Optogenetic Stimulation of the Central Amygdala Narrows and Intensifies Irrational Cocaine Pursuit

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Abstract

In addiction, motivation for drugs becomes simultaneously narrowed and intensified, often at the expense of ignoring other life rewards. Amygdala circuitry, through associative learning, may play a key part in amplifying motivation for rewards. Previous research has suggested that optogenetic stimulation of the central amygdala (CeA) but not basolateral amygdala (BLA) paired with an intravenous infusion of cocaine makes that particular cocaine infusion more pursued, as compared to an identical cocaine reward without CeA stimulation (Warlow, 2017). Here we examine the effect of optogenetic CeA stimulation in biasing motivation for a paired cocaine reward by pairing stimulation with earning one of two equal doses (either 0.3, 0.4, 0.5, or 0.6mg/kg/infusion). Rats were given free access to two different options for intravenous cocaine infusions. One cocaine infusion was paired with optogenetic CeA stimulation (*Laser+Cocaine*), while the other option delivered only cocaine (*Cocaine alone*). Rats preferred the CeA-paired option and ignored the identical alternative when earning all but 0.6mg/kg/infusion doses, and consumed more overall cocaine than control inactive virus rats. In a separate experiment, we paired earning optogenetic CeA stimulation with earning a lower dose of cocaine compared to a higher dose: the *Laser+Cocaine* infusion delivered half the amount of cocaine as compared to the *Cocaine alone* option. ChR2 CeA stimulation caused rats to irrationally prefer the lower dose cocaine option as opposed to the higher dose cocaine option, resulting in earning less cocaine overall than previously earned (and a similar amount compared to control virus rats). Our findings continue to support the CeA's role in narrowing and enhancing motivation for cocaine, to a seemingly irrational level which may mimic characteristics of addiction.

Key Words: optogenetic, central amygdala, cocaine, self-administration, addiction, irrational

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Introduction

Introduction to the Focused Area of Neuroscience

Neuroscience, a multidisciplinary branch of biology, is the scientific study of the nervous system. Within the field of neuroscience, there are specific subfields; one of which is biopsychology, also referred to as behavioral neuroscience. Biopsychological research aims to find the relationship between anatomical structures of the brain and the psychological states and behaviors it controls. Knight Dunlap, who was first to use the term "psychobiology" in the modern sense, described the field as "…bearing on the interconnection of mental and physiological functions" (Dunlap, 1914). Biopsychology explores the connection between body and mind as it neatly intertwines the fields of biology and psychology. More specifically, the anatomical locations of the brain are deeply rooted in the field of biology while the mental states are the primary focus of psychology. The field of biopsychology can be further subdivided into areas of study that focus on specific mental states. For example, affective neuroscience focuses specifically on the study of emotions.

Biopsychological studies share many fundamental elements, however, there still is plenty of variety with the methodology used. To begin, not all studies use humans as the subject of experimentation. Many biopsychological studies utilize animals, such as monkeys, mice, and rats, to investigate phenomena in the brain, performing methods which would otherwise be considered unethical to use on human subjects (Breedlove, Rosenzweig, & Watson, 2007). Although the 'animal' brain is vastly different from the human brain, it shares many brain structures and circuitries that can apply to human models. Furthermore, most biopsychological research involves two specific variables of interest: a biological variable and a psychological variable. These two variables can either be the dependent or independent variable depending on

the study. If the biological variable is chosen to be the independent variable in a study, manipulations can be conducted by eliminating or enhancing a component of the nervous system. As a response, the dependent psychological variable may show changes in emotional state, behavior, or even facial expression. Observing the specific changes allows us to make inferences about the relationship between the two variables. This experimental paradigm is clearly demonstrated in the study of Phineas Gage, which is arguably the poster child of biopsychological research. In 1848, Gage was a twenty-five year old who worked with a blasting crew removing debris for a developing rail road. A tamping iron accidentally drove through his head, entering through the left cheekbone, past his eye, and out the top of his skull. Ultimately, the injury removed a large portion of his frontal lobe. Following the accident, he showed significant behavioral changes such as notable signs of irritability and lacking self-control compared to before the incident (Harlow, 1993). Observing the story of Phineas Gage, we can clearly distinguish the three basic components of all biopsychological research: the biological variable (the frontal lobe), the manipulation (loss of function by removal), and the psychological variable (irritability and self-control). Using this information, we can infer that the functions of the frontal lobe involve executing self-control and maintaining temperament.

In order for inferences from biopsychological research to be conclusive, we must critically analyze the given results. For example, if we were to induce a neuronal manipulation in a rat and subsequently observe that the rat began to eat more food, what can we accurately conclude? It would be apparent that this manipulation impacts eating behavior, but how? Has the rat become hungrier? Does the rat ‘want’ more food? Have we found the area of the brain that controls the motor movement of chewing? We cannot infer that one answer is better than the other without critical investigation and more precise experimental manipulations. Biopsychology

embodies this approach of investigation to corroborate the age-old adage that correlation does not imply causation.

Researchers utilize several different methods to manipulate biological neuronal components of the brain. There have been multiple developments in the field that provide us with more precise and effective methods of brain manipulation other than forcing a tamping rod through the skull of the subject. Researchers can decrease or disable the function of a brain area using techniques both at the anatomical and cellular level. Lesion studies selectively remove or physically damage a region of the brain; enough so that the lesioned area can no longer function. Furthermore, neurochemical antagonists can be injected into the brain; these antagonists prevent brain function by disabling communication within the affected area. On the other hand, brain area function can also be enhanced or selectively activated during an experiment. At the cellular level, chemical agonists can be used to activate regions of the brain by enhancing or replacing endogenous neurotransmitters. While these agonists can effectively activate receptor-defined cells within particular regions of the brain, they are not precise enough, both spatially and temporally, to conduct certain experimental procedures. Fortunately, other methods, such as optogenetics, allow us to manipulate neuronal function precisely and transiently within an experiment.

Optogenetics is a relatively new development in the realm of experimental research methods. This technique takes advantage of cellular mechanisms that activate or deactivate neuronal cells in the brain. Neurons naturally activate based on the polarization of the cell. This polarization is regulated by specific ion channels, that when opened or closed, cause the cell to activate (depolarize) or deactivate (hyperpolarize). Optogenetics takes advantage of the natural mechanism of neuronal cells by using unique ion channels that, when exposed to a specific

wavelength of light, will open, allowing either positive or negative ions to rush into the cell, depending on the type of channel expressed (Gradinaru et al., 2010). By utilizing a viral vector, neuronal cells are infected with these light sensitive ion channels, and after approximately three weeks, the cells begin to express the channels on their own. Once expressed, the cells can be activated by exposing them to light via an optic fiber cable that is implanted in the brain. Depending on the type of channel, the cells can be activated or deactivated when exposed to the light. For example, Halorhodopsin is an optogenetic virus that inhibits a cell when exposed to yellow light. Conversely, Channelrhodopsin-2 (ChR2), the virus used in the study here, activates a cell when exposed to blue light. This method allows for high spatial and temporal resolution, allowing researchers to activate a specific area at a specific time, which is an invaluable tool for producing accurate and meaningful results.

The impact of biopsychology research extends to several facets of our daily lives. One of these is the phenomenon of addiction; a brain disorder characterized by compulsive engagement in rewarding stimuli, despite adverse consequences (Volkow, 2016). An individual can be diagnosed with an addiction when they meet a specific set of required symptoms as described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Research has shown that addictive behaviors are associated with hyper-reactive brain limbic areas, which are the same brain areas evolved to seek out natural rewards in the environment. These brain areas are thought to be ‘hijacked’ by drugs of abuse, such as cocaine and heroin, and other rewarding behaviors, such as sex and gambling. Over time, the limbic areas respond more intensely to cues in the environment that are associated with these rewards, which triggers intense urges/cravings to seek out the reward (Kelley & Berridge, 2002). Biopsychologists, specifically affective neuroscientists, conduct research that aims to reveal the brain mechanisms involved in

generating the many hallmarks of addiction, in hopes to explain and potentially cure this detrimental condition.

Critical Review of Relevant Literature

Motivation has become a prevalent and dynamic focus in behavioral neuroscience. Researchers are questioning what are the reasons and explanations behind our behaviors. Why do we make one choice over another? What can be done to influence the preference of different options? Theories to explain these behaviors have evolved significantly over time, all of them with the intention to help explain the reasoning behind our most basic actions. To begin, psychologist explained motivation based on homeostasis. In biology, homeostasis refers to a specific physiological set point; a built in base line that physiological processes aim to maintain. When there is a deviation from the homeostatic set point, a response is generated in order to bring the current state back to the desired set point (Cannon, 1939). Homeostatic mechanisms of the brain depend on several components including a set point, an error detector to identify physiological changes, and an error correction mechanism to counteract any deviations from the physiological set point (Epstein, 1976). When all three components are used together, their interactions help explain the reasoning behind our most basic motivated behaviors. For example, let's consider the homeostatic regulation of satiety. To begin, we know the body needs food in order to maintain an appropriate level of energy in order to function normally. The amount of energy required determines the physiological set point. Any deviation from this set point, such as food depletion, is detected by an error detection mechanism in the brain and must be corrected. This results in hunger, which motivates us to eat and return to our physiological set point.

Although homeostatic theory may explain motivation on the surface, there are other factors that need to be considered. To begin, homeostatic set points are not constant and the

homeostatic model alone does not provide a clear explanation to regulate all bodily needs (McEwen, 2000). Using the assumptions of homeostasis alone, it is difficult to explain and predict complex physiological mechanisms, such as thirst. To alleviate this problem, an intervening variable used to refine homeostatic motivations is necessary: drives. Homeostasis and drives are frequently referred to together as homeostatic drives. Drives, such as hunger, motivate our behaviors to ensure that our body maintains appropriate physiological levels in order to continue healthy function. Psychologists were quick to incorporate and accept drive theories as a fundamental component in explaining homeostatic behaviors. Drives allowed for a more 'reliable' model to predict choices and help explain what motivates individuals. From a behaviorist perspective, drives are explained as basic stimulus-response (S-R) relationships. The increase in a stimulus (i.e. water deprivation) has a direct impact to on the response drive (i.e. water drunk). Water deprivation, however, is not the only stimulus that may motivate drinking behavior. Other stimuli, such as blood salt concentration and heat, influence water consumption. Instead of creating individual 'drives' for each stimulus (e.g. The Blood Salt Drive vs. The Heat Drive) psychologists use 'drive' as an intervening variable between all stimuli and the response. This way, a single drive, such as this, can help explain how multiple stimuli influence a single motivated behavior in parallel. Stimuli such as water deprivation, blood salt level, and heat all intervene at the same point to generate the homeostatic drive of thirst, which then generates the motivated behavior response: drinking water (Johnson & Thunhorst, 1997).

Support for drive theory rested on the fundamental assumption that all drives are aversive and the lessening of deficit sensation (drive reduction) is rewarding. The end goal of motivated behavior is to reduce the aversive drive (Chatterton, 2016). This assumption held true and coincided with several motivated behaviors. For example, we are motivated to eat in order

reduce the hunger drive, we drink to reduce the thirst drive, we sleep to reduce the fatigue drive. In all scenarios, reducing the drive (hunger, thirst, fatigue, etc.) is a rewarding behavior. From an observational standpoint, drive theory seems to be a clear explanation as to when and why we demonstrate certain behaviors. However, drive theory did not hold true when analysis was focused at the neurobiological level. James Olds, an American psychologist credited for discovering the "pleasure center" of the brain, utilized electrode stimulation to activate brain regions in rats. His goal was to determine the relationships between specific anatomical brain areas and behavior. Based on prior research, Olds implanted electrodes in two distinct areas of the rat brain; the hunger and satiety centers. When activated, the hunger electrode would, not surprisingly, elicit the sensation of hunger and the satiety electrode would make the rat feel satiated. Rats were allowed 'free' brain stimulation, meaning that the rats could perform an action to activate these areas of the brain on their own will. Under normal conditions, hunger is the drive that aims to restore the state of satiety, implying that activation of the hunger electrode increases drive and activation of the satiety electrode decreases drive. Thus, following the guidelines of drive theory, the hunger electrode should be aversive (drive amplifier) and the satiety electrode should be rewarding (drive attenuator). Unexpectedly, the results supported that the opposite was true; the hunger electrode (drive amplifier) was more rewarding than the satiety electrode (drive attenuator) (Olds, 1973). These results made it clear that drive reduction could not be the explanation behind motivated behavior. The presence of a drive state should be aversive, not rewarding. Similarly, removal of a drive should be sought out and should be rewarding. This, however, was not demonstrated by Olds' rats. In order to be correctly understood, theories of motivation must be explained utilizing brain mechanisms beyond homeostatic drives.

Following the shortcoming of drive theory, hedonic reward theories emerged as a new approach to explain incentive motivation. Hedonic reward theories place emphasis on pleasure and reward in the brain in order to explain motivated behavior. The introduction of these theories was influenced by several psychologists, such as Pfaffmann. In the 1960's, Pfaffmann called for a better understanding of the relationship between pleasure and behavior (Pfaffmann, 1960). His analyses provided clear distinctions from drive behavior, as proposed by prior psychologist, and redefined them as pleasure responses. Succeeding Pfaffmann, Stellar continued the hedonic push, urging that the questions of feeling, sensation, and affect be considered when moving forward in the search for the explanation behind motivation (Stellar, 1980). No longer was the focus of motivation entirely on homeostatic drives. The increased attention on hedonic reward forged the introduction of more refined motivational concepts, such as incentive motivation.

The transition from drive reduction to incentive motivation theory did not occur all at once. The steps to refining the foundations of incentive motivation were built on the contributions of several psychologists, most notably Robert C. Bolles, Dalbir Bindra, and Fredrick Toates (Berridge, 2004). Bolles first suggested that motivation comes from incentive expectancies, not from drive reduction (Bolles et al., 1972). These expectancies are associations made between two stimuli (S-S* Associations). For example, a predictive neutral stimulus, such as a sound or a light flash, can be paired with a positive hedonic stimulus, such as a food reward. In a Pavlovian conditioning manner, presentation of the neutral stimulus (S) predicts the hedonic stimulus (S*), and thus the predictive S cue takes on the positive hedonic sensation of the expected S* reward. But if the neutral (S) stimulus induces the same sensation as the rewarding (S*) stimulus, why not settle for S alone? Why work for S*? Bindra responded to the claims made by Bolles with the intent to resolve the conflict between S and S*. He clarified that the

hedonic expectation induced by S is not the same as the one created by S* and that there is a unique inherent importance to the reward itself (S*) which is important for incentive motivation (Bindra, 1978). Bindra explained the positive sensations, induced by S, to simply being learned responses from classical conditioning with S*. Furthermore, S* induces its own unique physiological changes that S alone does not generate. S* therefore contains the source of the pleasure that S takes on, which is what reinforces the behavior to begin with. Critics of Bindra note that conditioned stimuli (S) do not remain incentives under all conditions. For example, the smell of food (S) acts as a stronger incentive when you are hungry than when you are satiated. To explain this, Toates suggested that physiological state can enhance or diminish the incentive value of the goal stimuli (Toates, 1986). Cognitive expectation can also influence the intensity of motivated behavior. In summary, incentive motivation generates goal oriented motivated behavior through the interaction between hedonic rewards and predictive cues. The strength of this interaction is dependent on the current physiological state and the cognitive expectations of the individual (Toates, 1986).

The Bolles-Bindra-Toates model of incentive motivation set the groundwork necessary to help create more refined theories of motivation. This refinement comes in the form of incentive salience, which separates the sensations of ‘wanting’ and ‘liking’. Although commonly used together when discussing reward, the sensations of ‘wanting’ and ‘liking’ are actually separate mechanisms in the brain (Berridge & Robinson, 2003). Dopamine, a neurotransmitter once thought to mediate sensory pleasure, has been intensely investigated to help differentiate the distinction between ‘wanting’ and ‘liking’ mechanisms. To clarify, ‘liking’ refers to the hedonic impact: the immediate pleasure sensation induced upon receipt of a reward. This is the unconditioned innate response to the S*. As suggested by Bindra, physiological responses of

‘liking’ can also be generated by a conditioned stimulus (S), but more importantly, the S stimulus acts as a cue which may induce ‘wanting’ of the S* reward. It is important to recognize that, unlike ‘liking’, ‘wanting’ is the motivational value of a reward, not the hedonic impact of it. ‘Wanting’, or desire, for a reward is referred to as incentive salience; in particular when the motivational cue (S) has taken on the value of the reward (S*) on its own. Considering that the sensations of ‘wanting’ and ‘liking’ are separate in the brain, it is possible to experience one without the other. This has been supported by experiments in which dopamine was suppressed in the brain. The motivational ‘wanting’ for pleasant incentives, such as food, was lost, while the hedonic ‘liking’ impact of the same incentive remained as observed by affective ‘liking’ facial reactions (Brauer et al., 1972). It is important to realize that concept of ‘wanting’ is not necessarily a cognitive want. An explicit want, “I want this.” does not need to exist to experience the ‘wanting’ sensation (Ledoux, 1994). This separation was observed in an experiment conducted by Fischman & Foltin (1992). In their experiment, cocaine addicts were given access to intravenous infusions of cocaine solutions. The solutions varied in concentration, some at concentrations considered to be a normal dose to a cocaine user, others at very low concentrations. The normal concentrations induced the sensations normally felt when using cocaine, while the very low concentrations were low enough that the normal sensations of cocaine could not be achieved. Trials were conducted allowing the cocaine users to select between the different dosages among several combinations. Not surprisingly, the normal dosages of cocaine were preferred over the low dosages. Participants would sample the two different concentrations, experience the hedonic ‘liking’ of the normal concentration, and generate a preference for that concentration. But what would happen if they had to select between the very low dosage cocaine and a saline placebo? Both options, as reported by the participants, did not

generate the normal sensations of cocaine use. However, the low concentration of cocaine was greatly preferred over the saline solution that contained no cocaine. They demonstrated no cognitive 'wanting' of the low dosage cocaine but did show a behavioral 'wanting' as observed by their selection bias. In fact, Fischman and Foltin noted that the cocaine users deemed the low concertation cocaine as "worthless". Yet, there still was an observable desire to use the low concentration cocaine over the placebo. There are limitations in this study in that the results are purely observations, but it does provide some insight into the behaviors of addicts and how motivation mechanisms can go awry as a result of drugs of abuse; which is the focus of this thesis.

With the distinction between 'wanting' and 'liking' made, theories to explain the mechanisms of addictions have emerged. Kent Berridge and Terry Robinson proposed the incentive-sensitization theory of addiction, which combines incentive salience concepts with the mechanisms of neuronal sensitization (Robinson & Berridge, 1993; Berridge & Robinson, 2016). Fundamental to this theory is the notion that incentive salience, 'wanting', can become sensitized over time, which is prevalent especially in addicts. Neuronal sensitization means that brain systems can be triggered into abnormally high levels of activity in response to drugs or other related stimuli. This is the opposite of drug tolerance. When addicts, for example cocaine addicts, abuse cocaine for an extended period of time, they build a tolerance to the drug requiring them to take more of the drug in order to experience the same level of the desired high (also known as drug tolerance). Simultaneously, particular areas of the brain (e.g. mesolimbic areas) will experience the opposite effect: neuronal sensitization (Berridge, 2012; Koob & Volkow, 2016). During sensitization, the same amount of the drug will induce greater and greater neuronal responses over time. These brain areas are overreacting to the amount of drug and are

considered hyper-reactive. It is important to make the distinction between hyper-reactive and hyper-active. Studies modeling addictive behavior in rats have demonstrated this differentiation. Researchers have observed the locomotion behavior of both normal rats and rats addicted to cocaine. Under normal conditions, the amount of locomotor activity was no different between the two groups of rats. However, when both the normal rats and rats previously exposed to cocaine were primed with a small amount of cocaine, the cocaine-exposed rats displayed a significant increase in locomotor activity as compared to the normal rats. This supports that although the cocaine-exposed rats were not hyper-active, as compared to the normal rats in normal conditions, the addicted rats were hyper-reactive to the cocaine (Deroche-Gamonet et al., 2004). This hyper-reactive response is not exclusive to the reward alone. Cues, such as the sounds and smells associated with the drugs of abuse, can trigger a strong sensitized 'wanting' behavior in addicts as well. As a result of the intense 'wanting' response generated, cues have the potential to trigger strong cravings and induce relapse in an addicted individual, even when they have the genuine desire to quit their addiction, and have even been able to abstain for several years (Berridge & Robinson, 2016).

One area of the brain that is vulnerable to neuronal sensitization is the mesolimbic pathway. As mentioned before, sensitization of this dopaminergic pathway causes it to become hyper-reactive to stimuli associated with the addicted drug or behavior (Koob & Simon, 2009). The primary connection of the mesolimbic pathway is between the ventral tegmentum area (VTA) and the nucleus accumbens, however, there are additional structures that mediate within the pathway, such as the hippocampus, amygdala, and pre-frontal cortex; all of which contribute a unique role in the mechanisms of addictions (Richard et al., 2013). Functional brain imaging studies on humans have demonstrated the incentive-sensitization theory of addiction and how

neuronal sensitization impacts several mesolimbic structures. Volkow et al. (2006) found that when presented with a drug related cue, cocaine addicts had increased activation in the dorsal striatum, nucleus accumbens, and prefrontal cortex, as compared to non-addicts. This finding supports that the mesolimbic circuitry of addicts have become sensitized to the drug cues, making them hyper-reactive as compared to non-addicts.

By individually isolating and manipulating areas of the brain associated with the mesolimbic pathway, both in human and animal studies, we can gain a better understanding of what may cause, maintain, and prevent the effects of addiction. Unfortunately, addiction studies are not easily conducted on humans. We cannot force a human to become an addict, nor can we efficiently predict a healthy individual's natural transition to becoming an addict. However, we can model aspects of addictive-type behaviors in non-human subjects, such as rats. Several studies have supported that addiction models in rats can be applied to characteristic behaviors seen in human addicts in order to better understand the mechanisms of addiction (Nesse & Berridge, 1997). Using several different procedures, researchers have been able to model the different characteristics of addiction, though usually one at a time and not fully grasping all characteristics in the same model. Some groups have been more successful than others at capturing the many different hallmarks of addiction in rats by using specific self-administration (SA) procedures. For example, the Deroche-Gamonet group showed that when rats are provided with the opportunity to self-administer intravenous infusions of cocaine, they will work for and continue to work for the drug (Deroche-Gamonet, 2004). Furthermore, drug use increased over time and the amount of work an addicted rat is willing to do for a cocaine infusion increases progressively after consistent infusions of the drug. From an observational standpoint, a rat's increased use of the drug of abuse supports the escalation of intake commonly seen in human

addicts, and is one of the hallmark criterion used in the DSM-V for diagnosing Substance Use Disorder. After 30 days of cocaine use, rats qualified for an addiction diagnosis as defined by the DSM-V, similar to humans. The specific diagnostic criteria that rats met included difficulty in stopping drug use, high motivation to take the drug, continued abuse despite harmful consequences (shocks), and other addiction-like behaviors. Addiction-like behavior in rats is similar to the addiction-like behavior in humans, which provides us with additional support that addiction mechanism studied in animals can be applied to humans as well. While models that accurately mimic human drug abuse are still being perfected and the type of model may not be identical, the preliminary findings in animal models help guide thought and research in understanding human addiction. Furthermore, the mesolimbic structures mentioned before are not exclusive to humans. The mesolimbic pathway, and the structures that integrate with it, are evolutionarily primitive components of the brain. As such, they are shared across many species allowing findings in one animal to be effectively applied to another (Saunders & Robinson, 2011).

Considering that mesolimbic circuitry interacts within multiple structures of the brain, the most efficient way to understand this complex system is to isolate and manipulate one structure at a time. This allows us to directly observe the contributions of a single structure. One such structure is the amygdala, which is the primary focus of this thesis. Primary investigations of the amygdala began with its contributions to associative learning and fear. After rats undergo Pavlovian fear conditioning, pairing an audible tone with a foot shock, presentation of the audible tone alone produces robust and replicable fear responses (i.e. freezing). Furthermore, during these fear responses, activity of the amygdala increases exclusively to the shock paired tone (LeDoux, 2003; Maren, 2005). These findings suggested that the learning of tone and fear is

maintained by the amygdala itself. Beyond fear, the amygdala also has unique contributions to other emotions in the brain, as observed in Klüver-Bucy Syndrome, which results from bilateral lesions of the medial temporal lobe including the amygdala nucleus. Experiments in rhesus monkeys and observations in affected humans support that, without an amygdala, individuals display several unusual behaviors. This includes hyper-orality, hyper-sexuality, and docility (reduced fear response) (Hayman et al., 1998). Additionally, the associative learning capacity of the amygdala extends beyond fear and into the realm of reward value, however, the mechanisms of associative fear and reward learning are not the same. In one study, monkeys were taught to associate a tone with an aversive stimulus. Intact animals with normal amygdala function learned that the tone will predict the punishment, while animals with impaired amygdala function did not make the association (Quirk, Armony, & LeDoux, 1997). However, when monkeys underwent Pavlovian reward conditioning, pairing of a tone with a food reward for example, monkeys still learned the reward association with or without amygdala function (Baxter & Murray, 2002). These results imply that the amygdala is needed for fear conditioning but not reward condition. While the evidence is compelling, it does not mean we should dismiss the role of the amygdala with reward. It is possible that other mechanisms of the brain, beyond the amygdala, allowed for simple auditory Pavlovian reward conditioning to occur. The amygdala then must contribute to associative reward learning by different means, which is why the association between tone and food reward could still be made by the monkeys. The experimental methods previously mentioned are demonstrations of first-order Pavlovian conditioning. During first-order Pavlovian conditioning, the tone conditioned stimulus (CS) is paired with the unconditioned reward stimulus (UCS). An incentive value is placed on the tone (CS) because it is directly paired with the reward (UCS), thus making the tone a first-order conditioned stimulus (FO-CS). Now

imagine if a new stimulus, for example a presentation of a light, is paired with the tone. After successful Pavlovian conditioning between the light and the tone, the light now predicts the tone, making the light a second-order conditioned stimulus (SO-CS). In summary, the light (SO-CS), predicts the tone (FO-CS), which predicts the reward (UCS). Experiments in rats demonstrate that both intact rats and rats lacking amygdala function can undergo first-order conditioning. However, when trained for second-order conditioning, only intact rats will demonstrate a learned association between the second-order stimulus and the original reward (Baxter & Murray, 2002). In successful second order conditioning, the reward value of the UCS is transferred to the FO-CS and then to the SO-CS. It appears that without appropriate amygdala function, the transfer of reward value from FO-CS to SO-CS cannot occur. This suggests that the amygdala plays a unique role in not necessarily *reward* conditioning, but rather reward *value* conditioning. This distinction is crucial in the model of addiction. Drugs of abuse can alter the normal function of the amygdala, thus the perceived reward value of said drug will be altered with it, disrupting normal function of the mesolimbic circuitry (Janak & Tye, 2015).

Up to this point, the amygdala has been referenced as one discrete unit. This, however, is not the reality of the structure. The amygdala is divided into two distinct units; the basolateral amygdala (BLA) and the central amygdala (CeA). Each of these structures have their own unique functions, as supported by double dissociation experimentation (Hsu, Schroeder, & Packard, 2002; Johnson, Gallagher, & Holland, 2009). Although their functions are different, they both have contributions in regards to reward value conditioning and are both serially connected in an observable macrosystem structure. The macrosystem arrangement between the BLA and CeA can be observed in multiple systems within the brain. This macrosystem, first proposed by Larry Swanson, in particular contains 3 levels: Cortical, Striatal, and Pallidal

components, each distinguished by their projection targets and inputs. Cortical structures send glutamatergic signals to striatal structures, which in turn, project GABAergic signals to pallidal structures, which then send out GABAergic signals to their targets (Zahm, 2006). At baseline, in a normally functioning brain, cortical structures in the brain suppress pallidal structures in a top-down fashion. That is, when a behavior needs to be produced, the cortical structures can release the inhibition of the striatal structures thus allowing the behavior to occur. Within the amygdala, due to its predominantly glutamatergic cell populations, the BLA is proposed to be positioned as a cortical structure, which sends projections to the CeA; and due to its predominantly GABAergic cell population, CeA is proposed to be striatal-like within the macrosystem (Reppucci & Petrovich, 2016). As a result, direct activation of the CeA increases the release of GABA, causing a decreased response in its pallidal targets (perhaps BNST in this proposed model), which ultimately increases the response in the end targets. Some of these end targets are located in mesolimbic circuitry. As such, it is thought that the CeA helps translate learning into motivation, enhancing incentive salience onto a specific learned reward cue. Mu-opioid activation of the CeA, as demonstrated by Mahler and Berridge (2012), makes a reward cue more attractive, noticeable, and liable to elicit appetitive and consummatory behaviors. Conversely, this effect is not observed with selective BLA activation, further supporting the notion that BLA and CeA, while serially connected, can also function in parallel to elicit motivated behaviors (Balleine & Killcross, 2006).

Furthermore, activation of the CeA via optogenetic ChR2 excitation has been shown to narrow incentive motivation to pursue one reward over another (Robinson, Warlow, & Berridge, 2014). Rats were given the opportunity to earn sucrose by pressing either of two levers, and one of these levers also delivered optogenetic CeA ChR2 stimulation. Through this experimentation,

there were some important findings. Rats not only narrowed their preference for the CeA-paired sucrose reward, they also showed increased motivation to earn that paired reward in a separate progressive ratio test of motivation. However, when the sucrose reward was removed and pressing the lever now only earned laser stimulation, rats stopped responding and laser stimulation alone did not maintain responding. Additionally, when given the opportunity, rats fail to self-stimulate for CeA activation alone. Both of these findings suggested that CeA stimulation was acting on its paired external reward to make it more attractive than an identical alternative.

This study also found that CeA stimulation, when paired with a reward that is not usually preferred, can make that reward preferable at equal levels. Specifically, rats were given access to earn either sucrose pellets or salt pellets in a similar choice task. Under normal circumstances, rats prefer to the sugar pellet to the salt (cracker-like) pellet. However, after pairing CeA stimulation with delivery of the salt pellet, rats began to choose equally between the two options. This implied that CeA activation increased the incentive value of the salt pellet reward to the same level as the sucrose pellet reward.

Recently, the CeA ChR2 effect has been extended to a substance more addictive than sucrose: cocaine. When presented with two levers that provide an infusion of intravenous cocaine, rats intensely pursue a cocaine reward paired with optogenetic CeA ChR2 stimulation while ignoring an alternative identical cocaine reward, resulting in increased overall cocaine consumption (Warlow, Robinson, & Berridge, 2017). As previously demonstrated, CeA ChR2 stimulation was still not rewarding when given alone. This further supports the findings that CeA ChR2 stimulation is transforming the value of the physical reward (whether it be sugar, salt, or cocaine) into something more desirable than its alternative, seemingly equal, reward. But by how

much does CeA ChR2 stimulation increase the value of a reward? Rats, when presented with two equal rewards, will select the reward paired with CeA excitation. Furthermore, CeA excitation can make two unequal different rewards, such as sucrose and salt, equally preferred. But what does this change of preference look like for two rewards of the same type, but delivered in two different amounts? Specifically, if CeA excitation can make unequal rewards such as salt vs. sugar equally preferred, would this effect extend to cocaine, in making two unequal doses (one high preferred dose vs. a low unpreferred dose) now equally preferred? And, if so, how much additional cocaine would the non-CeA ChR2 lever need to provide in order to counter the effects of CeA ChR2 activation, if possible at all?

Specific Goals of the Current Thesis

My proposed thesis intends to investigate how optogenetic stimulation of the central amygdala generates intense pursuit of cocaine in a dose-dependent fashion. Currently, it is understood that CeA ChR2 activation makes one cocaine reward preferred over another (Warlow, Robinson, & Berridge, 2017). However, the same dose was used for all prior studies (0.3mg/kg/infusion). My thesis aims to answer two questions. First, does the CeA effect of narrowing and amplifying incentive motivation for cocaine apply at other doses of cocaine? By using varying dosage of cocaine, we can observe any boundaries of the CeA's ability to focus incentive motivation; in other words, how much cocaine is too much, or too little to cause focused pursuit when paired with CeA stimulation? Second, can CeA excitation, when paired with an unpreferred dose of cocaine, make that cocaine more pursued than an alternative) higher dose option (more preferred? By raising the dosage of the non-laser cocaine infusion alternative, we can determine if there is a 'breaking point' at which the rats will switch from the laser-paired to the non-laser paired cocaine infusion choice. This 'breaking point' can tell us how powerful

the CeA ChR2 transformation can be. Conversely, lowering the dosage of the laser paired cocaine infusion will provide insight as to how small the reward can become while still maintaining a laser paired preference. Prior studies support that laser stimulation alone is not reinforcing and does not maintain a preferred response when cocaine is totally removed (Warlow, Robinson, & Berridge, 2017). However, we do not know if rats will continue to prefer smaller amounts of cocaine because they are paired with CeA ChR2 stimulation.

We predict that rats will bias their choice for a laser-paired cocaine infusion at each dose they are trained with (when the two options are equal). However, when cocaine options are unequal, we predict that CeA activation will reduce the discrepancy in preference between both options. More specifically, rats should switch to equally preferring both options when the alternative (non-CeA-paired) becomes more enticing (higher dose). This will be important in determining the role of amygdala circuitry in pursuit of drugs at the expense of alternate rewards.

Materials and Methods

Subjects

Female (n=12) Sprague Dawley rats, weighing 250-300g at surgery, were singly housed in a temperature controlled room at 21^0 C on a reverse 12hr light/dark cycle. Both food and water were available ad libitum throughout the entire experiment. All experimental procedures were approved by the University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan. The UCUCA protocol number is PRO00006178 and the authorized Principal Investigator is Dr. Kent Berridge.

Surgery: Optogenetic virus infusion and optic fiber implantation

Prior to surgery, all rats were anesthetized using isoflurane (Henry Schein, Wixom, MI; 5% for induction, and 2% during surgery) and received an intraperitoneal injection of atropine

(Sparhawk Laboratories, Shawnee Mission, KS; 0.04 mg/kg). During surgery, each rats also received subcutaneous injections of chloramphenicol sodium succinate (Henry Schein, Wixom, MI; 100mg/kg) to prevent infection, carprofen (Henry Schein, Wixom, MI; 5mg/kg) for pain relief, and 2.5ml of isotonic saline solution to prevent dehydration. Rats again received carprofen (Henry Schein, Wixom, MI; 5mg/kg) 24 and 48 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, Los Angeles, CA), and received bilateral infusions of an AAV ChR2 virus with human Synapsin promoter to infect neurons (AAV5-Hsyn-ChR2-eYFP). Virus infusions were targeted 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). A total of 1µl volume of virus per side was infused over 10 min at a constant rate (0.1µl/min), and the injector was subsequently left in place for an additional 10 min to allow for diffusion. Some rats (n=4) received infusions using an automatic syringe pump, while other rats (n=8) received infusions using a manual Hamilton syringe. Control rats received a control infusion of an optically inactive virus; identical except lacking the ChR2 gene (AAV5-Hsyn-eYFP; n=2). For all rats, bilateral optic fibers (200µm) were implanted during the same surgery, each aimed at 0.3mm dorsal to the rat's virus infusion.

Surgery: Intravenous catheter implantation

In a separate surgery approximately 3 weeks later, the same rats were implanted with a chronic intravenous jugular catheter for subsequent intravenous delivery of cocaine solution. After identical anesthesia and perioperative treatment as stated above, a silastic catheter (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 0.2ml isotonic saline solution containing 5mg/ml gentamicin sulfate and 0.1ml heparinized saline (Sigma-Aldrich, St. Louis, MO; 500IU/mL), to prevent infection and occlusions respectively. Catheter flushing continued every day throughout the duration of behavioral analyses. Catheter patency was tested before commencement of behavioral testing using an intravenous injection of 0.2ml methohexital sodium (20mg/ml in sterile water). Rats that became ataxic within 10s were considered to have a patent catheter and included in behavioral analyses.

Procedure: Instrumental choice of Laser+Cocaine versus Cocaine alone at the same dosage

Rats were trained to instrumentally earn intravenous cocaine infusions by performing a task on one of two instrument types: portholes or levers. Instrument designation was arbitrarily assigned to rats prior to training. Porthole instruments delivered cocaine infusions when the rat delivered a nose poke into either one of two portholes, of which they were allowed to choose between. The portholes were placed ~ 5 cm apart on the same wall, and detected nose pokes via beam breaks. Similarly, lever instruments delivered cocaine infusions when the rat pressed down on the lever. Levers were on the same wall, also 5cm apart and detected presses when a sufficient downward force was applied. Prior to behavioral analysis, rats were assigned a specific cocaine dosage measured in mg (weight of the salt) per kg (weight of the rat) dissolved in 0.9% sterile saline. The varying dosages included 0.3mg per kg (n=3), 0.4 mg per kg (n=3), 0.5 mg per kg (n=2), and 0.6mg per kg (n=4).

Both instrument types delivered cocaine infusions [(rat specific cocaine amount) mg per kg in 50µL volume infused over 2.8s]. Additionally, one instrument (*Laser+Cocaine* instrument)

arbitrarily designated for each rat, delivered an 8s optogenetic CeA ChR2 stimulation [5-8mW blue laser (473nm), 25Hz (15ms ON 25ms OFF for 8s train)], which began with the initial nose poke or lever press and continued during and immediately after the infusion. The other instrument (*Cocaine alone*) delivered an equal infusion of cocaine, but without any laser stimulation.

Each instrument's infusion was also accompanied by a distinctive 8s auditory cue (either white noise or tone, different between the two ports). All portholes (CeA ChR2 n=5; CeA control inactive virus n=2), and levers (CeA ChR2 n=5) were retractable. Either instrument was inserted one-at-a-time initially each day until cocaine was earned on each, and then both portholes/levers were inserted together for the remainder of each session.

MedAssociates chambers (30.5 x 24.1 x 21.0 cm) with clear Plexiglas floors 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.

Rats were trained for 10 days on a fixed ratio (FR-1) instrumental self-administration of intravenous cocaine, which will be referred to as Equal Dose (ED) training throughout this paper (CeA ChR2 virus: n=10; CeA control inactive virus: n=2). The first day began with a 1.5-hour session, and subsequent days were 1 hour sessions. Nose pokes or lever presses into/onto either porthole/lever earned an infusion of cocaine. Assignment of the laser-paired porthole and auditory cue versus cocaine-alone porthole and cue was always the same for a given rat across all days, but was counterbalanced between rats. For all instruments, a 20s timeout period was imposed after each cocaine infusion was earned, during which the instrument retracted and was

unable to be accessed. Rats were required to sample from both cocaine-delivering ports at least twice each day on the first two days. All rats met this criterion and continued to do so throughout the experiment. Subsequent sessions lasted 60 minutes or 40 infusions, whichever occurred first. Any rat that failed to respond (nose-poke/ lever press) at least 5 times per session over three consecutive sessions within the first 10 days was excluded from analyses (n=1).

Procedure: Instrumental choice of Laser+low-dose of Cocaine versus high-dose of Cocaine alone

To assess the "strength" of CeA stimulation in intensifying cocaine motivation, rats continued the same instrumental choice procedure but now the cocaine rewards became unequal – the *Cocaine alone* infusion was now double the dose of the *Laser+Cocaine* infusion, which will be referred to as the Unequal Dose (UD) paradigm throughout this paper. Following the 10-day ED training period where rats chose between *Laser+Cocaine* vs. *Cocaine alone* and cocaine infusions were the same dose, rats continued the same instrumental procedure with access to both nose-ports or levers. The assigned laser-paired instrument remained unchanged, relative to the training period, and each nose-poke or lever press continued to deliver an intravenous infusion of cocaine. However, the amount of cocaine delivered from the *Laser+Cocaine* instrument was reduced by half relative to the *Cocaine alone* instrument. Rats that were trained using 0.3mg per kg infusion concentrations received the same amount of cocaine per infusion from the laser-paired instrument, but twice the amount of cocaine per infusion from the non-laser paired instrument (relative to the ED training period). Rats that were trained using 0.4/0.5mg/0.6mg per kg infusion concentrations received the same amount of cocaine per infusion from the non-laser paired instrument, but half the amount of cocaine per infusion from the laser paired instrument (relative to the ED training period).

Because there was only one intravenous catheter port per rat, each rat could only have one cocaine source per session. In order to manipulate the amount of cocaine received per infusion, the concentration of the cocaine source remained constant, while the volume amount of cocaine per infusion was manipulated. All increases in non-laser-paired cocaine infusions were achieved by increasing the infusion from 50µL volume infused over 2.8s to 100µL volume infused over 5.6s; laser-paired cocaine infusions remained the same volume (50µL volume infused over 2.8s). All decreases in laser-paired cocaine infusions were achieved by diluting the concentration of the cocaine source by half, while maintaining the rate of infusion at 50µL volume infused over 2.8s. To compensate for the diluted cocaine solution, non-laser paired infusions were increased to 100µL volume infused over 5.6s, thus delivering the same amount of total cocaine (relative to the training period) over an extended period of time. In summary, all rats had their non-laser paired cocaine infusion delivered over a longer period of time and the only manipulation done to change the amount delivered was done by altering the concentration of cocaine.

Procedure: Conditioned Reinforcement

To test whether the associated cues (nose ports for some rats and levers for others plus auditory cues) had acquired incentive value, rats underwent a test of conditioned reinforcement after the first 10 days of ED training. On day 11, rats underwent a two-day procedure to assess the conditioned reinforcement of the cues associated with each instrument. Rats were placed in a different testing chamber from the training period; free of laser stimulation or cocaine infusion connections. The testing chambers contained the trained instruments (lever or nose-port) on the same wall of the chamber and the opposing instrument, unfamiliar to the rat, on the opposite wall. In the first session, instruments on the opposite wall were presented and the rat could either nose-

poke or press the instrument. Within each set of nose ports/levers, there were two instrument types: The active instrument and the inactive instrument. Pressing the active instrument delivered a 4s auditory cue (previously associated with either *Laser+Cocaine* or *Cocaine alone* responses) accompanied by presentation of that previous instrument (lever or nose port) on the opposite side. Presses/nose pokes during its presentation were also recorded. Pressing the inactive instrument had no consequences and served as a control for exploratory behaviors. Order of sessions involving *Laser+Cocaine* or *Cocaine alone* cues was counterbalanced between rats. Each of these sessions lasted 30 minutes.

Procedure: Cocaine Extinction

To assess if CeA ChR2 stimulation alone could act as a reinforcer by itself to maintain instrument responding, rats (n=10) were retrained for 2 days on the two-choice task (*Laser+Cocaine* versus *Cocaine alone*), using the same dosages of cocaine that were used in the initial ten-day training session. After the two days, Cocaine was discontinued from both instruments for the following test days. During the cocaine extinction tests, nose pokes and lever presses still earned their associated auditory cue and 8s laser stimulation for the appropriate *Laser+Cocaine* nose port or lever, but neither instrument delivered cocaine infusions. Essentially, rats underwent an identical procedure as the ED training period, but with the exclusion of cocaine infusions. In a separate group of rats (n=4), extinction tests also included extinction of laser stimulation, only delivering associated auditory cues, as a means of comparing rates of extinction between the laser and non-laser delivering groups.

Anatomical localization: Sites, virus expression, & Fos plume maps.

Following the completion of behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital (150-200mg/kg) and transcardially perfused. Following the

perfusion, the brains were immediately stored in 4% paraformaldehyde, transferred to 30% sucrose solution the following day, and sliced at 40µm in coronal sections for identification of optic fiber placements and virus expression. Sections were mounted, air dried, and cover slipped with ProLong Gold anti-fade reagent (Invitrogen, Carlsbad, CA).

Optic fiber sites and GFP virus expression was measured using images taken with a Leica microscope at 10x magnification. Using MCID Core 7 software (3 X 3; 10X magnification), multiple images were taken (~324) for a signal brain and compiled into one single image to map fiber placement. Infected cells were labeled with Green Florescent Protein (GFP) in both ChR2 and control inactive eYFP rats. This allowed for visualization of viral spread around the optic fiber tip. Fiber tip location was marked in Adobe Illustrator on a rat brain atlas (Paxinos & Watson, 2007).

Statistical Analyses

Results were analyzed using IBM SPSS Statistics software. Repeated-measures ANOVAs were used to examine responses and infusions for either port between groups for both the ED training and UD experimentation. Post hoc Bonferroni corrections were used for individual comparisons of *Laser+Cocaine* responses, *Cocaine alone* responses, and infusion amounts between groups on specific days of training. Furthermore, comparisons within groups, such as *Cocaine alone* vs. *Laser+Cocaine* responses, were done using paired *t*-test with Bonferroni corrections. For all analyses, the significance level was set at $p < 0.05$, two-tailed.

Results

CeA stimulation focuses pursuit of a particular cocaine reward

Histological analysis of GFP expression revealed zones of ChR2 infection, often filling most of the CeA. Virus expression and localization confirmed that the CeA target was successfully infected with AAV ChR2 virus (Figure 1).

During the 10 days of the Equal Dose (ED) two choice instrumental task, rats could choose between nose-poking/lever pressing at one port that delivered an intravenous infusion of cocaine (*Cocaine alone*) or an identical cocaine infusion also paired with CeA ChR2 laser illumination (*Laser+Cocaine*) (Figure 2). Laser pairing made ChR2 rats prefer their *Laser+Cocaine* option over the *Cocaine alone* option (Reponses: $F_{(1,9)}$= 1.678, p=.020) (Infusions: $F_{(1,9)}$= 1.678, p=.012; Figure 3A). ChR2 rats preferred the *Laser+Cocaine* port by ~x2 times by day 2 and this preference became significant by day 6 of training, ($t_{(8)}$= 1.678, p=.049, 95% CI [0.15, 34.9]), and remained for the final four days, where on day 10, rats preferred the *Laser+Cocaine* port 19-fold. By day 10 of training, CeA ChR2 rats were responding ~35 times at the *Laser+Cocaine* port compared to ~15 responses by control virus rats at the same port ($t_{(9)}$= 1.895, p=.091, 95% CI [-4.9, 55.2]), however this difference was not statistically significant different possibly due to low number of control virus rats (n=2).

CeA ChR2-pairing also resulted in rats earning more total cocaine infusions (*Laser+Cocaine* plus *Cocaine alone* infusions) compared to control virus rats by day 10 ($t_{(9)}$= -2.651, p=.026, 95% CI [-37.2, -2.9]). In conjunction with earning more infusions, ChR2 rats earned significantly more overall cocaine (mg/kg) than control rats on the last day of ED training

(ChR2: 14.16mg/kg/session; Control: 6.90mg/kg/session; $t_{(9)}$= -2.430, p=0.038, 95% CI [-14.03, -0.5]; Figure 3B).

Within the group of CeA ChR2 rats, rats trained with different doses of cocaine per infusion (either 0.3, 0.4, 0.5, and 0.6mg/kg/infusion. Each rat always earned the same infusion, but infusion dose was randomly assigned between rats. All rats, regardless of dose, preferred the *Laser+Cocaine* port over the *Cocaine alone*, as there was no significant effect of dose on *Laser+Cocaine* responses ($F_{(3,9)}$= 1.091, p=.389) or infusions ($F_{(3,9)}$= 1.028, p=.457; Figure 3C). However, rats receiving the highest dose of cocaine (0.6 mg/kg/infusion) earned roughly half the amount of overall infusions (*Laser+Cocaine* and *Cocaine alone*) than rats earning each of the other three doses, 0.3mg/kg/infusion (p=0.001, 95% CI [-30.439, -13.561]), 0.4 (p=0.001, 95% CI [-32.244, -13.756]), and 0.5 (p=0.001, 95% CI [-32.244, -13.7560]) on last day of training.

Placements of optic fiber tips were mapped on a rat brain atlas and labeled according to rats' *Laser+Cocaine* preference on the final day of ED training (% laser preference for the *Laser+Cocaine* port over the *Cocaine alone* port; Figure 4). Optic fiber tips were distributed evenly between the dorsal and ventral regions of the CeA. Additionally, most optic fiber placements fell towards the lateral edge of the CeA, bordering regions of the BLA. Localization of optic fibers confirmed successful placement in the CeA.

CeA ChR2 stimulation makes a lower cocaine dose preferred

After completing all 10 days of ED training, rats progressed to the Unequal Dose (UD) procedure, which was identical to the ED training, except now the *Laser+Cocaine* port delivered half the amount of cocaine per infusion as compared to the *Cocaine alone* port (Figure 5). Despite the greater amount of cocaine offered at the *Cocaine alone* port, CeA ChR2 rats

perseverated at the *Laser+Cocaine* port, preferring that over the *Cocaine alone* port (Reponses: $F_{(1,9)}$= 16.59, p=.004; Infusions: $F_{(1,9)}$= 17.817, p=.003; Figure 6A). The preference for *Laser+Cocaine* continued for all 5 days of the UD program, and by the last day they still preferred responded more at the *Laser+Cocaine* port (Day 5: $t_{(8)}$= 4.414, p=.002, 95% CI [14.061, 44.828]). Similar to ED training, preference for *Laser+Cocaine* vs. *Cocaine alone* did not differ as a result of the different doses per infusion between rats (Reponses: $F_{(3,9)}$= 0.466, p=.0.912; Infusions: $F_{(3,9)}$= 0.321, p=.976). While there was no significant difference in preference for either port between ChR2 rats and control rats during ED training, this was not the case for the UD program. By the final day of this training, ChR2 rats were responding at higher levels at the *Laser+Cocaine* port than control virus rats ($t_{(9)}$= -3.166, p=.011, 95% CI [-17.5,-2.9]), resulting in CeA ChR2 rats earning more total infusions than control rats ($t_{(9)}$= -2.663, p=0.026, 95% CI [-34.6, -2.8]). However, because majority of their infusions were earned via the *Laser+Cocaine* (lower dose) port, they actually earned the same amount of total cocaine (mg/kg) as control virus rats ($t_{(9)}$= -1.379, p=0.201, 95% CI [-4.8, 1.17]; Figure 6B). In fact, ChR2 rats earned significantly less cocaine during the UD program, than what they originally earned during ED training ($t_{(8)}$= 2.339, p=0.047, 95% CI [0.05, 7.4]). This was a result of ChR2 rats perseverating preference for the *Laser+Cocaine* port even when it was the lower dose.

While CeA ChR2 rats stayed responding at the *Laser+Cocaine* port (even though it now offered less cocaine), control virus rats switched over to the port that delivered more cocaine, and by end of the UD paradigm, were responding at the *Cocaine alone* (higher cocaine dose) port (~14 responses) more than their *Laser+Cocaine* port (~3 responses), and at higher levels than ChR2 rats at the same port ($t_{(9)}$= 2.449, p=0.037, 95% CI [-2.3, 58.1]).

While the number of total infusions differed, the amount of time to receive the infusions (total session duration) was the same for all rats, control and ChR2, during ED training ($T_{(9)}$= 1.274, p=0.235, 95% CI [-12.4, 44.7]). However, by the last day of the UD program, the ChR2 rats completed their sessions in significantly less time than the control rats ($T_{(9)}$= 2.361, p=0.043, 95% CI [1.36, 63.9]). This could be explained by the enhanced learned incentive salience as a result of more CeA activation by the fifteenth day of CeA laser stimulation. What dosage the rats receive also had an impact on total infusions. Rat who received the highest dose of cocaine (0.6 mg/kg) also had the lowest number of total infusions during both ED training (Reponses: $F_{(3,9)}$= 55.154, p=001) and the UD program (Reponses: $F_{(3,9)}$= 58.329, p=0.001; Figure 6C).

Conditioned Reinforcements

Following the end of ED training and before the start of the UD program, rats underwent testing for conditioned reinforcement. This test was used to determine if rats would perform a new task for 4s presentation of a cue associated with *Laser+Cocaine* outcome as compared to a cue associated with the *Cocaine alone* outcome (port was presented into chamber accompanied by associated auditory cue). Conditioned reinforcement was defined as active-inactive port responses. Nose pokes at both ports once they were presented were also recorded. For all rats, the mean levels of condition reinforcement (act-inact) were greater for the laser cue compared to the non-laser cue, however, this difference was not significant (Laser cue:~3.125; Non-Laser Cue:~1.125; $t_{(7)}$= 1.378, p=0.211, 95% CI [-1.4,5.4]). After working for the cue itself, the port responses in the same manner as they trained during ED training. The rat's response did not earn them any cocaine this time. During this response period, there was no difference in the total number of responses during cue presentation between the *Laser+Cocaine* and *Cocaine alone* cue ($t_{(7)}$= 0.557, p=0.595, 95% CI [-2.4, 3.9]).

CeA ChR2 stimulation alone does not maintain responding

Following completion of the UD program, rats were re-trained at the ED paradigm for one day, and doses of cocaine were identical (just like the initial tests). Starting day 2, cocaine was extinguished and nose pokes into either port no longer earned cocaine infusions. In one group of rats (n=7), rats could still earn Laser stimulation at the *Laser+Cocaine* port, rendering the options as *Laser alone* vs. *Nothing*. In a separate group (n=4), starting on day 2 rats earned no cocaine or laser at either port, rendering the options *Nothing* vs. *Nothing*. Within the group of rats that had only cocaine removed, there was a significant decrease in responses at the *Laser+Cocaine* port between the first day of extinction and day four ($t_{(7)}$= 2.546, p=0.038, 95% CI [1.4,38.57]), suggesting that laser stimulation alone does not maintain responding (Figure 7). On the fifth day, laser stimulation was removed for all rats. Between the fourth day with laser and the fifth day without laser, rats maintained the normal decline in responses at the *Laser+Cocaine* port (Day 4 w/ Laser Responses: ~17); (Day 5 w/o Laser Responses: ~13); ($t_{(7)}$= 1.507, p=0.175, 95% CI -2.0, 9.3]), suggesting that when laser is removed, rate of extinction is not altered. For all ChR2 rats, regardless of being in the group that lacked laser or both cocaine and laser, there was a significant decrease in overall responses as the extinction session progressed ($F_{(1,7)}$= 6.436, p=0.003). Furthermore, both groups decrease overall responding at similar rates ($F_{(1,3)}$= 2.176, p=0.126), suggesting that laser stimulation was not adding any extra reinforcing properties which would have maintained higher levels of responding.

Discussion

Central amygdala stimulation focuses cocaine pursuit

Our results demonstrate that pairing CeA stimulation with a cocaine infusion intensified rats' motivation to earn that specific cocaine option. The preference for the *Laser+Cocaine* instrument was so intense that an alternative and equal option for *Cocaine alone*, without laser stimulation, was almost completely ignored. By day ten of Equal Dose (ED) training, almost all responses for cocaine infusions were exclusive to the *Laser+Cocaine* option, which has also been observed in previous experimentation within the Berridge lab (Warlow, Robinson, & Berridge, 2017). This focused preference for a CeA paired reward over another identical reward suggests that CeA activation amplifies the perceived value of its paired reward, making the reward substantially more favorable. Indeed, even when rats could earn more cocaine by switching to the *Cocaine alone* option (when doses were made unequal), they persisted responding at the *Laser+Cocaine* option, earning less cocaine as a result. Furthermore, CeA stimulation caused rats to consume over 2.5 times more cocaine than control rats. The increased focus and increased overall cocaine intake is the result of providing CeA stimulation in tandem with administration of the reward, not just overall enhanced CeA activity in the brain. Considering the CeA's role in associative learning, we speculate that CeA stimulation and receipt of the reward must be in close temporal proximity (Baxter, 2002). Otherwise, the preference and increased response for *Laser+Cocaine* infusions would not have been observed.

Amplified CeA Activity Promotes Irrational Preference

After choosing a CeA laser-paired cocaine over and above an identical dose of cocaine, rats continued to pursue the *Laser+Cocaine* option, even when it delivered half the amount of

cocaine as compared to the *Cocaine alone* option. Previous research supports that rats will self-administer and demonstrate a preference for higher dosages of cocaine over a lower dosage alternative, but this was not the case when rats received CeA stimulation (Picetti et al., 2010). The intense ChR2 rats' preference for the *Laser+Cocaine* option persisted during the Unequal Dose (UD) paradigm despite the quantitative superiority of the *Cocaine alone* option. Interestingly, this preference was just as strong as the preference demonstrated during ED training; rats appeared to be unfazed by the discrepancy in the amount of cocaine offered as compared to their original infusions during ED training. This appears to not be due to pure persistence at the cocaine port, because response levels were higher than when cocaine was completely removed during cocaine extinction.

One possible explanation for the maintained preference for the *Laser+Cocaine* option could be that the response for the *Laser+Cocaine* option was simply a perseveration from the many days of training. That is, because ChR2 rats learned to focus their attention onto the *Laser+Cocaine* reward, by the time new experimentation with different dose options began, they simply repeated their learned behavior to prefer that option rather than switching their preference to the higher cocaine option. However, this is unlikely because in previous experiments in other labs, when rats were given the option to self-administer and select between low and high doses of cocaine, 95% of the rats preferred the higher dose option. This behavior was replicated and observed over multiple phases and dose combinations. In their experiment, once rats demonstrated a clear preference for the higher dose option, they progressed to a new phase of experimentation; increasing dosage from the previous phase's pairing. Rats learned which instrument provided the most cocaine and developed a new preference for that instrument, demonstrating that even after many training days, rats were flexible in their cocaine choice and

did not irrationally persevere (Picetti et al, 2010). If the options are made unequal (e.g. unequal dosages of cocaine) rats will learn to work for the quantitatively superior option. This was also demonstrated in our control rats here, who unlike the ChR2 rats, developed a strong preference for the higher cocaine dose.

In a sense, CeA ChR2 paired with cocaine caused rats to irrationally choose that paired option, even if they earned less overall cocaine (and worked even harder by responding more). It is unlikely, however, that rats did not have enough experience with new options to make a rational choice. In the beginning of each session for example, rats were required to sample both cocaine dose options at least twice before they had free section between the two. Due to this experimental design, we infer that ChR2 rats did not simply ignore the larger cocaine option because they never had the opportunity to try it. All rats did try both options, but only control rats demonstrated the rational selection of the higher dose option. Another possibility is that CeA ChR2 stimulation may have suppressed new learning of the different doses available to rats. Indeed, central amygdala's inhibitory output may have the ability to suppress learning (Reppucci & Petrovich, 2016). However, if learning was suppressed through CeA activation, responses would have stayed high during extinction when cocaine was removed but CeA laser remained. In fact, we observed decreased responding when cocaine was completely removed; indicating that CeA activation likely did not interrupt new learning of the available options.

CeA rather, through associative pairing, made the lower dose of cocaine a more attractive option than its alternative by transforming its value to be far and above the alternative. This likely formed an irrational preference for the lesser cocaine amount. Irrational preference for drugs at levels exceeding motivation for normal life rewards is a feature commonly observed in addiction, and CeA mechanisms may underlie this irrationality.

Complete Inversion of Unequal Reward Preference

Previous research supports that CeA stimulation makes unequal rewards equally preferred (Robinson, Warlow, & Berridge, 2014). For example, under normal conditions, rats prefer to earn a sucrose reward over salt reward. However, when the salt reward was paired with ChR2 CeA stimulation, the preference for the two rewards became equal. That is, the preference for two unequal rewards (sucrose and salt) changed as a result of ChR2 stimulation so that they were then equally desired. Rats in our experiment were also required to decide between two unequal rewards. However, unlike previous experiments, the reward options in our current experiment were qualitatively similar (both cocaine) but quantitatively unequal (different dosages). Here, when rewards were the same type but differed only by the amounts provided, CeA stimulation did not make the rewards equally preferred, but rather, the preference was inverted. The reward option paired with CeA stimulation becomes *the* preferred option, regardless of its innate quantitative value (i.e. amount of cocaine). It appears that if the two rewards are of the same type but delivered at varying amount, rats do not balance their preference between the two; the quantity of the reward is ignored because of CeA stimulation.

This observation should be tested further to ensure that the effect is not exclusive to cocaine options. There may be an interaction at the neurochemical level that prevents balancing reward preference. That is, since both cocaine options created the same endogenous neurochemical reaction in the brain, regardless of dose, the rewards appear to still be of equal value. It is uncertain if this inversion of preference would still occur for non-drug of abuse rewards (i.e. low amount of sucrose vs. high amount of sucrose) that lack this strong neurochemical effect like cocaine does.

CeA Stimulation Amplifies Cocaine Behavior Regardless of Dose

In our experiment, almost all ChR2 rats earned the maximum amount infusions per session regardless of the amount of the dose they received. The only deviation from this trend was within the 0.6mg/kg dosage group, of whom earned less infusions overall during both ED and UD training compared to other rats. Previous experiments suggest that large doses of cocaine are aversive to rats, resulting in avoidance of high dose cocaine infusions. However, this effect was only observed in doses above 2.5mg/kg, well above the maximum dose we offered our rats (0.6mg/kg; Mantsch, 2001). Here, the relatively low amount of infusions for the highest dose (0.6mg/kg) of cocaine offered may be due to the small amount of rats in this group, and future studies with more animals may help clarify this.

Apart from the 0.6mg/kg dose group, all other dose groups demonstrated statistically similar number of infusions during the session. Normally, there is an inverse relationship between self-administering behavior and cocaine dose; that is there is less self-administration as the dose of cocaine increases (Mantsch et al., 2001). On the contrary, in our experiment, rats self-administered cocaine the same number of times regardless of the dosages of cocaine as a result of CeA ChR2 stimulation. Unfortunately, these observations are limited because in our experiment, self-administration sessions ended after 40 total infusions were achieved or 60 minutes time. We cannot accurately predict what the total infusion amounts would look like without these restrictions. However, based off our observations, CeA stimulation caused rats to take the same number of total cocaine infusions for all doses, thus ignoring the normal effect that cocaine dose has on self-administering behavior. That is, because of CeA stimulation, the quantitative amount of each infusion was ignored. For an addict, if central amygdala circuitry were to become sensitized and hyper-reactive, the individual may continue to take a drug

consistently, regardless of the dose they are taking at each administration. This would lead to detrimental consequences, such as overdose, because the addict's motivation for cocaine is increased to a level beyond which is satisfied, just as our ChR2 rats demonstrated. This topic deserves further investigation with a larger sample size and longer session duration in order to determine if the phenomena of quantitative ignorance persists without strict session constraints.

ChR2 Laser Stimulation Amplifies Reward Value, but is not Inherently Rewarding

Laser stimulation of CeA paired with a particular cocaine reward intensely narrows pursuit for that reward over another, yet despite this intense effect, CeA ChR2 stimulation alone lacked any innate value. Previous studies have also support that CeA ChR2 stimulation alone is not rewarding. When provided the opportunity, rats did not self-stimulate for CeA ChR2 laser stimulation by itself and rats also did not demonstrate a place preference for locations that provided CeA ChR2 stimulation over locations that did not (Robinson, Warlow, & Berridge, 2014). In our current experiment, CeA stimulation alone failed to maintain the intense preference for the *Laser+Cocaine* instrument during the cocaine extinction procedure. That is, when cocaine was removed, but laser stimulation remained, response and preference for the *Laser+Cocaine* instrument declined when the instrument only provided laser. These results have been replicated in prior experiments, however to further investigate this phenomenon, a second extinction paradigm was used in our current experiment in which both laser stimulation and cocaine reward were removed. Now, responses at the original *Laser+Cocaine* port delivered nothing. Interestingly, the rate of decline for the *Laser+Cocaine* port was not statistically different between the two extinction groups. Responses at the original *Laser+Cocaine* instrument declined at similar rates despite if the instrument provided *Laser alone* or provided *Nothing*. This suggests that laser stimulation alone is virtually equal to receiving nothing; the

laser bears no inherent value. Thus, the increased preference for the CeA-paired cocaine was not due to an additive effect of laser reinforcement and cocaine. Rather, laser stimulation of the CeA uniquely transforms the represented value of its paired drug reward, making it the preferred reward.

Potential Roles of CeA in the Circuitry of Addiction

For an addicted individual, the increased desire to pursue a particular drug or reward can be so intense that it distracts the individual from other life rewards. Unfortunately, there are multiple regions of the brain that superintend the mechanism of addiction, creating a complicated challenge for addiction neuroscience. However, by individually isolating and examining the roles of different neuronal components of the addiction mechanism, such as the central amygdala, we can better understand their contributions and implications in addicted individuals. The current results demonstrate that optogenetic activation of CeA-related circuitry, when paired with a cocaine reward, causes intense focus and pursuit of that particular reward. This intense pursuit is strong enough to distract and prevent the acknowledgement of quantitatively superior rewards. The irrational behavior associated with CeA activation mimics the behavior seen in addicts; ignoring life's pleasures and responsibilities for that of taking drugs of abuse. Our findings continue to support that drugs of abuse, which hijack mesolimbic circuitry, can drastically affect the motivational behavior of an addict (Kelley & Berridge, 2002).

Sensitization of mesolimbic circuitry after consistent drug use can cause the CeA to become hyper-reactive, essentially replicating the effect of optogenetic stimulation of the CeA in a real-world scenario. Armed with the understanding that hyper-reactive responses of the CeA can make rewards more desirable, we can better focus our efforts in understanding addiction and

reduce addiction like behavior. Fundamentally, there are two potential solutions to addiction using the functions of the CeA; (1) aim to reduce the CeA's activity during experience with drugs of abuse (2) increase the effect of CeA on an alternative, normal life reward. Regarding the latter, the CeA's function could be utilized and directed towards healthier alternatives to distract an addict from the drug. For example, taking a rat addicted to cocaine, exposing them to a 'healthier' alternative such as sucrose, and selectively pairing CeA activation with the sucrose. This pairing may increase the value of the alternative sucrose reward, but may not reduce the value of original addicted substance; essentially generating a new separate addiction. Therefore, efforts in aiding addiction by use of the CeA should be focused on decreasing its association with drugs of abuse, rather than increasing its association with alternatives.

Recommendations for Future Investigation

Our current experiment has confirmed the results found in previous investigations and expanded the understanding of its effects in terms of relevant cocaine doses, and when making doses unequal. Further experimentation deserves to be conducted to refine and confirm our results. To begin, it would be worthwhile to replicate our experiment initially training the rats using the UD paradigm instead of the ED paradigm. By doing so, rats would be naïve when exposed to both the low and high cocaine dose options instead of already being familiar with the effects of cocaine. This would allow for a more genuine selection between the low (*Laser+Cocaine*) and high (*Cocaine alone*) options. Obviously, our current results would become stronger with a larger sample population, especially in regards to investigating effects of varying doses. It is not well understood if or how the dose of cocaine significantly impacts the value enhancing effects of the CeA, and knowing so would potentially reveal the extent and capacity of CeA stimulation. Extremely high doses of cocaine may be unaffected by CeA

stimulation and the same could be said about extremely low doses of cocaine. In other words, investigation into the upper and lower limits of the CeA effect would provide insight as to how large or small the reward can become, essentially revealing the ‘saturation point’ and limits of CeA value amplification.

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Figures

Figure 1: Histological Analysis of GFP Expression

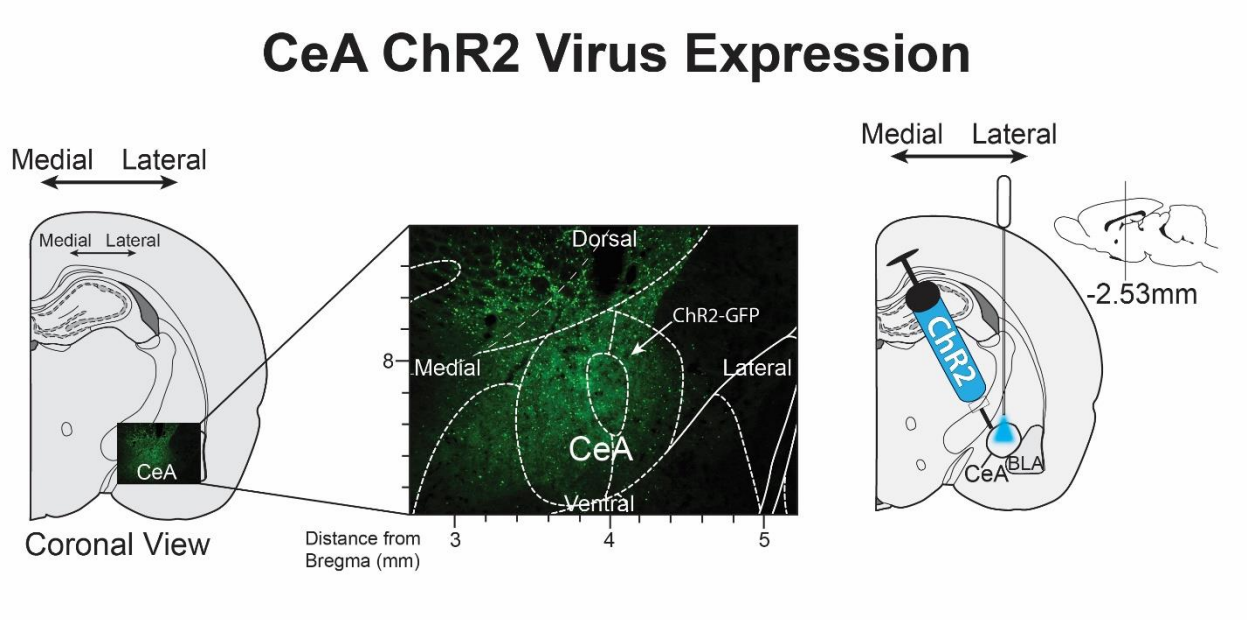

Figure 1: Histological analysis of GFP expression revealing areas of ChR2 infection. Brain regions infected with virus display florescent proteins as green plumes. GFP fills the CeA indicating successful viral infusion of target area.

Figure 2: Equal Dose Training

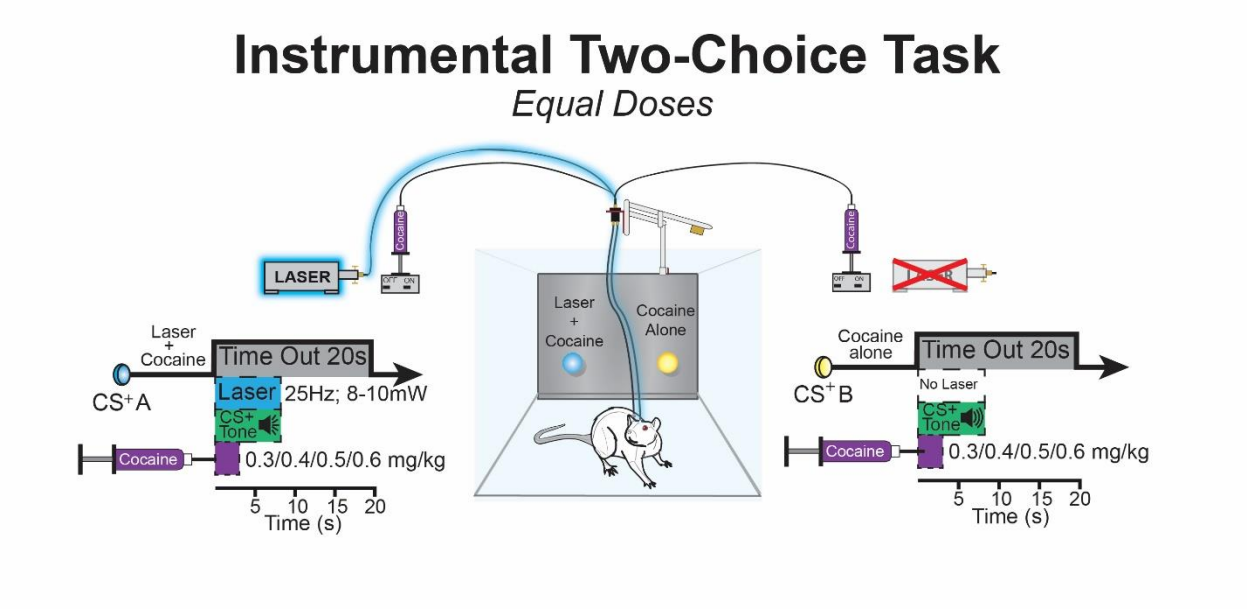

Figure 2: Visualization of the instrumental two-choice task paradigm with equal doses. Rats were trained to instrumentally earn intravenous cocaine infusions by performing a task on one of two instrument types: levers or portholes. Both options provided an intravenous infusion of cocaine, however, only one option also delivered optogenetic CeA stimulation via laser. Both the *Laser+Cocaine* and *Cocaine alone* options delivered an equal amount of cocaine per infusion infused over 2.8 seconds.

Figure 3: Equal Dose Training Results

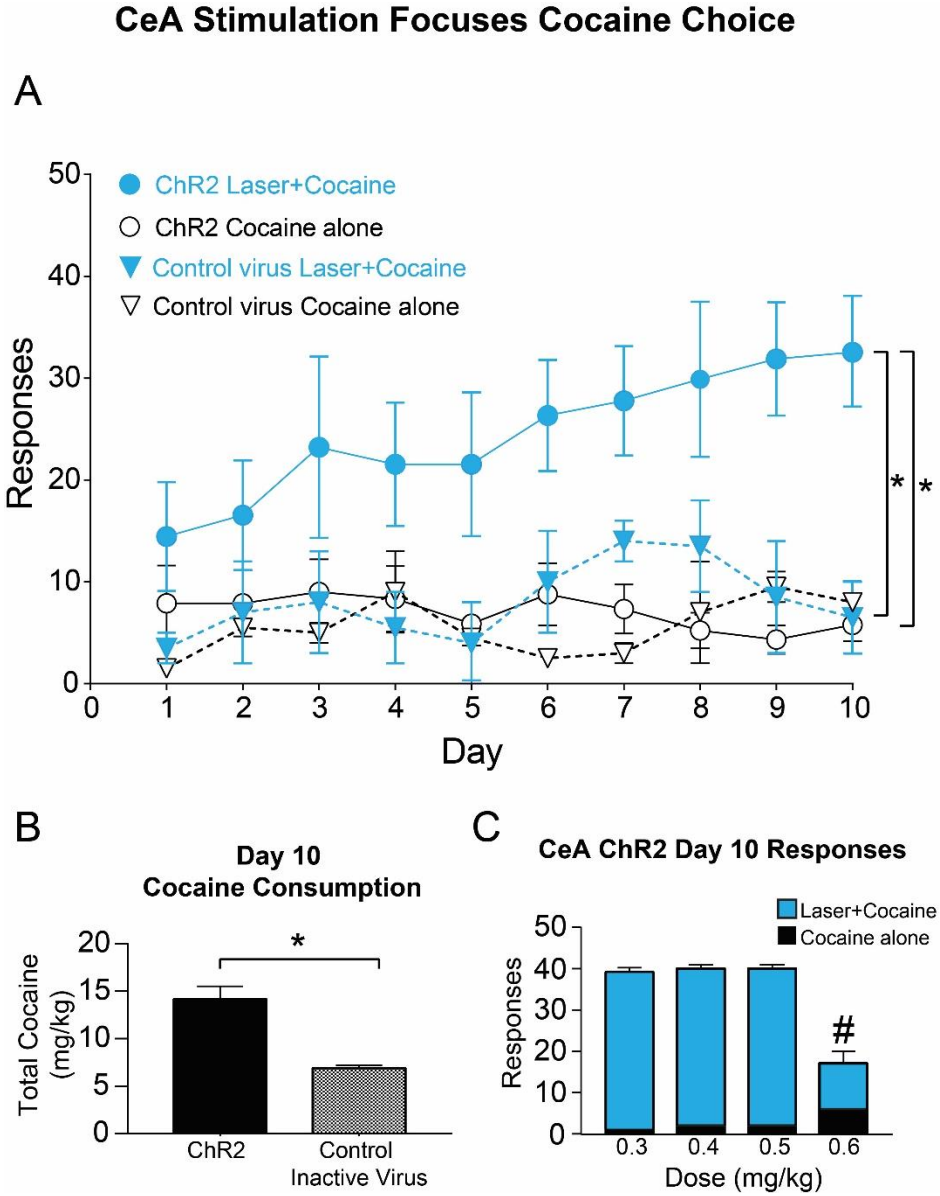

Figure 3: Optogenetic stimulation of the central amygdala focuses choice of laser paired cocaine reward. (***A***) During Equal Dose training, *Laser+Cocaine* responses (solid blue lines) significantly increased over the 10 days of training for CeA ChR2 rats (circles) as compared to the *Cocaine alone* (black solid lines) option, as well as compared to *Laser+Cocaine* responses by Control virus rats (triangles; *Laser+Cocaine*: blue dashed lines *Cocaine alone*: black dashed lines). (***B***) The total amount of cocaine infused on the last day of Equal Dose training (mg/kg) was significantly higher for ChR2 rats (black bar) compared to control inactive virus rats (checkered bar). (***C***) On the final day of Equal Dose training, total responses (*Laser+Cocaine* plus Cocaine alone) were similar for all doses except the 0.6 mg/kg dose. For all doses, there was a greater preference for the *Laser+Cocaine* option (blue bar) as compared to the *Cocaine alone* option (black bar). (# = posthoc comparison 0.6 vs. 0.3/0.4/0.5: $p < 0.01$; * $p < 0.05$; ** $p < 0.01$)

Figure 4: Localization of Function Map

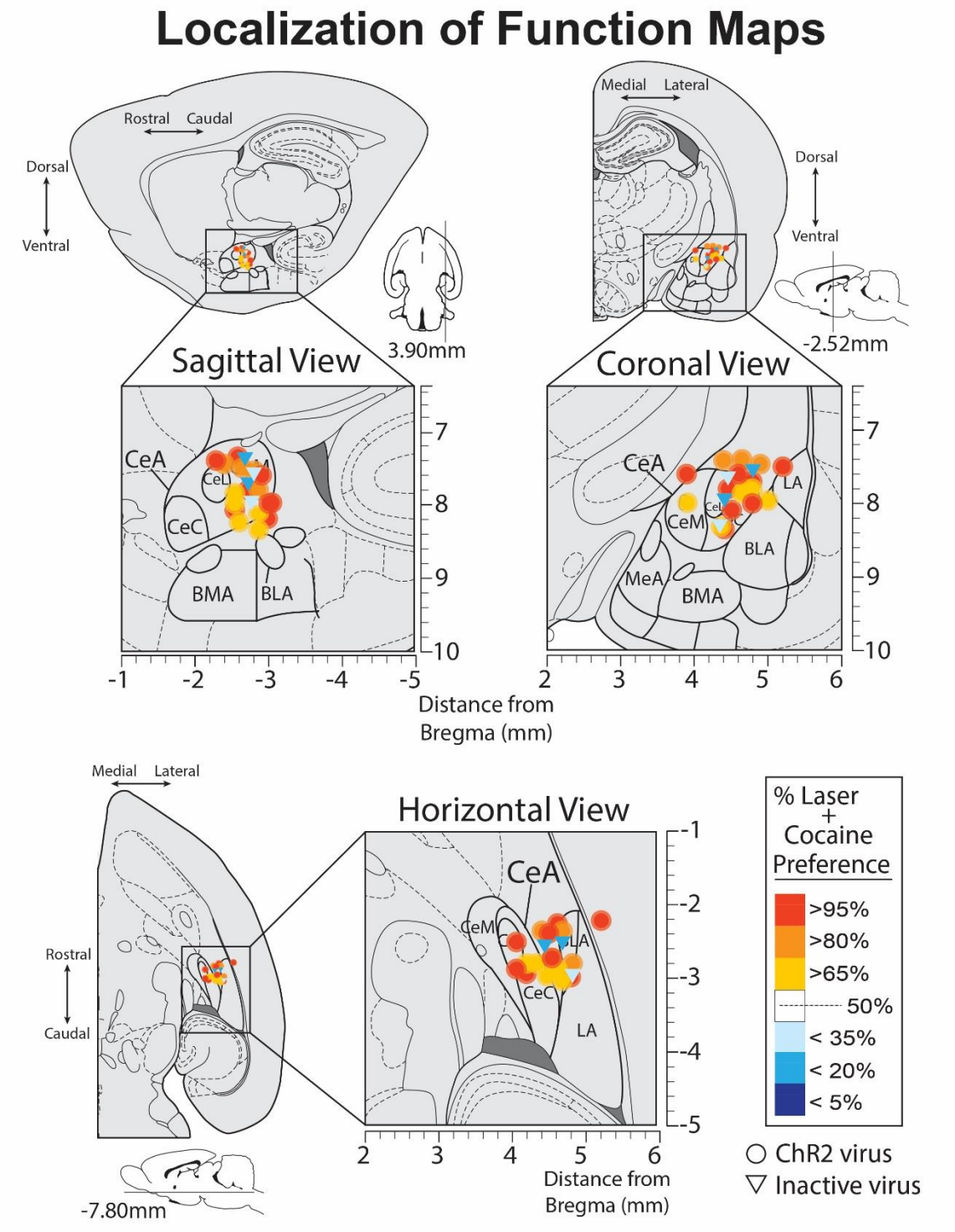

Figure 4: Localization of function maps for incentive enhancement. Maps show sites in CeA for ChR2 (circle) and control (triangle) rats' fiber optic placements. Color of each symbol in map depicts the behavioral consequence of ChR2 laser stimulation at that site during Equal Dose training (% laser preference for the *Laser+Cocaine* port over the *Cocaine alone* port).

Figure 5: Unequal Dose Paradigm

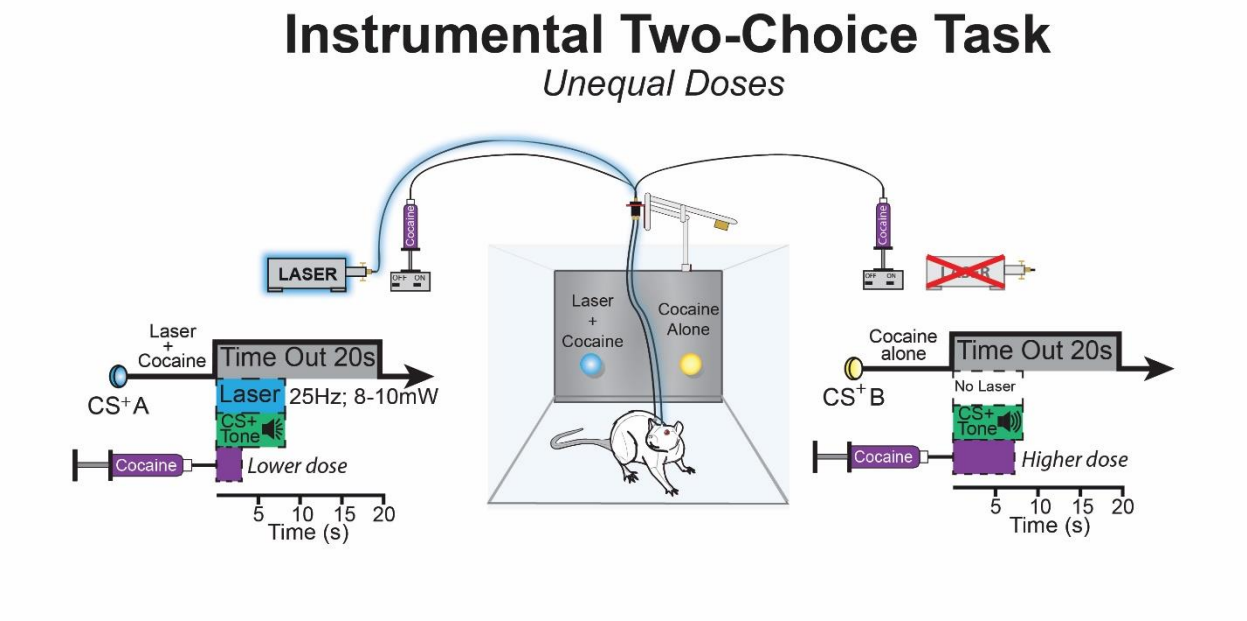

Figure 5: Visualization of the instrumental two-choice task paradigm with unequal doses. Following Equal Dose training, rats progressed to the Unequal Dose paradigm. Similar to the Equal Dose training, rats were able to self-administer cocaine through two options, one cocaine option was paired with CeA ChR2 laser stimulation (*Laser+Cocaine)* while the other option delivered *Cocaine alone*. The *Laser+Cocaine* option delivered a lower dose of cocaine compared to the *Cocaine alone* option, which delivered a higher dose by infusing cocaine over a longer duration of time.

Figure 6: Unequal Dose Paradigm Results

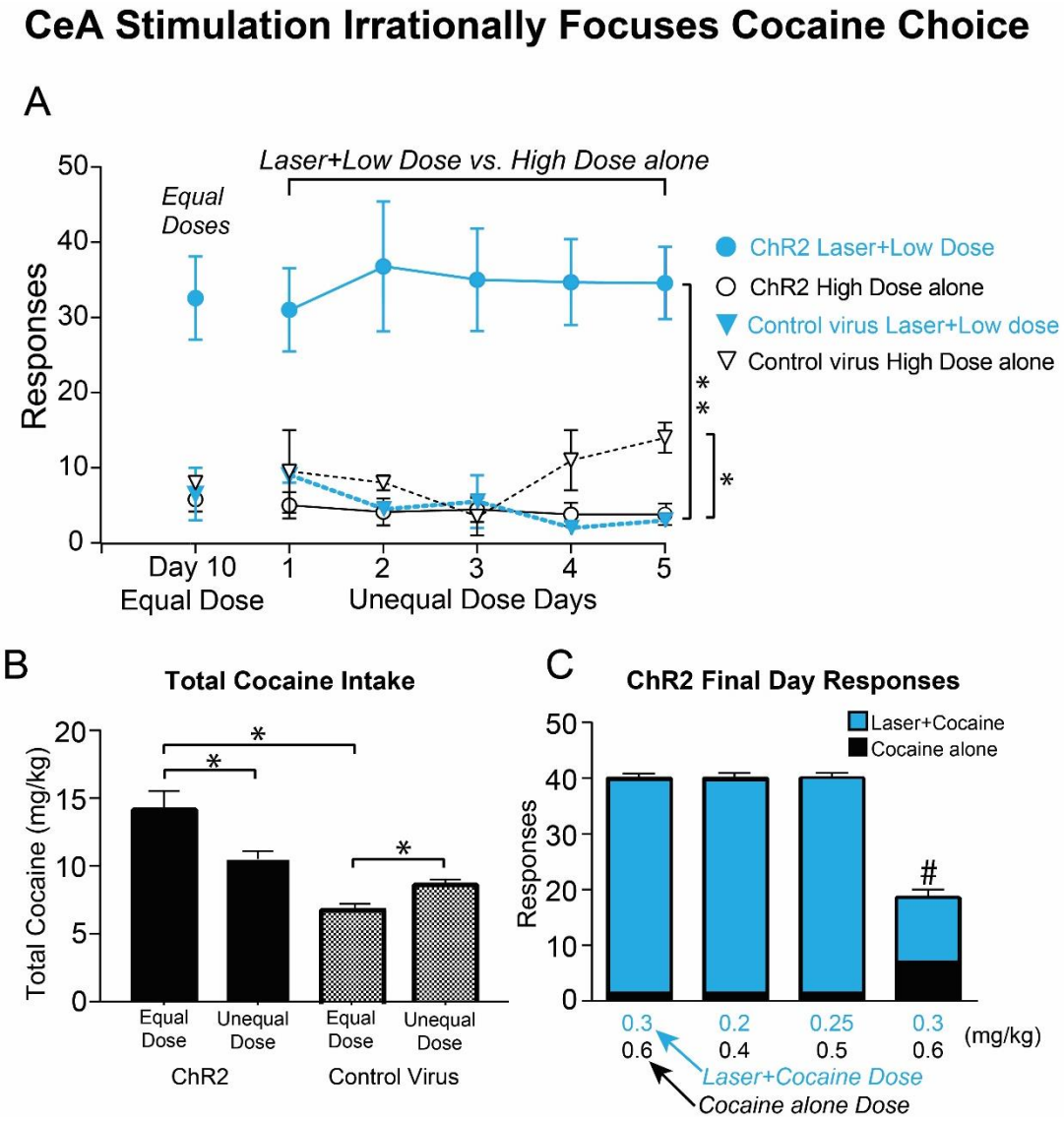

Figure 6: Optogenetic stimulation of the central amygdala continued to focus cocaine preference. (***A***) ChR2 rats' (circles) intense preference for the *Laser+Cocaine* reward (blue lines) continued through all five days of the Unequal Dose paradigm. Control virus rats demonstrated a significant preference for the quantitatively superior *Cocaine alone* option (open faced triangle). (***B***) The total amount of cocaine infused on the last day of Equal Dose training (mg/kg) was significantly higher for ChR2 rats (black bar) compared to control rats (checkered bar). However, from the equal Dose Training to the Unequal Dose paradigm, total cocaine intake decreased for ChR2 rats and increased for control rats. (***C***) On the final day of the Unequal Dose paradigm, rats performed similar amounts of self-administering responses for all doses excluding the 0.6 mg/kg dose, of which there was less. For all doses, there was a greater preference for the *Laser+Cocaine* option (blue bar) as compared to the *Cocaine alone* option (black bar). (# = posthoc comparison 0.6 vs. 0.3/0.4/0.5 $p < 0.01$; * $p < 0.05$; ** $p < 0.01$)

Figure 7: Extinction Paradigm Results

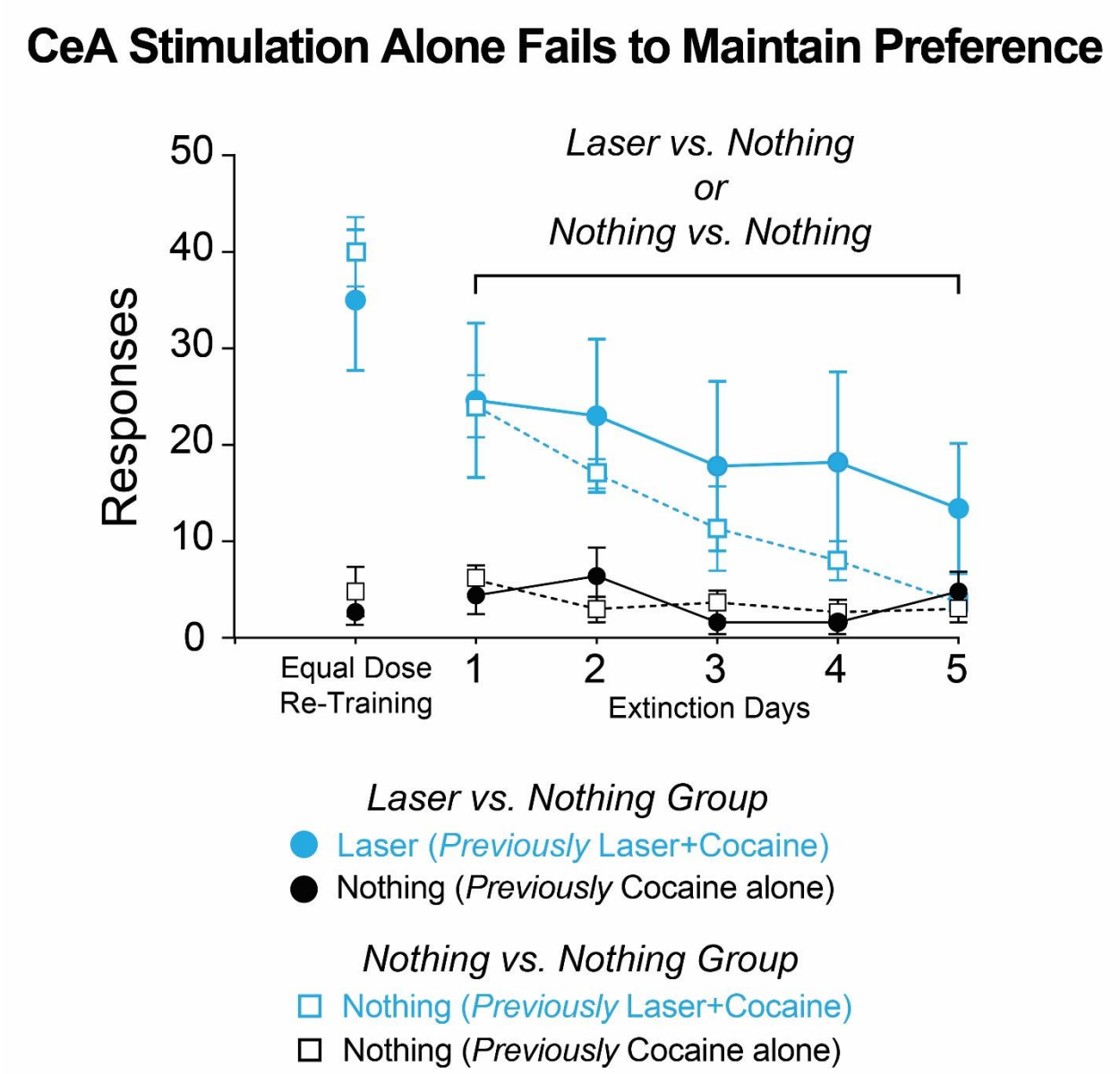

Figure 7: At the end of experimentation, rats underwent the extension paradigm; identical to the Equal Dose training except a variable (laser or cocaine) was removed. For one group of rats, only cocaine was removed (circles) and for the other group, cocaine and laser was removed (squares). Rats' responses at the *Laser+Cocaine* port (blue symbols) decreased during the extinction paradigm for both groups at a statistically similar rate. Responses at the *Cocaine alone* port (black symbols) remained statistically similar.