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LATERAL SEPTUM AND LATERAL HYPOTHALAMUS OREXIN NEURONS MEDIATE MOTIVATION FOR COCAINE THROUGH INTERACTIONS WITH THE MESOLIMBIC DOPAMINE SYSTEM

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ABSTRACT OF THE DISSERTATION

Lateral septum and lateral hypothalamus orexin neurons mediate motivation for cocaine through interactions with the mesolimbic dopamine system

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Drug addiction is a disorder characterized by pathological motivation for drug. Our laboratory has recently developed a behavioral economics (BE) paradigm that uniquely assesses demand for drugs of abuse. Animals' individual lever-pressing data can be fitted to demand curves to determine two important measures of cocaine demand: baseline consumption (Q0) and motivation (α). As α is the slope of the demand curve, it inversely scales with motivation, such that animals with greater demand elasticity (α) have lower motivation for cocaine. Here, we utilized our BE paradigm with pharmacological, morpholino antisense, and retrograde tracing approaches and determined that lateral septum (LS) and lateral hypothalamus (LH) orexin neurons are important neuronal populations for cocaine demand elasticity (α).

We demonstrate that inhibiting LS reduced motivation (increased demand elasticity; α) and that the benzodiazepine diazepam blocked this effect, pointing to opposing roles of the two manipulations on ventral tegmental area (VTA) dopamine signaling. The two manipulations were similarly found to be anxiolytic, indicating that changes in drug taking occurred independently of effects on anxiety. Similarly, we show that orexin signaling contributes to motivation for cocaine (α) and that the

involvement of orexin in motivated drug taking is mediated primarily by orexin neurons in LH. Animals sacrificed after BE had greater expression of orexin neurons in LH, and unilateral knockdown of LH orexin cells attenuated motivation during BE. The number of spared LH orexin neurons predicted α value in antisense-infused animals and in animals sacrificed two weeks after BE testing. We then blocked orexin-1 receptor signaling in VTA using the oreceptor antagonist SB-334867 (SB) and found that intra-VTA SB reduced motivation. The effects of SB specifically occurred in animals trained and tested on BE with cues and removing cocaine-paired cues reduced SB efficacy. Finally, using retrograde tract tracing, we observed that a greater proportion of LH orexin neurons project to VTA than do other subregions of the orexin cell field.

Collectively, these studies implicate two important neuronal populations in motivation for cocaine and indicate that both LS and LH orexin connections to the mesolimbic dopamine system mediate their effects on motivation.

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Table of Contents

List of Figures

Chapter 4: Orexin-1 receptor signaling mediates cue-associated cocaine demand

Chapter 5: Conclusions & Figure Directions

Chapter 1: Introduction

Addiction is a debilitating and chronic disorder with a high rate of relapse. The cost of addiction in the United States is about $740 billion annually, of which $193 billion is due to abuse of illicit drugs like marijuana and cocaine (NIDA, 2017). This financial cost includes loss of work productivity, healthcare, and crime attributed to drug-related offenses. According to the DSM-V, an individual can be diagnosed with a substance abuse disorder if he/she has impaired control over the drug used, social impairments due to substance use like neglect of societal responsibilities, risky use of the substance even with knowledge of health consequences, and pharmacological criteria like tolerance and withdrawal symptoms with chronic use (Association, 2013). A 2009 estimate from the Drug Abuse Warning Network determined that abuse of cocaine was the most common cause of emergency room visits due to illicit drugs, with roughly 420,000 of 1 million emergency room visits attributed to cocaine abuse (Substance Abuse and Mental Health Services Administration, 2010). About 2 million Americans aged 12 or older chronically use cocaine, which is about 0.7% of the population (Ahrnsbrak R, 2017).

Cocaine elevates heart rate, respiration, and blood pressure, and produces feelings of euphoria with initial use. Peak plasma concentrations of the drug occur 15-60 minutes after use (Van Dyke, Barash, Jatlow, & Byck, 1976). As brain cocaine concentrations decline, cocaine users will experience feelings of depression, anxiety, and exhaustion. Over time, cocaine users often progress to uncontrolled drug use, consisting of cycles of binging and abstinence (Ahmed &

Koob, 1998; Gawin, 1991). Unlike opioids or alcohol, cocaine does not produce physiological dependence, but rather a psychological need to pursue the drug during withdrawal (Gawin, 1991). In addition, therapies developed for the treatment of alcohol or opioid abuse are not as effective in cocaine addicts (Ling et al., 2016; Schottenfeld et al., 2014).

1.1. Challenges in developing treatments for cocaine addiction

There are currently no FDA-approved medications for the treatment of cocaine addiction. Several medications have shown promise in recent years, including oxytocin, baclofen, disulfiram, topiramate, and modafinil (Chiamulera, Padovani, & Corsi, 2017; Haile & Kosten, 2013). However, only certain individuals respond to these treatments, and this disparity may be due to genetic and/or environmental differences amongst patients (Badiani, Berridge, Heilig, Nutt, & Robinson, 2018). Consequently, the development of a panacea for psychostimulant addiction has been largely ineffective, and personalized medicine may be a more effective approach to treat the disorder. Another emerging strategy has been to use procedures like transcranial magnetic and deep brain stimulation to directly excite brain regions that may be hypofunctioning in addicted individuals (Bolloni, Badas, Corona, & Diana, 2018; Rachid, 2018). While several of these studies have yielded promising results, sample sizes have been limited to only a few patients. In addition, these procedures can be highly invasive to patients. As a result, most recovering addicts must turn to behavioral interventions like cognitive behavioral therapy, contingency management, motivational incentives to encourage abstinence, and 12-step programs.

Treatment options established in animal models of self-administration often fail when given to human addicts. Addiction is a multifaceted disorder with numerous symptoms. While sophisticated animal models have been developed to mirror drug seeking/taking in humans, these models are often limited to targeting one addiction endophenotype, such as propensity for relapse (Fernando & Robbins, 2011). Treatments developed in drug seeking paradigms, which measure operant responding in the drug-paired context in the absence of drug, may also not be effective in drug taking paradigms, and vice versa (Pilla et al., 1999; Roberts, Gabriele, & Zimmer, 2013). Thus, an ideal treatment for addiction must be effective across multiple endophenotypes. Another challenge for treatment is that addiction is a chronic disorder, and addicts can be dependent on the abused substance for many years before seeking treatment. In contrast, most models of addiction expose animals to drugs of abuse for only several weeks. Therefore, even if treatments can be proven effective in multiple animal models, the changes occurring in animals displaying addiction-like behaviors may not fully recapitulate the behavioral and neurobiological alterations that result from chronic drug use in human addicts.

The identification of addiction biomarkers, such as genetic factors or behavioral traits, that indicate propensity could aid in the treatment and prevention of the disorder. An established biomarker would permit more accurate modeling in animals and rapid screening of treatments in human patients. However, there is a considerable lack of established biomarkers of addiction susceptibility, which forces researchers to focus on the suitability of each animal model utilized and the

potential for translation across animal models and into humans (Markou, Chiamulera, Geyer, Tricklebank, & Steckler, 2009). It is well known that there is a strong hereditary component to addiction, and twin studies have revealed that addiction to cocaine is the most heritable of all abused substances (Goldman, Oroszi, & Ducci, 2005). While many genetic variants have been identified that associate addiction propensity (Bevilacqua & Goldman, 2009), it is also necessary to relate these variations to known endophenotypes in animal models and humans to confirm their causality in addiction pathology. Also, there is a clear environmental aspect to the development of addiction pathology in humans that cannot be explained solely through genetics [for example, (Allen et al., 2017; Crum, Lillie-Blanton, & Anthony, 1996)]. Consequently, it is imperative to elucidate a consistent biomarker in rodents and humans to understand the neurobiology underlying addiction.

1.2 Use of behavioral economics in animal models of addiction

One potential biomarker for addiction propensity is pathological motivation for drug. Human addicts have high demand (motivation) for their abused drug, which can be triggered even with prolonged withdrawal by the presence of drug-associated cues. Addicts are willing to expend more effort, and pay a larger price, to obtain drug, and sustain adverse consequences like loss of money, family, and career. Thus, addiction is thought to be a “disorder of choice”, such that addicts will exert greater effort to obtain drugs over other nondrug, socially acceptable reinforcers (Kalivas & Volkow, 2005). In addition, addicts persistently give high

reinforcing value to their drug of choice, which leads to suboptimal decision-making, termed reinforcer pathology (Bickel, Jarmolowicz, Mueller, Koffarnus, & Gatchalian, 2012).

One way to quantify reinforcer pathology is with behavioral economics (BE), which is the study of an individual's allocation of limited resources. According to consumer demand theory, individuals will decrease their motivation for a commodity as price increases (Hursh, Raslear, Shurtleff, Bauman, & Simmons, 1988). Price can be the time, effort, or monetary cost required to obtain the commodity. Addicted individuals excessively value their commodity (drug) and desire to immediately acquire it despite adverse consequences (Bickel, Jarmolowicz, Mueller, & Gatchalian, 2011). Thus, addicted individuals have greater demand for their commodity of choice, which leads to a greater allocation of resources towards obtaining drug and less sensitivity to changes in drug price (Bickel, Johnson, Koffarnus, MacKillop, & Murphy, 2014). Individuals whose consumption is less resistant to changes in price are considered to have inelastic demand, whereas those whose consumption declines as price increases have more elastic demand. Therefore, addicted individuals have more inelastic demand for the abused drug compared to unaffected, healthy individuals.

Drug demand can be measured using demand curves, in which an individual's level of consumption can be plotted as price increases. In clinical psychology, demand curves have been generated from human addicts using drug purchasing tasks. These questionnaires require participants to report their hypothetical consumption of a commodity as monetary price increases. Demand

curves can be created from these tasks across drugs of abuse, including nicotine, heroin, and alcohol (Jacobs & Bickel, 1999; Murphy, MacKillop, Skidmore, & Pederson, 2009). An individual's demand for drug can predict addiction severity and treatment outcomes (MacKillop & Murphy, 2007). Interestingly, self-reports of drug craving reveal only a minimal association between craving and demand parameters (MacKillop et al., 2012).

Addiction-related demand can also be modeled in animals, using price as the rate and magnitude of the reinforcer during operant responding on a fixed-ratio schedule of reinforcement (Bickel et al., 2014). Both within- and multiple-session procedures have been developed to plot cocaine self-administration behavior as a function of effort to determine individual demand for drug (Christensen, Kohut, Handler, Silberberg, & Riley, 2009; Christensen, Silberberg, Hursh, Huntsberry, & Riley, 2008; Espana et al., 2010; Oleson, Richardson, & Roberts, 2011; Zimmer, Oleson, & Roberts, 2012). In these paradigms, animals' lever-pressing behavior decreases with increased price to produce demand curves like those observed in human addicts' self-reports. Within-sessions procedures increase price within a single session, such that animals must lever-press progressively more throughout the session to obtain 1 mg of cocaine (Espana et al., 2010; Oleson et al., 2011; Zimmer et al., 2012). Until recently, this method relied predominantly on graphical estimation of baseline consumption (Q_0) and the maximum price paid for a commodity (Pmax). At prices to the left of Pmax, demand is inelastic, such that an increase in price produces an insubstantial (<1%) decrease in consumption. In contrast, prices to the right of Pmax produce elastic demand and a decrease in

consumption (>1%). This approach has several short-comings: as price is logarithmic, the accuracy of estimation is likely to decline at higher price points, and graphical calculation can only consider changes in demand at Pmax (Bentzley, Fender, & Aston-Jones, 2013). Therefore, while the within-session model is attractive in its manipulation of price within a single session, its graphical analysis lacks the precision to calculate subtle changes in demand.

The exponential demand equation developed by Hursh & Silberberg provides a quantitative measure of motivation, demand elasticity, reflected in the α parameter (Hursh & Silberberg, 2008):

$$\ln Q = \ln Q_0 + k(e^{-\alpha Q_0 C} - 1)$$

The α value is proposed to reflect the drug's essential value, or the animal's motivation to defend drug consumption as price increases (see Figure 1.1)(Chase, Mackillop, & Hogarth, 2013; MacKillop et al., 2010; Murphy et al., 2009). Motivational efficacy of drug reward scales inversely with α, such that animals with greater motivation for cocaine will display lower α values. Therefore, α is an attractive biomarker for addiction propensity. However, application of this equation has been limited to multi-session procedures, in which demand is determined at one price point across several sessions (Christensen et al., 2009; Christensen, Silberberg, Hursh, Roma, & Riley, 2008).

As α is normalized to changes in Q_0, it can be used to directly compare demand across different variables, including reinforcer type, that might be affected by differences in hedonic value (Q_0). Therefore, demand for drug can be compared to that for natural rewards to identify treatments for the former without impacting

the latter. For example, Christensen et al., (2008) reported that extended access to cocaine increased motivation (α) for the drug. This was not evident following extended access to food, indicating that experience can impact demand for drug, but not natural, rewards (Christensen, Silberberg, Hursh, Roma, et al., 2008). Previous research has also shown that α can be utilized to compare drug demand across species (Christensen et al., 2009). While the multi-session approach elucidated several interesting factors that contribute to demand, it is challenging to assess the effects of a short-acting treatment on demand in a paradigm that must occur across multiple days.

Our laboratory has recently developed a behavioral economics (BE) procedure using Hursh & Silberberg's multi-session exponential demand equation to calculate demand for cocaine in a single session (Bentzley & Aston-Jones, 2015; Bentzley et al., 2013; Bentzley, Jhou, & Aston-Jones, 2014). This paradigm can be used to calculate demand elasticity (α) and baseline consumption (Q_0). As α is normalized for different Q_0 values, it is not affected by changes in baseline cocaine consumption, such as with tolerance or sensitization, making it a powerful measure of motivation that is less confounded by baseline consumption than other measures. This paradigm has been applied to study demand for other drugs of abuse, including methamphetamine and remifentanil (Cox et al., 2017; Porter-Stransky, Bentzley, & Aston-Jones, 2017). Consequently, this paradigm can be utilized to investigate the neural systems involved in the hedonic (Q_0) and motivational (α) value of drugs of abuse.

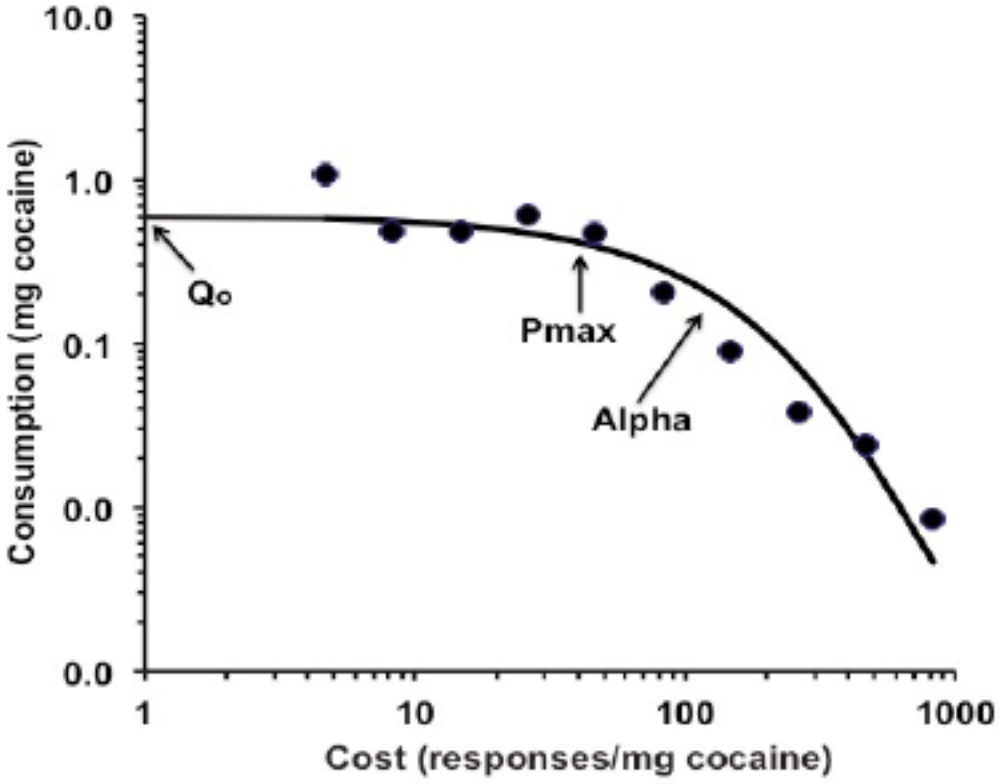

Figure 1.1. A representative demand curve generated from a single BE session. Data points are fit to the exponential demand equation to generate a demand curve for an animal during within-session BE cocaine self-administration. Baseline consumption (Q_0), maximum response (Pmax), and demand elasticity (α) can be determined from the curve.

1.3 Behavioral economics (BE) relates to other animal models of cocaine self-administration

Our behavioral economics (BE) paradigm is a unique measure of motivation that has several advantages over other models of motivation, like progressive ratio. Demand for cocaine in our BE paradigm also predicts addiction-like behaviors in

established models of relapse and compulsive drug taking. Recently, BE has also been utilized to investigate and compare the motivational changes that occur in paradigms of escalated drug use (long and intermittent access). Some animal models of addiction and their relationships to our BE procedure are discussed below.

1.3.1 BE is a more sensitive measure of motivation than other established paradigms

Fixed ratio

Fixed ratio (FR) responding is an operant paradigm commonly utilized to train animals to lever press for drug. Animals will receive an infusion of cocaine after reaching a predetermined number of lever presses, such as one reward for one lever press on an FR1 schedule. FR can also determine how contingency changes impact lever pressing, as when an animal previously trained on FR1 must now lever press five times to receive the same amount of reward (FR5). FR1 responding has been shown to be affected by the dose, rate, and volume of the infusion (Balster & Schuster, 1973; Downs & Woods, 1974; Fitch & Roberts, 1993; Panlilio et al., 1998).

It has been argued that FR responding cannot indicate the reinforcing value of drug and should not be used to measure motivation (Arnold & Roberts, 1997), as the rate of intake measured during FR1 is independent from its reinforcing efficacy. As an example, lesions to nucleus accumbens (NAc) did not affect the rate of intake of a dopamine agonist, yet response rates changed on a progressive

ratio schedule, which is a more sensitive measure of motivation (discussed below) (Roberts, Koob, Klonoff, & Fibiger, 1980). This distinction between “wanting” and “liking” the drug has become increasingly appreciated (Berridge & Robinson, 1998). The dichotomy is also evident in our BE paradigm, as the motivation to take reward (α) is independent from reward intake (Q_0), (Bentzley & Aston-Jones, 2015). Despite this limitation of FR schedules, they are critical procedures for operant learning before transitioning animals to more complicated behavioral paradigms.

Progressive ratio

One method utilized to assess the motivational efficacy of a reward is the progressive ratio schedule of reinforcement (PR), in which an animal must increase its lever pressing on a predetermined schedule to receive the same amount of reward. Each individual reaches a breakpoint, at which the effort required to receive the reward exceeds its attributed value. Thus, the breakpoint is thought to reflect the reinforcing value of the reward. PR demonstrates the maximum effort an individual will exert for a commodity and can determine the extent that responding persists for a reinforcer. PR is also sensitive to differences in drug dose and can assess shifts in the dose-response curve (Arnold & Roberts, 1997).

While PR is a highly motivating paradigm and can indicate an animal’s motivation for drug, it has several shortcomings. Under a PR schedule, the animal only receives drug when it has achieved the specified number of lever presses (ie 10 lever presses on FR10, 5 presses at FR5). Therefore, lever pressing is not

continuously reinforced with drug infusion, as it is during BE. PR also cannot determine motivation at any point other than at the breakpoint, whereas BE can measure effort across multiple price points. PR breakpoint is dependent on the schedule of reinforcement, and many different step functions of reinforcement (ie. arithmetic or exponential) are utilized. In addition, PR schedules may cause "ceiling" effects, as similar maximum breakpoints have been observed with different drugs of abuse (N. R. Richardson & Roberts, 1996). Ceiling effects are rare during BE, as highly motivated animals will continue to lever press throughout the paradigm if they continue to be reinforced with cocaine. Consequently, BE can determine motivation for drug across multiple price points, making it a more sensitive and thorough measure of motivated drug taking than PR.

1.3.2 Motivation for cocaine during BE predicts other addiction-like behaviors

Compulsive drug taking

Uncontrolled, compulsive drug taking distinguishes recreational from chronic drug use. Addicts will continue to impulsively seek and take drug despite the known adverse consequences. In animals, this behavior can be modeled by pairing drug self-administration with a stressor like a foot shock (Chen et al., 2013; Vanderschuren & Everitt, 2004). Previous research from our lab has adapted this model to account for individual differences in preferred intake (Q_0). By giving animals the same amount of drug paired with increasingly greater electrical charge in 10-minute bins, we have shown that the both α and Q_0 relate to the maximum

charge accepted for cocaine. Animals with greater demand (lower α) and baseline consumption (Q_0) will take drug paired with greater electrical charge than lower demand animals (Bentzley et al., 2014). These results indicate that highly motivated (low α) animals have greater compulsive drug taking and are more willing to sustain aversive consequences to pursue drug.

Extinction/abstinence

Recovering addicts will abstain from drug use and remove themselves from the environment previously associated with drug taking. In animals, two methods to model this behavior are abstinence or extinction training. During abstinence, animals previously taught to self-administer drug undergo a forced, drug-free period and are not re-exposed to the drug context. In contrast during extinction training, animals are placed in the drug context but lever pressing is no longer reinforced with drug. Over several days, animals will reduce lever pressing because the behavior is no longer reinforced by drug presentation. Therefore, extinction is synonymous with the loss of drug responding (Millan, Marchant, & McNally, 2011). Although extinction inhibits drug seeking, the propensity to seek drug remains, and animals often will reinstate lever pressing if stimuli associated with drug taking are present (discussed below). In our BE paradigm, high motivation animals had greater drug seeking on the first day of extinction (ED1), as indicated by the number of presses on the active lever, or the lever that previously resulted in drug infusion (Bentzley et al., 2014). These results demonstrate that animals with greater motivation for cocaine will continue to seek drug even when lever pressing is no longer reinforced.

A caveat to extinction/abstinence models is that they do not resemble the circumstances by which human addicts will abstain from drug use. In these models, animals are forced to refrain from drug use, whereas human addicts are often motivated by external factors to stop drug taking (Epstein, Preston, Stewart, & Shaham, 2006). In human addicts, the aversive consequences of pursuing drug taking also often outweigh or reduce the positive aspects the drug, and it is challenging to model this conflict in animals (Epstein et al., 2006; Peck & Ranaldi, 2014). However, when used in conjunction with reinstatement models, there can be stronger face validity in the extinction/abstinence models' capacity to promote relapse to drug seeking.

Reinstatement (stress-, primed-, cue-, context-induced)

Almost 90% of addicts will relapse within a year of abstaining from the drug (Koob, Kenneth Lloyd, & Mason, 2009). Human addicts report intense cravings when presented with cues associated with previous drug taking (Ehrman, Robbins, Childress, & O'Brien, 1992). Relapse can be modeled in animals trained to self-administer cocaine. Following a period of extinction or abstinence, animals are re-exposed to cocaine-associated cues (cue-induced reinstatement) or contexts (context-induced reinstatement), or given non-contingent injections of drug (cocaine primed-reinstatement). These cues are sufficient to reinstate lever-pressing for cocaine, even when drug is not present (de Wit & Stewart, 1981). Aversive stimuli like footshock or the pharmacological stressor yohimbine can similarly reinstate lever-pressing for drugs (stress-induced reinstatement). These models are particularly attractive because the factors that contribute to

reinstatement of lever pressing also trigger relapse in human addicts (Epstein & Preston, 2003; Shaham, Shalev, Lu, de Wit, & Stewart, 2003).

Reinstatement is the primary model for assessing relapse because of its strong face validity, or close modeling of the circumstances underlying relapse in human addicts. However, it often lacks predictive validity, or the capacity to predict treatment efficacy. It has been argued that human addicts do not experience acute drug cravings, as in reinstatement models, and that craving only in part predicts the rate of relapse (Epstein et al., 2006). These discrepancies between human relapse and animal reinstatement may explain why some therapies that show promise with these models have been ineffective in human patients (Grabowski et al., 1995; Nann-Vernotica, Donny, Bigelow, & Walsh, 2001). As a result, researchers have begun to develop behavioral procedures that model different facets of relapse, including reacquisition and incubation of craving (Marchant, Li, & Shaham, 2013). While reinstatement is a viable animal model for elucidating treatments for addiction, it cannot be the only animal model utilized to ascertain treatment efficacy.

Economic demand for psychostimulants has been shown to relate to the degree of reinstatement, making it an attractive complementary paradigm to utilize with reinstatement models that more accurately reflects drug seeking/taking in human addicts. Animals' motivation for cocaine predicts the degree of cue- and cocaine primed-reinstatement (Bentzley et al., 2014). Highly motivated animals (lower α) reinstate lever pressing more with exposure to drug and drug-paired cues. A similar relationship between motivation and cue reinstatement for

methamphetamine has also been shown in both males and females (Cox et al., 2017; Galuska, Banna, Willse, Yahyavi-Firouz-Abadi, & See, 2011). Therefore, BE elicits motivated behavior that is consistent with established models of relapse and using these paradigms in tandem may be more effective at screening treatments for relapse in human addicts.

1.3.3. BE highlights motivational changes that occur with extended drug access

Long (vs. short) access

The long-access (LgA) paradigm is frequently used to demonstrate the escalation of intake thought to transition individuals from drug use to addiction. In this paradigm, animals are given extended access (6 hours or more) to cocaine daily and are often compared to animals given short-access (1 hour) of cocaine. This paradigm can also be a standard 2-hour self-administration session, but with an extended number of self-administration days (Vanderschuren & Everitt, 2004). When compared to ShA animals, LgA animals have greater drug intake over self-administration sessions and enhanced reinstatement of drug-seeking following forced abstinence, indicating a change in hedonic set point and tolerance to the drug with increased exposure (Ahmed & Koob, 1998). The inability to maintain this elevated hedonic set point might induce a negative affective state and promote persistent drug seeking. Thus, compulsive drug taking in addicted individuals may result from negative reinforcement, in which the individual seeks to reduce this

negative affective state, rather than a desire for the positive reinforcing effects of cocaine (Ahmed & Koob, 2005).

Animals given extended access to cocaine recapitulate several endophenotypes evident in human addicts. Animals with extended access to cocaine are more motivated than ShA animals, as demonstrated by greater PR breakpoints (Oleson & Roberts, 2009) and reduced latency to traverse a runway to receive an infusion of cocaine (Ben-Shahar, Posthumus, Waldroup, & Ettenberg, 2008). LgA animals also reinstate their drug seeking to a greater extent in response to an injection of cocaine (Knackstedt & Kalivas, 2007) or cues previously associated with cocaine (Kippin, Fuchs, & See, 2006), and seek drug more during extinction than ShA animals (Ferrario et al., 2005). In addition, animals given prolonged access to cocaine continue to seek cocaine even when the drug is paired with an aversive shock, which is thought to indicate compulsive drug seeking (Vanderschuren & Everitt, 2004). Consistent with the negative reinforcement model of addiction (Ahmed & Koob, 1998, 2005), LgA animals also have increased anxiety-like behaviors, as measured in the elevated plus maze, and depression-like behaviors in the forced swim test (Valenza, Butelman, & Kreek, 2017). LgA animals may display more depression- or anxiety-like behaviors because of an inability to sustain an elevated hedonic drug set point.

Our lab has utilized the BE paradigm following LgA to determine how it can alter α and Q_0 values. Following a two-week period of LgA, animals had increased baseline consumption (Q_0) and demand for cocaine (α) compared to pre-LgA (Bentzley et al., 2014). However, the effects on demand were only due to changes

in high demand elasticity (low motivation) animals. Low demand elasticity (high motivation) animals did not alter their motivation for cocaine after LgA. As highly motivated animals more accurately reflect patterns of drug taking in addicted individuals, these studies highlight the limitations of the LgA model in fully recapitulating the persistent changes in motivation that occur with chronic drug use.

Intermittent access

It is becoming increasingly appreciated that the duration of drug access is not the only factor that can promote escalated drug intake and abuse. As discussed previously, human cocaine addicts display binge-abstinence cycles of intake. The intermittent access (IntA) paradigm models this pattern in a single 6-hour session. Cocaine is available for 5-minute periods when animals can load up on drug. These trials are separated by 25-minute timeout periods to produce a spiking pattern of intake (Zimmer et al., 2012). In contrast, LgA animals have consistent access to cocaine and will maintain a stable amount of brain cocaine. Cocaine is only available to IntA animals for 1 hour, and intake can be compared to ShA animals that undergo 1 hour of continuous cocaine self-administration.

Studies using BE have highlighted the pronounced changes in motivation following IntA, compared to LgA. Although both LgA and IntA animals had a higher Pmax (maximum price paid) than ShA animals, IntA animals had a significantly greater Pmax than LgA animals, even though LgA animals consumed more drug (Zimmer et al., 2012). These changes in motivation have also been demonstrated using PR breakpoints, as IntA animals had greater PR breakpoints with escalating

doses of cocaine compared to LgA animals (Allain, Bouayad-Gervais, & Samaha, 2018). IntA experience also increased Pmax and decreased motivation (α) when compared to pre-IntA values, demonstrating that this pattern of spiking cocaine intake can manifestly change reward/motivation processes (Kawa, Bentzley, & Robinson, 2016). These effects on demand are persistent and can last 50 days after IntA experience, unlike after LgA (James et al., 2018). Thus, IntA has gained increased attention in recent years as a method to significantly increase motivation for drugs of abuse, and this paradigm might more accurately reflect changes in reward circuitry that occur with extended access to cocaine in human addicts than LgA.

1.4 Motivational significance of drug-associated cues

As mentioned previously, cues and contexts associated with drug taking can trigger relapse in both animal models of addiction and human addicts. The reinforcing aspects of the drug facilitate learning these cues, which predict future drug availability (Hyman, Malenka, & Nestler, 2006). In animal models, these stimuli include the active lever that results in drug infusion, the light and tone that often accompany drug presentation, and/or the operant chamber in which drug taking occurs. It has been proposed that chronic drug use produces neuroadaptations that sensitize the individual to these stimuli, such that they acquire motivational significance similar to that of the drug itself (Robinson & Berridge, 1993). Thus, dysfunctional associative learning of the conditioned drug-cue relationship leads to an excessive valuation of these cues, termed incentive

salience. As a result, conditioned responses to the presentation of cocaine-associated cues may be similar in nature and degree to those following presentation of the drug, even without the drug present (Robinson, Yager, Cogan, & Saunders, 2014). Elevated reactivity to drug cues, such as physiological or emotional/motivational responses, has been observed in human addicts (Carter & Tiffany, 1999).

Excessive craving in response to drug cues could result from sensitized processes mediating drug "wanting". According to the Incentive Sensitization theory of addiction put forth by Robinson & Berridge (1993), there are dissociative neural systems for "wanting" and "liking" drugs of abuse, and repeated drug use specifically impacts drug wanting. In our BE paradigm, α and Q_0 could be thought to reflect wanting (α) and liking (Q_0) cocaine, as they are distinct (not correlated) processes that indicate the motivational (α) and hedonic (Q_0) aspects of the drug (Bentzley et al., 2013; Bentzley et al., 2014). Therefore, the neural substrates that mediate "wanting" (α) may be dysregulated in addicts to produce an augmented valuation and response to drug cues, leading to relapse. Robinson & Berridge attributed hyperactivity in the mesolimbic dopamine system, and its projections to the ventral striatum, as a key mediator of drug wanting (Robinson & Berridge, 1993).

1.5 Ventral tegmental area dopamine neurons as a locus for addiction pathology

Ventral tegmental area (VTA) and substantia nigra pars compata are the origins of DA neurons in the brain. VTA DA cells can be identified as

immunopositive for tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis, and have been shown to be either multipolar or fusiform neurons (Grace & Onn, 1989). These cells also have been identified electrophysologically as having one of three distinct profiles: exhibiting an inactive hyperpolarized state, single-spike tonic (2-10 Hz) or phasic/burst firing (Grace & Bunney, 1983). The electrophysiological profile of these cells has been thought to include pacemaker activity (indicative of tonic firing) and broad action potentials with long durations, hyperpolarization by D2 receptor agonists, and hyperpolarization-activated cation currents (Ih) (Grace & Onn, 1989). However, some studies have added complexity to this understanding, as some DA neurons lack an Ih current and fail to respond with DA application (Grace & Onn, 1989; Lammel, Lim, & Malenka, 2014). A subpopulation of DA cells are spontaneously active to mediate tonic DA release, while roughly 50% are normally silent through inactivation by GABA-mediated inhibitory post-synaptic potentials (for review, (Grace, Floresco, Goto, & Lodge, 2007)).

VTA afferents are numerous and arise from regions like cortex, lateral septum, bed nucleus of the stria terminalis (BNST), nucleus accumbens (NAc), lateral habenula, ventral pallidum, periaqueductal gray (PAG), and hypothalamus (Geisler & Zahm, 2005). WGA injections into VTA demonstrated that VTA receives predominantly ipsilateral glutamatergic input from the prelimbic cortex, but also the infralimbic, cingulate, and orbital cortices containing vesicular transporter-1 (VGlut1). Also, VGlut2+ cells in hypothalamus (lateral hypothalamic area (LHA), lateral preoptic area, and medial), ventral pallidum, and lateral habenula contact

VTA neurons (Geisler, Derst, Veh, & Zahm, 2007). In turn, VTA DA neurons project to NAc shell and core, ventral pallidum, and prefrontal cortex (Taylor et al., 2014). In particular, the VTA DA to NAc circuit has been implicated in addiction pathology, and there are topographic differences in VTA projections to NAc: DA neurons in lateral VTA projects to lateral NAc core and shell, while medial VTA DA neurons primarily connect to medial NAc shell (Baimel, Lau, Qiao, & Borgland, 2017).

Local glutamatergic and GABAergic cellular populations have also been identified in VTA and closely interact with VTA DA neurons. Glutamatergic neurons in VTA contain VGlut2, comprise about 2-3% of total VTA neurons (Nair-Roberts et al., 2008), and make local synapses onto VTA DA neurons (Dobi, Margolis, Wang, Harvey, & Morales, 2010). In lateral VTA, VGlut2 and TH label distinct cellular populations, but some cells in medial VTA co-express VGlut2 and TH, indicating that they may release both glutamate and DA (Hnasko et al., 2010). Both VTA glutamate and GABA cells have their own distinct afferents and efferents. Similar to VTA DA cells, VTA GABA neurons similarly receive glutamatergic input from BNST, mPFC, PAG, and lateral habenula, as well as from medium spiny neurons in NAc core. In turn, these cells project to NAc core and lateral habenula (for review, (Morales & Margolis, 2017)). In addition, VTA GABA neurons receive input primarily from the dorsal and median raphe, LH, caudate putamen, and ventral pallidum (Faget et al., 2016) and project to NAc core and lateral habenula (Morales & Margolis, 2017).

Multiple theories of addiction have posited that dysfunction in the mesocorticolimbic dopamine (DA) system either contributes to or underlies

addiction pathology. Most drugs of abuse have consistently been shown to directly or indirectly elevate dopamine influx in the nucleus accumbens (NAc) (Di Chiara & Imperato, 1988). Cocaine acts at the dopamine transporter (DAT) to block reuptake of DA at the synaptic cleft, and drugs of abuse induce changes in the synaptic plasticity of VTA DA neurons. Despite the clear evidence that dysregulated dopaminergic signaling contributes to addiction pathology, it remains unclear which stage(s) of the addiction cycle DA mediates.

In particular, VTA DA neurons undergo drug-induced plasticity that is glutamate-dependent. Alterations in glutamatergic signaling in VTA occur with exposure to drugs of abuse, and disruption to VTA glutamate signaling impacts addiction-like behaviors. Glutamate induces VTA DA neuron burst firing in vivo, which is thought to underlie drug-induced plasticity (Floresco, West, Ash, Moore, & Grace, 2003). In vitro recordings revealed that the synaptic strength of VTA DA neurons, reflected in greater AMPA/NMDA ratio, increased in cocaine-treated compared to saline-treated animals (Ungless, Whistler, Malenka, & Bonci, 2001). Similarly, over-expressing the AMPA receptor subunit GluR1 in VTA, which is necessary for LTP induction, enhanced the motivational and rewarding properties of drugs of abuse (Choi et al., 2011). In contrast, blocking NMDA receptor signaling in VTA prevented sensitization to psychostimulants (Vanderschuren & Kalivas, 2000). Drug-induced LTP in VTA DA neurons has been shown to enhance dopamine release in cortical and striatal regions like prefrontal cortex and NAc and reinstate drug seeking (Chen, Hopf, & Bonci, 2010; Pignatelli & Bonci, 2015). Therefore, plasticity of VTA DA neurons, mediated by glutamate signaling,

contributes to addiction pathology, yet the behavioral significance of LTP in VTA DA neurons remains unclear.

Early self-administration experiments concluded that VTA DA mediates the acute rewarding effects of drugs of abuse under low-effort conditions like FR responding. Low doses of DA antagonists increased lever pressing, whereas high doses blocked responding for both amphetamine and cocaine rewards (De Wit & Wise, 1977; Yokel & Wise, 1975). Neither observation was evident following administration of norepinephrine antagonists. Increased responding was thought to reflect decreased reinforcing efficacy of cocaine and amphetamine, such that more lever pressing was required to obtain the same amount of reward. However, others argued that these effects revealed a purely motoric role for DA, and NAc DA only contributed to reinforcement in that it stimulated movement to obtain reward (Salamone, Cousins, & Snyder, 1997). As well, drugs known to elevate DA in NAc did not increase lever pressing, adding complexity to a potential role for DA in the rewarding effects of drugs of abuse (Jasinski, 2000; Volkow et al., 2009).

Despite conflicting evidence that VTA DA neurons contribute to reward, it is clear that these neurons mediate several processes implicated in addiction pathology. These processes include reward learning, effort-based decision making, and impulsivity, all of which combine processing the motivational significance of reward and generating a behavioral output towards obtaining it.

VTA DA neurons have also been implicated in promoting effortful decision-making, which integrates both the reinforcing value of the reward and the physical work necessary to receive it (Salamone & Correa, 2012; Salamone, Correa, Yang,

Rotolo, & Presby, 2018). Therefore, the decision to allocate effort and promote motor output towards obtaining reward could be considered similar to motivation (Salamone & Correa, 2012). Depleting or blocking DA signaling promotes executing actions that require low effort (Denk et al., 2005). In contrast, elevating NAc DA signaling increased responding for food on a progressive ratio schedule (Boekhoudt et al., 2018; Trifilieff et al., 2013) and chemogenetic activation of VTA DA neurons increased progressive ratio for sucrose, leading to the conclusion that VTA DA promotes action initiation (Boekhoudt et al., 2018).

The proposed role for VTA DA neurons in action initiation may explain the system's involvement in driving impulsivity, which is the tendency to act prematurely without foresight and reflects impaired decision-making and top-down cognitive control over one's actions (Dalley, Everitt, & Robbins, 2011). Impulsivity is a personality trait that may predispose an individual to develop addiction or is a state that results from addiction to abused drugs (Dalley et al., 2011; Molander et al., 2011; Winstanley, Olausson, Taylor, & Jentsch, 2010). In addicted individuals, impulsivity can contribute to compulsive drug seeking and subsequent relapse. Animal models of impulsivity include procedures that measure the ability to inhibit responding for a reward (stop signal reaction time task), the rate of premature responding for a predicted reward (5-choice serial reaction time task), or the extent to which an individual will delay choosing a larger reward for an immediate smaller one (delayed discounting).

DAergic input from VTA to both dorsal and ventral striatum, as well as prefrontal cortex, are important circuits for promoting impulsive behavior. In the 5-

choice serial reaction time task, increased premature responding for reward is thought to reflect impulsive behavior. Chemogenetic activation of VTA DA reduced the latency to make premature responses and collect reward, and decreased trial omissions (Boekhoudt et al., 2017), whereas chemogenetic inactivation increased trial omissions, the time to elicit a correct response, and the time to collect reward, indicating that premature response rate and perseverative responses decreased with VTA inhibition (Fitzpatrick et al., 2019). DA loss in NAc and dorsal striatum with 6-OHDA increased the number of trial omissions and response latencies, indicative of reduced impulsivity (Baunez & Robbins, 1999; Cole & Robbins, 1989). Similarly, blocking kappa opioid receptors on VTA DA neurons reduced DA release and induced DA burst responding in prefrontal cortex (PFC), which produced more premature responses in a response inhibition task (Abraham et al., 2018; Sokolowski & Salamone, 1994). Consequently, activation of VTA DA neurons is necessary for promoting impulsive-like behaviors, which may increase propensity to engage in drug seeking behaviors.

Similarly, VTA DA neurons contribute to reward learning and may be responsible for attributing motivational significance to reward-paired cues over time and generating output towards obtaining reward in response to cues. These neurons phasically respond to unpredicted rewards, as well as rewarding and reward-predicting cues (Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Schultz, 1998; Schultz, Dayan, & Montague, 1997; Wise, 2004). In addition, phasic firing of VTA DA neurons is necessary for conditioning to reward-paired cues (Tsai et al., 2009; Yun, Wakabayashi, Fields, & Nicola, 2004). Broadly, VTA DA neurons are

thought to encode reward prediction (Keiflin & Janak, 2015), particularly the temporal specificity of the reward (Takahashi, Langdon, Niv, & Schoenbaum, 2016). Calcium transients in the VTA to NAc circuit were elevated preceding entry into a conditioned cocaine-paired context, and these data were observed in both male and female animals (Calipari et al., 2017). Thus, VTA DA activation may be critical for processing conditioned cues paired with reward and drive goal-directed behavior towards obtaining the reward.

Thus, there is clear evidence that VTA DA is necessary for both low and high effort responding for cocaine. Our lab has found that manipulating VTA DA neurons can impact both motivation (α) and free consumption (Q_0) of cocaine in our BE paradigm (Mahler et al., 2018). Transgenic rats expressing Cre in tyrosine hydroxylase cells (TH-cre) were infused with either G_q, G_s, or G_i Designer Receptor Exclusively Activated by Designer Drugs (DREADDs). This approach permitted excitation (G_q), modulation (G_s), or inhibition (G_i) of VTA DA neurons prior to BE. Stimulation of VTA DA neurons (G_q DREADD) decreased baseline consumption (Q_0) and increased motivation (decreased α). Both findings point to increased reinforcing value of cocaine (Figure 1.2). In contrast, inhibition of VTA DA neurons (G_i DREADD) increased baseline consumption (Q_0) and decreased motivation (increased α). These results indicate that animals required more drug to reach satiety in early bins (Q_0) and were less willing to work for cocaine under high effort conditions (α) when VTA DA neurons were inhibited.

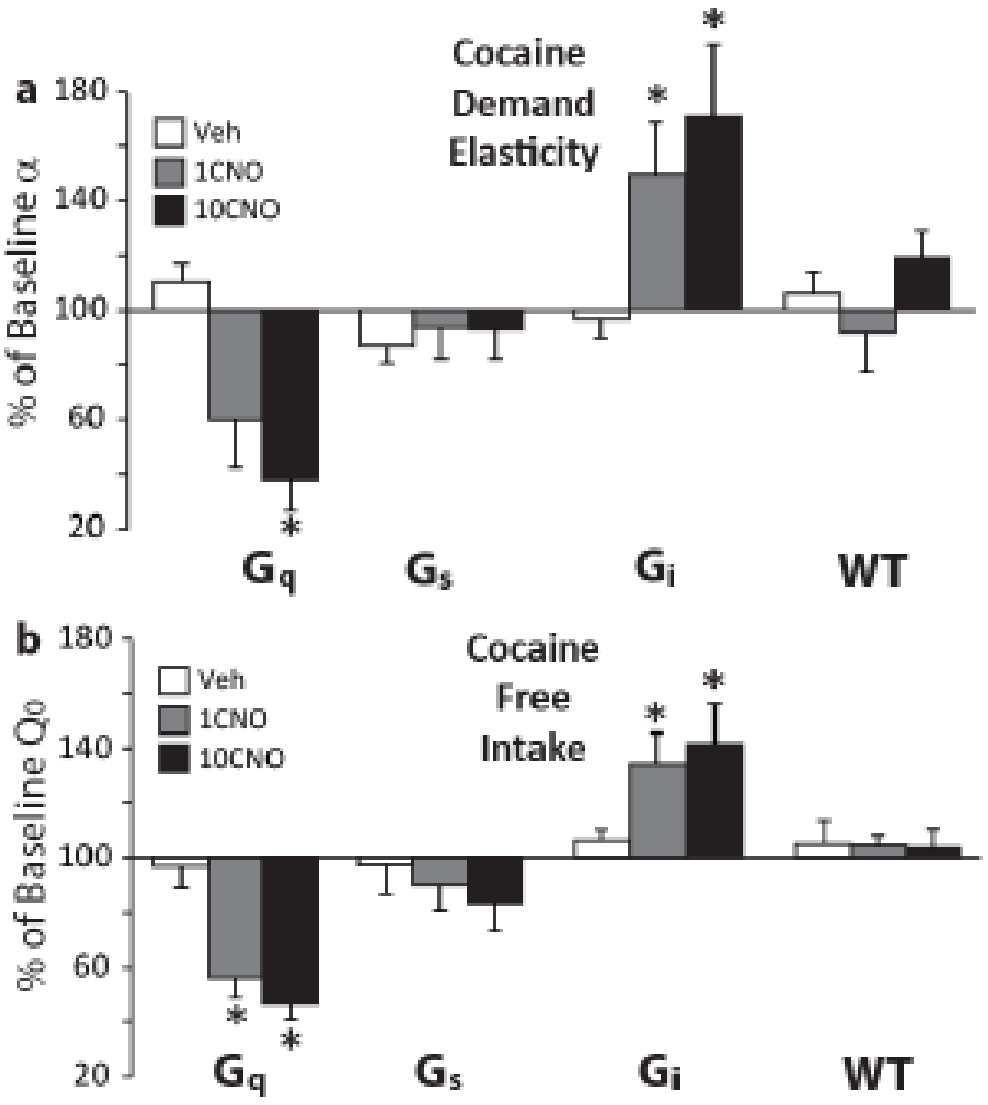

Figure 1.2. Effects of VTA DA activation (Gq), modulation (Gs), or inhibition (Gi) using DREADDs on behavioral economics parameters (from (Mahler et al., 2018)). A. Stimulation of VTA DA neurons decreased α (increased motivation; Gq), whereas inhibition increased α (decreased motivation; Gi) following systemic injection of the inert ligand for the DREADD receptor, clozapine N-oxide (CNO). B. Stimulation of VTA DA neurons decreased baseline consumption of cocaine (Q_0), and inhibition increased consumption. *p<0.05.

Based on these data, it remains unknown what key inputs to VTA DA neurons mediate these effects on cocaine demand. Because VTA DA respond to reward-predictive cues and are necessary for cue learning, these studies investigated two neuronal populations with known connections to VTA: lateral septum and lateral hypothalamus orexin neurons. Both of these cellular populations have established roles in cue-dependent addiction-like behaviors, suggesting that their input to VTA is necessary for promoting cocaine demand paired with cues.

1.6 Lateral septum neurons mediate cue-associated cocaine seeking

Lateral septum is a region largely associated with anxiety, yet its connectivity with the mesocorticolimbic dopamine system strongly suggests that it may contribute to reward-guided behavior. Lateral septum (LS) contains predominantly GABAergic medium spiny neurons that inhibit both extraseptal targets and neighboring subnuclei in LS. LS neurons also co-express several neuropeptides like enkephalin, somatostatin, and neurotensin, and LS can be divided into dorsal, intermediate, and ventral regions based on the topographic organization of these neuropeptides (Risold & Swanson, 1997a). LS receives input from regions associated with reward, learning, and memory, such as the hippocampus and prelimbic and anterior cingulate cortices (Beckstead, 1979). This connectivity makes LS ideally suited to integrate cognitive information about a reward, such as reward-associated contexts or cues, to promote reward-seeking behaviors (Sheehan, Chambers, & Russell, 2004). The region also receives input

from areas associated with affect, such as BNST and amygdala. Thus, LS may also receive information about the valence associated with reward to drive motivated behavior.

Several studies have linked LS activity to motivated behaviors such as feeding, movement, and aggression that seem to depend on LS relaying information from cognitive areas like hippocampus or PFC to hypothalamus. LS contains receptors for several hormones like oxytocin (Guzman et al., 2013), vasopressin (De Vries & Buijs, 1983), and corticotrophin-releasing factor (Anthony et al., 2014), and the significance of these populations for behavior has begun to be appreciated. In particular, LS is important for male aggression through signaling to ventromedial hypothalamus, and optogenetic inhibition of this circuit suppresses aggression (Wong et al., 2016). Recently, this circuit was shown to require hippocampal area CA2 release of vasopressin in LS, and silencing the CA2 to LS circuit inhibited input to ventromedial hypothalamus and aggression (Leroy et al., 2018). Similarly, glutamatergic input from ventral hippocampus to LS, and LS input to lateral hypothalamus, suppressed feeding, and inhibiting either circuit promoted feeding (Sweeney & Yang, 2015, 2016). In contrast, gamma-rhythmic input from medial PFC to LS improved performance during learning for food reward, and projections from somatostatin-containing LS neurons to hypothalamus were necessary for this behavior (Carus-Cadavieco et al., 2017). Finally, theta oscillations from hippocampus to LS promote general movement and depend on LS signaling to hypothalamus (Bender et al., 2015). Consequently, LS acts as an

important relay between cognitive and limbic regions to mediate numerous physiological behaviors.

LS has been implicated in seeking drugs of abuse like cocaine (Harasta et al., 2015; Luo, Tahsili-Fahadan, Wise, Lupica, & Aston-Jones, 2011; McGlinchey & Aston-Jones, 2017; Sartor & Aston-Jones, 2012), and the region's interconnectivity with the mesolimbic DA system may contribute to its role in drug seeking. LS has a bidirectional connection to VTA, and LS neurons have both D1- and D2-like receptors that may make the region responsive to VTA dopamine innervation (Swanson, 1982). Local microinjections of cocaine elevate DA in LS which can be inhibited by stimulating glucagon-like peptide 1 receptor-containing neurons in LS (Reddy et al., 2016).

In turn, LS acts on the mesolimbic DA system; the region relays information from area CA3 to VTA, which is important for context-induced reinstatement of cocaine seeking (Luo et al., 2011). A recent study determined that LS GABA neurons can disinhibit VTA DA neurons through local VTA GABA interneurons (Vega-Quiroga, Yarur, & Gysling, 2017). LS also is necessary for the DA-elevating effect of alcohol in NAc, as *in vivo* microdialysis of tetrodotoxin into LS reduces DA influx in NAc after alcohol perfusion (Jonsson, Morud, Stomberg, Ericson, & Soderpalm, 2017). Interestingly, LS is interconnected with the rostral dorsal NAc shell, which has been referred to as a hedonic hotspot (Pecina & Berridge, 2005) and projections from both regions almost entirely overlap in lateral preoptic area (Zahm, Parsley, Schwartz, & Cheng, 2013), further supporting the notion that LS forms a functional network with neurons in NAc. Consequently, LS appears to be

important for driving motivated behaviors and is influenced with exposure to drugs of abuse, indicating that it may mediate drug-induced changes in motivation.

1.7 Hypothalamic orexin neurons contribute to cocaine seeking behaviors paired with cues

Another neuronal population with known projections to VTA is orexin neurons in LHA. The neuropeptide orexin (also called hypocretin) is produced only in the hypothalamus and is involved in many physiological processes. Orexin A and B (also called hypocretin 1 and 2 respectively) are synthesized in the hypothalamus from prepro-orexin (de Lecea et al., 1998; Sakurai et al., 1998). Orexin A binds to both the orexin-1 and -2 receptors, whereas orexin B preferentially binds to the orexin-2 receptor (Zhu et al., 2003). The orexin field can be divided into dorsomedial (DMH), perifornical (Pef), and lateral hypothalamus (LH) within the lateral hypothalamic area.

Orexin neurons project throughout the brain and spinal cord. Some of the most predominant inputs to orexin neurons include LS, BNST, amygdala, NAc shell, and periaqueductal grey (Yoshida, McCormack, Espana, Crocker, & Scammell, 2006), whereas key output regions, as indicated by a high density of orexin fibers or retrogradely labeled orexin cells, include paraventricular nucleus of the thalamus, ventral pallidum, medial NAc, locus coeruleus, BNST, and VTA (Fadel & Deutch, 2002; Peyron et al., 1998). Orexin neurons promote physiological behaviors through interactions with numerous neurotransmitter systems, including the cholinergic, histaminergic, dopaminergic, and noradrenergic systems (Baimel

et al., 2015; de Lecea & Huerta, 2014; Huang et al., 2001; James, Mahler, Moorman, & Aston-Jones, 2017; Schone & Burdakov, 2017; Yamanaka, Muraki, Tsujino, Goto, & Sakurai, 2003).

Orexin has an established role in stimulating physiological behaviors important for energy homeostasis. One of the most well understood is promoting arousal. Early studies demonstrated that orexin deficiency underlies narcolepsy and indicated a role for orexin in the sleep/wake transition (Adamantidis, Zhang, Aravanis, Deisseroth, & de Lecea, 2007; Lin et al., 1999; Nishino, Ripley, Overeem, Lammers, & Mignot, 2000). Orexin also drives motivated behavior such as feeding, as intracerebroventricular injections of the peptide stimulated feeding (Sakurai et al., 1998) and systemic injections of the orexin-1 receptor antagonist SB-334867 (SB) reduced food consumption (Haynes et al., 2000). In addition, appetitive hormones like glucose, ghrelin, and leptin influence the activity of orexin neurons (Burdakov, Gerasimenko, & Verkhratsky, 2005; Yamanaka, Beuckmann, et al., 2003). Orexin's role in motivated behavior extends to stimulating addiction-like behaviors for drugs of abuse (James, Mahler, et al., 2017; Mahler, Moorman, Smith, James, & Aston-Jones, 2014; Mahler, Smith, Moorman, Sartor, & Aston-Jones, 2012), and the specific roles of orexin in addiction is discussed in Chapters 3 and 4. Finally, orexin acts on stress pathways through corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus; orexin neurons activate CRF cells to stimulate the hypothalamic-pituitary-adrenal (HPA) axis and elevates corticosterone levels (James, Campbell, & Dayas, 2017; Russell et al., 2001).

Orexin neurons can be considered heterogenous and have been characterized based on differences in physiology or associated behaviors. Our lab has proposed a functional dichotomy of orexin function, in which orexin neurons in lateral hypothalamus (LH) promote stress and those in perifornical/dorsomedial (Pef/DMH) stimulate arousal/stress (Harris & Aston-Jones, 2006; Harris, Wimmer, & Aston-Jones, 2005; Harris, Wimmer, Randall-Thompson, & Aston-Jones, 2007). In brief, orexin neurons in LH, and not other hypothalamic nuclei, are Fos-activated in proportion to preference for drugs of abuse, alcohol, and natural rewards, as well as to cues associated with food or drug reward (Aston-Jones et al., 2010; Brown, Woodworth, & Leinninger, 2015; Mahler et al., 2012). In contrast, Fos activation of DMH, but not LH, orexin neurons increased during waking hours or with foot shock (Estabrooke et al., 2001). DMH orexin neurons also receive input from other hypothalamic regions like the preoptic area and anterior hypothalamus that regulate homeostatic function and arousal (Yoshida et al., 2006).

Other groups have proposed alternative theories about distinct orexin neuron subpopulations. Orexin neurons have been categorized based on their electrophysiological responses to glucose application (Williams, Alexopoulos, Jensen, Fugger, & Burdakov, 2008), referred to as Type-D or Type-H orexin cells. Type-D are thought to be adaptive glucose-sensing cells in that they are transiently inhibited by glucose and restore firing through closure of leak-like K+ channels, whereas Type-H cells display sustained hyperpolarization. Still, another study characterized gene expression of 48 distinct genes in orexin neurons and did not find a consistent subpopulation based on genetic profile (Mickelsen et al., 2017).

Consequently, further investigation into the diversity of orexin neurons is warranted to determine which cells may be recruited during motivated behaviors.

1.8 Overview

These studies seek to elucidate key inputs to VTA neurons in cocaine demand using our BE paradigm. As discussed above, VTA DA neurons are critical for both cocaine demand elasticity (α) and baseline consumption of cocaine (Q_0). Both LS and LH orexin neurons have established reciprocal connections to VTA DA neurons (Fadel & Deutch, 2002; Swanson & Cowan, 1979). In addition, both LS and LH orexin input to VTA are known to be important for cue-dependent drug seeking behaviors (Luo et al., 2011; K. A. Richardson & Aston-Jones, 2012). Therefore, LS and LH orexin neurons should be critical brain regions that influence VTA DA neurons to drive cocaine demand. Consequently, these experiments investigated if LS (Chapter 2) or LH orexin neurons (Chapter 3) contributed to cocaine demand, and whether their connectivity to the mesolimbic DA system might mediate these effects (Chapter 2 and 4).

Chapter 2: Lateral septum inhibition reduces motivation for cocaine: reversal by diazepam

This chapter has been adapted from the manuscript, Pantazis CB & Aston-Jones G. “Lateral septum inhibition reduces motivation for cocaine: reversal by diazepam” (2019). Accepted for publication at Addiction Biology.

Abstract

The lateral septum (LS) is a brain region implicated in motivation, addiction, anxiety, and affect. We recently found that LS is necessary for cocaine-seeking behaviors including conditioned place preference and reinstatement of extinguished drug seeking, which involve LS input to limbic regions including ventral tegmental area (VTA) and orexin neurons in hypothalamus. Here, we microinjected baclofen-muscimol (B-M) in LS prior to testing in a behavioral economics (BE) paradigm. We found that intra-LS B-M decreased motivation (increased demand elasticity; α) for cocaine but did not change consumption at low effort (Q0). We also compared the effects of LS inhibition to effects of treatment with the benzodiazepine diazepam, which has been shown to facilitate reward pathways and disinhibit VTA dopamine neurons. Pretreatment with diazepam blocked the effects of LS inhibition and restored cocaine demand to that following vehicle treatment. These changes in cocaine demand after LS inhibition or diazepam were not due to effects on anxiety, as both manipulations produced similar effects on anxiety measures but opposing effects on drug taking.

Collectively, these studies point to LS as a critical region driving motivation for cocaine, likely through its interactions with the mesolimbic dopamine system.

Introduction

Drug addiction is a chronic and debilitating disorder with a high incidence of relapse. The lateral septum (LS) has been linked to the effects of abused drugs, but it is often overlooked in drug addiction research. Early intracranial self-stimulation experiments revealed that LS stimulation is highly reinforcing, as animals learn and maintain operant responding for electrical LS stimulation (Olds & Milner, 1954). LS projects to several limbic regions, including lateral hypothalamus and ventral tegmental area (VTA), and has been shown to regulate the mesolimbic dopamine (DA) system in both VTA and nucleus accumbens (Jonsson et al., 2017; Luo et al., 2011; Vega-Quiroga et al., 2017). Recently, it has been shown that inhibition of $GABA_{A\alpha1}$ receptor-containing VTA GABA neurons is necessary for LS to disinhibit VTA DA neurons (Vega-Quiroga et al., 2017).

Several studies indicate that LS neurons contribute to the rewarding (hedonic) and reinforcing effects of cocaine. LS neurons are Fos-activated following non-contingent injections of cocaine, cocaine self-administration, or exposure to cocaine-paired contexts (Franklin & Druhan, 2000; Zahm et al., 2010). We have shown that inhibition of LS neurons reduces cocaine conditioned place preference (CPP) and context- or cue-induced reinstatement of cocaine seeking (McGlinchey & Aston-Jones, 2017; Sartor & Aston-Jones, 2012). We have also reported that LS input to VTA DA neurons is an important circuit for reinstatement

of extinguished cocaine seeking (Luo et al., 2011). The role of LS in addiction appears to be complex, as knockdown of glucagon-like peptide-1 receptor (GLP-1R)-containing neurons in LS promotes cocaine CPP (Harasta et al., 2015). These studies reveal that LS neurons are active during addiction-like behaviors and necessary for cocaine seeking.

However, LS neurons have also been shown to promote anxiety and interact with stress circuits (Anthony et al., 2014; Sheehan et al., 2004) Recently, several subpopulations of LS neurons have been identified that drive stress/anxiety. Optogenetic inhibition of Crfr2-containing LS neurons reduces anxiogenic behaviors and decreases plasma corticosterone levels (Anthony et al., 2014). Similarly, knockdown of oxytocin receptor-containing LS neurons reduces a fear conditioned response following social defeat stress (Guzman et al., 2013). Finally, blocking GLP-1R signaling in LS attenuates stress-induced hypophagia (Terrill, Maske, & Williams, 2018). Therefore, it is unclear if changes in drug seeking following LS manipulations are due to alterations in reward or anxiety.

Here we show that LS inhibition increased demand elasticity (decreased motivation) for cocaine in our BE paradigm without affecting consumption at low effort. The effects of LS inhibition on motivation were blocked by the benzodiazepine diazepam, which has been shown to disinhibit VTA DA neurons, through inhibition of $GABA_{A\alpha1}$ receptor-containing VTA GABA neurons, and stimulate reward pathways (Heikkinen, Moykkynen, & Korpi, 2009; Straub, Carlezon, & Rudolph, 2010; Tan et al., 2010). Diazepam and LS inhibition similarly decreased anxiety but had opposing effects on drug taking, indicating that changes

in motivation by LS inhibition were likely not due to changes in anxiety. Consequently, we conclude that LS regulates the motivational properties of cocaine during drug taking independently of effects on anxiety.

Methods

Animals

Adult male Sprague-Dawley rats (n=88) weighing 325-350 grams were pair-housed on a 12:12 hour light:dark cycle in a temperature- and humidity-controlled animal facility with ad libitum access to standard rat chow and water. Animals were allowed to acclimate for 2 days, followed by handling for 3 days. All protocols and animal care procedures were approved by the Institutional Animal Care and Use Committee at Rutgers University.

Drugs

Diazepam (Sigma Aldrich, St. Louis, MO) was dissolved in 45% 2-hydroxypropyl-beta-cyclodextrin in sterile saline, and 1 or 2 mg/kg was given in a volume of 2 ml/kg (intraperitoneally). Each injection was given 30 minutes prior to the start of the behavioral economics (BE) paradigm. Cocaine HCl powder was provided by the National Institute of Drug Abuse (Research Triangle Park, NC) and was dissolved in 0.9% sterile saline.

Intravenous catheter surgery

Rats were anesthetized with ketamine/xylazine (56.5/8.7 mg/kg, i.p., respectively) and given an analgesic (rimadyl 5 mg/kg, s.c.). Rats were implanted with chronic indwelling catheters into the jugular vein. Intracranial surgeries were

performed following catheterization. Catheters were flushed with cefazolin (0.1 ml; 100 mg/ml) and heparin (0.1 ml; 100 U/ml) after surgery, and daily beginning 2 days after surgery and after each self-administration session. Rats were allowed to recover for 1 week following surgery before self-administration training.

Stereotaxic surgery

Immediately following catheter surgery, animals were placed in a stereotactic frame (Kopf, Tujunga, CA, USA) and implanted with bilateral stainless steel guide cannulae (26 gauge, 5 mm, Plastics One, Roanoke, VA, USA) 2 mm dorsal to rostral LS (coordinates relative to bregma from skull surface: +1.1 mm AP, $\pm$ 0.5 mm ML, -4.0 mm DV). Guide cannulae were secured to the skull using acrylic cement and jeweler screws. Rats were allowed to recover for 1 week after surgery before behavioral training began.

Intracranial microinjections

Control microinjections were performed first to ensure that behavioral changes following baclofen-muscimol were not due to dorsal diffusion up the cannula tract. Injectors projecting 0.2 mm below the tip of the guide cannula were used to deliver the $GABA_{A/B}$ agonists baclofen plus muscimol (0.3/0.03 nmol in artificial CSF, 0.5 ul) 1.8 mm dorsal to LS.

For LS microinjections, injectors were lowered 2 mm below the guide cannulae into LS to infuse artificial CSF (aCSF) or baclofen-muscimol (B-M). The injector cannulae were kept in place for 1 minute after infusion to allow for drug diffusion. A counterbalanced within-subjects design was used in bilateral LS microinjection experiments. Animals received no more than 6 microinjections

during testing. Animals with misplaced cannula (n= 10 for LS B-M experiment, n=5 for LS B-M x diazepam experiment) were excluded from analyses. Animals with misplaced cannula in the lateral ventricle (n=6) failed to lever press following B-M microinjections. All other animals (n=9) had cannulae dorsal to LS or off-center in LS were analyzed separately.

Cocaine self-administration

Rats were trained on an FR1 cocaine self-administration paradigm (20-second timeout post-infusion) for 2 hours/session, 1 session/day. Sessions occurred in operant chambers in sound-attenuating boxes using Med-PC IV software (Med Associates). During training sessions, cocaine infusions (0.19 mg cocaine/infusion) were paired with discrete light and tone cues (white stimulus light above the active lever; 78-dB, 2900-Hz tone). After reaching criteria ($\geq$ 10 infusions/session for 10 sessions), animals were trained on the within-session threshold BE procedure.

BE procedure

Following FR-1 training, rats were trained on a within-session threshold BE procedure, as described previously (Bentzley et al., 2013; Bentzley et al., 2014). During the 110-minute session, the dose of cocaine decreased by decreasing the duration of cocaine infusion per lever press in successive 10-minute intervals on a quarter logarithmic scale (383.5, 215.6, 121.3, 68.2, 38.3, 21.6, 12.1, 6.8, 3.8, 2.2, 1.2 ug cocaine per infusion), such that animals had to work progressively harder (produce more lever presses) to receive the same amount of cocaine. Animals were trained for a minimum of six days (mean $\pm$ SEM of all tested animals: 7.3 $\pm$

0.4 days, range: 6-15 days). When animals displayed stable behavior (Q_0 and α values ≤ 30% variability across the last three sessions), they received a microinjection of baclofen-muscimol (B-M) above LS (control) or a microinjection of B-M or aCSF into LS (n=12), a systemic injection of diazepam or vehicle (n=11), or both (n=10). Animals were tested again when BE performance re-stabilized (after at least three BE daily sessions, mean ± SEM: 4..2 ± 0.2 days, range: 3-12 days). Animals completed testing on the BE procedure after receiving all treatments (mean ± SEM: 22.7 ± 0.9 days, range: 15-37 days).

Demand curve analysis

Lever pressing responses during each BE session were fit to a demand curve to calculate two demand parameters (Q_0 and α), as described in previous papers from our lab (Bentzley et al., 2013). Q_0 represents baseline consumption at low effort, and α indicates demand elasticity or the rate of decline in consumption as price increases. Therefore, Q_0 is a measure of low effort consumption of the drug, and α is a measure of motivation for drug. As α is the slope of the demand curve, it inversely scales with motivation, such that animals with high motivation for cocaine will have lower demand elasticity and α values. Following each testing session, Q_0 and α values were compared to those the day previously to determine the percent change with LS B-M or diazepam treatment.

Sucrose self-administration

To determine if LS or diazepam manipulations impacted the motor ability of animals to lever press for cocaine, animals (n=15) were trained on sucrose self-administration. Separate cohorts of animals were used for LS B-M (n=7) and

diazepam (n=8) experiments with sucrose self-administration; these also were separate animals from those studied with cocaine self-administration. Animals were trained on FR-1 sucrose self-administration for 2 hours/day for a minimum of 5 days, as described in previous publications from our laboratory (Cason & Aston-Jones, 2014). Responses on the active lever resulted in a sucrose pellet (45 mg, Test Diet), with a 20 s timeout after each reward. Animals were tested when they displayed stable active lever responses across the last three days (≤ 25% variability).

Locomotor testing

Animals (n=25) trained on the threshold BE procedure underwent general locomotor activity testing with B-M/aCSF microinjected into LS (n=8), diazepam/vehicle given (n=8), or both (n=9) to determine if effects on motivation were due to sedation. All animals had previously undergone BE testing for cocaine, except animals used for the combined LS B-M/diazepam experiment, in which a separate cohort of animals was used. At least 24 hours following the final BE testing session, rats were habituated to locomotor boxes (clear acrylic, 40 x 40 x 30 cm) equipped with Digiscan monitors (AccuScan Instruments) for 2 hours/day for 3 days. After three days of habituation, rats received a microinjection of aCSF or B-M into LS and/or a systemic injection of diazepam/vehicle prior to testing. Rats received each drug once in a randomized and counterbalanced fashion. In between each testing day, rats were given a 1-day washout session, in which they were placed in the locomotor box but did not receive any treatment. Total locomotor activity and center time were recorded using beam breaks, and the

amount of time spent within an 8x8 in square matrix in the center of the chamber was determined with Fusion SuperFlex software.

Elevated plus maze

Animals were tested on an elevated plus maze (EPM) to measure anxiety-like behavior following LS inhibition (n=5/group) or diazepam treatment (n=7/group). LS inhibition animals had previously undergone BE testing. To obtain enough animals for the three diazepam treatments (vehicle, 1 or 2 mg/diazepam), we tested animals that had undergone BE testing with diazepam treatment (n=7) and an additional cohort of cocaine-experienced animals (n=14). The EPM (Med Associates, Inc.) was made of black, opaque Plexiglas elevated 60 cm above the floor of the testing room. The apparatus consisted of four arms (10 x 50 cm) perpendicular to one another: two opposing closed arms (with 40-cm walls) and two opposing open arms (with no walls). Two infrared photo-beams were positioned at the entrance of each of the four runways of the maze to track subjects' movements and record entry time, based on when subjects broke and released both infrared photo-beams. Animals were placed at the junction of the open and closed runways 5 minutes after intracranial injections or 30 minutes after systemic diazepam treatment. Animals explored the apparatus for 15 minutes, and the percent time that the animals spent in the open, closed, and junction areas was recorded by Med-PC IV software.

Localization of microinjections

Following the last behavioral test, animals were deeply anesthetized with isoflurane and decapitated. Brains were dissected, flash-frozen in 2-methylbutane,

and sectioned into 40 um sections on a cryostat. Sections were slide-mounted, Nissl-stained with neutral red, and coverslipped with DPX mounting medium to localize injection sites. We excluded data from animals that had damage near the injection site as seen with Nissl staining (n=2).

Data analysis

Statistical analyses were performed with GraphPad Prism 7. BE data were analyzed using repeated measures one-way analyses of variance (ANOVA) with Bonferroni's multiple comparisons test as appropriate. Locomotor, elevated plus maze, and sucrose self-administration tests were analyzed using paired samples t-tests to compare performance following aCSF or B-M microinjections. Non-parametric Friedman or Kruskal-Wallis test with post-hoc Dunn's test, or Wilcoxon matched-pairs signed rank tests, was used when data were not normally distributed, as determined by a Shapiro-Wilk normality test. All statistics were two-tailed.

Results

Animals implanted with guide cannulae above LS (Figure 2.1A) were trained on cocaine self-administration and the within-session BE procedure. There were no significant differences in the animals' baseline α (Shapiro Wilk normality test, $W=0.76$, $p<0.01$; Friedman test, $Q=0.500$, $p=0.779$) or Q_0 values (one-way repeated measures ANOVA, $F_{2,22}=1.81$, $p=0.188$) preceding each of the three treatments. Inhibition of LS with B-M increased demand elasticity (α; decreased motivation) compared to baseline (one-way repeated measures ANOVA, $F_{2,22}=9.89$, $p<0.01$; Figure 2.1B). No changes were observed following

microinjections of aCSF into LS, or of B-M 1.8 mm dorsal to LS. A one-way repeated measures ANOVA determined that none of the intracranial manipulations altered cocaine consumption at low effort (Q_0; one-way repeated measures ANOVA, $F_{2,22}$=0.23, p= 0.76; Figure 2.1C). Representative demand curves for LS aCSF and B-M manipulations are shown in Figure 2.1D. These effects were not due to sedation, as there was a trend towards increased locomotor activity during open field testing (paired t test; t_7= 2.05, p=0.08; Figure 2.1E). When locomotor data were analyzed in 5min bins, there was a significant effect of time (two-way repeated measures ANOVA, $F_{23,161}$=6.99, p<0.0001; Figure 2.1F) and a time x treatment interaction ($F_{23,161}$=1.94, p<0.01), but no effect of treatment ($F_{1,7}$=4.19, p=0.08). Animals had greater locomotor activity following LS B-M microinjections at 40, 45, and 50 minutes (p<0.05), and at 60 minutes (p<0.01) of testing.

As an additional motor control, we trained a separate cohort of animals on FR1 responding for sucrose pellets. We found that B-M microinfusions into LS increased active lever pressing for sucrose, confirming that the effect of LS B-M microinfusions to increase demand elasticity for cocaine was not due to decreased lever pressing abilities (Shapiro-Wilk test for normality: W=0.73, p<0.01; Wilcoxon matched-pairs signed rank test; z= 1.68, p<0.05; Figure 2.1G).

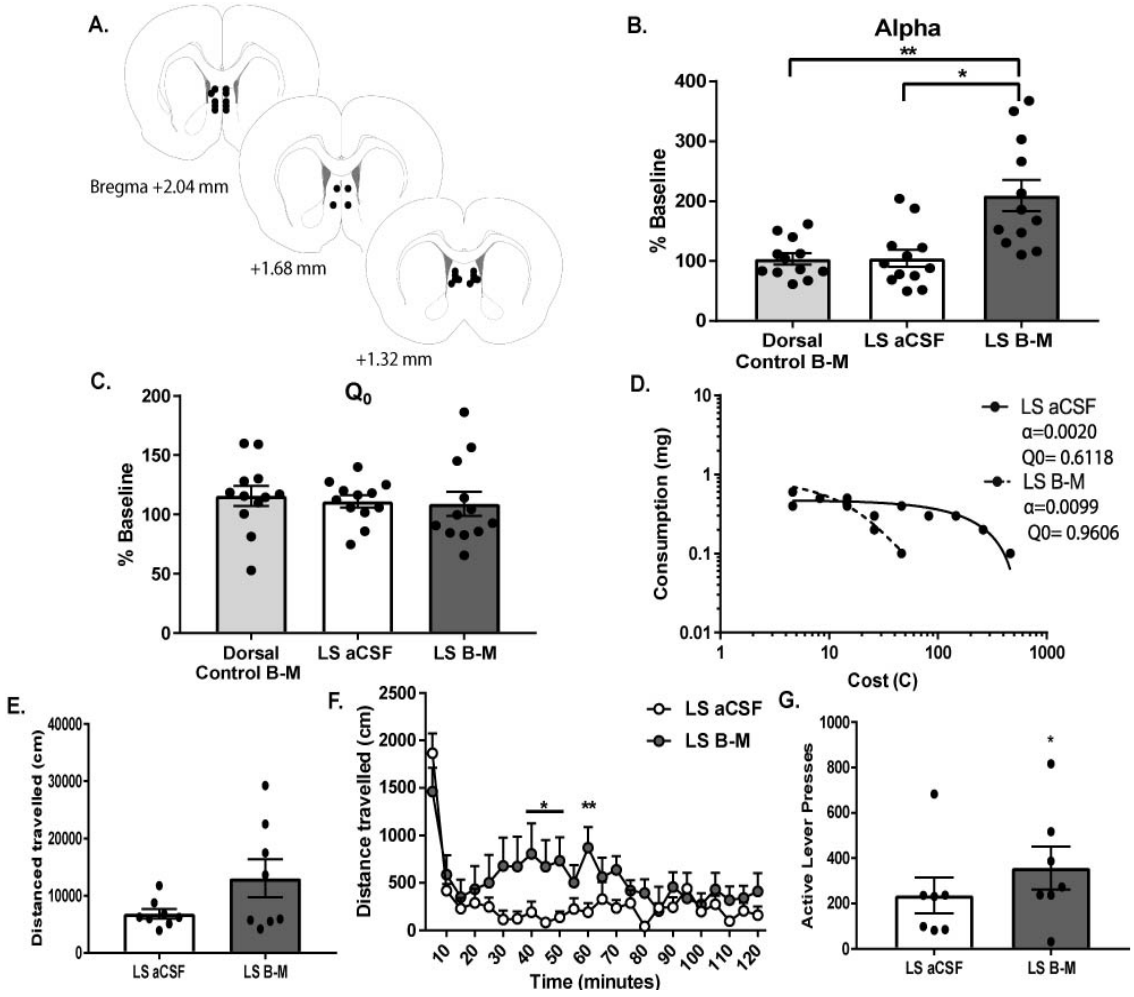

Figure 2.1. Intra-lateral septum (LS) baclofen-muscimol (B-M) microinjections reduced motivation for cocaine. (A) Schematic of bilateral microinjector placements in rostral LS in animals tested on behavioral economics (n=12). (B) B-M microinjection into LS increased α compared to dorsal control B-M intra-LS aCSF microinjections (*p<0.05, **p<0.01). (C) None of these treatments altered low effort consumption of cocaine (Q0; p>0.05). (D) Representative demand curves constructed from an animal's BE performance following microinfusion of aCSF (solid line) or B-M (dotted line) into LS. (E) B-M microinjections did not significantly affect locomotor activity (p>0.05). (F) Binned data from locomotor activity testing. Locomotor activity following B-M

microinjections was higher than after aCSF microinfusions between 40-50 minutes and at 60 minutes of testing (*p<0.05, **p<0.01). (G) B-M microinjections increased active lever presses for sucrose pellets during FR1 sucrose self-administration (*p<0.05). Bar graphs and error bars indicate mean + SEM.

LS has an established role in anxiety, and therefore we sought to determine whether LS inhibition impacted anxiety in our animals. BE-experienced animals underwent locomotor testing in an open field, and the time spent in the center was determined following either microinjections of aCSF or B-M into LS. Intra-LS B-M microinfusions increased total center time, compared to aCSF microinjections (Shapiro-Wilk test: $W=0.81$, $p<0.05$; Wilcoxon matched-pairs signed rank test; $z=2.42$, $p<0.01$; Figure 2.2A). Microinfusions of B-M into LS also increased the percent time spent in the open arms of an elevated plus maze (unpaired t-test; $t_8=3.56$, $p<0.01$; Figure 2.2B).

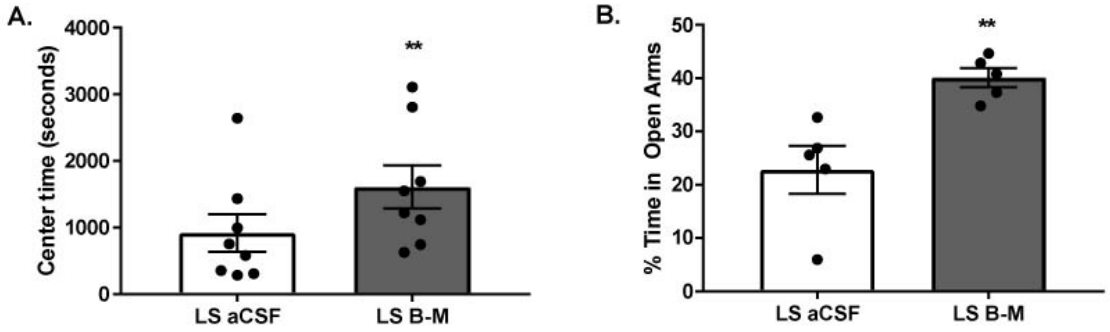

Figure 2.2. Inhibition of LS reduced anxiety-like behaviors. (A) B-M microinjections into lateral septum (LS B-M) increased total center time during locomotor testing, compared to aCSF (**p<0.01). (B) LS B-M increased the percentage of time animals spent in the open arms of an elevated plus maze (**p<0.01). Bar graphs and error bars indicate mean ± SEM.

Diazepam has been shown to increase drug intake during cocaine self-administration (David, Gold, Koob, & Cazala, 2001; Maier, Ledesma, Seiwell, & Duvauchelle, 2008). Although these effects were primarily attributed to reduced anxiety, previous research has shown that benzodiazepines also stimulate reward pathways via disinhibition of VTA DA neurons (Heikkinen et al., 2009; Tan et al., 2010). To determine if diazepam altered cocaine demand (α and Q_0 values), we trained animals on the BE procedure. When animals displayed stable baseline α and Q_0 values, they were given systemic injections of diazepam (1 or 2 mg/kg) or vehicle IP thirty minutes prior to testing. There were no differences in animals' baseline α (Shapiro-Wilk normality test, $W=0.77$, $p<0.01$; Friedman test, $Q=0.00$, $p=0.99$) or Q_0 (one-way repeated measures ANOVA, $F_{2,20}=0.022$, $p=0.978$) values preceding the three treatments. Diazepam significantly reduced α (increased motivation for cocaine) compared to vehicle treatment (one-way repeated measures ANOVA; $F_{2,18}= 4.20$; $p<0.05$; Figure 2.3A), and this effect was specifically at the 2 mg/kg dose (Bonferroni's multiple comparisons test, $p<0.01$). We also observed a trend towards a decrease in Q_0, but this failed to reach statistical significance (one-way repeated measures ANOVA; $F_{2,18}=3.24$, $p=0.051$; Figure 2.3B). A representative demand curve illustrating changes in α and Q_0 is given in Figure 2.3C.

Benzodiazepines also have been shown to have sedative properties (Rudolph et al., 1999). Both doses of diazepam caused a significant reduction in locomotor activity (Shapiro Wilk, $W=0.82$, $p<0.05$; Friedman test, $Q=12.00$, $p<0.01$; Figure 2.3D). When locomotor activity was binned in 5-minute intervals,

there was a significant effect of time (two-way repeated measures ANOVA; $F_{23,161}$=23.46, p<0.0001), treatment ($F_{2,14}$=12.78, p<0.01), and a treatment x time interaction ($F_{46,322}$=3.32, p<0.0001; Figure 2.3E). Animals had lower total locomotor activity following 1 or 2 mg/kg diazepam compared to vehicle during the first 5min (Bonferroni multiple comparisons test, p<0.0001) and 10min of testing (p<0.0001). Animals also significantly increased FR1 responding for sucrose following diazepam treatment (Shapiro-Wilk normality test, W=0.822, p<0.05; Friedman test: Q=9.25, p<0.01; Figure 2.3F). Therefore, although diazepam reduced habituated locomotor activity, it did not impair lever-pressing behavior.

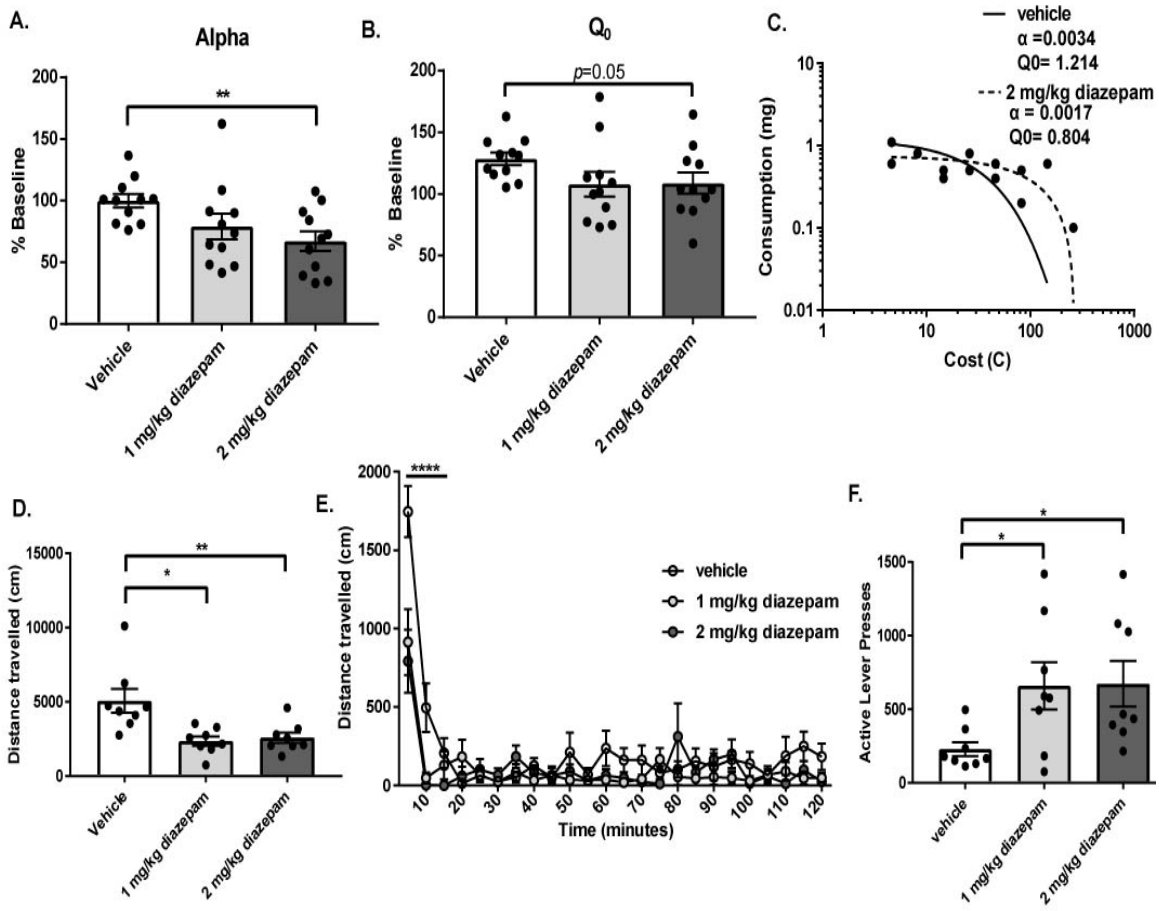

Figure 2.3 Systemic diazepam increased motivation for cocaine. The day after animals displayed stable BE behavior, they were given vehicle, 1 or 2 mg/kg diazepam ip 30min prior to testing. (A) The 2 mg/kg dose of diazepam decreased cocaine demand elasticity (α; increased motivation) compared to vehicle (**p<0.01 relative to vehicle). No significant change in demand elasticity was observed following the 1 mg/kg dose (p>0.05). (B) There was a trend towards a decrease in Q_0 following 2 mg/kg diazepam, compared to vehicle (p=0.05). No significant effects were observed at the 1 mg/kg dose (p>0.05) (C) Representative demand curves from an animal showing a reduction in α (increase in motivation) and in Q_0 following 2 mg/kg diazepam (dotted line) compared to vehicle (solid line). (D) Both

doses of diazepam decreased locomotor activity compared to vehicle (1 mg/kg dose: *p<0.05; 2 mg/kg dose: **p<0.01). (E) Both 1 and 2 mg/kg diazepam reduced locomotor activity in the first 5 and 10 minutes of testing (****p<0.0001) (F) Both doses of diazepam increased the number of active lever presses during FR1 responding for sucrose pellets (*p<0.05) compared to vehicle. Bar graphs and error bars indicate mean ± SEM.

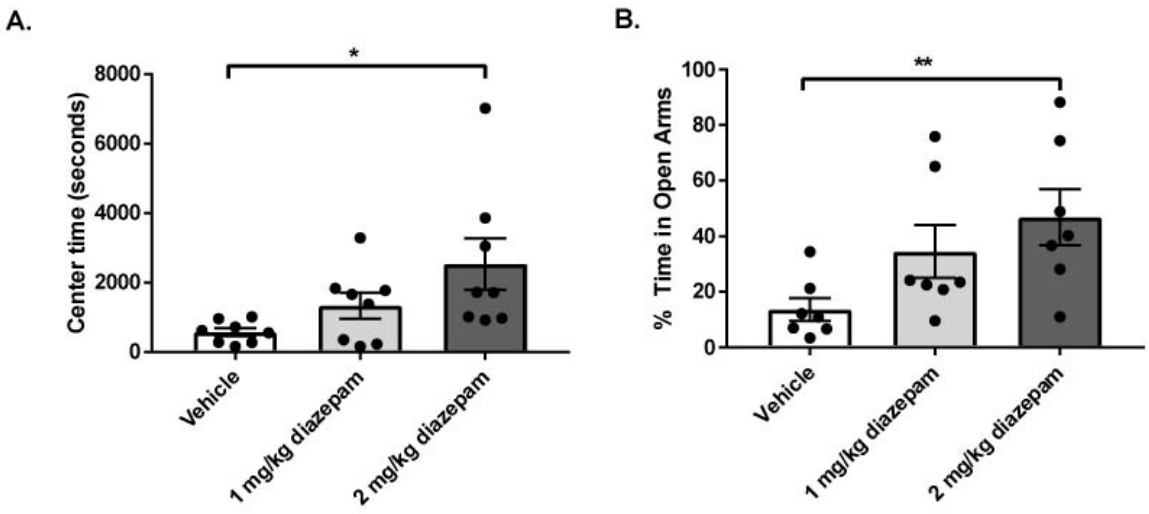

Figure 2.4 Diazepam was anxiolytic. (A) 2 mg/kg dose of diazepam increased center time during open field testing (*p<0.05) and (B) increased the percentage of time spent in the open arms of the elevated plus maze (*p<0.05) relative to vehicle. No changes were observed at the 1 mg/kg dose in either open field or elevated plus maze testing (p>0.05). Bar graphs and error bars indicate mean $\pm$ SEM.

We then tested animals on general anxiety measures to confirm that diazepam was anxiolytic. Diazepam increased center time in an open field (Shapiro-Wilk test, $W=0.80$, $p<0.05$; Friedman test, $Q=6.25$, $p<0.05$, Figure 2.4A) and this was specifically at the 2 mg/kg dose (Dunn's multiple comparisons test, $p<0.05$). Diazepam also increased percent time in the open arms of an EPM (Shapiro-Wilk test, $W=0.79$, $p<0.05$; Kruskal-Wallis test; $H_{3.21}=8.29$, $p<0.05$; Figure 2.4B), and this effect was only for the 2 mg/kg dose (Dunn's multiple comparisons test, $p<0.01$). Therefore, consistent with our observations following LS inhibition, diazepam treatment was anxiolytic during general anxiety measures.

The above tests show that LS inhibition and diazepam are both anxiolytic but have opposite effects on motivation for cocaine. Both LS stimulation and diazepam have opposing effects on VTA DA neurons (Tan et al., 2010; Vega-Quiroga et al., 2017). To test whether diazepam could reverse the effects of LS inhibition on cocaine demand elasticity, we gave animals with stable BE behavior either 2 mg/kg diazepam or vehicle IP, followed by an intracranial microinjection of B-M or aCSF into LS 30 minutes later; animals were re-tested on a BE paradigm immediately after the intracranial injection. Animals' baseline α and Q_0 values did not differ across the four treatments (α: Shapiro-Wilk test, $W=0.7$, $p<0.01$; Friedman test, $Q=2.28$, $p=0.52$; Q_0: one-way repeated measures ANOVA, $F_{9,27}=1.68$, $p=0.20$). Microinjections of aCSF into LS and systemic diazepam (LS aCSF/diazepam) increased motivation, whereas LS B-M and systemic vehicle treatment (LS B-M/vehicle) decreased motivation relative to vehicle, consistent with our observations above (one-way repeated measures ANOVA; $F_{3,27}=13.4$,

$p<0.01$; Figure 2.5A). However, we found that motivation following LS B-M microinjections and systemic diazepam treatment (LS B-M/diazepam) did not differ from that following vehicle treatment (LS aCSF/vehicle; Bonferroni's multiple comparisons test, $p=0.99$). None of the manipulations altered Q_0 (one-way repeated measures ANOVA; $F_{3,27}=2.70$, $p=0.085$; Figure 2.5B).

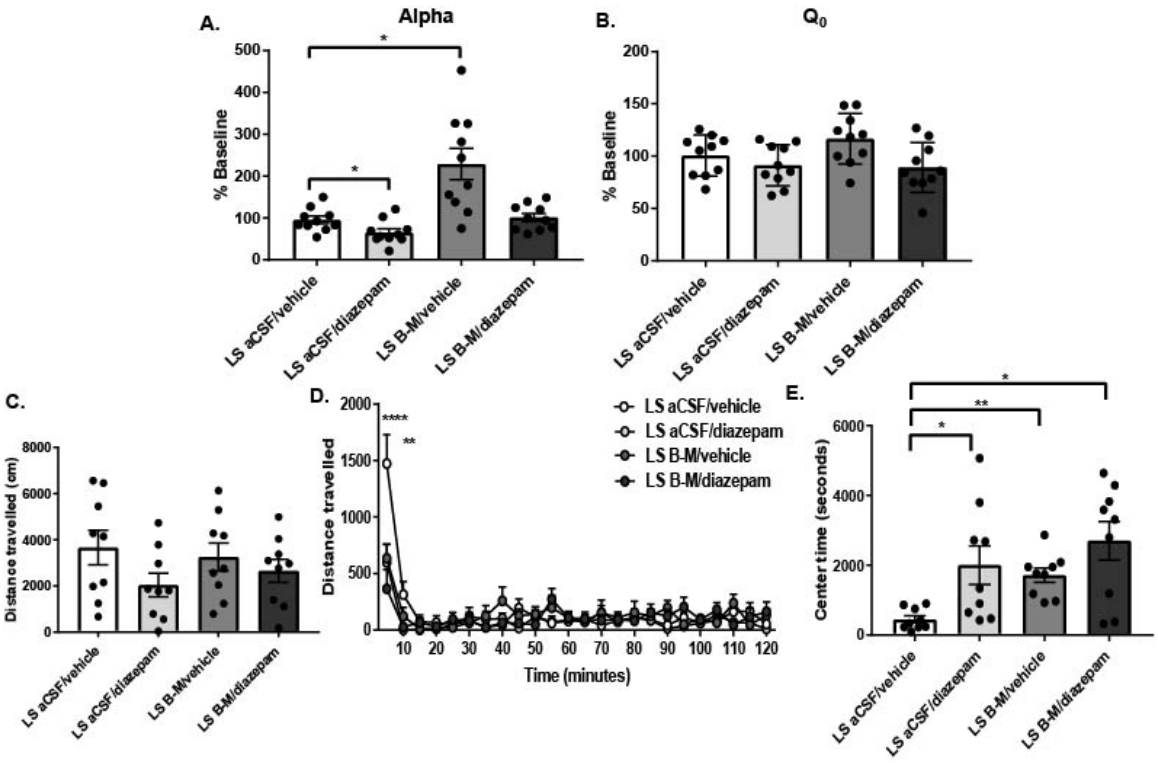

Figure 2.5. Diazepam reversed the effects of LS inhibition on motivation for cocaine, but not on anxiety. (A) Pretreatment with 2 mg/kg diazepam decreased cocaine demand elasticity (increased motivation; *p<0.05), whereas intra-LS B-M increased cocaine demand elasticity (decreased motivation; *p<0.05) compared to vehicle. In contrast, combined LS B-M/diazepam treatment did not significantly differ from vehicle treatment. (p>0.05) (B) No effects on free consumption (Q_0) were observed following any of these treatments. (C) None of the manipulations altered the total distance animals traveled during locomotor testing. (D) LS aCSF/diazepam, LS B-M/vehicle, and LS B-M/diazepam reduced locomotor activity in the first 5 minutes of testing (**p<0.01, ****p<0.0001). LS aCSF/diazepam and LS B-M/diazepam also reduced locomotor activity in the first 10 minutes of testing (**p<0.01). (E) Both diazepam (*p<0.05) and intra-LS B-M microinjections (***p<0.001) significantly increased total center time, as did the

combined treatment (**p<0.01). There was no difference between the combined treatment and LS aCSF/diazepam or LS B-M/vehicle (p>0.05). Bar graphs and error bars indicate mean ± SEM.

These manipulations also did not alter locomotor activity in a separate cohort of animals (one-way repeated measures ANOVA; $F_{3,24}$=2.36, p=0.10; Figure 2.5C). When locomotor data was binned in 5min time intervals, there was a significant effect of time ($F_{23,184}$= 18.5, p<0.0001) and an interaction between time x treatment ($F_{69, 184}$=3.37, p<0.0001, Figure 2.5D). In the first 5min of testing, animals had higher locomotor activity following LS aCSF/vehicle than after LS aCSF/diazepam (Bonferroni's multiple comparison test, p<0.0001), LS B-M/vehicle (p<0.0001), or LS B-M/diazepam (p<0.0001), or after 10 minutes with LS aCSF/diazepam (p<0.01) or LS B-M diazepam (p<0.01). All three manipulations significantly increased open field center time compared to vehicle (one-way repeated measures ANOVA; $F_{3,24}$=5.48; p<0.05; Figure 2.5E), although there was no additive effect of the combined LS B-M/diazepam treatment on center time when compared to either LS aCSF/diazepam (Bonferroni's multiple comparisons test, p=0.99) or LS B-M/vehicle (Bonferroni's multiple comparisons test, p=0.70).

Discussion

Here, we extend recent work on the role of LS in addiction to show that LS is a critical region for effortful responding for cocaine. We found that pharmacological inhibition of LS reduced motivation for cocaine, as measured by

demand elasticity, in the BE paradigm. These effects were not present after intra-LS microinjections of aCSF or B-M dorsal to LS, indicating that changes in motivation were due specifically to inhibition of LS neurons. LS B-M microinjections did not alter free consumption of cocaine (Q_0). Effects of LS inhibition were not likely due to anxiolytic actions, as the anxiolytic diazepam and LS inhibition had similar effects on anxiety but opposing effects on motivation for cocaine. The reduction in cocaine motivation following LS inhibition was blocked with pretreatment of diazepam. These results demonstrate that LS inhibition reduces cocaine demand elasticity by decreasing motivation for the drug.

LS inhibition increases cocaine demand elasticity

Previous studies reported that LS is an important region for cocaine seeking, yet it was unclear how the region contributes to this behavior. Our lab and others have implicated LS in stimulus-associated drug seeking including cue- or context-induced reinstatement of extinguished cocaine seeking, or CPP (Franklin & Druhan, 2000; Harasta et al., 2015; Luo et al., 2011; McGlinchey & Aston-Jones, 2017; Sartor & Aston-Jones, 2012). Most recently, our lab found that inhibiting LS with B-M (as in in the present studies) attenuated both context- and cue-induced reinstatement of cocaine seeking. We also showed that hippocampal input to LS drove context-induced reinstatement behavior (McGlinchey and Aston-Jones, 2017), implicating LS in cue- and context-associated cocaine seeking and relapse. To our knowledge, ours is the first study to demonstrate that LS also specifically

mediates motivation for cocaine. In light of these studies, we hypothesize that inhibition of the region reduces the motivational properties of cocaine.

We saw no effect of LS B-M on general locomotor activity, indicating that the effects of LS inhibition on motivation were not due to sedation. These results are also consistent with a previous study from our lab showing no change in general locomotor activity following intra-LS B-M microinjections (McGlinchey & Aston-Jones, 2017). We tested animals on FR-1 responding for sucrose to determine the effects of inhibition on cocaine demand elasticity were due to impaired lever-pressing. We observed no decrease (and in fact an increase) in active lever responding for sucrose, indicating that LS inhibition did not compromise animals' ability to lever-press. These results agree with recent studies showing that LS inhibition promotes feeding behavior (Sweeney & Yang, 2015, 2016). Our data support a role for LS in motivation for cocaine; future studies are needed to examine whether LS is also involved in effortful responding for natural rewards.

Pretreatment with diazepam increases motivation for cocaine

Human cocaine addicts will take benzodiazepines to alleviate the anxiogenic effects of cocaine withdrawal (Motta-Ochoa, Bertrand, Arruda, Jutras-Aswad, & Roy, 2017). However, benzodiazepines have their own addictive properties, which make them poorly suited to treat addiction. In contrast to the effects on drug taking following LS inhibition, we observed that diazepam at the 2 mg/kg dose decreased α (increased motivation) and Q_0 in the BE paradigm. These

results may be due either to diazepam's ability to ameliorate cocaine's anxiogenic properties or to its ability to facilitate reward (Ettenberg & Geist, 1991; Geist & Ettenberg, 1997; Paine, Jackman, & Olmstead, 2002; Reynolds et al., 2012; Straub et al., 2010). Our results support the latter hypothesis for two reasons. If diazepam reduced anxiety during BE performance, we would expect to see enhanced consumption in early BE bins, reflected by increased Q_0. However, the trend towards a reduction in Q_0 after diazepam indicates that animals need less drug to reach satiety, pointing to elevated rewarding effects of cocaine (Caine & Koob, 1994; Koob, Le, & Creese, 1987; Maldonado, Robledo, Chover, Caine, & Koob, 1993). Also, if LS inhibition and diazepam treatment both acted on cocaine demand elasticity by reducing anxiety, we would expect to see similar effects on drug taking as both were found to be anxiolytic. However, the two had opposing effects on demand elasticity and similar effects on general anxiety. Our results do not discount that diazepam reduces drug-induced anxiety but provide support for the idea that the increased motivation it causes is due instead to enhanced activation of dopaminergic reward circuits following benzodiazepine treatment.

Animal studies have predominantly attributed diazepam's actions on cocaine self-administration to its role as an anxiolytic. It has been reported that diazepam increases cocaine self-administration across sessions or within a single session (David et al., 2001; Maier et al., 2008). In addition, diazepam has been shown to decrease the latency to start the self-administration session or to enter a goal box to receive cocaine infusions (Ettenberg & Geist, 1991; Geist & Ettenberg, 1997; Maier et al., 2008). Still, other studies have shown that benzodiazepines

decrease cocaine intake (Augier, Vouillac, & Ahmed, 2012; Goeders, McNulty, & Guerin, 1993; Weerts, Froestl, & Griffiths, 2005). These differences may be dependent on dose or the type of benzodiazepine administered. Lower doses of diazepam similar to those utilized in our study have consistently been shown to increase cocaine self-administration (David et al., 2001; Maier et al., 2008). We only observed changes in cocaine motivation and anxiety with a higher dose (2 mg/kg) of diazepam, which has also been shown to increase addiction-like behaviors without producing excessive sedation (Geist & Ettenberg, 1997; Molander et al., 2011). We administered diazepam acutely, which is sufficient to increase activity of VTA DA neurons (Heikkinen et al., 2009). It remains unclear how chronic treatment would impact performance in the BE paradigm. Previous studies highlighting diazepam's role as an anxiolytic have shown the largest increases in cocaine intake occur late in the self-administration session (David et al., 2001) and the greatest decreases in latency to start the self-administration session after two days of diazepam treatment (Maier et al., 2008). Therefore, investigation of the effects of chronic treatment on cocaine intake, particularly in the early bins (and in Q_0), or the latency to start the session, might point to an anxiolytic role for diazepam in effects on motivation for cocaine.

Increased lever pressing during BE following diazepam treatment is not due to sedation

Diazepam also has sedative properties and can attenuate locomotor activity (Soderpalm, Svensson, Hulthe, Johannessen, & Engel, 1991). Although both

doses of diazepam used in this study reduced general locomotor activity, FR-1 responding for sucrose increased. Moreover, following diazepam treatment the number of presses for sucrose was greater than during BE, indicating that diazepam did not prevent lever-pressing behavior. The increase in lever-pressing for sucrose is consistent with previous studies demonstrating that diazepam increases sucrose palatability and consumption (Pecina & Berridge, 1996; Treit & Berridge, 1990), which is opioid-dependent and can be blocked with the opioid receptor antagonist naltrexone (D. K. Richardson, Reynolds, Cooper, & Berridge, 2005). Taken together, these results indicate that although sedation by diazepam reduces general locomotor activity, it does not interfere with lever pressing.

Diazepam reverses the effects of LS inhibition on cocaine demand elasticity

LS inhibition and diazepam treatment produced opposing effects on drug taking, and diazepam pretreatment blocked the effects of LS inhibition on motivation for cocaine. A possible mechanism for these differences is through different effects on the activity of VTA DA neurons. Diazepam's rewarding/reinforcing effects are thought to occur through its binding to the $GABA_{A\alpha1}$ receptor on VTA GABAergic neurons, thereby disinhibiting VTA DA neurons (Heikkinen et al., 2009; Tan et al., 2010). Other receptor subunits of the $GABA_A$ receptor, including the α2 and α3 subunits, have also been implicated in diazepam's rewarding effects (Reynolds et al., 2012). (Mahler et al., 2018). Recently, LS stimulation was found to promote disinhibition of DA neurons via the $GABA_{A\alpha1}$ receptor on VTA GABAergic neurons (Vega-Quiroga et al., 2017). Our

laboratory found that inhibiting LS input to VTA reduces context-induced reinstatement, indicating that LS disinhibition of VTA DA neurons is an important connection that drives drug seeking (Luo et al., 2011). Therefore, we hypothesize that the inhibitory LS to VTA GABAergic interneuron pathway may be downregulated following LS inhibition, but potentiated by diazepam treatment.

LS inhibition and diazepam similarly reduce anxiety

Both LS inhibition and diazepam decreased anxiety-like behaviors, as indicated by increased center time in the open field and open arm time in the elevated plus maze, and combined treatment did not have an additive effect on anxiolytic behaviors. Experiments investigating the role of LS on anxiety have yielded differing results. Optogenetic inhibition of LS neurons containing the Crf2 receptor was anxiolytic and reduced corticosterone levels (Anthony et al., 2014). However, other studies found that inhibiting or lesioning LS was anxiogenic, giving rise to the conclusion that LS neuronal activity is anxiolytic (Sheehan et al., 2004). These differences across studies may result from the subpopulations of LS neurons impacted by each study's manipulation. Notably, early experiments linked the anxiolytic properties of LS to those seen after benzodiazepine treatment (Clarke & File, 1982; Drugan, Skolnick, Paul, & Crawley, 1986; Yadin, Thomas, Grishkat, & Strickland, 1993). We observed a similar but non-additive effect of LS inhibition and diazepam on anxiety-like behaviors, indicating that diazepam may act on LS neurons to reduce anxiety. Although it is possible that LS neurons

mediate some of the anxiogenic properties of cocaine, our results provide strong evidence for a role for LS in motivation for cocaine.

Taken together, our study implicates LS in promoting high effort cocaine seeking, and points to an important role for LS in motivation for cocaine. Inhibiting LS prior to BE testing reduced motivation for cocaine (increased α) without impacting low effort consumption (Q_0) or general locomotor activity. These findings add to a growing literature that LS neurons contribute to cue-dependent, motivated drug seeking. The benzodiazepine diazepam blocked the effects of LS inhibition on motivation for cocaine, pointing to opposing roles for the two manipulations on VTA DA neurons. Collectively, these studies indicate that LS regulates motivated drug taking, making it an attractive region to manipulate for the treatment of addiction.

Chapter 3: The number of lateral hypothalamus orexin/hypocretin neurons contributes to individual differences in cocaine demand

This chapter has been adapted from the manuscript, Pantazis CB; James, MH; Bentzley, BS; & Aston-Jones G. “The number of lateral hypothalamus orexin/hypocretin neurons contributes to individual differences in cocaine demand” (2019). In revision at Addiction Biology and available on bioRxiv.

Abstract

Lateral hypothalamus (LH) orexin neuron signaling has been implicated in the motivation to seek and take drugs of abuse. The number of LH orexin neurons has been shown to vary with behavioral state and can be upregulated with exposure to drugs of abuse. We sought to determine if the number of LH orexin neurons related to individual differences in motivation (demand) for cocaine in our behavioral economics (BE) paradigm, and whether knockdown of these cells predicted changes in economic demand. We quantified LH orexin cell numbers in animals immediately following our BE paradigm, as well as BE-experienced animals after a two-week period of abstinence to relate the number of LH orexin cells to economic demand for cocaine. We also unilaterally knocked down LH orexin expression prior to BE with an orexin morpholino antisense to determine how orexin knockdown impacted cocaine demand. We found that animals with greater motivation for cocaine (lower demand elasticity) had more LH orexin neurons. Following a two-week abstinence from BE, the number of LH orexin neurons predicted demand for cocaine prior to abstinence. Reducing LH orexin cell numbers with antisense decreased motivation for cocaine (increased demand

elasticity) without affecting baseline consumption. In addition, the number of spared LH orexin neurons correlated with individual demand for cocaine. These studies point to a role for the endogenous number of LH orexin neurons as a critical mediator of individual differences in motivation for cocaine.

Introduction

Orexin A and B (also called hypocretin 1 and 2) are peptides synthesized by a small population of neurons in posterior hypothalamus, ranging mediolaterally from dorsomedial hypothalamic nucleus (DMH), through perifornical hypothalamus (Pef), to lateral hypothalamus (LH) (de Lecea et al., 1998; Sakurai et al., 1998). Orexin A binds both orexin-1 and -2 receptors (OxR1 and OxR2), whereas orexin B preferentially binds OxR2 (Zhu et al., 2003). Orexin neurons interact closely with the mesolimbic dopamine (DA) system to drive motivation and addiction (Borgland, Taha, Sarti, Fields, & Bonci, 2006; Espana et al., 2010; Fadel & Deutch, 2002; James et al., 2011; Mahler et al., 2014; Mahler, Smith, & Aston-Jones, 2013; Mahler et al., 2012). Many studies now indicate that blockade of OxR1 signaling attenuates cocaine seeking behaviors, particularly under high effort conditions (for review, (James, Mahler, et al., 2017), indicating a role for OxR1 signaling in the motivational properties of cocaine.

A major goal of addiction research is to elucidate the neural substrates that contribute to addiction vulnerability. We previously found that the degree to which drug-associated cues and contexts activate the LH subpopulation of orexin neurons predicts individual differences in drug seeking. Fos activation of LH, but

not Pef/DMH, orexin neurons correlated with reinstatement of extinguished cocaine or morphine preference (Harris et al., 2005). LH orexin Fos expression also correlated with preference for, and context-induced reinstatement of, ethanol seeking (Moorman, James, Kilroy, & Aston-Jones, 2016). Further, rats that exhibited a multifaceted addiction phenotype following intermittent access (IntA) to cocaine exhibited higher Fos expression selectively in LH orexin neurons following re-exposure to the self-administration context relative to short access control rats (M. H. James, C. M. Stopper, et al., 2018). OxR1 blockade is particularly effective at reducing drug-seeking in highly motivated animals (James, Bowrey, Stopper, & Aston-Jones, 2018; M. H. James, C. M. Stopper, et al., 2018; Jupp, Krstew, Dezsi, & Lawrence, 2011; Lawrence, Cowen, Yang, Chen, & Oldfield, 2006; Lopez, Moorman, Aston-Jones, & Becker, 2016; Moorman & Aston-Jones, 2009; Moorman, James, Kilroy, & Aston-Jones, 2017), further indicating that individuals with high propensity for drug seeking may have elevated stimulus-driven LH orexin cell activity.

Although it is clear that the activity of LH orexin neurons is important for drug seeking, emerging evidence indicates that upregulation of orexin expression may also be involved. We reported that the persistent IntA-induced addiction phenotype was associated with higher numbers of orexin-expressing neurons in LH but not Pef/DMH (M. H. James, C. M. Stopper, et al., 2018). Another recent study found that postmortem tissue from heroin addicts contains higher numbers of orexin-expressing neurons compared to healthy controls, and mice exposed to chronic non-contingent morphine injections had increases in the number of orexin

neurons particularly in LH (Thannickal et al., 2018). These studies are consistent with an earlier study that reported that chronic alcohol consumption increased the area of prepro-orexin mRNA expression preferentially in LH of alcohol-preferring rats (Lawrence et al., 2006). Changes in the number of orexin-expressing LH neurons might reflect individual differences in drug seeking that result from 'state' factors like extended or binge-like drug access. However, it is unclear whether variability in the baseline (non-stimulated) numbers of orexin-expressing LH neurons contributes to trait differences in addiction behavior.

We sought to determine if differences in the numbers of LH orexin neurons contributes to individual differences in baseline or 'trait' motivation for cocaine using our within-session behavioral economics (BE) paradigm (Bentzley et al., 2013; Bentzley et al., 2014). In this paradigm, cocaine consumption is measured at increasing price points. By fitting the resulting data using an exponential demand equation (Hursh & Silberberg, 2008), it is possible to calculate demand elasticity (α), or the extent to which consumption changes with price. We previously reported that α predicts several addiction-relevant behaviors, including compulsive (punished) responding for cocaine, drug seeking during initial abstinence, and cue-induced reinstatement of extinguished drug seeking (Bentzley et al., 2014; M. H. James, H. E. Bowrey, et al., 2018). Here, we show that the number of orexin neurons in LH, but not Pef/DMH, correlates with motivation for cocaine (α), and this relationship persists after abstinence. In addition, unilateral LH orexin knockdown with orexin-A morpholino antisense is sufficient to increase demand elasticity (reduce motivation), and the degree of knockdown predicts the extent of

motivation reduction. Collectively, these studies indicate that the number of LH orexin neurons is a potent predictor of addiction vulnerability.

Methods

Animals

Adult male Sprague-Dawley rats (300-325 grams) were pair-housed on a reverse 12-hour light:dark cycle in a temperature- and humidity-controlled animal facility at Rutgers University or Medical University of South Carolina (MUSC) with ad libitum access to standard rat chow and water. Upon arrival, animals were acclimated to the colony room for 2 days and handled for at least 3 days prior to surgery. All protocols and animal care procedures were approved by the Institutional Animal Care and Use Committee at Rutgers University-New Brunswick or Medical University of South Carolina

Drugs

Cocaine HCl powder was obtained from the National Institute of Drug Abuse (Research Triangle Park, NC) and dissolved in 0.9% sterile saline.

Intravenous catheter surgery

After handling for a minimum of 3 days, rats were anesthetized with a ketamine/xylazine (56.5/8.7 mg/kg, i.p., respectively) mixture and also given an analgesic (rimadyl at 5 mg/kg or meloxicam at 1 mg/kg, s.c.). Rats were implanted with indwelling catheters into the jugular vein for iv infusion of cocaine. For morpholino experiments, cannulae implantation occurred immediately followed catheterization. Cefazolin (0.1 ml; 100 mg/ml) and heparin (0.1 ml; 100 U/ml) were

flushed through the iv catheter after surgery, and after each self-administration session. Following a one-week recovery after surgery, animals were trained to self-administer iv cocaine.

Cocaine self-administration

Rats were trained on a fixed ratio-1 (FR-1) cocaine self-administration paradigm (20-second timeout post-infusion) as previously described (McGlinchey, James, Mahler, Pantazis, & Aston-Jones, 2016). Sessions occurred in operant chambers contained in sound-attenuating boxes with Med-PC IV software (Med Associates). During training sessions, cocaine infusions (0.19 mg cocaine/infusion) were paired with discrete light and tone cues (white stimulus light above the active lever; 78-dB, 2900-Hz tone). After reaching criteria, ($\geq$ 10 infusions/session for 10 sessions), animals were trained on a BE procedure.

Behavioral Economics

Following FR-1 training, rats were trained on a within-session BE procedure, as described previously (Bentzley et al., 2013; Bentzley et al., 2014). During the 110-minute session, the price of cocaine increased in successive 10-minute intervals on a quarter logarithmic scale (383.5, 215.6, 121.3, 68.2, 38.3, 21.6, 12.1, 6.8, 3.8, 2.2, 1.2 μg cocaine per infusion). Lever pressing responses were fit to a demand curve, as described in a previous paper from our lab (Bentzley et al., 2013). From the demand curve, we derive consumption at low effort (Q_0) and demand elasticity (α, the rate of decline in consumption as price increases). Q_0 is low effort consumption extrapolated to the y-axis of the demand curve, where cocaine price approaches null; α is the decay constant of this curve. Thus, α scales

inversely with motivation, so high motivation animals have lower α values. Animals were trained on BE for a minimum of six days. Testing occurred when animals displayed stable behavior on the BE paradigm (Q_o and α values $\leq$ 30% variability across the last three sessions).

Tissue preparation for immunohistochemistry

Animals were deeply anesthetized with a ketamine/xylazine mixture and transcardially perfused with 0.9% sterile saline then 4% paraformaldehyde. Brains were dissected and postfixed in 4% paraformaldehyde, then 20% sucrose-PBS azide solution. Brains were frozen with dry ice and sectioned at 40 um on a cryostat. Sections were collected in PBS azide.

Immunohistochemistry

To visualize orexin neurons, hypothalamic tissue was incubated in goat anti-orexin A (1:500, Santa Cruz Biotechnology) in 5% NDS at room temperature overnight. The following day, tissue was incubated in biotinylated donkey anti-goat secondary antibody (1:500, Jackson ImmunoResearch Laboratories) for 1.5 hours, followed by avidin-biotic complex (1:500) for 1.5 hours. After washing, sections were stained with DAB (Sigma) in Tris buffer. Tissue was mounted on slides, dehydrated, and coverslipped with DPX mounting medium (Electron Microscopy Sciences).

Cell quantification

Coronal lateral hypothalamus images were taken across the orexin cell field (2.5-3.8 mm caudal to bregma) with a Zeiss Axio Zoom V16 microscope (Paxinos and Watson, 2007). Tiled photographs were compiled at 20x magnification using

Zen 2 imaging software (Carl Zeiss Microscopy). Orexin A- and Fos-immunopositive neurons were quantified in both hemispheres with Adobe Illustrator by an observer blind to experimental conditions. Three sections were taken per animal, as in our previous studies (M. H. James, C. M. Stopper, et al., 2018; Mahler & Aston-Jones, 2012; Moorman et al., 2016). The sum of neurons counted in both hemispheres in each section were determined and averaged across the three sections. To determine the extent of orexin A knockdown, the percent change in injected vs. non-injected hemispheres for each section was averaged across the three sections.

Experiment 1: Relationship between orexin cell numbers and economic demand for cocaine

We first sought to examine the relationship between endogenous orexin cell numbers and baseline economic demand for cocaine. To do this, animals were trained on the BE paradigm as above until they displayed stable behavior. The following day, rats were again tested on the BE paradigm and perfused 90 min after the point of maximum responding (Pmax), defined as the price at which maximum responding occurred. Tissue was processed for orexin A immunohistochemistry and cell quantification; cell counts were compared to demand measures from the final BE test session.

Experiment 2: Effect of orexin cell knockdown on economic demand for cocaine

Stereotaxic surgery

We next investigated the impact of knocking down LH orexin expression using orexin-A morpholino antisense on demand for cocaine. Immediately following catheter implantation (described above), animals were secured in a stereotactic frame (Kopf, Tujunga, CA, USA) and implanted with a unilateral stainless steel guide cannula (22 gauge, 11 mm, Plastics One, Roanoke, VA, USA) 2 mm dorsal to LH (coordinates relative to bregma: -3.0 mm AP, +/- 2.6 mm ML, -6.8 to -7.4 mm DV). We varied the dorsal-ventral cannula coordinates to produce varying degrees of knockdown in morpholino-infused animals. The implanted hemisphere was counterbalanced across all tested animals, such that an equal number of animals were implanted in the left vs. right hemisphere. Acrylic cement and jeweler screws were used to secure the cannula to the skull surface.

Orexin morpholino antisense microinjections

One day after animals displayed stable behavior on the BE paradigm, an injector cannula was unilaterally lowered into LH (2 mm below the guide cannula) to infuse orexin-A morpholino antisense (Vivo-Morpholino; 150nmol/0.3 uL in 0.5mM phosphate buffer, Gene Tools, 5'-GTATCTTCGGTGCAGTGGTCCAAAT-3') or an inverted orexin control missense (Vivo-Morpholino, 5'-TAAACCTGGTGACGTGGCTTCTATG-3'). The injector cannula was kept in place for 1 minute before infusing the morpholino for 2 min. Injectors were then kept in place for 1 minute to reduce diffusion of the morpholino. All animals were infused between 14:00-15:00h, during their active period. Animals were tested on BE for 6 d following morpholino microinjection, and perfused on day 6 when peak orexin knockdown occurs (Reissner et al., 2012; Sartor & Aston-Jones, 2012). Animals

were perfused with fixative, brains were sectioned, sections were stained for the orexin A protein, and orexin-positive cells were quantified as above. By restricting morpholino injections to a single hemisphere, we were able to simultaneously: i) confirm the efficacy of the antisense by comparing the number of orexin cells in the injected and uninjected hemispheres; ii) examine the effect of reducing overall orexin numbers on demand for cocaine; and iii) examine the relationship between endogenous orexin cell numbers (in the uninjected hemisphere) with demand prior to antisense injections.

Brain sections adjacent to those used for orexin A staining were dehydrated and Nissl-stained with neutral red. Sections were coverslipped with DPX mounting medium (Electron Microscopy Sciences) to determine cannula location.

Experiment 3: Relationship between LH orexin cell number and demand following abstinence

To determine whether the relationship between LH orexin cell numbers and demand persisted after abstinence, we sacrificed animals with a history of cocaine self-administration two weeks after their final BE test. Animals were first trained on the BE procedure until behavior was stable, as above. Cocaine self-administration was then discontinued, and rats were then tested for locomotor activity in locomotor chambers (clear acrylic, 40 x 40 x 30 cm) equipped with Digiscan monitors (AccuScan Instruments) in 2 h sessions, 5d/wk for 2 wk. During these 2 wks, rats received ip injections of up to 3 compounds (propranolol, prazosin, clonidine) as a control for a separate study. At least 2d washout was given between

treatments and before the final sacrifice. Horizontal, vertical, and total locomotor activity was recorded using beam beaks. Rats were perfused immediately after the final habituated locomotor test for orexin cell quantification.

Data analysis

Statistical analyses were performed with GraphPad Prism 7, except for multivariate regressions, which were performed using SPSS Statistics (Version 22). Baseline α and Q_0 values were determined by averaging performance across the three days preceding testing. In Experiment 1, a median split of α was used to determine high demand elasticity vs. low demand elasticity animals, and a median split of Q_0 identified high takers vs. low takers. Unpaired samples t-tests were used to compare differences in LH orexin neuron numbers between high and low demand elasticity, or high and low Q_0 animals. In Experiment 2, a two-way ANOVA was used to compare BE performance following morpholino treatments. Demand associations with orexin cell counts were determined using multiple linear regression with log10(α) and log10(Q_0) set as independent variables with cell count as the dependent variable. In Experiment 3, an unpaired samples t-test was used to compare LH orexin neuron numbers in animals subjected to BE vs. locomotor testing. Pearson correlations were used for all univariate relations to compare orexin neuron numbers with total locomotor activity or α values. All statistics were two-tailed. A Shapiro-Wilk normality test determined that all data were parametric.

Results

Experiment 1: Animals with high motivation for cocaine have more LH orexin-expressing neurons.

Animals (n=12) were trained on the BE paradigm and divided into high vs. low demand elasticity based on a median split of α values (representative demand curves shown in Figure 3.1A). Low demand elasticity (high motivation) animals had significantly lower α values than high demand elasticity (low motivation) animals (Figure 3.1B, t_{10}=3.53, $p<0.01$). Low demand elasticity animals also had more orexin-immunopositive cells in lateral hypothalamus (LH), compared to high demand elasticity animals (Figure 3.1D, t_{10}= 2.32, $p<0.05$). No such differences were observed for perifornical area/dorsomedial hypothalamus (Pef/DMH) orexin neurons (Figure 3.1E, t_{10}=1.138 p=0.282). When animals were instead divided based on their preferred level of consumption at low effort (Q_0), there was no difference in the number of LH (Figure 3.1F, t_{10}=0.29, p=0.776) or Pef/DMH (Figure 3.1G, t_{10}=0.068 p=0.947) orexin neurons between high- and low-takers.

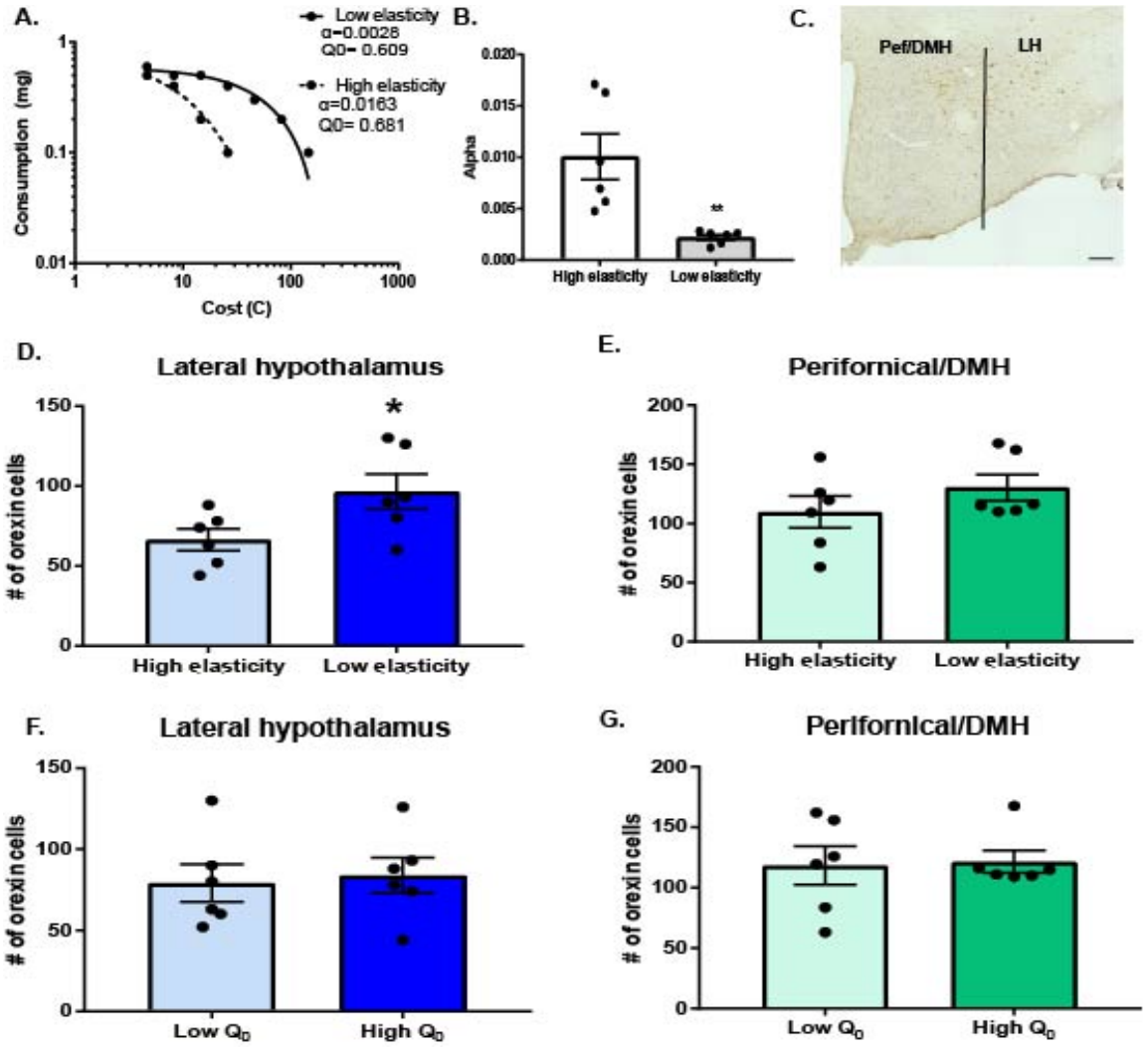

Figure 3.1. Number of LH orexin neurons is elevated in low demand elasticity (high motivation) animals. A. Representative demand curves from high (dashed line) and low (solid line) demand elasticity animals with similar baseline consumption (Q0). B. Low demand elasticity (high motivation) animals had significantly lower α values than high demand elasticity animals (unpaired samples t-test, **p<0.01). C. Representative section of hypothalamus stained for orexin from an animal sacrificed 90 minutes post-Pmax. Scale bar denotes 200 μm. D. Low demand elasticity (high motivation) animals had more LH orexin neurons, compared to high demand elasticity (low motivation) animals (n=6/group, unpaired

samples t-test, *p<0.05). Bilateral orexin neurons in Pef/DMH and LH were counted separately and averaged across three sections per animal, representing the rostral-caudal extent of the orexin cell field for this panel and for panels E-G. E. High and low demand elasticity animals had similar numbers of Pef/DMH orexin cells (unpaired samples t-test, ns). F. No differences were observed in LH orexin numbers in high Q0 compared to low low Q0 (unpaired samples t-test, ns). G. High and low Q0 animals did not differ in the number of Pef/DMH orexin cells (unpaired samples t-test, ns).

Experiment 2: Unilateral knockdown of LH orexin neurons reduces cocaine demand elasticity

We next examined whether the number of LH orexin neurons plays a causal role in demand by testing whether LH orexin knockdown would reduce motivation for cocaine (increase α). In a separate cohort of animals, we unilaterally infused an orexin-A morpholino antisense (n=9) or control missense (n=8) into LH after animals displayed stable BE behavior. Injection sites are depicted in Figure 3.2A.

Unilateral infusion of orexin antisense reduced orexin cell numbers

In rats with LH morpholino injection, we observed a significant reduction (~35%) in the number of orexin-expressing neurons in LH (Figure 3.2B,C; unpaired t-test; t_{15}=3.55; $p<0.01$), but no change in orexin expression in Pef/DMH (Figure 3.2B,D; unpaired t-test; t_{15}=0.49; ns). The number of orexin neurons in the uninjected hemisphere of morpholino-infused animals was similar to that observed in animals from Experiment 1, indicating no compensatory effects from morpholino treatment in the contralateral hemisphere (data not shown; unpaired t-test; t_{19}=1.084; ns). Orexin-A morpholino antisense microinjections produced varying degrees of knockdown in these animals, depending on the location of cannula. We observed that antisense-infused animals with cannula targeted dorsal to the top of the fornix had a knockdown of ~25% of orexin cell numbers compared to the uninjected hemisphere, and this was statistically significant (n=5) (data not shown, unpaired t-test, t_8= 2.69, $p<0.05$). This knockdown was proportional to the knockdown observed in our previous publication (M. H. James, C. M. Stopper, et al., 2018) where injections were made bilaterally into the same dorsal region. The

degree of orexin knockdown was slightly greater in animals with injections directed ventral to fornix.

Unilateral infusion of antisense reduced motivation

A representative demand curve 6d following orexin-A morpholino antisense in LH unilaterally is compared to the baseline demand (prior to antisense injection; within-subject) in Figure 3.2E. Overall, unilateral orexin-A antisense into LH significantly increased α (decreased motivation) (Figure 3.2F, two-way RM ANOVA, main effect of treatment: $F_{1,16}$= 16.61, $p<0.001$; interaction of treatment x morpholino type: $F_{1,16}$=5.52, $p<0.05$). No changes were observed in Q_0 for either orexin-A antisense or control missense-infused animals (Figure 3.2G, two-way RM ANOVA, ns). Of note, the change in α observed here following unilateral injections of morpholino was roughly half that reported in our previous study using bilateral LH orexin knockdown, and this trended towards significance (data not shown, paired samples t-test, t_4=2.67, $p=0.056$).

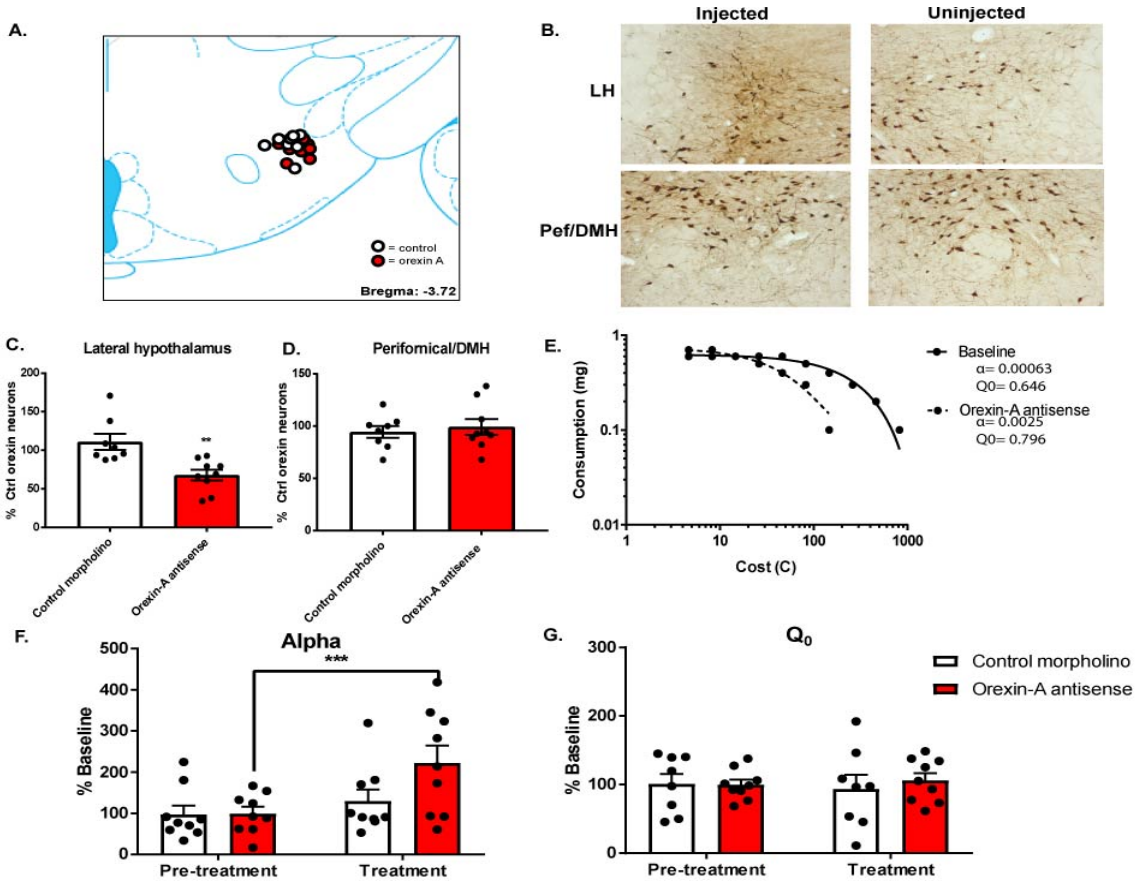

Figure 3.2. Knockdown of LH orexin-expressing neurons with unilateral morpholino antisense decreases demand for cocaine. A. Schematic representation of LH cannula placements for control missense- (n=8, white circles) and orexin-A antisense- (n=9, red circles) infused animals. B. Representative images of hypothalamus stained for orexin-A (brown) following a unilateral orexin-A antisense-infusion. The uninjected hemisphere was used as a within-subjects control. The top left panel indicates LH orexin cells in the injected hemisphere, top right LH orexin cells in the uninjected hemisphere. The bottom left panel indicates Pef/DMH cells in the injected hemisphere, whereas the bottom right panel shows those cells in the uninjected hemisphere. Scale bar denotes 200 μm. C. The percent reduction in orexin expression in orexin-A antisense-infused animals was

significantly greater than in the control missense-infused animals (unpaired t-test, $p<0.01$). D. Pef/DMH orexin expression was unaffected in orexin-A antisense and control missense-infused animals, compared to the uninjected hemisphere (unpaired t-test, ns). E. Sample demand curves for an antisense-infused animal at baseline (solid line) and following morpholino treatment (dashed line). F. Microinjection of orexin-A antisense increased α (decreased motivation) compared to baseline (red bars; Sidak's multiple comparisons test, ***$p<0.001$). No change was observed in control missense-infused animals (white bars; ns). G. There was no effect of treatment on Q0 in orexin-A antisense- (red bars) or control missense- (white bars) infused animals (Sidak's multiple comparisons test, ns).

The number of LH orexin neurons correlated with motivation for cocaine

Within antisense-infused animals, we observed a negative correlation between overall LH orexin cell numbers (summed across injected and non-injected hemispheres) and α values, such that animals with higher motivation (lower α values) had greater LH orexin+ neurons (Figure 3.3A, β=-0.62, $p<0.05$). In contrast, Q_0 values were not significantly correlated with the number of LH orexin cells (Figure 3.3B, β= 0.41, p=0.09). No associations were observed between the number of Pef/DMH orexin neurons and α (Figure 3.3C, β=-0.35, p=0.41) or Q_0 (Figure 3.3D, β=0.29, p=0.49).

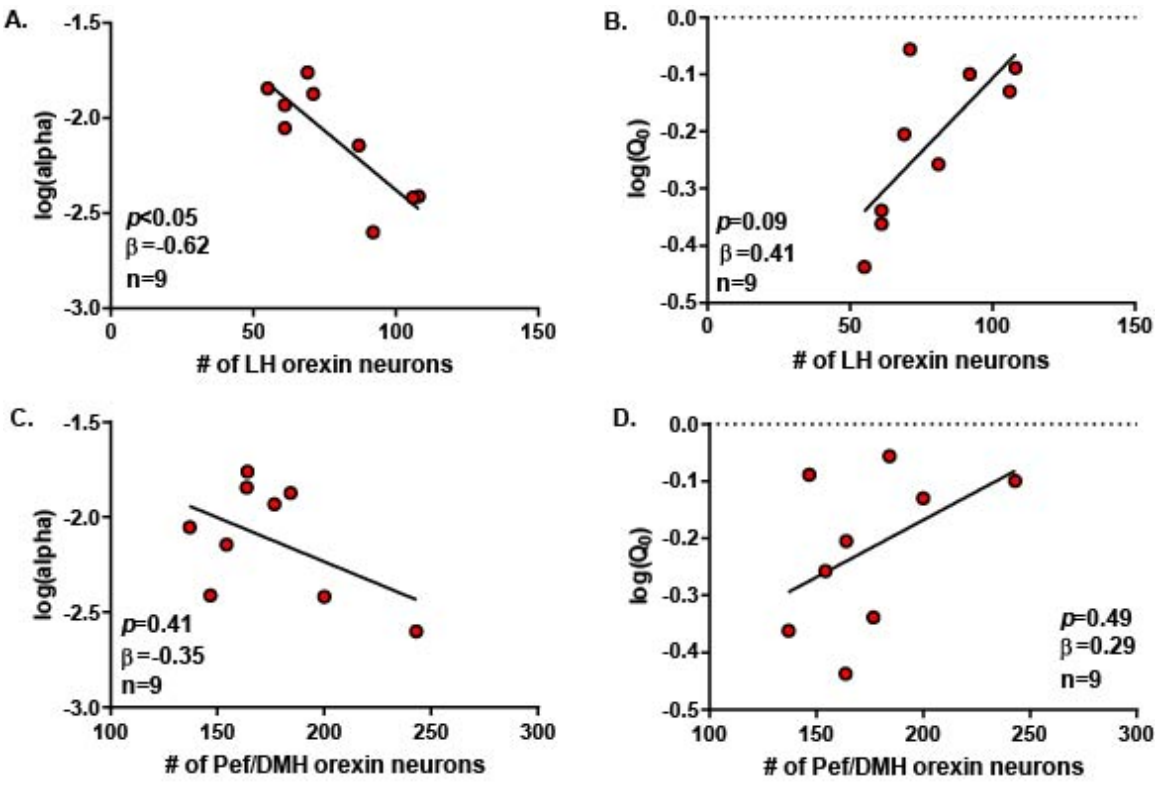

Figure 3.3 LH orexin neuron number predicts cocaine demand elasticity (α) and baseline consumption (Q0). A. The number of LH orexin neurons predicted α (multiple linear regression, $p<0.05$) but not B. Q0 values (multiple linear regression, ns). C. There was no relationship between Pef/DMH orexin cell number and α (multiple linear regression, ns) D. or Q0 values (multiple linear regression, ns).

We found that the percentage change in α correlated with the amount of LH orexin knockdown in antisense-injected animals when compared to the uninjected hemisphere, such that animals with a greater change in baseline α had a larger LH orexin cell knockdown (Figure 3.4A, β=-0.71, $p<0.05$). There was no such association between the change in Q_0 and LH orexin knockdown (Figure 3.4B, β=0.09, p=0.78).

Finally, we correlated baseline (pre-morpholino injection) cocaine demand elasticity (α) with the number of orexin-expressing neurons in the uninjected hemisphere (as a measure of endogenous orexin levels). Consistent with Experiment 1, there was a strong trend towards a negative correlation between these two measures, although this failed to reach statistical significance (Figure 3.4C, β=-0.40, p=0.33). There was no association between baseline Q_0 and LH orexin neurons in the uninjected hemisphere (Figure 3.4D, β=0.24, p=0.55).

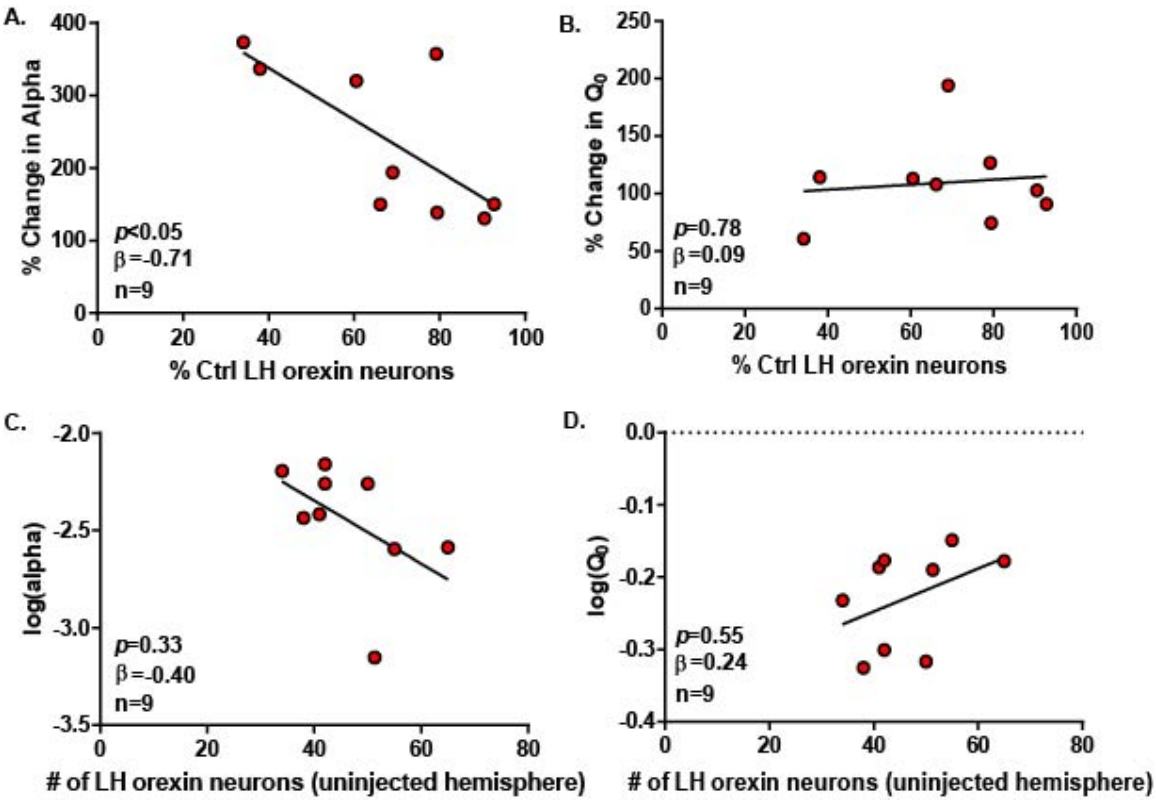

Figure 3.4. LH orexin neuron knockdown predicts antisense-induced changes in cocaine demand elasticity. A. Animals with greater LH orexin knockdown following antisense infusion, when orexin neurons in the injected hemisphere were compared to the uninjected hemisphere, had a larger change in α value (multiple linear regression, *p<0.05). B. The degree of LH orexin knockdown did not predict changes in Q0 post-antisense microinjection (multiple linear regression, ns). C. In antisense-infused animals, there was a non-significant relationship between the numbers of LH orexin neurons in the uninjected hemisphere and baseline (pre-antisense injection) α values (multiple linear regression, ns). D. The number of LH orexin neurons did not predict baseline Q0 in the uninjected hemisphere (multiple linear regression, ns).

Experiment 3: Relationship between LH orexin cell number and demand following abstinence

To determine if the relationship between LH orexin expression and demand persisted after abstinence or with locomotor activity, we quantified LH and Pef/DMH orexin cells in BE-experienced animals sacrificed immediately after locomotor testing which followed 2 wk of abstinence from cocaine self-administration (n=8). LH orexin cell number inversely correlated with animals' baseline α (measured 2 wk prior to sacrifice; Figure 3.5A, Pearson correlation, $p<0.05$), whereas Pef/DMH orexin expression did not (Figure 3.5B, Pearson correlation, $p= 0.770$). This demonstrates that LH orexin cell numbers during abstinence reflected prior individual differences in motivation for cocaine. We also observed that the number of LH orexin cells did not correlate with total locomotor activity in the test prior to sacrifice (Figure 3.5C, Pearson correlation, $p=0.982$). This reveals that individual differences in numbers of orexin-expressing neurons in Experiments 1 and 2 were not likely due to differences in general arousal/motor activity. We also found no difference in the number of LH orexin neurons in animals perfused following locomotor testing and animals Experiment 1 (Figure 3.5D, unpaired samples t-test, $t_{18}=0.123$, $p=0.903$).

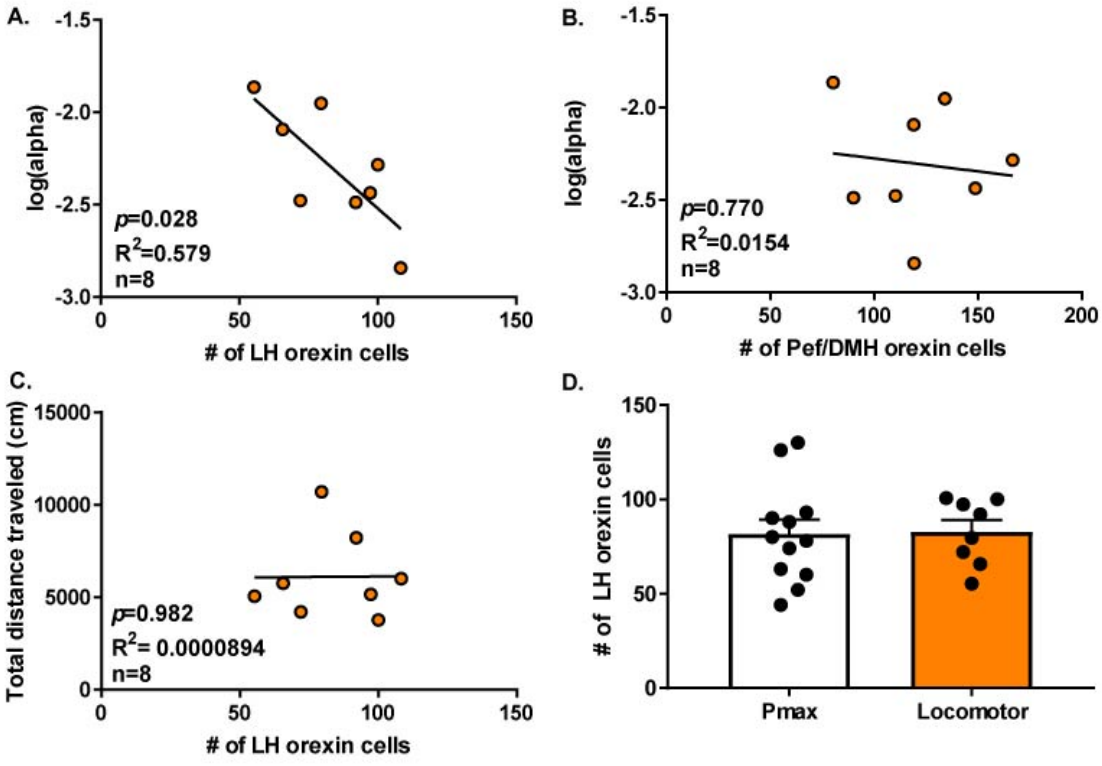

Figure 3.5. Numbers of LH orexin neurons predict individual differences in α values following abstinence from cocaine. A. LH orexin cell numbers at 2 weeks of cocaine abstinence correlated with animals' baseline α values measured two weeks previously (Pearson correlation, $p<0.05$). B. No relationship was observed between baseline α values and the number of Pef/DMH orexin cells (Pearson correlation, ns). C. The number of LH orexin neurons in locomotor-tested animals did not predict habituated locomotor activity (Pearson correlation, ns). D. Animals sacrificed immediately following locomotor testing (locomotor) had similar numbers of LH orexin neurons compared to animals sacrificed 90 minutes post-Pmax (unpaired samples t-test, ns).

Discussion

Here, we show that the number of orexin cells in LH predicts motivation (demand elasticity) for cocaine. A similar relationship was observed when tissue was collected from rats that underwent 2wk abstinence from cocaine. Differences in LH orexin number were not due to variability in arousal state, as orexin expression did not correlate with general locomotor activity. In addition, knockdown of orexin signaling reduced demand (α) but not low effort consumption of cocaine (Q_0), and degree of knockdown correlated with the change in α following knockdown, consistent with other studies from our lab (Bentzley & Aston-Jones, 2015; M. H. James, C. M. Stopper, et al., 2018). These studies are consistent with the view that orexin mediates the motivational properties of cocaine without influencing the hedonic value of the drug (James, Mahler, et al., 2017; Mahler et al., 2014). Taken together, our results indicate that the number of orexin-expressing neurons in LH is a determinant of individual differences in cocaine motivation.

High motivation animals have greater orexin expression in lateral hypothalamus

Across all three experiments, animals with more LH orexin cells had higher motivation for cocaine. In Experiment 1, high motivation (low demand elasticity) animals had greater LH orexin expression than low motivation animals (high demand elasticity) animals. In Experiment 2, the number of remaining LH orexin cells following orexin-A morpholino antisense knockdown correlated with demand

elasticity for cocaine, and the change in LH orexin cell number correlated with the change in α value. The relationship between demand elasticity and LH orexin neuron number does not require the presence of the drug or drug-paired context because in Experiment 3, the number of LH orexin cells in animals sacrificed immediately after general locomotor testing correlated inversely with cocaine demand elasticity 2 wk prior. Although other studies reported that chronic exposure to drugs of abuse upregulated numbers of LH orexin-expressing cells (M. H. James, C. M. Stopper, et al., 2018; Lawrence et al., 2006; Thannickal et al., 2018), to our knowledge our results are the first to show a relationship between the endogenous number of LH orexin cells and baseline addiction propensity. Taken together, these results are consistent with our hypothesis that orexin signaling is particularly important in highly motivated individuals (M. H. James, H. E. Bowrey, et al., 2018), and that the number of LH orexin neurons could serve as a biomarker of addiction susceptibility.

We proposed that orexin signaling translates motivational drive into behavioral output (Mahler et al., 2014), particularly in response to drug-associated cues and contexts (Bentzley & Aston-Jones, 2015; Smith, See, & Aston-Jones, 2009; Smith, Tahsili-Fahadan, & Aston-Jones, 2010; L. Zhou et al., 2012). Our lab has shown that LH orexin neurons in particular mediate reinstatement of drug seeking elicited by drug-associated stimuli (Harris et al., 2005; James, Mahler, et al., 2017; Mahler et al., 2012; Sartor & Aston-Jones, 2012). Thus, higher expression and/or activation of these cells may attach greater motivational significance to cocaine-associated stimuli and result in greater motivation

(demand) for cocaine. A recent study in hypocretin/orexin knockout mice demonstrated that animals lacking the neuropeptide have reduced incubation of cocaine craving and do not reinstate drug seeking in response to cocaine-paired cues (Steiner et al., 2018). Greater numbers of cells promoting cue reactivity in high motivation animals may also explain our previous finding that animals with low α measured in BE have greater cue-induced reinstatement of cocaine seeking (Bentzley et al., 2014). Therefore, a greater number of LH orexin-expressing cells may result in higher cue reactivity and greater drug seeking in highly motivated animals.

Although we did not directly assess the pathways through which orexin mediates motivation for cocaine, other studies indicate that VTA is likely an important target region. Significant data indicates that orexin neurons drive motivational processes via projections to ventral tegmental area (VTA) (Mahler et al., 2014). Orexin potentiates cocaine-induced plasticity of VTA DA neurons in vitro (Borgland, Storm, & Bonci, 2008; Borgland et al., 2006), and orexin-glutamate interactions in VTA are necessary for cue-associated cocaine seeking (Mahler et al., 2013). Blocking orexin-1 receptor signaling in VTA reduces cocaine seeking (James et al., 2011; Mahler et al., 2013) or effortful self-administration (Espana et al., 2010), whereas infusion of orexin A into VTA reinstates extinguished cocaine seeking (Harris et al., 2005; Wang, You, & Wise, 2009). Orexin input to VTA increases DA release in nucleus accumbens (NAc) (Baimel et al., 2017; Espana, Melchior, Roberts, & Jones, 2011; Prince, Rau, Yorgason, & Espana, 2015), which is associated with goal-directed behavior (Saddoris, Sugam, Cacciapaglia, &

Carelli, 2013). Greater LH orexin input to the mesolimbic dopamine system may drive greater cocaine taking in high motivation animals, and future experiments could seek to determine if greater LH orexin input to VTA circuit specifically mediates motivation for cocaine.

Consistent with a selective role for the orexin system in motivated responding for cocaine, we generally saw no relationship between orexin cell numbers and low-effort cocaine intake. We did not observe a difference in the number of orexin cells between high and low takers (high and low Q_0) in Experiment 1. Also, we observed no change in Q_0 following antisense injections (Experiment 2), indicating no causal relationship between orexin cell numbers and low-effort cocaine intake. These findings are consistent with previous studies from our lab and others indicating that blockade of orexin-1 receptor signaling has no effect on low effort (FR1) cocaine consumption (Borgland et al., 2009; Espana et al., 2011; Espana et al., 2010; Hollander, Pham, Fowler, & Kenny, 2012; Smith et al., 2009).

Unilateral LH orexin knockdown is sufficient to reduce cocaine demand

Unilateral orexin knockdown with orexin-A morpholino antisense was sufficient to reduce motivation for cocaine (increase demand elasticity), but not low effort consumption (Q_0). Peak selective knockdown of orexin A protein occurs six days post-antisense without affecting expression of interdigitated neurons containing melanin-concentrating hormone (M. H. James, C. M. Stopper, et al., 2018; Reissner et al., 2012; Sartor & Aston-Jones, 2012). Although other studies

have shown no effect of unilateral orexin inhibition or knockdown on behavior (Mahler et al., 2013; Sartor & Aston-Jones, 2012), ours is the first to examine unilateral orexin knockdown during BE performance requiring high levels of motivation. It may be that orexin neurons bilaterally project to reward-associated areas like VTA, and unilateral knockdown reduces orexin input to both hemispheres the target brain area. Orexin collateralization in reward-associated areas has largely been unexplored. Thus, future anatomical studies should investigate the extent to which orexin inputs collateralize in reward-associated regions like VTA.

Lateral hypothalamus orexin neuron number correlates with motivational and not arousal state

Our findings revealed that the number of orexin neurons in LH, but not in Pef/DMH, predicted motivation for cocaine as assessed by the BE paradigm (demand elasticity). Our lab also recently determined that knockdown of orexin in LH, but not in Pef/DMH, reduced motivation for cocaine (M. H. James, C. M. Stopper, et al., 2018). We also observed that the number of LH orexin neurons did not correlate with locomotor activity, indicating that differences in cell number were not related to non-specific changes in, e.g., arousal state. Taken together, these results support our lab's hypothesis that LH orexin cells are preferentially involved in regulating motivational processes and are less important for arousal/stress regulation (Aston-Jones et al., 2010; Harris & Aston-Jones, 2006; James, Mahler, et al., 2017).

Our results are consistent with several studies from our lab and others indicating that individual differences in the LH orexin system are associated with reward/motivation levels. LH orexin neurons and mRNA expression is upregulated in animals with high motivation for drug (M. H. James, C. M. Stopper, et al., 2018; Lawrence et al., 2006; Thannickal et al., 2018). In addition, Fos expression specifically in LH orexin cells correlates with preference for both morphine and cocaine (Harris et al., 2005; Harris et al., 2007; Lasheras, Laorden, Milanes, & Nunez, 2015; K. A. Richardson & Aston-Jones, 2012; Sartor & Aston-Jones, 2012). The preferential involvement of LH orexin cells in drug seeking may be mediated by distinct inputs or outputs compared to Pef/DMH cells (Fadel & Deutch, 2002; Gonzalez, Jensen, Fugger, & Burdakov, 2012), though anatomical evidence thus far remains unclear.

Collectively, these studies demonstrate that the number of LH orexin neurons is a significant factor contributing to individual differences in motivation for cocaine and may be a previously unrecognized but important element in addiction pathology.

Chapter 4: Orexin-1 receptor signaling in the ventral tegmental area mediates cue-associated demand for cocaine

Abstract

The ventral tegmental area (VTA) is a critical area for reward and drug addiction. Orexin-1 receptor signaling in VTA contributes to highly motivating and cue-dependent addiction-like behaviors. Here, we investigated the role of orexin-1 receptor signaling in demand for cocaine by infusing the receptor antagonist SB-334867 (SB) into VTA prior to behavioral economics (BE) testing. We found that SB reduced motivation for cocaine (α) without affecting baseline consumption (Q_0) or general locomotor activity. In addition, SB only reduced motivation when animals were trained and tested on BE with cues, and removal of cocaine-associated cues reduced SB efficacy. Similarly, SB did not impact motivation when animals were trained on FR-1 or BE without cues. To ascertain the source of orexin input to VTA, we performed retrograde tract tracing in VTA and observed that a larger proportion of lateral hypothalamus (LH) orexin neurons project to VTA than do orexin cells in perifornical/dorsomedial hypothalamus (Pef/DMH). These studies demonstrate a role of orexin signaling in VTA specifically when cues predict drug reward and point to LH orexin neurons as key mediators of orexin's effects on VTA neurons.

Introduction

Drug-associated cues acquire motivational significance over time and serve as triggers of relapse, even in the absence of drug. The hypothalamic neuropeptide orexin (also called hypocretin) has been linked to cue-dependent and highly motivating cocaine seeking behaviors [for review, (James, Mahler, et al., 2017; Mahler et al., 2014)]. Ventral tegmental area (VTA) is a critical target of orexin neurons, and reducing orexin-1 receptor signaling to VTA attenuates responding for cocaine under high effort conditions (Bernstein, Badve, Barson, Bass, & Espana, 2017; Espana et al., 2011; Espana et al., 2010) or in the presence of drug-associated cues (James et al., 2011; Mahler et al., 2013). In particular, orexin potentiates glutamatergic input onto VTA dopamine (DA) neurons (Moorman & Aston-Jones, 2010), which is necessary for their drug-induced plasticity (Baimel & Borgland, 2015). These studies and others indicate that orexin-1 receptor signaling in VTA may mediate motivation for cocaine by regulating glutamatergic signaling in response to drug-associated cues (Mahler et al., 2012).

Our lab has established a role for orexin and VTA DA neurons in motivation for cocaine in our behavioral economics (BE) paradigm. In this paradigm, lever-pressing data is fitted to an exponential demand equation (Hursh & Silberberg, 2008) to generate two parameters of cocaine demand: demand elasticity (α), which inversely scales with motivation, and baseline consumption (Q_0). In this paradigm, we have shown that cocaine-associated cues increased motivation (decreased α), and that blocking orexin-1 receptor signaling with SB-334867 (SB) attenuated motivation without impacting baseline consumption only when discrete cues were

present (Bentzley & Aston-Jones, 2015). Similarly, knockdown of orexin neurons specifically in lateral hypothalamus (LH), and not perifornical/dorsomedial hypothalamus (Pef/DMH), reduced motivation for cocaine (M. H. James, C. M. Stopper, et al., 2018). More recently, we have shown that chemogenetic inhibition of VTA DA neurons lowered motivation for cocaine (Mahler et al., 2018). Collectively, these studies have implicated the orexin and DA systems in motivation and point to the LH orexin to VTA circuit as a driver of high effort, cue-dependent drug seeking.

Here, we investigated the role of orexin-1 receptor signaling in VTA during effortful responding for cocaine in our lab's BE paradigm. Our results mirror those of previous studies showing that orexin-1 receptor signaling mediates the motivational effects of cocaine (Bentzley & Aston-Jones, 2015; Morgan H James et al., 2018), as local microinjections of SB into VTA lowered α, but not Q_0 values. Building on recent studies from our lab, we also show that intra-VTA SB microinjections are effective at reducing motivation for cocaine when animals are trained and tested on BE with cues present, and removing cues reduces the effect of SB on motivation. Finally, using retrograde tract tracing, we show that a greater proportion of orexin neurons in LH innervate VTA. Collectively, these studies point to a role for orexin signaling in VTA in driving cue-dependent, motivated drug taking.

Methods

Animals

Adult male Sprague-Dawley rats (weighing 300-325 grams) were paired-housed on a 12-hour reversed light: dark cycle in a temperature- and humidity-controlled animal facility at Rutgers University-New Brunswick. All animals were given ad libitum access to water and standard rat chow. Animals were acclimated for 2 days to the animal facility upon arrival and were handled for at least 3 days prior to surgery. All protocols and animal care procedures were approved by the Institutional Animal Care and Use Committee at Rutgers University-New Brunswick.

Drugs

Cocaine-HCl powder was obtained from the National Institute of Drug Abuse (Research Triangle Park, NC) and dissolved in 0.9% sterile saline. For intracranial microinjections SB-334867 (SB) was suspended in artificial cerebrospinal fluid (aCSF) at a 1mM concentration. This concentration is a relatively low dose and has been utilized in several intracranial injection studies from our lab and others (James et al., 2011; Mahler et al., 2013). The retrograde tracer cholera toxin β subunit (CTb; Sigma) was dissolved at a 0.5% concentration in 0.1 M PBS.

Intravenous catheter surgery

Rats were anesthetized with a ketamine/xylazine mixture (56.5/8.7 mg/kg, i.p., respectively), followed by rimadyl (5 mg/kg, s.c.). Rats were implanted with indwelling catheters into the jugular vein. VTA cannula implantation occurred

immediately after catheterization. Cefazolin (0.1 ml; 100 mg/ml) and heparin (0.1 ml; 100 U/ml) were flushed through the catheter after both surgeries, daily for 2 days after surgery, and following each self-administration session. Following a 1-week recovery period, animals underwent cocaine self-administration training.

Stereotaxic surgery

Animals were secured in the stereotactic frame (Kopf, Tujunga, CA) and implanted bilaterally with stainless steel guide cannulae (22 gauge, 11 mm, Plastics One, Roanoke, VA) 2 mm dorsal to VTA (-5.5 mm AP, +/- 2.0 mm ML, -7.4 mm DV, relative to bregma). Cannula were secured to skull surface with acrylic cement and jeweler screws.

Cocaine self-administration

Rats were trained on a fixed ratio-1 (FR-1) self-administration paradigm for 2 hours/day. Sessions occurred in operant chambers enclosed in sound-attenuating boxes with Med-PC IV software (Med Associates). Animals trained on FR-1 with cues received cocaine infusions (0.19 mg cocaine/infusion) paired with discrete light and tone cues (white light above active lever; 78 dB, 2900-Hz tone). Animals received a 20-second time-out post-infusion. After reaching criteria ($\geq$ 10 infusions/sessions for 10 sessions), animals were trained on the behavioral economics (BE) procedure.

BE procedure

Rats were trained for a minimum of 6 days on a within-session threshold BE procedure described elsewhere (Bentzley et al., 2013; Bentzley et al., 2014). During the session, the duration of cocaine infusion decreased in successive 10-

minute intervals on a quarter logarithmic scale (383.5, 215.6, 121.3, 68.2, 38.3, 21.6, 12.1, 6.8, 3.8, 2.2, 1.2 ug per infusion). For cue BE sessions, each infusion was paired with a light and tone cue, as above. When animals displayed stable behavior ($\leq$30% variability in Q_0 and α values across the last three sessions), they received a microinjection of SB 2 mm dorsal to VTA, or aCSF or SB into VTA. Animals were re-stabilized for a minimum of three sessions on BE before testing again.

Lever pressing was fit to demand curves using an exponential demand equation to calculate Q_0 (consumption at low effort) and α (demand elasticity) values, as described previously (Bentzley et al., 2013). Low α values indicate greater motivation for cocaine, as α is the slope of the demand curve and scales inversely with motivation. Both α and Q_0 values were averaged across the three days preceding testing and used as baseline values. After each BE test, α and Q_0 were compared to these baseline values to determine the percent change following treatment.

Locomotor Activity

Following testing on the BE procedure, animals were habituated to a locomotor chamber (clear acrylic, 40 x 40 x 30 cm) equipped with Digiscan monitors (AccuScan Instruments). Habituation sessions occurred for 2 hours/day for 3 days. After habituation, rats received an intra-VTA microinjection of aCSF or SB and underwent locomotor testing. The next day, rats underwent a 1-day washout period, in which they were placed in the locomotor chamber without any treatment. Rats then received either aCSF or SB the following day in a final testing

session. Each animal received aCSF or SB once in a randomized and counterbalanced fashion.

Sucrose self-administration

Animals were trained to lever press for sucrose on a fixed ratio-1 (FR1) sucrose self-administration schedule for 2 hours/day for a minimum of 5 days, as described previously (Cason & Aston-Jones, 2014). Active lever presses yielded one sucrose pellet (45 mg, Test Diet), and a 20 s timeout proceeded each reward. Animals were trained until active lever responses across three days were $\leq$25% variability. Animals received intra-VTA SB or aCSF in a counterbalanced design.

Intracranial microinjections

Control microinjections were performed to ensure that effects following intracranial microinjections were not due to diffusion of liquid up the cannula tract. Injectors projecting 0.2 mm past the bottom tip of the guide cannula were inserted to infuse the SB-334867 (SB) dorsal of VTA (0.3 ul; 1 mM concentration dissolved in artificial cerebrospinal fluid (aCSF).

All other microinjections were performed using injectors extending 2 mm past the guide cannula to infuse SB or aCSF into VTA. The injector cannula was kept in place for 1 minute to allow for drug diffusion. All tests used a counterbalanced and within-subjects design.

Localization of microinjections

Cannula-implanted animals were deeply anaesthetized with isoflurane and decapitated. Brains were dissected, flash frozen in 2-methyl butane, and dissected

on a cryostat at 40 um. Sections were slide-mounted, Nissl stained with neutral red, and coverslipped with mounting medium.

Experimental BE cue/no cue design

Three groups of animals were utilized to investigate the role of orexin-1 receptor signaling in cocaine demand with cues. Figure 1 outlines the procedure for behavioral testing. Briefly, animals were trained to self-administer cocaine with (Groups 1 and 2) or without (Group 3) cues. Animals were then trained on BE with (Group 1) or without (Groups 2 and 3) cues for a minimum of 6 days until criteria was reached. Animals were tested on SB/aCSF, after which they were restabilized on BE with (Groups 2 and 3) or without (Group 1) cues and were tested on BE with SB/aCSF. Animals received four microinjections: SB/aCSF during cue BE and SB/aCSF during no cue BE except if catheter patency failed during testing (Group 2 n=2; Group 3 n=4). We included data from these animals in all analyses.

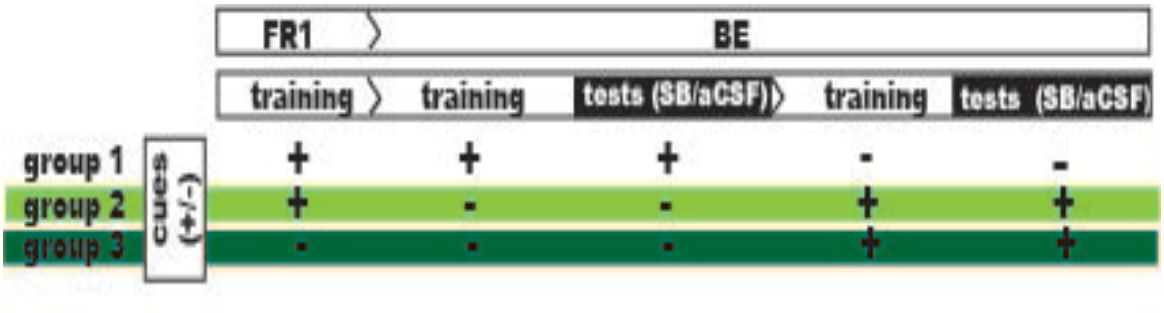

Figure 4.1. Schematic of cue/no cue behavioral testing. Groups 1 and 2 were trained on FR1 with cues, and group 3 without cues. Group 1 then underwent BE training and testing with cues, while groups 2 and 3 were trained and tested on BE without cues. Once animals had been tested with aCSF and SB microinjections into VTA, they then were trained on BE with (groups 2 and 3) or without (group 1) cues.

Tract-tracing

For tract-tracing experiments, animals (n=12) were anesthetized as described above and received pressure injections of ~40 nl of CTb in a stereotaxic holder using a Picospritzer (General Valve, Inc), as described previously (Mahler et al., 2013). Injections were made into VTA (-5.5 mm AP, +0.8 mm ML, -8.3 mm DV) through a glass pipette (internal diameter 10-15 uM) over 5 minutes. Pipettes were left in place for 10 minutes to minimize diffusion. After waiting at least one week, animals were perfused for cell quantification. Animals with CTb diffusion up the pipette track or outside of VTA (n=4) were excluded from analysis.

Tissue preparation for immunohistochemistry

CTb- and orexin shRNA/scRNA-infused animals were anesthetized with ketamine/xylazine and transcardially perfused with 0.9% saline, then 4% paraformaldehyde. Brains were removed and postfixed overnight. The next day, brains were transferred to a 20% sucrose-azide solution. Sections were cut on a cryostat at 40 um and collected in PBS-Azide.

Immunohistochemistry

For retrograde tracing experiments, tissue was processed for both orexin A and cholera toxin β subunit (CTb) proteins. Briefly, sections were incubated in 5% normal donkey serum (NDS) in phosphate buffered saline with Triton (PBST) for 2 hours then transferred into primary goat or mouse anti-orexin A antibody (1:500; Santa Cruz) in 2% NDS overnight at room temperature. The following day, sections were washed and incubated for 2 hours in donkey anti-goat 488 fluorescent secondary antibody (1:500; Invitrogen). Sections were washed with PBST and

transferred to PBS-Azide for an hour. Tissue was then incubated overnight in primary CTb antibody (1:10,000; Sigma) in 2% NDS. The next day, brains were washed and incubated in donkey anti-mouse 594 fluorescent secondary antibody (1:500; Invitrogen). Sections were mounted on slides with fluorescent mounting medium with DAPI (Abcam).

Cell quantification

Coronal lateral hypothalamus images were taken across the orexin cell field (2.5-3.8 mm caudal to bregma) using a Zeiss Axio Zoom V16 microscope. Tiled photographs were compiled at a 20x magnification using Zen 2 imaging software (Carl Zeiss Microscopy). Cell counts were done in Adobe Illustrator by an observer blind to experimental conditions. For CTb experiments, the number of cells expressing CTb, orexin, or both were determined in the ipsilateral and contralateral hemisphere of the retrograde tracer injection. LH and Pef/DMH were delineated as described in other studies (Harris et al., 2005; M. H. James, C. M. Stopper, et al., 2018). Three sections were taken per animal, and cell counts were averaged across the three sections.

Data analysis

Statistical analyses were performed with GraphPad Prism 7. BE data and binned locomotor data were analyzed using repeated measures ANOVA with Bonferroni's multiple comparisons tests when appropriate. Total locomotor data and sucrose self-administration results were analyzed as paired t-tests. Non-parametric Kruskal-Wallis tests with post-hoc Dunn's tests were used when data

were not normally distributed, as determined by a Shapiro-Wilk normality test. All statistics were two-tailed.

Results

Animals were first tested to determine whether blocking orexin-1 receptor signaling in VTA impacted cocaine demand. Figure 4.2A shows a schematic of VTA cannula placements. Intra-VTA SB microinjections reduced cocaine demand elasticity (increased α; Figure 4.2B) when compared to VTA aCSF or dorsal SB microinjections (Figure 4.2C; Shapiro-Wilk normality test, $W=0.80$, $p<0.05$; Friedman test, $H_{3,9}=10.89$, $p<0.01$). Intra-VTA SB significantly increased cocaine demand elasticity (increased α) compared to intra-VTA aCSF (Dunn's multiple comparisons, $p<0.05$) or dorsal VTA SB ($p<0.01$) microinjections. There was no effect of VTA SB on Q_0 (Figure 4.2D; Shapiro-Wilk normality test, $W=0.76$, $p<0.01$; Friedman test, $H_{3,9}=2.89$, $p=0.28$).

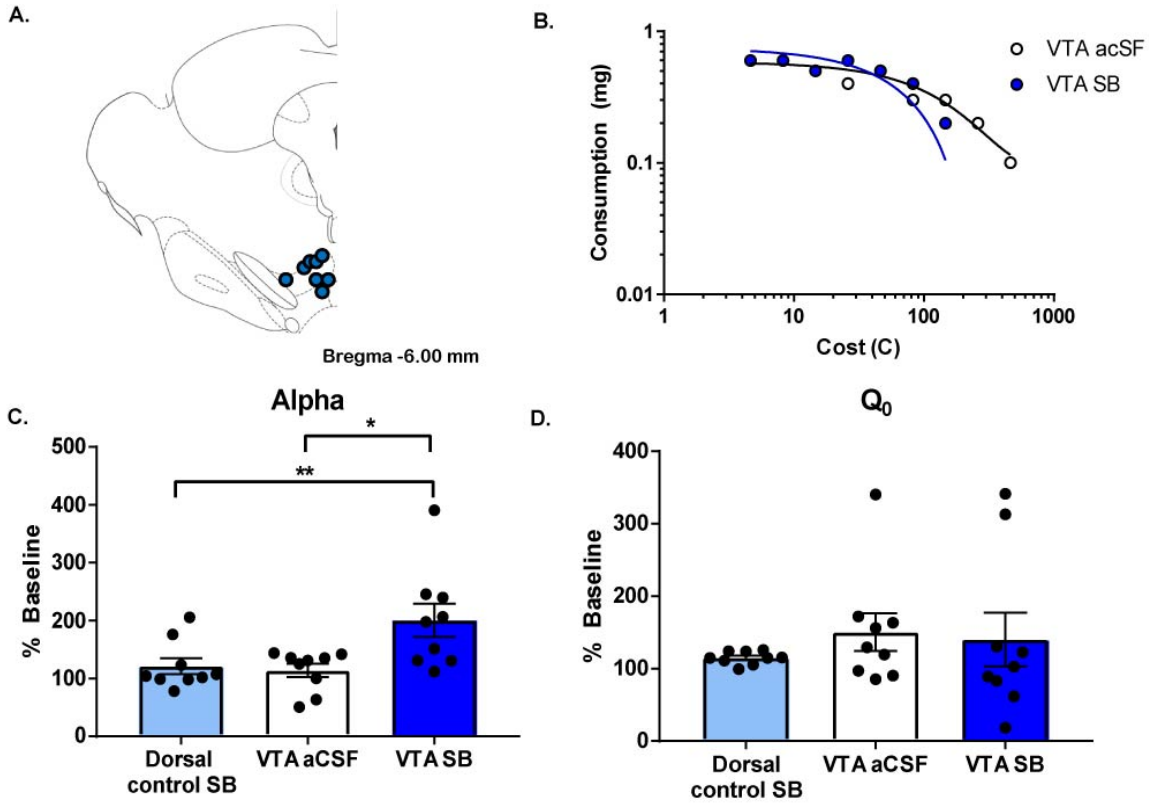

Figure 4.2. Intra-VTA microinjections of the orexin-1 receptor antagonist SB-334867 (SB) attenuates cocaine demand elasticity. A. Schematic of cannulae placements for intra-VTA SB microinfusions. B. A representative demand curve for a single animal following VTA aCSF (white circles) or SB (blue circles). C. VTA SB microinjections increased demand elasticity (increased α) compared to dorsal control SB or VTA aCSF microinjections (*$p<0.05$, **$p<0.01$). D. None of the treatments impacted baseline consumption.

Animals were then tested for locomotor activity or sucrose self-administration to determine if intra-VTA SB effects were due to motor impairments. There were no differences during locomotor testing between VTA aCSF and SB microinjections (Figure 4.3A; paired t test; t_9= 0.387; p=0.709). When we binned locomotor data in 5-minute intervals, there was no effect of treatment on distance travelled at any time point (two-way RM ANOVA; $F_{(1,8)}$=0.018; p=0.896). Similarly, there was no effect of treatment on fixed ratio-1 responding for sucrose (Figure 4.3B; paired t test; t_6= 0.362; p=0.732). Therefore, intra-VTA SB reduced cocaine demand elasticity without impacting general locomotor abilities.

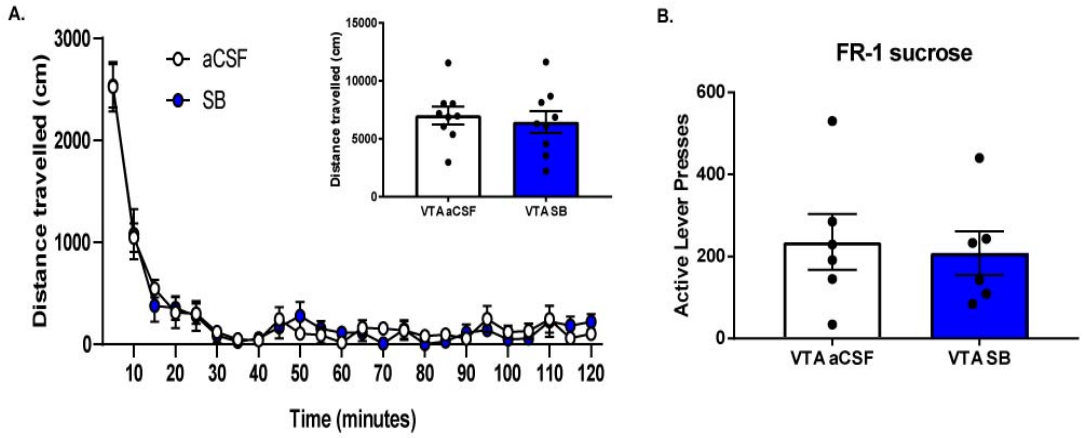

Figure 4.3. Intra-VTA SB does not produce sedation. A. No changes in general locomotor activity were observed following intra-VTA SB compared to aCSF microinjections. B. SB did not impact FR-1 responding for sucrose pellets.

Next, we determined whether cues impacted the efficacy of SB to decrease motivation. To do this, we trained three groups of animals, as outlined in Figure 4.1, on FR-1 and BE. We observed a significant effect of day (two-way RM ANOVA, $F_{9,243}$= 8.50, p<0.0001; Figure 4.4A), but no group differences ($F_{2,27}$=0.08, p=0.92) in the number of infusions across FR-1 training days. There were also no group differences in the number of active lever presses (two-way RM ANOVA, $F_{2,27}$=0.10, p=0.91, Figure 4.4B) or in inactive lever presses (two-way RM ANOVA, $F_{2,27}$=0.47, p=0.63, Figure 4.4C), though there was a significant effect of day ($F_{9,243}$= 9.02, p<0.0001) and a group x time interaction ($F_{18,243}$=2.10, p<0.01). During BE training, there was a trend towards a group difference on baseline α (two-way RM ANOVA, $F_{2,25}$=2.86, p=0.09, Figure 4.4D) and no significant effect on Q_0 values ($F_{2,25}$=0.33, p=0.72, Figure 4.4E).

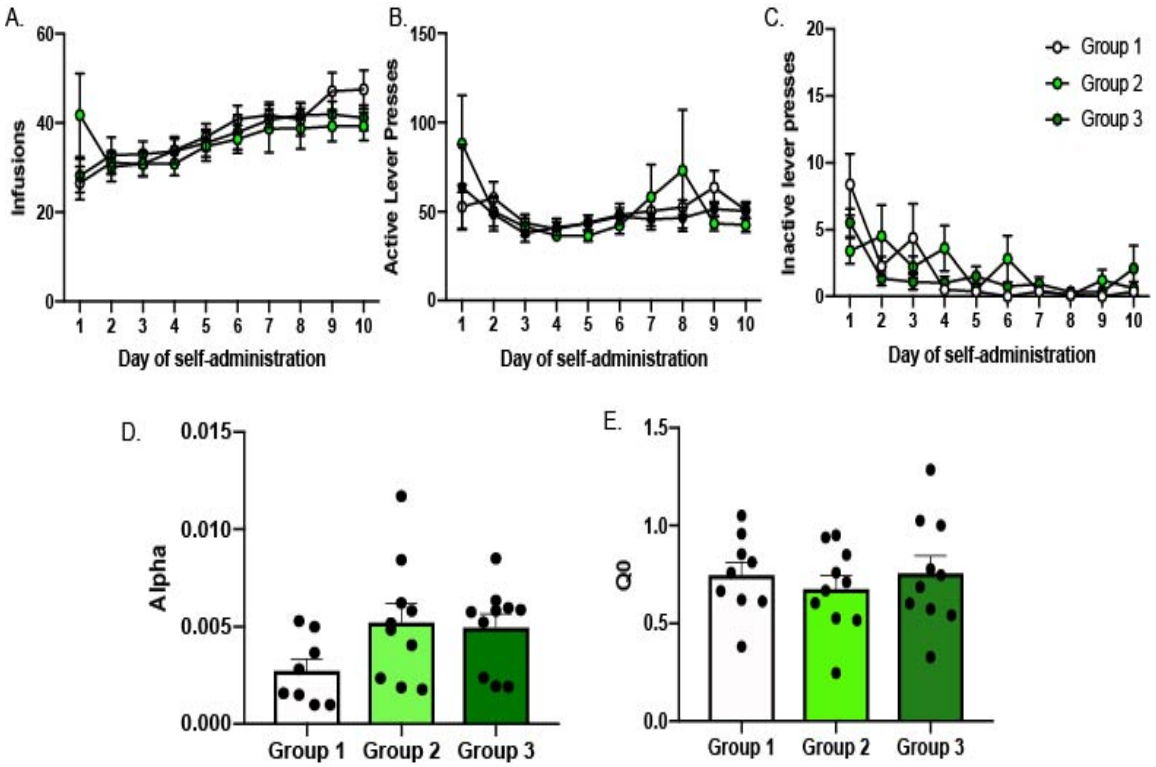

Figure 4.4. Cues do not significantly impact behavior during self-administration or BE training. No group differences were observed in A. infusions, B. active lever presses, or C. inactive lever presses over the 10 days of self-administration training. D. There was a trend towards a group difference in baseline α values, but this failed to reach statistical significance. E. No group differences were observed on baseline Q_0 values.

Next, we sought to determine how removing (Group 1) or adding (Groups 2 and 3) cues impacted cocaine demand. Briefly, we compared animals' last baseline demand parameters with (Group 1) or without (Groups 2 and 3) cues to the first baseline parameters with (Group 2 and 3) or without (Group 1) cues to capture behavioral effects from the cue-to-no cue (Group 1) or no cue-to-cue (Groups 2 and 3) transition. In Group 1, removing the cues significantly increased α (decreased motivation; Wilcoxon matched-pairs signed rank test, W=28.0, $p<0.05$, Figure 5A) but did not impact Q_0 (paired t-test, $t_6=1.07$, p=0.33, Figure 4.5B). In contrast in Group 2, adding cues did not impact α (paired t-test, $t_7=0.63$, p=0.55, Figure 4.5C) or Q_0 (paired t-test, $t_7=0.65$, Figure 4.5D). Similarly in Group 3, cues did not alter α (Wilcoxon matched-pairs signed rank test, W=14, p=0.30, Figure 5E) or Q_0 (Wilcoxon matched-pairs signed rank test, W=12, p=0.38, Figure 4.5F).

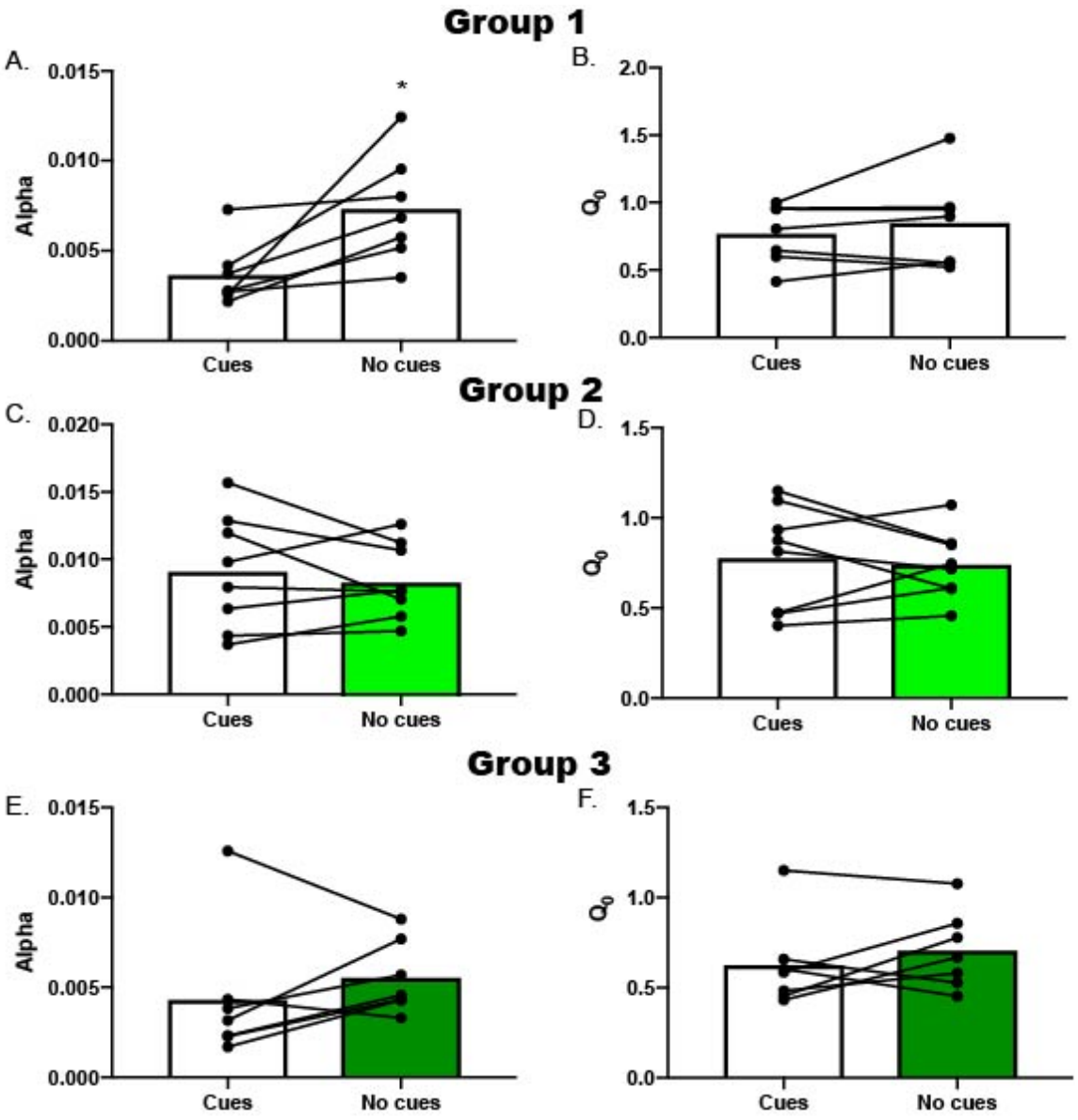

Figure 4.5. Removing cues in animals trained on BE with cues reduces motivation. A. Animals trained on FR-1 and BE with cues had decreased motivation (increased α) when cues were removed (*$p<0.05$). B. Removing cues did not impact Q_0. Adding cues in the BE paradigm animals trained on FR-1 with cues did not impact baseline C. α or D. baseline Q_0 values. Adding cues in the BE paradigm for animals trained on FR-1 without cues did not impact baseline E. α or F. Q_0 values. *$p<0.05$

Finally, we determined if SB impacted cocaine demand in any of the groups. In Group 1 animals, there was a significant main effect of treatment on α value (two-way RM ANOVA, $F_{1,5}=9.82$, $p<0.05$, Figure 4.6A) and a treatment x cue interaction ($F_{1,5}=8.58$, $p<0.05$). SB reduced motivation only when animals were tested on BE with cues (Bonferroni's multiple comparisons test, $p<0.05$). There was no interaction between treatment x cue with Q_0 (two-way RM ANOVA, $F_{1,5}=1.36$, $p=0.30$, Figure 4.6B). Similarly, in Group 2, animals there was a significant treatment x cue interaction (mixed-effects model, $F_{1,1}=23.43$, $p<0.01$, Figure 4.6C), and SB significantly impacted motivation when animals were tested on BE with cues (Bonferroni's multiple comparisons test, $p<0.05$). There was no significant treatment x cue interaction with Q_0 (mixed-effects model, $F_{1,4}=1.45$, $p=0.90$, Figure 4.6D). In Group 3, we did not observe a significant treatment x cue interaction on α (mixed effects model, $F_{1,1}=0.35$, $p=0.66$, Figure 4.6E), although there was a significant effect of cue that trended towards significance ($F_{1,8}=4.89$, $p=0.058$). There was also no treatment x cue interaction on Q_0 (mixed-effects model, $F_{1,25}=0.35$, $p=0.85$, Figure 4.6F).

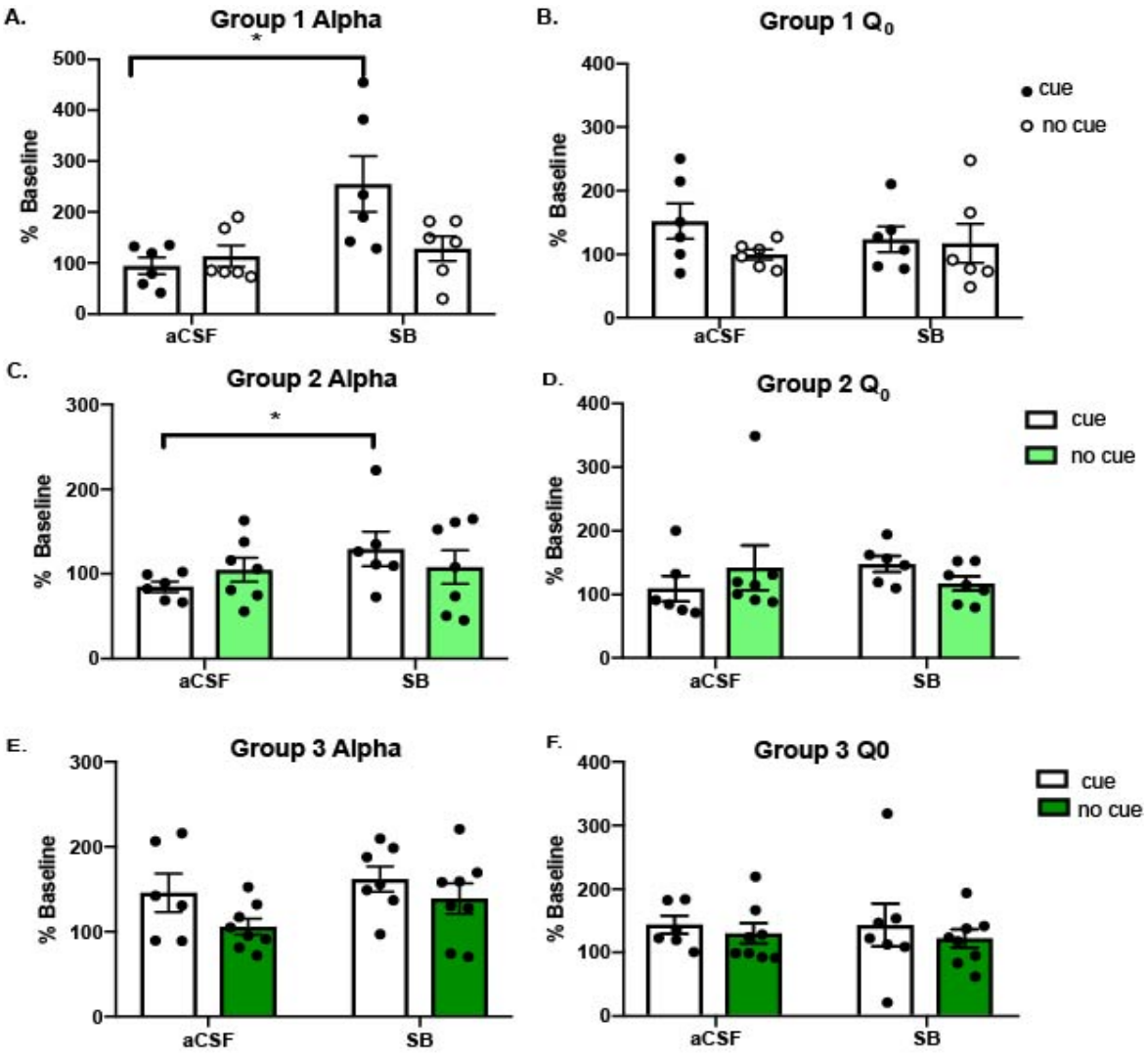

Figure 4.6. SB reduces motivation for cocaine paired with discrete cues. A. When animals were trained on FR-1 and BE with cues, then tested on BE with cues, SB reduced motivation (increased α). SB did not impact motivation in the absence of cues. B. SB did not impact Q_0 in this group of animals. C. In animals trained on FR-1 with cues, then tested on BE without cues, SB did not change motivation. When animals were then tested on BE with cues, SB reduced motivation. D. SB did not impact Q_0 in this group of animals. E. In animals trained

on FR-1 without cues, SB did not impact motivation when animals were tested on BE with or without cues. *p<0.05. Bar graphs indicate mean ± SEM.

We then investigated the potential source of orexin input to VTA using microinjections of the retrograde tracer into VTA. A representative image of a CTb microinjection is depicted in Figure 4.7A, and a schematic of all microinjection sites is presented in Figure 4.7B. We quantified CTb+, orexin+, and CTb+/orexin+, as represented in Figure 4.7C. In both perifornical/dorsomedial hypothalamus (Pef/DMH) and lateral hypothalamus (LH), we observed CTb-positive cells in the hemisphere ipsi- and contralateral to the injection site. In Pef/DMH, we observed significantly more CTb+ cells in the ipsilateral hemisphere compared to the contralateral (two-way RM ANOVA, cell type x hemisphere interaction: $F_{2,21}$=41.62, p<0.0001; Bonferroni's multiple comparisons test, p<0.0001; Figure 4.7D), and no differences were observed in the number of orexin+ (p>0.999) or CTb+/orexin+ cells (p>0.999) between hemispheres. Similarly, in LH we observed significantly more CTb+ cells in the ipsilateral hemisphere compared to the contralateral (two-way RM ANOVA, cell type x hemisphere interaction: $F_{2,21}$=23.92, p<0.0001; Bonferroni's multiple comparison test, p<0.0001).

When we compared bilateral expression of CTb, orexin, and co-labelled cells in Pef/DMH vs. LH, we observed that Pef/DMH contained more immunopositive cells (two-way RM ANOVA, cell type x subregion interaction: $F_{2,21}$=7.79; Figure 4.7E). There were significantly more orexin+ cells (Bonferroni's multiple comparison's test, p<0.0001) and a trend towards significantly more CTb+

cells ($p=0.053$). However, there was no significant difference in the number of co-labeled cells in the two subregions ($p>0.999$). As a result, the percentage of orexin neurons that co-expressed CTb was greater in LH than Pef/DMH (paired t-test, $t_7=4.08$, $p<0.01$, Figure 4.7F).

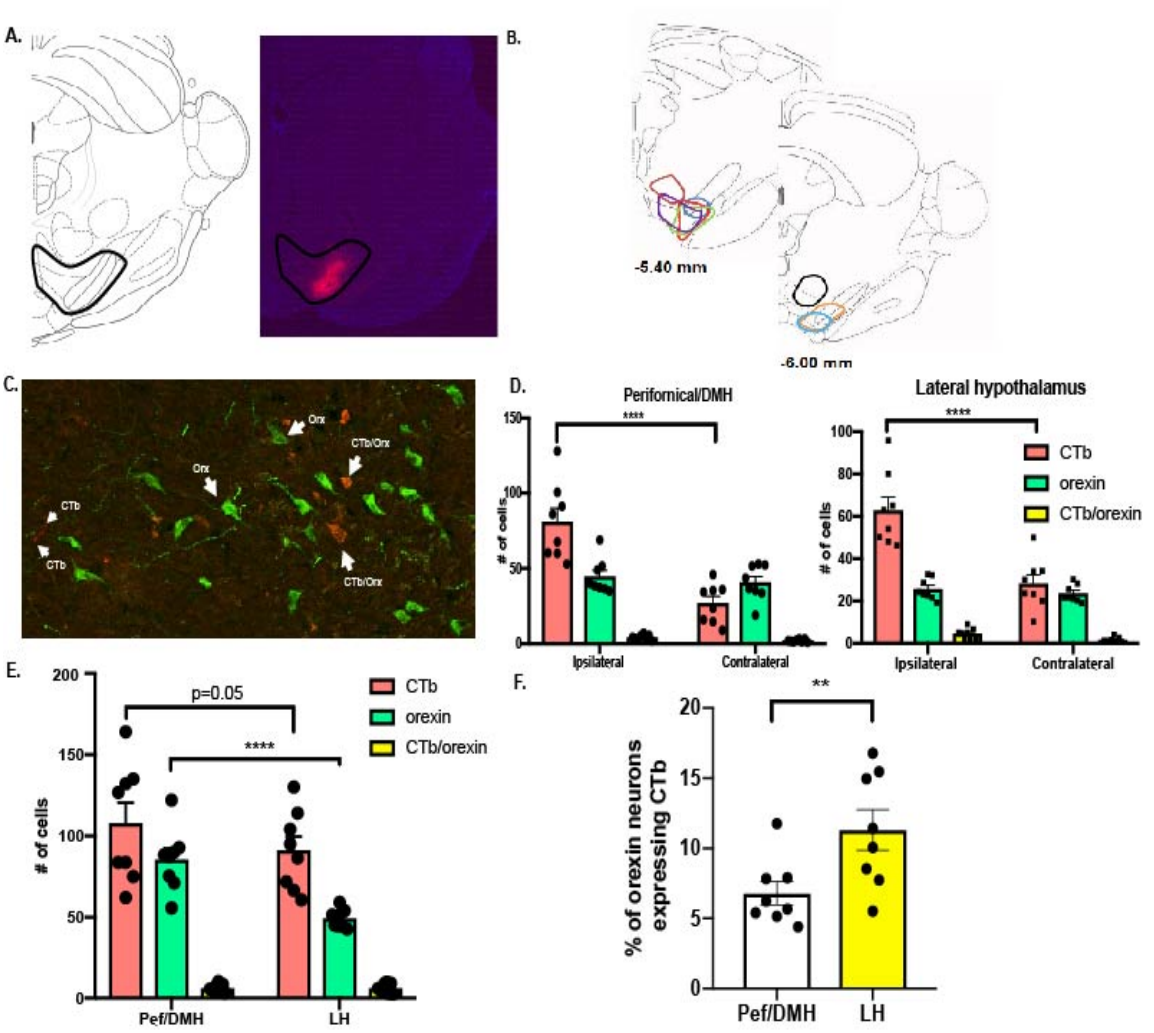

Figure 4.7. VTA receives input from both Pef/DMH and LH orexin neurons. A. Representative image of VTA CTb microinjection. CTb expression is labeled in red, and the boundaries of VTA are outlined in black. B. Schematic of the area of CTb expression in VTA. C. Representative image of hypothalamus stained for CTb (red), orexin A (green), and co-localization (yellow). Scale bar denotes 200 μm. D. Both Pef/DMH and LH subregions of the orexin cell field had greater CTb expression in the hemisphere ipsilateral to CTb microinjection than contralateral. E. Bilateral expression of CTb and orexin were greater in Pef/DMH than in LH. F.

A greater percentage of LH orexin neurons project to VTA than do Pef/DMH orexin neurons. **p<0.01, ****p<0.0001. Bar graphs indicate mean ± SEM.

Discussion

Here, we show that orexin-1 receptor signaling in VTA is necessary for motivation for cocaine when discrete drug-associated cues are paired with cocaine delivery. Microinjections of the orexin-1 receptor SB reduced motivation (increased demand elasticity; α) but did not impact Q_0, general locomotor activity, or fixed-ratio responding for sucrose, indicating that SB effects on motivation were not due to sedation. In addition, dorsal microinjections of SB did not impact motivation, so the ability of SB to decrease demand elasticity was not caused by actions outside of VTA. We also observed that SB significantly impacted motivation only when animals were trained and tested on BE with cues, and removing cues reduced the efficacy of SB on motivation. In addition, SB was still effective if animals were tested on BE without cues first, as long as they had been trained on FR-1 with cues. Finally, we observed that orexin neurons across the cell field project to VTA but to a greater extent in the LH orexin cell population than in Pef/DMH. Collectively, these studies indicate that orexin-1 receptor signaling to VTA drives cue-associated motivation for cocaine.

Orexin-1 receptor signaling mediated high effort drug taking

We observed the microinjections of the orexin-1 receptor antagonist SB attenuated motivation (increased α), and these effects were not observed following

microinjections of aCSF into VTA or dorsal SB microinjections. These results are consistent with studies that demonstrate that orexin-1 receptor signaling is necessary for high effort drug taking (Bentzley & Aston-Jones, 2015; Bernstein et al., 2017; Brodnik, Bernstein, Prince, & Espana, 2015; Espana et al., 2011; Espana et al., 2010; Porter-Stransky et al., 2017) and that VTA is a critical target of orexin (Bernstein et al., 2017; Borgland et al., 2009; Espana et al., 2011; Espana et al., 2010). In addition, we observed no change in Q_0 following SB microinjections in the same animals, demonstrating that orexin-1 receptor signaling does not mediate low-effort consumption of cocaine. Other studies have similarly shown that SB does not affect FR-1 responding for cocaine (Espana et al., 2011; Espana et al., 2010; Smith et al., 2009). Collectively, our study adds to a growing literature that orexin-1 receptor signaling promotes high effort drug taking through actions on VTA DA neurons.

SB effects on motivation were not due to sedation

We assessed effects of SB on sedation using two measures of locomotor activity: the total distance animals travelled in a locomotor chamber and the rate of responding for sucrose under low-effort (FR1). We observed no change in total locomotor activity, and when locomotor activity was grouped in 5-minute bins, SB did not alter activity in any of the bins. Similarly, SB did not impact the number of active lever presses for sucrose, indicating that animals could still maintain a high rate of lever pressing. Therefore, SB did not impact motivated drug taking by reducing arousal. Our lab and others have similarly shown that systemic or local

injections of SB do not interfere with locomotor activity (Borgland et al., 2009; James et al., 2011; Smith et al., 2009).

While SB did not attenuate low-effort responding for sucrose, orexin A has been shown to mediate the hedonic value of sucrose and promote motivation to consume natural rewards (Borgland et al., 2009; Castro, Terry, & Berridge, 2016; Terrill et al., 2016). However, our lab has shown that blocking orexin-1 receptor signaling systemically with SB reduces consumption of sucrose pellets on fixed or progressive ratio schedules only in food-restricted animals (Cason & Aston-Jones, 2013, 2014). One possible reason for these discrepancies is that orexin A binds to both the orexin-1 and -2 receptors, and thus the hedonic value of natural rewards may be mediated by the orexin-2 receptor. Future studies could investigate whether intra-VTA SB impacts motivation for food to determine if orexin-1 receptor signaling specifically reduces motivation for drugs, and not food.

SB specifically reduced motivation when cocaine is paired with discrete cues

To elucidate how orexin promotes cue-associated motivation, we microinjected SB into three groups of animals that were trained and tested on FR-1 and BE with or without cues. When animals were trained on FR-1 with cues (Groups 1 and 2), we observed that SB only reduced motivation when cues were present, and removing these cues reduced the efficacy of SB. These effects were not dependent on the order in which animals were trained on BE with cues, as Group 1 was trained on BE with cues first, while Group 2 were first trained on BE without cues. In contrast, Group 3 was trained on FR-1 without cues, and there

was no effect of SB during BE with or without cues in these animals. Our results are consistent with a previous study from our lab showing that systemically blocking orexin-1 receptor signaling reduces motivation only when animals are trained and tested with cues (Bentzley & Aston-Jones, 2015) and indicate that the initial learning of discrete reward-paired cues may be particularly important for orexin involvement in motivation.

Orexin has been implicated in translating motivational drive into behavioral output in response to external stimuli like cues associated with drug taking (Mahler et al., 2014). Orexin-1 receptor signaling has been shown to be necessary for cue-dependent drug seeking and less important for behaviors that do not require cues, such as drug primed reinstatement (James et al., 2011; Mahler et al., 2013; Smith et al., 2010). Our study and others have demonstrated that blocking orexin-1 receptor signaling does not attenuate motivation in the absence of cues (Bentzley & Aston-Jones, 2015), indicating that orexin does not mediate the reinforcing effects of drugs of abuse. Rather, blocking orexin input to VTA seems to reduce the ability of conditioned cues to trigger motivated behavior.

Orexin-1 receptor signaling potentiates glutamatergic input onto VTA DA neurons, which has been shown to be necessary for promoting drug seeking. Orexin A promoted NMDA receptor recruitment to VTA DA synapses and enhanced excitatory transmission, and SB application blocked the drug-induced plasticity of VTA DA neurons (Borgland et al., 2006). Similarly, orexin A induced excitatory postsynaptic currents in VTA DA neurons, and this effect was augmented following cocaine self-administration (Borgland et al., 2009). Blocking

both orexin-1 and AMPA receptor signaling simultaneously reduced cue-induced reinstatement (Mahler et al., 2013). These studies highlight that both orexin and glutamate systems are necessary for promoting drug seeking. The source of the glutamatergic input to VTA may come from regions like hypothalamus or medial prefrontal cortex (mPFC) that densely innervate VTA (Geisler et al., 2007). In particular, our lab has shown that orexin modulates mPFC input to VTA DA neurons (Moorman & Aston-Jones, 2010), which may be an important circuit for driving orexin involvement in cue-associated motivation.

Cocaine-paired cues increase motivation for the drug

We compared animals' stable baseline α and Q_0 during their last test, before switching to the new paradigm, to their stable baseline α and Q_0 before their first test on the new paradigm. Using this approach, we sought to determine how the cue-to-no cue (Group 1) or no cue-to-cue (Groups 2 and 3) transition impacted stable motivation and baseline consumption during BE. Consistent with our previous study, we found that removing cues in animals trained on FR-1 and BE with cues (Group 1) reduced motivation (increased α) (Bentzley & Aston-Jones, 2015). In addition, adding cues did not alter motivation when animals were trained on FR-1 without cues (Group 3). However, when cues were added to the BE paradigm in animals trained on FR-1 with cues (Group 2), we did not observe an increase in motivation, though we did observe an effect of SB in these animals only with cues present. One possible explanation is that in the absence of discrete cues, these animals relied on other external stimuli (ie. the operant chamber or

active lever) that signal drug availability. Therefore, once animals learned the BE paradigm without discrete cues, the cues alone might not be reinforcing enough to further increase motivation. Another possibility is that it may be more physically challenging to increase effort for reward than to decrease it, and cues may still hold motivational significance without producing greater effort, which may explain why SB still impacted motivation in these animals.

A recent study showed that cues do not impact the price at which male animals will maximally respond for cocaine (Pmax) or baseline consumption, but adding cues does increase Pmax in estrus females (Johnson et al., 2019). One possible explanation for the discrepancy in our results is the paradigms for training on BE; the authors put animals through four days of transitions between cue and no cue BE, whereas in our study, animals were stabilized for a minimum of three days on BE before testing. Therefore, we may be analyzing different aspects of cue learning and the motivational significance animals attribute to cocaine-paired cues over time.

A greater proportion of LH orexin neurons project to VTA

Using the retrograde tracer CTb into VTA, we observed both ipsilateral and contralateral projections from the orexin cell field to VTA. However, we observed fewer VTA-projecting orexin neurons in the contralateral hemisphere in both LH and Pef/DMH. To our knowledge, this is the first study to demonstrate that orexin neurons collateralize in VTA, though orexin neurons have been shown to collateralize in other brain regions. Retrograde labeling in structures mediating

arousal revealed that there are substantial bilateral orexin projections to locus coerulus, whereas basal forebrain receives predominantly ipsilateral input (Espana, Reis, Valentino, & Berridge, 2005). In addition, retrograde Fluoro-Gold injections revealed that input to VTA is largely bilateral, and that the lateral hypothalamic area, which contains the orexin cell field, has a large contralateral input to VTA (Geisler & Zahm, 2005). Therefore, it is unsurprising that orexin neurons project bilaterally to VTA and may explain why unilateral infusions of SB into VTA can attenuate drug seeking (James et al., 2011; Narita et al., 2006).

When we compared the number of CTb, orexin, and CTb/orexin -expressing neurons in LH and Pef/DMH, the number of CTb- or orexin-containing cells was greater in Pef/DMH. We previously showed that Pef, DMH, and LH have similar numbers of CTb-labelled cells in animals following cued reinstatement (Mahler & Aston-Jones, 2012). We pooled the total number of immuno-labeled cells in Pef and DMH, as DMH contains a very small population of orexin neurons, and our previous study analyzed DMH and Pef separately, which likely explains the discrepancy in our data. However, we observed similar numbers of CTb-expressing orexin neurons in LH and Pef/DMH, so a greater percentage of LH orexin neurons project to VTA. LH orexin neurons project preferentially to VTA using combined retro- and anterograde tracing in rats, and the entire antero-posterior VTA receives orexinergic input (Fadel & Deutch, 2002). Still, other studies have shown that there are no topographic differences in orexin projections to VTA with injection or retrobeads into VTA in mice (Gonzalez et al., 2012). This may be due to the anatomical tracer or species utilized in each study. In addition,

these studies highlight the importance of developing tools to manipulate specific orexin circuits to VTA to determine their functional importance.

Collectively, these studies demonstrate that orexin-1 receptor signaling in VTA mediates the motivational properties of drug-associated cues. In addition, targeting LH orexin projections specifically in VTA may reduce propensity for relapse in respond to drug-associated cues.

Chapter 5: Conclusions & Future Directions

These studies implicate both LS and LH orexin neuron input to the mesolimbic DA system as critical connections that drive motivation for cocaine. In Chapter 2, we showed that LS inhibition increased demand elasticity (α) but did not impact baseline consumption (Q_0), and these effects could be reversed with pretreatment of diazepam. These studies demonstrated that LS mediates demand elasticity by stimulating reward, and not anxiety, pathways. In Chapter 3, we found that the number of LH orexin neurons predicts motivation for cocaine, and knockdown of LH orexin cells attenuated motivation. Finally, in Chapter 4, orexin input to VTA reduced α but not Q_0 values. These studies and previous research from our lab (Morgan H James et al., 2018) indicate that orexin neurons in LH mediate orexin's effects on motivation for cocaine.

Both LS and LH orexin neurons have been shown to mediate cue-dependent cocaine seeking behaviors. While we did not directly test the impact of LS inhibition in the absence of cues, we did observe that orexin signaling in VTA is cue-dependent (Chapter 4). In particular, orexin specifically mediates motivation for cocaine when animals are trained and tested on BE with cues. Removing cues reduces motivation and the efficacy of the orexin-1 receptor antagonist SB to decrease motivation. Collectively, these studies point to LS and LH orexin neurons as critical brain regions to target for the treatment of addiction.

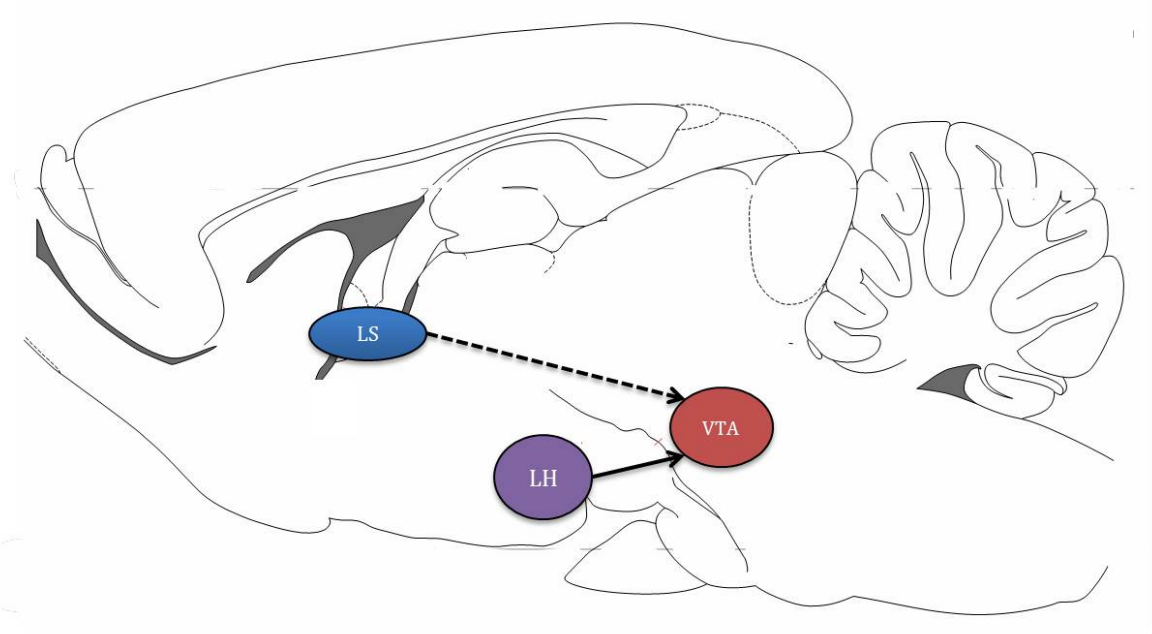

Figure 5.1. Key inputs to the ventral tegmental area (VTA) from lateral septum (LS; Chapter 2) and lateral hypothalamus (LH) orexin neurons (Chapters 3 and 4) that are necessary for cocaine demand. Both LS and LH orexin neurons have been implicated in processing cocaine-associated cues and may be necessary for linking stimulus (cocaine cues/context) to reward (drug seeking/taking).

5.1 LS and LH orexin neurons mediate cue processing in addiction

Both LS and LH orexin neurons have established roles in cue-dependent drug seeking behaviors, yet it remains unclear how these neuronal populations mediate cue processing in addiction. These studies highlight that both populations of neurons are necessary to drive motivation for cocaine when cues are present.

5.1.1. LH orexin neurons

As discussed in Chapter 4, orexin signaling is particularly important for drug seeking when cues are paired with drug delivery. Orexin has been proposed to translate motivational drive into behavioral output in response to external cues (Mahler et al., 2014). Therefore, orexin seems to be important for reactivity to cues and, in doing so, trigger relapse to drug taking. Blocking orexin-1 receptor signaling is particularly effective at reducing drug taking/seeking in highly motivated animals (M. H. James, H. E. Bowrey, et al., 2018; Morgan H James et al., 2018; Lopez et al., 2016; Moorman & Aston-Jones, 2009; Moorman et al., 2017). Greater LH orexin neuron expression may indicate greater reactivity to cocaine cues and subsequently, higher motivational drive and drug taking.

5.1.2. Lateral septum

Lateral septum receives input from regions associated with cognition, like prefrontal cortex and hippocampus and projects to reward-associated areas, indicating that it may integrate cognitive information associated with reward delivery to promote behaviors towards obtaining the reward. It has been proposed that LS encodes valence associated with sensory stimuli and promotes an affective state based on stimuli valence (Sheehan et al., 2004). It is thought that cocaine

induces both positive and negative affective states that co-occur and oppose one another (Ettenberg, 2004; Geist & Ettenberg, 1997; Solomon & Corbit, 1974). As cues paired with cocaine may acquire both reinforcing/positive (Berridge & Robinson, 1998; Robinson & Berridge, 1993; Robinson et al., 2014) and/or aversive/negative (Koob, 2008; Wheeler et al., 2015) associations over time, inhibiting LS may shift the opponent process theory to bias one (or neither) affective state. Our data support a role for LS in the positively reinforcing properties of cocaine, so a lack of this affective state may reduce drug taking by biasing the negative/anxiogenic properties of the drug.

Like the LH orexin cell population, it is clear that LS mediates cue-dependent behaviors (Harasta et al., 2015; Luo et al., 2011; Mahler & Aston-Jones, 2012; McGlinchey & Aston-Jones, 2017). However, unlike the LH orexin neurons, LS has not been studied during drug taking when cues are not present, such as with drug primed reinstatement. Therefore, while there is an established role for LS in cue-associated drug taking, it is unclear if its role is dependent on cues. In addition, there has not been a study conducted on whether LS inhibition impacts low effort drug taking, such as during FR-1. Consequently, future studies would illuminate whether LS promote the reinforcing properties of cocaine, cocaine-associated cues, or both, and whether LS involvement in drug addiction varies with effort.

5.2. Possible interconnectivity between LS and LH orexin neurons for motivation for cocaine

LS and LH orexin neurons were found to have similar roles in promoting high effort drug taking, as reducing input from these two regions attenuated motivation for cocaine. As discussed in Chapter 2, inhibitory LS input to VTA GABAergic neurons may disinhibit VTA DA neurons and promote drug seeking. Similarly, orexin-1 receptor signaling in VTA drives highly motivating, cue-dependent drug seeking behaviors, and local injections of SB reduced motivation for cocaine. These results indicate that the two neuronal populations may interact, either in VTA or with each other, to drive drug taking.

LS neurons may act on orexin neurons, as well as VTA DA neurons, for motivation. Several studies have shown that LS connects to orexin neurons and other subpopulations of hypothalamic cells to drive motivated behavior for natural and drug rewards (Bender et al., 2015; Carus-Cadavieco et al., 2017; Sartor & Aston-Jones, 2012; Sweeney & Yang, 2016). LS neurons also regulate the hypothalamic-pituitary-adrenal (HPA) axis through input to paraventricular nucleus, and LS stimulation elevates corticosterone levels (Anthony et al., 2014). Therefore, LS neurons may also act on orexin neurons either through direct stimulation or disinhibition of local hypothalamic GABAergic neurons. In particular, a recent study showed that GABAergic input from the preoptic area (POA) inhibits orexin neurons (Saito et al., 2018), and medial POA is densely interconnected with ventral LS (Risold & Swanson, 1997b). Orexin neurons are also locally inhibited by hypothalamic GABAergic interneurons, as optogenetic inhibition of Vgat+ hypothalamic neurons induced inhibitory post-synaptic currents in orexin neurons (Ferrari et al., 2018). Consequently, there is evidence that LS may also act on

orexin neurons to influence their activity, and this connectivity may be an important driver for orexin's role in motivation for cocaine.

LS and LH orexin input may also converge in VTA to promote drug taking. In particular, LH orexin potentiates glutamatergic input onto VTA DA neurons, and this input is necessary for drug taking (Borgland et al., 2009; Borgland et al., 2006; Mahler et al., 2013). However, as mentioned in the Introduction, roughly half of VTA DA neurons are silenced by local VTA GABA neurons, and stimulating LS can disinhibit VTA DA neurons. Consequently, both LS disinhibition of VTA DA neurons and LH orexin potentiation of glutamate input may be necessary to promote drug-induced changes in VTA DA neurons that can trigger motivated behavior in response to cues.

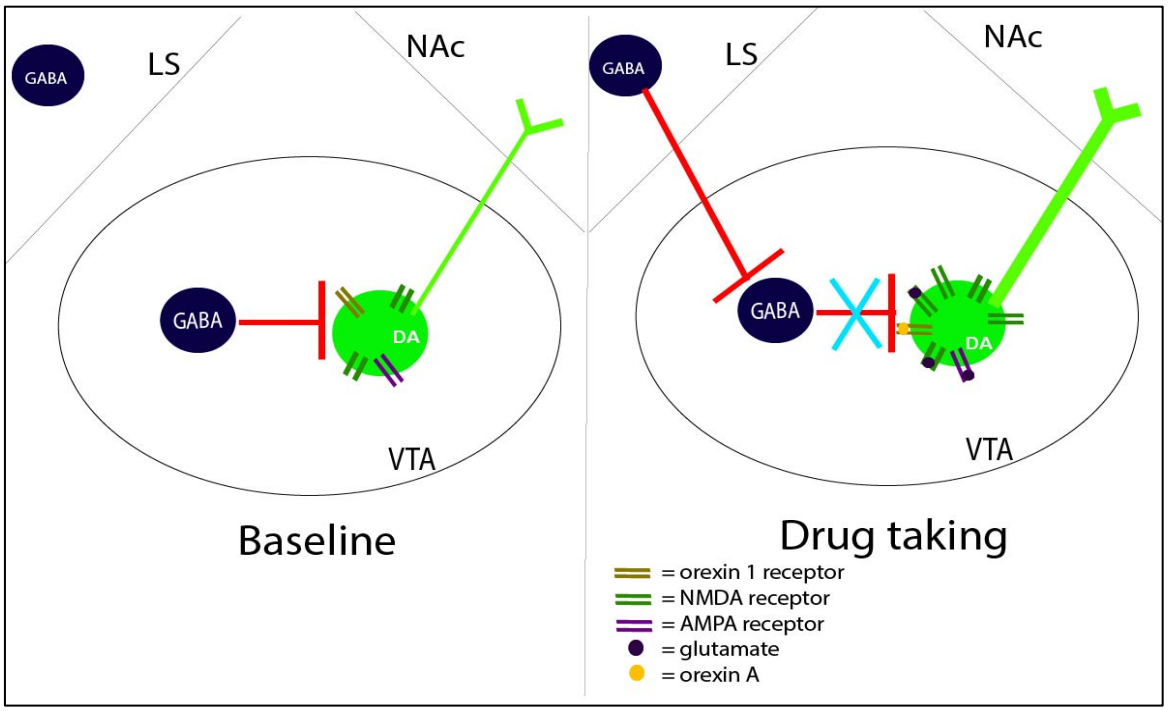

Figure 5.2. Proposed mechanism for the integration of LS and LH orexin input in VTA. At baseline, VTA DA neurons are inhibited by local VTA GABA neurons. During drug taking, LS neurons disinhibit these GABAergic neurons to permit VTA DA firing. Orexin binding to the orexin-1 receptor potentiates glutamate input by increasing NMDA receptor surface expression. These processes induce LTP in VTA DA projections to NAc.

5.3 Future Directions

5.3.1. Identification of LS and LH orexin circuits important for BE

Our studies utilized local microinjections of pharmacological, antisense, or retrograde tracing approaches to ascertain brain regions and potential circuits that contribute to motivation for cocaine. Future studies should be directed towards manipulating specific circuits in which LS, LH orexin, or VTA participate to further elucidate the regions' contributions to cocaine demand. One approach would be to utilize DREADDs and inject the inert ligand of the receptor, clozapine N-oxide (CNO), into known targets through local microinjections. Our lab recently manipulated VTA DA neurons using DREADDs into TH-cre transgenic rats and observed that inhibition with the inhibitory (Gi-coupled) DREADD reduced both motivation (α) and increased baseline consumption (Q_0). In addition, when animals expressing the excitatory (Gq-coupled) DREADD into VTA and CNO was injected into the medial prefrontal cortex (mPFC) or basolateral amygdala (BLA), stimulation of the VTA DA-mPFC or -BLA pathway augmented reinstatement of drug seeking to cues (Mahler et al., 2018). Using this approach or optogenetics (Corre et al., 2018) during BE could elucidate important projection targets of each of these regions for their respective roles in cue-associated motivation for cocaine.

However, there are several challenges when manipulating either LS or LH orexin neurons using these approaches in rats. Our lab has observed that Gi-coupled DREADD virus transduction under a ubiquitous synapsin promoter expresses in LS but does not produce behavioral effects during either reinstatement or BE (unpublished data). This may indicate that the Gi DREADD is

ineffective in LS. One possible explanation for this is that LS is highly connected to medial septum, and this septal interconnectivity may be able to compensate for DREADD inhibition (Montagnese, Zachar, Balint, & Csillag, 2008; Swanson & Cowan, 1979). Thus, targeting certain neuronal populations in LS with a cell-type specific promoter may be more effective. Optogenetic methods have been employed in LS using a Cre-dependent adeno-associated virus into Crfr2-Cre mice to specifically target Crfr2-containing neurons (Anthony et al., 2014). In particular, glucagon-like peptide-1 receptor-expressing (GLP1R) neurons in LS have been linked to addiction and motivation to seek drugs of abuse (Harasta et al., 2015; Reddy et al., 2016), and Glp1r may be an effective promoter to drive DREADD or optogenetic virus expression in LS neurons. Alternatively, developing transgenic rats expressing Cre in certain cell types enriched in LS may be another strategy to interrogate the importance of LS circuits in addiction.

Similarly, circuit-level manipulation of orexin neurons in rats has proved challenging, possibly because orexin protein expression is dynamic and fluctuates in response to external stimuli [(Morgan H James et al., 2018; Lawrence et al., 2006; McGregor, Wu, Barber, Ramanathan, & Siegel, 2011; Thannickal et al., 2018) and Chapter 3]. Several studies knocked down orexin neurons using morpholino (Morgan H James et al., 2018; Prasad & McNally, 2014; Reissner et al., 2012; Sartor & Aston-Jones, 2012) or viral vector (Schmeichel et al., 2018) approaches, or orexin receptor expression in known target regions (Bernstein et al., 2017; Chen et al., 2010). In addition, chemogenetic manipulation of orexin circuits has been successful in orexin-cre transgenic mice (Stanojlovic, Pallais

Yllescas, Mavanji, & Kotz, 2019; W. Zhou et al., 2018), as well as through optogenetic methods [for review, (de Lecea, 2015), yet application to rats has been limited. Consequently, it is necessary to develop viral vectors that causally link orexin neuron manipulation to changes in drug taking.

5.3.2. Are LS or LH orexin effects on motivation drug-specific?

Both LS and orexin neurons have opposing roles on feeding behavior; LS inhibition promotes feeding (Sweeney & Yang, 2015, 2016), whereas blocking orexin-1 receptor signaling decreases motivated consumption of sucrose (Cason & Aston-Jones, 2013; Richards et al., 2008) or high fat food (Borgland et al., 2009; Nair, Golden, & Shaham, 2008). In Chapter 2, our studies provided support for a role for LS in feeding, as inhibition of the region increased FR-1 consumption of sucrose pellets. As therapies directed towards treating addiction should decrease motivation for drug, and not natural rewards, future studies should investigate whether any of the manipulations utilized in our studies impacted motivated feeding behavior in addition to drug seeking. BE has been utilized to compare motivation for food and drugs of abuse (Christensen et al., 2009; Christensen, Silberberg, Hursh, Roma, et al., 2008), so these manipulations could be performed during food BE to determine if the changes were observed on demand elasticity were specific to cocaine or also extend to natural rewards.

5.4 Summary

Here we found in our BE paradigm that LS inhibition reduced motivation for cocaine (increased demand elasticity, α) without impacting baseline consumption

(Q_0) and that stimulating reward pathways with the benzodiazepine diazepam reversed the effects of LS inhibition on motivation. These results indicate that LS likely promotes motivation through its connection to the mesolimbic DA system. In addition, we also observed that blocking orexin-1 receptor signaling with SB-334867 (SB) in VTA also reduces motivation for cocaine, and that SB effects on motivation are dependent on the presence of cues during training. Orexin neurons in LH may mediate orexin's effects, as a greater proportion of LH orexin neurons project to VTA and the number of LH orexin neurons predicts individual differences in motivation for cocaine immediately after BE testing or after two weeks of abstinence from cocaine taking. Collectively, the results of this dissertation implicate LS and LH orexin neurons connectivity to the mesolimbic DA system as important for economic demand and potential therapeutic targets for addiction treatment.

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