum can enhance the incentive salience of specific food cues (DiFeliceantonio & Berridge, 2016), vagal-evoked dopamine release in dorsal neostriatum might similarly promote 'wanting' to eat evoked by particular food cues associated with vagal stimulation. This would be an incentive motivation mechanism, probably maximally triggered by particular foods that are both caloric and palatable, rather than a behaviorist response stamping-in mechanism, and would not be confined to habits but could promote eating even if food seeking required novel responses or if the food cues moved to new settings. But this does not mean that hedonic processes do not play a role. While 'wanting' may much more largely contribute to maladaptive motivations, it's important to state that 'liking' may still play a smaller role. People don't overcome disgusting foods. In fact, highly palatable foods become targets

sought out with compulsive motivations, suggesting that whole incentive sensitization may more robustly contribute to overeating or maladaptive drug taking, some hedonic contributions may also be needed.

So what is the function of 'liking' in reward? From an evolutionary standpoint, robust affective responses could serve as early signals from the brain that we've encountered something in the environment potentially important for our survival and well-being. These affective responses could enable a cascade of incentive and learning signals that will ultimately continue to guide us towards these rewards in the future. A similar idea has been proposed by Peter Dayan, who notes that 'liking' processes may offer immediate and editable predictions about the long-term worth of stimuli in our environments (Dayan, 2022). Over time, environmental contexts, learning, and potentially noxious experiences that lead to malaise can ultimately override these initial hedonic calculations. This is consistent with the idea that incentive motivation and hedonic processes are not static across time. 'Liking' and 'wanting' change with experience, internal states, environmental conditions, and emotional reactivity. For example, conditioned taste aversions are clear examples of how normally palatable foods may not remain 'liked' if they've led to sickness and malaise. 'Liking' can also be magnified by hunger and satiety states through a phenomenon known as alliesthesia (Cabanac, 1971, 1979). This is why foods become more pleasant when we're hungry, and less appealing when we're full (Berridge, 1991; Cabanac, 1971, 1979; Cabanac & Lafrance, 1990). Thus, hedonic hotspot may be brain sites of alliesthesia modulation, that increase the hedonic impact of foods when we're hungry, and potentially this is dampened by increased coldspot activity as we reach satiety. Alliesthesia processes may require important interactions between 'wanting' and 'liking' systems with hypothalamic circuitry to promote appetite and motivation, and understanding how these systems

interact is an enduring but exciting quest with the potential to help us better understand how the brain regulates emotion and motivation.

Conclusions

Mesocorticolimbic systems that control 'liking' and 'wanting' for reward are dissociable. Small hedonic hotspots are localized subregions of nucleus accumbens medial shell, caudal ventral pallidum, rostromedial orbitofrontal cortex, caudal insula, and mid cingulate cortex where various forms of neuronal manipulations can causally amplify 'liking' reactions to pleasant stimuli. The present dissertation used optogenetic techniques as an alternative method to drug microinjections to probe the robustness of hedonic hotspot control of 'liking' reactions. We report that 'liking' reactions can be effectively amplified via ChR2 stimulations in rostromedial OFC, caudal insula, cingulate cortex, and rostral ventral pallidum similar to previous microinjection studies, suggesting that hotspot sites are robust neurofunctional entities of exerting hedonic control. The same structures that contain hotspots often also contain separable hedonic Coldspots where the same optogenetic stimulations suppress 'liking'. These hotspots are nestled within larger mesocorticostriatal 'wanting' circuitry, such as in central amygdala, where many forms of stimulation robustly generate intense cue-triggered incentive salience, amplifying motivation to seek and consume natural and drug rewards, whether or not 'liking' is simultaneously enhanced.

The distinguishable identities of brain systems for 'liking' versus 'wanting' has implications for understanding hedonic dysfunctions in affective disorders, at least some cases of human obesity, binge eating, and addiction. Future research in this area will continue to extend understanding of how mesocorticolimbic systems differentially control 'liking' vs. 'wanting' and how specific dysregulations may contribute to various neuropsychiatric disorders.

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