Optogenetic Insights into Central Amygdala Motivational Mechanisms

by

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DEDICATION

In loving memory of Elizabeth Halfacre and Jeremy Clark.

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ABSTRACT

A characteristic hallmark of addiction is the focused pursuit of drugs at the expense of other life rewards. Focusing motivation towards the appropriate target and at appropriate times is an adaptive strategy, and helpful in directing motivation towards targets such as food or sex through forming of associations with neutral environmental stimuli. However, this strategy can be hijacked by drugs of abuse to cause pathological pursuit of drugs and their related cues rather than normal life rewards in an intense fashion. Amygdala circuitry may be a crucial mechanism by which focused motivation for normal life rewards can become hijacked by drugs of abuse to create excessive motivation focused on pursuing drugs of abuse. The experiments described here used optogenetic techniques to dissect the psychological and neurobiological mechanisms by which amygdala circuitry generates incentive motivation focused onto particular targets.

We first paired optogenetic CeA excitation with earning a particular sugar reward, when rats were choosing between that and an identical sugar reward lacking CeA excitation. CeA excitation made its paired reward the sole target of pursuit - both narrowing and enhancing motivation for that reward, even though both rewards available were identical. Similarly, CeA excitation paired with a cocaine reward made that particular cocaine infusion the sole target of motivation compared to an identical cocaine infusion available, which lacked CeA excitation. In both cases, CeA excitation was not reinforcing alone, indicating that CeA excitation was directly transforming the brain's representation of its associated reward (sucrose or cocaine) to make it more 'wanted'. Using taste reactivity techniques, we show that CeA excitation does not alter hedonic 'liking' of sucrose, and thus is not the reason rats 'want' rewards paired with CeA excitation (they do not 'like' it more).

Further, we demonstrate that we can gain control of which reward gets 'wanted' most by pairing CeA excitation arbitrarily with either sucrose or cocaine, when rats are choosing between the two different rewards. Finally, pasting CeA excitation onto a known aversive target enhances attraction and investigation of that stimulus while also reducing defensive behaviors.

In all situations, CeA excitation being temporally pasted onto a motivationally relevant stimulus, makes that stimulus the sole object of desire. Evidence within each experiment supports the notion that CeA excitation is likely doing so by enhancing the attribution of incentive salience to those stimuli and their related cues. These findings demonstrate that a hijacked CeA circuitry is sufficient to control reward 'wanting', even to irrational and dangerous levels, highlighting a potentially crucial role for amygdala-related circuitry in focused and irrational pursuit occurring in addiction.

CHAPTER I. Introduction

Escalated motivation focused exclusively on pursuing drugs while ignoring other life rewards is a characteristic hallmark of addiction. While directing motivation towards appropriate targets at particular times is adaptive for seeking out rewards crucial to survival, this strategy can be hijacked by drugs of abuse to produce pathological motivation irrationally focused on pursuing drugs at the expense of normal life rewards. A major question in addiction neuroscience is how the brain generates such focused motivation for a particular 'drug of choice', making that drug and its cues a motivational magnet and the sole target of pursuit.

To first answer this question, it is important to understand how brain circuitry generates focused pursuit of natural rewards in an appropriate manner. Substantial research has demonstrated that amygdala circuitry is crucial for integrating previously learned associations with in-the-moment motivation to guide behavior appropriately. However, previous work has relied on enhancement of motivation towards an already preferred target based on Pavlovian associations. To better understand how the brain *creates* focused motivation for rewards, the experiments described here sought to gain control of the focus of motivation from the beginning, while learning was taking place.

This dissertation will explore the possibility that amygdala circuitry plays a crucial role in such focused pursuit seen in addiction by making motivationally significant stimuli attractive. Further, it explores the psychological mechanisms by which amygdala circuitry acts to promote excessive desire, whether acting to enhance pleasure 'liking' of rewards or reward 'wanting'.

Psychological components of reward

The brain is responsible for generating motivation for rewards, such as food, sex, and social relationships. This motivation for reward can be further parsed into several different psychological components, that each serve their own independent function (Berridge and Robinson, 2003). Specifically, a reward is usually both liked and wanted, and the value associated with that reward elicits learning to help guide future behavior. In most situations, liking, wanting, and learning seamlessly function together to influence motivation. For example, when one thinks about what they want to eat for dinner, a number of pleasurable meals that have

been eaten in the past are instantly recalled. In this way, 'liking' and 'wanting' naturally act in tandem to generate motivation to seek out and obtain the rewards which produce pleasure. However, each of these functions is attributed to distinct but overlapping brain mechanisms. Separate brain systems means that in certain situations, these components can diverge. Whereas people typically 'want' what we 'like' and 'like' what we 'want', a person can at times develop excessive motivation for rewards known to evoke only moderate amounts of expected pleasure, or even generate excessive craving for rewards which have in the past been repeatedly paired with unpleasant consequences. Such is the case in the transition from recreational drug use to addiction.

Both 'wanting' and 'liking' do not necessarily have to be consciously experienced to influence behavior. For instance, subliminally presented pictures of either happy or angry faces can influence incentive motivation for drinking a beverage without those thirsty participants reporting any changes in conscious feelings (Winkielman et al., 2005). Even with drug rewards, wanting and liking can occur below consciousness. For example, recovering cocaine addicts will consistently choose a very low dose of cocaine over an injection of saline, despite reporting no more subjective feelings of pleasure than with saline, no cardiovascular responses and indicating that they thought they were sampling both options equally (Fischman and Foltin, 1992). Furthermore, presenting cocaine addicts with brief (33 millisecond) images of drug-related stimuli (e.g., a pipe) or sexual stimuli, that are masked by the longer presentation of another subsequent image to prevent conscious perception, activates similar reward brain circuitry and causes an enhancement of that brain's reactivity to a later, consciously-seen drug stimulus (Childress et al., 2008). Thus, drug craving ('wanting'), can occur completely unconsciously, and as such we refer to 'wanting' as incentive salience and 'liking' as hedonic impact in quotations to distinguish those objective processes, which can occur either unconsciously or consciously, from subjective feelings of wanting and liking that are necessarily conscious.

Pleasure 'Liking' as a Separate Psychological Component

Pleasure is more than a property of a physical reward stimulus; it is actively generated by the brain as one of the components of an experienced reward (Berridge, 2009; Dai et al., 2010; Litt et al., 2010). 'Liking' refers to the objective hedonic impact, measurable in affective reactions, derived from a pleasant reward. Although rewards such as food, drink or sex comprise multiple sensory properties that elicit pleasurable reactions, 'liking' is a distinct psychological

component that goes beyond the mere sensory qualities of a reward. The sensory properties of a reward such as the sweetness of ice cream remain constant, yet the pleasurable sensation associated with the reward itself can be dramatically eliminated if it was previously paired with the nausea of visceral sickness (Garcia et al., 1985; Rozin, 2000; Berridge et al., 2010). Conversely, the bitter taste of beer or coffee can become not only desired but also positively enjoyed for many people, when repeatedly paired with the pharmacological properties of alcohol and caffeine. Furthermore, sudden changes in internal physiological state can produce a dynamic shift in hedonic tone known as "alliesthesia" (Cabanac, 1971). And whereas hunger can make foods more 'liked' (Cabanac, 1971; Cabanac and Lafrance, 1990; Kaplan et al., 2000), satiation can dampen the pleasure elicited by chocolate, even in self-proclaimed "chocoholics" (Small et al., 2001; Lemmens et al., 2009).

'Liking' as an affective response to hedonic stimuli can be measured in behavior and physiology even in the absence of subjective liking. Orofacial hedonic reactions to sweet versus bitter tastes were first measured in human infants by Jacob Steiner (Steiner, 1973), and subsequently extended to rats in the taste reactivity test (Grill and Norgren, 1978), which measures orofacial reactions elicited in response to different tastes. These include objective patterns of hedonic reactions such as lip licking and rhythmic tongue protrusions in response to 'liked' tastes such as sweet sugars, and negative gapes and headshakes in response to 'disliked' tastes such as bitter quinine. These reactions are highly conserved and homologous across species including humans, rats and apes (Steiner et al., 2001; Berridge and Kringelbach, 2008). Importantly, these hedonic orofacial reactions to pleasantness are separable from just sweet or bitter sensation, and track the hedonic impact of the taste rather than its sensory properties. For example, a once 'liked' sweet taste that elicits tongue protrusion 'liking' reactions, can become 'disliked', causing gaping 'disliking' reactions when paired with sickness (Delamater and McNamara, 1986; Parker, 2014; Itoga et al., 2016). Orofacial affective reactions also may provide an accurate measure of hedonic impact that are less influenced by one's cognitive framework, desire to be consistent in ratings, or previous hedonic framing experiences, than are subjective ratings (Bartoshuk, 2014; Berridge and Kringelbach, 2015). For example, subjective ratings of pleasure and pain don't take into account differences in sensation between individuals, as prior experience with extreme pains such as traumatic injuries can influence the intensity of perceived pleasure or pain to a later stimulus (Bartoshuk, 2014). By contrast, those who are

relatively inexperienced with extreme pains may experience pain more strongly. On the other hand, a limiting feature of orofacial affective reactions is that the measure is limited to taste pleasures. However, evidence suggests there is extensive overlap in brain circuitry responsive to different types of pleasures (i.e, food, sex, or music pleasures), opening a possibility that orofacial affective reactions to taste pleasure can be used as a means of providing insight into brain mechanisms of pleasure as a whole (Salimpoor et al., 2011; Xu et al., 2011; Cacioppo et al., 2012; Georgiadis and Kringelbach, 2012).

Neuroanatomy of 'Liking': Hedonic Hotspots

Where in the brain is pleasure generated? Using the taste reactivity test that measures changes in hedonic orofacial reactions in response to passively infused tastes, researchers in our laboratory have identified small cubic millimeter sized zones in which neurochemicals such as opioids, endocannabinoids, and orexin, but not dopamine, can enhance pleasure. These small zones, located in distinct sites within larger brain structures, are referred to as hedonic hotspots because of their unique capacity to cause hedonic enhancements of sweetness pleasure (Berridge and Robinson, 2003; Smith and Berridge, 2007). Thus far, identified hedonic hotspots include zones within the rostral portion of the medial shell of nucleus accumbens (Peciña and Berridge, 2000, 2005; Castro and Berridge, 2014; Castro et al., 2016), the ventral pallidum (whose hotspot is crucial for 'liking') (Cromwell and Berridge, 1993; Smith and Berridge, 2005; Ho and Berridge, 2013), cortical regions such as orbitofrontal cortex and insula (Georgiadis and Kringelbach, 2012; Castro and Berridge, 2017), and parabrachial nucleus of the brainstem (Söderpalm and Berridge, 2000). These hotspots seem to function as a cooperative network that requires a unanimous vote to engender a 'liking' response. While stimulation of just one hotspot will typically recruit others, pharmacologically inhibiting activity in one hot spot will prevent an enhancement of 'liking' from opioid stimulation in one of the other hotspots (Smith and Berridge, 2007; Castro and Berridge, 2017).

'Wanting' and the attribution of incentive salience

While 'liking' refers to the pleasure derived from rewards, 'wanting' refers to incentive salience, a specific motivation process underlying the desire to obtain and seek out those rewards. This motivational desire given to a reward can be conferred to learned cues and objects associated with that reward (Bindra, 1978), transforming them into 'wanted' incentives. Reward-related cues have the powerful ability to trigger bursts of motivation and reward-seeking,

mediated by mesolimbic circuitry involving dopamine and other neurotransmitters in Nucleus Accumbens (NAc) (Holmes et al., 2010; Peciña and Berridge, 2013). For example, the enticing smell of freshly baked cookies or the aroma of freshly brewed coffee can elicit consumption even in the absence of any need. However, in cases of pathological motivation, such as addiction, cues can become powerful enough to trigger intense cravings for rewards that may not even be consciously wanted or may have adverse consequences. Even an addict who has been able to abstain for many years may encounter a drug-related cue such as a particular location, drug paraphernalia, or smell the odor of an alcoholic drink, which then causes intense cravings that become hard to ignore, possibly resulting in relapse.

Through repeated pairings with a particular reward, cues become imbued with incentive salience, making them attractive targets of attention and desire (Hickey and Peelen, 2015). 'Wanted' reward-related cues are able to attract approach and invigorate actions. Experimentally, the attribution of incentive salience to cues can be measured using a variety of tests. Pavlovian sign-tracking or autoshaping, assesses how attractive the cue has become by examining whether an animal will sniff, nibble or even bite inedible objects such as a protruding metal lever because it has been previously paired with a reward (Brown and Jenkins, 1968; Uslaner et al., 2006; DiFeliceantonio and Berridge, 2012; Mahler and Berridge, 2012). Whether the cue has become a valued and desired object on its own is measured using the conditioned reinforcement test (Robbins, 1976), and whether presence of the reward-paired cue has the ability to invigorate seeking of the reward itself can be established using Pavlovian-to-Instrumental Transfer (PIT), a measure of cue-triggered bursts of 'wanting' (Zhou et al., 2012; Peciña and Berridge, 2013; Ostlund et al., 2014). Importantly, these tests can be used in affective neuroscience studies to provide insight into the brain mechanisms involved in generating 'wanting' for either rewards themselves or for reward-paired cues.

The ability of reward-related cues to invigorate 'wanting' is dependent on two major components, the reward cue's predictive and incentive value. The predictive value of a cue is dependent on how well it predicts the presence of reward, yet this does not necessarily imbue the cue with incentive value (Robinson and Flagel, 2009; Berridge, 2012; Anselme and Robinson, 2013). Only when the cue also carries incentive value does it become powerfully able to motivate reward seeking. The attribution of incentive value to a cue by an individual can be measured using autoshaping. Two broad behavioral phenotypes emerge through training. Sign-

trackers assign incentive value to the reward predictive cue, a lever, and are thus attracted to and engage with the cue. In contrast, goal-trackers assign only predictive value to the same cue, and instead use it as a signal to approach the location where the reward will be delivered (Boakes et al., 1978; Flagel et al., 2009; Flagel and Robinson, 2017). This distinction between predictive and incentive value of reward cues can be further exemplified by modulating the strength of the predictive value. For example, reward uncertainty, in which the cue predicts delivery of the reward only 50% of the time, degrades a cue's predictive value, and yet increases the amount to which that cue is attributed with incentive value (Anselme and Robinson, 2013; Robinson et al., 2015). Similarly, incentive value of a cue can persist even when the cue's predictive value has declined by changing reward contingencies to omit the delivery of rewards when the lever is pressed. Evidence shows that although rats learn to stop pressing the lever (the cue in this situation), they continue to show appetitive approach behaviors toward that lever, at the same rate as another group who never experienced the change in predictive value. This would indicate that the cue (the lever) has acquired incentive value, able to motivate actions such as sniffing and biting, which persist beyond the cue's predictive ability (Chang and Smith, 2016). As such, there is a clear distinction between predictive and incentive value that a cue acquires, with the latter being most important for intensifying 'wanting'. This is of particular interest to addiction, since the incentive value attributed to a cue can determine its ability to trigger bouts of craving and drug-seeking.

However, it is important to note that the intensity of 'wanting' triggered by a predictive cue not only depends on the cue's incentive value, but also the current dopamine-related brain state of the individual (Zhang et al., 2009; Berridge and Robinson, 2016). The motivation triggered by a reward-related cue can be exponentially increased in the moment by current brain dopamine levels. Physiological states such as stress, relevant appetites, intoxication or excitement (Robinson and Berridge, 2013; Sinha, 2013; Anselme, 2016; Preston et al., 2018) which heighten the dopaminergic brain reactivity state, can combine with the cue's presentation to raise the intensity of triggered bursts of 'wanting'. This mirrors real-world situations of addictive relapse, where an abstinent addict can successfully resist a cue multiple times without succumbing to relapse, but subsequently upon a single presentation of that cue under conditions of higher stress or excitement, can suddenly heighten the incentive value and drug 'wanting' triggered by that cue to a point where temptation is overwhelming and results in relapse. Thus

incentive salience is thought to integrate two separate factors - current neurobiological state plus a cue's incentive value - which are integrated together to determine the level of 'wanting' triggered at that moment by the cue (Berridge, 2012).

Brain generators of 'wanting'

'Wanting' generators in the brain are much more robust and diffuse than those brain mechanisms generating pleasure 'liking'. 'Wanting' includes dopaminergic (and opioid, glutamatergic and other) systems across mesocorticolimbic structures. Dopamine neurons residing in the midbrain ventral tegmental area send projections and release dopamine in limbic structures such as the nucleus accumbens and prefrontal cortex, interacting with other structures such as the amygdala, ventral pallidum, and lateral hypothalamus to enhance motivation for rewards (both natural and drug rewards) and reward-paired cues (Cameron et al., 2014; Castro et al., 2015). In laboratory experiments, stimulations of these structures (for example, by infusing an opioid or dopamine agonist, or by optogenetic stimulation of neurons) can increase 'wanting' to consume rewards, as well as enhance the focus of cue-triggered reward seeking and approach of reward cues (Smith and Berridge, 2005; Smith et al., 2011; DiFeliceantonio and Berridge, 2012; Mahler and Berridge, 2012; Peciña and Berridge, 2013; Castro and Berridge, 2014). In contrast, drugs that block dopamine transmission, such as the dopamine antagonist pimozide, or treatments (i.e., 6-OHDA) that destroy over 99 % of mesolimbic and neostriatal dopamine afferents disrupt 'wanting', in that the animals lack the motivation to feed themselves and display life-threatening aphagia and adipsia (Berridge et al., 1989). In humans, drugs that block dopamine function completely fail to reduce the subjective ratings of pleasure people give to an addictive drug, such as amphetamine, cocaine or methamphetamine, yet diminish craving to take more drug (Brauer and De Wit, 1997; Wachtel et al., 2002; Leyton et al., 2007), and diminish cue-induced craving (Berger et al., 1996). Similarly, studies in which dopamine transmission was decreased by interfering with dopamine synthesis (acute phenylalanine/tyrosine depletion; APTD) show that the subjective pleasure ratings and mood altering effects of a wide range of abused substances, such as alcohol (Leyton et al., 2000; Barrett et al., 2008), tobacco (Casey et al., 2006; Munafò et al., 2007), amphetamine (Leyton et al., 2007), and cocaine (Leyton et al., 2005), remain intact, while subjective ratings of drug 'wanting' and cocaine-induced confidence are reduced (Leyton et al., 2005).

Natural rewards such as food, water, and sex all generate pleasure, while also triggering the release of mesolimbic dopamine and activating our 'wanting' system (Hernandez and Hoebel, 1988; Pfaus et al., 1990). In drug, food, and gambling addictions, we see evidence of hypersensitive 'wanting' systems taking incentive salience attribution to maladaptive levels, often with very little change in pleasure responding (Robinson and Berridge, 2008; Rømer Thomsen et al., 2014; Robinson et al., 2015).

Sensitized 'wanting' in Incentive Sensitization Theory

While mesocorticolimbic brain structures evolved to generate 'wanting' for natural rewards crucial to survival, they are especially heavily activated by modern drugs of abuse, hyperpalatable foods, and other artificial rewards such as gambling. Activation of 'wanting' circuitry by these rewarding stimuli or incentive cues related to them, causes surges of dopamine to be released and hyper-reactivity to those cues in target structures. Over time, drugs can induce particular neural changes called sensitization in mesolimbic circuitry, which once formed may be extremely long lasting. Sensitization causes mesocorticolimbic activation to become increasingly responsive to those particular rewards and their related cues, such that higher levels of dopamine are released and higher neural responses are evoked in target structures when cues are encountered. This is especially true in a subset of individuals partaking in particular binge/purge patterns of drug use, who are particularly vulnerable to sensitization due to their genes, steroid hormones, previous stress experiences, etc (Piazza et al., 1990; Rougé-Pont et al., 1993; Kawa et al., 2016). In sensitized individuals, presentation of the reward or reward-related cue causes an enhanced release of dopamine among mesocorticolimbic brain structures responsible for generating reward 'wanting'. Further, the magnitude of incentive salience evoked by a cue can be augmented even further by current states of intoxication or stress (Berridge, 2012). Thus, being primed with drug consumption, stress or emotional excitement states, and then encountering incentive cues can cause intense cravings for the drug and heightened motivation to seek out that particular drug. In addiction, this could mean that temptation for the drug becomes overwhelming enough to cause relapse.

According to the Incentive Sensitization theory of addiction first proposed by Robinson and Berridge (Robinson and Berridge, 1993), drug-induced sensitization causes brain mesolimbic structures to become hyper-reactive to the addict's drug of choice and its cues. This hyper-reactivity caused by drugs or drug-paired cues triggers an increase in cravings for that

particular drug of abuse, and results in patterns of excessive drug use. In individuals recovering from drug abuse, these cravings can become so overwhelmingly tempting (especially in times of stress or excitement) as to result in relapse.

Over the years there has been a substantial amount of evidence accumulated to support this theory. For example, drugs of abuse cause anatomical and morphological sensitization (i.e., increased dendritic spines and amount of dendrites capable of responding to drug) in mesolimbic brain structures, and this morphological sensitization can result in behavioral sensitization to that drug (Robinson and Kolb, 2004; Singer et al., 2009; Wolf, 2010; Steketee and Kalivas, 2011). Furthermore, history and pattern of drug use play a key role in sensitized dopaminergic response to drugs of abuse or their paired cues (Robinson and Becker, 1986). For example, sensitization especially occurs in cases of drug binges and patterns of intermittency (Robinson and Becker, 1986; Kalivas and Stewart, 1991; Kawa et al., 2016). As a result, addicts show heightened mesolimbic activation in response to drugs (Boileau et al., 2007; Cox et al., 2009).

Not only does a sensitized mesolimbic structure become hyper-reactive to the drug of choice itself, but it also becomes hyper-reactive to drug-related cues and contexts that have been paired with drug-taking. For example, a heightened brain response in limbic circuitry is caused by reward-related cues after sensitization (Tindell et al., 2005) and drug paraphernalia in human addicts (Cox et al., 2009; Vezina and Leyton, 2009; Kühn and Gallinat, 2011; Leyton and Vezina, 2013) . Furthermore, cue reactivity in mesolimbic ventral striatum correlates with years of cocaine use such that the more years of use, the greater the brain activation (Prisciandaro et al., 2014). Additionally, time-dependent increases in cue-induced craving have been observed in methamphetamine addicts (Wang et al., 2013) as well as in alcoholics (Li et al., 2015a), and these time-dependent increases in cue-induced craving (also referred to as incubation) are dependent on mesolimbic structures such as the central amygdala (Lu et al., 2005, 2007; Li et al., 2015b) and nucleus accumbens (Xi et al., 2013). A study of recovering cocaine addicts showed that those who reported sensitization of other drug-related effects, such as paranoid psychosis, were also more likely to relapse (Bartlett et al., 1997). This suggests that sensitization of drug effects is linked to the risk of relapse, even when sensitization simultaneously increases the intensity and occurrence of adverse effects of the drug, such as drug-induced paranoia. Combined, this evidence suggests a possible reason for how drug craving increases over time to

a point where an addict's drug of choice is almost impossible to ignore, and where motivation to obtain that reward, even after withdrawal, is so high that relapse becomes a recurrent problem.

Sensitized 'wanting' is also focused temporally to particular moments in time. Rather than creating an overall hyperactive dopaminergic state, implying that those brain areas are always driving intense 'wanting', incentive sensitization creates a hyper*re*active response to particular stimuli, causing heightened activity in response to drugs or drug-related cues, but not at other times. Incentive sensitization theory posits that it is the hyper*re*activity to drugs and their cues that triggers intense motivation and cravings leading to relapse - not any baseline hyperactivity or constant drive in these brain mesolimbic structures.

Focusing of Drug 'Wanting'

However, if sensitization appears to promote dopaminergic activity, then why aren't addicts addicted to all drugs of abuse, even across reward-types such as gambling, food, or sex? Indeed, a degree of comorbidity does exist between various addictions, where human drug addicts 'want' several different drugs, may be hypersexual, and are prone to other compulsions (Washton and Stone-Washton, 1993; Benotsch et al., 1999; Leeman and Potenza, 2012). However, incentive-sensitization may be quite focused on one particular reward target, and need not generalize to other rewards. Evidence from our lab shows that the narrowed focusing of 'wanting' to one particular target in addiction may involve dopamine-circuitry interactions with the amygdala (Koob and Volkow, 2010; DiFeliceantonio and Berridge, 2012; Mahler and Berridge, 2012; Venniro et al., 2016). Amygdala focusing of incentive salience on particular learned targets results in a narrow focus of reward seeking on to one particular reward.

In human addicts, the amygdala consistently shows heightened activity in response to cues related to their drug of choice, but not to other drug or sexual cues, and these cues lead to self-reports of craving for their preferred drug of choice (Childress et al., 2008; Kühn and Gallinat, 2011). In rats, mu-opioid stimulation of central amygdala enhances appetitive behaviors towards a prepotent cue (either towards the food cup in goal trackers or the lever in sign trackers) (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012). Similarly, the same stimulation temporally focuses instrumental sucrose seeking only during periods of time when a Pavlovian cue is present and suppresses seeking during periods when the cue is not present (Mahler and Berridge, 2012). This work has suggested that central amygdala not only magnifies 'wanting', but also more narrowly focuses it onto a prepotent target. Though this has provided

some clue as to how the brain appropriately focuses motivation onto a particular target at a particular time, these manipulations were made after the learning had taken place and after a preferred target had been created. In order to gain better insight into how the brain creates the target of motivation, it is crucial to be able to control the narrowing of focus. The temporally precise features of optogenetics allow us the opportunity to co-opt amygdala circuitry to control the directional target of motivation.

Amygdala anatomy: Subdivisions and circuitry

Although initially described as a solitary structure, the amygdala is composed of functionally and morphologically heterogeneous subnuclei displaying complex interconnectivity (Pitkänen et al., 2000; Kim et al., 2017). Two of the major subdivisions of amygdala are the basolateral (BLA) and central nucleus (CeA). Within a macrosystem framework of the brain, the BLA and CeA are positioned at different levels within cortico-striatal-pallidal networks (Swanson, 2003). Several distinctions between these two nuclei have led to the proposition that BLA should be considered similar to a cortical structure and the CeA is essentially a subcortical, striatal-like structure (similar to those in ventral striatum) (Alheid and Heimer, 1988; Swanson, 2003). For example, BLA consists of primarily glutamatergic neurons (Carlsen, 1988) while CeA consists of primarily GABAergic medium spiny neurons (McDonald, 1982). As a cortical-like structure, BLA sends dense glutamatergic projections to CeA, similar to cortico-striatal-pallidal networks. Additionally, amygdala nuclei and basic circuit connections are conserved across mammalian species (Janak and Tye, 2015). However, while CeA size and proportion has stayed relatively the same, BLA is enlarged in primates compared to rodents, possibly due to the increased size of cortical regions that communicate with BLA (Freese and Amaral, 2005; Chareyron et al., 2011).

Similar to cortical structures, BLA receives sensory input from thalamus and sends glutamatergic projections to other cortical structures and subcortical structures, such as striatum (McDonald and Culberson, 1986; Unal et al., 2014). BLA consists of lateral, basolateral, and basomedial portions, with the lateral portion receiving thalamic sensory information and projecting to the CeA through the intercalated nuclei. This pathway is especially crucial for expression of fear memory (Maren and Quirk, 2004; Paré et al., 2004; Ponnusamy et al., 2007). BLA additionally projects to known reward-related regions such as Nucleus Accumbens (NAc) Shell and Core as well as medial prefrontal cortex. Via these projections, BLA can influence

dopamine release in NAc and mPFC (Ahn and Phillips, 2002), as well as dopamine signaling in response to reward-related stimuli (Ambroggi et al., 2008). This projection is especially crucial for a previously paired cocaine cue to reinstate cocaine seeking (Stefanik and Kalivas, 2013). Additionally, BLA sends serial projections directly to the CeA, which in turn sends output projections through its medial portion (CeM) (Beyeler et al., 2018). Previous optogenetic studies have compared BLA-CeA and BLA-NAc projections and have proposed evidence to suggest that BLA-NAc projections regulate positively valenced behaviors and promote reward while BLA-CeA projections mediate negatively valenced behavior and promote aversion (Namburi et al., 2015; Beyeler et al., 2018).

In addition to receiving heavy input from BLA, CeA contains its own unique inputs and outputs. For example, the CeA also receives substantial input from other cortical sites such as insula, and this pathway is crucial for incubation of drug seeking (Venniro et al., 2016). Additionally, it is reciprocally connected with several brainstem sites such as parabrachial nucleus and nucleus of the solitary tract , where these connections are proposed to be implicated in autonomic responses such as unconditioned freezing (Petrovich and Swanson, 1997). The CeA also sends heavy projections to reward-related regions such as perifornical lateral hypothalamus, substantia nigra pars compacta, ventral pallidum, and ventral tegmental area (Zahm et al., 1999; Pitkänen et al., 2000), and some have explored the role of these projections in reward-related behaviors. For example, the serial projection from CeA to substantia nigra, which in turn projects to dorsolateral striatum, has been implicated in habitual food and drug taking behaviors (Lingawi and Balleine, 2012; Murray et al., 2015).

Amygdala and incentive motivation

While BLA and CeA are serially connected, their unique cell types and projections have led researchers to directly compare their roles in motivated behavior. In the case of unconditioned eating, the two sites have been contrasted. While CeA inactivation via GABA agonist muscimol blocks food intake, BLA inactivation only blocks food intake that is normally increased by mu-opioid stimulation of NAc (Will et al., 2004). On the other hand, CeA mu opioid activation via DAMGO potentiates food intake above normal levels, whereas DAMGO in BLA does not increase food intake above baseline levels (Mahler and Berridge, 2012). Thus in simple unconditioned eating, CeA and BLA have dissociable roles, with both being necessary and just CeA being sufficient.

CeA and BLA also play dissociable roles in incentive motivation. For example, mu-opioid stimulation of CeA via DAMGO enhances approach of and consummatory behaviors towards Pavlovian food cues while BLA mu-opioid stimulation does not (Mahler and Berridge, 2009). Similarly CeA and BLA play dissociable roles in cue-triggered food seeking. During a Pavlovian-instrumental-transfer test (PIT), previously paired food cues have the ability to cause surges of 'wanting' in the form of instrumental lever pressing. Mu-opioid stimulation of CeA, but not BLA, enhances these peaks of 'wanting' in the presence of cues (Mahler and Berridge, 2012). Others have also shown that lesions of the BLA abolish US-specific PIT while CeA lesions abolish general, but not US-specific, PIT (Corbit and Balleine, 2005). For these reasons, BLA and CeA may play separate roles in cue-triggered food and drug seeking, with BLA being responsible for establishing sensory-specific CS-US associations while CeA may be more important for establishing the association of a CS with general affective properties (Balleine and Killcross, 2006).

Thus, the CeA, not unlike other striatal structures such as NAc, has the privileged role within amygdala of generating intense motivation for rewards and their related cues. However, in all of these previous experiments, CeA manipulations have relied on pre-existing preferences. It is unclear whether CeA circuitry can create a preferred target of motivation. Is CeA circuitry a mechanism by which one particular outcome becomes pursued over other outcomes? And, in addiction, can CeA circuitry be hijacked towards pursuing drugs of abuse in excess over other normal life rewards? My dissertation has focused on this question and has leveraged optogenetic tools to paste optogenetic CeA stimulation onto one particular outcome (reward+associated cue) while rats are choosing between two outcomes. My overall hypothesis here is that amygdala circuitry, particularly involving the central nucleus of amygdala (CeA), is a crucial mechanism that hijacks motivation and narrows choice to create pathological pursuit of drugs at the cost of normal life goals.

Summary of the Present Experiments

The experiments described in this dissertation aim to explore the role played by amygdala-circuitry in reward 'wanting' and 'liking'. We paired optogenetic CeA or BLA stimulation with earning a particular reward (natural or drug) or during an aversive event and found that CeA, but not BLA, stimulation is capable of generating intense motivation for a

paired sucrose or cocaine reward, as well as amplifying attraction towards aversive stimuli, and that this attraction may be due to the enhancement of incentive salience attribution.

Chapter 2: Optogenetic central amygdala stimulation narrows and amplifies motivation for sucrose

This chapter examines the role of CeA in instrumental motivation for a sucrose reward. We paired earning a particular sucrose pellet with optogenetic CeA excitation versus earning an identical sucrose pellet lacking CeA excitation in an instrumental two-choice task. We showed that rats pursued the CeA-paired sucrose pellet almost exclusively, while ignoring the identical alternative. Rats were also more motivated to earn that paired pellet in a progressive ratio test. Further, we explored the psychological processes that may mediate this amplifying and narrowing of motivation for sucrose, including probing the possibility of CeA stimulation being rewarding on its own.

Chapter 3: Optogenetic central amygdala stimulation narrows and amplifies sucrose 'wanting' without altering 'liking'

We next tested the possibility that CeA enhances the pleasure derived from the sucrose itself. This would in turn create a preferred sucrose reward over the alternative sucrose lacking CeA excitation as reported in the previous chapter. Using the taste reactivity test, we measured affective orofacial reactions in response to sucrose or bitter quinine in the presence of CeA laser stimulation. While we observed no alterations of hedonic impact by CeA laser, the same rats showed a narrowing of incentive motivation for sucrose in a two-choice operant task.

Chapter 4: Optogenetic central amygdala stimulation narrows and amplifies motivation for cocaine

Chapter 4 extends the findings from the previous chapter to drugs of abuse as the target of pursuit. I gave rats a choice between earning intravenous cocaine infusions by making nose pokes into either of two portholes. One port earned cocaine plus CeA ChR2 laser stimulation, whereas the other port earned an equal cocaine infusion alone, but without any CeA laser. When subsequently given a choice between both options simultaneously, the rats chose and intensely pursued only the CeA-laser cocaine option, while nearly ignoring the alternative cocaine-alone option. Furthermore, and similar to Chapter 2, rats were more motivated to earn the CeA-paired cocaine infusion, reaching higher breakpoints in a progressive ration session. Focused pursuit of

the CeA-paired cocaine infusion was uniquely accompanied by consummatory nibbles, bites, and sniffs of the Laser+Cocaine associated retractable port.

Chapter 5: Central amygdala circuitry controls choice between sucrose and cocaine

The previous chapters imply a shared underlying circuitry mediating intense motivation for different rewards, potentially shared between 'cocaine addiction' and 'sucrose addiction'. However, this needs to be explicitly tested. Thus, chapter 5 tests whether the CeA narrow-and-intensify effect is powerful enough to control the directional focus of addictive-like motivation between a drug-of-abuse vs. natural sucrose. Specifically, can CeA-excitation when paired with a sucrose reward, create a rat that pursues only sucrose and not cocaine? Conversely, when paired with a cocaine reward, can CeA stimulation create a rat that pursues only cocaine, and ignores sucrose? I found that CeA-pairing was indeed powerful enough to create a reward, whether sucrose or cocaine, which was highly preferred over the alternative, un-paired reward.

Chapter 6: Dangerous desire: Central amygdala circuitry amplifies attraction towards aversive stimuli

Finally, chapter 6 sought to examine the effect of pairing CeA-stimulation with an aversive target. Being famous for its role in fear, it was important to understand how CeA stimulation would regulate behavior when rats had the opportunity to interact with a known aversive target such as a shock rod. Here, we paired optogenetic CeA excitation with touching of a shock-delivering rod. CeA-pairing with the shock rod surprisingly induced attraction towards it, where rats spent more time near the rod and touched it more than control rats. Rather than defensively burying the rod, CeA stimulation caused vigorous chewing and biting of the rod. This CeA-induced attraction may have involved enhanced incentive salience attribution, as a sound cue associated with the shock rod became valuable. Fos protein quantification in several brain regions revealed that CeA-induced attraction towards an aversive stimulus was associated with recruitment of mesolimbic circuitry.

CHAPTER II. Optogenetic excitation of central amygdala amplifies and narrows motivation for sucrose

Introduction

Amygdala-related circuitry plays an important role in motivation and learning, including incentive motivation for rewards as well as fear of threats (Baxter and Murray, 2002; Will et al., 2004; Balleine and Killcross, 2006; LeDoux, 2007; Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012). Learning a preference for one particular reward is often adaptive in guiding normal choice and pursuit. But pathological amplification and narrowing in the focus of incentive motivation may produce drug addiction, binge eating, gambling or related compulsive pursuit disorders. When single-minded pursuit of an addicted reward occurs at the expense of other life goals, such a winner-take-all narrowing of motivation preference may involve dysfunction in brain circuitry involving the amygdala (Claus et al., 2011; Lesscher and Vanderschuren, 2012).

Within the amygdala, the basolateral nucleus (BLA) and central nucleus (CeA) are arranged partly in series, but also each possess independent inputs and outputs that allow the two nuclei to act in parallel (Parkinson et al., 2000; Corbit and Balleine, 2005; Balleine and Killcross, 2006; LeDoux, 2007; Mahler and Berridge, 2009; Lingawi and Balleine, 2012).

Here, with lead author Mike J.F. Robinson, we used optogenetic stimulation in CeA versus BLA to compare their roles (Tye et al., 2011), and reveal new aspects of amygdala-related control of incentive motivation and focused pursuit. Our results reveal a CeA mechanism able to both narrow and amplify learned incentive motivation in an addictive-like fashion. 'Wanting' to pursue one reward that is associatively paired with CeA stimulation becomes specifically amplified, even at the expense of earning another alternative reward of comparable value.

Materials and Methods

Animals

Female Sprague-Dawley rats (250-325g, N = 31) were housed in a reverse 12h light/dark cycle at 21°C constant temperature. Purina chow pellets (15-20g per day) and ad-lib water were provided. The University Committee on the Use and Care of Animals of the University of Michigan approved all experimental methods performed in this research.

Surgery

Rats were anesthetized with ketamine (100 mg/kg, IP) and xylazine (7 mg/kg, IP), given atropine (0.04 mg/kg, IP) to protect respiration. Each rat was surgically infused bilaterally into the central nucleus of amygdala (CeA rats; N =14) or the basolateral nucleus of amygdala (BLA rats N =8) with 2 µl of a CAG channelrhodopsin virus (AAV5-CAG-ChR2-GFP), or with an optically-inactive control virus lacking ChR2 as inactive-virus control rats (AAV5-CAG-GFP; N = 5). Finally, an additional set of control rats received no virus microinjection to serve as a baseline control group (N = 4). Bilateral infusions were made through microinjection cannula stereotaxically aimed at *CeA*: A/P: -2.4; M/L: 4; D/V: -7.6 (with mouth bar set at -3.3) or at *BLA*: A/P: -1.6; M/L: 4.9; D/V: -7.8, infused over 10 minutes at constant rate (0.2 µl/min), followed by an additional 10 minutes in place for diffusion. Bilateral optic fibers (200 µm) were also implanted in either central amygdala or basolateral amygdala during the same surgery, aimed 0.2 mm dorsal to the location of virus injection. Rats were post-operatively treated with the antibiotic chloramphenicol (60 mg/kg, SC) and carprofen (5 mg/kg, SC) as an analgesic, and given at least 3 weeks to recover and to allow for optimal virus expression.

Apparatus

Instrumental training in the two-sucrose choice test and in the progressive ratio one-choice test was carried out in Med-Associates chambers (30.5 X 24.1 X 21.0 cm) with clear plexiglass floors (Figure 1). The chamber walls were equipped with four illuminated retractable levers (4.5 x 2 cm), auditory speakers (for tone/white noise components of CSs) and a magazine for sucrose pellet delivery. Two contact liquid sippers (located on the back wall) and a grid floor were inserted into the chambers for self-stimulation tests and removed when not in use. A video camera placed below the transparent chamber floor recorded the animal's behavior.

Optogenetic self-stimulation tests (without sucrose or other food rewards) were conducted in separate chambers. The first self-stimulation test employed a novel place-preference versus place-avoidance measure, using a 'Sensorat' apparatus designed in-house,

which allowed rats to self-administer CeA laser illumination by going to a particular place (constituting ¼ of the chamber area), or to avoid illumination by remaining in any other place within the chamber (i.e., ¾ of the chamber). The apparatus consisted of a 4-corner square plexiglass chamber with bedding on the floor (38 x 38 cm) with a plexiglass cylinder that occluded the center (20 cm diameter) so that rats could be only in the periphery or corners. Each of the four corners contained its own motion detector 46 cm above the floor to sense if the rat entered its location below, with all data recorded by a Matlab computer program. Laser illumination was always paired with entry into one corner (either a 3-sec pulse or 8-sec pulse for different rats; 25 Hz; 8-10 mW), serially re-activated by any continuing movement within that corner, and terminated by exit from that corner. The same corner was always used for a given individual rat, but different rats were each assigned to their own arbitrarily-chosen corners.

The second self-stimulation test allowed rats to self-administer CeA laser stimulation by simply touching a particular object (an empty water spout). The apparatus used two metal spouts that protruded from the wall, approximately 5 cm apart, both empty, but physically identical to drinking spouts familiar to rats from their home cage. Touching of one designated spout earned brief laser illumination of the rat's amygdala (either a 1-sec or 8 sec pulse for different rats; 25 Hz; 8-10 mW) on an FR1 schedule. Touching of the other spout earned nothing. Assignment of spouts as laser-paired or inactive control was balanced across different rats.

Procedures

Making an equal choice unequal: Instrumental choice of Laser + Sucrose vs. Sucrose Alone

During instrumental training, rats (CeA: N = 10; BLA: N = 8; Control virus: N = 5) were presented two illuminated levers, one on either side of the magazine (Figure 2.1). Depressions of one lever (*Laser + Sucrose* Lever) led to instrumental delivery of a sucrose pellet plus 8 seconds of 25 Hz (15 msec ON, 25 msec OFF) blue (473 nm) laser stimulation at 8-10 mW, accompanied by a distinctive 8-second auditory cue (white noise or tone; always the same paired with this outcome for a particular rat, but counterbalanced assignments across rats). In contrast, pressing the other lever (*Sucrose Alone* Lever) delivered a single sucrose pellet accompanied by its own distinct 8-second auditory cue (tone or white noise; whichever was not paired with laser-sucrose), but no laser illumination. For both levers, presses during the 8 seconds after sucrose delivery had no further consequence. After two days of initial acquisition, each daily session

began with a single lever presented alone to allow opportunity to earn its associated reward (either *Laser + Sucrose* or *Sucrose Alone*), after which the lever was retracted. Then the alternative lever was presented by itself to allow opportunity to earn the other reward. Each lever was presented again alone for a second cycle, to ensure that the rat sampled both reward outcomes. Those single-choice exposures were intended to help learn the association between each lever and its particular outcome. Finally, both levers together were extended for the remainder of the session (30 min total), allowing the rat to freely choose between the two levers and to earn respective rewards in any ratio it chose. In addition, a third lever (inactive control) was constantly extended from the opposite back wall, on which presses earned nothing, and simply served as a control measure of general activity that resulted in lever responses. Whenever the number of lever presses required by a day's schedule was completed on either lever (FR1, FR4, RR4, RR6), its sucrose pellet was immediately delivered, accompanied by 8 seconds of the appropriate auditory cue that labeled the particular level and its outcome (white noise or tone). For the *Laser + Sucrose* lever, delivery of the sucrose pellet was also accompanied by additional simultaneous laser stimulation (8-sec pulse; 25 Hz; 8-10 mW). During those 8 seconds, animals typically rapidly retrieved the sucrose pellet and then resumed responding on either of the two active levers.

Progressive ratio: Laser + Sucrose vs. Sucrose Alone

On day 9, a progressive ratio test was given with either the *Laser + Sucrose* instrumental lever together with CeA illumination or with the *Sucrose Alone* instrumental lever without any laser (order of test conditions was balanced across rats). On Day 10, the progressive ratio test was repeated for each rat with its other lever and other laser condition (CeA: N = 7; Control virus: N = 5). The number of presses required to produce the next reward delivery increased after each reward, according to an exponential progression (Progressive Ratio Schedule = 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268,…) derived from the formula PR = $[5e^{(reward\ number\ x\ 0.2}]$ and rounded to the nearest integer (Richardson and Roberts, 1996; Saunders and Robinson, 2011). To examine whether any preference in responding was the result of increased workload, animals were given a final FR1 session on day 11, identical to the initial day of training.

Laser self-stimulation (CeA laser self-administration without sucrose)

Rats that had shown CeA ChR2 incentive facilitation (plus control CeA inactive-virus rats) were allowed to self-administer laser illumination by performing a simple new response in two situations. In the first, by going to a particular corner location in a 4-corner chamber the rat could earn pulses of CeA laser illumination: an easy way to gain photo-excitation that requires no additional active behavioral responses, which was modeled on the original Olds & Milner demonstration of electrode self-stimulation by going to a location (Olds and Milner, 1954). Rats were placed in a plexiglass arena (38 cm x 38 cm; height: 47 cm) in which they could wander the periphery and enter any of 4 corners. The center of the arena was occluded by a plexiglass cylinder (20 cm diameter x 30 cm height) to restrict rats to the outer rim. Each corner of the chamber had its own motion detector (Visonic) placed above to detect entries. One of the four corners was assigned for self-stimulation (assignment balanced across rats): entries into that corner triggered laser stimulation each time the motion sensor detected movement (25 Hz; 15 msec ON 8-10mW, 25 msec OFF; either a 3-sec pulse (N = 5) or a 8-sec pulse (N = 4) for different rats). The 30 min session was repeated with the same corner assignment on three consecutive days.

In a second self-stimulation situation, Med Associates operant chambers were equipped with two empty liquid sippers on the back wall of the chamber and with grid floors, wired to detect body contacts that closed a circuit with the floor. Contacts on one of the two sippers (assignment counterbalanced between rats) delivered a brief CeA laser stimulation (25 Hz; 15 msec ON, 25 ms OFF; either a 1-sec train for 10 rats [similar to other optogenetic self-stimulation studies (Witten et al., 2011)] or a 8-sec train for 4 rats [similar to duration in sucrose experiments above]). Contacts on the other sipper produced no consequence and served as a control measure for exploration touching or habitual spout-approach. Physical contacts with the two empty sipper tubes were recorded over a 30-minute session, and repeated for 3 days. A separate group of rats received similar 1-sec pulse training with the only additional feature being that contact with each sipper was accompanied by its distinctive 1-sec sound to serve as an auditory label that contact had been achieved, in case that sensory label was helpful to learn the laser versus non-laser discrimination (tone or white noise counterbalanced across spouts).

Fos immunofluorescence and viral expression

Following training, animals were returned to the training context and given a final 30-minute training session (RR6) in the presence or absence of laser stimulation. Following

behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital and were perfused. Brains were then subsequently stored in 4% paraformaldehyde, cryoprotected in 30% sucrose, and then sliced at 40 μm. Slices were blocked in 5% normal donkey serum/0.2% Triton-X solution for 30 mns, before being incubated for 24 h in a polyclonal goat anti-c-fos IgG primary antibody (Santa Cruz), followed two days later by 2 h in Alexa Fluor 594 donkey-anti-goat IgG (Invitrogen) (Paxinos and Watson, 2007; Faure et al., 2008). Sections were mounted, air-dried, and cover-slipped with ProLong Gold antifade re-agent (Invitrogen). To identify fiber tip locations and assess viral spread, relevant sections were examined using a Leica microscope, and results were marked on a coronal schematic in Adobe Illustrator using the rat brain atlas (Paxinos and Watson, 2007). Images were also taken using a color camera coupled to the Leica microscope at 10x and 40x magnification for Fos protein analysis. Nine images were compiled using MCID Core 7 software (3x3; 10x magnification) into one single image centered on the fiber tip. The procedure used for measuring Fos plumes surrounding a fiberoptic site that are induced by light stimulation was modified slightly from one described previously for counting around the site of drug microinfusion (Peciña and Berridge, 2000). Immunoreactivity for Fos-like protein was visualized using a fluorescent microscopy filter with an excitation band at 515-545 nm for Fos-positive cells. For analysis of laser stimulation spread, Fos plume images were taken in the areas surrounding the fiber optic tip. Fos-labeled cells were individually counted within successive blocks (50 x 50μm), along eight radial arms emanating from just under the fiber optic tip, with 10x magnification. If at least two sequential blocks lacked any Fos-labeled cells, then no subsequent blocks further along the arm were counted. Zones of Fos elevation in neurons surrounding fiber optic sites (or 'plumes') were assessed as previously described.

Statistical Analysis

Results were analyzed using repeated measures ANOVAs, to examine the response preference for either lever, followed by t-tests for individual comparisons. Effect sizes were calculated using Cohen's *d*. When necessary a Wilcoxon sign-ranked test was run for non-parametric tests. For all analyses the significance level was set at $p \leq 0.05$, two-tailed.

Results

Two-choice instrumental task.

When presented with a choice of two levers that each earned a sucrose pellet, rats trained without any amygdala laser illumination showed essentially an equal preference between the two levers, and essentially selected randomly (mean 53%:47% preference ratio; N= 4; $F_{1,3} = 0.02$; $p > 0.05$). However, adding CeA photo-excitation associatively to the final press of one lever and to its sucrose delivery in rats with CeA channelrhodopsin (CeA ChR2 rats), caused those rats to become narrowly focused on the particular laser-associated lever by the 5th day of training, choosing and intensely pressing and nibbling on the *Laser + Sucrose* lever while ignoring the alternative *Sucrose Alone* lever that was not paired with laser (day 5 comparison: $t_{1,5}= 3.01$, $p < 0.05$, Cohen's d=1.78; Figure 2.2). By the 8th day when effort levels were highest (requiring roughly 6 presses for each reward) CeA ChR2 rats reached over a 24:1 preference ratio to favor the laser-paired lever/reward combination over the no-laser alternative, resulting in overall effect of laser throughout the 8 days of training ($F_{1,6} = 12.86$, $p < 0.05$; Figure 2.2). CeA ChR2 rats also showed numerous intense consummatory actions directed specifically toward the laser-paired lever: rapidly sniffing, nibbling and biting that particular metal object as though the laser pairing had strengthened its incentive salience properties as an attractive Pavlovian food-related cue. Even when CeA ChR2 rats actually received their sucrose reward, they typically paused pressing and nibbling the CeA-associated lever only momentarily, just long enough to recover and consume the sucrose pellet (i.e., 1-2 sec), before then immediately returning to the laser-paired lever again and resuming a frenzied bout of pressing and nibbling of the metal lever (even though no further sucrose could be earned for at least the 8-sec time-out period while the laser was still illuminated). Consequently, CeA ChR2 rats worked 50% harder than needed for each sucrose reward earned by the laser-paired lever (compared to sucrose alone reward: $F_{1,19} = 7.34$, $p < 0.05$), and exerted nearly all of that extra effort in the few seconds while still receiving CeA illumination. In contrast, CeA ChR2 rats typically ignored the non-laser lever that earned sucrose alone, leaving it alone for up to 5-10 minutes at a time. As a result, CeA ChR2 rats earned over 90 pellet rewards on the CeA *Laser + Sucrose* lever during the 30 minute session (95% preference), compared to earning only 3 sucrose rewards (only 5% of total rewards) on the *Sucrose Alone* lever ($t_{1,9} = 9.88$, $p \leq 0.001$, d=4.46). The skewing of preference towards the laser-paired lever and its outcome when a simultaneous choice was given, and consequent dramatic slowing of approach to the non-laser lever, indicates that increased attraction to the *Laser +*

Sucrose choice situation was at the expense of reduced attraction to the *Sucrose Alone* lever (*Sucrose Alone* for laser-trained vs. no-laser controls: $t_{1,16} = 3.22$, $p < 0.01$, d=1.99).

The CeA ChR2 laser-induced bias grew over 8 consecutive days from an initial 62% on the first day to a 94% preference by the 8th day (Figure 2.2). Preference strengthened even though the effort requirement also grew from 1 press per outcome (FR1) to a random average of 6 presses per outcome during those days (RR6; range 4-8 presses) ($F_{7,42} = 9.86$, $p < 0.001$). Preference growth probably was due primarily to time and increasing experience with *Laser + Sucrose* choices, rather than to the additional effort requirement, because a second group (N = 4) of separate CeA rats kept at a lower FR1 effort demand for all 8 days also showed a pronounced growth in preference over days leading to at least a 5:1 preference ratio (Day x Laser: $F_{7,21} = 6.03$, $p < 0.01$; Group: $F_{1,12} = 1.81$, $p > 0.05$). However on day 8, this group's ceiling preference of 82% was still slightly lower than the 94% ceiling of the rising effort group ($t_{1,12} = 2.59$, $p < 0.05$, d=1.63). Overall, all CeA ChR2 rats in both groups virtually ignored their *Sucrose Alone* alternative choice by the final day, suggesting an intense degree of preference and motivation focused on the laser-paired lever.

By comparison, inactive-virus control rats that similarly received CeA virus microinjections and CeA laser, but whose virus contained only the GFP gene while lacking the ChR2 gene for photoreceptors, emitted only half the number of presses on the *Laser + Sucrose* lever as CeA ChR2 rats ($F_{1,6} = 2.63$, $p < 0.05$; Figure 2.2). Inactive-virus rats showed a modest preference for their CeA laser-paired lever over their no-laser lever, suggesting either that slight reinforcing effects were exerted by light reaching the retina from external reflection or from intra-cranial diffusion, or by other local effects of CeA illumination that did not depend on ChR2 photoreceptor expression. However, control rats with inactive-virus in CeA never pressed with the avid intensity described above for CeA ChR2 rats, and instead dramatically fell below the levels of CeA ChR2 rats, especially during the last three days of testing as effort requirement rose from 4 presses on average to 6 presses on average for each reward ($t_{1,34} = 2.91$, $p < 0.01$, d=1.04). Consequently, by the final day, inactive-virus CeA rats had only a 3:1 preference for the laser-paired lever, while CeA ChR2 rats reached roughly a 24:1 preference ($t_{1,10} = 5.74$, $p < 0.001$, d=3.31).

Indicating localization of function, BLA ChR2 rats completely failed to prefer their laser-paired lever over the sucrose-alone lever that lacked laser illumination ($t_{1,7} = 0.79$, $p > 0.05$; Figure 2.2). If anything, the reverse: BLA ChR2 rats tended by 3:2 to prefer the non-laser lever that earned *Sucrose Alone* over the lever that earned *Sucrose + BLA Laser*. However, the BLA ChR2 preference for non-laser outcome did not reach statistical significance ($F_{7,49} = 0.57$, $p > 0.05$), so future work would be required to assess whether BLA ChR2 rats express a preference or simply choose randomly. In any case, BLA ChR2 rats were far below CeA ChR2 rats in effort on the laser-paired lever, especially by the final days ($F_{1,16} = 21.92$, $p < 0.001$). BLA ChR2 rats did not differ from inactive-virus CeA control rats that received illumination ($F_{1,11} = 1.72$, $p > 0.05$), suggesting that both groups were similarly weak in laser effects on preference among sucrose outcomes. By contrast, CeA ChR2 rats pressed far more on the laser-paired lever by the final days than either BLA ChR2 rats ($t_{1,43} = 5.13$, $p < 0.001$, d=1.57) or CeA inactive-virus rats which also received illuminations ($t_{1,34} = 2.91$, $p < 0.01$, $d = 1.03$). That pattern indicates it was the combination of ChR2 photoreceptor-mediated excitation plus the CeA site for location of laser illumination that was especially crucial to produce such an intense 24:1 preference for laser-paired lever and sucrose outcome.

Finally, a narrowing in the focus of preference by CeA ChR2 laser was also evident by comparing groups' presses on the non-laser-paired lever that earned only *Sucrose Alone*. By the final day, CeA ChR2 rats were actually pressing less on the non-laser lever than either CeA inactive virus control rats ($t_{1,10} = 3.18$, $p < 0.01$, d=1.83) or BLA ChR2 rats ($t_{1,13}$= 4.02, $p < 0.01$, $d = 2.37$), despite the generally much higher and more frenzied level of effort overall by CeA ChR2 rats. Thus, CeA ChR2 laser-pairing appeared not only to magnify but also narrowly focus all effort nearly exclusively on the sole *Laser + Sucrose* option, at the expense of less effort being directed toward earning the alternative *Sucrose Alone* option.

The failure of BLA sites to control preference or amount of lever pressing also indicates that the success of CeA sites was not due to extraneous features of the optogenetic test procedure that would have been shared by both sites (e.g., reinforcement by visible blue light diffusion from laser), but rather specifically due to CeA optogenetic stimulation.

Anatomical localization of function: CeA enhancement vs. BLA failure

Histological analysis indicated that virus expression for the rats showing the incentive enhancement effects described above filled most of the medial-lateral extent of CeA (Figure 4). Optic fibers were placed in roughly equal proportions in medial and lateral divisions of CeA across individual rats (Figure 2.4), with the tips of fibers placed mostly in the dorsal half of CeA, and primarily in the rostral half of the central nucleus. Observations of Fos plumes, expressed as elevations of protein in neurons surrounding an optic fiber tip after ChR2 infection, suggested that laser illumination (beginning 75 min prior to sacrifice) activated Fos expression in surrounding neurons, primarily placed immediately below the fiber tip, extending roughly 0.3 mm in radius from the fiber tip (e.g., 150% elevation compared to 100% Fos levels observed surrounding a fiber tip in control rats that had received inactive virus but also received laser illumination; Figure 2.3). The descending shape of observed Fos plumes below the tip of optic fiber in CeA rats seemed consistent with the possibility that neuronal activation might have reached into the ventral half of CeA. However, further analyses of Fos plumes comparisons to other control conditions would be useful in future to confirm how deeply neuronal activation extends below a fiber tip. In any case, in CeA ChR2 rats, Fos was observed both in neurons that co-expressed virus GFP and in other neurons that were not infected by virus, and which presumably were indirectly modulated across synapses via local circuit interaction. The anatomical extent of Fos spread suggested that optogenetic stimulation in CeA rats probably modulated neurons filling most of the rostral half of the CeA. Optic fiber sites were distributed in both the medial division and the lateral division of CeA, and the spread of activation suggested that there also may have been some cross-division spillover; that is, a fiber in one division may have induced activation that extended also into the other division.

Given that neurons in both medial and lateral divisions of CeA were likely to be affected by many of our sites, it seems plausible to conclude that these CeA behavioral effects were primarily driven by activation of output projection neurons located in the medial division of CeA. That's because the medial division is the final common path for CeA outputs. Behavioral effects were similar across CeA rats, regardless of division placement as far as could be told, and any co-activation of both lateral and medial divisions together would presumably be dominated behaviorally by the neuronal contribution originating in the medial division. By comparison, BLA sites of virus and optic fiber were located similarly dorsally, in the dorsal half of BLA, but more laterally, ventrally and posterior to CeA sites, with BLA fiber tips clustered together in the

dorsal half and caudal two-thirds of the BLA (Figure 2.4). This pattern of placements suggested that BLA stimulations here may have excited neurons mostly contained in the posterior two-thirds of the BLA.

CeA ChR2 laser increases breakpoint: working harder for a single outcome.

To independently assess whether CeA stimulation amplified the intensity of motivation to work on its paired lever/sucrose combination, we turned to an instrumental breakpoint or progressive ratio test of incentive motivation in which a rat faced only a single lever (Figure 2.5). This test measures the breakpoint or the maximum effort price rats are willing to pay for an outcome, when the price grows progressively over a session. On one day, the available outcome was *Laser + Sucrose* (using the particular lever with sound label previously associated with *Laser + Sucrose* in the 2-choice task); on another day the outcome was *Sucrose Alone* without any laser (using the other lever location plus different sound previously associated with *Sucrose Alone*). This allowed the effect of adding CeA laser to sucrose to be assessed by comparing a rat's effort levels across the two days (order was counterbalanced across rats). During each 30 min session, the effort requirement escalated systematically from 1 press per sucrose pellet to over 200 presses per pellet. On the laser day, CeA laser stimulation onset began with each final instrumental press that earned a sucrose pellet, and continued for 8 sec while the sucrose was retrieved and consumed, similarly to above (*Laser + Sucrose*). On the non-laser day, to allow comparison to a non-laser control or baseline condition, each CeA ChR2 rat or CeA inactive-virus control rat was tested while earning sucrose pellets alone without any laser following the same progressive ratio schedule.

Breakpoint results showed that CeA ChR2 laser amplified effort to earn sucrose by over 250% compared to the same rats' performance in the baseline day that earned sucrose pellets without any laser ($t_{1,12} = 3.85$, $p < 0.01$, d=2.21; Figure 2.5). CeA-ChR2 rats pressed on average up to 133 times for a single laser-paired sucrose pellet on the CeA laser day, in contrast to only 57 times for sucrose alone on the non-laser day. That is, CeA-ChR2 laser stimulation made rats press more than twice as much ($t_{1,12} = 3.17$, $p < 0.01$, d=1.74), and consequently earned over 140% more sucrose pellets than the same rats did in their *Sucrose Alone* session ($t_{1,12} = 3.71$, $p < 0.01$, d=1.95; the number of pellets earned rose less than effort because the progressive ratio schedule demanded many more presses for later rewards). The CeA ChR2 laser magnification of

effort actually grew in percentage terms over the 30-min session as effort demands increased, from 283% elevation in the first minutes during low initial effort (1:1 to 12:1 press:reward ratio) to 1720% elevation at the end of the session for higher effort ratios (77:1 to 200:1) (Interaction: $F_{(1,13)} = 14.75$, $p < 0.001$). Facilitation of breakpoint by CeA ChR2 laser was specific to the day it was actually illuminated: even those ChR2 rats that were tested on a first day with CeA illumination, and on a subsequent day without laser illumination, showed the enhancement only on the first day ($t_{1,2}= 4.5$ $p < 0.05$, d=2.67), and were no longer elevated on the second day, (which remained comparable to the no-laser day of other rats that were tested first in that no-laser condition) ($t_{1,5} = 1.02$, $p > 0.05$).

By contrast, in inactive-virus control rats CeA laser illumination failed to increase the breakpoint price, and the two days did not differ in effort for sucrose ($t_{1,8} = 0.75$, $p > 0.05$). Directly comparing across groups, CeA ChR2 rats pressed nearly twice as much as control CeA inactive-virus rats on their respective CeA laser day ($F_{laser*virus}$=14.8, p<.01). Consequently, CeA laser illumination made CeA ChR2 rats earn roughly twice as many sucrose pellets as control rats with CeA inactive-virus on the laser-paired day ($t_{1,10}$=3.42, $p < 0.01$, d=2.0). Finally, an additional control group of no-laser rats was tested on both days without CeA laser in order to assess between-session breakpoint stability. These no-laser control rats were willing to pay on average only half (70 presses) the laser-induced CeA ChR2 breakpoint price (133 presses; $t_{1,9}$= 2.87, $p < 0.05$), which was only as much as control CeA inactive-virus rats paid on their laser day, and as the CeA-ChR2 rats paid on their non-laser day (64-76 presses; $t_{1,6}$= 0.50, $p > 0.05$).

Absence of pure Self-Stimulation of CeA

To further assess if CeA ChR2 photo-excitation itself was a goal, or independent reward or reinforcer, which rats would work to gain (i.e., self-stimulate their CeA laser), we used two simple self-stimulation tasks. In these self-stimulation tasks, a rat could earn CeA photo-excitation by performing an easily-acquired new action: either approaching a particular location or contacting a particular object. First, our location-approach task was similar to the original procedure used by Olds and Milner to discover the phenomenon of deep brain electrode self-stimulation reward (Olds and Milner, 1954):a rat could earn CeA ChR2 laser illumination every time it approached one designated corner of a 4-corner chamber. In the location-approach task rats could obtain brief pulses of CeA laser stimulation (8 sec for some rats; 3 sec for other rats)

simply by entering one arbitrarily-designated corner of a four-cornered square chamber, in which the circular center of the chamber was occluded to encourage visiting of corners (corner assignment for CeA self-stimulation was balanced across rats, but remained consistent for each rat during testing). A pulse of CeA illumination (3 sec or 8 sec; 25 Hz; 8-10 mW) was triggered by entry into that corner, activating a positioned infrared motion detector, and additional illumination pulses were earned by any further movements that activated the detector while the rat remained in that corner.

Results showed that CeA ChR2 laser stimulation failed to produce any self-stimulation or induce any preference (nor avoidance) for the paired corner on this location task ($F_{3,12} = 0.53$, $p > 0.05$; Figure 2.6). No preference for the laser-delivering corner or avoidance of that corner was observed in either CeA inactive-virus rats ($F_{6,24} = 0.26$, $p > 0.05$) or CeA ChR2 rats ($F_{3,12}= 0.08$, $p > 0.05$) whether or not they earned a 3-sec or 8-sec pulse of laser illumination ($F_{3,7} = 0.49$, $p > 0.05$). Even CeA ChR 2 rats that had earlier robustly worked for CeA-paired sucrose at a 20:1 preference ratio failed to show any preference for their laser corner, not even after three repeated days of testing ($F_{6,24} = 0.66$, $p > 0.05$; Figure 2.6).

To confirm the lack of support for self-stimulation by CeA illumination, rats were also tested in another independent and equally simple self-administration task (designated spout-touch), which has been shown to reveal optogenetic self-stimulation of other brain systems (Kravitz et al., 2012). In this spout-touch task, rats could earn CeA laser stimulation by merely approaching and touching a particular empty metal sipper-spout inserted through a side wall of the chamber (Kravitz et al., 2012). The laser-delivering spout was always available for self-stimulation throughout the entire 30 min session, and was empty though similar in appearance to water spouts in the home cage. A second empty spout was also present but earned no laser, serving as a control object for contact comparison. Each physical contact with the laser-delivering spout closed a circuit that delivered a short pulse (either 1 sec or 8 sec in separate rats; 25 Hz; 8-10 mW) of laser stimulation to bilateral CeA (instrumental FR1 schedule). For some rats, each touch of the laser-designated spout was additionally tagged with a distinctive 1-sec auditory cue to provide a further sensory label that CeA stimulation was being administered (for these rats the other spout produced a different sound; tone or white noise; counterbalanced across rats). For rats in the no tone condition both spouts remained silent even when laser was delivered. All groups received 3 consecutive days of daily 30 min training/test sessions. Results

again indicated failure to establish any self-stimulation for CeA ChR2 laser illumination (nor conversely, any specific avoidance of CeA illumination). CeA ChR2 rats touched each spout approximately 17 times per session (33 times in the presence of tones), but failed to specifically touch the laser-earning spout any more or less than the alternative spout, even after three days of repeated experiences (F's < 1.38, p's > 0.05; Figure 7), whether or not rats were additionally informed of internal CeA ChR2 stimulation by a distinct external auditory label ($F_{1,13}$= 1.61, p > 0.05), and whether or not they earned a 1-sec pulse or an 8-sec pulse of laser illumination ($F_{1,9}$ = 0.32, p > 0.05). Control rats with CeA inactive-virus similarly failed to show any preference or avoidance for the laser-delivering spout compared to the other spout ($F_{2,26}$ = 0.001, p > 0.05).

Discussion

Optogenetic (ChR2) stimulation of the central amygdala (CeA) amplified the intensity of incentive motivation to pursue a paired external food reward. Simultaneously, CeA ChR2 stimulation narrowed the focus of that motivation to the particular act of earning sucrose that had been associatively paired with CeA photo-excitation, pulling motivation away from the alternative lever that earned comparable sucrose but lacked CeA laser. CeA ChR2 rats preferred their CeA laser-paired lever earning sucrose by 24:1 over their alternative but otherwise-identical sucrose-alone lever. Similarly, in a separate breakpoint test of incentive motivation intensity, CeA ChR2 stimulation made rats willing to pay up to a seventeen-fold higher price for their sucrose reward on a progressive ratio task that required increasingly higher levels of effort as the session continued. These findings demonstrate that associative pairing of CeA photo-excitation can hijack learned choice and amplify motivation mechanisms to create a single-minded intense pursuit of the designated target.

In short, pairing with laser stimulation of the CeA seemed to have specifically amplified the motivational attractiveness or incentive value of its associatively-paired reward representation (or act of earning it), raising that incentive value by at least several times. Such intense but narrow enhancement of learned motivation for a single associated target by CeA stimulation complements previous demonstrations of broader motivation effects, such as reduction of anxiety by optogenetic excitation of glutamate terminals in CeA (Tye et al., 2011), or reward-related self-stimulation and associative unblocking by optogenetic excitation of mesolimbic dopamine neurons (Witten et al., 2011; Kravitz et al., 2012). Our observation of

CeA ChR2 focusing of intense food-related consummatory actions, such as avid nibbling and sniffing of the associated metal lever, is also reminiscent of similar enhancements of metal Pavlovian cues into intense motivational magnets, which elicit consummatory nibbling as well as approach, by CeA opioid stimulation induced by DAMGO microinjection (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012). However, those pharmacological manipulations acted to intensify motivation for a previously learned reward, after learning was completed, whereas the present study employed associative pairings during training to intensify learned motivation for a particular reward. Future studies will be needed to further disentangle the roles of learning versus post-learning enhancements of motivation in CeA ChR2 effects. Further studies will also be needed to identify whether it is a central representation of the paired sucrose outcome, the associated metal lever as object, or the act of earning the outcome that is the chief target for incentive enhancement by CeA ChR2 stimulation.

CeA versus BLA localization of function

Only sites within CeA for ChR2 stimulation were highly effective here in intensely enhancing incentive motivation. By contrast, ChR2 sites in basolateral amygdala (BLA) completely failed to have any detectable enhancement effect at all. That anatomical site difference within amygdala indicates potential localization of function to CeA for incentive motivation enhancement achieved in this way. Although BLA and CeA are serially connected and sometimes play similar roles in learned incentive motivation (Ahn and Phillips, 2002; Stuber et al., 2011; Wassum et al., 2011), the two nuclei also have their own separate inputs and outputs, and often play different or parallel roles (Parkinson et al., 2000; Corbit and Balleine, 2005). Further, since sites in the medial division of CeA, which serves as origin for CeA outputs, were as effective here as sites in the lateral CeA that projects to medial CeA, it appears plausible that activation of medial CeA output projection neurons may have been especially important in enhancing learning and incentive motivation (Ciocchi et al., 2010). This anatomical hypothesis would be valuable to test further in future studies.

Our observations of local Fos plumes surrounding the ChR2 laser sites indicated that photo-excitation in CeA succeeded in stimulating neurons located within a roughly 0.3 mm radius of the optic fiber tip, perhaps especially in a ventral direction. CeA neurons within the 0.3 mm radius appeared to include both those directly stimulated (also expressing GFP virus,

reflecting ChR2 infection) and indirectly stimulated (showing Fos but not GFP/ChR2 virus). This suggests that ChR2 directly stimulated a subset of CeA neurons that became infected by virus, and that additional neurons were also recruited by laser to express Fos, such as through local synaptic circuitry interactions. While the fiber optic tips were placed more dorsally within CeA and BLA, it is conceivable that the laser illumination at each tip extended more ventrally, as is consistent with our initial Fos plume analysis, and is an issue that can be given future attention. The general CAG promoter used here might also have led to virus infection of other cells in CeA, including glia, so it will be of interest for future studies to more specifically examine the roles of particular CeA neuron subtypes in incentive motivation, as well as of particular anatomical point-to-point connections of CeA with other structures.

CeA enhancement needs an external target (sensory reward)

It may be an important observation that CeA ChR2 enhancement of incentive learning and motivation required the presence of an *external* target here (i.e., earning a sweet or salty food as sensory reward, paired with CeA stimulation). As an internal state by itself, CeA photo-excitation failed to establish any self-stimulation behavior. Conversely, it seems noteworthy that CeA stimulation by itself was never avoided by rats in the self-stimulation test, which allowed assessment of both appetitive and aversive effects (e.g., no avoidance of laser-location or compared to non-laser alternatives in the place test). That suggests further that CeA stimulation here did not simply produce a strongly aversive or stressful type of internal state that motivated food seeking as an escape from distress.

Overall, our pattern of results suggests that the role of CeA photo-excitation was to selectively magnify the pursuit of the particular associated external reward. It was striking that CeA excitation produced such intense and focused enhancement of its paired sucrose or salt incentive, yet completely failed to reinforce self-stimulation to obtain the laser by itself. Although self-stimulation states can certainly be produced by optogenetic excitation of other brain structures (Rossi et al., 2013; Steinberg and Janak, 2013), we surmise that a function of CeA-related circuitry revealed here specifically relates to external targets (perceptual cues and associative representations of external rewards and actions). That is CeA excitation controlled learning to ‘want’ particular rewards as external incentives. This does not necessarily rule out the possibility that self-stimulation someday might be produced by different CeA excitation

parameters than used here, but it shows that the enhancement of food incentive value found here was not simply due to pursuit of internal CeA excitation as an independent target. Instead our results reveal a CeA bias toward external events in the world that may be important for understanding amygdala-related function in learning and motivation.

Figures

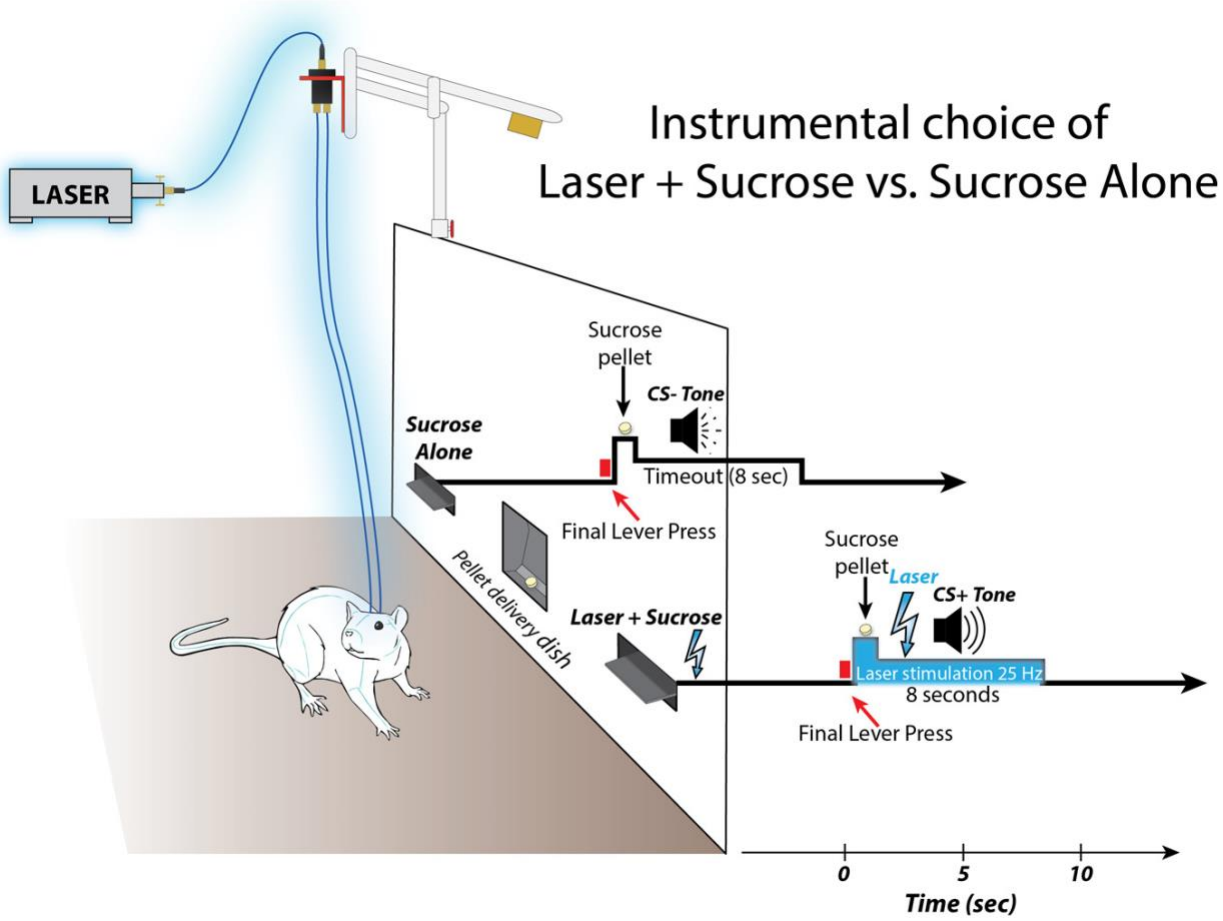


Figure 2.1: Procedure for enhancement of laser-paired sucrose in 2-sucrose choice test. Schematic shows apparatus and event timeline for simultaneous choice tests. Two levers protruded on either side of sucrose dish in center. Pressing either lever earned an equivalent sucrose pellet, and a distinctive 8-sec sound which marked that lever's identity, but only one lever's pellet and sound was also paired simultaneously with onset of 8-sec laser pulse (473nm; 25Hz; 8-10mW).

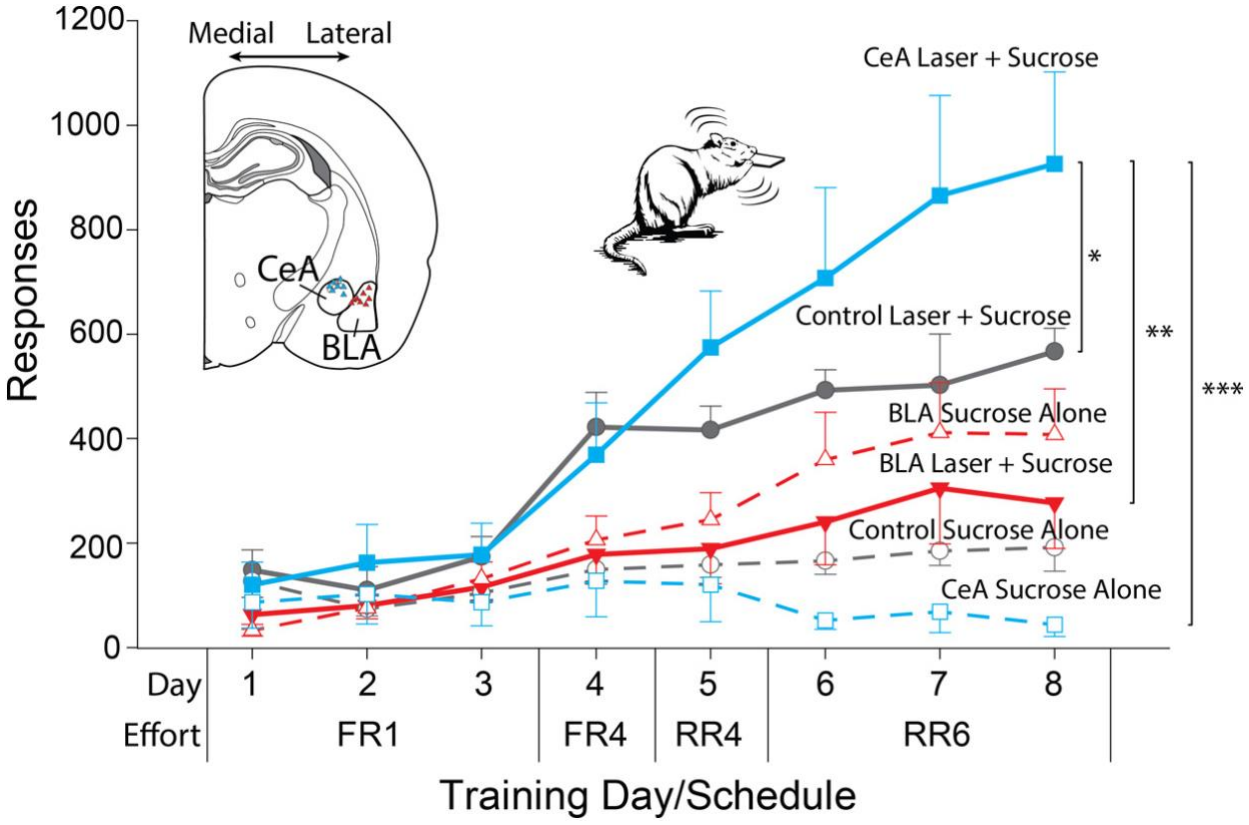


Figure 2.2: Optogenetic stimulation of the central amygdala focuses choice on laser-paired sucrose reward. Rats (CeA: N = 7) developed a powerfully distorted choice towards one of two equivalent sucrose rewards over 8 days when its delivery was associated with CeA ChR2 laser stimulation (8 sec; 25 Hz). A strong preference developed across 8 days of training (30 min sessions) with increasing effort requirements (FR1 → RR6) showing a sharp increase in responding (lever presses) for the laser-paired sucrose reward (*CeA Laser + Sucrose;* blue solid line & squares) over an otherwise equivalent sucrose reward (*CeA Sucrose Alone;* blue dashed line & squares) for CeA ChR2 rats. Control CeA rats (N = 5) with inactive virus in CeA showed a much lower preference for laser-paired sucrose (*Control Laser + Sucrose;* gray solid line & circles) over Sucrose Alone (*Control Sucrose Alone;* gray dashed line & circles). By contrast, in BLA ChR2 rats, BLA sites produced no preference for paired *Laser + Sucrose* (red solid line & triangles), and if anything instead a nonsignificant trend toward choosing the *Sucrose Alone* option (red dashed line & triangles). Data are shown as mean ± SEM. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

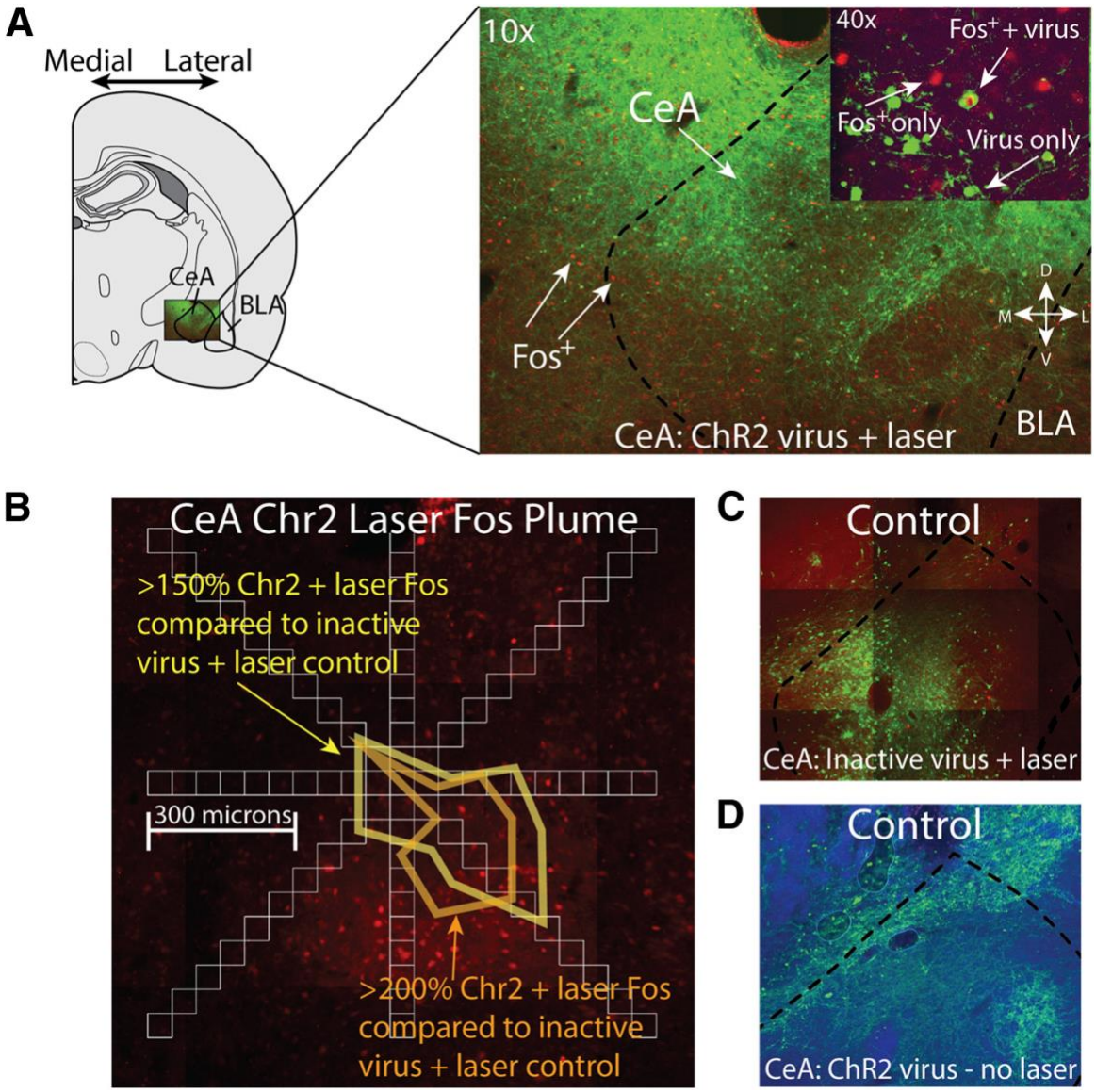


Figure 2.3. Viral expression and laser-induced Fos plume maps. Photomicrographs show double-label immunohistochemistry results depicting neuronal viral infection (green fluorescent protein; GFP) and Fos protein elevation (red; Fos^+) induced by photo-excitation in CeA ChR2 (A) and inactive virus rats (C) (laser illumination of optic fiber conducted 75 min prior to sacrifice, using stimulation parameters identical to behavioral choice tests), and in the absence of photo-excitation (D). A 150% elevation of Fos protein-expressing neurons was observed and extended 0.3mm away from the implanted fiber optic tip in CeA ChR2 rats that received laser illumination compared to rats expressing inactive control virus that received laser illumination whereas a 200% elevation extended roughly 0.2mm (B).

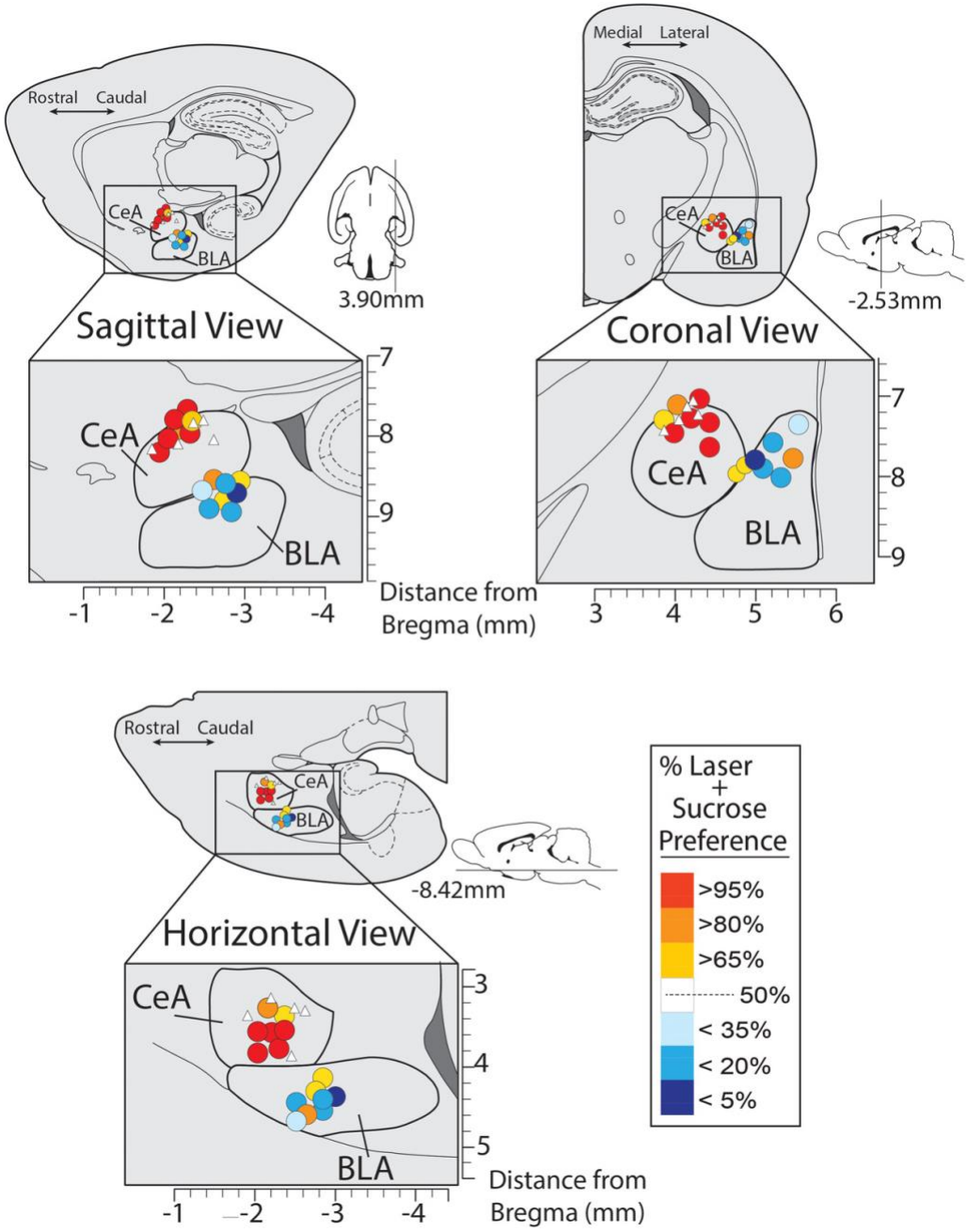


Figure 2.4. Localization of function maps for incentive enhancement. Maps show sites in CeA and BLA corresponding to data in Figure 2 for ChR2 enhancement of sucrose choice. Color of each symbol in map depicts the behavioral consequence of ChR2 laser stimulation at that site in the 2-sucrose choice test (% laser preference for the *Laser + Sucrose* lever over the *Sucrose Alone* lever). Sizes of symbols from both CeA ChR2 and BLA ChR2 rats are scaled to represent the average Fos plume observations of CeA ChR2 rats who received laser, shown in Figure 3 (.25mm). White triangles depict placements of inactive virus controls.

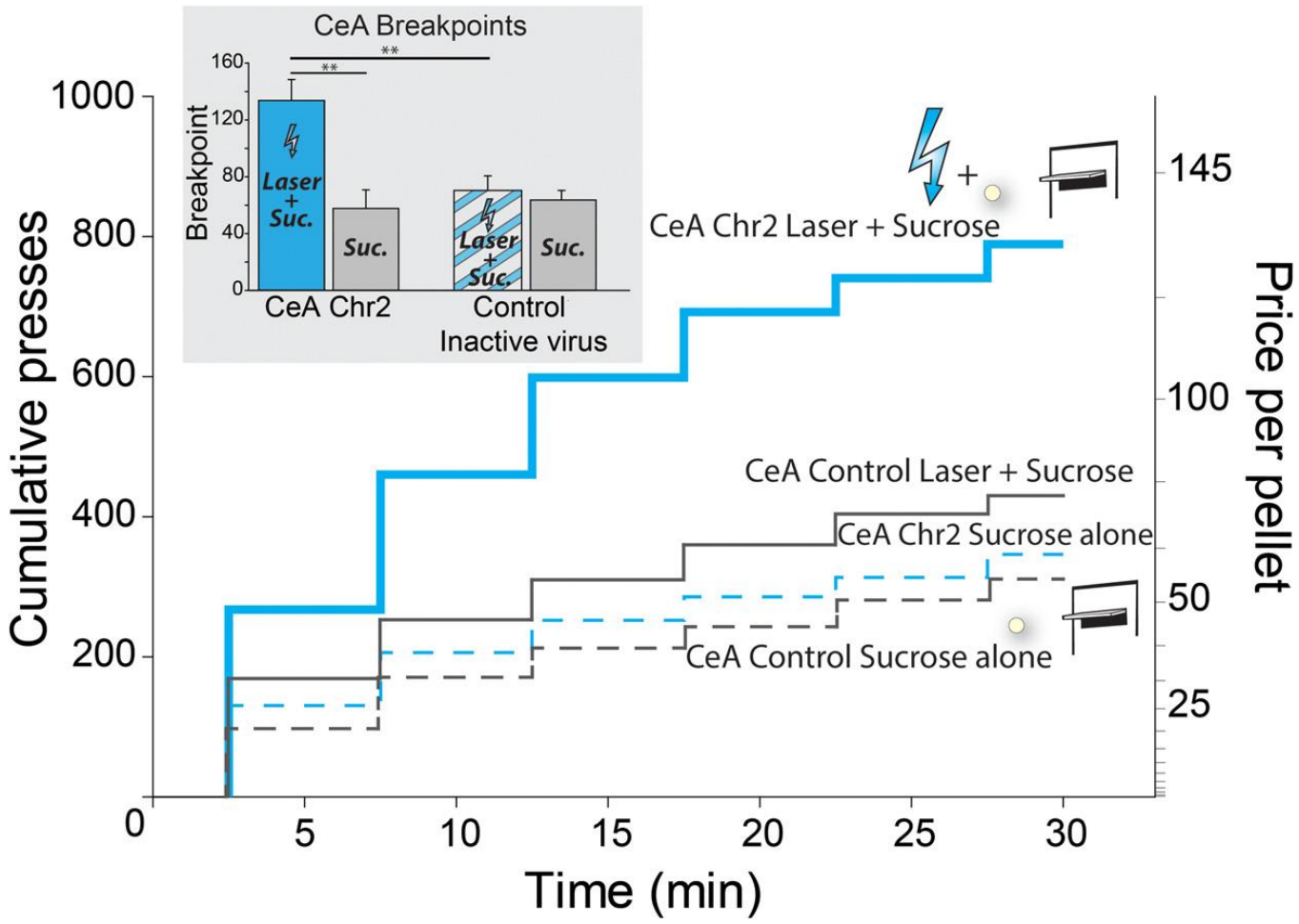


Figure 2.5: Breakpoint enhancement of motivation intensity. CeA ChR2 Laser increases breakpoint and makes rats work harder to earn sucrose. Laser stimulation of CeA made CeA ChR2 rats (N=7) press more as effort requirement increased over the session, compared to baseline pressing by the same rats on a different day when laser was not illuminated. The 'breakpoint' or highest effort price that rats were willing to pay for another sucrose pellet was accordingly increased on the day of CeA ChR2 photo-excitation, compared to all other conditions (No laser day, or control virus animals (N = 5) depicted by inset figure: *CeA Breakpoints*). Data are shown as mean ± SEM. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

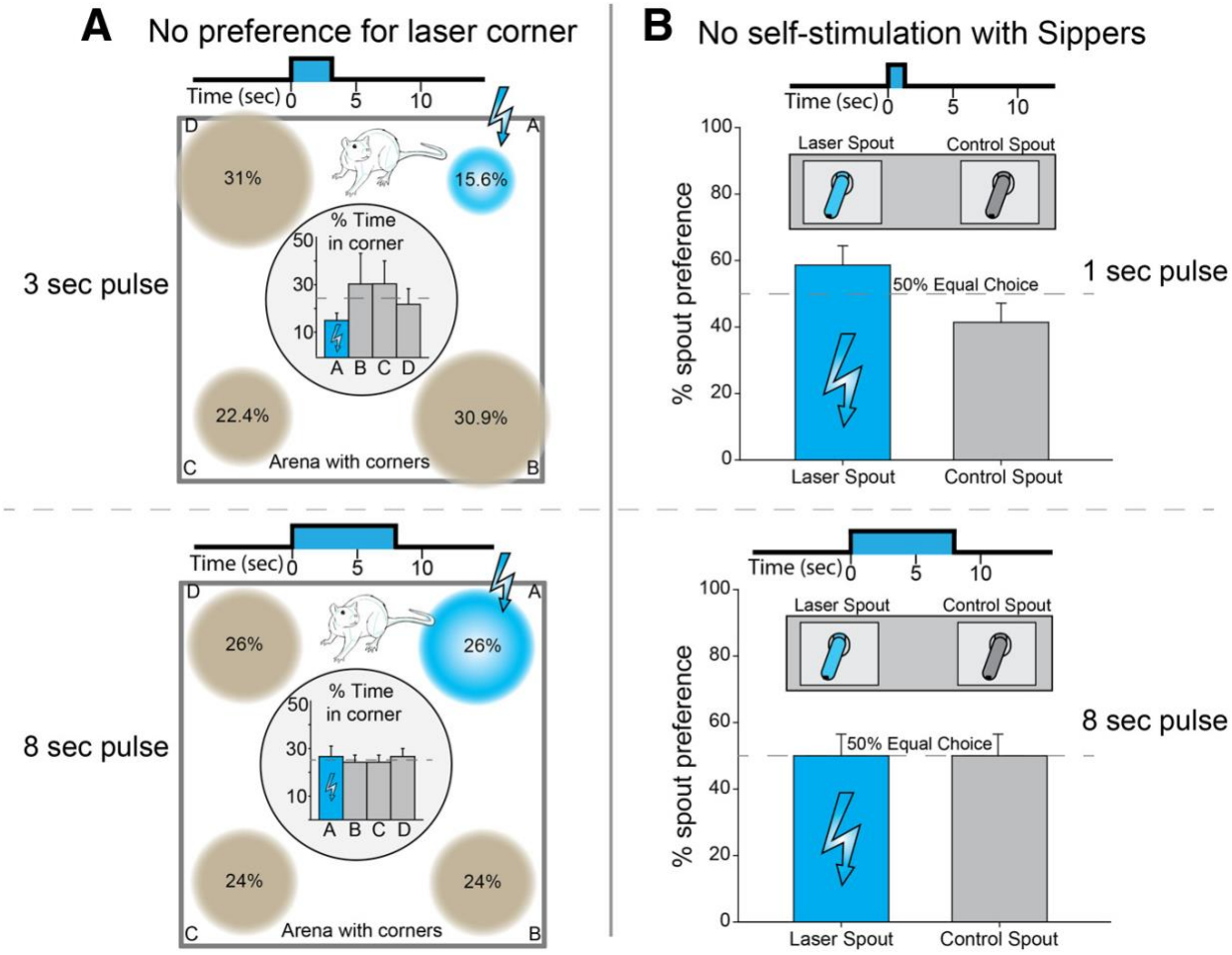


Figure 2.6: No self-stimulation for CeA ChR2 laser by itself. Rats would not perform easy responses to earn laser stimulation of CeA when laser occurred in the absence of any external food reward. A) In a place self-administration test, rats neither preferred the particular corner location where CeA ChR2 excitation was delivered (either 3-sec (N = 5) or 8-sec (N = 5)), nor avoided that location compared to the other corners. B) In a spout-touch self-administration test, rats did not touch the empty metal spout that earned a brief pulse of CeA ChR2 excitation (either 1-sec (N=10) or 8-sec (N=4)) any more than the other metal spout that delivered nothing.

CHAPTER III. Optogenetic central amygdala stimulation narrows and amplifies sucrose 'wanting' without altering 'liking'

Introduction

The amygdala plays a crucial role in motivation and while it is most famous for its role in fear (LeDoux, 2007), amygdala circuitry also functions in reward (Baxter and Murray, 2002; Balleine and Killcross, 2006; Averbeck and Costa, 2017). However, the amygdala is a heterogeneous structure containing several subdivisions, which play dissociable roles in motivation (Corbit and Balleine, 2005; Balleine and Killcross, 2006). In particular, two of the major subdivisions of amygdala are the basolateral (BLA) and central nucleus (CeA). Previous work has highlighted a special role for CeA and not BLA in generating incentive motivation for sucrose and food rewards. For example, activation of CeA, and not BLA, via mu-opioid agonist DAMGO enhances appetitive behaviors (nibbles, sniffs, and bites) towards a sucrose predictive cue, and increases intake of sweet foods (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012). Building on this work, we have found that CeA optogenetic excitation causes rats to intensely pursue a sucrose pellet paired with CeA excitation over another identical sucrose pellet, a bias that persists for many days even after the excitation has stopped (Robinson et al., 2014). Interestingly, rats would not work for optogenetic stimulation of CeA alone, but rather the presence of the sucrose pellet was necessary to maintain their behavior. This suggests that the CeA stimulation during ingestion of sucrose was transforming the brain representation of the sucrose chemosensory reward.

This transformation may possibly have involved enhancing the hedonic value of that sucrose and making it more 'liked', which in turn influenced a strong bias for earning that sucrose above another identical sucrose pellet. Alternatively, the CeA stimulation may have preserved hedonic impact or 'liking' unchanged, and instead transformed the downstream computation of incentive value of the paired chemosensory identity (i.e. taste 'wanting' without taste 'liking').

Indeed, as a key forebrain site of gustatory-limbic interaction, the CeA is well positioned to modulate the hedonic value of incoming chemosensory information. Specifically, the CeA receives direct projections from gustatory brainstem nuclei such as Parabrachial nucleus (PBN) and Nucleus of the solitary tract (NST), while it also provides descending projections back to each of these taste relay centers (Norgren, 1976; van der Kooy et al., 1984; Bernard et al., 1993; Krukoff et al., 1993), both of which play a functional role in taste responsivity (Norgren and Pfaffmann, 1975; Norgren, 1983). CeA additionally receives taste relevant information from the gustatory cortex within the insula, forming a point of connection between the dorsal and ventral streams that joins them into a connected loop (Allen et al., 1991; Schiff et al., 2018). This evidence supports CeA as a potential key player in mediating the valuation of taste chemosensation. Indeed, both lesion and electrical stimulation studies have implicated CeA in processing taste information about several types of gustatory stimuli. However, they have found mixed effects: some have found no change in gustatory function (Galaverna et al., 1993), while others revealed an increase in aversive reactions to aversive tastes (Touzani et al., 1997; Riley and King, 2013; Ross et al., 2016) or even to sucrose (Ross et al., 2016).

To assess whether previously demonstrated CeA-induced narrowing and enhancement of motivation for sucrose (Robinson et al., 2014) was due to enhanced 'liking', here we used the taste reactivity test while optogenetically stimulating CeA neurons. The taste reactivity test is based on hedonic orofacial reactions (Steiner et al., 2001), which are homologous across rodents, apes, and humans and track the hedonic impact (pleasantness or unpleasantness) of a taste rather than its sensory properties (Cabanac, 1971; Spector et al., 1988; Steiner et al., 2001). Importantly, the taste reactivity test provides experimenter control over the gustatory stimulus without needing to rely on the subject's willingness to ingest it. This is crucial for testing whether CeA stimulation selectively alters 'liking' as a first step in enhancing subsequent motivation for sucrose. We found that optogenetic CeA channelrhodopsin (ChR2) stimulation did not alter hedonic reactions to sucrose or quinine, even though in the same rats it narrowed and enhanced motivation for sucrose in an operant task. This suggests that CeA ChR2 stimulation acts selectively to enhance reward 'wanting' without enhancing reward 'liking'.

Materials and Methods

Animals

Female Sprague-Dawley rats (250-300g, n=18) were housed in a reverse 12h light/dark cycle at 21°C constant temperature. All rats had *ad libitum* access to both food and water. All procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Viral delivery and optic fiber implantation

Rats were anesthetized with 5% isoflurane and given atropine (0.04 mg/kg, IP), and were then maintained at 2-3% isoflurane during surgery. Rats received bilateral 1 µl infusions of either AAV5-hsyn-ChR2-eYFP (*n* =13) or AAV5-hsyn-eYFP (n=5) into the central nucleus of amygdala. Infusions were made through microinjection cannula stereotaxically aimed at CeA: A/P: −2.4; M/L: +/- 4; D/V: −7.6 (with mouth bar set at −3.3), infused over 10 min at constant rate (0.1 µl/min), followed by an additional 10 min in place for diffusion. Rats were postoperatively treated with the antibiotic chloramphenicol (60 mg/kg, s.c.) and carprofen (5 mg/kg, s.c.) as an analgesic and given at least 3 weeks to recover and to allow for optimal virus expression.

Oral cannula implant surgery

For subsequent taste reactivity testing, rats were anesthetized three weeks later with ketamine (100 mg/kg, IP) and xylazine (7 mg/kg, IP), and given atropine (0.04 mg/kg, IP) to help with respiration. They were implanted in the same surgery with bilateral oral cannulae [polyethylene -100 tubing] to permit oral infusions of sucrose or quinine solutions. Oral cannulae entered the mouth in the upper cheek pouch lateral to the first maxillary molar, traveling beneath the zygomatic arch, and exited the skin at the dorsal head cap. Stylets were inserted into the cannulae to prevent blockage due to ingested food or saliva. Oral cannulae did not disrupt normal eating. In the same surgery, bilateral optic fibers (200 µm) were also implanted in central amygdala at the same coordinates as viral delivery, except aimed 0.2 mm dorsal to the location of virus injection. After surgery, each rat received subcutaneous injections of chloramphenicol sodium succinate (60mg/kg) to prevent infection and carprofen (5mg/kg) for pain relief. Rats received carprofen again 24 h later and monitored daily until testing 3 weeks later.

Taste reactivity testing

The taste reactivity test was used to measure affective orofacial reactions of rats to a 1 ml volume of solution infused into the mouth via oral cannula. Tests occurred during 1-min infusions administered during 8-10 mW laser stimulation of CeA (5 Hz, 25 Hz, 40Hz, or 5 Hz

frequency in cycles of 5 s ON 15 s OFF). To infuse sucrose solution into the mouth, a syringe containing sucrose or quinine in a syringe pump (Sucrose: 1.0%, 0.029 M, 1 ml per test; Quinine: $3x10^{-3}$M, 1 ml per test) was attached via hollow tubing (PE-50 connected to a PE-10 delivery nozzle) to a rat's oral cannula. A 1 ml volume of solution was infused evenly over a period of 1 min duration. A subset of rats (n=9) was tested on test reactivity before and after operant training for sucrose (described below). Orofacial taste reactivity responses were video recorded via close-up lens and an angled mirror placed underneath the transparent floor for subsequent slow-motion video analysis.

Taste reactivity video scoring

Hedonic, aversive, and neutral taste reactivity patters were scored off-line in slow motion (1/30 s frame-by-frame to 1/10th actual speed). Hedonic responses were classified as rhythmic midline tongue protrusions, lateral tongue protrusions, and paw licks. Aversive responses were classified as gapes, head shakes, face washes, forelimb flails, and chin rubs. Neutral responses were classified as passive dripping of solution out of the mouth, ordinary grooming, and rhythmic mouth movements. A time-bin scoring procedure was used to ensure that taste reactivity components of different relative frequencies still contribute equally to final affective hedonic/aversive totals, and that frequent components such as rhythmic tongue protrusions do not swamp rare but equally informative components, such as lateral tongue protrusions. Specifically, rhythmic mouth movements, passive dripping, and paw licking reactions, which occur in long bouts, were scored in 5 s time bins (e.g., 5 s continuous paw licking behavior equals one bout occurrence). Rhythmic midline tongue protrusions and chin rubs, which occur in shorter bouts, were scored in 2 s time bins. Lateral tongue protrusions, gapes, forelimb flails, and head shakes, which typically occur as discrete events, were scored as single occurrences each time they occurred (e.g., one gape equals one occurrence). Individual totals were calculated for hedonic versus aversive categories. A hedonic reaction total was quantified as the sum of scores for lateral tongue protrusion, rhythmic tongue protrusion, and paw lick scores. An aversive reaction total was quantified as the sum of gape, head shake, face wash, forelimb flail, and chin rub scores.

Operant training and progressive ratio testing

Operant training was carried out in Med-Associates chambers (30.5 X 24.1 X 21 cm) with clear plexiglas floors, which were equipped with four illuminated retractable levers (4.5 x 2cm),

auditory speakers (for tone/white noise components of CSs) and a magazine for sucrose pellet delivery. Lever presses, magazine entries into the goal dish and sipper contacts were automatically recorded using MedPC® software and Med Associates® hardware. Med-Associates chambers were placed in cabinets whose doors were shut during the training sessions in order to ensure reduced ambient light and noise. Red LED house lights were mounted to the ceiling and floor of the cabinets and turned on during the training sessions. A video camera placed below the chamber recorded the animal's behavior at all times.

During instrumental training, rats (n=9) were presented with two illuminated levers, one on either side of the magazine. Responses on one lever (*Laser + Sucrose* Lever) led to the delivery of a sucrose pellet plus 8s of 25Hz (15ms ON, 25ms OFF) blue (473nm) laser stimulation at 8-10mW and an 8 s auditory cue (white noise or tone; always the same paired with this outcome for a particular rat, but counterbalanced assignments across rats). Laser delivery started on the first day of training. In contrast, response on the other lever (*Sucrose Alone* Lever) delivered an auditory cue (tone or white noise) and instrumentally delivered a single sugar pellet, but never delivered laser stimulation. For both levers, presses during the 8 s after sucrose delivery had no further consequence. After 2 d of initial acquisition, each daily session began with a single lever presented alone to allow opportunity to earn its associated reward (either *Laser + Sucrose* or *Sucrose Alone*), after which the lever was retracted. Then, the alternative lever was presented by itself to allow opportunity to earn the other reward. Each lever was presented again alone for a second cycle to ensure that the rat sampled both reward outcomes. Those single-choice exposures were intended to help the rat learn the association between each lever and its particular outcome. Finally, both levers together were extended for the remainder of the session (30 min total), allowing the rat to freely choose between the two levers and to earn respective rewards in any ratio it chose. In addition, a third lever (inactive control) was constantly extended from the opposite back wall, on which presses earned nothing and simply served as a control measure of general activity that resulted in lever responses. Whenever the number of lever presses required by a day's schedule was completed on either lever (FR1, FR4, RR4, RR6), its sucrose pellet was immediately delivered, accompanied by 8 s of the appropriate auditory cue that labeled the particular lever and its outcome (white noise or tone). For the Laser + Sucrose lever, delivery of the sucrose pellet was also accompanied by additional simultaneous laser stimulation (8 s pulse;

25 Hz; 8–10 mW). During those 8 s, rats typically rapidly retrieved the sucrose pellet and then resumed responding on either of the two active levers.

Progressive ratio: Laser + Sucrose vs. Sucrose Alone

On day 10 of testing, a progressive ratio test was given with either the *Laser + Sucrose* instrumental lever or with the *Sucrose Alone* instrumental lever (counterbalanced across rats). On Day 11, the progressive ratio test was repeated for each rat with its other lever. The number of responses required to produce the next reward delivery increased after each reward, according to an exponential progression (Progressive Ratio Schedule = 1,2,4,6,9,12,15,20,25,32,40…) derived from the formula PR = $(5 \times e^{0.2n}) - 5$) and rounded to the nearest integer (Richardson and Roberts, 1996; Saunders and Robinson, 2011).

Optic fiber placement and viral spread verification

After behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital and perfused. Brains were then subsequently stored in 4% paraformaldehyde, cryoprotected in 30% sucrose, and then sliced at 40 μm. Sections were mounted, air-dried, and coverslipped with ProLong Gold antifade reagent (Invitrogen). To identify fiber tip locations and assess viral spread, relevant sections were examined using a Leica microscope and results were marked on a coronal schematic in Adobe Illustrator using the rat brain atlas (Paxinos and Watson, 2007).

Statistical analysis

Wilcoxon signed rank tests was used for nonparametric analysis of positive and negative orofacial reactions between non-laser and laser sessions, as well as between control virus and ChR2 virus groups. Results from operant two-choice and progressive ratio tasks were analyzed using repeated-measures ANOVAs to examine the response preference for either lever, followed by *t* tests for individual comparisons. Effect sizes were calculated using Cohen's *d*. For all analyses, the significance level was set at $p = 0.05$, two-tailed.

Results

CeA optogenetic stimulation does not alter hedonic impact of taste

During the taste reactivity test, orofacial movements in response to sweet solutions as well as bitter solutions were measured in the presence and absence of optogenetic CeA ChR2 stimulation (Figure 3.1). First, a 1% sucrose solution was delivered intraorally for 1 min. Intraoral sucrose typically elicits a suite of positive 'liking' reactions such as rhythmic tongue

protrusions, lateral tongue protrusions, and bouts of paw licking. Overall, there was no change in positive 'liking' reactions in response to intraorally delivered sucrose orofacial responses between CeA laser and non-laser sessions among ChR2 rats (Wilcoxon Signed Rank Test: Z=-.49, p=.62) (Figure 3.2A, inset). No significant differences were observed in either of the specific behaviors within the suite of positive 'liking' reactions. Specifically, tongue protrusion bouts were similar between baseline and laser sessions (Z=-1.2, p=.23), amount of paw licking was similar between sessions (Z=-.96, p=0.34), and amount of lateral tongue protrusions were similar across laser and non-laser sessions (Z=.00, p=1.0) (Figure 1A). Rats emitted essentially zero negative reactions (i.e., gapes, forelimb flails, head shakes) in both laser and non-laser sessions (Z = -.37, p=.71). Similarly, rats containing inactive control virus in CeA (lacking ChR2) elicited similar levels of positive reactions in laser and non-laser conditions (Z=-.82, p=.41), with virtually zero negative reactions in either session (Z=-1.0, p=.32). Furthermore, CeA laser did not alter positive (Z=-.17, p=.87) or negative (Z=-.41, p=.68) reactions in ChR2 rats differently from control virus rats (Figure 3.2B).

We next tested whether CeA ChR2 stimulation altered aversive reactions to a bitter quinine solution. After sucrose delivery, a bitter quinine solution was delivered intraorally for 1 min. In response to this solution, rats typically emit a suite of negative orofacial reactions that reflect 'disiking' such as gapes, forelimb flails, and chin rubbing. We did not observe any difference in this suite of disliking reactions between laser and non-laser sessions (Z=-.07, p=.94). Nor did CeA stimulation enhance the appearance of any positive liking reactions above a low baseline amount (Z=0, p=1.0) (Figure 3.3A). Further, ChR2 rats showed no difference in negative reactions to sucrose during their laser session compared to control virus rats (Z=-.84, p=.4). Among control virus rats, negative reactions to quinine were elicited in equal amounts between laser and non-laser sessions (Z=-.54, p=.59) (Figure 3.3B).

Previous studies have reported increased motor movements of the mouth during electrical stimulation of CeA, even in the absence of a gustatory stimulus (Riley and King, 2013). It is unlikely that optogenetic CeA ChR2 stimulation was causing any changes in mouth movements separate from hedonic impact of taste. No difference in mouth movements (neutral and unrelated to hedonic impact) was observed between laser and non-laser sessions during either sucrose (Z=0, p=1.0) or quinine delivery (Z=-.63, p=.53). Further, mouth movements were similar between ChR2 and control virus groups (Z=-1.55, p=.21). Thus, CeA ChR2 stimulation did not

cause any change in mouth movements unrelated to the hedonic impact of taste.

We next tested whether 'liking' reactions may be altered at different frequencies of laser stimulation. In addition to the stimulation parameters used in the initial tests to match those used to amplify motivation for sugar in operant settings, we also used parameters that have been shown by others to amplify orofacial responses in other brain regions known as hedonic hotspots that amplify 'liking' of sweet tastes. Positive 'liking' reactions to sucrose did not differ between laser and non-laser sessions at lower frequencies of 5 Hz (Z=-1.1, p=.26), 25 Hz frequency (Z=-.09, p=.93), or higher frequencies such as 40 Hz (Z=-.84, p=.39). Even laser being delivered at a low 5 Hz frequency in cycles of 5 s pulses did not alter positive reactions to sucrose from baseline (Z=-.7, p=.48). Nor was there any difference between frequency stimulations on positive 'liking' reactions (Friedman Test: X^2=2.96, p=.39) (Figure 3.4). Similarly, no negative 'disliking' reactions emerged as a result of stimulation at any of the frequencies (X^2=4.3, p=.23).

CeA stimulation narrows and amplifies motivation for sucrose

In the same group of ChR2 rats that showed no change in orofacial responses during CeA excitation, CeA laser excitation produced an intense preference for a sucrose pellet paired with laser stimulation over an identical sucrose. Rats intensely biased their choice in an operant task where they would press a lever to earn a sucrose pellet paired with CeA laser stimulation far more than another lever that delivered an identical sucrose pellet but in the absence of laser stimulation ($F_{1,8}$=39.13, p=.000) (Figure 3.5A). This narrowing of preference for the laser-paired sucrose grew over days ($F_{8,64}$=3.32, p=.003).

Rats were additionally more motivated to pursue this laser-paired sucrose compared to the sucrose alone, when in a progressive ratio or breakpoint session, they were willing to exert greater effort to earn that laser-paired pellet. ChR2 rats responded more than 365 times for their laser-paired sucrose compared to only ~178 times for their sucrose alone (t_8=4.2, p=.003, 95% CI: 84, 290, Cohen's *d*=1.96). As a result, rats reached twice as high of breakpoints for their laser-paired sucrose pellet compared to a sucrose pellet alone (~82 vs. ~41; t_8=4.9, p=.001, 95%CI: 22, 60; *d*=2.33) (Figure 3.5B).

To test whether the experience of working to earn sucrose and earning multiple laser paired sucrose pellets made that outcome a more pleasant option over time, we returned rats to taste reactivity tests to again test the effect of CeA ChR2 stimulation on hedonic impact. After the experience of instrumentally working to earn sucrose paired with CeA ChR2 stimulation,

CeA stimulation still did not alter positive reactions to sucrose from baseline non-laser conditions (Z=-.37, p=.72). Similarly, no change in negative reactions to sucrose was observed (Z=-1.34, p=.18). Further, reactions during CeA laser stimulation were not different from pre-operant training reactions for both positive reactions to sucrose (Z=.00, p=1.0), as well as for negative reactions (Z=.00, p=1.0) (Figure 3.5C).

Discussion

We found that CeA excitation was unable to alter orofacial responses to sweet or bitter tastes but instead was capable of amplifying motivation to pursue sucrose ('wanting' without 'liking'). While infusing a sucrose solution, CeA laser did not alter either positive or negative orofacial reactions from baseline non-laser conditions. Similarly, while infusing a bitter quinine solution, CeA laser did not reduce or enhance aversive reactions that normally occur (nor did CeA stimulation cause any positive reactions to emerge). Even at several different frequencies of CeA laser stimulation, no alterations in reactions to sucrose (positive or negative) were observed. Yet, in the same rats that showed no CeA-induced changes in hedonic impact, CeA ChR2 stimulation biased choice for a paired sucrose. In an operant task, when rats were choosing between pressing a particular lever to earn sucrose and pressing a different lever in the same session to earn sucrose paired with CeA ChR2 stimulation, rats focused their pursuit to exclusively earn their CeA ChR2-paired sucrose while ignoring the sucrose alone option. Furthermore, rats were more motivated to earn that paired sucrose pellet in a progressive ratio test, reaching higher breakpoints for that CeA-paired sucrose pellet. These findings indicate that CeA computations of gustatory evaluation are occurring downstream of the basic hedonic component of taste, and are potentially independent.

CeA ChR2 enhancement of incentive motivation for sucrose is consistent with our previous demonstration of CeA, but not BLA, enhancement of incentive motivation (Robinson et al., 2014). Here we confirm that CeA can generate intense motivation for sucrose, and further show that this intense motivation is unlikely due to CeA-induced enhancement of the pleasure associated with that sucrose. In other words, rats did not 'want' the sucrose paired with CeA stimulation because they 'liked' it more. Enhancement of sucrose 'liking' was not the reason here for CeA enhancement of 'wanting', and thus is unlikely the reason for 'wanting' in our previous demonstration. In our previous findings, we also demonstrated that CeA stimulation alone was not sought after in the same rats that showed biasing of motivation for sucrose, as they

would not self-stimulate laser in multiple settings. Thus, neither the simple reinforcing effects of CeA stimulation or enhancement of sucrose 'liking' were possible mediators of amplified motivation. Future studies should explore the possibility that CeA ChR2 stimulation is instead acting to bias and increase motivation by enhancing incentive salience attribution to reward-related cues (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012).

Our findings here are inconsistent with other studies using electrical stimulation. For example, previous work has demonstrated that electrical stimulation of CeA increased aversive reactions to a bitter taste (Riley and King, 2013; Ross et al., 2016) and even increased aversive reactions to normally 'liked' sucrose (Ross et al., 2016). However, electrical stimulation targets fibers of passage through CeA in addition to actual CeA cell bodies, and that would include gustatory afferent fibers from CeA to PBN (or those from PBN to CeA). Indeed, brainstem gustatory nuclei such as PBN and NST are the first relays in taste processing and may also be involved in mediating the hedonic impact of tastes (Söderpalm and Berridge, 2000). Electrical stimulation could have targeted these projections to alter hedonic impact, or even to modify motor movements within the mouth, which could have led to the interpretation that CeA alters the hedonic impact of taste. Our manipulations here only specifically excited neurons within CeA, and the behavioral consequence was only due to any effects downstream of stimulating CeA neurons. This suggests that any valuation of hedonic impact of taste is likely occurring upstream of CeA, and not occurring within CeA itself.

Failure of 'liking' alteration here also somewhat contradicts previous lesion studies. CeA lesions have either found no dramatic change of gustatory function when CeA was lesioned as measured by assessing oral motor responses to different taste stimuli (Galaverna et al., 1993), or they have shown that CeA lesions amplified the aversive impact of an already aversive taste as measured by amount consumed (Touzani et al., 1997). In combination with our findings that CeA stimulation did not alter the hedonic impact of taste highlights the possibility that CeA may be necessary for normal hedonic processing, but is not sufficient to alter pleasure beyond normal conditions, and still cannot be the reason by which rats preferred to earn sucrose paired with CeA ChR2 stimulation in the two-choice instrumental task.

Our findings here rule out the possibility that experience with working to earn sucrose paired with CeA stimulation and experiencing many CeA laser-paired sucrose rewards made that paired sucrose more 'liked' over time. Here, after demonstrating that CeA ChR2 stimulation can

bias pursuit and enhance motivation for a paired sucrose pellet above an identical sucrose lacking CeA stimulation, the same rats were again tested for 'liking' in the taste reactivity test under laser and non-laser conditions. Having experience working for the paired sucrose pellet did not then render the ability for CeA ChR2 stimulation to alter the hedonic impact differently from before operant training. Additionally, rats didn't learn to 'like' the sucrose more after repeatedly working to earn it or simply just earning laser stimulation repeatedly.

The ability for CeA stimulation to selectively enhance 'wanting' without altering 'liking' is consistent with previous CeA stimulation studies. For example, Mahler & Berridge found that mu-opioid stimulation of CeA enhanced food intake, or 'wanting' without enhancing 'liking' (Mahler and Berridge, 2012). Instead, they showed that CeA mu-opioid stimulation decreased hedonic reactions to sucrose below baseline, making sucrose less 'liked'. Our findings are not consistent with this reduction in 'liking'. One possible explanation could be that mechanisms of mu-opioid stimulation are not completely identical to CeA ChR2 stimulation. Mu-opioid receptors are predominantly inhibitory g-protein coupled receptors, and stimulation of these receptors may inhibit rather than excite cells. Furthermore mu-opioid agonists act on intrinsic enkephalin neurons within CeA (Poulin et al., 2006; Le Merrer et al., 2009), however, it is unclear whether mu-opioid receptors populate inhibitory interneurons or principal cells within CeA which could have opposing effects to CeA ChR2 stimulation. Thus, while optogenetic CeA ChR2 stimulation using human synapsin promoter may have more general targeting of CeA neuronal activity, mu-opioid stimulation is most likely more selective. In this situation, mu-opioid stimulation could have selectively targeted specific neurons within CeA to suppress 'liking'. Thus, future studies could explore cell-specific modulation of sucrose 'liking'.

Brain generators of 'wanting' without 'liking' have been described within other brain regions within mesolimbic circuitry, such as Nucleus Accumbens Shell and Ventral Pallidum (Peciña and Berridge, 2000; Smith and Berridge, 2005; Castro and Berridge, 2014). Specifically, same manipulation in these sites usually produces 'wanting' (as measured by enhancement in food intake) throughout that structure, while only producing enhancements of 'liking' within smaller more restricted zones referred to as hedonic hotspots (Castro et al., 2015; Olney et al., 2018). Here, placements within CeA were scattered evenly throughout rostral-caudal, dorsal-ventral, and medial-lateral portions, and yet no pattern of enhanced 'liking' was ever detected among these portions. Rather, all sites produced enhanced 'wanting'. Future studies may

strategically target anterior vs. posterior sites of CeA to more thoroughly assess the effect of CeA ChR2 stimulation on hedonic impact and the possibility of a defined hedonic hotspot.

Our demonstration here that CeA ChR2 stimulation selectively enhanced sucrose 'wanting' without altering 'liking' may reflect the dissociation between 'wanting' and 'liking' brain mechanisms that occur in addiction. Specifically, brain systems mediating 'wanting' for drugs and their related cues can sensitize over time, becoming hyper-reactive (Robinson and Berridge, 1993, 2008; Berridge and Robinson, 2016). By contrast, the pleasure ('liking') derived from drug-taking stays relatively the same over time, thus a dissociation between 'liking' and 'wanting' systems occurs. Sensitized 'wanting' systems render the brain hyper-reactive to drugs and drug-related cues, triggering relapse in the presence of them, even after long periods of abstinence. In addiction, CeA circuitry may be hijacked by drugs of abuse to selectively enhance 'wanting', and could be a potential target for treatment.

Figures

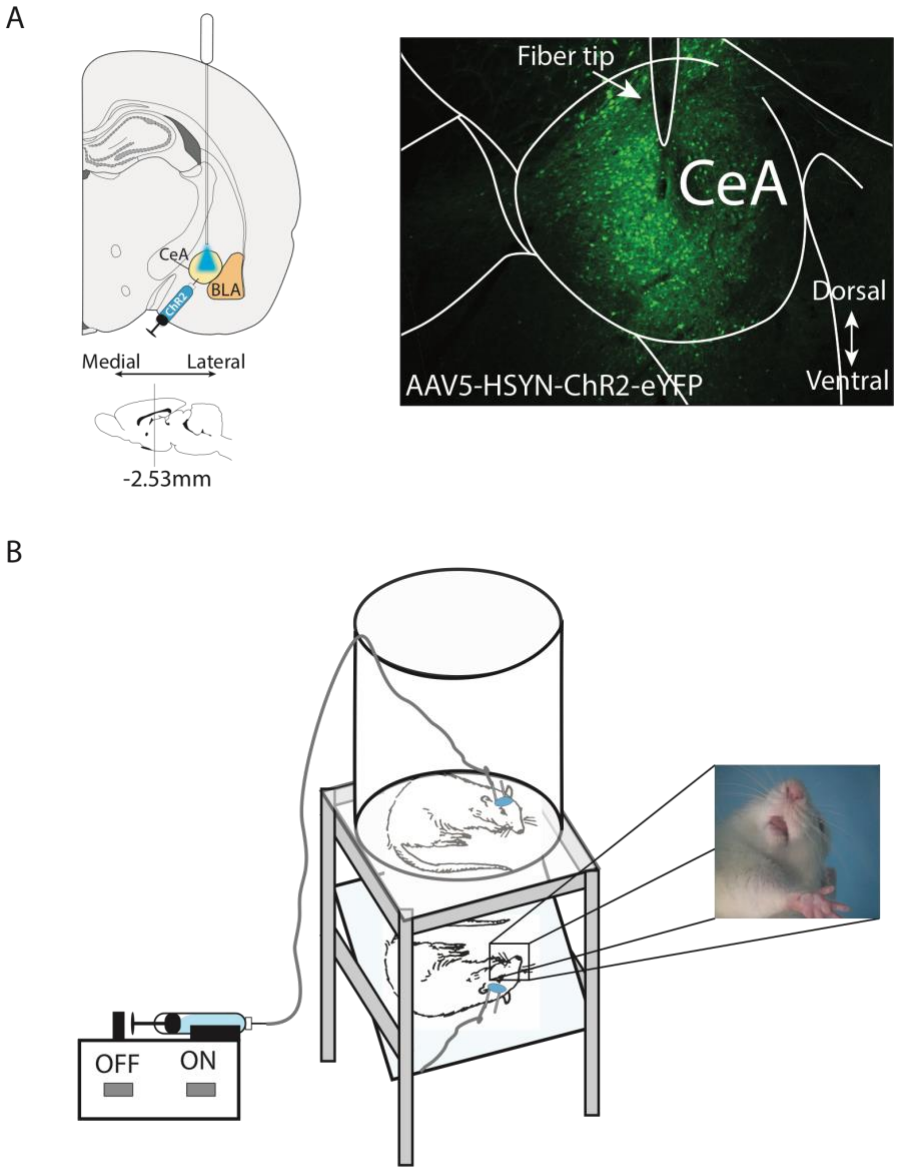
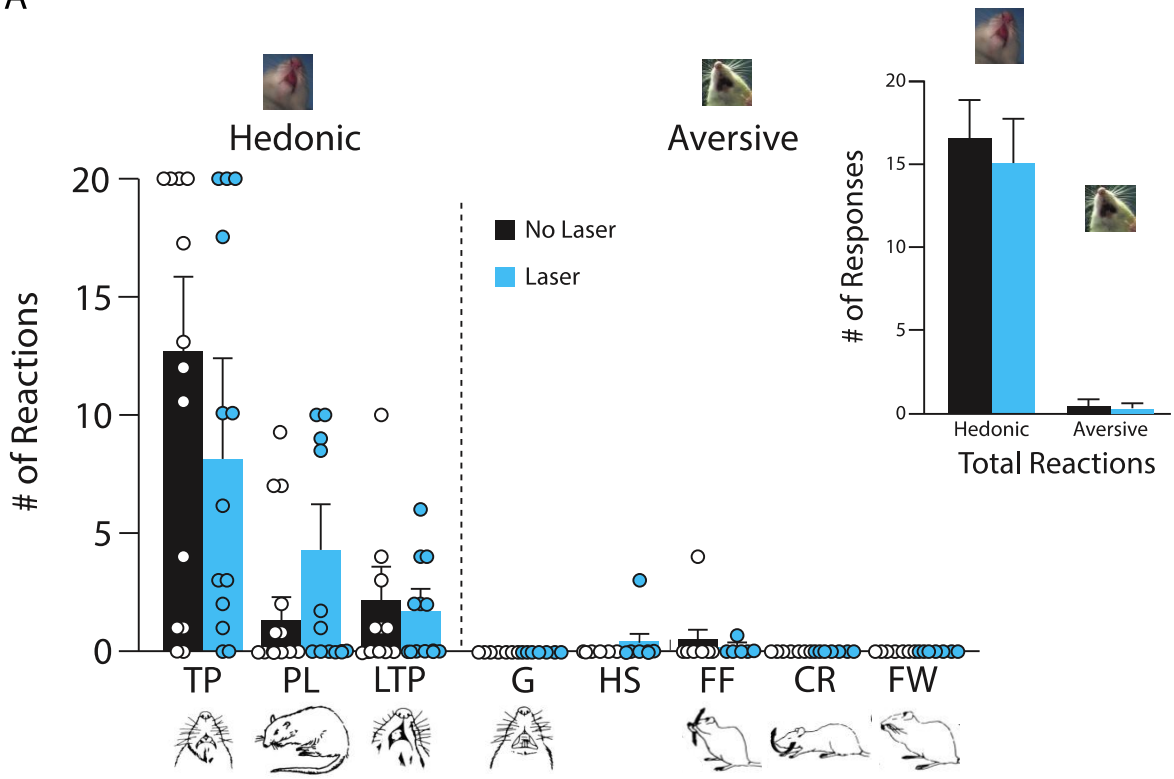


Figure 3.1
ChR2 virus expression in CeA and Behavioral Paradigm. (A) ChR2 virus or control eYFP virus was infused into the central amygdala (CeA). Photo shows coronal view of Central amygdala, and representative photo at 10 x magnification depicts an example viral spread of a ChR2-transfected rat (eYFP fluorescence). (B) Depicts the taste reactivity testing chamber. Rats are placed into a plexiglas chamber containing a clear floor. A mirror below the chamber is tilted up towards the floor of the chamber to make mouths of rats visible to a camera aimed at the mirror. Tastant solutions are infused through PE 10 tubing connected to previously implanted intraoral cannulas via a microinjection pump at a rate of 1 mL/min.

CeA ChR2 stimulation does not change orofacial responses to sucrose

A



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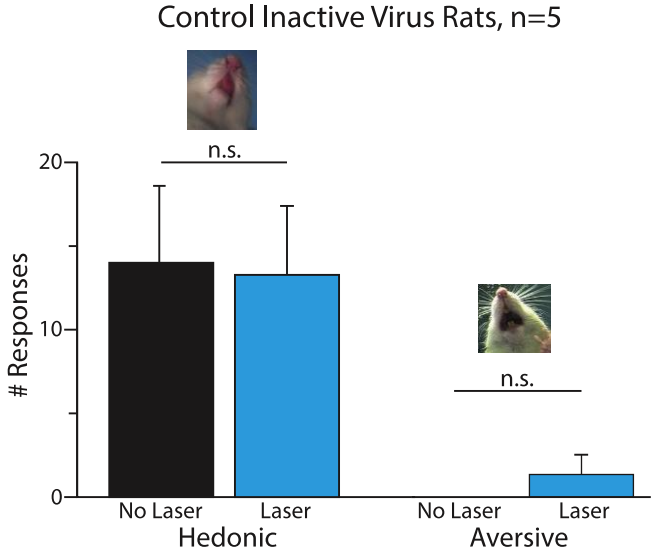


Figure 3.2
CeA ChR2 stimulation does not change orofacial responses to sucrose. (A) # Total Hedonic and Aversive Reactions are shown for ChR2 rats during no laser (black bars) and laser (blue bars) sessions for (inset graph). Average reactions for each behavior belonging to hedonic category (TP, tongue protrusions; PL, paw licking; LTP, lateral tongue protrusions) and those belonging to the aversive category (G, gapes; HS, head shakes; FF, forelimb flails; CR, chin rubbing; FW, face washing) are shown for both non laser (black bars) and laser sessions (blue bars). Circles represent values for each individual rat on no laser (white circles) and laser (blue circles) sessions. (B) # Total hedonic and aversive reactions are shown for control virus eYFP rats during no laser (black bars) and laser (blue bars) sessions. Data represent means and standard error.

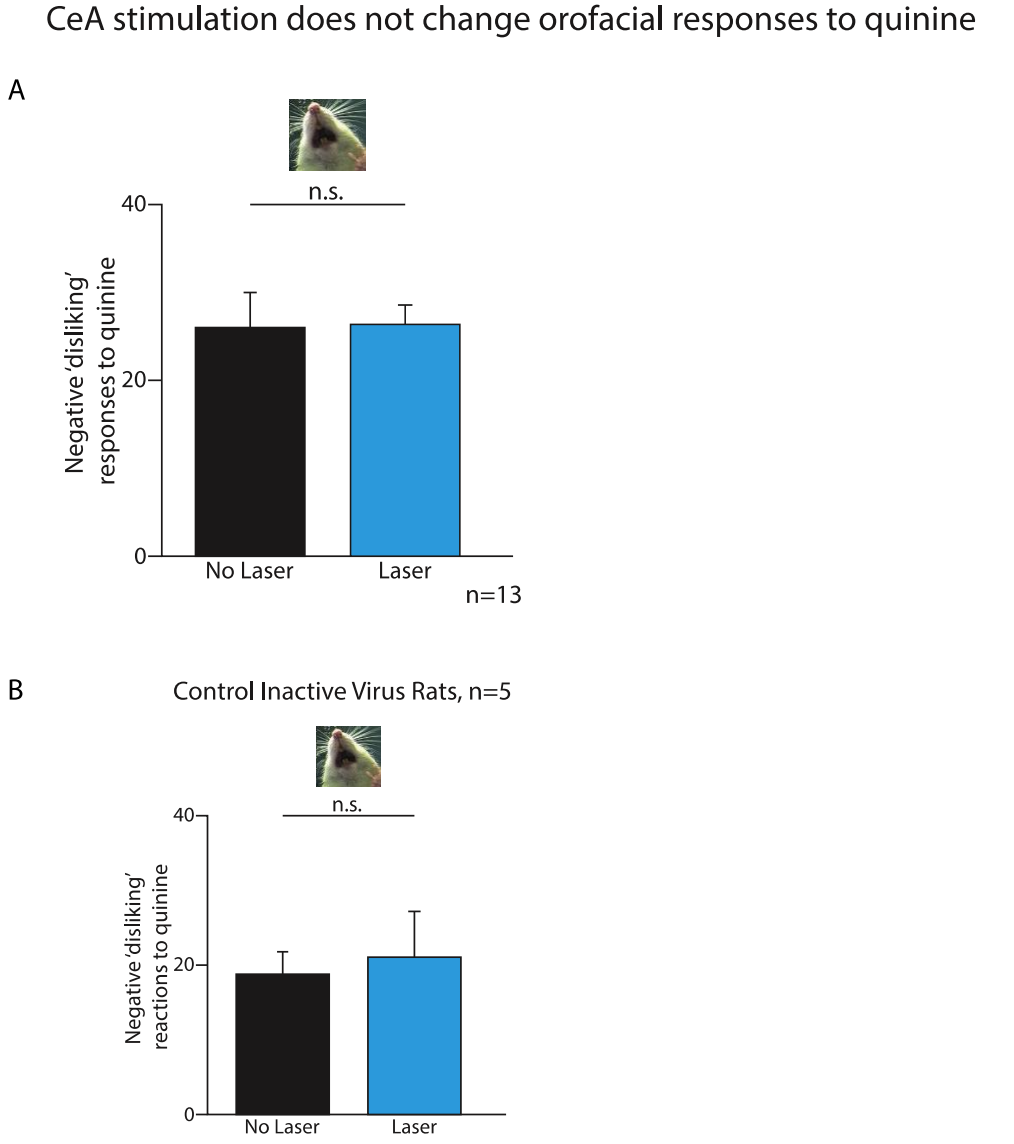


Figure 3.3
CeA stimulation does not change orofacial responses to quinine. (A) # Total negative 'disliking' reactions are shown for ChR2 rats during no laser (black bars) and laser (blue bars) sessions. (B) # Total negative 'disliking' reactions are shown for control virus eYFP rats during no laser (black bars) and laser (blue bars) sessions. Data represent means and standard error.

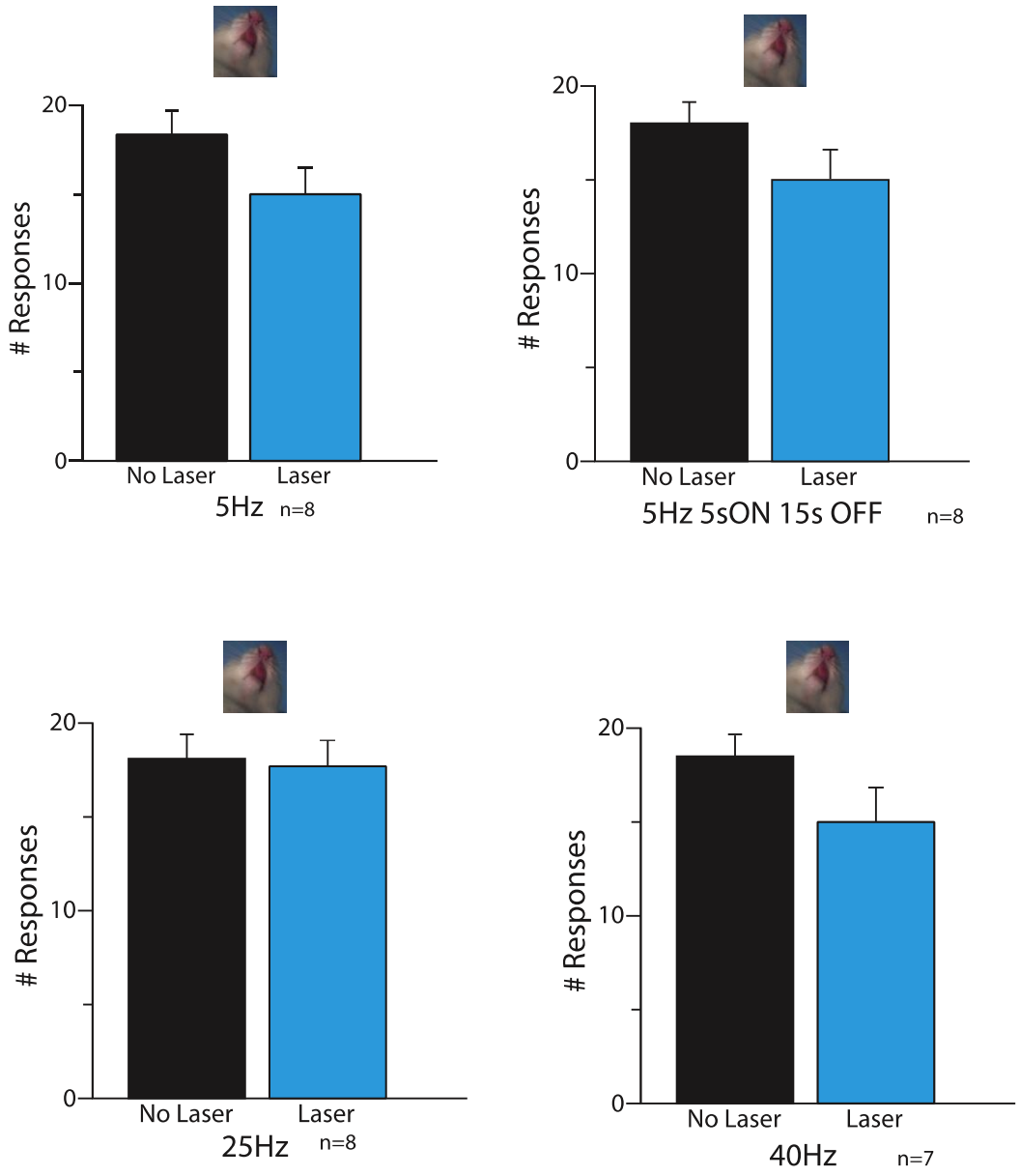
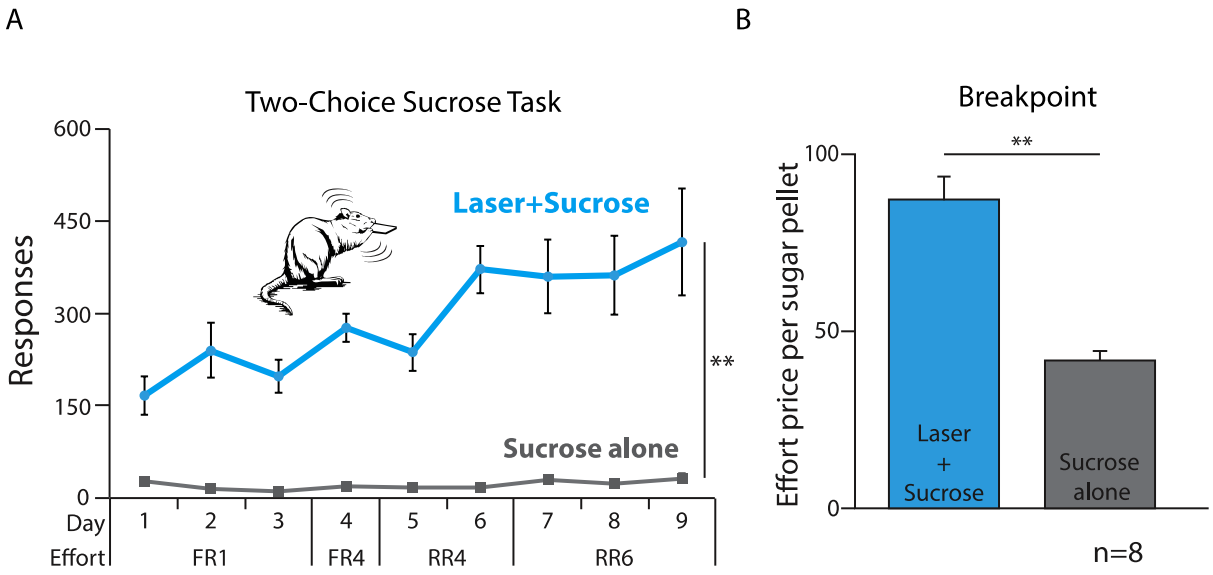


Figure 3.4
CeA stimulation at different stimulation parameters does not alter hedonic reactions to sucrose. # Total hedonic reactions are shown for no laser (black bars) and laser (blue bars) sessions when varying frequencies of laser delivery are used (5 Hz, top left; 5 Hz in cycles of 5 s ON and 15 s OFF, top right; 25 Hz, bottom left; and 40 Hz, bottom right). Data represent means and standard error.

CeA stimulation biases choice and increases effort for sucrose



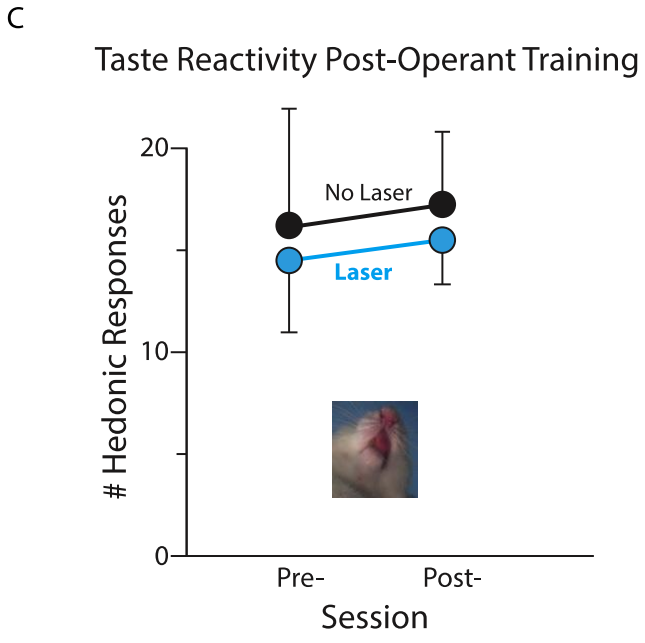


Figure 3.5

CeA stimulation biases choice and increases effort for sucrose. (A) During instrumental two-choice tasks ChR2 rats could respond by pressing a lever to earn sucrose paired with CeA laser (Laser +Sucrose, blue line and blue circles) or sucrose alone (grey line and grey squares) in each session for 9 consecutive days, where effort increased over days from FR1 ratio (fixed ratio 1) to RR6 (random ratio 6) days 7-9. (B) In a separate progressive ratio (breakpoint) test of motivation, rats were trained on separate days to earn either their Laser+ Sucrose (blue bar) or their Sucrose alone (grey bar). Effort for each subsequent pellet increased progressively throughout the session, and breakpoint represents the ratio reached for each session. (C) Taste reactivity testing was again performed after operant training and no difference was observed in hedonic reactions to sucrose during no laser sessions (black circles and black lines) and during laser sessions (blue circles and line) from pre-operant training to post-operant training. Data represent means and standard error. **p<.01

CeA Functional sites of 'liking' vs. 'wanting'

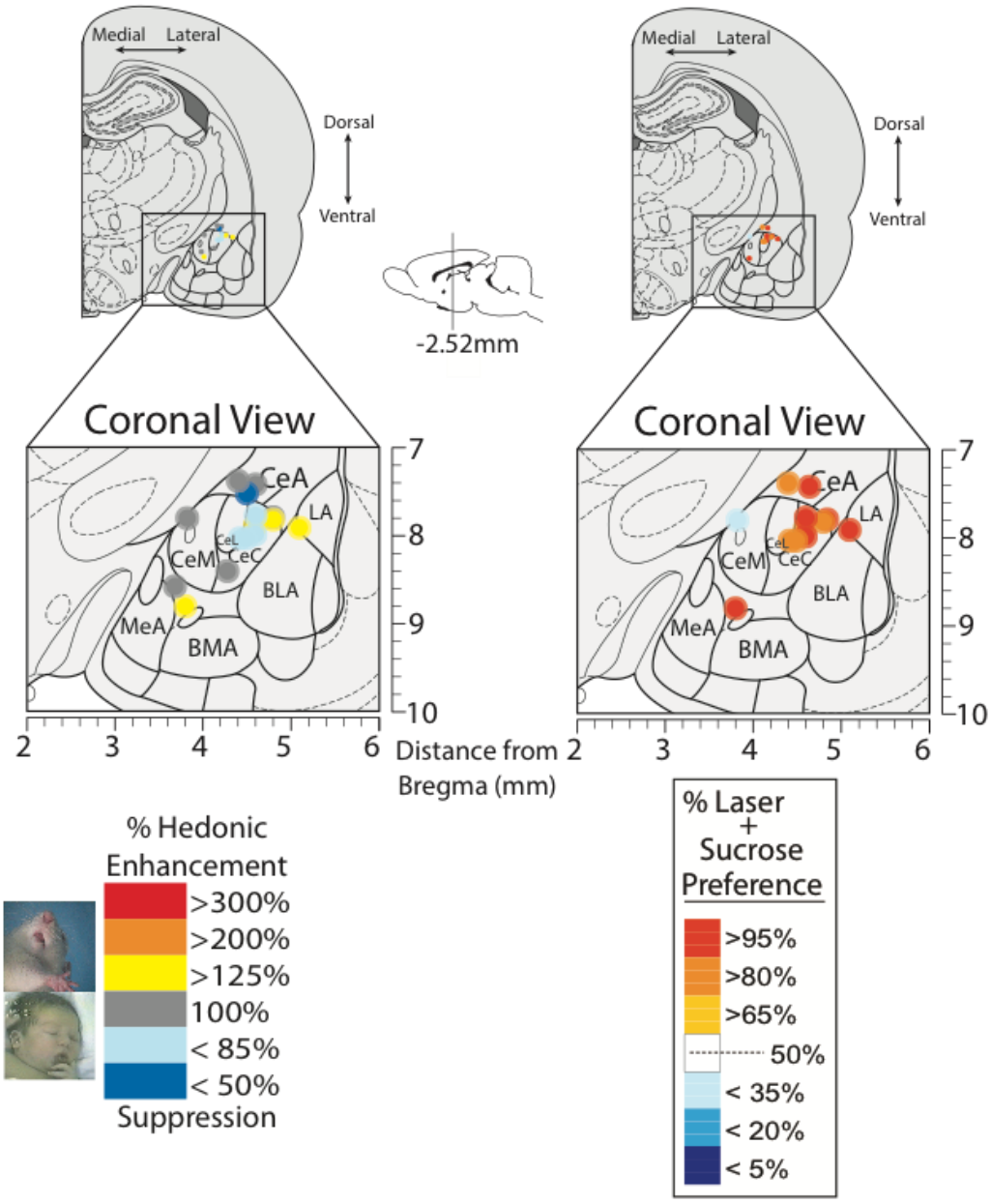


Figure 3.6

CeA Functional sites of 'liking' vs. 'wanting'. Coronal sections of CeA are based on rat atlas from Paxinos and Watson (2007) at -2.52 mm from Bregma. Each coronal view depicts placement of optic fibers within CeA (circles) among ChR2 rats. Size of circles correspond to previously reported Fos plume spread after the same laser stimulation parameters in CeA (Warlow et al, 2017). Color of circles in left coronal map represent % hedonic enhancement to sucrose during the taste reactivity test (% enhancement of total hedonic reactions in laser session compared to no laser session) for that individual rat. Color of circles in right coronal map represent % Laser+sucrose preference during instrumental two-choice test (% Laser+sucrose responses compared to sucrose alone responses on last day of training) for that individual rat.

CHAPTER IV. Optogenetic central amygdala stimulation narrows and amplifies motivation for cocaine

Introduction

In addiction, intense motivation often becomes narrowly focused on an addictive reward target, while other alternative rewards may be relatively ignored. How does limbic circuitry narrow the focus of incentive motivation to a single drug target, making that target more intensely 'wanted', at the expense of other alternatives?

Amygdala-related circuitry assigns motivational significance to particular stimuli based partly on associative information (LeDoux, 2000; Balleine and Killcross, 2006; Mahler and Berridge, 2009; Haubensak et al., 2010; Berridge, 2012; DiFeliceantonio and Berridge, 2012; Peck et al., 2013; Janak and Tye, 2015; Yasoshima et al., 2015). In human addicts, drug cues can activate amygdala and related mesocorticolimbic circuitry, and trigger urges to take drugs (Tang et al., 2012; Volkow et al., 2013). Within the amygdala, the basolateral nucleus (BLA) and the central nucleus (CeA) are serially connected. However, the CeA may be especially effective in generating intense incentive motivation that is narrowly focused on a particular stimulus target, when neurochemically or optogenetically stimulated (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012; Robinson et al., 2014; Seo et al., 2016). The CeA is also implicated in the strengthening of cue-triggered craving for addictive drugs during incubation in animals (Lu et al., 2005, 2007; Li et al., 2015b), which might reflect incentive-sensitization. The special role of CeA in generating intense motivation may be related to its status as a GABAergic 'striatum-level structure' within a cortico-striato-pallidal macrosystem framework (Alheid and Heimer, 1988; Swanson and Petrovich, 1998; Zahm, 2006).

In a previous study, we reported that associative pairing of brief optogenetic ChR2 stimulations of CeA with pressing a particular lever to obtain sucrose pellet rewards, caused rats to intensely and narrowly pursue that sole sucrose option, while ignoring an alternative lever that earned equal sucrose but without laser (Robinson et al., 2014). Yet, seemingly paradoxically, the

CeA ChR2 stimulation by itself appeared worthless as a reinforcer to the same rats, indicated by their failure to self-stimulate for CeA laser illumination by itself. This discrepancy suggested that value was not simply transferred from CeA ChR2 activation as an additive reinforcement signal, but rather that CeA ChR2 actively transformed the motivational value specifically of the laser-paired lever or its sucrose reward, to make that reward option exclusively and intensely more attractive than the alternative (Robinson et al., 2014).

Our hypothesis is that stimulation of CeA circuitry narrows the focus of incentive motivation to its paired reward target, and simultaneously raises the intensity of attraction, as happens in addiction. If so, then CeA ChR2 control of incentive motivation should also apply to earning an addictive drug reward, such as intravenous cocaine. Here we tested whether CeA ChR2 pairing would intensify and narrow pursuit to a single paired cocaine self-administration option, while making rats ignore another alternative and equally good cocaine option. Our results confirmed that prediction, and showed further that intense consummatory oral responses, including bites, became directed toward the paired metal cocaine-associated porthole, as though unusually intense incentive salience were attributed to that paired cue. Further, CeA ChR2 stimulation also intensified the breakpoint effort price rats were willing to pay for cocaine, which further indicates amplification of incentive motivation. Conversely, normal CeA function appeared needed for cocaine self-administration acquisition in this task, as CeA pharmacological inhibition completely prevented self-administration acquisition in our two-choice task, and halorhodopsin inhibition of CeA slightly suppressed cocaine intake. Yet CeA ChR2 excitation alone completely failed to support any self-stimulation behavior, even in the same rats in which it controlled cocaine motivation. That is, rats refused to touch a spout to earn laser illumination, nor would perform nosepokes or return to a place to self-stimulate CeA ChR2 photo-excitations. Thus, CeA ChR2 stimulation intensified and narrowed motivation for its paired cocaine option to produce single-minded pursuit and consumption of the paired drug reward, despite having no independent reinforcement value. These results suggest CeA circuitry can enhance the attractiveness of a particular drug reward and associated stimuli, and narrow the focus of incentive motivation specifically to that reward, in a way that may be relevant to addiction.

Materials and Methods

Subjects

Female Sprague Dawley rats (n=55), weighing 250-300g at surgery, were housed at ~21°C constant temperature on a reverse 12 hr light/dark cycle. Estrus cycle was monitored via cervical smear. All rats had ad libitum access to both food and water in their home cage throughout the experiment. 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 optic fiber implant

Rats were anesthetized with intraperitoneal injections of ketamine (100 mg/kg; Henry Schein, Dublin, OH), xylazine (7 mg/kg; Henry Schein), and received atropine (0.04 mg/kg; Henry Schein) prior to surgery. At surgery, each rat also received subcutaneous injections of chloramphenicol sodium succinate (60 mg/kg, Henry Schein) to prevent infection, and carprofen (5 mg/kg, Henry Schein) for pain relief. Rats again received carprofen (5 mg/kg) 24 hours later, and were allowed at least 3 weeks for recovery and viral expression before behavioral testing began. Each rat was placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and received bilateral infusions of an AAV ChR2 virus with human Synapsin promoter to infect neurons (AAV5-Hsyn-ChR2-eYFP, UNC Vector Core, Chapel Hill, NC). Virus infusions were targeted either into the central nucleus of amygdala (CeA; Bregma A/P: -2.4, M/L: +/-4.0, D/V: -7.6; mouth bar set to -3.3; n=15) or into the basolateral amygdala as an anatomical comparison site (BLA; A/P: -1.92, M/L: +/-5.0, D/V: -8.0; mouth bar set to -3.3; n=6). A total of 1µl volume of virus per side was infused over 10 min at a constant rate (0.1 µl/min), and the injector was subsequently left in place for an additional 10 min to allow for diffusion. To test the effects of optogenetic inhibition in a separate group of rats (n=7), a 1 µl volume of the inhibitory AAV halorhodopsin virus under the human synapsin promoter was infused into CeA using the same coordinates and at the same rate (AAV5-HSYN-eNpHR3.0-eYFP, UNC Vector Core, Chapel Hill, NC). To serve as inactive-virus controls, other rats received an infusion of optically inactive control virus, identical except lacking either the ChR2 or eNpHR3.0 gene (n=10; AAV5-Hsyn-eYFP, UNC Vector Core, Chapel Hill, NC). For all rats receiving virus in CeA, bilateral optic fibers (200 µm) were implanted during the same surgery, each aimed at 0.3 mm dorsal to the rat's virus infusion.

To test the effects of pharmacological inhibition of CeA, and assess normal function contribution to cocaine motivation via muscimol/baclofen microinjections, a separate group of

rats (n=9) were implanted with microinjection guide cannulae bilaterally with tips located 1 mm above CeA (22-gauge stainless steel, 11 mm long; Plastics One, Inc., Roanoke, VA; AP~-2, ML ~4, DV~6.8). Dummy cannulas were inserted to prevent occlusion.

Finally, we tested the combination of optogenetic stimulation plus pharmacological inhibition at the same CeA site, to assess if muscimol/baclofen microinjections would prevent CeA ChR2 optogenetic stimulation from enhancing cocaine motivation. A separate group of rats initially received only bilateral CeA ChR2 virus (AAV5-Hsyn-ChR2-eYFP). During intra-jugular catheter implantation three weeks later, these same animals were also implanted with bilateral chronic guide cannula with tips implanted 1 mm above the same CeA virus sites (n=8).

Intravenous catheter implantation

Chronic intravenous jugular catheters for subsequent intravenous delivery of cocaine solutions were implanted in a separate surgery approx. 3 weeks later (Crombag et al., 2001). Briefly, after similar anesthesia and perioperative treatment as above, a Silastic intra-jugular catheter (Plastics One, Inc., Roanoke, VA, internal diameter=0.28 mm; external diameter=0.61 mm; dead volume=12 µl) was threaded into the right jugular vein. The outer end was passed under the skin toward the head along the dorsal neck, and exited from a secure subcutaneous anchor at the mid scapular region. Rats were allowed to recover for 7-10 days after surgery prior to beginning behavioral training. Catheters were flushed daily with a 0.2 ml isotonic saline solution containing 5 mg/ml gentamicin sulfate (Sparhawk, KS) to prevent infection and occlusions for the first 10 days. After 10 days, catheters continued to be flushed daily with 0.2 ml sterile isotonic saline alone (without gentamicin). Catheter patency was tested once before behavioral testing, and again after the end of all tests, by making an intravenous injection of 0.2 ml methohexital sodium to induce anesthesia (20 mg/ml in sterile water, JHP, MI). Rats that became ataxic within 10s were considered to have a patent catheter and included in behavioral analyses.

Behavioral Procedures

Instrumental choice of Laser+Cocaine versus cocaine alone

Excitation of Amygdala. Briefly, each rat was trained to instrumentally earn intravenous cocaine infusions by making nose pokes into either one of two portholes, and then allowed to

choose between them. The portholes were on the same wall ~ 5 cm apart, and detected nose pokes via beam breaks. Both portholes delivered cocaine infusions [0.3 mg (weight of the salt) per kg weight of the rat in 50 µL volume infused over 2.8 s] (NIDA, MD) dissolved in 0.9% sterile saline on a fixed ratio 1 (FR1) schedule. Additionally, one porthole (*Laser+Cocaine* port) arbitrarily designated for each rat, delivered an 8 s optogenetic CeA ChR2 or BLA ChR2 stimulation [8-10 mW blue laser (473 nm), 25 Hz (15 ms ON 25 ms OFF for 8 s train)], which began with the final nose poke and continued during and immediately after the infusion. Stimulation parameters were based on (Kravitz and Kreitzer, 2011; Robinson et al., 2014). The 8 s duration was aimed to paste photoexcitation onto the auditory cue at time of reward delivery. The other porthole (*Cocaine alone*) delivered an equal infusion of cocaine alone, but without any laser illumination. Each port's infusion was also accompanied by a distinctive 8 s auditory cue (either white noise or tone, counterbalanced between the two ports). Both portholes were permanently fixed in position for some rats (n=9 CeA ChR2; n=2 BLA ChR2), and always present in the chamber: each was illuminated whenever it was active and able to earn cocaine. Both portholes were movable for other rats (n=5 CeA ChR2: 4 bilateral CeA illumination; 1 unilateral CeA illumination; n=4 BLA ChR2): movable portholes were usually kept retracted into the wall, and only inserted into the chamber when actively available to earn cocaine. At the beginning of each session, for both porthole types, initially only one porthole was actively available to earn cocaine (illuminated if fixed in place; inserted into the chamber if movable). Next, only the alternative porthole was activated (illuminated or inserted), while the first porthole was dimmed or retracted. This initial one-at-a-time presentation ensured that a rat was exposed to each porthole and its outcome every day. Subsequently both portholes were inserted or illuminated together for the remainder of each session, allowing the rat to freely choose between the two portholes for the rest of the hour. Med Associates chambers (Med Associates, Inc, St. Albans, VT) with clear Plexiglas floors (30.5 x 24.1 x 21.0 cm) were used for training and testing instrumental cocaine self-administration, and contained an auditory speaker (for tone/white noise components of CSs). An infusion pump was located outside the sound attenuated chamber to allow for cocaine delivery. A video camera placed below the transparent chamber floor recorded the rat's behavior for subsequent analysis of consummatory behaviors and stereotypy.

Rats were trained and tested for 10 days on instrumental self-administration of intravenous cocaine (CeA ChR2 virus: n=15; BLA ChR2 virus: n=6; CeA/BLA control inactive virus: n=8; CeA eNpHR (halorhodopsin) inhibitory opsin virus: n=7). The first day began with a 2 hour session, and subsequent days were 1 hour sessions. Nose pokes into either porthole earned a 0.3 mg/kg infusion of cocaine hydrochloride. The session always began with one porthole illuminated or inserted and available to earn cocaine (either *Laser+Cocaine* or *Cocaine alone*; order counterbalanced across rats). Once cocaine was earned, the other porthole was activated next until cocaine was earned on it. This one-at-a-time presentation cycle was repeated a second time. Subsequently, both portholes remained equally available to earn cocaine for the rest of the session. Assignment of an auditory cue to laser-paired porthole versus the different auditory cue to the cocaine-alone porthole was always the same for a given rat across all days, but was counterbalanced between rats. For both portholes, a 20 s timeout period was imposed after each cocaine infusion earned, during which subsequent nose pokes into either hole had no consequence (See Figure 4.1). Rats were required to sample from both cocaine-delivering ports at least twice each day on the first two days, and all rats met this criterion and so continued in the experiment: 85% of rats (11/13) within the first hour on the first day and the remaining 15% during the second hour of the first day, and 100% in the first hour on the second day. All subsequent sessions lasted 60 minutes or 40 infusions, whichever occurred first. Any rat that failed to nose poke at least 5 times per session over three consecutive sessions within the first 10 days was excluded from analyses (n=2). For all rats, every nose poke earned a cocaine infusion on an FR1 schedule throughout Days 1-10 (i.e., one nosepoke earned an infusion, followed by 20 s timeout).

Inhibition of Amygdala (halorhodopsin or muscimol/baclofen). For rats receiving halorhodopsin inhibition of CeA for loss of function studies (n=7), all procedures were identical except that a constant yellow laser [8-10 mW (592 nm) was bilaterally substituted for CeA illumination in place of blue laser during training. The yellow laser (592 nm; 8 s constant duration; 8-10 mW) was associatively paired with each time cocaine was earned on one of the two cocaine portholes. CeA halorhodopsin rats were trained either with fixed nosepoke ports (n=2 rats) or retractable ports that were usually retracted, but phasically inserted separately for initial one-at-a time self-administration opportunities each session, or together to allow choice between the two for the remainder of each session (n=5 rats). Training at an FR1 schedule

continued for 10 days as above, with 8 s yellow laser bins paired with one cocaine option (counter-balanced across rats). On an additional day 11 test, the yellow laser was illuminated constantly for the entire 1 hr session (592 nm, 8-10 mW), while rats received the same schedule of cocaine options as on days 1-10, to detect whether constant optogenetic CeA inhibition would suppress cocaine consumption.

For rats receiving pharmacological GABAergic inhibition of CeA daily during training (muscimol/baclofen: n=5 rats; vehicle: n=4 rats), bilateral microinjections were delivered 15 min before the rat was placed in the self-administration chamber beginning on Day 3, and for each day following. To give microinjections, a rat was gently cradled by the experimenter's arms, and microinjectors were inserted into each bilateral guide cannula (microinjectors = 21 mm long, so as to extend 1 mm below ventral tip of guide cannula; Plastics One, Inc). Bilateral microinjections of a mixture of $GABA_A$ agonist muscimol (Tocris, Inc.) and $GABA_B$ agonist baclofen (Tocris, Inc.) [0.1 µg/0.2 µL of each combined in a single 0.5 µL volume of artificial cerebrospinal fluid (ACSF)] was delivered per side. Vehicle control rats received identical volume microinjections of ACSF alone. Doses and volumes were based on prior studies using muscimol/baclofen combinations in amygdala (Mahler and Berridge, 2009; Ho and Berridge, 2014). Microinjections were delivered by syringe pump at a rate of 0.5 µL over 1 min, and the microinjector was left in place for an extra minute to allow for drug diffusion. For the first 2 days of training, rats were only hooked up to intravenous catheter to allow habituation. Afterwards, from days 3-10, an optic fiber and cable was additionally attached to the headcap to match test conditions for optogenetic rats as described above.

Combined CeA ChR2 excitation + muscimol/baclofen inhibition. To test whether GABAergic inhibition would block CeA ChR2 enhancement of motivation, this group of rats received optogenetic CeA ChR2 excitation combined with pharmacological inhibition of the same CeA sites (n=4). Rats first received microinjections of muscimol/baclofen 15 min before each session from Days 1-4. Following microinfusion, microinjector tips were removed and replaced by optogenetic fibers, secured in place by being screwed on to the cannula guide thread. At the end of each session, optogenetic fibers were removed and replaced by dummy cannulas. On every day, rats were trained with CeA ChR2 stimulation in the two-choice task with identical procedures as for the CeA ChR2 group above. In order to test whether any CeA inhibition effects on behavior seen on Days 1-4 were permanent or state-dependent, no muscimol/baclofen

microinjections were administered before sessions on days 5-6. Each rat had either fixed portholes throughout all days (n=2 muscimol/baclofen, n=2 vehicle rats) or retractable portholes throughout all days (n=2 muscimol/baclofen, n=2 vehicle rats).

Progressive ratio test of breakpoint: Does CeA ChR2 amplify effort to obtain cocaine?

To independently assess if CeA ChR2 stimulation amplified the intensity of incentive motivation to earn cocaine, a progressive ratio test of instrumental effort was used to obtain a breakpoint measure on two successive days. On one day (order counter-balanced across rats), only the *Laser+Cocaine* instrumental nose port and outcome was offered (CeA ChR2: n=9 unilateral and n=3 bilateral; CeA control virus: n=5). On the other day, only the *Cocaine alone* port and outcome was offered. The alternative nose port was removed each day and replaced with wall inserts. Within each session, the number of nose pokes required to earn the next cocaine infusion (0.3 mg/kg) was increased after every cocaine reward, according to an exponential progressive ratio schedule (1,2,4,6,9,12,15,20,25,32,40,50,62,77,95,118,145,…) derived from the formula PR = $[5e^{(reward\ number\ x\ 0.2)}] - 5$ and rounded to the nearest integer (Richardson and Roberts, 1996; Saunders and Robinson, 2011; Robinson et al., 2014). Each cocaine infusion was followed by a 20 s timeout identical to that imposed during the instrumental two-choice test.

Laser self-stimulation (CeA laser self-administration without cocaine)

To more directly probe whether CeA ChR2 excitation was an independent reinforcer, the same rats from above could earn CeA laser self-stimulation pulses alone by performing new responses in three different situations. In an 'active-response' self-stimulation task, rats could earn CeA laser illuminations by simply touching one of two empty water spouts. In a second 'active response' self-stimulation task, using an instrumental response more similar to that used in our cocaine self-administration task, drug-naïve rats could earn CeA laser illuminations by making a nosepoke into a fixed porthole on two consecutive days. In a third 'passive response' self-stimulation task, rats could earn CeA laser illuminations by simply going to a particular location, or even just remaining in that location.

In the spout self-stimulation active task (spout-touch task), rats were placed into Med Associates operant chambers equipped with two novel and empty sipper spouts on the back wall of the chamber, positioned ~5 cm apart. A metal grid floor was wired to close a circuit to detect

contacts at each spout. Touching one spout (designated as 'active spout'; spout assignment counterbalanced between rats) delivered CeA laser stimulation [25 Hz; 8-10 mW; FR1 schedule; some rats always earned a 1 s pulse (n=6), and other rats always earned an 8 s pulse (n=7); no auditory cue]. The 1 s pulse was used because it has supported optogenetic self-stimulation in previous studies (Witten et al., 2011; Kravitz et al., 2012) .The 8 s pulse was used for other rats because it was identical in parameters used to amplify motivation for cocaine in our drug self-administration task above. Touching the other available spout produced no consequence, and its contacts simply served as a control measure for exploratory touches, general motor activity, or habitual spout investigation. Each self-stimulation test lasted 30 min, and each rat was repeatedly tested on 3 consecutive days.

In the nose-poke self-stimulation active task, procedures were similar to the spout task above, but with portholes rather than two spouts (n=6). The active porthole (arbitrarily assigned) earned either 1 s or 8 s pulses of 8 mW, 25 Hz laser illuminations (pulse duration was counterbalanced across rats, but always the same for an entire 1 hr session on a given day).

In the place-based self-stimulation passive task, a rat could earn real-time CeA laser stimulation simply by going to a particular corner location within a 4-corner chamber to trigger a motion detector, or simply by making any further movement while remaining in that corner (Robinson et al., 2014). This place-based self-stimulation task was modeled on the original Olds and Milner demonstration of deep brain electrode self-stimulation, in which a rat earned electrode stimulation simply by going to a particular corner of a table (Olds and Milner, 1954). The apparatus consisted of a 4-corner square Plexiglas chamber (38 x 38 cm) with bedding on the floor. A Plexiglas cylinder occluded the center of the chamber (20 cm diameter) so that rats were confined to the outer perimeter, where they could freely perambulate to enter or leave any of the four corners of the chamber. Each corner contained an infrared motion detector (Visonic, Bloomfield, CT) 46 cm above the floor that was triggered if the rat entered, and by any subsequent movement within the corner. Entry into a designated corner delivered a pulse of CeA ChR2 laser illumination lasting either 1 s or 8 s duration for different rats (25 Hz, 8-10 mW). Exit from that corner always terminated laser. The same corner was always used for a given individual rat on every session, but different rats were randomly assigned to different corners. Each self-stimulation session lasted 30 min, and each rat received 3 repeated sessions, one per day.

Switch from laser + cocaine to laser alone: can CeA ChR2 laser maintain responses during drug-extinction?

As a final test of laser self-stimulation, we assessed if CeA ChR2 stimulation by itself would at least *maintain* instrumental nosepoke responding after a substantial level of this response already had been well established by *Laser+Cocaine* combination. To test this, CeA ChR2 rats from the cocaine self-administration group above (n=7) were retrained for 2 days on the two-choice task (*Laser+Cocaine* versus *Cocaine alone*; FR1 schedule; days 20-21) to re-establish and confirm their nosepoke response. Then cocaine was discontinued from both portholes for four successive test days (days 22-25) to convert the task to pure CeA ChR2 laser self-stimulation (now without cocaine: cocaine-extinction). On these cocaine-extinction days, nosepokes into the original *Laser+Cocaine* porthole still earned 8 s bins of laser alone as before (8 s, 25 Hz, 8-10 mW), and both portholes delivered their associated auditory cues, but neither porthole delivered cocaine infusions. In other words, on days 22-25 rats had a choice between *Laser alone* vs. *Nothing* while performing the same instrumental responses that were previously reinforced by cocaine. For the next three days (days 26-28), laser was discontinued in addition to cocaine, and nosepokes into either port only delivered their associated auditory cues (*Nothing vs. Nothing*).

Anatomical localization: Sites, virus expression, Fos plumes, & localization of function maps.

At the end of behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital (150-200 mg/kg) and transcardially perfused 75 min after receiving laser. For ChR2 experiments, blue laser (8-10mW) was administered at 25 Hz, 8 s ON 22 s OFF cycle for 30 min duration immediately before anesthetization. For halorhodopsin (eNpHR) experiments, constant yellow laser (8-10 mW) was bilaterally illuminated continuously for 30 min duration before anesthetization. Brains were stored in 4% paraformaldehyde, cryoprotected in 30% sucrose, and sliced at 40 µm coronal sections for identification of optic fiber placements and virus expression. Brains were additionally processed for Fos protein expression and for Fos plumes surrounding the illuminated optic fiber (CeA ChR2: n=5; BLA ChR2: n=5; CeA eNpHR: n=6). Slices were blocked in 5% normal donkey serum/0.2% Triton-X solution for 30 min before being incubated for 24 hours in a polyclonal goat anti-c-fos IgG primary antibody (Santa Cruz

Biotechnology; 1:1000 dilution; Lot# K0415, RRID: AB_2106783), followed 1 day later by 2 hours in Alexa Fluor 594 donkey anti-rabbit IgG secondary antibody (Life Technologies; 3:1000 dilution; Lot# 1668652, RRID: AB_141637)(Faure et al., 2008). Sections were mounted, air dried, and cover slipped with ProLong Gold anti-fade reagent (Invitrogen). Control virus rats that also received laser illumination prior to perfusion (Total n = 10; CeA Blue laser: n=4; BLA Blue laser: n=3; CeA Yellow laser: n=3) served as controls to compare baseline effects of laser illumination on local Fos plumes (Stujenske et al., 2015). Additional un-operated rats (no surgery; completely naïve) served as normal baseline controls to determine spontaneous baseline levels of Fos expression in CeA and BLA (n=6).

Optic fiber sites, GFP virus expression, and Fos expression was measured using images taken with a Leica microscope (Leica Microsystems, Inc., Buffalo Grove, IL) at 10x and 40x magnification, and marked in Adobe Illustrator (RRID: SCR:014198) on a rat brain atlas (Paxinos & Watson, 2007). For each CeA site, nine images (3 X 3; 10X magnification) were compiled using Oasis Surveyor software (Objective Imaging, Inc., Kansasville, WI; RRID: SCR014433) into one single image centered on the fiber tip, and spread/intensity of virus and neuronal Fos expression surrounding the tip was mapped. Our procedure used for measuring Fos plumes surrounding a fiber optic tip induced by ChR2 photostimulation was modified from (Robinson et al., 2014). Immunoreactivity for Fos-like protein was visualized using a fluorescent microscopy filter with an excitation band at 515–545 nm for Fos-positive cells. The number of Fos-expressing cells was counted within successive blocks (50 x 50 µm) along eight radial arms emanating from the fiber optic tip at 10x magnification. Counts continued outward along an arm until at least two sequential blocks contained zero Fos-labeled cells, which was taken as marking the radius of the Fos plume along that arm. Intensities of Fos elevation in neurons were calculated in terms of percent change from either of two baselines: 1) *Inactive-virus control baseline*: CeA tissue from rats with an optically-inactive control virus containing GFP gene but lacking ChR2 gene, which received laser illumination prior to perfusion similarly to ChR2 rats. 2) *Normal tissue baseline:* CeA or BLA counts of Fos from un-operated control brains of normal rats. Elevations were denoted in increments of > 200% elevation above the respective two baselines, or higher > 300% elevation above. Inhibitory suppressions were denoted in decrements of <0.75 fraction relative to the two baselines (i.e., >25% suppressions) and <0.50 fractions (i.e., > 50% suppressions). The local region surrounding a fiber tip that expressed Fos

elevation was considered to constitute the local stimulated 'Fos plume', and its diameter and volume reflected the extent of local neuronal activation induced by laser illumination in CeA ChR2 rats. Conversely, the local region surrounding a fiber tip that expressed Fos suppression was considered to constitute the local inhibited 'Fos anti-plume', with its diameter and volume reflecting the extent to which neuronal activation was suppressed using halorhodopsin. The size of measured stimulated Fos plume was used to assign the scaled size of symbols expressing behavioral consequences in brain atlas maps showing localization of function, in which the color of symbols represented behavioral effects produced at particular sites (Figure 3).

For quantification of viral spread around the optic fiber tip, GFP-labeled cells were individually counted within successive blocks (50 x 50 µm) along eight radial arms emanating from the fiber optic tip at 10x magnification (Robinson et al., 2014). The number of GFP-labeled cells in each block was counted to assess the intensity of virus expression at each point, and to measure the radius of GFP expression along each arm. Intensity of virus infection was mapped as elevations over normal zero baselines.

Statistical analysis

Results were analyzed in SPSS software (RRID: SCR:002865) using repeated-measures ANOVAs to examine responses for either nose port over training days, followed by *t*-tests for individual comparisons on specific days with Bonferroni corrections. For non-normally distributed data including all progressive ratio tests, Friedman's two-way ANOVAs were used as nonparametric within-subject tests and Kruskal-Wallis one-way ANOVAs for between subject tests, followed by Wilcoxon sign-ranked/ Mann-Whitney tests for individual comparisons. Effect sizes for parametric tests were calculated using Cohen's d and for nonparametric tests using $r = \frac{Z}{\sqrt{N_1+N_2}}$. For all analyses, the significance level was set at $p < 0.05$, two-tailed.

Results

Two-choice instrumental task

CeA ChR2 laser pairing made rats prefer their *Laser+Cocaine* option by roughly 2:1 over the *Cocaine Alone* option as early as the 1st day in the two-choice cocaine self-administration task, where rats could choose between earning either intravenous infusions of cocaine alone (*Cocaine alone*) or cocaine plus unilateral CeA ChR2 laser illumination (*Laser+Cocaine*)

(Figure 4.1; Movie 1). By the third day, CeA ChR2 preference reached a 4:1 ratio and statistical significance (n=10; t_9=2.52, p=.03, Cohen's d=1.23, 95% C.I. [.99, 18.4]), and continued to rise to 10:1 or more by the 10th day of FR1 training (t_9=3.79, p=.004, d=1.8, 95% C.I. [7.5, 30]; Figure 2A).

Bilateral delivery of CeA ChR2 laser stimulation (n=5 rats) magnified the strength of *Laser+Cocaine* preference even more than unilateral CeA laser (n=10 rats; Laser x Uni/bilateral: $F_{1,13}$=4.48, p=.027; all rats received bilateral CeA ChR2 virus microinjections and bilateral implantation of optic fibers, but a single fiber coupling failed in some rats, resulting in unilateral CeA ChR2 stimulation). For example, on day 1 bilateral CeA ChR2 stimulation produced a 4:1 preference for the *Laser+Cocaine* option, compared to 2:1 for unilateral stimulation (Laser x Uni/bilateral: $F_{1,13}$=2.15, p=.16). By day 10, bilateral stimulation produced an 18:1 preference (t_4=59.0, p=.000, 95% C.I. [33, 37], d=1.73) compared to only 10:1 for unilateral stimulation (see above; Laser x Uni/bilateral: $F_{1,13}$=5.49, p=.036). Further, bilateral CeA ChR2 rats took nearly double the amount of cocaine via their *Laser+Cocaine* option (11.4 mg/kg total cocaine; maximum of 39 infusions per day on Days 6-10) compared to unilateral rats (7 mg/kg total cocaine; 23 infusions on day 10) (t_{12}=2.7, p=.019, 95% C.I. [-8.8,-1], d=-2.34).

By contrast, rats with ChR2 virus in basolateral amygdala (BLA) failed to develop any consistent preference between *Laser+Cocaine* versus *Cocaine alone* (Figure 4.2C; n=6; $F_{1,5}$=4.253, p=.095), and did not differ from inactive-virus control rats in random *Laser+Cocaine* preference ($F_{1,9}$=0.003, p=.96). Thus, BLA ChR2 rats chose more or less equally between the two options, and were significantly different from CeA ChR2 rats, which preferred *Laser+Cocaine* ($F_{1,17}$=11.4, p=.004). CeA ChR2 rats (both bilateral and unilateral) also consumed more total cocaine overall on day 10 than did BLA ChR2 rats (29 vs. 9 total infusions or 3 mg/kg total cocaine; Figure 2B; t_{18}=3.54, p=.002, 95%C.I. [2.42, 9.5]). Total nosepoke responses (*Laser+Cocaine* and *Cocaine alone* combined) by BLA ChR2 rats were actually less than control virus rats ($F_{1,9}$=33.6, p=.000). Within BLA ChR2 rats, 4 rats earned bilateral stimulation and 2 rats earned unilateral stimulation, but these BLA ChR2 bilateral and unilateral stimulation conditions did not differ in outcome ($F_{1,4}$=.122, p=.74), nor was there any interaction between port preference and bilateral vs. unilateral stimulation condition ($F_{1,4}$=.28, p=.63).

CeA inactive-virus control rats, with illuminated optic fibers in CeA but infected with optically-inactive GFP virus that lacked the ChR2 gene, failed to develop any consistent preference for their *Laser+Cocaine* option over *Cocaine alone,* and continued to choose equally between the two options for all 10 days (Figure 4.2D; n=5; $F_{1,4}$=.77, p=.44). As a result, inactive-virus control rats (choosing randomly) differed from all CeA ChR2 rats (which preferred *Laser+Cocaine)* ($F_{1,19}$=6.52, p=.02). For example, on day 10, inactive virus rats poked less at their *Laser+Cocaine* port than either unilateral or bilateral CeA ChR2 rats (t_{19}=2.6, p=.01, d=1.79, 95% CI [-33.6, -3.4]), and poked more at their *Cocaine alone* port than CeA ChR2 rats (t_{17}=3.1, p=.006, d=1.4, 95% C.I. [3, 15.6]). However, overall, inactive-virus rats consumed less cocaine than CeA ChR2 rats (5 mg/kg cocaine total in 17 infusions; vs. 7 mg/kg unilateral CeA ChR2 and 11.4 mg/kg bilateral; Figure 4.2B; $F_{2,22}$=6.35, p=.002).

Total responding for cocaine on the last day (*Laser+Cocaine* and *Cocaine alone*) did not differ across phases of the estrus cycle, neither at the beginning of training (unilateral CeA rats: $F_{1,4}$=2.35, p=.2; bilateral CeA rats: $F_{1,3}$=.6, p=.5; BLA rats $F_{1,3}$=.01, p=.92),) nor at the end of training for any rats (unilateral rats: $F_{1,4}$=.02, p=.89; bilateral CeA rats: $F_{1,3}$=.6, p=.5; BLA rats $F_{1,3}$=.01, p=.92).

Fos plumes versus virus infections: Fos plume sites determine localization of function

ChR2 Fos plumes were much smaller than CeA or BLA zones of virus infection, and therefore the mean Fos plume radius (0.2 mm outer radius of doubled elevation) was used to set the size of individual map symbols in Figure 4.3. Histological analysis of GFP expression revealed zones of ChR2 virus infection averaging ~1.8 mm diameter in either CeA or BLA, forming a roughly spherical volume of 2.6 mm^3, and often filling most of either CeA (CeA volume is 2.4 mm^3) or BLA. In CeA, optic fiber tips were concentrated mostly in the caudal and middle A-P zones of CeA (Figure 4.3), and distributed mediolaterally in roughly equal numbers in the medial subdivision of CeA (CeM; n=5), lateral subdivision of CeA (CeL; n=5) and capsular subdivision of CeA (CeC; n=4). In BLA, sites were scattered throughout mid-rostral BLA extent and more laterally. Mapping of CeA fiber sites that were behaviorally effective in the two-choice task revealed that sites in CeM subdivision as well as in CeL comparably support CeA ChR2 induction of a 25:1 preference ratio for *Laser + Cocaine* (Figure 3). Two sites in CeC capsular nucleus, slightly dorsal to CeM or CeL, were also effective at amplifying motivation for

cocaine. By contrast, BLA sites were generally ineffective at enhancing cocaine pursuit. In fact, a few BLA sites appeared to produce negative avoidance of their laser-paired cocaine option by at least 3:1 to 5:1 ratios (though not significant for the entire BLA Ch2 group), and further actually suppressed total cocaine intake slightly compared to inactive virus controls ($F_{1,9}$=33.6, p=.000).

Laser illumination of fibers in CeA ChR2 rats produced 0.02mm^3 Fos plumes of elevated expression surrounding the optic fiber tips, compared either to control levels measured in inactive-virus rats (0.14 mm radius; $F_{1,6}$=19.2, p=.000, 95% C.I.[-.173, -.063]), or to CeA tissue from normal un-operated rats ($F_{1,3}$= 15.6, p=.001, 95% C.I.[-.189, -.061]). Fos plumes contained a tiny < 0.01 mm^3 center of intense > 300% Fos elevation (3.4±.2.1 Fos+ neurons), compared to illumination in control rats with inactive-virus (0.0114±.11 Fos+ neurons) (also > 300% above CeA levels in un-operated control brains: 0.0042±.065 Fos+ neurons). The intense center was surrounded by a larger 0.02 mm^3 volume outer plume of less-intense 200% Fos elevation, arrowhead-shaped and extending farthest 0.21 mm ventrally beneath the illuminated tip (range 0.07 to 0.2 mm in all directions), and elevation declined from 150% toward baseline within 0.2 to 4 mm below optic fiber tip (Figure 4.4). CeA ChR2 laser illumination induced Fos plumes of similar sizes, regardless of whether the optic fiber was placed in CeM, CeL, or CeC subdivisions ($F_{2,5}$=.373, p=.706). By contrast, laser produced no Fos plumes in control inactive-virus rats (p=1.0, 95% C.I. [-.08, .07]).

It seems noteworthy that the average Fos plume produced by CeA ChR2 laser stimulation was less than ~2% of the volume of average CeA virus infection (Figure 4.4). Further, each 0.02 mm^3 Fos plume corresponded to only ~1% of the entire anatomical volume of CeA. This observation suggests that optogenetic illumination of ChR2 directly modulates Fos expression of neurons within a tiny fraction of CeA, and a much smaller anatomical zone than the total range of virus infection, but is still sufficient to induce powerful behavioral effects, and this was true whether sites were in CeL or CeM. Further suggesting that the small Fos plumes were more important than total virus infection, the size of larger volumes of virus infection did not correlate with degree of *Laser+Cocaine* preference (Spearman's rho, r_s= .018, p=.969).

Laser illumination of fibers in BLA ChR2 rats produced similarly small 0.03 mm^3 Fos plume of elevated expression surrounding the optic fiber tips compared to control levels

measured in inactive-virus rats (.16 mm radius; $F_{1,8}$=22.9, p=.000, 95% C.I. [.2, .6]), and compared to BLA tissue from normal un-operated rats (.25mm radius; $F_{1,7}$=9.31, p=0.003, 95% C.I. [.8, 1.27]). Similarly to CeA ChR2 plumes, BLA ChR2 Fos plumes also contained a smaller .01 mm^3 center of intense > 300% Fos elevation (26.5±8.6 Fos+ neurons) over Fos levels in control rats with inactive-virus (6.5±3.5 Fos+ neurons) (Figure 5).

Halorhodopsin in CeA, when illuminated by yellow laser, suppressed Fos expression by at least 25% below control levels: creating 0.016 mm^3 inhibitory plumes with approximately 0.25 mm radius (i.e., which we call anti-plumes). These Fos levels were only <0.75 of control levels (i.e., >25% suppression) compared to baselines of either inactive-virus control rats that also received yellow laser in CeA ($F_{2,9}$=60.9, p=.000, 95% C.I. [.21, .13]), or of normal un-operated rats that received no laser (0.14 mm radius; $F_{2,8}$=22.9, p=0.001, 95% C.I. [.21, .1]). Halorhodopsin antiplumes also contained smaller 0.003 mm^3 centers of even more intense suppression to levels between 0.1 to 0.5 of inactive-virus baseline levels (i.e., >50% suppression to >90% suppression), and these centers had nearly a 0.1 mm radius (1±.39 Fos+ neurons), compared to normal un-operated tissue (11±1 Fos+ neurons; Figure 4.4). Thus, halorhodopsin illumination by yellow laser produced opposite neurobiological effects on local neuronal Fos expression than ChR2 illumination by blue laser in CeA. The opposite effects of illuminating halorhodopsin versus ChR2 confirm that these manipulations have opposite neurobiological effects on neuronal Fos expression in CeA, even though Fos changes do not always correspond to electrical depolarization versus hyperpolarization. The comparable sizes of ChR2 Fos plumes versus halorhodopsin Fos anti-plumes further indicate that both manipulations primarily affect neurons within a 0.1 mm (center) to 0.3 mm (outer plume) radius of the optic fiber tip, even if virus expression or light extends further from the tip. That relatively small radius suggests that infected neurons may have a threshold of illumination required to alter Fos expression, which is met only by neurons lying within that radius with these laser parameters.

Nose-poke duration and consummatory responses to porthole

Beyond making CeA ChR2 rats choose *Laser+Cocaine* far more often than *Cocaine alone*, laser pairing in CeA ChR2 rats also tripled the duration of each nose poke they made into that *Laser+Cocaine* porthole (3 s), compared to poke duration in the alternative *Cocaine alone* porthole (1 s) (t_8=4.92, p=.001, 95%C.I.[.88, 2.44], d=2.374). By comparison, inactive-virus

control rats made pokes of merely 1s durations in both their *Laser+Cocaine* and alternative portholes (t_{11}=2.91, p=.014, 95% C.I. [-2.9,-.4], d=3.5). This ChR2 duration extension of the consummatory nosepoke for *Laser+Cocaine* was found in both bilateral and unilateral CeA ChR2 rats (p=.396, 95% C.I. [-.47, 1.72]). The prolongation of nose poke duration appeared related to the induction of oral consummatory behaviors emitted during the nosepoke toward their metal laser-paired porthole.

Specifically, laser pairing made CeA ChR2 rats display novel active consummatory reactions of nibbling and biting of the metal rim of their laser-paired porthole, in addition to perseverative sniffs that most rats commonly display toward Pavlovian cocaine cues. During nibbles, a rat's upper teeth at least contacted the metal rim for a brief moment (< 0.5 s), sometimes repeatedly within 1-2 s. In bites, a CeA ChR2 rat's upper and lower incisors closed completely on the outer rim of its *Laser+Cocaine* porthole, remaining closed for up to 1 s. A bite often was accompanied by a strong pulling motion of the head and neck away from the wall, which sometimes actually succeeded in moving the metal porthole rim a millimeter or so further out from its anchored position. Approximately 80% of CeA ChR2 rats nibbled and/or bit their *Laser+Cocaine* retractable porthole, by comparison to 0% of inactive-virus control rats or 0% of BLA ChR2 rats (Figure 4.6A). Nibbles and bites were specifically directed to the *Laser+Cocaine* porthole, as CeA ChR2 rats were never observed to nibble or bite their *Cocaine alone* porthole, even when they made nosepokes into it. Bites and nibbles toward the laser-paired porthole were observed both in CeA ChR2 rats that received unilateral laser illumination (n=1) or bilateral laser illumination (n=4).

Actual intoxication with cocaine emerged as a further facilitating or necessary factor for CeA ChR2 consummatory bites and nibbles of the laser-paired retractable porthole: For example, bites and nibbles often did not emerge until the second half of a 60 min session for CeA ChR2 rats, after the rat had already consumed about 1.5 mg/kg to 3.3 mg/kg cocaine (Bites during 1st half vs. 2nd half of session: t_4=3.68, p=.02, 95%C.I.[-16.1,-2.3], d=-3.5). Some rats that had previously bitten their porthole late in a session, subsequently emitted a few bites in the first half of a session on following days, but still tended to emit most bites later in the session (Bites during 1st half vs. 2nd half of session: t_4=3.8, p=.02, 95%C.I.[-13.4,-2.2], d=-3.8).

Finally, it also appeared necessary that the porthole actively move in and out of the chamber, as only moving or retractable portholes elicited bites and nibbles from CeA ChR2 rats when paired with laser (n=5), and not fixed or immovable nose ports that always remained constantly in the chamber (n=10) (bites: $F_{1,9}$=6.5, p=.04; nibbles: $F_{1,9}$=9.0, p=.02). Fixed portholes failed to elicit consummatory bites even when paired with bilateral laser stimulation in CeA ChR2 rats (n=1). Pavlovian cue features are known to be important as unconditioned reward features in determining a conditioned response, as for example when a rat responds to another rat that predicts food with pro-social behaviors (Timberlake and Grant, 1975), but to a rolling ball bearing that predicts food with predatory pounces (Timberlake et al., 1982). Thus, the sudden appearing and disappearing features of the moving porthole, its cocaine reinforcement, and its associative pairing with CeA ChR2 laser stimulation, all likely combined together to produce the consummatory bites emitted toward the metal object. Still, a retractable porthole only elicited bites here when it had *Laser+Cocaine* status, and the same CeA ChR2 rats never bit their retractable *Cocaine Alone* porthole. Finally, inactive-control virus rats and BLA ChR2 rats never displayed bite or nibble consummatory responses, not even to a retractable cocaine porthole paired with laser ($F_{2,7}$=32.2, p=.000; Figure 4.6B and 4.6C).

Nibble or bite consummatory responses toward a metal cocaine cue by rats have never been reported before to our knowledge. Instead rats are reported typically to emit approach and sniff responses toward a Pavlovian light, location or object CS for cocaine in autoshaping or sign-tracking studies, but not to bite or nibble the CS object with mouth or teeth (Kearns and Weiss, 2004; Uslaner et al., 2006). Instead, only food-associated CSs are known to evoke nibbles and bites as consummatory responses (DiFeliceantonio and Berridge, 2012; Mahler and Berridge, 2009; Tomie, 1989). We note that CeA ChR2 stimulation similarly increased consummatory bites and nibbles of a sucrose-associated metal lever paired with laser in our earlier study (Robinson et al., 2014). Bites and nibbles of the cocaine laser-paired cue here might reflect ingestive incentive motivation, or conceivably a different CeA ChR2-induced motivation, such as predation or aggression, or some unspecified incentive attractiveness that made the metal porthole more able to elicit the urge to bite, nibble and orally grasp.

By contrast, control and BLA rats always merely sniffed their portholes, the more typical consummatory response to a cocaine cue (Kearns and Weiss, 2004; Uslaner et al., 2006). CeA ChR2 rats also emitted consummatory sniffs toward their portholes (both fixed and retractable

ports), in addition to nibbles and bites, and in fact, emitted twice as many sniffs to their *Laser+Cocaine* porthole as control inactive-virus rats did toward either porthole (mean + SEM: 5 ±1 vs. 2 ±.8 sniffs; t_{11}=3.22, p=.008, 95% C.I.[.85, 4.5], d=1.82). CeA ChR2 rats also emitted ~6 times more sniffs to their *Laser+Cocaine* porthole than to their *Cocaine Alone* porthole (t_8 =6.83, p=.000, 95%C.I.[1.5, 3], d=1.45). Even among rats with retractable portholes, sniffs were ~2 times greater to their *Laser+Cocaine* porthole than to their *Cocaine alone* porthole (t_3 =3.3, p=.04, 95%C.I. [1.0, 4.1], d=2.36; Figure 4.6D). Thus CeA ChR2 pairing potentiated sniffing, in addition to creating novel bites and nibbles as consummatory response, and enhanced responses were always directed specifically toward the *Laser+Cocaine* porthole.

CeA ChR2 perseveration during 20s time-out after cocaine

As soon as a cocaine infusion actually began, control and BLA rats typically stopped making frequent nose-pokes into the porthole they had chosen [randomly overall] ($F_{1,5}$=2.3, p=.19), but CeA ChR2 rats perseverated with longer pokes into their *Laser+Cocaine* porthole that continued during an infusion, and even afterwards during the 20 s timeout when no further cocaine could be earned ($F_{1,13}$= 8.9, p=.01). For example, CeA ChR2 rats in this sense wasted 50% more instrumental nosepokes than inactive-virus control rats, making pokes that could earn nothing during time-outs on the final four days of training (57% vs 38% of total nose pokes in each session were made during the 20s timeout; $F_{2,15}$=4.36, p=.03). CeA ChR2 perseveration after infusion was shown by both bilateral and unilateral CeA ChR2 rats ($F_{1,13}$=.29, p=.6).

Loss of CeA function: Optogenetic CeA inhibition does not alter choice, but pharmacological CeA inhibition abolishes cocaine pursuit and laser preference

In the inhibitory or loss of function group, CeA halorhodopsin rats that had 8 s illuminations of constant yellow CeA laser (592 nm, 8-10 mW) paired with one of two cocaine options (n=7), suppressing Fos to 10% to 50% normal levels within a 0.25 mm anti-plume radius, did not alter preference and simply chose equally between the two cocaine options when given a choice (Figure 4.7A; $F_{1,6}$=0.27, p=.62). However, overall cocaine consumption appeared marginally reduced for CeA halorhodopsin rats, a suppression which became significant if we raised the criterion for control rats from 5 infusions per day for three days to 6 infusions per day for three days (eliminating one low outlier from inactive-virus control rats and leaving a control n=2), ($F_{1,7}$=10.5, p=.01), though not if we dropped all inclusion criteria ($F_{1,8}$=1.7, p=.23).

Applying the stricter inclusion criterion, halorhodopsin rats took only about half as much cocaine as control rats on day 1, (t_7=4.3, p=.003, 95%CI[-21, -6], d=-3.02), and were still taking less on day 5 (t_7=3.5, p=.01, 95%CI[-39, -7.5], d=-3.7). However, turning the yellow laser on for the entire 60 min session on day 11 did not further alter that pattern or induce any further suppression of intake (t_7=1.3, p=.23, 95%CI[-39, 11]).

CeA inhibition by GABAergic microinjections

By contrast, rats that received pharmacological inhibition of CeA via bilateral microinjections of a muscimol/baclofen cocktail from Day 1 of training nearly completely failed to self-administer cocaine once they were connected to headcap cables on the 3rd day (cables were needed for laser optic fiber connections in optogenetic studies, and so were added here to keep conditions similar even though no laser was given to the microinjection-only group). On the first two days of training, CeA muscimol/baclofen rats were not connected to head cables, and did self-administer cocaine. On the first day, muscimol/baclofen rats had lower cocaine self-administration levels than vehicle control rats, though not significant (t_6=2.4, p=.05). However, on the 2nd no-cable day, consumption levels did not differ statistically (t_6=.14, p=.89). A closer look at individuals revealed that 2 of 6 CeA muscimol/baclofen rats began to robustly self-administer 3 to 5 daily infusions on the first day, and rose to 7 to 11 on the 2nd day, while 3 other CeA muscimol/baclofen rats remained at 0 to 1 infusions on both days (control vehicle rats all consumed 5 to 15 infusions on both days). However, beginning on day 3, once headcaps were connected to optic fiber cables for all rats, CeA muscimol/baclofen microinjections apparently combined with the additional challenge of mild head tension from head cables, nearly completely prevented any cocaine-self-administration: zero or one infusion for all CeA muscimol/baclofen rats versus on average 12 infusions for control CeA vehicle microinjection rats. CeA muscimol/baclofen rats all remained at zero or 1 daily infusion from Days 3 to Day 10, whereas control rats rose gradually to ~20 infusions by Day 10 (Figure 4.7B; day x group interaction: $F_{7,42}$=2.5, p=.03). We note that CeA muscimol/baclofen microinjections may induce some degree of general motor suppression (Mahler and Berridge, 2009), which could have contributed to disrupting cocaine self-administration here. However, clearly motor suppression was not so great as to by itself disrupt self-administration, given that no disruption of self-administration was seen following muscimol/baclofen microinjections on Days 1 and 2, but only emerged when headcaps were first connected to optic fiber tethers on Day 3. At that point, CeA

muscimol/baclofen suppression of motor, sensory, motivational, and/or associative functions apparently only then became sufficient to disrupt the more challenging task demands presented by having to make nose-pokes while head-tethered in to self-administer cocaine.

The relatively severe disruption of cocaine self-administration by CeA muscimol/baclofen microinjections (when combined with head cables) versus much milder effects of CeA halorhodopsin illumination might possibly reflect less neuronal inhibition by halorhodopsin than by muscimol/baclofen microinjections. This would be consistent with Fos plume data, as halorhodopsin here induced anti-plumes in which the zone of suppression to 50% of normal Fos levels extended approximately 0.25 mm in radius from optic fiber tip. By comparison, CeA muscimol microinjections have produced larger anti-plumes in which the zone of 50% Fos suppression extended 0.75 mm from microinjector tip (Mahler and Berridge, 2009). A 3-fold difference in radius would correspond to >25-fold difference in volumes of CeA tissue inhibited by halorhodopsin illumination versus muscimol microinjection (0.06 mm^3 to 1.76 mm^3; assuming roughly spherical shapes). Also supporting the possibility that halorhodopsin causes only mild neurobiological inhibitions, electrophysiological studies have reported that in infralimbic cortex 70% of affected neurons were inhibited, but 30% of neurons oppositely excited, by halorhodopsin (Smith and Graybiel, 2013). Similarly, in ventral pallidum 80% of neurons were unaffected by halorhodopsin, while in the affected 20% subgroup 2 out of 3 neurons were inhibited and the remaining 1 neuron was excited (Chang et al., 2017). If comparably weak electrophysiological effects occurred in CeA, this might also help explain why behavioral effects of CeA halorhodopsin were relatively weak.

CeA muscimol/baclofen inhibition prevents CeA ChR2 control of motivation

In a final CeA inhibition group, we tested whether muscimol/baclofen CeA inhibition during training would block the ability of simultaneous CeA ChR2 excitation to intensify cocaine motivation and establish preference for the *Laser+Cocaine* option. CeA inhibition/excitation rats received muscimol/baclofen microinjections prior to sessions on each of the first four days of training. In each session, they received CeA ChR2 laser pairings with one cocaine option, as in CeA ChR2 groups above. Results showed that CeA muscimol/baclofen microinjections completely prevented CeA ChR2 laser pairing from generating any detectable enhancement in motivation for cocaine or establishing any detectable preference for the

Laser+Cocaine option over *Cocaine Alone* option (Figure 4.7C; $F_{1,3}$=1.0, p=.39). Instead these rats made only zero to one nosepokes per session, randomly distributed between the two options. On the fifth and sixth day, muscimol/baclofen microinjections were omitted, to assess whether the suppression of self-administration was enduring or instead was CeA state-dependent. Without simultaneous CeA inhibition, cocaine intake immediately jumped on the 5th day to about 30 total infusions, and a 3:1 preference immediately emerged so that CeA ChR2 rats now made ~25 nosepokes for their *Laser+Cocaine* option vs. about 6 nosepokes for *Cocaine alone* (t_3=3.7, p=.03, 95%CI[2.4, 35], d=2.19). This level of effort was ~20 times higher than on the previous day 4, when muscimol/baclofen microinjections had been received (t_3=4.14, p=.03, 95%CI[-43,-5], d=-3.7), even though responses at the *Cocaine alone* port did not significantly rise from days 4-5 (t_3=2.3, p=.11, 95%CI[-19,3.3]. This renewed self-administration pattern remained stable on Day 6 when muscimol/baclofen microinjections again were omitted, and CeA ChR2 preference for *Laser+Cocaine* continued at about 26 entries in the *Laser+Cocaine* port vs. 8 in the *Cocaine alone* port (t_3=4.9, p=.02, 95%CI[6, 29], d=2.8).

Progressive Ratio: CeA ChR2 stimulation amplifies incentive motivation breakpoint

Does CeA ChR2 stimulation actually increase the intensity of incentive motivation for cocaine? We ran a progressive ratio or breakpoint test to independently assess if laser would increase effort rats were willing to exert to obtain cocaine, in a 2-day within-subject comparison. Rats were presented with only one cocaine option per day (order counterbalanced across rats): either *Cocaine alone* or *Laser+Cocaine*. On each day, the available porthole earned their customary outcome, either cocaine infusions plus 8s laser illuminations and its auditory cue (*Laser+Cocaine*), or only cocaine infusions plus its distinctive auditory cue (*Cocaine alone*). However, the effort required to earn each reward progressively increased after each infusion during a session. Overall, CeA ChR2 rats were willing to work about 8 times harder on their *Laser+Cocaine* day (breakpoint = 75) than on their *Cocaine alone* day (breakpoint = 9; n=11; Wilcoxon Signed Ranks Test, Z=-2.93, p=.003, r=0.88; Figure 4.8A). Further, among CeA ChR2 *Laser+Cocaine* sessions, rats with bilateral CeA illumination had breakpoints nearly twice those of rats receiving unilateral illumination (130 vs. 54; n=3 bilateral, n=9 unilateral), although with only 3 bilateral rats this difference was not statistically significant (X^2=-5.29, p=.24). Overall, the total cumulative number of pokes per day rose under *Laser+Cocaine* day by an order of magnitude to 348 ±140 from 25 ±10 on *Cocaine alone* day (Z=-2.93 p=.003, r=0.88;

Figure 4.8B) for the same rats. This difference was evident within the first 5 min of each day's session (23 *Laser+Cocaine* vs. 3 *Cocaine alone* pokes; Z=-2.37, p=.018, r=0.71), and continued robustly for the remainder of the 60min session (Z=-2.93, p=.003, r=0.88; Figure 4.8C). By contrast, breakpoints of control inactive-virus rats were insensitive to the presence of CeA laser (breakpoint = 21 & 16; n=4; Z=-.37, p=.715). Inactive virus control rats also made similar total numbers of nose pokes on both days (*Laser+Cocaine*=60*; Cocaine Alone*=48; Z=-.405, p=.686), and fewer overall than CeA ChR2 rats (Mann Whitney U Test, Z=-2.03, p=.04, r=0.61). Order of *Laser+Cocaine* versus days did not influence responding (Mann Whitney U test, Z=-1.14, p=.26).

CeA ChR2 laser by itself fails to support self-stimulation in same rats

In three separate tests of laser self-stimulation, CeA ChR2 illumination by itself failed to reinforce responses even though laser had previously potentiated cocaine pursuit in the same rats: an active response task (object-touch self-administration), a passive response task (place-based real-time self-stimulation), and an instrumental nose-poke response task (minus cocaine delivery).

In a spout-touch self-stimulation task requiring active responses, every touch on a designated metal spout (empty drinking spout) with paw or mouth closed an electrical contact and earned a brief pulse of laser stimulation (either 1 s or 8 s duration for different rats; 25 Hz, 8-10 mW, blue laser) (Kravitz et al., 2012), whereas touching an alternative spout earned nothing. Results showed that CeA ChR2 rats failed to touch the active spout any more than the inactive spout (n=12; $F_{1,10}$=.013, p=.91; Figure 4.9A). No detectable self-stimulation behavior was obtained at either 1 s pulse duration (n=6; $F_{1,5}$=.046, p=.84) or 8 s pulse durations (n=6; $F_{1,5}$=.075, p=.79). Rats touched both spouts between 20-70 times per session for each of three consecutive sessions (possibly attracted because spouts resembled water drinking spouts in home cage), but with roughly equal frequency ($F_{2,20}$=.72, p=.5). On the first two days, rats touched both spouts less frequently if earning 8 s duration CeA pulses than 1 s pulses, possibly suggesting a slight general deterrent effect of longer CeA pulses or general arousing effect of shorter pulses (first day 65 touches 1 s vs. 12 touches 8 s; t_2=3.2, p=.01, 95%CI [16.1, 89.5], d=2.35; second day t_{10}=2.5, p=.03, 95%CI [-17.9, 36.9], d=.45). However, even that difference disappeared by the third day (t_{10}=.87, p=.4).

In a second active-response task, spout-touching was replaced by nose-poking as the instrumental response, similar to the instrumental response used for cocaine self-administration. This was to test the alternative interpretation that nosepokes were simply an easier response for rats to acquire, and that a difference in task ease explained why CeA laser controlled cocaine self-administration but not laser self-stimulation in the spout task. Drug-naïve CeA ChR2 rats that had never earned cocaine encountered two portholes (both were retractable, or both were fixed for different rats). Nosepokes into one arbitrarily designated porthole earned either 1 s bins (typical duration for self-stimulation studies) or 8 s bins (similar to cocaine two-choice bins) (balanced across different rats) of CeA blue laser illumination (25 Hz, 8 mW), whereas a second porthole delivered nothing and was counted simply as a control for general activity. Results showed that CeA ChR2 rats failed to nosepoke into their laser-delivering porthole any more frequently than into the inactive porthole, neither for 1 s laser bins (n=6; t_5=1.23, p=.273, 95%CI[-21.6, 16.1]), or for 8 s laser bins (n=6; t_5=1.34, p=.24, 95%CI[-11, 34]). Overall, nosepokes also did not differ between 1 s and 8 s laser bins ($F_{1,5}$=1.18, p=.33), nor was there any interaction between porthole preference and bin duration ($F_{1,5}$=1.1, p=.35). Thus cocaine-naïve CeA ChR2 rats failed to nosepoke to self-stimulate CeA laser, indicating that CeA excitation by itself is not sufficient to serve as an independent reinforcer in our 2-porthole nosepoke task.

In the passive response or location-based task, where CeA ChR2 stimulation could be earned even more easily by entering or just remaining in a particular corner while making small movements (Olds and Milner, 1954), CeA ChR2 rats failed to show any self-stimulation or preference for their laser-delivering corner over the other 3 corners (n=7; $F_{3,9}$=.19, p=.901; Figure 4.9B). Equally, CeA ChR2 rats did not avoid their laser-corner, regardless of whether laser illumination was earned in 1 s duration pulses (n=4; $F_{3,9}$=2.3, p=.145) or in 8 s duration pulses (n=3; $F_{3,6}$=.079, p=.97). Instead, rats appeared oblivious to the location of CeA ChR2 stimulation delivered by itself, simply exploring the chamber at random. Lack of self-stimulation suggests that CeA ChR2 laser does not add a prediction error or reinforcement signal able to 'stamp-in' a preceding action.

CeA ChR2 laser fails to maintain responding after cocaine is discontinued

In a final test of whether CeA ChR2 laser illumination by itself can be sufficient at least to *maintain* instrumental nosepoke responding (if not to establish nosepokes), we examined the

effect of turning off cocaine infusions in CeA ChR2 rats that had already acquired nosepoke responding for the combination of *Laser+Cocaine.* In other words, could CeA ChR2 laser by itself at least *maintain* nosepokes in rats that were already responding at a high level for cocaine accompanied by CeA laser? After two days of refresher training on the original two-choice task for *Laser+Cocaine* and *Cocaine alone* (FR1 schedule), cocaine was suddenly discontinued for the next 4 days while laser could still be earned by itself (n=7). That is, the customary porthole still delivered laser as before, and both portholes delivered their associated auditory cues, but neither delivered cocaine (*Laser alone* vs. *Nothing*). Finally, after 4 days of laser-alone, the laser was also discontinued for the next three days to examine whether its removal dropped responding further (*Nothing* vs. *Nothing*; 7 rats). Results showed that by the second day of laser-alone, CeA ChR2 rats lost their preference for the former *Laser+Cocaine* option that now delivered *Laser alone,* and chose equally at low levels between that and *Nothing* (t_6=1.01; p=.35, 95% CI[-6.6, 15.7]; Figure 4.10). CeA ChR2 rats failed to self-stimulate on their *Laser alone* porthole, even though previously they had responded vigorously when it also earned cocaine ($F_{4,24}$=10.22, p=.000). Both unilateral laser and bilateral laser rats showed similar declines when only laser by itself was available (n=3 unilateral and n=4 bilateral; $F_{1,5}$=.012, p=.92). Finally, when CeA laser was additionally discontinued several days later, there was no further drop from *Laser alone* to *nothing* (5 ±2 vs. 4 ±2 responses on days 5 and 8, respectively; t_6=.36; p=.73, 95% C.I. [-3.3, 4.4]). Thus, without cocaine, CeA ChR2 laser failed to maintain any detectable preference or instrumental responding above complete extinction levels. Instead CeA laser appeared entirely worthless to the same rats in which it had established intense and narrowly focused motivation for cocaine.

Discussion

Our results demonstrate that pairing CeA ChR2 stimulation with a particular option to earn intravenous cocaine 1) narrowly focused amplified motivation solely to its paired cocaine option - at the expense of nearly ignoring the alternative cocaine option, and 2) intensified the level of incentive motivation to obtain cocaine, resulting in greater cocaine consumption. In independent progressive ratio tests of intensity of incentive motivation, CeA ChR2 stimulation further amplified the breakpoint effort price that rats were willing to pay for cocaine by more than eight times. This pattern closely resembles the CeA ChR2 amplification and narrowing of sucrose motivation that we previously reported (Robinson et al., 2014). Conversely, CeA

inhibition by optogenetic halorhodopsin here mildly impaired cocaine consumption, and stronger GABAergic inhibition by muscimol/baclofen microinjections nearly abolished self-administration by tethered rats, and prevented CeA ChR2 enhancement or focusing of motivation. These results indicate that CeA-related circuitry can powerfully control the amplitude and targeting of motivation for cocaine.

CeA ChR2 pairing also generated intense consummatory bite and nibble reactions directed specifically toward the metal Laser+Cocaine porthole, in addition to increasing consummatory sniffing of that laser-associated cocaine cue (a more typical conditioned response to cocaine cues). Bites and nibbles are common rat consummatory behaviors elicited by cues for sucrose or food rewards, but to our knowledge have not been reported for cocaine cues. Here, bites and nibbles were never emitted by control rats earning cocaine, nor by BLA ChR2 rats, or even by CeA ChR2 rats toward their *Cocaine Alone* porthole. Consummatory bites emerged only when several conditions were simultaneously met: 1) CeA ChR2 laser was paired with the target cocaine porthole, 2) the metal porthole actively moved, appearing abruptly into the chamber, and retracting back out after cocaine was earned, and 3) cocaine was actually earned by that option. Further, a fourth condition of cocaine intoxication dramatically facilitated oral consummatory behaviors: bites, nibbles and sniffs all became most intense near the end of a session when CeA ChR2 rats had already consumed a substantial dose of cocaine. Consummatory responses are a signature feature of incentive salience when attributed toward a Pavlovian reward cue, to the extent that individuals sometimes try to consume a 'wanted' cue as though it were the reward (Rosse et al., 1993). Here, we hypothesize that CeA ChR2 pairing with cocaine amplified the incentive salience of its associated cue, adding a new motivation status that made it more attractively biteable.

Amplified cocaine value, not transferred laser value

Yet, despite powerfully intensifying and narrowing motivation to take cocaine and making its cue more attractive, CeA ChR2 stimulation apparently lacked any reinforcement value on its own without cocaine. Even the same rats which had pursued only their *Laser+ cocaine* option, completely failed to show laser preferences when given opportunities to earn self-stimulation with CeA ChR2 laser alone. CeA ChR2 rats neither touched a spout nor made nosepokes in a porthole to earn laser, and did not even remain or return to a location where laser

was delivered. Further, CeA ChR2 laser by itself also failed to maintain instrumental nosepoke responding that had previously been intensely established by pairing that laser with cocaine. Thus by itself, CeA ChR2 laser appeared worthless to these rats. Although CeA ChR2 self-stimulation might be conceivably found in future using different situations, it seems clear that CeA ChR2 laser was not a potent reinforcer at our parameters.

Lack of independent reinforcement by laser rules out the possibility that CeA ChR2 stimulation acted as a prediction-error teaching signal to create a learned expectation of greater reward, or that the laser acted to strengthen stimulus-response habit associations, or that laser was ever sought by our rats as an independent hedonic reward. We conclude that CeA ChR2 enhancement of cocaine motivation cannot be explained by mere transfer of any additive laser reinforcement/reward signal to its paired cocaine. Instead CeA ChR2 enhancement of cocaine motivation was greater than the sum of its two separate parts, namely laser stimulation value and cocaine value, assessed separately. We suggest CeA ChR2 laser specifically transforms the motivational value of earning its paired sensory reward, and enhances incentive salience of its cocaine cue. This CeA ChR2 value transformation seems unable to act in vacuo on any relatively neutral stimulus (spout, porthole, location), but rather requires some motivationally salient sensation on which to act, such as a cocaine reward or sucrose reward.

Localization of function in CeA for enhanced motivation

Effective sites for amplifying motivation for paired cocaine were nearly all clustered within CeA, or slightly above so downward-projecting light would penetrate CeA. By contrast, nearly all BLA ChR2 sites were ineffective.

Laser-induced Fos plumes were much smaller (0.1 to 0.3 mm radius) than GFP virus infection zones (about 1 mm radius). That difference suggests ChR2-infected CeA neurons may need to be within 0.3 mm of an 8-10 mW optic fiber tip in order to receive sufficient light to alter neuronal function, induce Fos translation, and presumably increase firing. Based on this assumption to aid localization of function, effective sites were evenly spread in most CeA subdivisions, including both the medial half of central amygdala (CeM) and the lateral half of central amgydala (CeL), as well as the capsular division (CeC). We note that it has been suggested that CeL mediates appetitive motivation for reward (Cai et al., 2014) and reduces anxiety (Tye et al., 2011), whereas CeM mediates fearful or defensive behaviors (Haubensak et

al., 2010; Namburi et al., 2015). However, our results imply that neurons in CeM as well as CeL likely contributed to CeA ChR2 enhancement of cocaine motivation here. As a caveat, though, we acknowledge that many of our CeM Fos plumes may also have partly penetrated into CeL, and thus not have been fully contained in CeM. Future work could examine anatomical subdivisions or neuronal subpopulation roles more specifically either by creating smaller Fos plumes or by probing neurochemical subpopulations within CeM and CeL (Gafford and Ressler, 2015).

Although BLA has been implicated in both reward-related and in fearful behaviors, often by lesion or related loss of function studies (Kochli et al., 2015; McGaugh, 2015; Wassum and Izquierdo, 2015), BLA was not effective for ChR2 enhancement of cocaine motivation here. That CeA vs. BLA difference replicates the pattern we previously reported for CeA ChR2 amplification of motivation to earn sucrose, indicating similar anatomical specificity for optogenetic control of a natural sensory reward and a drug reward (Robinson et al., 2014). CeA is also typically more effective than BLA for pharmacological microinjection enhancements of incentive motivation (Corbit and Balleine, 2005; DiFeliceantonio and Berridge, 2012; Holland and Hsu, 2014). CeA is also important in incubation of cue-triggered drug craving (Lu et al., 2005, 2007; Shaham and Hope, 2005; Funk et al., 2016). A CeA advantage for generating intense incentive motivation may be related to CeA's macrosystem status as a 'striatal-level structure' (e.g., containing mostly GABAergic neurons), similar to several other striatal structures where stimulations also may generate intense motivation for rewards (e.g., nucleus accumbens; some regions of neostriatum). By contrast, BLA has the status of a 'cortical-level structure' (e.g., mostly glutamatergic neurons, which project in turn to CeA) in the same macrosystem framework (Alheid and Heimer, 1988; Swanson and Petrovich, 1998; Swanson, 2003; Zahm, 2006; Heimer, 2008).

Regarding larger mesocorticolimbic circuitry, CeA is known to modulate mesolimbic dopamine systems in tegmentum and nucleus accumbens activity (Ahn and Phillips, 2002) via outputs to intermediary ventral pallidum, lateral hypothalamus, ventral tegmentum and other targets (Yoshida et al., 2006; Heimer, 2008; Janak and Tye, 2015; Reppucci and Petrovich, 2016) (Heimer, 2008; Janak and Tye, 2015; Reppucci and Petrovich, 2016; Yoshida et al., 2006). CeA ChR2 recruitment of VTA, mesolimbic dopamine projections and related limbic circuitry may well be part of the mechanism that enhanced incentive motivation here.

Our results confirm that optogenetic activation of CeA-related circuitry, paired with one particular reward option, produces narrowly focused yet intense motivation, and extends this phenomenon to intravenous cocaine reward. Our results also demonstrate a potentially 'irrational' feature of this CeA-generated intense motivation that might be shared with addiction, in that motivation enhancement was far greater than the sum of its constituent reinforcer elements (i.e., cocaine alone plus CeA ChR2 laser alone).

Figures

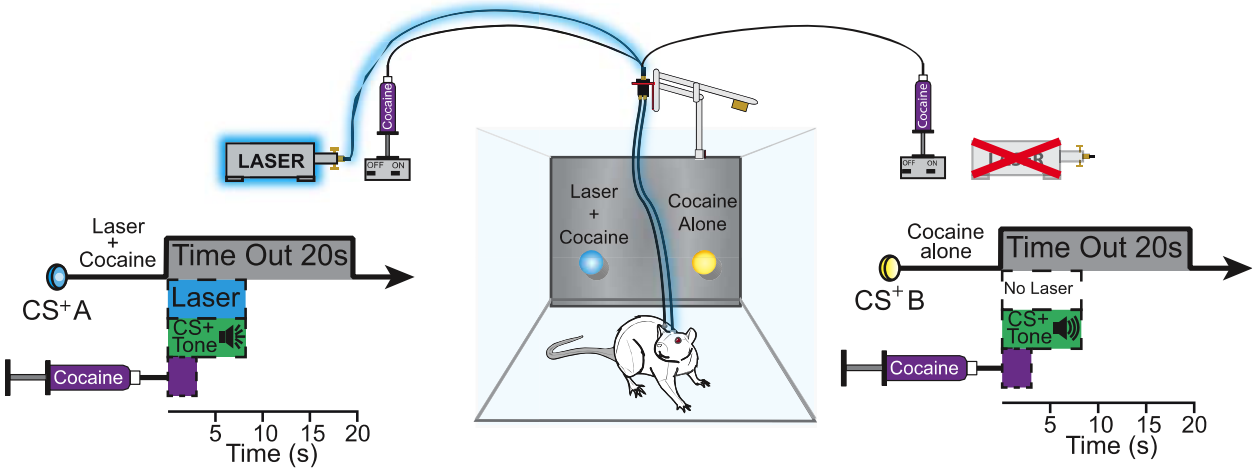


Figure 4.1
Instrumental two-choice task. Rats instrumentally nose poked into two different ports, first one at a time and then allowed to choose between the two for the remainder of each session. One port earned a cocaine infusion (0.3 mg/kg in 50 µl, 2.8 s duration; FR1 schedule) accompanied by a discrete 8 s tone and additional blue laser stimulation (25 Hz, 8-10 mW, 8 s) ("*Laser+Cocaine*"). Nose poking into a second port located on the same wall earned an identical cocaine infusion (0.3 mg/kg in 50 µl, 2.8 s duration) accompanied by a different 8 s tone ("*Cocaine alone*"). Both choices resulted in a 20 s timeout after cocaine infusion.

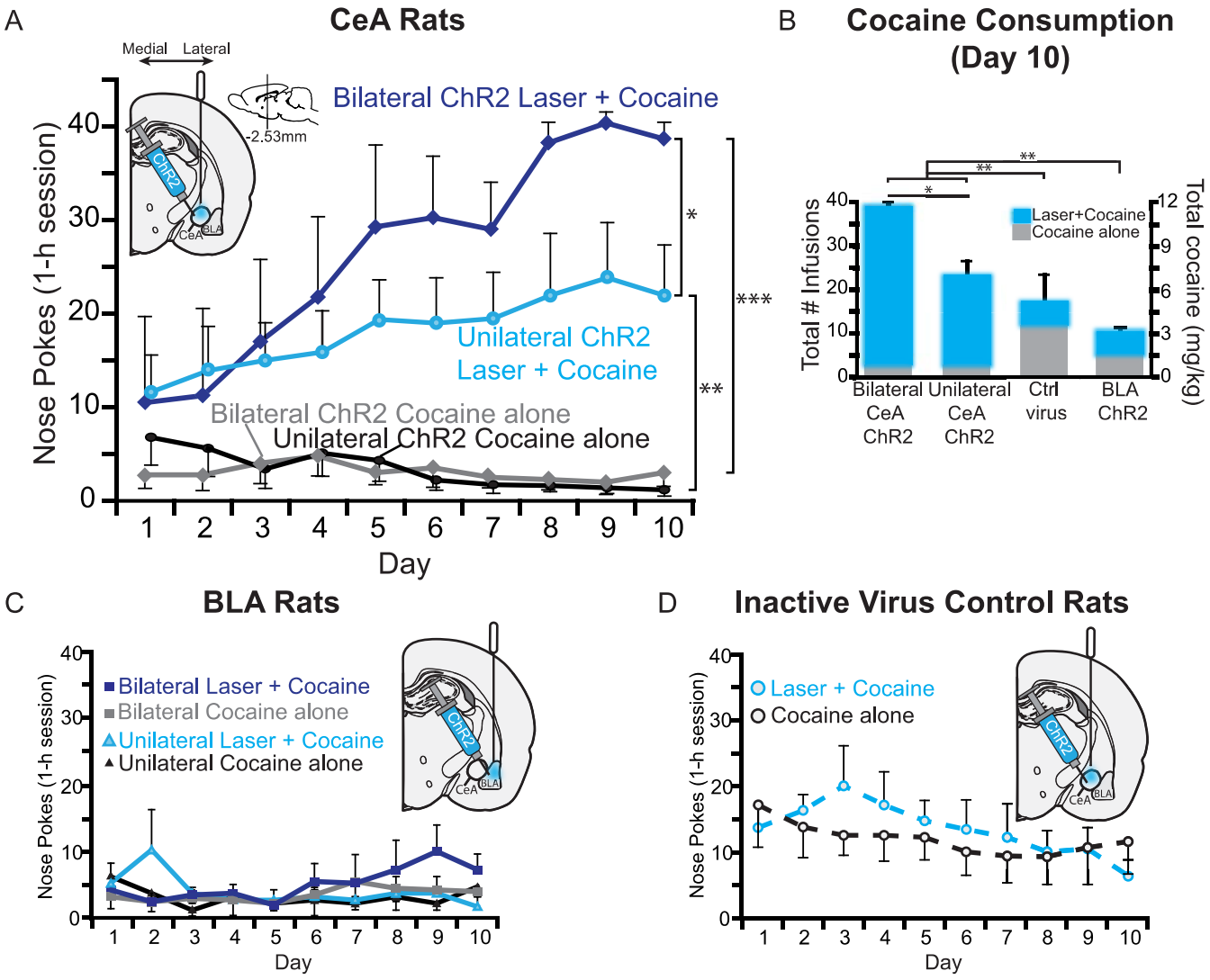


Figure 4.2

CeA ChR2 stimulation captures choice for a cocaine reward. (A) Rats exclusively pursued their cocaine reward paired with CeA optogenetic ChR2 stimulation: either unilateral CeA laser illumination ("Unilateral ChR2 *Laser+Cocaine*"; solid light blue line with circle symbols) or bilateral CeA illumination ("Bilateral ChR2 *Laser +Cocaine*"; solid dark blue line with diamond symbols). The *Cocaine alone* option became relatively ignored ("Unilateral ChR2 Cocaine alone"; solid black line with circle symbols; "Bilateral ChR2 *Cocaine alone*"; solid grey line with diamond symbols). (B) CeA laser resulted in increased cocaine consumption compared to control inactive virus rats and BLA ChR2 rats by last day of training (Day 10). (C) By contrast, basolateral amygdala ChR2 simulation failed to enhance cocaine preference whether bilateral stimulation was earned ("Bilateral ChR2 "*Laser+Cocaine*"; solid blue lines with blue squares vs. Bilateral "*Cocaine alone*"; solid grey lines with grey squares) or unilateral stimulation was earned ("Unilateral ChR2 "*Laser+Cocaine*"; solid blue lines with blue triangles vs. Unilateral "*Cocaine alone*"; solid black lines with black triangles). (D) Similarly, Control inactive-virus rats lacking ChR2 gene chose equally between the two cocaine options ("*Laser+Cocaine*"; dashed blue line with grey circles and blue outline vs. "*Cocaine alone"*; dashed black line with grey circles and black outline). CeA ChR2: n=15, BLA ChR2: n=6, CeA Control virus: n=5. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

CeA ChR2 and BLA ChR2 Sites

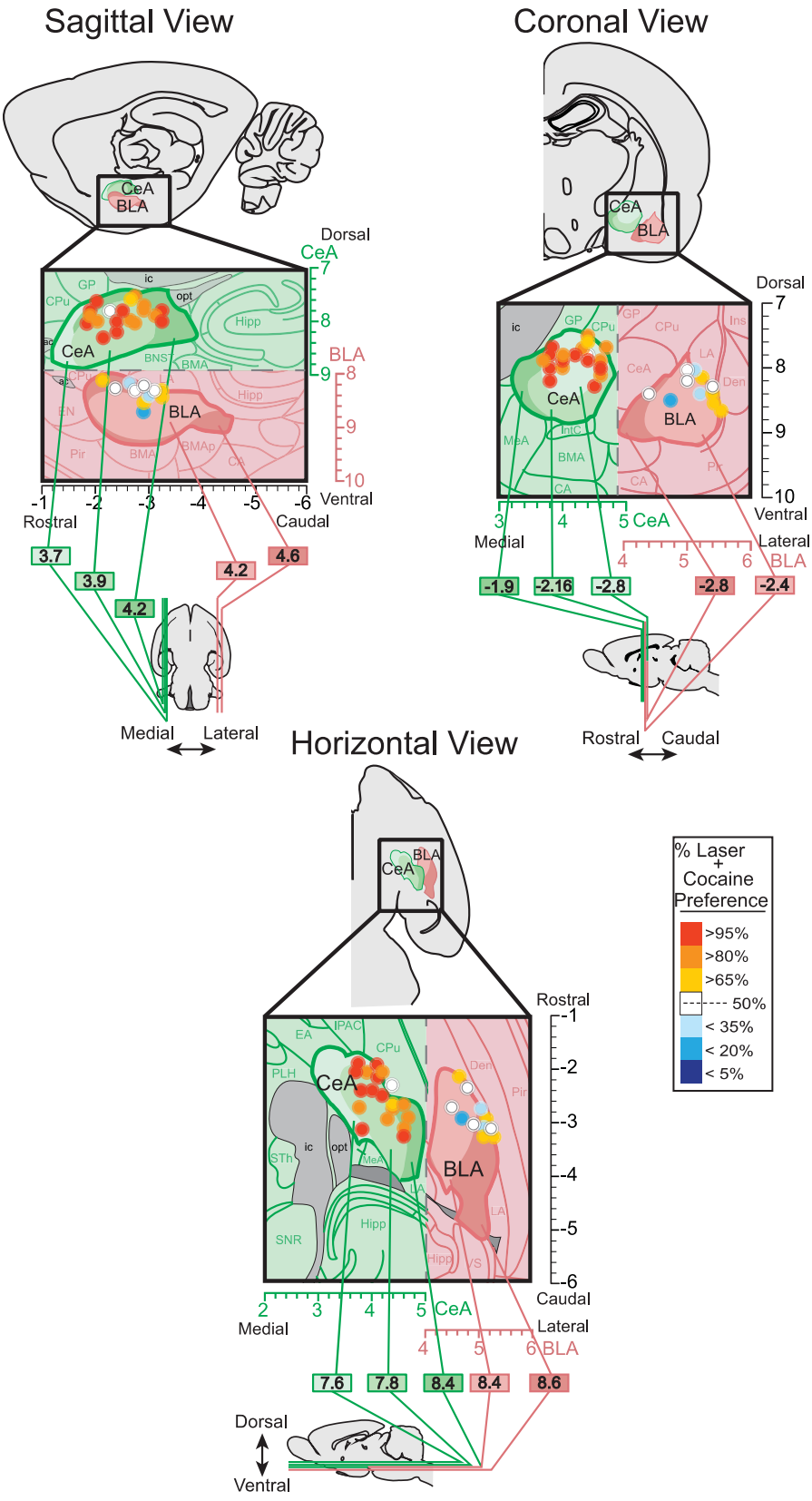
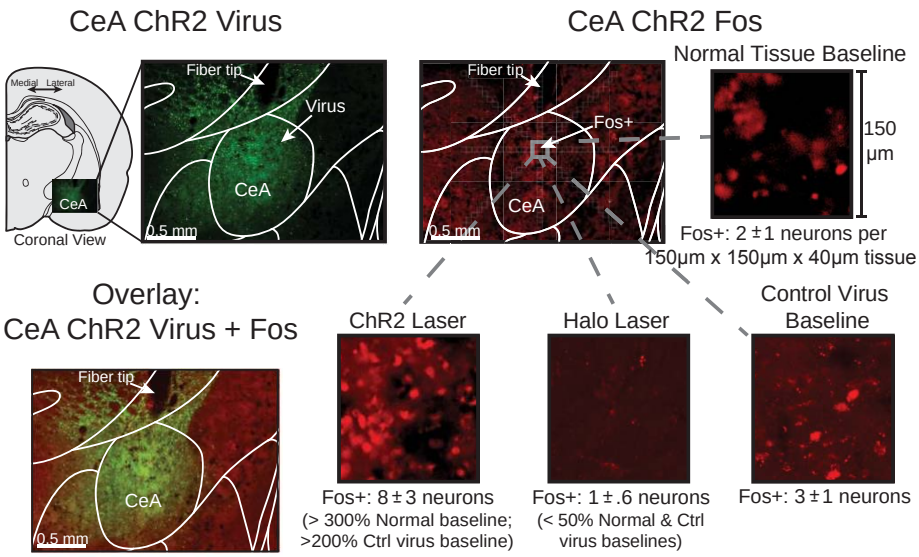
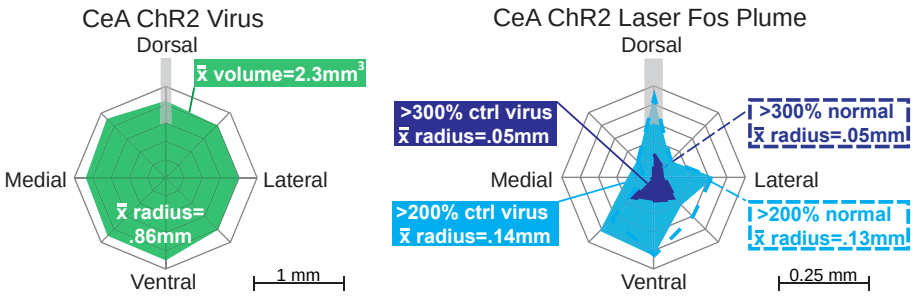
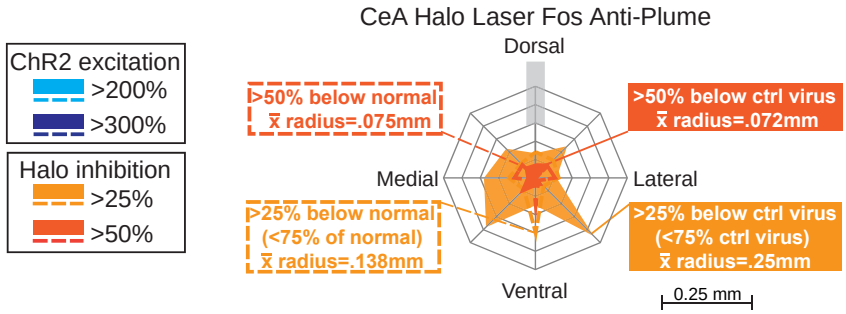


Figure 4.3

Localization of function maps. Maps show behavioral effects in the 2-choice task of corresponding amygdala sites in Figure 2. At each sagittal, coronal and horizontal view, the outermost boundaries of CeA and BLA are shown at varying medial/lateral levels (Sagittal view; ML 3.7 to 4.6 mm from Bregma), rostral/caudal levels (Coronal View; AP -1.9 to -2.8mm from Bregma), or dorsal/ventral levels (Horizontal View; DV -7.6 to -8.6 mm from Bregma). The unique boundaries at each level within CeA or BLA are shown as lighter to darker shades (shades of green for CeA and shades of pink for BLA). CeA but not BLA sites of optic fibers enhanced preference for cocaine. Colors depict the preference intensity for *Laser+Cocaine* caused by ChR2 laser pairing at that site, expressed as percentage of choice over *Cocaine alone* during the last two sessions of the 2-choice test. Sizes of symbols represent the average Fos plume diameter observed in CeA ChR2 and BLA ChR2 after laser stimulation (inner plumes scaled to represent Fos plume elevations > 200% above control virus baseline Fos levels [0.2 mm radius], and outer plumes represent Fos plume elevations > 150% above baseline Fos levels [~.3 mm radius]).


CeA ChR2 Virus
CeA ChR2 Fos
Normal Tissue Baseline
Medial Lateral
Fiber tip
Virus
CeA
Coronal View
0.5 mm
Fos+
150 µm
Fos+: 2 ± 1 neurons per 150µm x 150µm x 40µm tissue
Overlay:
CeA ChR2 Virus + Fos
ChR2 Laser
Halo Laser
Control Virus Baseline
Fos+: 8 ± 3 neurons
(> 300% Normal baseline; >200% Ctrl virus baseline)
Fos+: 1 ± .6 neurons
(< 50% Normal & Ctrl virus baselines)
Fos+: 3 ± 1 neurons


CeA ChR2 Virus
Dorsal
x̄ volume=2.3mm3
Medial
Lateral
x̄ radius= .86mm
Ventral
1 mm
CeA ChR2 Laser Fos Plume
>300% ctrl virus x̄ radius=.05mm
>300% normal x̄ radius=.05mm
>200% ctrl virus x̄ radius=.14mm
>200% normal x̄ radius=.13mm
0.25 mm


ChR2 excitation
>200%
>300%
Halo inhibition
>25%
>50%
CeA Halo Laser Fos Anti-Plume
Dorsal
>50% below normal x̄ radius=.075mm
>50% below ctrl virus x̄ radius=.072mm
Medial
Lateral
>25% below normal (<75% of normal) x̄ radius=.138mm
>25% below ctrl virus (<75% ctrl virus) x̄ radius=.25mm
Ventral
0.25 mm

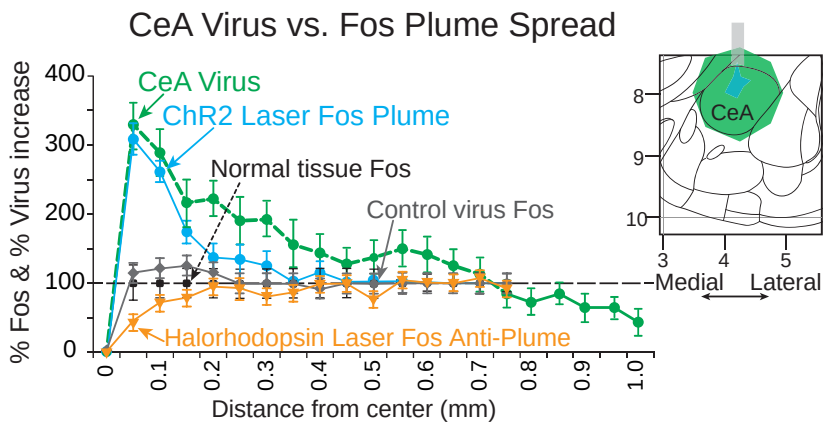

CeA Virus vs. Fos Plume Spread
% Fos & % Virus increase
CeA Virus
ChR2 Laser Fos Plume
Normal tissue Fos
Control virus Fos
Halorhodopsin Laser Fos Anti-Plume
Distance from center (mm)
CeA
Medial
Lateral

Figure 4.4

Anatomical spread of laser-induced CeA Fos plumes and of virus expression. (Top) Photomicrographs of CeA show green channelrhodopsin (ChR2) virus expression (CeA ChR2 Virus), red Fos protein immunohistochemistry expression stimulated around an optic fiber tip, induced by laser illumination in a CeA ChR2 rat prior to euthanasia (CeA ChR2 Laser Fos), and overlay photo combining GFP virus infection plus laser-induced Fos plume in the same CeA ChR2 rat (Overlay: CeA ChR2 Virus + Fos). Blow-ups of 150μm x 150μm x 40μm CeA tissue from immediately below the optic fiber tip depict sample numbers of Fos-expressing neurons in each condition, which were used to help determine % Fos intensity within local plumes induced by laser stimulation. Groups include Normal tissue Baseline (spontaneous Fos in CeA of a normal un-operated rat), Control Virus Baseline (a control rat with inactive-virus in CeA, after local blue laser illumination), CeA ChR2 Laser Fos plume (a CeA ChR2 rat after blue laser illumination), and CeA Halorhodopsin Laser (a CeA halorhodopsin rat after yellow laser illumination). For each blow up, the mean number ± standard error of Fos^+ neurons per 150μm x 150μm x 40μm tissue sample for that entire condition's group at same anatomical position within CeA plume (relative to fiber tip) is also shown; such numbers were used to calculate % change in local Fos expression at various positions within Fos plume, and to establish the borders of laser-induced Fos plumes). (Middle) Map on left (CeA ChR2 Virus) shows average mean radius of ChR2 virus spread in CeA from center of infection (virus radius = 0.86 mm, spherical volume = 2.63 mm^3). Right blue map shows laser-induced CeA ChR2 Fos plume (light blue outer zone = extent of > 200% Fos elevation induced by laser illumination (measured relative to baseline levels in control inactive-virus rats after similar CeA laser; ~0.14 mm mean radius). Dark blue inner zone = extent of higher > 300% Fos elevation over inactive-virus levels. Note that Fos plumes may extend maximally straight below optic fiber tip, reflecting downward path of light beam (0.35 mm) Dotted blue lines indicate similar ChR2 elevations relative to normal CeA tissue baselines (illumination of control virus may induce mild Fos elevation over normal tissue levels, perhaps due to heat, virus infection, or surgical penetration). Yellow map below shows Fos suppression caused by yellow laser illumination in CeA Halorhodopsin rats (i.e., Anti-plume). Outer solid orange plume = extent of >25% Fos suppression (i.e., Fos reduction to below 75% control level) compared to control inactive virus condition after yellow laser (0.25 mm mean radius). Inner solid dark orange plume shows zones of more intense >50% Fos suppression over inactive virus levels (0.072 mm mean radius). Dotted lines depict zones of suppression compared to normal baseline tissue. (Bottom) Quantitative comparison of virus infection vs. Fos elevation intensity as a function of distance beneath center of optic fiber tip. Fos is plotted as % change from normal tissue Fos baseline (= 100%) (CeA ChR2 Laser Fos Plume: blue circles & solid blue lines; Halorhodopsin Laser Fos: orange downward triangles & solid orange lines; Control virus Fos: solid grey diamonds). ChR2 GFP virus intensity plotted as % change from un-injected tissue (baseline = 0 +1; CeA Virus: green circles with green dashed line). All data are shown as mean ± SEM. Note that Fos elevation falls from peak levels more rapidly with distance than ChR2 virus infection, perhaps reflecting thresholds of light intensity needed to induce Fos in infected neurons.

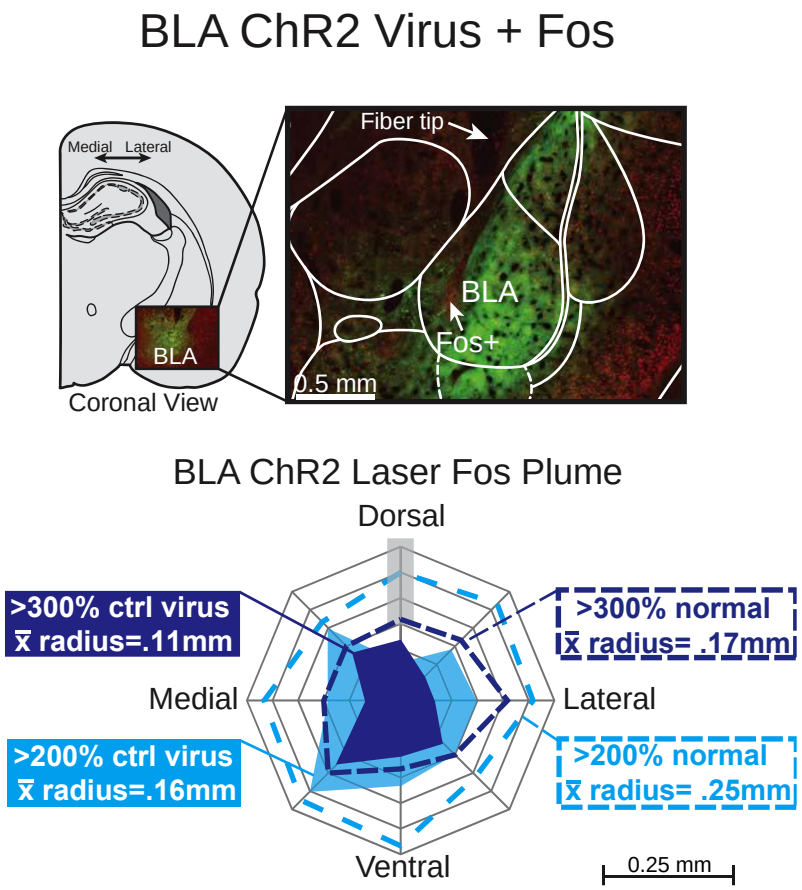


Figure 4.5
BLA Virus spread and Fos Plume. Photomicrograph shows BLA ChR2 Fos protein immunohistochemistry and virus expression in BLA rats, similar to Figure 4 around an optic fiber tip, induced by laser illumination prior to euthanasia (red= Fos, green= ChR2 GFP virus). Outer solid light blue plume = >200% elevation in Fos plume expression relative to control inactive-virus condition after similar BLA laser (0.16 mm mean radius). Inner solid dark blue plume shows >300% Fos elevation over inactive virus levels (0.11 mm mean radius). Dotted lines indicate Fos elevations calculated relative to normal BLA tissue baseline (spontaneous Fos baseline in BLA of un-operated rats).

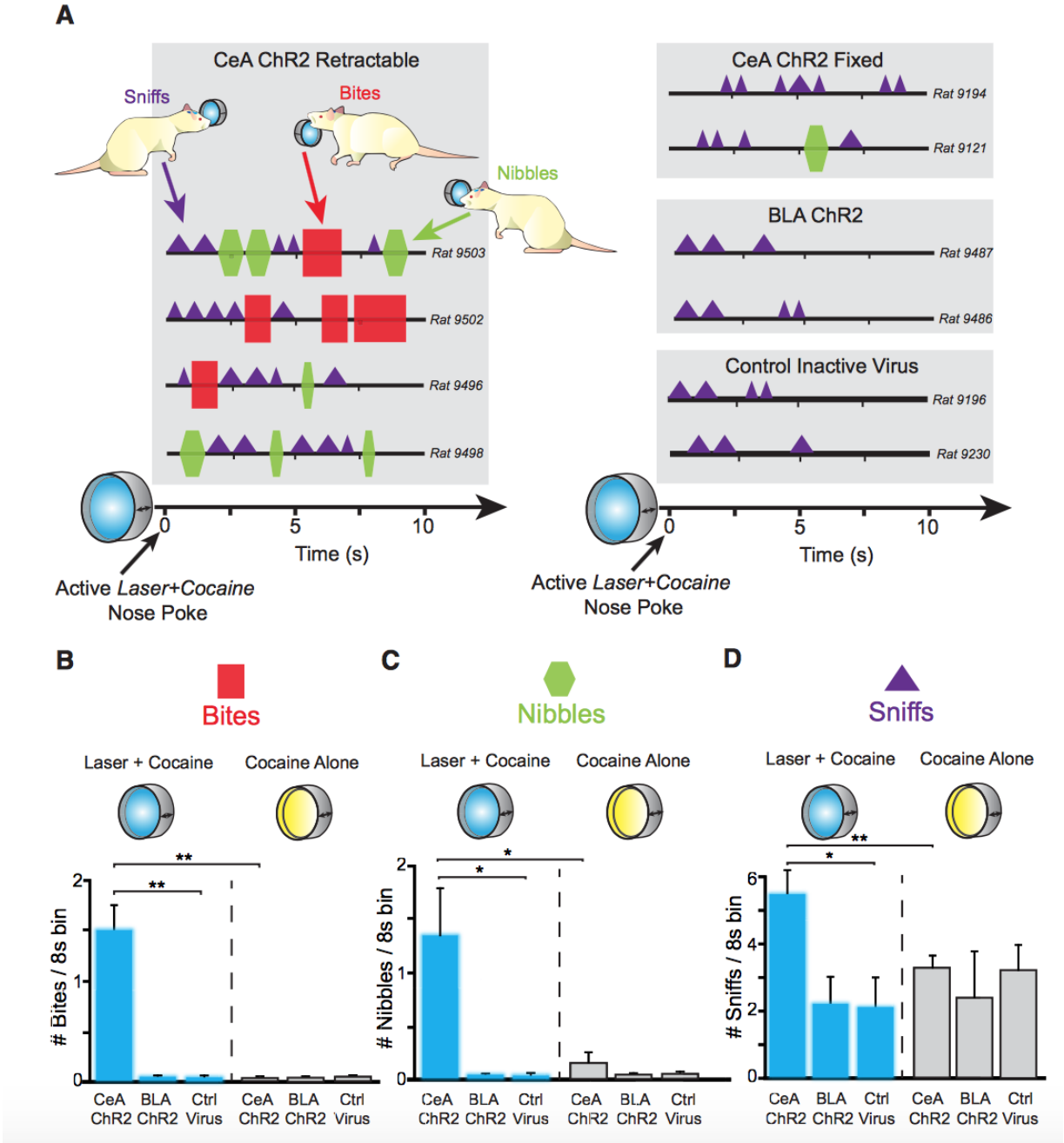
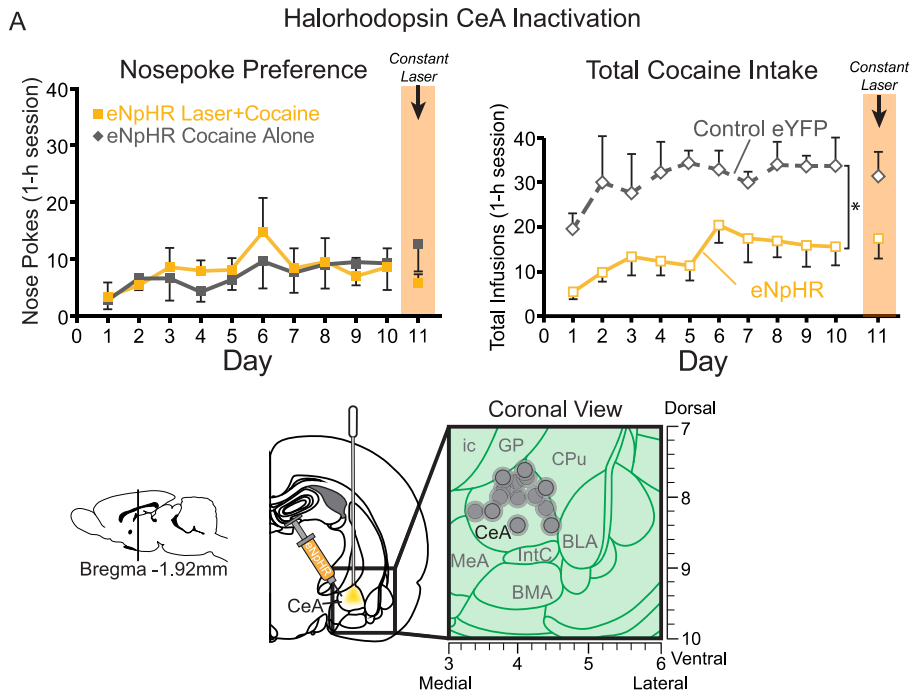
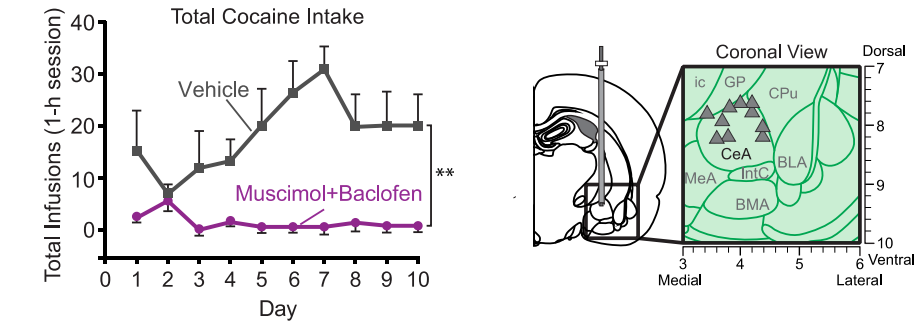

A
CeA ChR2 Retractable
Sniffs
Bites
Nibbles
Rat 9503
Rat 9502
Rat 9496
Rat 9498
0
5
10
Time (s)
Active Laser+Cocaine
Nose Poke
CeA ChR2 Fixed
Rat 9194
Rat 9121
BLA ChR2
Rat 9487
Rat 9486
Control Inactive Virus
Rat 9196
Rat 9230
0
5
10
Time (s)
Active Laser+Cocaine
Nose Poke
B
Bites
Laser + Cocaine
Cocaine Alone
Bites / 8s bin
CeA ChR2
BLA ChR2
Ctrl Virus
C
Nibbles
Laser + Cocaine
Cocaine Alone
Nibbles / 8s bin
D
Sniffs
Laser + Cocaine
Cocaine Alone
Sniffs / 8s bin

Figure 4.6

Consummatory bites, nibbles, and sniffs at porthole. (A) Depicts topography of behavior towards the *Laser+Cocaine* porthole among representative rats from each group (CeA ChR2 rats towards a retractable or fixed port, BLA ChR2, and control inactive virus rats). Each choreograph shows a 'typical' response, about 30 min into a session, at a porthole during the 8 s after a successful nose poke that earns a cocaine infusion and laser illumination plus accompanying auditory cue. Bites (red squares) and nibbles (green hexagons) predominantly occurred only in CeA ChR2 rats toward their *Laser+Cocaine* retractable port, whereas sniffs (purple triangles) were more common towards fixed ports and in other groups. CeA ChR2 rats on average bit (B), nibbled (C), and sniffed (D) at greater numbers during each 8 s bin after successful nose poke of their *Laser+Cocaine* retractable porthole (blue bars) compared to both their *Cocaine alone* port (grey bars) or compared to BLA ChR2 and Control inactive virus rats at their *Laser+Cocaine* retractable port (blue bars). Data are shown as mean ± SEM. *$p<0.05$ **$p<0.01$.


A
Halorhodopsin CeA Inactivation
Nosepoke Preference
Constant Laser
eNpHR Laser+Cocaine
eNpHR Cocaine Alone
Nose Pokes (1-h session)
Day
Total Cocaine Intake
Control eYFP
eNpHR
Total Infusions (1-h session)
Bregma -1.92mm
Coronal View
Dorsal
Ventral
Medial
Lateral
ic
GP
CPu
CeA
BLA
MeA
IntC
BMA


B
Muscimol/Baclofen CeA Inactivation
Total Cocaine Intake
Vehicle
Muscimol+Baclofen
Total Infusions (1-h session)
Day
Coronal View
Dorsal
Ventral
Medial
Lateral

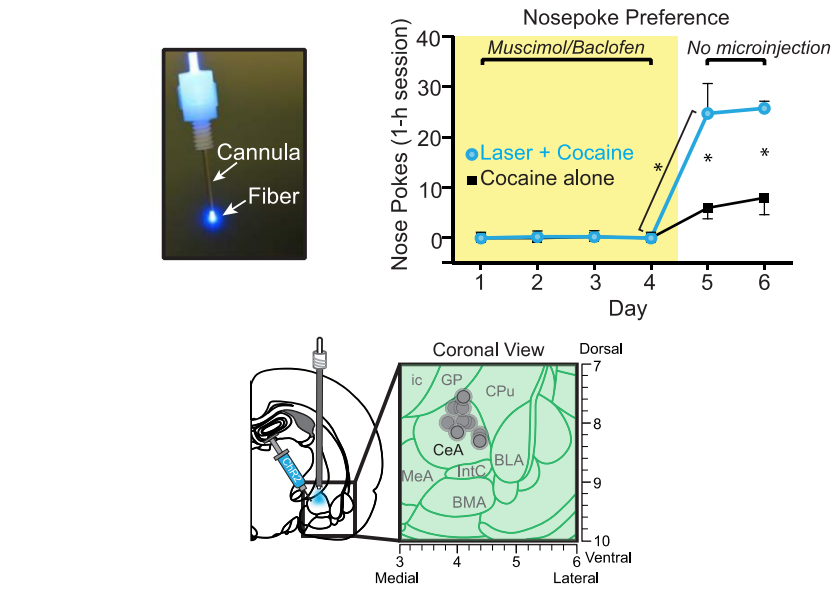

C
CeA muscimol/baclofen prevents Laser+Cocaine preference
Cannula
Fiber
Nosepoke Preference
Muscimol/Baclofen
No microinjection
Laser + Cocaine
Cocaine alone
Nose Pokes (1-h session)
Day
Coronal View
Dorsal
Ventral
Medial
Lateral

Figure 4.7

Loss of CeA function: Optogenetic CeA inhibition and pharmacological CeA inhibition. (A) Optogenetic CeA inhibition paired with earning one cocaine reward failed to alter nosepoke preference, as CeA halorhodopsin rats ("eNpHR"; n=7) chose equally between the *Laser+Cocaine* (solid orange lines with orange filled squares) and *Cocaine alone* (solid grey line with grey filled squares). However, over the course of the 10 days, total cocaine intake graph on right shows that halorhodopsin rats ("eNpHR"; solid orange lines with open squares outlined in orange) self-administered less cocaine infusions compared to control inactive virus rats ("Control eYFP"; n=2; dashed grey lines with open diamonds outlined in grey). (B) *Muscimol/Baclofen CeA inactivation.* Rats receiving microinjections into CeA of muscimol/baclofen ("Muscimol+Baclofen"; n=5; solid purple lines with filled purple circles) self-administered fewer infusions than rats receiving vehicle ("Vehicle"; n=4; solid grey lines with filled grey circles). (C) *CeA muscimol/baclofen prevents Laser+Cocaine preference.* When CeA microinjections of muscimol/baclofen were administered for the first four days of training, cocaine responding was completely suppressed for both cocaine options in the 2-choice task. As soon as microinjections ceased (beginning days 5-6), CeA ChR2 rats (n=4) exclusively chose the *Laser+Cocaine* option (solid blue lines with blue filled circles) over and above their *Cocaine alone* option (solid black lines with black filled squares), as well as above the prior day when receiving muscimol/baclofen. Data are shown as mean ± SEM. *$p<0.05$ **$p<0.01$.

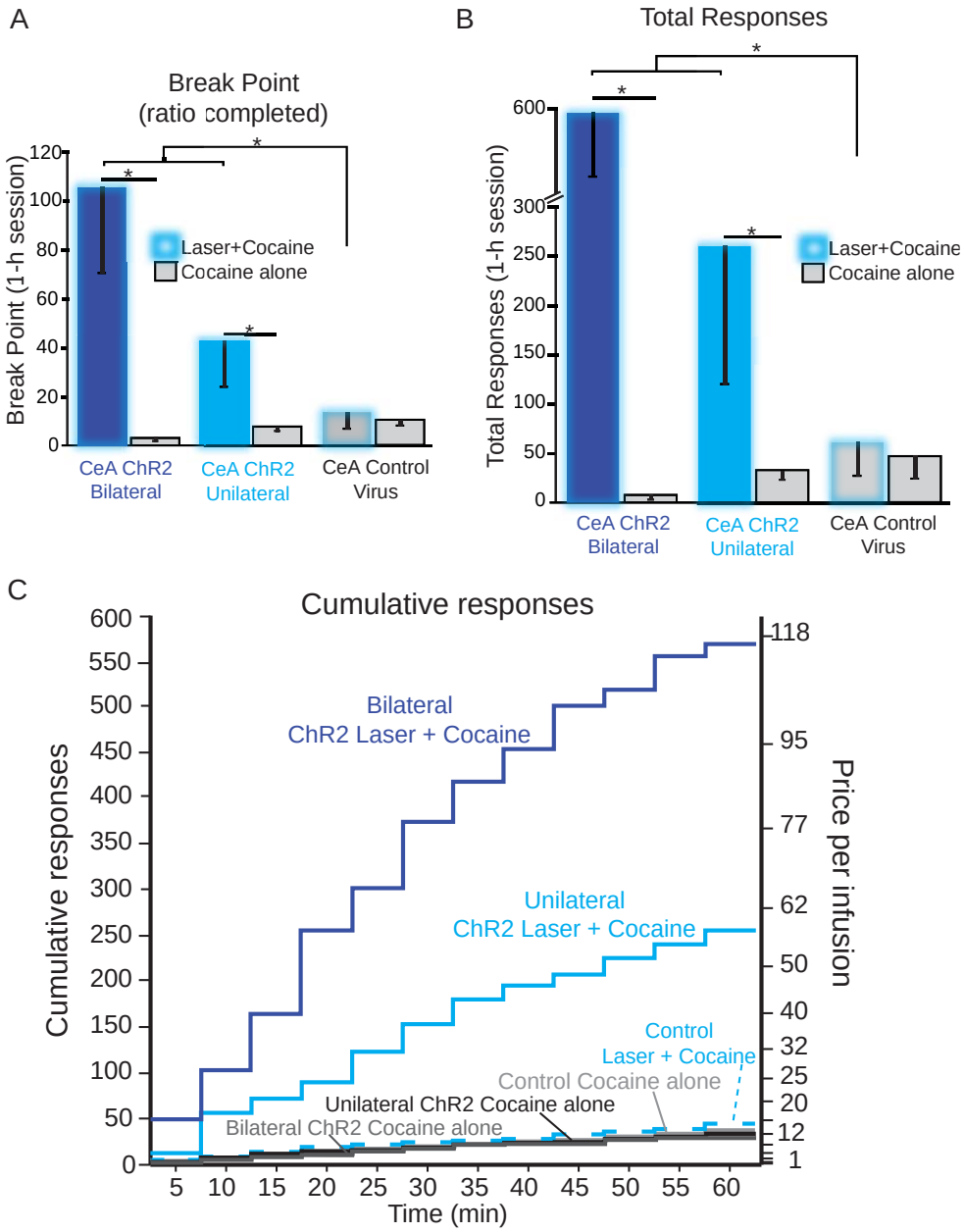


Figure 4.8

CeA ChR2 stimulation amplifies breakpoint motivation. A progressive ratio test of breakpoint was given on two consecutive days (counter-balanced order). On one day, rats earned *Laser+Cocaine* accompanied by its 8 s auditory cue. On the other day, rats earned *Cocaine alone* plus its own 8 s auditory cue. On each day, effort required to obtain the next cocaine infusion increased exponentially after each earned infusion. (A) CeA ChR2 rats reached higher breakpoints (maximum effort price rats were willing to pay) for *Laser+Cocaine* than for *Cocaine alone*: making more nosepokes overall (B) and (C). Bilateral amygdala laser illumination increased motivation more than unilateral laser illumination in CeA ChR2 rats ("CeA ChR2 Bilateral"; dark blue bars; n=3; "CeA ChR2 Unilateral"; light blue bars; n=8). In contrast, control virus rats worked equally hard for cocaine regardless of laser condition, and at much lower levels than CeA ChR2 rats did for *Laser+Cocaine* ("CeA Control Virus"; grey bars; n=5). Data are shown as mean ± SEM. *p<0.05.

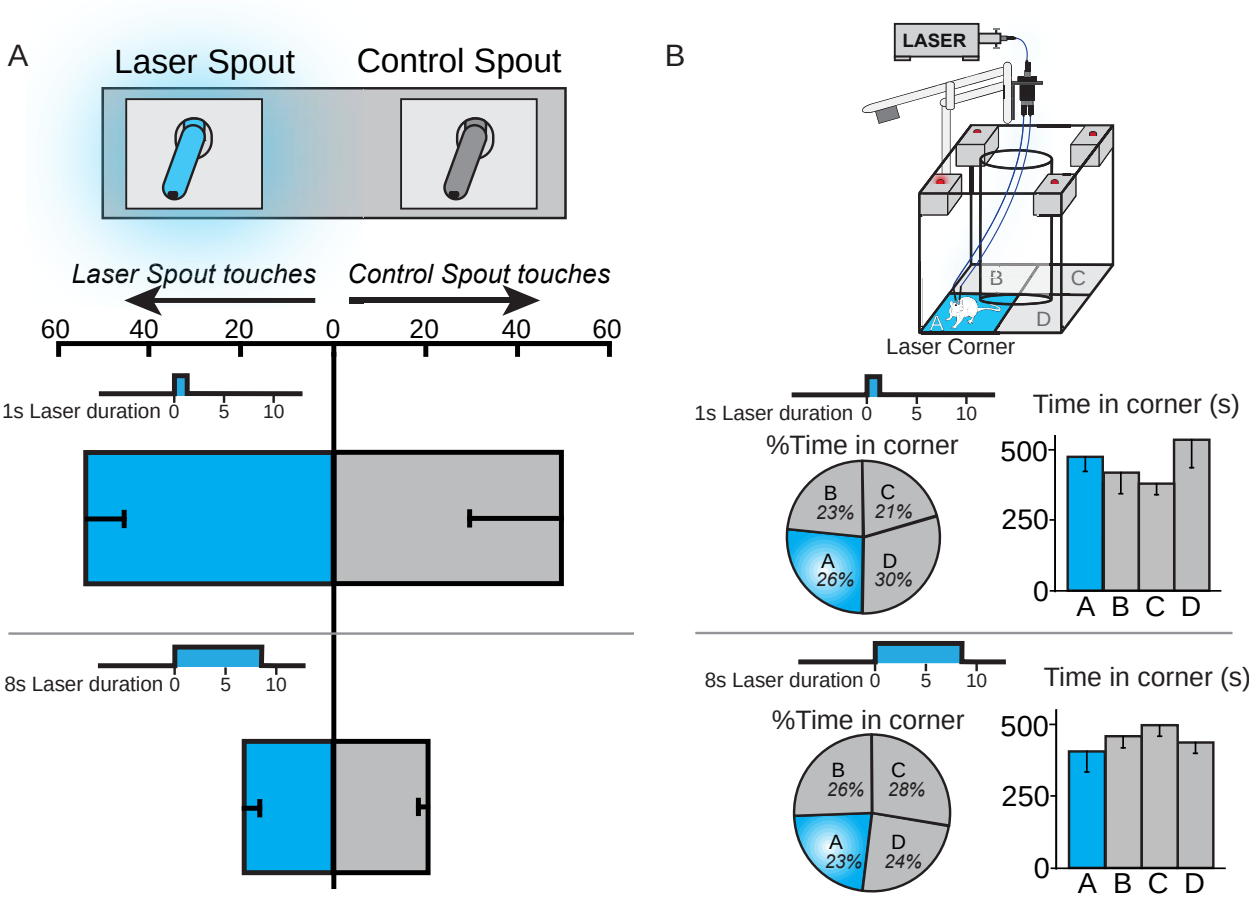


Figure 4.9
CeA ChR2 laser fails to support self-stimulation. (A) In an active spout-touch self-stimulation test, rats failed to touch the spout delivering CeA ChR2 stimulation (25 Hz, 8-10 mW, 1 s and 8 s durations, n=6 and n=3 respectively) any more than the other control spout that delivered nothing. (B) In a passive location-based self-stimulation test, rats neither preferred nor avoided the corner location where CeA ChR2 stimulation was delivered, compared to other three corners that lacked laser. In other words, rats simply ignored the laser location (blue laser, 25 Hz, 8-10 mW, 1 s and 8 s durations) (n=4 and n=3 respectively). Data are shown as mean ± SEM.

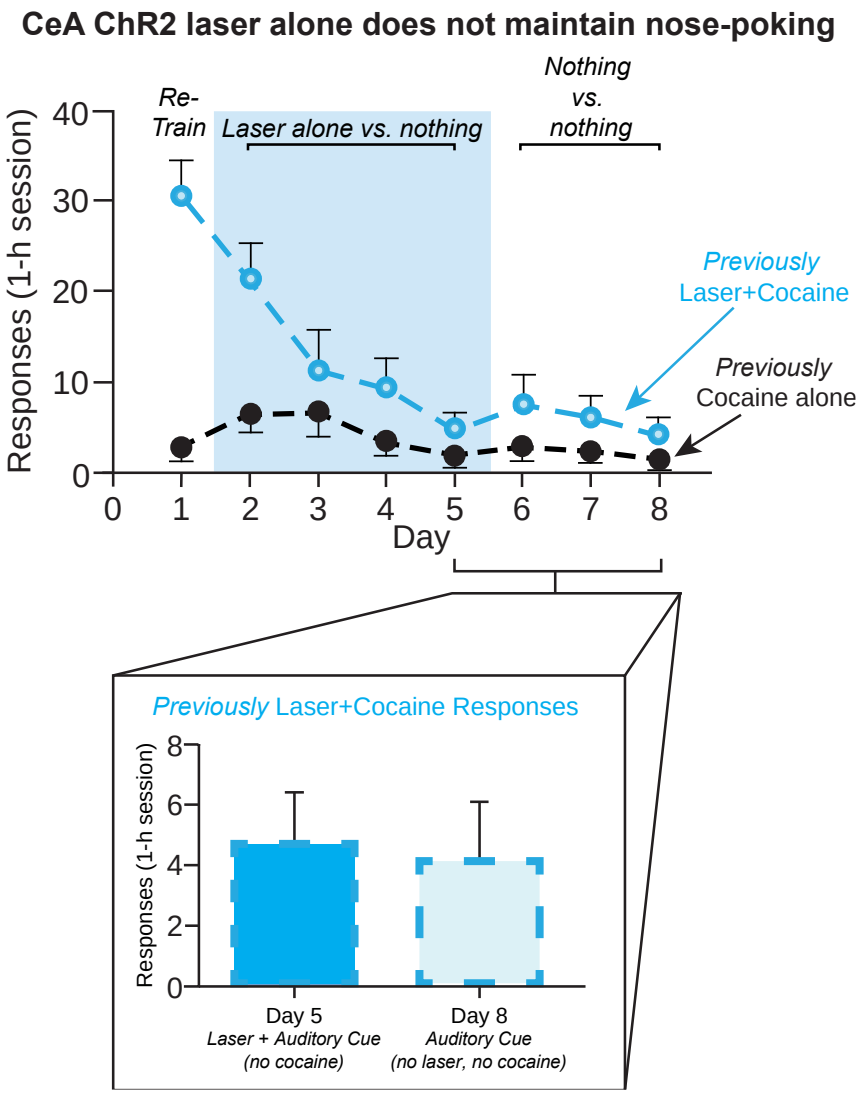


Figure 4.10
CeA ChR2 laser alone does not maintain nose-poking. Rats were re-trained on the 2-choice task to choose between *Laser+Cocaine* vs. *Cocaine alone*. Starting day 2, cocaine was removed, but rats could still earn laser stimulation by poking into their previous *Laser+Cocaine* port ("*Previously Laser+Cocaine*"; dashed blue line with blue filled circles). Pokes into their previous *Cocaine alone* port earned nothing ("*Previously Cocaine alone*"; dashed black lines with black filled circles). When laser stimulation was offered alone, responding declined and rats no longer preferred the Laser-delivering port. On subsequent days 6-8, laser was removed and no further decline in responding was observed. A comparison of responding on the last day of Laser alone (Day 5) and after three days of responding for neither laser nor cocaine (nothing; Day 8) showed no difference (inset).

CHAPTER V. Optogenetic Central amygdala excitation controls choice between sucrose and cocaine

Introduction

The amygdala is thought to assign motivational significance to environmental stimuli on the basis of learned associations (Baxter and Murray, 2002; LeDoux, 2007; Peck et al., 2013; Averbeck and Costa, 2017). As such, many studies have implicated a role for the amygdala in assigning motivational significance to cues that predict both rewards or threats. The amygdala is a heterogeneous structure with several different subdivisions, including the central nucleus (CeA) and basolateral nucleus (BLA). According to a macrosystem view of the brain, the BLA is more cortical-like due to its predominant glutamatergic output, whereas the CeA is considered to be more striatal-like in nature due to its GABA-ergic output (Alheid and Heimer, 1988; Swanson, 2003). Striatal-level structures are known to generate intense motivations (either positively or negatively valenced). In line with this, pharmacological mu-opioid stimulation of the CeA but not BLA can enhance food consumption, conditioned responses during autoshaping tasks, and instrumental food-seeking in the presence of a Pavlovian food cue (Holland and Gallagher, 2003; Mahler and Berridge, 2009, 2012; DiFeliceantonio and Berridge, 2012).

Similarly, we've previously shown a role for the CeA in narrowing and amplifying incentive motivation for sucrose, as well as for cocaine. Specifically, we gave rats a choice between earning sucrose by pressing a lever and earning sucrose paired with optogenetic CeA stimulation by pressing a different lever in the same session. Channelrhodopsin (ChR2) rats narrowed their focus to earning the CeA-paired sucrose pellet while ignoring the alternative identical sucrose option that lacked CeA stimulation (Robinson et al., 2014). ChR2 rats were also more motivated to earn that sucrose pellet in a separate progressive ratio test. Importantly, CeA ChR2 stimulation was not reinforcing on its own, as rats would not self-stimulate CeA laser in three separate tests. This indicated that CeA ChR2 laser being paired with a reward such as sucrose made that sucrose the sole object of desire and a motivational magnet. Similarly, pairing CeA ChR2 stimulation with earning an intravenous cocaine reward, when rats can choose

between that or cocaine alone, make rats narrow their focus to that CeA-paired cocaine, as well as increase their motivation to earn that cocaine in a separate progressive ratio test (Warlow et al., 2017). Given CeA's ability to create a motivational magnet through its associative pairing, we wanted to know whether CeA can control the target of pursuit when the rewards are different? Specifically, whether CeA-pairing can create a preference for sucrose over cocaine, or for cocaine over sucrose, when rats are choosing between the two rewards.

Here, we paired optogenetic CeA ChR2 stimulation with earning either sucrose or cocaine when rats were choosing between the two rewards via nose-poking during instrumental choice sessions and intermittent access sessions. We found that CeA ChR2 stimulation amplified attraction towards whichever rewarding (sucrose or cocaine) stimulus it was paired with, and rats were motivated to earn the laser-paired reward in a separate intermittent access session.

Materials and Methods

Subjects

The subjects in this experiment were male and female Sprague Dawley (n=19) that weighed between 250 g and 300 g before surgery. Rats were housed in a room maintained at ~21^{o}C on a reverse 12 hour light/dark cycle. The rats had *ad libidum* access to both chow (Purina Lab Chow) and water. Before performing surgery, each rat was handled on average 5 days for 10 minutes each day. The University of Michigan's Committee on the Use and Care of Animals approved all procedures.

Surgeries

Optogenetic virus infusion and optic fiber implant

Each rat received an optogenetic surgery and were infused with one of two viruses: either the experimental virus (AAV5-HSYN-ChR2-eYFP, n=14) or the inactive virus (AAV5-HSYN-eYFP, n=5). Optogenetic virus was injected bilaterally into the CeA (A/P from Bregma: -2.4, M/L: 4, D/V: -7.6 with mouth bar set to -3.3), followed by implantation of chronic dwelling optic fibers lowered to 0.3 mm above virus placement and secured via dental acrylic (info). Prior to the surgery, the rats were anesthetized with 5% isoflurane anesthesia, and received atropine (0.04 mg/kg; Henry Schein). During surgery, rats were maintained at 2-3% isoflurane. Additionally, rats were subcutaneously injected with chloramphenicol sodium succinate (60 mg/kg, Henry Schein) to prevent infection, and carprofen (5 mg/kg, Henry Schein) as an analgesic.

Intrajugular catheter implantation

In addition to the optogenetic surgery, rats underwent an intravenous catheter implantation two weeks later so that liquid cocaine could be administered during the behavioral experiments (Crombag et al., 2001). A Silastic intra-jugular catheter (Plastics One, Inc., Roanoke, VA, internal diameter=0.28 mm; external diameter=0.61 mm; dead volume=12 µl) was inserted into the right jugular vein and then woven along the underside of the skin of the rats' back toward the dorsal neck. The catheter was then secured via a subcutaneous anchor located in the dorsal mid-scapular region. Rats were allowed ten days for recovery before beginning behavioral tests during which their catheters were flushed daily with 0.2 ml isotonic saline solution containing 5 mg/ml gentamicin sulfate (Sparhawk, KS) for two weeks followed by flushing with saline solution daily thereafter. The flushing of the catheters was done to ensure no clogging or infections occurred during this recovery period. Catheter patency was tested once before behavioral testing, and again after the end of all tests, by making an intravenous injection of 0.2ml methohexital sodium to induce anesthesia (20mg/ml in sterile water, JHP, MI). Rats that became ataxic within 10s were considered to have a patent catheter and included in behavioral analyses.

Apparatuses

Instrumental sucrose vs. cocaine training and testing were performed in MedAssociates chambers (30.5 x 24.1 x 21.0 cm) with clear Plexiglas floors. Chambers contained speakers for auditory cues (tone or white noise). An infusion pump was fixed on the outside allowing for intravenous liquid cocaine delivery. A video camera placed below the transparent chamber floor recorded behavior during sessions for analysis of consummatory behaviors.

Procedures

Sucrose vs. Cocaine Instrumental Choice

Rats were initially trained on multiple days that alternated between earning sucrose or cocaine exclusively. For some rats, the sucrose pellet they earned was a 45 mg sucrose (n=8; LabTabs^{TM}, TestDiet, Richmond, IN) and for other rats, it was a highly palatable sucrose pellet (n=8; AIN-76A, TestDiet, Richmond, IN). Training to earn sucrose via nose-poking alternated with training sessions to earn intravenous cocaine based on protocol designed (Lenoir et al., 2013a). The rewards for each rat were counterbalanced with some starting on training to earn their sugar pellet and others starting by training to earn a cocaine infusion (0.3 mg (weight of the salt) per kg (weight of the rat) dissolved in 0.9% saline totaling 50 µL volume infused over 2.8 s

per infusion) (NIDA, MD) by nose-poking into one of two portholes. A discrete 8 s auditory cue (tone or white noise) accompanied each reward delivery, and assignment of auditory cue to sucrose or cocaine was randomized. Furthermore, optogenetic CeA excitation via blue laser (473 nm, 10mW, 25Hz, 8 s duration) was paired when rats earned one of the two rewards (Laser +Sucrose: n= 5 ChR2 and n=2 control eYFP; Laser+Cocaine: n=4 ChR2 and n=3 control eYFP). These training sessions lasted 60 minutes and were alternated for each rat (i.e. day one receiving sucrose, day two receiving cocaine, etc.) until each rat attained 50 of each reward. The preliminary sessions allowed for the rats to learn each outcome prior to the choice sessions that would allow for outcomes to be chosen.

Once rats received 50 rewards of each (sucrose and cocaine) over the training sessions, choice sessions began. During choice sessions, rats were presented with both rewards on an FR1 schedule (two portholes concurrently). During choice sessions, laser-reward pairing for each rat was the same as their training sessions. Each test session lasted 2 hours and began similar to the training sessions with only one porthole being presented at a time (a sampling, forced choice period). During the forced choice sampling period, rats were required to earn each reward one-at-a-time twice before commencing the choice session with both portholes being presented. The portholes were retracted for an imposed 20 second time-out during the one-at-a-time period. This procedure was used to guarantee that the rats understood the options from each respective porthole before making a choice between the two for the remainder of the session. Following forced choice trials, both options became available, signaled by protrusion of nosports back into the chamber at the same time. Nose ports stayed out until rats made their choice. Once their choice was made, that outcome was delivered (sucrose pellet or 0.3mg/kg/infusion) accompanied by its paired auditory cue. For the laser-paired reward, laser stimulation was concurrently delivered at the same parameters as during training (473 nm, 10mW, 25Hz, 8 s duration) (Figure 1). After each choice was made, a 10 min timeout was imposed to allow for the locomotive-inducing effects of cocaine to not bias reward choice in subsequent trials (Lenoir et al., 2013a).

Sucrose vs. Cocaine Intermittent Access

To test the level of motivation for each outcome (Laser+reward or alternate reward alone), modified intermittent access procedures were used on separated days following choice sessions (Zimmer et al., 2012). On separate days, ChR2 rats (n=5) were allowed to nose poke for either their laser-paired reward or for their reward alone. Each intermittent access session

consisted of 4 blocks of 'reward available' periods followed by 'reward unavailable periods'. 'Reward available periods' were signaled by insertion of nose ports into the chamber for 5 minutes. During these 5 min periods, rats could nose poke to earn that particular outcome (laser-paired reward or reward alone, depending on which session it was) at an FR1 schedule. Outcomes were identical to training and choice session outcomes, where laser stimulation (473 nm, 10 mW, 25 Hz, 8 s duration) co-occurred with delivery of the same reward as in choice sessions plus its own distinct auditory cue that occurred during choice sessions. Rather than imposing timeouts, rewards were continuously available throughout the entire 5 min period. Following the 5 min 'reward available period', a 20 min 'reward unavailable' period was imposed, and was signaled by retraction of the nose ports back into the wall of the chamber. Each intermittent access session consisted of 4 blocks of 'reward available' and 'reward unavailable' periods, totalling 2 hr per session.

Virus and Optic Fiber placement verification

Rats were injected with an overdose of sodium pentobarbital (150-200 mg/kg) to deeply anesthetize and were subsequently transcardially perfused. An additional group (n=4) had no manipulations and were injected directly from their home cage to serve as a baseline, naive tissue group. After the perfusion, the brains were stored in a solution of 4% paraformaldehyde for 24 hours prior to being cryoprotected in 30% sucrose. To identify the optic fiber placement and virus expression, the brains were sliced at 40 µm coronal sections. The coronal slices were subsequently processed for Fos protein visualization using immunohistochemical procedures previously described (Warlow et al., 2017). Once processed, sections were mounted onto slides, air dried, and then cover slipped using ProLong Gold anti-fade reagent (Invitrogen) in order to preserve the brain slices.

A Leica microscope (Leica Microsystems, Inc., Buffalo Grove, IL) was used to analyze the optic fiber sites and GFP virus expression of the brain slices at a magnification of 10x. The virus and optic fiber placement were illuminated in Adobe Illustrator (RRID: SCR:014198) on a rat brain atlas (Paxinos and Watson, 2007). Using Oasis Surveyor software (Objective Imaging, Inc., Kansasville, WI; RRID: SCR014433), nine images of the central amygdala site were assembled into one image that were centered at the fiber tip with mapping of virus intensity around the fiber tip.

Statistical Analysis

Using SPSS software (RRID: SCR:002865), parametric paired t-tests and independent t-tests were used to analyze within group data and between group differences in regards to laser-pairing versus non-laser pairing, control group versus experimental group, and cocaine reward versus sucrose reward. Certain measures were not normally distributed, and were thus analyzed using non-parametric paired t-tests. Each of the tests used a confidence interval of 95% with a significance level of $p<0.05$, two-tailed. Cohen's *d* was used to calculate effect sizes.

Results

Sucrose vs. Cocaine Two-choice task

When CeA ChR2 laser was paired with earning a reward (either sucrose or cocaine; Figure 5.1), that reward was highly preferred to the alternate reward lacking laser stimulation. Specifically, those rats choosing between laser-paired sucrose or alternatively a cocaine infusion alone, preferred the laser-paired sucrose reward, choosing it on average 11 times compared to only earning 3 cocaine rewards (t_3=6.2, p=.009, 95%CI: -11.7, -3.8, *d*=3.18). Similarly, when CeA ChR2 stimulation was paired with earning cocaine compared to earning a sucrose reward alone in the same session, the laser-paired cocaine became the sole object of desire, with rats earning an average of 12 laser-paired cocaine rewards as opposed to 3 sucrose rewards earned in the same session (t_4=9.87, p=.0006, 95%CI: -11.0, -6.2, *d*=4.48) (Figure 5.2A). In short, rats chose to exclusively earn their laser-paired reward, earning barely any more rewards than when they were forced to sample each option in the beginning of the session. Furthermore, rats were quicker to make their choice, showing shorter latencies to actually make their laser-paired reward choice - between the nose port first extending and when the rat made its active poke to receive the reward, on average rats took 4 seconds to make their laser-paired reward choice versus 62 seconds to make their reward alone choice (Wilcoxon Signed Ranks Test, Z_5=-2.2, p=.02, 5.92, 17.18; effect size=2.45), presumably so long because they were waiting for the laser-paired option to appear and after waiting over 4 minutes, decided to just nose poke for their reward alone option (Figure 5.2B). Control virus rats ('eYFP Controls') chose equally between their laser-paired reward and reward alone options, choosing each on average ~5 times per session (t_4=.18, p=.87) (Figure 5.2C).

CeA ChR2 rats showed similar amount of days to train and to earn each laser-paired and non-laser paired reward - taking the same amount of days to earn 50 rewards of each, around 4 days to earn 50 laser-paired rewards and 5 days on average to earn 50 rewards not paired with

laser (# of training days laser-reward vs # training days for non-laser reward: t_8=1.82, p=.107). Furthermore, amount of days training did not differ between CeA ChR2 rats and control inactive virus rats ($F_{1,12}$=1.49, p=.25).

Virus spread and optic fiber placements

ChR2 and eYFP control virus was spread throughout medial and lateral portions of CeA (CeL and CeM, respectively) (Figure 5.3B). Optic fiber placements were predominantly located in middle to posterior divisions of CeA (n=6), while fewer placements were in sites anterior of -2.0 from bregma. Preference for laser-paired reward was high in virtually all rats, with no apparent anatomical segregation of function (Figure 5.3B).

Intermittent access

We next gave rats a chance to earn their laser-paired reward or reward alone in two separate sessions and during a different pattern of self-administration. Specifically, we gave rats intermittent access to their reward, using design based on (Zimmer et al., 2012). Sessions consisted of four 5 minute blocks of reward availability where rats could continuously nose poke for their reward alone or laser-paired reward without incurring any time-outs. Following each 5 min reward availability period was a 25 min 'reward unavailable' period. Each session allowed access to one reward (laser-paired reward or reward alone) and order of reward type was counterbalanced between rats.

Cumulative responses throughout the session showed an effect of Laser such that laser-paired reward responding was higher than reward alone responding (F_1=23.23, p=.017), and responses specifically grew just for the laser-paired reward (interaction between bin and laser: $F_{3,9}$=10.11, p=.003) (Figure 5.4). This enhancement of motivation in the intermittent access paradigm was mostly driven by the rats whose laser was paired with sucrose rather than if it were paired with cocaine (interaction between laser*reward type: $F_{1,3}$=13.51, p=.035). For instance, total responses by rats for their laser-paired sucrose were higher than the cocaine alone in the intermittent access test (t_2=7.01, p=.02, 95%CI: 50.9, 212.4; d = 6.64) while total responses by rats for their laser-paired cocaine did not significantly differ from responding for their sucrose alone reward (t_1=0.27, p=0.8).

Discussion

CeA controls choice between sucrose and cocaine

When CeA ChR2 stimulation was paired with earning either intravenous cocaine, sucrose, that reward became the target of pursuit. Additionally, rats were faster to make their choice, suggesting they preferred to earn that reward above and beyond the alternative. Furthermore, in the intermittent access sessions where there was continuous access to a reward during certain periods of time, rats similarly showed increased responses for their laser-paired reward compared to the reward that was unpaired with laser stimulation. Our results suggest that CeA excitation can powerfully control which reward gets preferred when rats are able to choose between two different reward types, and during different patterns of reward-taking.

CeA stimulation creates learned preference for a paired reward

Here, CeA stimulations were paired with the reward from the very beginning of training, and continued through choice sessions. Thus, CeA stimulation was acting on associations (between the action of nose poking and a particular sucrose or cocaine outcome) while learning was taking place, and resulted in making that reward more preferred. Even if rats completed their training sessions at similar rates for their laser-paired reward and reward alone, a significant preference emerged during their choice session. This suggests that CeA excitation was interacting with learning to create a preferred target across multiple days of training. This also suggests that CeA stimulation may not create a preference in-the-moment for its paired reward. For example, CeA laser being applied after learning had taken place may not have been effective in biasing choice here (but future work could directly test this). This finding is consistent with previous enhancement of motivation by CeA photoexcitation (Robinson et al., 2014; Warlow et al., 2017), where preference for a particular reward only emerged after several days of training. Further, preference for those laser-paired rewards persisted several days after laser stimulation was removed. Our evidence here combined with previous demonstrations suggest that the ability for CeA excitation to focus motivation onto a particular target depends somewhat on learning. Indeed, CeA is crucial for several forms of appetitive learning (El-Amamy and Holland, 2007; Mahler and Berridge, 2009, 2012; Esber et al., 2015; Fadok et al., 2018), and excitation here could have altered associative learning in such a way that increased the perceived value of that paired reward, making it a preferred option. This would result in a strong preference as soon as rats were able to choose between rewards (as seen in greater responses during the choice session), and would also explain why rats barely hesitated to make their laser-paired reward choice when both were available (as seen in reduced latencies).

CeA stimulation may overcome any baseline preference for sucrose

Here we showed that no matter the type of reward, CeA stimulation was able to control the focus of motivation. Others using the same procedures have demonstrated that virtually all rats highly prefer sucrose rewards over intravenous cocaine or other drugs (i.e., nicotine or methamphetamine) (Lenoir et al., 2007; Lesage, 2009; Cantin et al., 2010; Galuska et al., 2011; Kerstetter et al., 2012). While our observation that CeA biasing of preference for a paired sucrose reward would be somewhat not surprising given previous reports of sucrose preference, we also demonstrate that CeA-pairing produces a cocaine-preferring rat, even if they would have preferred sucrose under normal conditions.

It is worth noting that preference for sucrose over drugs can be manipulated by several factors, such as extended history of drug self-administration (Lenoir et al., 2013b) or choice sessions that allow shorter time out intervals between choices (Lenoir et al., 2013a; Ahmed, 2017), which lead to a shift in preference for drug over sugar. However, these factors were controlled for in our design, and we still observed preference for whichever reward was paired with CeA stimulation. Further, in some situations, intensely sweet substances such as saccharine solution can override these factors and saccharine is still highly preferred by rats. For example, saccharin solution is highly preferred by all rats over an alternative cocaine reward during the same choice procedure used here (Lenoir et al., 2007), even in individuals with history of extended access to cocaine self-administration. While our stimulation demonstrates a powerful ability for CeA stimulation to induce preference between sucrose and cocaine, this does not necessarily extend to intensely sweet, non-caloric, saccharine. Future work could examine CeA control of choice between drugs and other types of rewards such as saccharine or with other highly palatable food (Calu et al., 2013; Krasnova et al., 2014).

Here we demonstrate that hijacking CeA circuitry to arbitrarily paste excitation onto a particular reward makes that reward exclusively pursued. The fact that excitation of amygdala circuitry amplified motivation for a sucrose or for a cocaine reward implies overlapping circuitry which mediates motivation for both rewards. This would suggest that while mesolimbic circuitry may have evolved for seeking out natural rewards, it can be hijacked by drugs of abuse to produce pathological ‘wanting’ for drugs as seen in addiction (Nesse and Berridge, 1997). Indeed, CeA circuitry may be a potential target that is hijacked during drug exposure in some individuals to produce narrowed motivation for drugs at the expense of other life rewards

(Robinson and Berridge, 1993; Ahmed, 2010; Berridge and Robinson, 2016), and could provide further insight to explain why addicts ‘want’ only one reward.

Figures

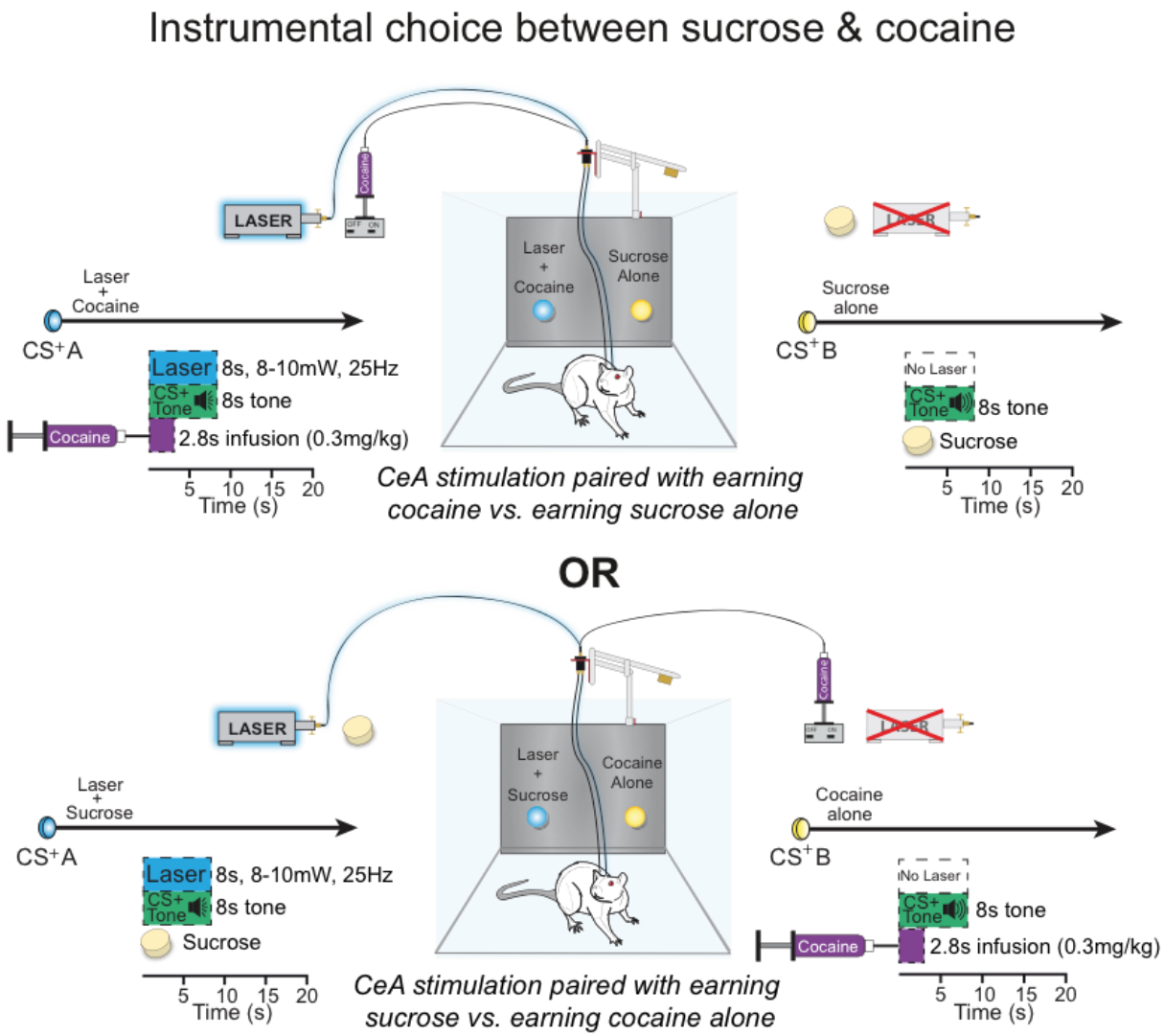
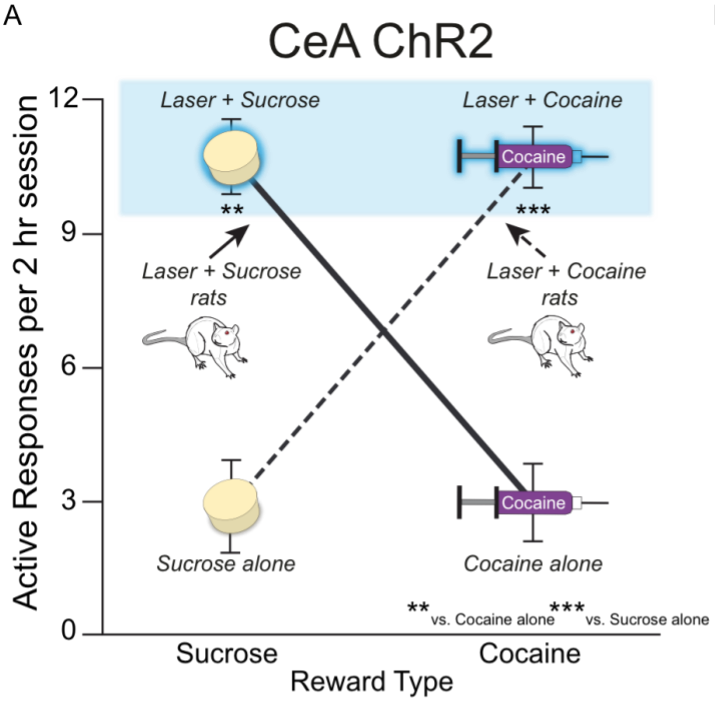
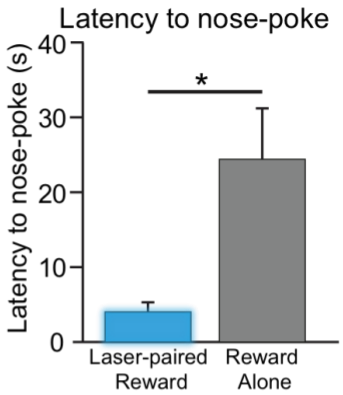


Figure 5.1
Instrumental choice between sucrose and cocaine
Choice sessions allowed rats to choose between earning a 45 mg sucrose pellet or earning a cocaine infusion (0.3 mg/kg/infusion) by nose poking into portholes. In some rats, nose poking into one porthole earned a cocaine infusion accompanied by a 8 s auditory cue, plus CeA laser stimulation (473 nm, 8-10 mW intensity, 25 Hz frequency: 25 ms ON, 15 ms OFF; "Laser + Cocaine"), while nose poking into a different porthole in the same session earned a 45 mg sucrose pellet accompanied by its own distinct 8 s auditory cue (and lacking CeA laser; "Sucrose alone"). In a separate group of rats, the sucrose and cocaine were still options that could be earned via nose poking, but CeA laser stimulation accompanied sucrose pellet delivery ("Laser + Sucrose) and not the cocaine infusion ("Cocaine alone"). After each choice was made and that particular reward was earned, a 10 min timeout was imposed before both options became available again.

A


CeA ChR2
Laser + Sucrose
Laser + Cocaine
Cocaine
**
***
Laser + Sucrose rats
Laser + Cocaine rats
Cocaine
Sucrose alone
Cocaine alone
** vs. Cocaine alone
*** vs. Sucrose alone
Active Responses per 2 hr session
12
9
6
3
0
Sucrose
Cocaine
Reward Type

B


Latency to nose-poke
Latency to nose-poke (s)
40
30
20
10
0
*
Laser-paired Reward
Reward Alone

C

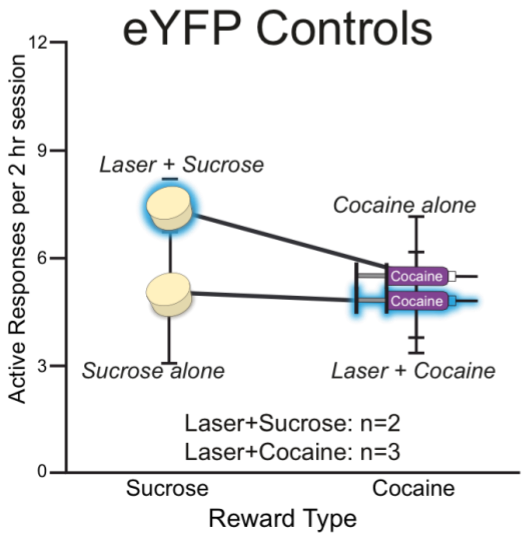

eYFP Controls
Active Responses per 2 hr session
12
9
6
3
0
Laser + Sucrose
Cocaine alone
Cocaine
Cocaine
Sucrose alone
Laser + Cocaine
Laser+Sucrose: n=2
Laser+Cocaine: n=3
Sucrose
Cocaine
Reward Type

Figure 5.2
CeA stimulation controls choice between sucrose and cocaine. (A) CeA ChR2 rats who had CeA laser paired with earning sucrose ("Laser + Sucrose") chose to exclusively earn that reward compared to a cocaine infusion lacking CeA laser ("Cocaine alone") in the same 2 hr session, responding at higher amounts for the Laser + Sucrose option (group of rats indicated by solid black line). CeA ChR2 rats who had CeA laser paired with earning cocaine ("Laser + Cocaine") similarly chose to exclusively earn that paired cocaine reward above and beyond a sucrose pellet lacking CeA excitation ("Sucrose alone"), making greater responses for the Laser-paired Cocaine reward (group of rats indicated by dashed line). (B) Furthermore, CeA ChR2 rats were quicker to choose the laser-paired reward (blue bar) compared to the reward lacking CeA laser ("reward alone"; grey bar) during forced choice trials in the beginning of the session when one reward was available at a time. (C) By contrast, control virus rats chose equally between laser-paired rewards and rewards alone ("sucrose alone" and "cocaine alone") in their choice sessions, whether laser-was paired with sucrose ("Laser+Sucrose") or with cocaine ("Laser+Cocaine"). Data represents mean +/- SEM. **p<.01 ***p<.001

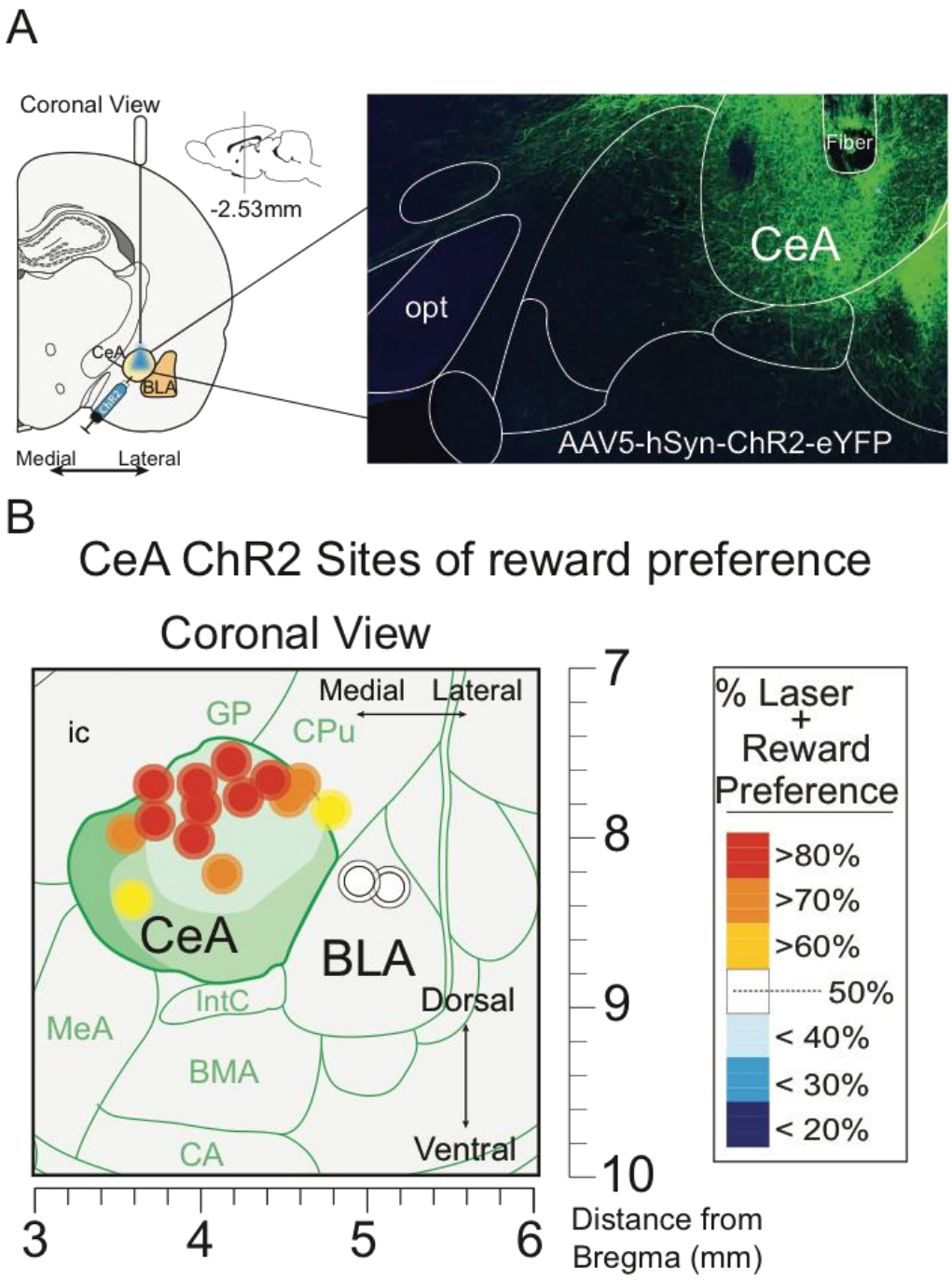


Figure 5.3
ChR2 virus expression and placement map. (A) Photomicrograph taken at 10x magnification depicts representative ChR2 virus (AAV5-hsyn-ChR2-eYFP) transfection within CeA based on rat atlas modified from Paxinos and Watson (2007). ChR2 virus fluoresces green against a DAPI stained blue background. (B) *CeA ChR2 sites of reward preference*. Placements of optic fiber implants for each ChR2 rat were plotted onto a Coronal view of amygdala modified from Paxinos and Watson (2007). Size of each placement was based on previously reported Fos plume sizes as a result of CeA laser stimulation at the same stimulation parameters (Warlow et al, 2017). Each placement's color represents the percentage preference for the Laser-paired reward during the sucrose vs. cocaine choice sessions.

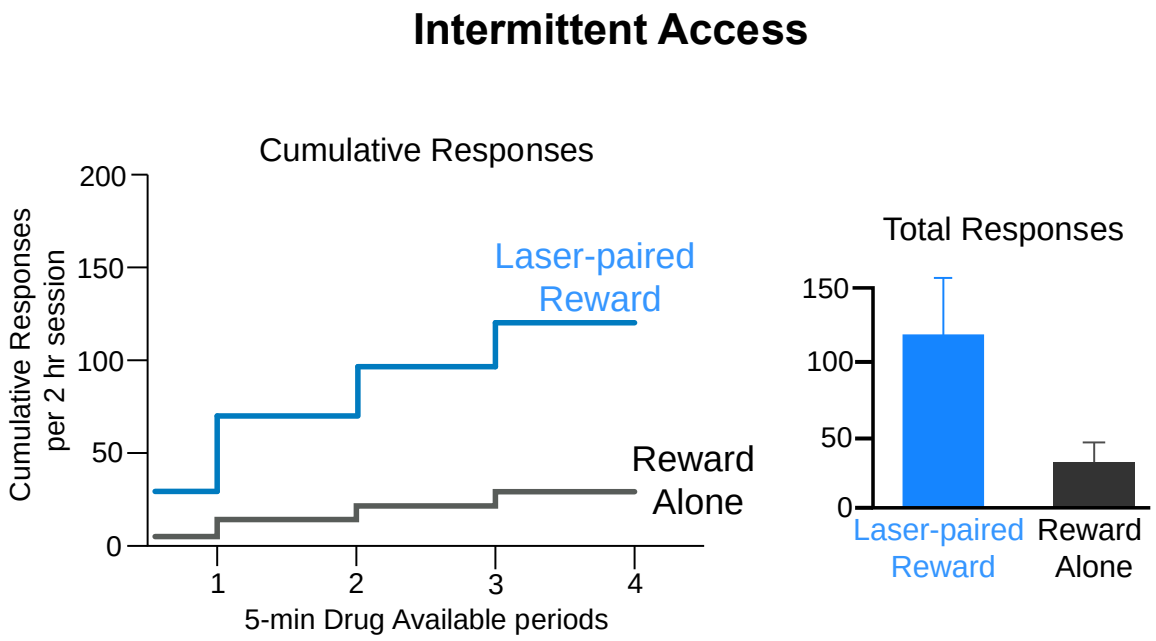


Figure 5.4
Intermittent Access Paradigm. Rats were tested on two days of intermittent access sessions, where they had access to one reward on each day ("Laser-paired reward" or "reward alone"; order of sessions counterbalanced between rats). Each session consisted of 4 5-min reward available periods, each followed by a 25-min reward unavailable period. ChR2 rats showed increased responding for their laser-paired reward (solid blue line) compared to responding for the reward alone (solid grey line) as early as the first 5-min reward available block, and this increase continued throughout the session, resulting in greater total responses for the laser-paired reward (blue bar) than for reward alone (black bar) Data represents mean +/- SEM. **p<.01 ***p<.001

CHAPTER VI. Dangerous desire: Central amygdala circuitry amplifies attraction towards aversive stimuli

Introduction

Central amygdala (CeA) is a crucial node for fear expression, and substantial evidence supports CeA in regulating freezing during fear conditioning, as well as promoting avoidance behaviors (Haubensak et al., 2010; Wolff et al., 2014; Campbell-Smith et al., 2015; Kochli et al., 2015; Namburi et al., 2015). Though most widely known for its role in fear, central amygdala circuitry can promote reward (Robinson et al., 2014; Seo et al., 2016; Kim et al., 2017; Warlow et al., 2017). Previous work has demonstrated that CeA excitation focuses and intensifies motivation for a particular sucrose reward (Robinson et al., 2014). Similarly, CeA excitation narrows choice and enhances motivation for intravenous cocaine (Warlow et al., 2017). If CeA excitation was able to powerfully bias preference for a particular reward over and above an identical alternative, this suggested that CeA excitation was exponentially increasing the perceived incentive value of that reward, making it irrationally 'wanted'. If CeA excitation were able to increase perceived incentive value of a reward, then the question remains whether the same CeA excitation could be powerful enough to flip an aversive target into one that may be perceived as rewarding. Thus, explored the effects if pairing optogenetic CeA excitation with a known aversive target. Would CeA-pairing be powerful enough to transform an aversive stimulus into an attractive stimulus?

Here, we paired CeA ChR2 stimulation with an electrified shock rod upon interaction. We found that CeA ChR2 stimulation amplified attraction towards rather than avoidance of it. Importantly, CeA ChR2 stimulation was not reinforcing on its own, as rats did not self-stimulate laser in two laser self-administration tests. CeA-induced attraction was accompanied by consummatory biting and chewing of the target, and conditioned reinforcement of a paired cue, suggesting enhancement of incentive salience attribution caused by CeA ChR2 stimulation. Furthermore, attraction was associated with enhancement of Fos protein expression among

several mesolimbic brain regions suggesting CeA stimulation recruits mesolimbic brain circuitry to amplify attraction towards rewarding and aversive stimuli.

Materials and Methods

Subjects

The subjects in this experiment were male and female Sprague Dawley (n=18), male Sprague Dawley (n=6) and female Long Evans Hooded rats (n=12) weighing between 250g and 300g before surgery. Rats were housed in a room maintained at ~21ºC on a reverse 12 hour light/dark cycle. The rats had *ad libidum* access to both chow (Purina Lab Chow) and water. Before performing surgery, each rat was handled on average 5 days for 10 minutes each day. The University of Michigan's Committee on the Use and Care of Animals approved all procedures.

Optogenetic virus infusion and optic fiber implant

Each rat received an optogenetic surgery in order to be infused with one of two viruses: either the experimental virus (AAV5-HSYN-ChR2-eYFP, n=25) or the inactive virus (AAV5-HSYN-eYFP, n=11). Optogenetic virus was injected bilaterally into the CeA (A/P from Bregma: -2.4, M/L: 4, D/V: -7.6 with mouth bar set to -3.3), followed by implantation of chronic dwelling optic fibers lowered to 0.3 mm above virus placement and secured via dental acrylic. Prior to the surgery, the rats were anesthetized with 5% isoflurane anesthesia, and received atropine (0.04 mg/kg; Henry Schein). During surgery, rats were maintained at 2-3% isoflurane. Additionally, rats were subcutaneously injected with chloramphenicol sodium succinate (60 mg/kg, Henry Schein) to prevent infection, and carprofen (5 mg/kg, Henry Schein) as an analgesic.

Testing Apparatuses

Shock rod testing was performed in Plexiglas chambers (38 cm width x 38 cm length x 48 cm height). An electrified shock probe was kept inserted into one side of the chamber at 5 cm high and protruded 9 cm into the chamber. Corncob bedding identical to the bedding in the rats' home cage (Bed'O'Cobs, Andersons Inc., Maumee, OH), was placed in the bottom of the chamber and filled the entire floor and was 2 cm deep. The shock rod (1.5 x 1.5 x 9 cm) contained wiring all around the entirety of the probe and delivered a 0.1 mA shock when touched at any location. A video camera was placed in front of the chamber and recorded each session.

Conditioned reinforcement tests were performed in modified MedAssociates chambers. Chambers were equipped with fixed portholes on either side of the same wall plus auditory

speakers to play different auditory cues (tone or white noise). Nose poking into either porthole was detected by infrared beams and recorded by MedPC software.

Self-stimulation spout based tests were performed in modified MedAssociates chambers. On one side of the wall, two empty liquid spouts were placed into the chamber – one on either side of the same wall. Plexiglas floors of the chamber were replaced by grid floors, which, upon activation of either spout, closed a circuit to trigger a response, which was recorded by MedPC.

Self-stimulation location-based tests were performed in modified Plexiglas chambers identical to shock rod tests. Floors of chamber, again, contained bedding (2 cm deep) and were equipped with movement sensors placed above each of the 4 corners of the chamber. Sensors detected movement and triggered a MATLAB program.

Odor place preference tests were performed in a modified conditioned place preference chamber (21 x 23 x 70 cm). The chamber contained three different compartments distinguished by different grated floors and either striped or polka dotted wall sides. One side of the chamber contained walls with a striped pattern and a separate side of the chamber displayed a polka dot pattern on the walls, with a third middle compartment containing a neutral gray color. Each side could be additionally distinguished by different floors, one side with larger grating and the other side with smaller grating. A video camera was placed on top of the chamber and recorded each session.

Procedures

Laser-paired Aversive Shock Rod

We paired CeA ChR2 stimulation with contacting an aversive shock rod, which protrudes 9 cm into one side of a Plexiglas chamber containing bedding on the floor. The use of shock rods is based in models of anxiety and active expression of fear, as rats perceive the shock rod as a threat and subsequently attempt to bury it by defensively treading (Treit et al., 1981; Reynolds and Berridge, 2001; De Boer and Koolhaas, 2003) (Figure 6.1). At the start of each session, rats (ChR2: n= 25; eYFP Control: n=11), were attached to cables and placed into the middle of the chamber and allowed to freely walk around the chamber. Upon contacting the shock rod at any part, a low-intensity shock (0.25 mA) is delivered. Laser stimulation (473 nm, 10 mW, 40 Hz (5 ms ON, 25 ms OFF)) was triggered via MATLAB program whenever rats were within an inch of the shock rod, and lasted for as long as they stayed within that perimeter (on average between 3 and 8 s duration). Each session lasted 20 minutes, and was video recorded for scoring offline

later using Noldus Observer Software. On the fourth day of training, sessions were identical to the previous days except that laser stimulation was no longer delivered at all during the entire session.

Conditioned Reinforcement Test

To test whether a sound cue associated with the shock rod had gained value as a result of association, a conditioned reinforcement test was administered after training sessions with rats. In a subset of rats (ChR2: n=8, eYFP Control: n=6), training sessions with the shock rod involved presentation of an auditory cue (tone or white noise, counterbalanced between rats) whenever rats were within an inch of the shock rod (same criteria for delivering laser illumination). In a separate MedAssociates chamber, conditioned reinforcement tests were administered on 2 separate days. Two portholes (fixed into the same wall of chamber) were available for nosepoking. Nose poking into one of the portholes triggered delivery of the shock rod-paired auditory cue (CS+) to play for 4 seconds (either tone or white noise; 'Active poke'), while nose poking into the other porthole was recorded but had no consequences ('Inactive poke'). On a separate day, nosepoking into the 'active port' delivered an auditory cue that was never paired with the shock-rod, but instead was played in a separate neutral environment (CS-; amount of time was matched to the same amount of times the CS+ was played for each rat in their shock rod context). Each session lasted 30 minutes, and order of paired and unpaired cue delivery was counterbalanced.

Food Intake: Laser stimulation and general motivation to eat

We explored the effect of CeA laser stimulation on voluntary food consumption in a 60 min free-intake test. Food intake tests took place in a familiar home cage environment with bedding on the floor, where ChR2 rats (n=8) had continuous access to pre-weighed quantities of Purina Lab Chow (~20 g), while also having constant access to water. At the end of each session, remaining chow was re-weighed to calculate the amount consumed. Intake test days were repeated on 3 consecutive days, and laser stimulation was administered only on one day, occurring either on day 2 or 3 (counterbalanced across rats). Laser stimulation was delivered in 15 s pulses once every 30 s (40 Hz; 20 ms ON, 5 ms OFF; 10 mW; 15 s ON pulse duration followed by 9 s OFF). Control baseline intake was measured in the absence of laser stimulation during days 2 or 3, whichever day that lacked laser stimulation. During each test day, a pre-weighed wooden block (~18 g) was additionally placed into the testing chamber to test the

effects of CeA stimulation on chewing behavior of a neutral object, and was weighed at the end of each test to calculate amount consumed. Time spent chewing during laser and non-laser sessions was also scored offline using Noldus Observer software.

Self-stimulation Tests

Rats showing CeA ChR2-induced attraction towards either a paired sucrose or cocaine reward, or towards the aversive shock rod were allowed to self-administer laser stimulation by performing a simple response in two different self-stimulation tests. In the first, a location-based tested modeled after the original Olds & Milner demonstration of electrode self-stimulation (Olds and Milner, 1954), they could walk into one side of a plexiglas chamber (38 cm x 38 cm x 47 cm height) and earn pulses of CeA laser stimulation. Each side of the chamber had 2 motion detectors (Visonic) placed above the chamber which detected entries. One of the sides was assigned for laser stimulation (sides counterbalanced between rats), and rats (n=9) could earn laser stimuation by staying in that side and continuing to move to trigger the sensors (40 Hz, 10 mW, 3 s duration). Each session lasted 30 min and was repeated with the same side assignment on 2 consecutive days.

A second self-stimulation test, CeA ChR2 rats were allowed to self-administer laser illumination by touching a spout. Rats were placed in MedAssociates chambers equipped with two empty liquid spouts on the back wall of the chamber containg grid floors, which were wired to detect body contacts that closed a circuit with the floor. Contacts on one of the two spouts delivered brief CeA laser stimulation (assignment of spout counterbalanced between rats) at the same stimulation parameters as during the shock rod experiments (40 Hz, 10 mW, 1 s duration: n=11, or 8 s duration: n=7). Touching the other spout produced no consequences, serving as a control for baseline exploratory behavior. Each session lasted 30 min, and was repeated for 2 consecutive days, with laser spout assignments staying the same for each rat across days.

Pavlovian Fear Conditioning

Rats (CeA ChR2: n=8; CeA eYFP control virus: n=5) were trained for 3 consecutive days. During training on the first day, where after a 3-min baseline of being in the chamber, they were subsequently exposed to 3 CS-US pairings separated by 60 s fixed intervals, where the CS+ was a 10s tone (75 db) plus blue laser illumination (10s, 40Hz, 10mW), and the US was a shock scrambled across a grid floor (500 ms, 0.75 mA). After 3 CS-US pairings on day 1, rats were exposed to 2 CS-US pairings on day 2 (identical to day 1 conditions), and on day 3 were exposed

to 1 CS-US pairing again identical. During each training day, a distinct odor (almond or lemon essence, type of odor counterbalanced between rats) was placed onto 2 delicate task wipes (KimTech Science), and were used to wipe the Plexiglas chamber. Following training days, rats were tested in a distinctly different chamber, consisting of a different odor (versaclean), a house light, and a plexiglas floor. Testing sessions consisted of a 1 min baseline followed by 10 CS+ tone presentations separated by 60 s fixed intervals. For 5 of these presentations (order randomized), CeA laser illumination occurred concurrently with the 10 s CS+ tone (10 s, 40 Hz, 10 mW). Following test sessions, on a separate day, rats were tested for preference or avoidance of the odor CS+ that was paired with shock context or for a CS- odor that was separately presented for a similar amount of time but in a neutral home setting with no shock delivery. Animals were placed into modified conditioned place preference chambers that contained separate sides that were distinguished by different patterns displayed on the walls (stripes or polka dots). Task wipes (Kimtech Science) containing CS+ odor were placed under one compartment while wipes containing CS- were placed under the other compartment (sides of CS+ odor assignment were counterbalanced between rats). Time spent in each compartment was scored offline using Noldus Observer Software.

Virus and Optic Fiber placement verification plus distant Fos-protein analysis

The same rats used in the experiments above were subjected to either of the following conditions: 1) a shock rod session identical to procedures used during the first three days of training (n=18), or 2) a session where laser stimulation was delivered non-contingently in the same Plexiglas chambers for 20 min (40 Hz, 10 mW, 6 s ON 24 s OFF cycle for 20 min; n=6). 75 minutes after the beginning of either of these conditions, rats were injected with an overdose of sodium pentobarbital (150-200 mg/kg) to deeply anesthetize and were subsequently transcardially perfused. An additional group (n=4) had no manipulations and were injected directly from their home cage to serve as a baseline, naive tissue group. After the perfusion, the brains were stored in a solution of 4% paraformaldehyde for 24 hours prior to being cryoprotected in 30% sucrose. To identify the optic fiber placement and virus expression, the brains were sliced at 40 µm coronal sections. The coronal slices were subsequently processed for Fos protein visualization using immunohistochemical procedures previously described (Warlow et al., 2017). Once processed, sections were mounted onto slides, air dried, and then cover slipped using ProLong Gold anti-fade reagent (Invitrogen) in order to preserve the brain slices.

A Leica microscope (Leica Microsystems, Inc., Buffalo Grove, IL) was used to analyze the optic fiber sites and GFP virus expression of the brain slices at a magnification of 10x. The virus and optic fiber placement were illuminated in Adobe Illustrator (RRID: SCR:014198) on a rat brain atlas (Paxinos and Watson, 2007). Using Oasis Surveyor software (Objective Imaging, Inc., Kansasville, WI; RRID: SCR014433), nine images of the central amygdala site were assembled into one image that were centered at the fiber tip with mapping of virus intensity around the fiber tip.

For distant Fos quantification, Oasis Surveyor software was used to capture tiled images of whole brain coronal section at 10x magnification pre-determined by Paxinos & Watson brain atlas and using the TXRED filter cube to visualize Fos protein. Brain images were used to count Fos protein in various brain regions. For each brain region, anterior, posterior, and middle regions were counted. For each region (at each anterior-posterior site), three 100 x 100 um boxes were placed onto the coronal brain image in Adobe Photoshop software. In order to make sure box placements for each region were consistent between rats, placement of boxes for that location was based on the placement of boxes on Atlas pages as a template.

Statistical Analysis

Using SPSS software (RRID: SCR:002865), parametric paired t-tests and independent t-tests were used to analyze within group data and between group differences in regards to laser-pairing versus non-laser pairing, control group versus experimental group on each day. Certain measures were not normally distributed, and were thus analyzed using non-parametric paired t-tests. Each of the tests used a confidence interval of 95% with a significance level of $p \leq 0.05$, two-tailed. Cohen's *d* was used to calculate effect sizes.

Results

CeA ChR2 stimulation paired with an aversive shock rod

We paired CeA ChR2 stimulation with an aversive shock rod that delivers a small shock upon touching with snout or forepaws. Shock rod experiments have been classically used to measure levels of anxiety and active forms of fear (Treit et al., 1981; De Boer and Koolhaas, 2003; Pinel et al., 2013). Every time rats interacted with the shock rod, CeA laser stimulation was delivered (40 Hz: 5 ms ON, 25 ms OFF, 10 mW) (Figure 6.1).

Starting on the first day, CeA ChR2 rats made slightly greater number of shock rod touches (on average 5 times) than control inactive virus rats who touched the rod on average 2

times (t_{26}=-2.06, p=.05, 95%CI: -5.5, .002, *d*=-1.2). CeA ChR2 rats in fact increased their touching of the shock rod over the next two test days from 5 to 9 touches (this increase was not significant: F_2=2.95, p=.07), averaging ~7 touches vs. ~2 touches by control virus rats on day 2 (t_{23}=3.8, p=.001, 95%CI:-7.8, -2.2, *d*=-1.12) and ~8 touches vs. ~1 touch by control virus rats on day 3 (t_{23}=3.6, p=.002, 95%CI:-11.8, -3.2, 11, *d*=-1.07) during each 20 minute session (Figure 6.2A).

Ethovision software was used to create heat maps of each individual rat's location throughout each session, and depict where rats spent the majority of their time. The location of each rat's predominant location (indicated by darkest red color on each heat map) showed that ChR2 rats spent the majority of their time 5 +/- 2 cm away from the tip of the shock rod whereas control virus rats spent the majority of their time in a given session 21 +/- 2 cm away from the shock rod (t_{18}=-3.8, p=.001, 95% CI: -25, -7.2, *d*=2.11) (Figure 5.2B). This indicates that ChR2 rats were more attracted to the rod, spending most of their time near it, while control virus rats were averse to the shock rod, spending most of their time as far away from it as possible (the wall opposite of the shock rod is 29 cm away from the tip of the rod and control virus rats spent most of their time 21 cm away, which is basically positioned on the opposite wall).

In addition to touching the shock-delivering rod at greater numbers and being located nearer to it, CeA ChR2 rats displayed ingestive-like chewing of the rod. Although being shocked upon contact of the shock rod, CeA rats receiving CeA stimulation paired with the shock rod spent on average 60 seconds vigorously chewing the shock rod (either at the end or along the sides) on the first day (20 minute session) This was in comparison to virtually no chewing among control virus rats (t_{20}=-2.6, p=.02, 95%CI: -67, -7.5, *d*=0.8). By day 2, CeA ChR2 rats were spending roughly 75 seconds chewing the rod vs. 0 seconds by control virus rats (t_{20}=-4.4, p=.000, 95%CI: -111, -40, *d*=1.37). On day 3 CeA ChR2 rats spent an average of ~85 seconds chewing the shock rod compared to 0 seconds by control virus rats (t_{18}=-3.3, p=.004, 95%CI: -140, -30, *d*=1.06). Chewing was not limited to a few rats but rather on the first test day 14/21 rats chewed the rod at least once vs. the 0/8 control virus rats (Figure 6.3).

Instead of chewing the shock-delivering rod, control virus rats emitted defensive treading towards it. Defensive treading is an anti-predator action usually elicited by rodents towards a perceived threat (i.e. snakes in the wild) (Owings and Coss, 1977; De Boer and Koolhaas, 2003). In laboratory settings, defensive treading involves pushing of bedding with forepaws towards a

perceived threat (i.e., an experimenter or salient aversive stimulus) (Bolles and Fanselow, 1980; Treit et al., 1981; Reynolds and Berridge, 2001; Pinel et al., 2013). Here, treading was observed in control rats towards the shock rod which increased over test days, and was very rarely observed among CeA ChR2 rats. Specifically, on day 1, control virus rats spent double the time treading towards the shock rod compared to ChR2 rats (~120 seconds vs. ~70 seconds; t_{26}=-.72, p=.47). By day 2, time spent treading by control virus rats significantly exceeded CeA ChR2 rats with control virus rats treading ~150 seconds and CeA ChR2 spending only ~9 seconds treading (t_{28}=2.66, p=.01, 95%CI: 32, 245, *d*=0.81). By the third day of testing, CeA ChR2 rats were similarly emitting low levels of treading, only spending ~13 seconds of the 20 minute session while control virus rats spent on average 186 seconds treading towards the shock rod (t_{21}=-2.4, p=.03, 95%CI: 29, 410, *d*=0.72) (Figure 6.4A). Furthermore, while 6/8 control virus rats emitted treading during the test session, less than half of CeA ChR2 rats (9/22) treaded towards the shock rod, and 3 of those 9 rats spent under 5 seconds doing so (From day 2).

Treading, or defensive burying, often resulted in mounds that sometimes completely buried the shock rod. 100% of control rats had formed some semblance of a mound by the end of their session, while only 4/19 ChR2 rats created a mound by the end of their session. Mounds created via defensive burying were always around the shock rod and never created in other areas of the chamber. Control rats created mounds which were on average 5 cm height x 6 cm length x 19 cm width, whereas mounds created by ChR2 rats were much smaller and barely visible (2.5 cm height x 0.9 cm length x 2.4 cm width) (height: t_{26}=-4.3, p=.000, 95%CI: -3.5, -1.2, *d*=1.73); length: t_{26}=-5.4, p=.000, 95%CI: -6.9, -3.6, *d*=2.09); width: t_{26}=-6.5, p=.000, 95%CI: -22.2, -11.6, *d*=2.34) (Figure 6.4B). As a reference, the top of the shock rod sat at 6 cm high, so control rats were creating mounds on average that were right up to the rod (5 cm), whereas the average height of mounds created by the few ChR2 rats were less than half the height of the shock rod (2.5 cm high).

On day 4, when CeA laser was no longer paired with the shock rod (rats did not receive any CeA stimulation during the entire 20 min session), touches dropped to control virus levels with CeA ChR2 rats touching the rod an average of 3 times vs. Control virus rats touching the rod on average less than 1 time (t_{11}=1.18, p=.26). Similarly, while treading continued among control virus rats (spending ~80 s treading), ChR2 rats started to tread only slightly (averaging ~5 s), although this difference was not significant perhaps due to high variation among rats (t_{4}=-

1.69, p=.16). ChR2 rats also ceased chewing of the rod, and spent on average less than 4 s total chewing it, which was comparable to control virus rats who did not chew at all (t_{11}=.9, p=.38).

To test how robust this shock rod attraction was, we delivered laser stimulation at varying frequencies on different test days, order of frequency randomized between rats, such that each rats was tested on at least 3 of the 4 different stimulation parameters: 10 Hz (10 ms ON 90 ms OFF), 25 Hz (15 ms ON 25 ms OFF), 1 mW constant illumination, or 40 Hz (5 ms ON 20 ms OFF). We found that measures of shock rod attraction such as touches among ChR2 rats were similarly high across the different parameters, ranging from 4 +/-2 at 1 mW constant duration to 8+/-3 touches at 25 Hz, and 6 +/-2 touches at 40 Hz stimulation ($F_{3,43}$=.60, p=.62) (Figure 6.5). Time spent treading at each of the stimulation parameters was quite low and always below 5 s (comparable to the first 3 days of training among ChR2 rats). There were no differences in time spent treading between different parameters ($F_{3,43}$=1.15, p=.34).

Conditioned Reinforcement

To test whether a cue associated with the shock rod had gained value because of association, a conditioned reinforcement test was administered after training sessions with rats. On one day, nose poking into one of the fixed portholes delivered a sound cue (tone or white noise) that had been previously paired with shock rod while nose poking into the other porthole delivered nothing. On a separate day, nose poking into the active port delivered a sound that had not been paired with a shock rod, but instead was played a similar amount of times in a neutral setting while rats were in their home cage (amount of times was yoked to each rat's amount of times they heard the shock-rod paired sound cue). When rats could nose poke to earn their shock rod-associated sound, ChR2 rats nose poked to hear their shock rod associated sound ~15 times more than nose poking into the inactive port and this was compared to control virus rats that nose poked ~0.2 times less for the active port than the inactive port (t_{12}=2.4, p=.03, 95%CI: 1.5, 29.6, *d*=1.35) (Figure 6.6). By contrast, when able to nose poke to hear a neutral, unpaired sound, the difference between active and inactive pokes among ChR2 and control virus rats was similarly even (4 nose pokes higher for the active port in both groups; t_{12}=-.06, p=.95).

Spout based laser self-administration

To test whether the CeA ChR2-induced shock rod attraction was due to simple reinforcing effects of CeA ChR2 stimulation, two separate laser self-administration tests were used. In the first test, a spout based test, empty water spouts were available to touch in a

modified operant chamber. Touching one spout delivered laser stimulation at the same parameters ('Active spout':40 Hz, 10 mW, 1 s or 8 s), while touching the other spout had no consequences ('Inactive spout'). ChR2 rats did not touch the active, laser-delivering spout any more than the inactive spout (t_{11}=1.65, p=.13) (Figure 6.7A) when laser duration was in shorter 1 s bursts. Even if laser stimulation was offered in longer 8 s bursts, ChR2 rats did not touch the active spout any more than the inactive spout (t_6=1.6, p=.16).

There were a subset of rats (n=4/19) who did self-stimulate laser in the spout based test. Using the criteria of at least 100 touches at the 'active' laser-delivering spout revealed that 3 rats self-stimulated CeA laser. But, even if these self-stimulating rats were removed from statistical tests during the shock rod experiments, measures of shock rod attraction were still significant, including touching the rod more than control virus rats (t_{18}=2.5, p=.02, 95%CI: .52, 5.6, *d*=1.03), chewing for longer periods of time than control virus rats (t_{15}=2.33, p=.03, 95%CI: 4.3, 93.9, *d*=0.9), and treading for less periods of time than control virus rats (t_{20}=-2.31, p=.03, 95%CI: -295, -15, *d*=0.86).

Location based laser self-administration

A second, location-based test was used to assess whether CeA ChR2 rats 'wanted' laser stimulation by itself. In this test, rats could earn laser stimulation by simply walking into one side of a chamber and continuing to move in that side to trigger the laser at the same parameters as during the shock rod experiments (3 s duration, 10 mW, 40 Hz). When laser stimulation was offered in 3 s pulses, ChR2 rats spent equal time in the laser-delivering side and in the opposite side (t_8=0.77, p=.46), and triggering the detectors on each side at equal amounts (t_8=1.99, p=.08) (Figure 6.7B).

Food Intake: Laser stimulation and general motivation to eat

We explored the effect of CeA laser stimulation on voluntary food consumption in a 60 min free-intake test in order to see whether the above chewing and biting incentive-enhancing effects with the shock rod were mediated by increased appetite or general mouth motor effects. Here we tested rats on two separate days where they had access to lab chow and an inedible wooden block. On one day, laser stimulation was delivered throughout the session (40 Hz; 20 ms ON, 5 ms OFF; 10 mW; 15 s ON pulse duration followed by 9 s OFF), and on a separate baseline day, no laser stimulation was delivered. In each 60 min session, rats ate small amounts of lab chow (~1 grams eaten), and there was no difference in amount eaten between laser and

non-laser days (laser session: 1.1 ± 0.4 g, nonlaser session: 1.2 ± 0.6 g; t_7=-.19, p=.86). Laser stimulation did not cause generalized eating of inedible objects such as a wooden block, as rats did not consume the wooden block on either baseline or laser sessions (~0 g each day; t_7=0, p=1.0).

CeA stimulation during Pavlovian Fear Conditioning

To test whether the same CeA-induced attraction to a localizable shock rod source also extended to more classical models of fear expression, we next paired CeA excitation with a tone CS+ during Pavlovian Fear Conditioning. Following 3 training days, rats were placed into a novel context where after 1 min the CS+ was presented alone (no shock delivery) 10 times with 1 min inter trial intervals (ITI) (Figure 6.8A). During this test session, all rats showed enhanced freezing during their first trial compared to baseline with rats on average freezing between 0 and 10% during baseline, and subsequently freezing between 50-70% as soon as the first CS+ tone is presented (baseline vs. first trial, CeA ChR2 rats: t_7=-3.8, p=.007, 95%CI: -75.89, -17.86, *d*=1.9; CeA eYFP control virus: t_4=-3.57, p=.02; 95%CI: -101, -12.6; *d*=2.0). Baseline freezing was significantly lower in the CeA ChR2 group than the eYFP control group (t_{11}=-3.12, p=.01, 95%CI: -17.98, -3.1; d=1.52), thus the two groups were not compared on subsequent freezing levels. In eYFP rats, freezing during the CS+ tone throughout the session did not significantly differ from freezing during the ITI's between tone presentations (F_{period}=5.25, p=.08). Similarly there was no difference in enhanced freezing to the tone as a result of laser stimulation trials in eYFP rats ($F_{period*laser}$=5.5, p=0.94). By contrast CeA ChR2 rats displayed higher freezing levels specifically during the CS+ tone as opposed to the ITI throughout the session (F_{period}=13.1, p=.009). There was a slight trend towards this CS+ focused freezing to be more pronounced during laser stimulation trials ($F_{period*laser}$=5.5, p=.05), and this enhancement of freezing during laser trials was most pronounced when comparing the very first laser and non-laser trials, with freezing levels at near 50% for the first laser trial and 15% for the first non-laser trial (t_7=2.44, p=.04, 95%CI: .98, 64; *d*=1.21). Freezing during all of laser and non-laser trials did not differ among CeA ChR2 rats (F_{laser}=.035, p=.858) or CeA eYFP rats (F_{laser}=.70, p=.45), thus CeA enhancement of freezing appears to be amplified during the first laser trial compared to the first non-laser trial, but afterwards freezing is similar across both trial types (Figure 6.8B).

To test the effect of CeA ChR2 stimulation on forming an association between a specific odor CS+ and shock delivery, we also paired a distinct odor with the shock grid context, and

subsequently tested rats in a place preference chamber. Here, one compartment contained an odor paired with shock (CS+), and the other compartment contained an odor paired with a neutral environment (CS-). CeA ChR2 rats displayed a significant avoidance of the CS+ odor compartment (t_7=-3.53, p=.01, 95%CI: -393, -77, *d*=-1.25) whereas eYFP control virus rats spent equal time in both compartments (t_4=.24, p=.01) (Figure 6.8C). Failure of eYFP controls to acquire an odor avoidance could relate to the timing of the odor presentations. Previous work demonstrates strong odor aversions when odor is discretely presented over the course of 10-15 s as a predictive CS+ of shock, rather than presenting it throughout the conditioning session here (Camp and Rudy, 1988; Boulanger Bertolus et al., 2014). Here, our effect of CeA-induced avoidance could be interpreted as enhancing formation of associations with shock that wouldn't usually be as robust to cause avoidance. Combined with freezing levels, these findings suggest that CeA stimulation during more classical fear conditioning causes a sharpening of fear expression focused onto a particular time (only to the CS+ tone), and enhancement of the formation of associations with both the tone and even to an odor that is paired with shock.

CeA ChR2 induced shock rod attraction recruits Fos expression in reward circuitry

We next assessed which downstream structures were recruited differently by CeA ChR2 stimulation to cause attraction towards the shock rod. Fos protein expression was measured in several sites known to be directly or indirectly innervated by CeA, and compared between ChR2 rats showing laser-induced attraction towards the shock rod and control virus rats showing fearful defensive treading towards the shock rod.

CeA induced attraction was associated with a ~250% enhancement of Fos protein in medial orbitofrontal cortex (mOFC) (350% of control virus levels; t_{11}=-2.94, p=.01, 95%CI: -19, -3, *d*=-2.7) (Figure 6.9). By contrast, other cortical sites showed similar Fos protein levels between groups, such as infralimbic (120%; t_{14}=-1.8, p=.1) and prelimbic (106% of control virus levels; t_{12}=-.19, p=.85) cortices. Interestingly, CeA-induced attraction was associated with suppressed Fos expression in anterior Insula (70% of control virus levels; t_{14}=1.8, p=.09, 95%CI: -1.8, 19, *d*=-.8), but enhanced Fos expression in posterior Insula (234%; t_{16}=-1.6, p=.13, *d*=1.04), though neither enhancement reached statistical significance.

Beyond cortical structures, CeA-induced attraction was associated with elevated Fos protein expression in the rostral portion of nucleus accumbens medial shell (NAc shell; 180%; t_{14}=-2.2, p=.04, 95%CI: -4.5, -.05), *d*=-1.11), but no difference in Fos levels was observed in the

caudal portion (<116%; t_{14}=-0.5, p=.62). Similarly, Fos protein levels were equal between attracted and fearful rats in nucleus accumbens core (108% of control virus levels; t_{16}=-.36, p=.73). Further, Fos protein levels were roughly similar between groups in both rostral ventral pallidum (VP; 102% of control virus; t_{16}=-.06, p=.95) and caudal VP (83% of control virus; t_{14}=.45, p=.66). Within lateral hypothalamus (LH), Fos protein levels were similar between groups in rostral (82% of control; t_{16}=.67, p=.51) and middle portions (95% of control; t_{14}=.27, p=.79), but were elevated by 200% of control virus rats in perifornical region of LH (where orexin neurons reside) (t_4=1.37, p=.24, *d*=-1.05). Further, dorsolateral striatum Fos expression was similar between groups (t_{16}=1.8, p=.08).

Further, Fos protein was elevated among ChR2 rats compared to control virus rats in several midbrain sites. CeA-induced attraction was associated with a 400% enhancement of Fos protein exclusively in the caudal portion of ventral tegmental area (VTA) (t_2=1.73, p=.33, *d*=-1.2), and suppressed slightly in the rostral portion (72% of control virus; t_6=1.4, p=.23). Elevated Fos protein among ChR2 rats was also observed in nearby Substantia Nigra pars compacta (SNc) (250% of control virus; t_2=1.4, p=.2).

By contrast, shock rod-induced fear among control virus rats was associated with enhanced Fos expression compared to ChR2-induced attraction. For example, Fos protein was enhanced by over 240% in control virus rats compared to ChR2 in basolateral amygdala (40% suppression among ChR2 rats; t_{13}=3.23, p=.02, 95%CI: 1.74, 15.3, *d*=2.04). Similarly, Fos elevation was almost doubled in control virus rats throughout bed nucleus stria terminalis (BNST; 45% suppression among ChR2 rats, t_{12}=2.15, p=.05, *d*=1.12). Fos expression was doubled among control virus rats compared to ChR2 rats in ventrolateral periaqueductal gray (t_2=4.24, p=.05, 95%CI: -.04, 6, *d*=4.22).

Discussion

Here, we demonstrate that pairing CeA ChR2 excitation upon interaction with an aversive shock rod induced attraction towards it rather than eliciting defensive treading. Not only did ChR2 rats spend most of their time near the shock rod, as opposed to control virus rats spending most of their time in a given session away from it, but ChR2 rats touched the shock rod at greater numbers across days while control virus rats touched the rod only 1-2 times before completely avoiding it.

Our findings suggest that the stimulus paired with CeA ChR2 stimulation needs to be motivationally salient to induce attraction. Pairing CeA stimulation with innocuous stimuli did not cause attraction to them. For example, food intake of chow was not altered when CeA stimulation was delivered among *ad libidum* fed rats. Furthermore, CeA-pairing with exposure to a wood block did not induce attraction towards it, or chewing of it in the same way CeA stimulation caused chewing of shock rod here or in previously reported pairings with cocaine associated ports (Warlow et al., 2017). Thus, in order for CeA stimulation to enhance attraction towards a stimulus, that stimulus must be motivationally significant, whether positively or negatively valenced. This is in line with more classical views of striatal circuitry, such that striatal-like structures are thought to generate intense motivation, and that the amygdala is positioned to assign that generated motivation to particular environmental stimuli.

CeA-induced attraction not due to simple reinforcing effects of laser

It may have been possible that CeA-enhancement of attraction towards rewarding and aversive stimuli was due to the simple fact that CeA stimulation is rewarding on its own. Here, we gave rats the chance to earn laser stimulation on its own, either by touching an empty spout or by walking into one side of a chamber. We found that rats did not self-stimulate CeA laser at the same stimulation parameters delivered during instrumental sucrose vs. cocaine choice sessions or during shock rod sessions. Even when longer or shorter durations of CeA stimulation were offered, the majority of rats were indifferent to CeA laser on its own. This is consistent with previous findings showing lack of CeA self-stimulation (Robinson et al., 2014; Warlow et al., 2017). Although CeA may be a site for self-stimulation using different parameters or under different conditions, it does not appear to be the reason the same rats were attracted to both the rewarding and the aversive stimuli presented in these experiments.

CeA-induced attraction may involve incentive salience of cues

CeA ChR2 rats elicited vigorous chewing and biting of the shock rod when paired with laser stimulation. Consummatory nibbles and bites are a hallmark of incentive salience (Bindra, 1978; Tomie et al., 2008). Although these behaviors are usually observed towards stimuli that would normally be ingested and not to intravenously delivered drug (Uslaner et al., 2006), here we show that CeA laser transforms cues associated with un-ingested items such as aversive shock rods into something that rats attempt to ingest, similar to food-predictive cues. Additionally, a sound paired with shock rod interaction became a conditioned reinforcer. In other

words, rats found a cue paired with shock rod interaction attractive enough that it reinforced a new nose-poking behavior on its own. The ability for a reward-paired cue to become attractive on its own and sufficient to reinforce new behavior is another key feature of incentive salience attribution (Robbins, 1976; Robinson and Flagel, 2009). Cues imbued with incentive salience have the powerful capability of eliciting approach towards them, spurring ingestive/ consummatory-like behaviors, and reinforcing new behaviors. Thus, it is likely that CeA-pairing with aversive stimuli could have involved enhanced attribution of incentive salience towards its associated cue.

We also showed here that the same CeA stimulation causing attraction to an aversive shock rod also enhanced fear expression during a more classical Pavlovian fear conditioning procedure. This enhancement of fear conditioning is in line with previous research implicating CeA as crucial for fear expression (Ponnusamy et al., 2007; Campbell-Smith et al., 2015; Kochli et al., 2015) as well as other optogenetic enhancements of fear in CeA (Haubensak et al., 2010; Wolff et al., 2014). That the same stimulation even in the same rats was able to produce opposing effects may be related to several factors. One factor could include the shock source itself. The shock rod here was a localizable source that rats have the power to interact with or not. Shock rods typically elicit active forms of fear (avoidance and defensive treading) (De Boer and Koolhaas, 2003; Blanchard et al., 2011). The shock grid condition is a situation lacking a localizable source of fear and elicits passive forms of fear (freezing) (Fanselow, 1980). These forms of fear are mediated by opposing microcircuitry within CeA that are mutually inhibitory (Fadok et al., 2017). Our global stimulations here could have potentially activated more passive forms of fear, which may in turn have inhibited active fear circuitry. However, future work will disentangle cell-types mediating shock rod attraction vs. fear conditioning enhancement. A second potential factor influencing opposing effects of attraction vs. fear may relate to the environment in which testing occurred. Localized shock rods were stimuli rats could choose to interact with, and may have translated into perceived control, whereas Pavlovian paired shocks were uncontrollable, and thus could have elicited greater fear at baseline. This environmental influence would relate to other demonstrations that the same stimulations in other striatal sites (i.e., Nucleus Accumbens) generate either fear or desire and can be modulated by environment (Reynolds and Berridge, 2008; Richard et al., 2013).

CeA-induced attraction recruits mesolimbic circuitry

We found that CeA ChR2 stimulation may cause attraction to the aversive shock rod by increasing activity in mesolimbic site such as VTA, substantia nigra, and lateral hypothalamus. Increased activity here could have increased overall dopaminergic activity in some downstream sites such as nucleus accumbens or dorsal striatum. This would be in line with our additional observation of heightened Fos protein expression in the rostral portion of NAc medial shell, a site previously implicated in generating intense desire for food and enhancement of cue-triggered food-seeking (Peciña and Berridge, 2000, 2013; Richard and Berridge, 2011). Separately, we observed elevations in Fos among medial OFC and posterior Insula, but not in other cortical sites. Previous research has shown divergent roles in motivation between cortical sites. For example, medial OFC activation can expand the sites within NAc shell that promote feeding vs. fear, whereas infralimbic activation suppressed both feeding and fear and prelimbic cortex had no effect (Richard and Berridge, 2013). By contrast, shock rod avoiding control virus rats had heightened activation among proposed anxiety-inducing brain regions such as BNST and periaqueductal gray. Interestingly, we observed BLA Fos elevation in control virus rats compared to ChR2 rats. These findings suggest that CeA ChR2 stimulation may recruit mesocorticolimbic circuitry to induce attraction towards aversive targets, while suppressing activity in other brainstem and extended amygdala generators of anxiety.

Figures

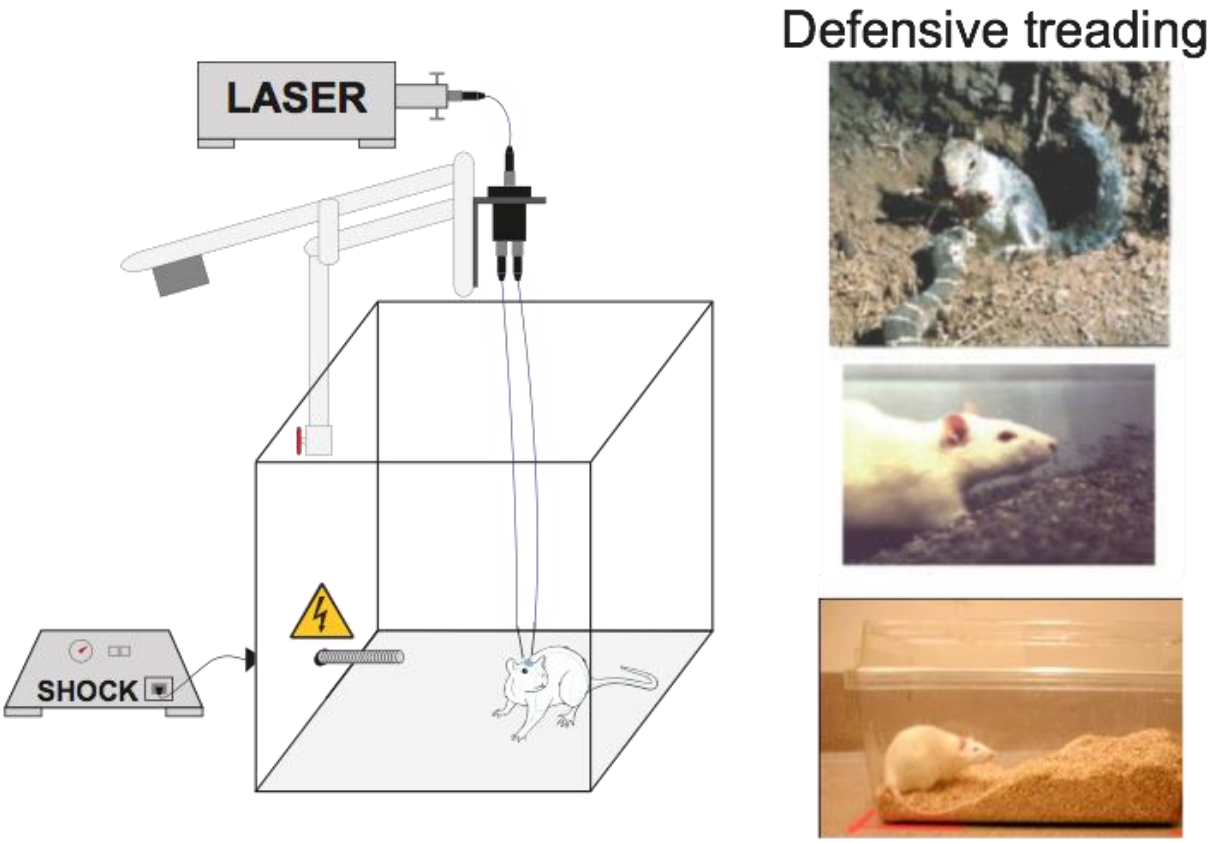


Figure 6.1
CeA pairing with aversive shock rod. An electrified shock rod protruded ~9 cm into one side of a plexiglas chamber. Touching the rod at any location with the forepaws delivered a low-intensity shock (0.25mA). Additionally, laser illumination (473 nm, 40 Hz: 20 ms ON 5 ms OFF) was delivered when rats were within a 1 inch perimeter of the shock-delivering rod. Photographs depict examples of defensive treading elicited both in the wild towards predators such as snakes, but also in laboratory settings towards perceived threats (such as towards shock rods or experimenters). Defensive treading involves pushing up dirt or bedding with the forepaws, and can result in large piles in an attempt to bury the perceived threat (as seen in the bottom right photo).

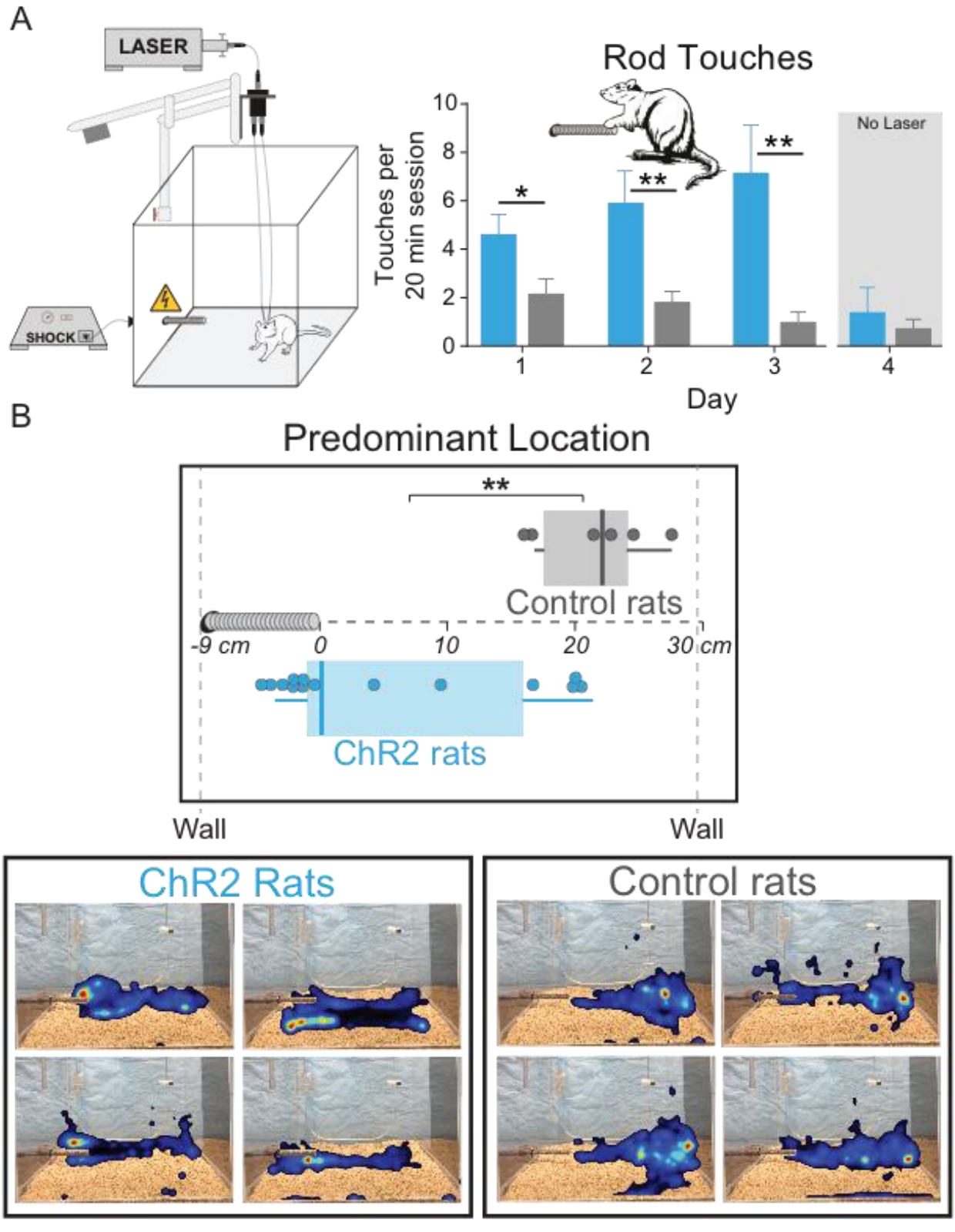

A
LASER
SHOCK
Rod Touches
Touches per 20 min session
No Laser
Day
B
Predominant Location
Control rats
-9 cm
0
10
20
30 cm
ChR2 rats
Wall
Wall
ChR2 Rats
Control rats

Figure 6.2

CeA stimulation amplifies attraction towards an aversive shock rod. (A) CeA ChR2 rats (blue bars) touch the shock rod at greater numbers than control virus rats (grey bars), on all three days when laser stimulation is paired with shock rod interaction. On day 4, when laser pairing is removed, touches decrease to control virus levels. Data represent means and standard error. (B) Using heatmaps compiled to display where each rat spent most of their time during a 20 min session, boxplots depict here that ChR2 rats (blue box) spent the majority of their time closer to the tip of the shock rod than did control virus rats (grey box). Each individual rat's predominant location is represented by circles (ChR2 rats by blue circles, and control virus rats by grey circles). Thick lines in the middle of each box depict median of each group (ChR2 or control virus), and lines extending to the left and right of each box represent the 1st and 3rd quartiles of each group, respectively. Photos below depict representative heatmaps for individual rats in a given 20 min session within each group (ChR2 or control virus). *p<.05 **p<.01

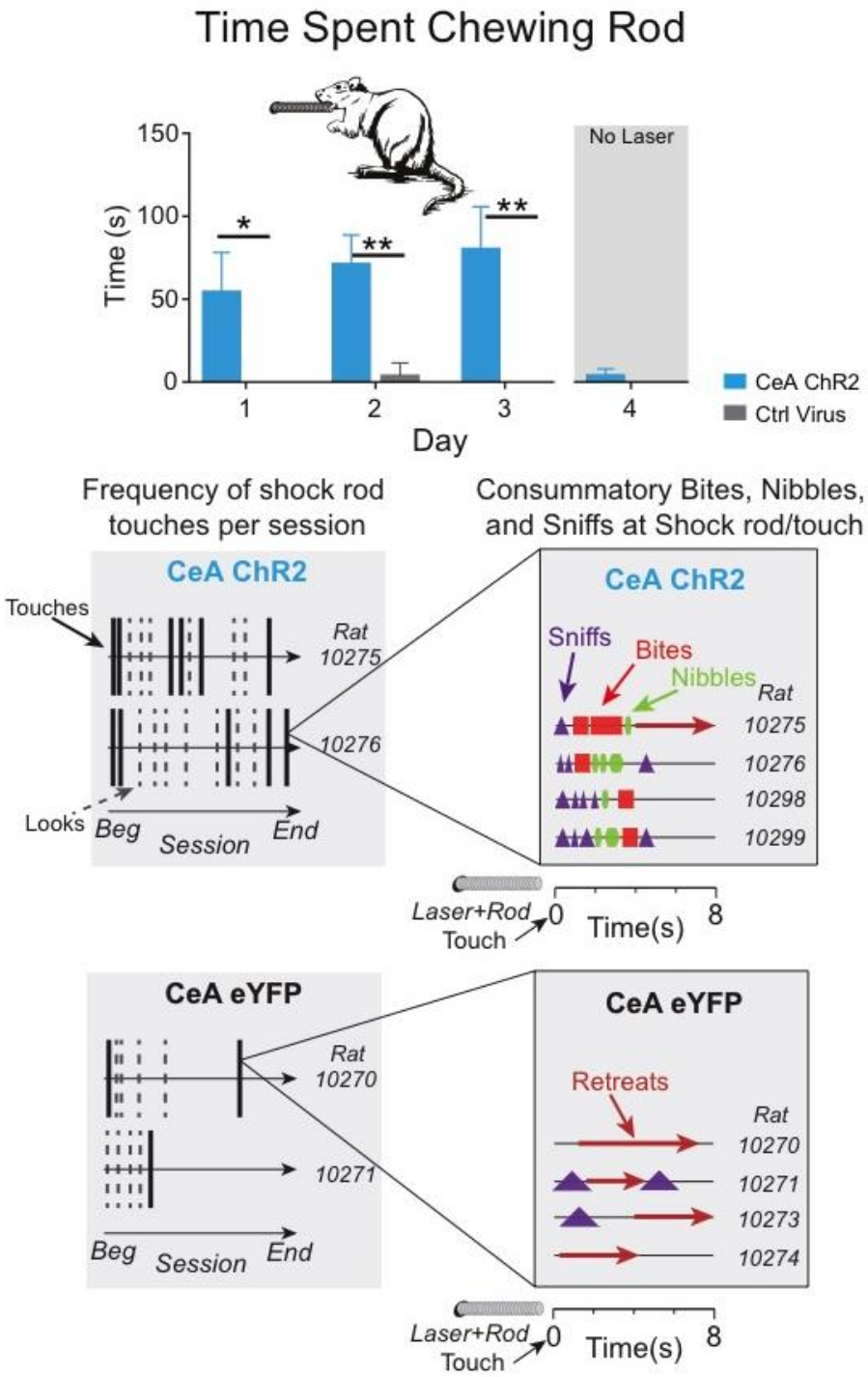


Figure 6.3

Consummatory bites, nibbles, and sniffs accompany CeA-induced shock rod attraction. Above graph shows that CeA ChR2 rats (blue bars) spent more time chewing the shock rod than control virus rats (grey bars) on days when laser stimulation was offered but not when it was removed. Data represent means and standard error. Frequency of shock rod touches (solid lines) and looks (dashed lines) within a 20-min session are shown in representative ChR2 (above grey box) and control virus rats (below grey box). Boxes on the right show consummatory behaviors (nibbles: green hexagons, sniffs: purple triangles, bites: red squares, retreats from shock rod: dark red arrow) towards the shock rod for the 8 s duration following when the shock rod is touched among several representative ChR2 rats (above box) and control virus rats (below box).

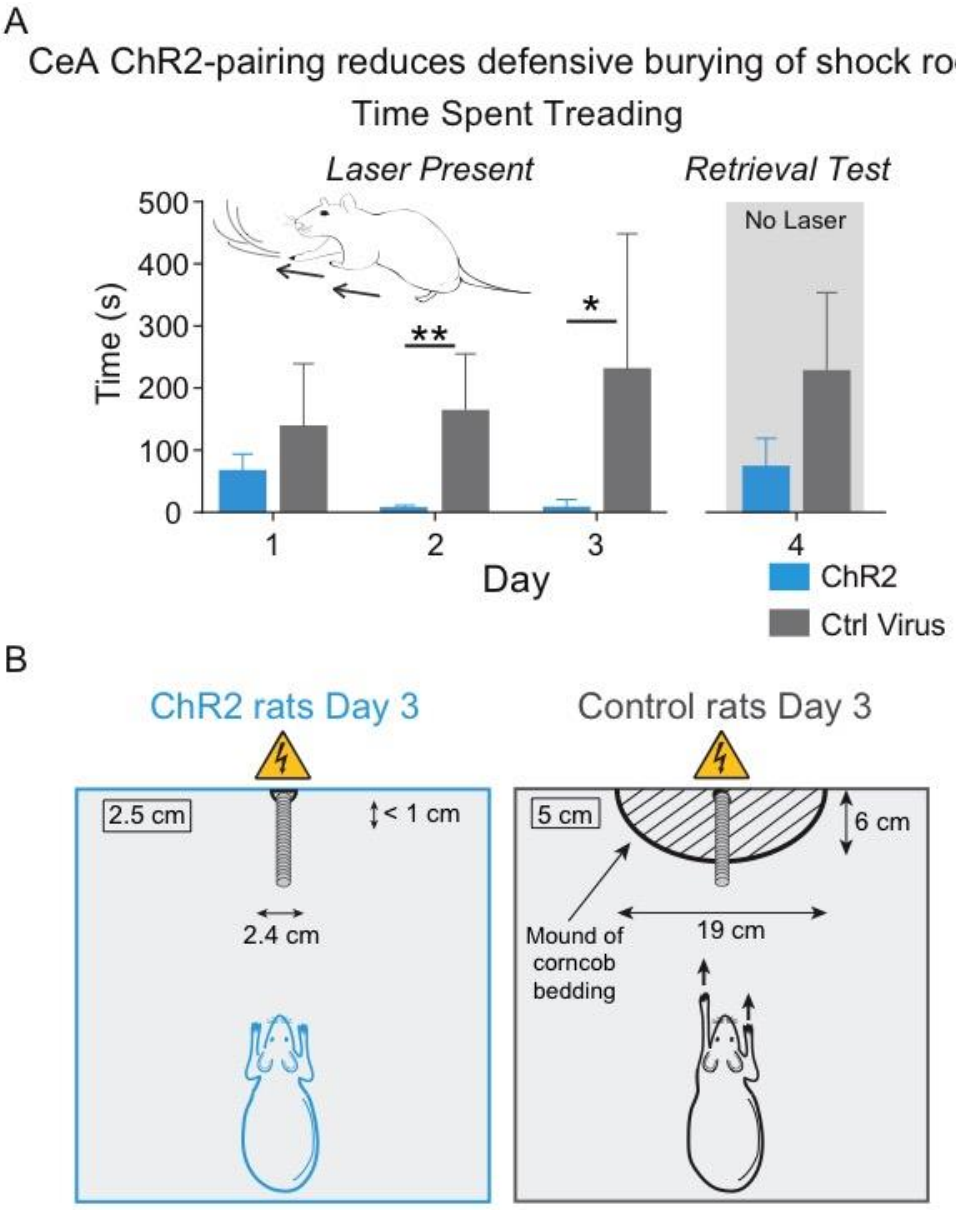


Figure 6.4

CeA ChR2-pairing defensive burying of shock rod. (A) Time spent eliciting defensive treading towards the aversive shock rod was reduced in CeA ChR2 rats (blue bars) compared to control virus rats (grey bars) when laser stimulation was delivered on days 2 and 3 but not when it was removed on day 4. Data shown as means and standard error. *p<.05 **p<.01 (B) While control virus rats (right) attempted to defensively bury the shock rod by creating mounds that were on average 5 cm high x 6 cm long x 19 cm wide around the shock rod (diameter of the mound of corncob bedding depicted by striped lines), ChR2 rats (left) created much smaller (if any) mounds of bedding (2.5 cm x 1 cm x 2.4 cm).

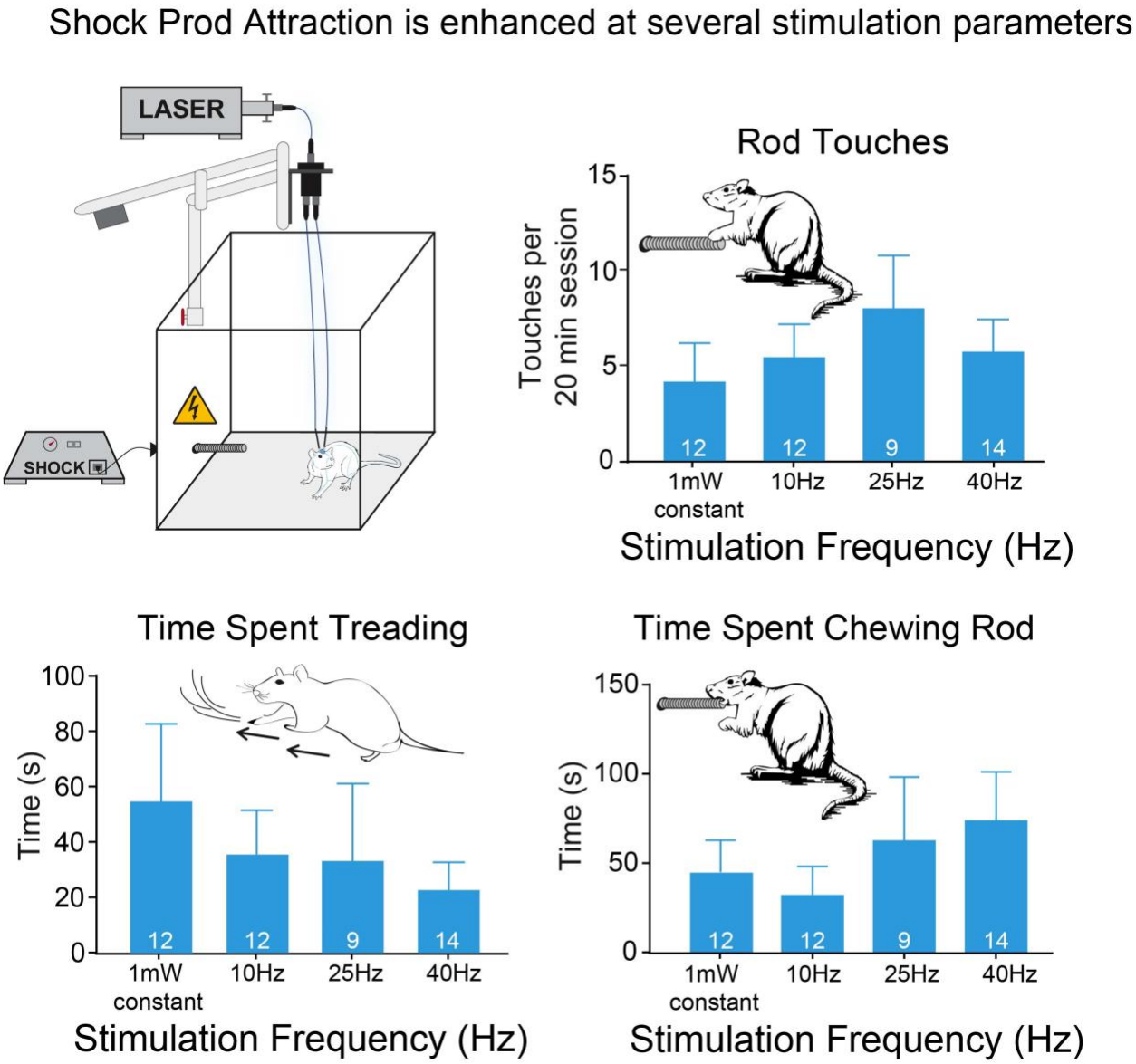


Figure 6.5

Shock prod attraction is enhanced at several stimulation parameters. Rats were tested in shock rod experiment during different sessions where different stimulation parameters of CeA laser were paired with shock rod interaction (1 mw constant, 10 Hz, 25 Hz, or 40 Hz). Rod touches, time spent defensively treading, and time spent chewing the shock rod are displayed for each 20 min session at each stimulation parameter. White numbers inside each blue bar represent the number of rats in that particular group. Data represent mean and standard error.

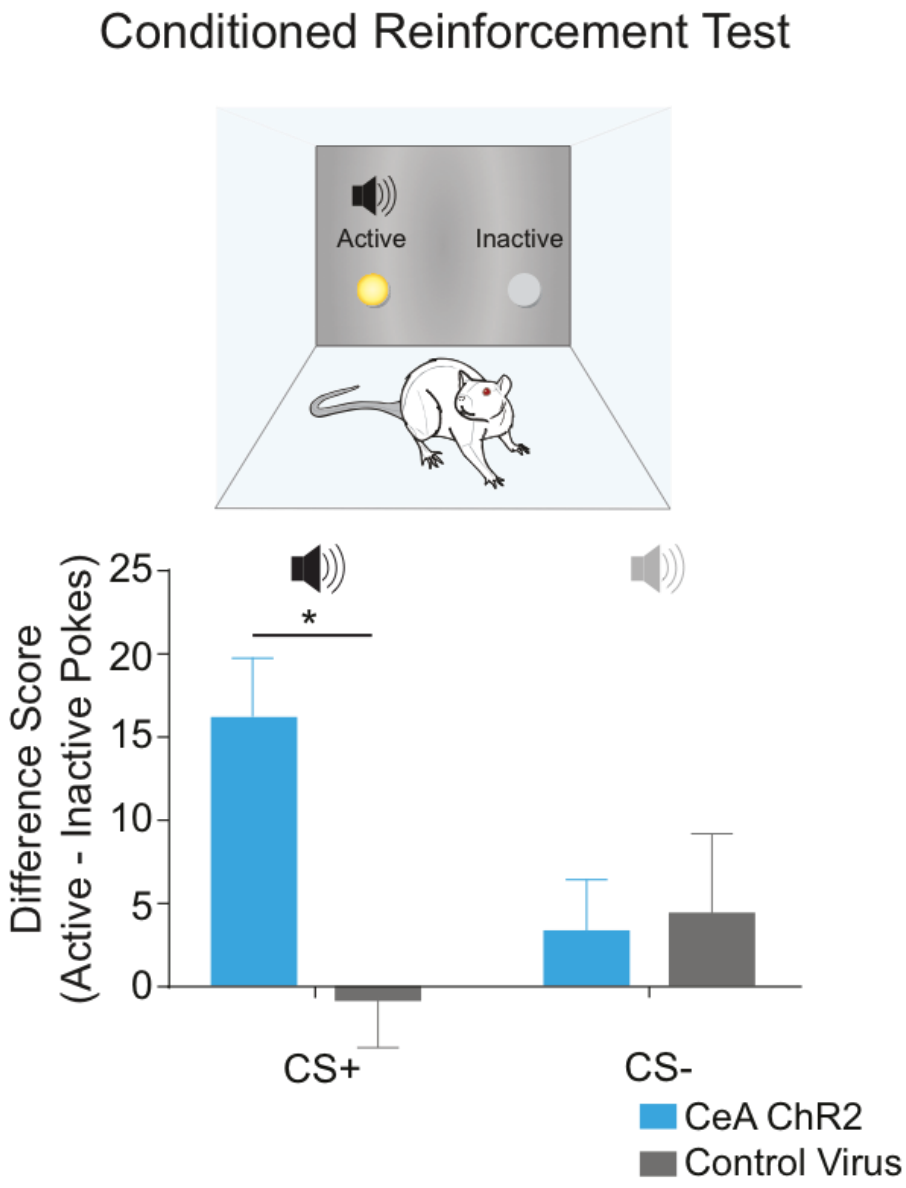


Figure 6.6

Shock rod is sought as a conditioned reinforcer. During two separate conditioned reinforcement tests, rats could nosepoke into the active port (black auditory cue symbol) which delivered the auditory cue paired with the shock rod (CS+) or in a separate session a neutral auditory cue never paired with the shock rod (CS-). Rats could nose poke into the inactive port which had no consequences (grey auditory cue symbol).Data depict the difference between active and inactive port responses during CS+ sessions in CeA ChR2 rats (blue bars) and control virus rats (grey bars). Data represent mean and standard error. *p<.05

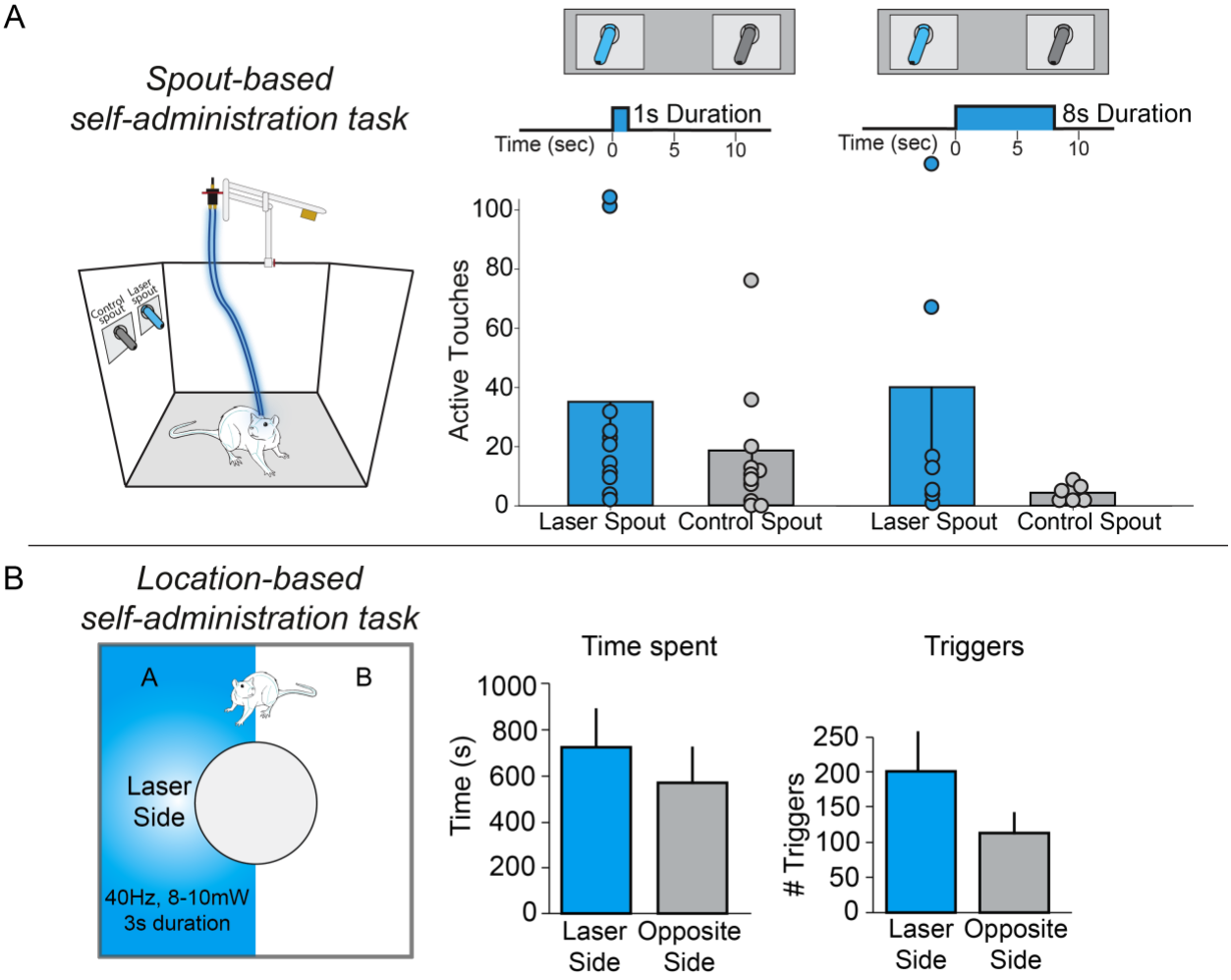


Figure 6.7

CeA stimulation is not reinforcing alone. The same rats showing shock rod attraction did not self-stimulate CeA laser in two different self-stimulation tests. (A) In a spout-based laser self-administration task, touching one spout earned CeA laser (473 nm, 40 Hz, 10 mw; "Laser Spout") and touching another spout earned nothing ("Control spout"). Rats did not touch the laser spout any more than the control spout when offered in 1 s (left) or 8 s durations (right) (individual rats indicated by blue circles for laser spout touches and grey circles for control spout touches). (B) In a separate self-stimulation test, rats could walk into one side of a plexiglas chamber to earn laser stimulation (473 nm, 40 Hz, 8-10 mW, 3 s duration). ChR2 rats did not spend more time (left graph) in the laser-delivering corner (blue bar) than the non-laser corner (grey bar), nor did they trigger the laser sensor (blue bar) any more than the non-laser sensor (grey bar). Data represent mean and standard error.

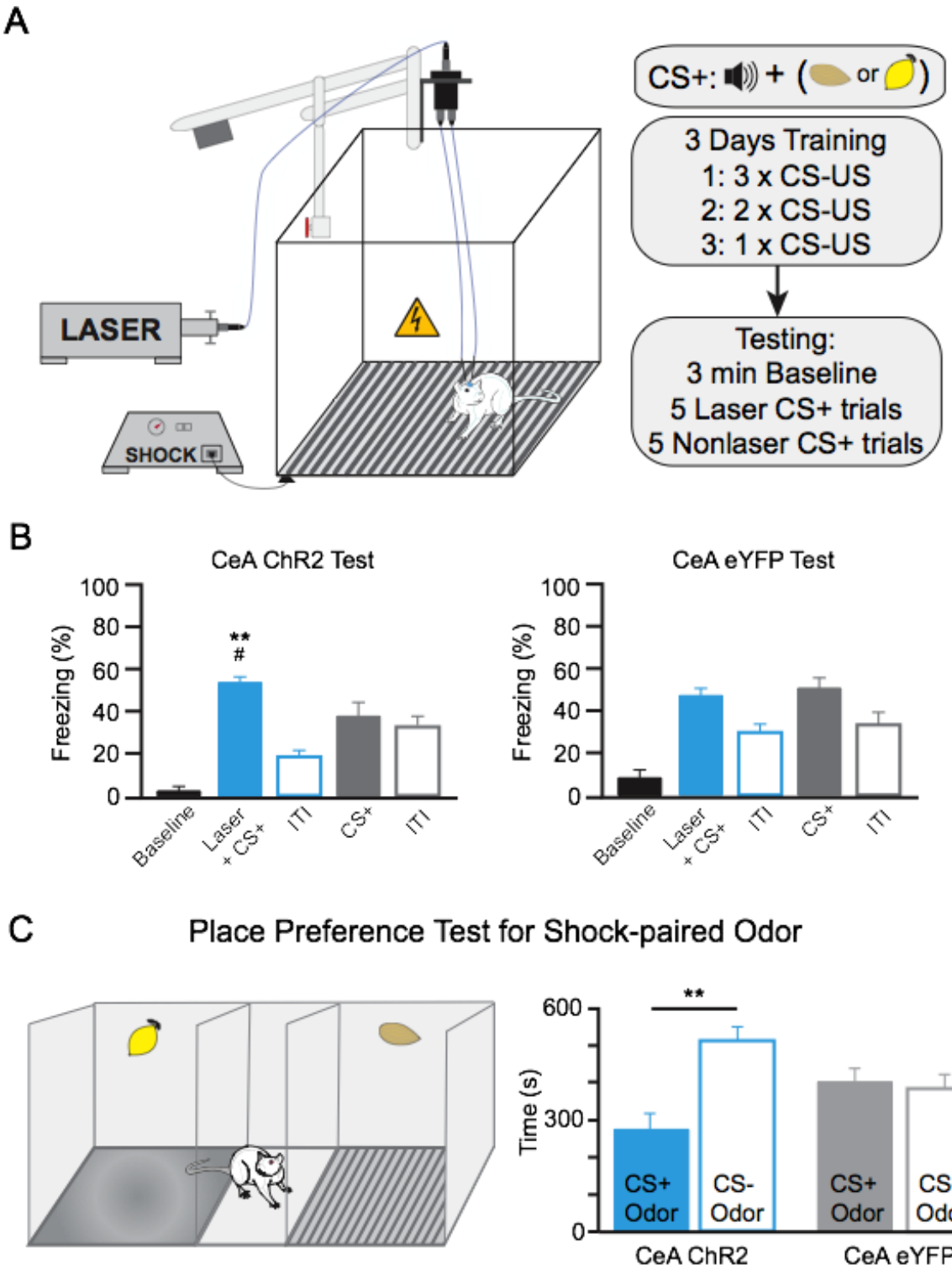

A
LASER
SHOCK
CS+: + (or)
3 Days Training
1: 3 x CS-US
2: 2 x CS-US
3: 1 x CS-US
Testing:
3 min Baseline
5 Laser CS+ trials
5 Nonlaser CS+ trials
B
CeA ChR2 Test
CeA eYFP Test
Freezing (%)
100
80
60
40
20
0
**
#
Baseline
Laser + CS+
ITI
CS+
ITI
C
Place Preference Test for Shock-paired Odor
Time (s)
600
300
0
**
CS+ Odor
CS- Odor
CeA ChR2
CeA eYFP

Figure 6.8

CeA stimulation during Pavlovian Fear conditioning. (A) Rats were placed into a standard MedAssociates chamber with a grid floor allowing for scrambled shock delivery. During 3 days of training, a discrete 10 s tone (CS+) was presented prior to shock delivery (500 ms, 0.75 mA). A distinct odor was also present (almond or lemon scent). During testing, rats were tested in a novel context (different box, plexiglas floor, different odor, and presence of house light), where the CS+ tone was presented on 10 different trials without the US (shock). (B) ChR2 rats displayed greater freezing levels during the test trials that also delivered CeA laser (blue bars; Laser+CS+) than at baseline (black bars) and compared to their intertrial intervals where no CS+ was played (ITI; open blue bars). By contrast, among CeA ChR2 showed no enhancement of freezing to a CS+ without laser (grey bars) compared to the ITI (open grey bar) following those trials. (C) Among control virus (CeA eYFP) rats, freezing during the CS+ trials were equal between laser (blue bars) and no laser (grey bar) trials, and enhanced compared to baseline freezing (black bar), but not significantly higher than either laser ITI (open blue bar) or no laser ITI (open grey bar). **$p<.01$ compared to baseline; #$p\leq.05$ compared to ITI (D) In a separate test of odor CS+ avoidance, rats were allowed to walk into separate compartments of a modified CPP chamber, where each compartment was distinguishable by the pattern on the wall and byb the texture of the floor. Further, one compartment contained the CS+ odor (that had been paired with shock) and one side of the compartment contained a CS- odor (that had been paired with a neutral environment) (side of odor pairing was counterbalanced between rats). CeA ChR2 rats avoided the CS+ odor side (blue bar), spending more time in the side containing the CS- odor (grey bar), while control virus (CeA eYFP) rats spent equal time in both CS+ (grey bar) and CS- (open grey bar) sides. Data represent mean and standard error. **$p<.01$

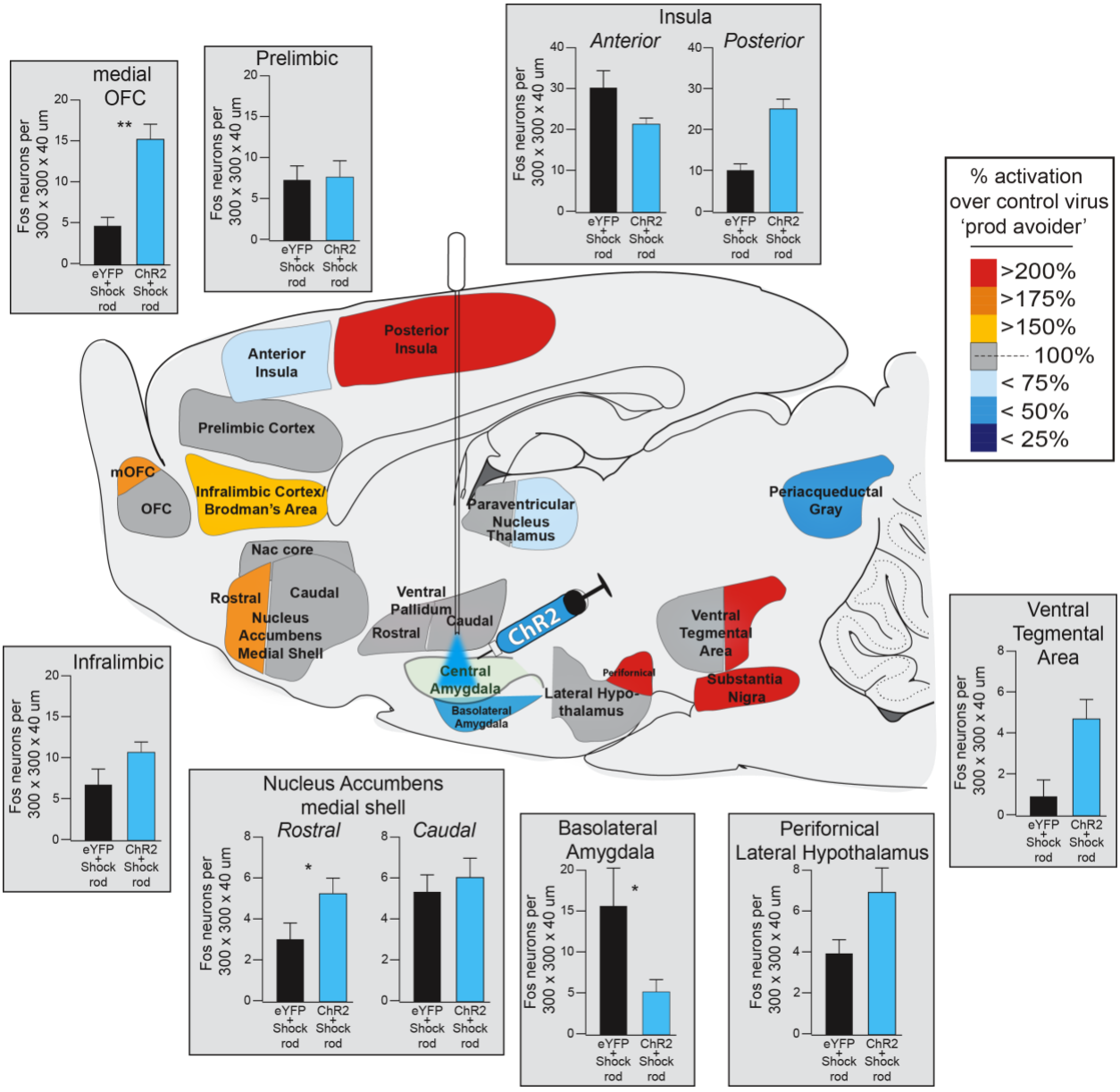


Figure 6.9

CeA ChR2-induced attraction recruits mesocorticolimbic circuitry. Sagittal view of the rat brain based on Paxinos and Watson rat atlas (2007) highlights brain regions where Fos protein was quantified. Color of region indicates the % Fos activation among ChR2 rats showing attraction towards an aversive shock rod, compared to control virus rats showing aversion. Bar graphs for each region depict the mean +/- standard error of Fos neurons per 300 x 300 x 40 micron sampled tissue within that region of interest (ChR2 rats: blue bars, eYFP control virus rats: black bars). *p<.05 **p<.01

CHAPTER VII. General Discussion

Synopsis

A characteristic hallmark of addiction is the focused pursuit of drugs at the expense of other life rewards. Focusing motivation towards the appropriate target at the appropriate time adaptive for directing motivation towards targets such as food or sex. However, this strategy can be hijacked by drugs of abuse to pathologically pursue drugs and their related cues rather than normal life rewards in an intense fashion. Amygdala circuitry is thought to integrate previously learned associations with in-the-moment motivation to guide behavior appropriately. Here, we used optogenetic techniques to test the hypothesis that amygdala circuitry, particularly involving the central nucleus of amygdala (CeA), is a crucial mechanism that hijacks motivation and narrows choice to create pathological pursuit of drugs at the cost of normal life goals.

CeA excitation narrows and amplifies motivation for sucrose

Previous work has demonstrated that mu-opioid stimulation of CeA and not basolateral amygdala (BLA) can direct incentive salience onto prepotent reward-associated stimuli based on previous Pavlovian associations (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012). Enhanced 'wanting' by CeA stimulation preferentially occurred towards learned stimuli (approaching a food cup or approaching a lever) suggesting that CeA plays a unique role in integrating learned associations with in-the-moment motivation. Here, we sought to gain control of the focus of motivation from the beginning, while learning was taking place. We paired CeA excitation with earning a particular sucrose reward from the beginning of training when rats were choosing between two sucrose rewards. Pairing CeA excitation with one sucrose made that the preferred reward while the other identical reward was ignored. By contrast, rats chose equally between both rewards if BLA stimulation was offered. Importantly, CeA excitation was not sought after on its own and the sucrose reward was needed in order to enhance instrumental motivation. This suggests that CeA excitation acted to transform the brain's representation of the paired sucrose, making it more 'wanted'.

Central Amygdala excitation narrows and amplifies sucrose 'wanting' without altering 'liking'

Though it was demonstrated in the previous chapter that CeA excitation enhances motivation focused onto a particular sucrose reward, the question remained *how* CeA excitation was acting to transform the sucrose into a more 'wanted' target above and beyond the alternative identical sucrose reward. Our self-stimulation tests revealed that CeA excitation was neither acting as a pure reinforcer nor was it stamping in the habit of pressing the lever. Instead, the sucrose pellet was necessary in order to enhance motivation (rats were indifferent to laser excitation alone).

One possibility left unanswered in our original demonstration was that CeA excitation amplified sucrose 'wanting' by making that associated sucrose more 'liked'. This would in turn make that sucrose more preferred than its alternative sucrose lacking CeA excitation. Previous work has found mixed effects of CeA manipulations on hedonic 'liking', with some finding no role (Galaverna et al., 1993), and others finding suppression of 'liking' (Mahler and Berridge, 2012; Riley and King, 2013; Ross et al., 2016). However, a majority of these studies used electrical stimulation of CeA, which could have also excited fibers of passage to gustatory brainstem sites. Here, we used optogenetics to target CeA neurons during the taste reactivity test to assess whether CeA excitation alters hedonic impact (or 'liking'). The taste reactivity test is based on hedonic orofacial reactions which track the pleasantness of a taste rather than the sensory properties. It crucially does not rely on the willingness of the rat to ingest the tastant by allowing experimenters to directly infuse tastes into the mouths of rats via intraoral cannulas (Grill and Norgren, 1978; Spector et al., 1988; Berridge and Kringelbach, 2015). This allowed us to remove the confound of motivation in our two-choice task or any voluntary intake test, and rather to simply measure their reactions to sweet or bitter tastes in the presence or absence of CeA laser.

We found that CeA ChR2 stimulation did not alter hedonic orofacial reactions to sucrose or to quinine, but was still capable of amplifying and narrowing motivation for sucrose in the same rats. These findings replicated the previous experiments, showing CeA ChR2-induced enhancement of motivation. Further, because we used a more specific neuronal promoter of ChR2 transfection (human synapsin) instead of the more general cell promoter (CAG), we confirmed that motivational enhancement was due to CeA neuronal stimulation rather than other cell types (glia, etc). Importantly, we extended the previous results to demonstrate that CeA is

acting to focus excessive motivation for sucrose by selectively enhancing 'wanting' and not 'liking'.

Central Amygdala excitation narrows and amplifies motivation for cocaine

The incentive sensitization theory proposes that brain 'wanting' systems sensitize with repeated exposure to drugs among some individuals, whereas brain 'liking' systems stay relatively the same or even decrease over time (Robinson and Berridge, 1993). As such, these 'wanting' systems become hyper-reactive to drugs and their related cues, and trigger intense cravings which can result in relapse. That CeA excitation selectively enhanced 'wanting' without altering 'liking' suggests that this same mechanism may underlie the dissociation between 'wanting' and 'liking' that occurs in the transition to addiction. If so, then the same amplifying and narrowing of motivation caused by CeA circuitry should extend to drugs of abuse. Indeed, we found that pairing CeA excitation with earning a particular intravenous cocaine infusion (0.3 mg/kg) made that particular cocaine reward exclusively pursued. By contrast, when BLA excitation was paired with earning cocaine, rats chose equally between the laser and non-laser cocaine infusions. Rats were additionally more motivated to earn that CeA laser-paired cocaine reward in a separate breakpoint test of motivation. Similar to when CeA excitation was paired with a sucrose reward, rats would not self-stimulate CeA laser alone, and cocaine was required to maintain responding. By contrast optogenetic or pharmacological inhibition of CeA suppressed overall cocaine intake. These findings demonstrate that CeA circuitry can focus motivation for cocaine onto a particular target, building on our previous demonstration with natural sugar. This implies that the same mechanism underlying focused motivation for natural rewards can be hijacked by drugs of abuse to focus pursuit for drugs at the expense of other rewards in cases of addiction.

Central Amygdala controls choice between sucrose and cocaine

The previous experiments support the hypothesis of a common mechanism of CeA in biasing choice for cocaine reward and sucrose reward. This implies a shared underlying circuitry mediating intense motivation for different rewards, potentially shared between 'cocaine addiction' and 'sucrose addiction'. However, this needed to be explicitly tested. Is the CeA narrow-and-intensify effect powerful enough to control the directional focus of addictive-like motivation between a drug-of-abuse vs. natural sucrose? We presented rats with an opportunity to choose between two sensory rewards (natural sucrose vs. intravenous cocaine). When CeA

stimulation was paired with earning either sucrose or cocaine, rats intensely preferred the paired reward almost 10-fold compared to an alternative reward not paired with CeA stimulation. Further, they were quicker to make their laser-paired reward choice. In a separate intermittent access paradigm, where rats had continuous access to each reward on separate days for four 5-min blocks of time, rats responded at higher amounts for their laser-paired reward than the reward lacking CeA excitation. These findings demonstrate that pairing CeA excitation with a particular reward makes that reward the target of pursuit, and is relevant to explaining why addicts 'want' one reward more than others.

Central Amygdala amplifies attraction towards an aversive target

In the prior experiments, we showed that CeA excitation can powerfully control the directional target of motivation for rewards. This implies that CeA excitation is transforming the brain's representation of a particular reward into the most attractive option beyond other identical options. Is it possible that CeA circuitry is powerful enough to transform an aversive target into one that becomes attractive? Here, we paired CeA ChR2 stimulation with touching an aversive shock rod that delivered a mild, low-intensity shock to the paw or snout when touched. Control inactive virus rats, upon touching the rod, learned very quickly to avoid it (spent more time on the opposite side of the chamber), and even elicited anti-predator defensive treading towards it. However, in CeA ChR2 rats, pairing CeA stimulation with this shock-delivering rod made the rod more attractive to rats, as they spent more time on that side of the chamber and engaged in biting and sniffing of the rod. Further, CeA ChR2 stimulation failed to support self-stimulation in a spout task and location-based task in the same rats showing intense desire for the shock rod. Finally, we measured and compared Fos protein expression in CeA output circuitry (such as lateral hypothalamus, ventral pallidum, periaqueductal gray, and ventral tegmental area). Our findings suggest that CeA ChR2 stimulation recruits mesocorticolimbic circuitry to amplify attraction toward rewarding events and to transform avoidance of an aversive event into attraction towards it, but needs a motivationally salient event on which to act.

CeA-mediated focused pursuit of drugs

A common question in addiction neuroscience, is why addicts aren't addicted to all drugs of abuse and why they don't show addictive behaviors globally to all rewards such as food, sex, or gambling. If dopamine activity is heightened in addicts, and dopamine mediates motivation across all reward types, then why don't addicts 'want' several drugs? Although human addicts

report using and craving several different drugs or engaging in many forms of compulsive behavior (Washton and Stone-Washton, 1993; Benotsch et al., 1999; Leeman and Potenza, 2012), incentive sensitization can also be focused onto one particular reward target. In many situations, addicts usually crave just one drug or one compulsion (Vanderschuren and Everitt, 2005). A possible reason for such focused craving may arise from pre-existing differences. For example, when rats were pretreated with a sensitizing dose of amphetamine (one that causes psychomotor sensitization), this did not globally enhance future appetitive behaviors such as food or sexual seeking (Nocjar and Panksepp, 2002). Rather, drug-experienced animals either showed heightened food seeking or showed amphetamine place preference, but not both. This suggested that drug pre-exposure had distinct sensitization effects, sensitizing 'wanting' for one type of reward over another (drug vs. food vs. sex). In addition, differences in brain reactivity when associations are being formed between the drug and its context or cues may result in a preferred motivational target (Tindell et al., 2005).

In human addicts, the amygdala consistently shows heightened activity in response to cues related to their drug of choice, but not to other drug or sexual cues, and these cues lead to self-reports of craving for their preferred drug of choice (Childress et al., 2008; Kühn and Gallinat, 2011). Laboratory work has also implicated amygdala in focused motivation. Mu-opioid stimulation of central amygdala is capable of enhancing appetitive behaviors towards a prepotent cue (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012). Similarly, the same stimulation temporally focuses instrumental sucrose seeking only during periods of time when a Pavlovian cue is present and suppresses seeking during periods when the cue is not present (Mahler and Berridge, 2012). These findings have provided some clues as to how the brain appropriately focuses motivation onto a particular target at a particular time, but all brain manipulations were made after the learning had taken place. Here we attempted to provide new insight into why and how addicts 'want' only one thing. We gained control of the target of motivation by temporally pairing optogenetic CeA excitation with one reward and not the other, and from the beginning of training while learned responses are still being formed. By pasting CeA excitation arbitrarily onto one target from the very beginning, we were able to create focused pursuit of one reward above another, even if both rewards were identical (or despite any pre-existing preferences). Similar to previous work, CeA excitation did not generally increase

'wanting' for all rewards available, and we show for the first time that we can *create* focused pursuit arbitrarily even when it is not appropriate or rational.

CeA circuitry and subdivisions

Our finding that CeA but not BLA stimulation produced narrowing of motivation for both sucrose and cocaine may be related to its standing as a striatal-level structure within a macrosystem framework (Swanson and Petrovich, 1998; Swanson, 2003). BLA, due to its predominant glutamatergic projections to CeA, is more cortical-like while CeA and its predominant GABAergic population of neurons is similar to a striatal structure (Alheid and Heimer, 1988; Zahm, 2006). As such, CeA is be more similar to other striatal structures such as Nucleus Accumbens or dorsal striatum in its ability to generate intense motivations across many reward types (Peciña and Berridge, 2000; Reynolds and Berridge, 2001). Though CeA is the primary output region of the amygdala (receiving input from BLA), BLA and CeA contain several distinct afferents and efferents. For example, BLA receives predominant input from sensory regions and projects directly to Nucleus Accumbens Core (Sesack and Pickel, 1990; O'Donnell and Grace, 1995) bypassing CeA. By contrast CeA receives its own distinct projections from some brainstem structures (parabrachial nucleus and nucleus of the solitary tract) as well as other cortical sites such as Anterior Insula (Allen et al., 1991; Bernard et al., 1993; Schiff et al., 2018). In turn, CeA sends direct projections back to these brainstem structures, and also to other nodes within the more classical reward circuitry such as to perifornical regions of lateral hypothalamus, ventral tegmental area, ventral pallidum, and substantia nigra (van der Kooy et al., 1984; Petrovich and Swanson, 1997; Zahm et al., 1999; Reppucci and Petrovich, 2016). Through these projections, CeA can indirectly modulate dopaminergic tone in striatal structures such as in Nucleus Accumbens and dorsolateral striatum (Ahn and Phillips, 2002; Fudge and Emiliano, 2003; El-Amamy and Holland, 2007). While our stimulations were globally altering CeA-related circuitry, our dissociable BLA and CeA findings suggest we were altering circuitry separate from the classical serial model of amygdala output that projects from BLA through CeA (Balleine and Killcross, 2006). Here our Fos quantifications provide preliminary evidence to suggest that CeA ChR2-induces attraction towards motivationally relevant stimuli by enhancing activity in VTA and lateral hypothalamus as well as substantia nigra. Through this enhancement, dopaminergic tone in striatal structures

would have been elevated, which is consistent with our observation of Fos elevation in Nucleus Accumbens medial shell.

Our stimulations targeted sites spread roughly evenly throughout central amygdala subdivisions, with some optic fiber sites placed within medial (CeM) or within lateral (CeL) portions, a even a few sites within the capsular portion (CeC). CeM is the predominant output region within amygdala, where it receives serial input from BLA via CeL. Our localized Fos plumes suggest that sites within CeL as well as within CeM are capable of producing focused motivation, which may be a function of the specific cell-types activated rather than anatomical location. Medial vs. lateral segregation of CeA has been proposed to mediate opposing roles, with CeM promoting anxiety-related behaviors and CeL suppressing anxiety (Tye et al., 2011). However, functional differences in motivation can arise due to specific cell types even if they span both lateral and medial portions (Kim et al., 2017; Fadok et al., 2018). For example, GABAergic neurons within both CeL and CeM contain partially overlapping genetic identities. CeL GABAergic neurons contain a mixture of corticotropin-releasing hormone (Crh) neurons, dopamine receptor 1 containing (D1r) neurons, and dopamine receptor 2 containing (D2r) neurons (and several other cell types), while GABAergic neurons within CeM contain a mixture of neurotensin, somatostatin, D1r and D2r neurons. Selective targeting using optogenetics in transgenic lines has revealed that specific cell types within CeA promote opposing roles in reward and aversion. For example, D1r neurons within CeA can promote appetitive behaviors. Direct stimulation of these neurons enhances food seeking, and even supports self-stimulation (Kim et al., 2017). Further, incubation of drug seeking is associated with increased D1r neuron activity in CeA (Venniro et al., 2017). By contrast D2r neurons within CeA are associated with suppression of these behaviors (Kim et al., 2017; Venniro et al., 2017). Future work could explore which cell types within CeA are predominantly mediating our amplifying of incentive motivation effects either by co-labeling with Fos protein or by directly stimulating these neurons. Knowing candidate cell types would also inform possible output pathways of CeA to target in the future.

Central Amygdala Necessity vs. Sufficiency

Experiments here have focused on sufficiency of CeA circuitry in producing irrational pursuit of several incentives. Optogenetic CeA excitation was capable of generating intense incentive motivation irrationally focused onto a particular target. CeA inhibition (either

optogenetic or pharmacological) suppressed overall motivation for cocaine, but did not produce aversion of the laser-paired cocaine reward in the two-choice task (rats chose equally between the two options). Instead, CeA inhibition left rats behaving 'rationally' in the sense that they chose equally between two identical rewards. This demonstrates that CeA excitation being paired with a reward is a selectively sufficient mechanism to produce irrationally focused pursuit of particular incentives. However, CeA necessity has previously been demonstrated in related tasks. For example, CeA lesions prevent acquisition of conditioned approach to Pavlovian cues (Gallagher et al., 1990; Parkinson et al., 2000), and block the ability of Pavlovian cues to trigger food- seeking (Hall et al., 2001; Holland and Gallagher, 2003; Corbit and Balleine, 2005; Mahler and Berridge, 2012) or drug related cues to trigger drug-seeking (Lu et al., 2005; Li et al., 2008, 2015b). These tasks rely on the ability for an animal to integrate knowledge of a Pavlovian cue with in-the-moment motivation to guide behavior toward the cue or the cue's representation of its associated reward. Designing future experiments that inhibit CeA while instrumentally choosing between a known preferred reward and an alternative un-preferred reward (such as a preferred concentration of sucrose compared to an un-preferred concentration) could provide better insight to whether CeA is necessary for demonstrating narrowing of motivation under normal circumstances.

Focused 'wanting' involves some learning component, but doesn't rely on it

CeA circuitry is crucial for appetitive and aversive learning. For example, both expression of conditioned fear (Pitkänen et al., 1997; Paré et al., 2004; LeDoux, 2007; Kochli et al., 2015) and conditioned responses to food-related stimuli (Gallagher et al., 1990; Cardinal et al., 2002; Lee et al., 2011) rely on CeA circuitry. Our findings provide further evidence to suggest that CeA circuitry integrates learned associations with in-the-moment motivation to produce amplified motivation focused onto a particular target. For example, narrowing of reward preference for sucrose or cocaine did not emerge until the second or third day. This laser-paired reward preference lasted several days after laser stimulation was removed. Further, CeA-induced shock rod attraction grew over multiple days, as ChR2 rats touched the rod at increasing amounts each day, reaching their highest levels by day 3. This evidence suggests that CeA stimulation delivered from the beginning of training is transforming the brain's representation of a paired incentive, which in turn is amplifying motivation towards it. However, learning was not a crucial component for CeA stimulation to cause some behaviors reported here. For example, in our

cocaine two-choice task when CeA was inhibited via muscimol/baclofen microinjections during training, cocaine intake was completely suppressed. But, as soon as inhibition was removed and rats were allowed to choose between a laser-paired cocaine reward or a cocaine alone reward, they showed a dramatic preference just on that first day (without having to learn each outcome first). This demonstrates the ability for CeA stimulation to boost in-the-moment motivation exclusively for its paired cocaine reward, without having to be on board during training. Thus, evidence presented here reveals the ability for CeA excitation to alter learning to produce focused motivation for specific rewards and even aversive stimuli, but also demonstrates that learning was not necessary to create focused motivation.

What psychological component is CeA circuitry acting on to amplify attraction?

We showed that we can control the directional target of motivation with CeA excitation, but there were several psychological components CeA excitation could be acting on to cause narrowed and amplified 'wanting'. Our initial two-choice tasks for sucrose and for cocaine were somewhat confounded by the involvement of many different psychological components, each capable of causing biasing of choice and an increase in motivation.

One possibility was that CeA excitation is reinforcing on its own. This would in turn cause rats to seek out the stimulation-paired with sucrose and/or cocaine. This could similarly explain why rats were willing to endure being shocked - in order to receive CeA laser (Pascoli et al., 2015). We showed here that when given the opportunity to self-stimulate CeA laser, the same rats demonstrating focused pursuit were indifferent to laser alone. They did not self-stimulate CeA laser, even when it was relatively easy to do so (e.g., walk into a corner and stay there). Thus, CeA ChR2 stimulation does not appear to be an incentive reward on its own (unlike ChR2 in other limbic structures that support optogenetic self-stimulation such as dopamine projections, nucleus accumbens, neostriatum, etc) (Kravitz et al., 2012; Pascoli et al., 2015; Yoo et al., 2017). While self-stimulation and place preference may be possible, especially through targeting of cell-specific circuitry within CeA (Douglass et al., 2017; Kim et al., 2017), it is not the reason by which rats showed increased incentive motivation here. Nor does CeA ChR2 stimulation appear to induce an aversive state in which rats are then motivated to seek out rewards to reduce this state; rats did not avoid a corner delivering laser stimulation, but rather they spent equal time in all four corners. This is an important distinction, as CeA circuitry and corticotropin releasing hormone signaling within CeA has been implicated in inducing aversion. This aversive state has

been proposed to subsequently affect drug seeking and can cause future drug relapse (Koob and Volkow, 2010; Koob, 2013). Our evidence suggests that CeA excitation does not induce aversion, so cannot be the reason for focusing and amplifying of incentive motivation here.

Further, we demonstrated that CeA laser was not sufficient to maintain the same behavior that had previously earned laser paired with reward but now just earned laser (rats would not continue nose poking or lever pressing if the external reward was removed). This evidence rules out the possibility that CeA was generating intense incentive motivation by strengthening the habit of pressing a lever, nose-poking, or continuing to touch an aversive shock rod. Rather, as soon as the target (sucrose or cocaine, or aversive shock rod) was removed, rats became indifferent to CeA stimulation alone. CeA therefore may be unable to create stimulus-response habits by itself, even though it intensifies actions associated with a paired motivational target. Our findings here contradict findings by others that implicate CeA circuitry, specifically involving substantia nigra and dorsolateral striatum, in stimulus-response habit formation (Lingawi and Balleine, 2012; Murray et al., 2015). For example, lesions of anterior CeA disrupt normal habit acquisition of lever pressing for food, such that, when the outcome is devalued (i.e, through lithium chloride pairings), CeA lesioned rats remain sensitive to this devaluation (stop pressing the lever), compared to sham lesioned rats who are insensitive due to normal habit formation (Lingawi and Balleine, 2012). While CeA may be necessary for habit formation to occur as shown in lesion studies, especially in behavioral paradigms that encourage overtraining and emergence of habits (Everitt and Robbins, 2005), strengthening of habit acquisition was not the reason by which incentives became intensely pursued here.

A second possible psychological component being recruited by CeA to amplify attraction is that CeA excitation was enhancing the hedonic impact or the pleasure derived from the associated incentive. We tested that possibility using the taste reactivity test in the presence or absence of CeA ChR2 stimulation, and found no alterations in hedonic impact even though the same stimulation produced focused motivation for sucrose in the same rats. Thus, CeA modulation of 'liking' was not a reason for the intense incentive motivation we reported here. Though 'liking' for drugs is hard to assess in rats, the same phenotype of enhanced motivation and narrowing of cocaine choice suggests that 'wanting' may also be selectively enhanced for cocaine without altering pleasure derived from cocaine consumption.

A more promising possibility is that CeA circuitry focuses motivation for particular targets by enhancing the attribution of incentive salience to a particular reward and its related cues. Through Pavlovian learning, cues not only become learned predictors, but they also acquire incentive motivational properties, becoming imbued with incentive salience (Robinson and Flagel, 2009). Attribution of incentive salience makes an otherwise neutral stimulus (i.e., metal lever) an attractive and 'wanted' stimulus (Robinson and Berridge, 1993; Berridge, 2012). Cues imbued with incentive salience can become reinforcing on their own even in the absence of reward (Robbins, 1976), and they have the ability to trigger strong urges of desire for their associated reward (Tomie, 1996; Kruzich et al., 2001; Tomie et al., 2008). In addicts, drug associated cues can trigger intense drug cravings that lead to relapse (Volkow et al., 2006; Goldman et al., 2013; Sinha, 2013; Preston et al., 2018). Behaviors toward a cue imbued with incentive salience often take the form of the behavior directed towards the reward itself. For example, rats will engage in nibbling, sniffing, and biting of a lever that predicts food (Brown and Jenkins, 1968; Jenkins and Moore, 1973; Kearns and Weiss, 2004; Tomie et al., 2008). In other situations, cue-elicited behaviors can take a form more appropriate to the cue's properties, such as when the conditioned stimulus and the reward it predicts support conflicting behaviors. For example, when presentation of another rat predicts food, behaviors towards that rat are more social in nature (anogenital sniffing, and playful behaviors) (Timberlake and Grant, 1975).

Previous research using both lesion and pharmacological approaches have revealed that CeA circuitry plays a crucial role in incentive salience attribution. CeA mu-opioid stimulation enhances approach and consummatory behaviors directed towards a Pavlovian food cue that is preferred (either a lever or a food dish) (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012). Further, the same stimulation enhances Pavlovian cue-triggered food seeking during PIT tests (Mahler and Berridge, 2012), suggesting that CeA stimulation can tap into and heighten the expression of incentive salience. CeA is also necessary for incentive salience attribution to occur (Gallagher et al., 1990; Parkinson et al., 2000), and similarly for cue-triggered food-seeking in PIT (Corbit and Balleine, 2005; Chang et al., 2012). Further, the ability of previous drug cues (cocaine, or methamphetamine) to reinstate drug seeking after protracted withdrawal is regulated by CeA (and not BLA) circuitry (Kruzich and See, 2001; Lu et al., 2005; Li et al., 2008, 2015b).

Our findings here also support CeA in enhancing attribution of incentive salience to reward-related cues. We reported enhanced consummatory behaviors such as sniffing, biting, and nibbling towards cues associated with the laser-paired reward across several experiments. We also observed novel consummatory biting behaviors not typically observed to cocaine cues (Kearns and Weiss, 2004; Uslaner et al., 2006), which were additionally observed toward the CeA laser-paired aversive shock rod. Further, we demonstrated that the shock rod-associated auditory cue became an attractive stimulus among ChR2 rats, able to reinforce new nose poking behavior in a conditioned reinforcement test. This evidence combined suggests that CeA-induced focusing of incentive motivation likely involves incentive salience attribution to that particular reward's associated cues (i.e., metal portholes and sounds). Future work will directly test this possibility using more traditional tests that allow incentive salience attribution to occur to Pavlovian cues such as autoshaping or during PIT. Pasting CeA excitation onto one of two reward cues could answer whether CeA enhances incentive salience attribution above normal levels (similar to mu-opioid stimulations). It is also worth noting that the outcome representation of the laser-paired incentive could have been altered here by excitation, making it better than the identical alternative (Balleine and Killcross, 2006). We do not discount this possibility, and future work should disentangle this from the incentive salience possibility.

Fear vs. 'wanting' roles of Central Amygdala Circuitry

One remaining question arising from experiments described here, is why did CeA excitation have mostly pro-rewarding effects, causing attraction even towards something known to be aversive? Though others are beginning to acknowledge its role in reward, the amygdala is and its output circuitry is by far most famous for its role in fear expression (Paré et al., 2004; LeDoux, 2007; Ponnusamy et al., 2007; Haubensak et al., 2010). By most accounts, excitation here would have been expected to produce behaviors such as freezing and avoidance. However, we did not observe avoidance nor active fear when we paired excitation with rewards and with the aversive shock rod. Perhaps a better way of defining the amygdala's role in motivation is that it integrates sensory properties of a stimulus with its affective properties and uses that information to guide subsequent behavior appropriately. In times when an animal is hungry, the amygdala may act to make stimuli associated with food become especially attractive. In times where there is a perceived threat, the amygdala might be acting to enhance the salience of smells, sounds, or visual stimuli associated with threats and guide behavior to avoid those situations.

Thus, rather than mediating just one valence of affect (positive or negative), the amygdala may mediate one type over another in certain motivated states (hunger, sexual deprivation, or threatful). This hypothesis would still be consistent with the classical interpretations proposed by Weiskrantz (Weiskrantz, 1956). Weiskrantz found that the constellation of unusual emotional behaviors observed in Kluver-Bucy syndrome, such as attempting to eat rocks or increased sexual drive towards unusual partners, were due to lesions of the amygdala. That amygdala lesions resulted in indiscriminate behavior towards motivationally-relevant objects led to his proposal that amygdala associates the affective or reinforcing properties of stimuli (both positive and negative) with their sensory representations (Weiskrantz, 1956; LeDoux, 2003).

While amygdala can regulate focused motivation for rewards, it may be especially tuned to detect threats because not detecting them can be detrimental in a much more urgent way (i.e., in the case of predators). Because of this, amygdala involvement in enhancing fear may have historically been easier to detect in laboratory settings (LeDoux, 2000). For example, learning about cues that predict a shock occurs after only one trial while learning to predict food cues can take several days and many trials. It is no doubt the reason why learning and memory researchers use fear conditioning as their prime model - it is fast and robust.

Our finding that CeA stimulation caused attraction to an aversive shock rod, but that the same stimulation produced enhancement of classically conditioned freezing supports the notion that the same circuitry can support differently valenced motivations. However, it is still unclear how this is happening. One possibility is that the danger signals received from the instrumental shock rod paradigm were perceived differently than those from the classical conditioning shock grid paradigm. Danger can be perceived differently and produce fearful reactions that vary depending on the type of fear-eliciting stimulus. A shock grid, lacking a localizable source, becomes a perceived fearful situation and produces immobility, which is thought to be a passive form of fear (Fanselow, 1980). By contrast, shock rods are discrete, approachable, and localizable stimuli that also elicit fear but produce avoidance and defensive treading, which are considered active forms of fear (Blanchard et al., 2011). Pasting of CeA excitation onto the discrete localizable source may have been able to alter the percept of that source in a way that made it ‘wanted’ while CeA excitation in the fearful situation of shock grid could have interacted with a different percept of fear, resulting in fear enhancement. In both situations, however, and in the sucrose and cocaine experiments here, CeA excitation produced sharpening of motivation:

freezing was enhanced only during the shock-associated cue and not generally throughout the session just as motivation was focused for one sucrose or for one cocaine, and not generally for all rewards.

Another possibility is that the environment of testing may have influenced the observed differences between shock rod and shock grid experiments. Other striatal-level structures generate differently valenced motivations (eating vs. fear), and these motivations are typically mediated by segregated portions within the medial shell of Nucleus Accumbens (anterior vs. posterior, respectively) (Reynolds and Berridge, 2002). However, the environment can be influential such that more comfortable home environments expand the sites within NAc shell that enhance feeding and by contrast stressful environments expand the sites within NAc shell that enhance fearful behaviors (Reynolds and Berridge, 2008). This gradient of desire and dread may also apply to CeA. Indeed, several groups have identified segregated cell populations within CeA that promote fear or promote reward (and also follow an anterior - posterior gradient like NAc shell) (Kim et al., 2017). Furthermore, environment can flexibly switch the activity of mutually inhibitory CeA neurons to produce active or passive forms of fear (Wolff et al., 2014; Fadok et al., 2017). Thus, environment could have interacted with the type of paradigms (rod vs. grid vs. sucrose) to influence the fear vs. desire observed here, potentially through recruitment of specific cell types (i.e., D1r vs. Crh vs. D2r neurons). Future studies could explore whether the same cells involved in shock rod attraction were also involved in shock grid fear, or other forms of attraction.

Irrational 'wanting' and addiction

The transition to addiction involves a progressive neglect of alternative behaviors in favor of drug procurement and results in many opportunity costs (opportunities for marriage, job promotions, and social relationships). This type of behavior is seemingly irrational because addicts are behaving against their best interests and judgments (Bechara, 2005; Redish et al., 2008). Irrational choice in addiction has been described for several decades and importantly has been distinguished from compulsivity and other features of addiction (Heyman and Dunn, 2002). Although irrational 'wanting' can lend itself to compulsive drug seeking, the two can be distinguished. Irrational behavior refers to choosing one option over another competing option for reasons that don't reflect the value associated with those options (i.e., choosing to pay more for a brand product rather a generic product even if they contain the exact same composition of

ingredients). Compulsive behavior refers to behavior that persists despite negative consequences. While 'wanting' something irrationally can lead to compulsive behavior, it can occur separately. In a 2010 review, Serge Ahmed highlighted the need to understand the "underlying neurobiological dysfunctions of this apparent loss of ability to make rational and voluntary choices" (Ahmed, 2010). Here we used optogenetic CeA excitation to produce irrational 'wanting' by making equal rewards become unequal, and making even aversive stimuli 'wanted' as if they were rewards. We show for the first time a brain mechanism within amygdala capable of producing this type of irrational 'wanting'. Our manipulations here could highlight brain circuitry that gets hijacked during drug exposure to produce such irrational 'wanting' in addiction.

Future directions

Building on these experiments, it will be important to understand the circuitry mediating focused incentive motivation and amplified attraction. We have attempted to gain some insight by quantifying Fos protein among various brain regions. Using this information and known direct output pathways of CeA will help future studies use more targeted approaches to directly stimulate specific CeA circuitry during any of the previous experiments. Furthermore, as CeA is a heterogeneous structure with many different cell types often playing opposing roles in motivation, it will be important to target circuitry in a more cell-specific fashion (i.e., stimulating Crh neurons and their projections from CeL to VTA). Assessing the role of dopamine in CeA-induced attraction would be crucial to understanding how motivation becomes focused onto a particular target. One hypothesis is that CeA can influence dopamine firing via projections to ventral tegmental area, subsequently enhancing dopamine signaling within Nucleus Accumbens (Ahn and Phillips, 2002). Directly assessing this pathway could be a crucial next step.

Our experiments have primarily involved only one form of natural reward (sucrose). However, it would be useful to understand if CeA circuitry can induce attraction towards a social or sexual stimulus and their related cues (Hong et al., 2014; Trezza et al., 2014). It may be possible that the same underlying mechanism mediating focused motivation for sucrose would also mediate focused motivation for social play or for sex just as it does for drugs of abuse, but would need to be directly tested.

Conclusion

Here, we showed that temporally pairing optogenetic stimulation of central amygdala circuitry with motivationally relevant stimuli makes those incentives the sole object of desire. Evidence suggests that CeA excitation is likely doing so by enhancing the attribution of incentive salience to cues associated with those stimuli and not by reinforcing actions alone, or by making the stimulus more pleasant. These findings demonstrate that a hijacked CeA circuitry is sufficient to control reward 'wanting', even to irrational and dangerous levels, highlighting a potentially crucial role for amygdala-related circuitry in focused and irrational pursuit occurring in addiction.

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