THE ROLE OF THE CENTRAL GLP-1 SYSTEM IN COCAINE-SEEKING BEHAVIOR

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To my fellow underrepresented students, may you prosper.

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ABSTRACT

THE ROLE OF THE CENTRAL GLP-1 SYSTEM IN COCAINE SEEKING BEHAVIOR

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Cocaine use disorder continues be a significant public health concern with no available FDA-approved pharmacotherapies despite decades of research focused on treatment and the neurobiological mechanisms underlying drug addiction. Thus, there remains a critical need for conceptually innovative approaches toward identifying new medications to treat this disease. Current preclinical literature show that analogs of the neuroendocrine hormone glucagon-like peptide-1 (GLP-1) have promising clinical potential, as GLP-1 receptor (GLP-1R) activation reduces the rewarding and reinforcing effects of cocaine in animal models of drug addiction. To expand our knowledge of the neural circuitry and neurobiological mechanisms that underly the effects of GLP-1 on cocaine addiction, the research presented in this doctoral dissertation studies the role of the central GLP-1 system in regulating the reinstatement of cocaine-seeking behavior, an animal model of relapse. In Chapter 2, I establish that the GLP-1R agonist exendin-4 reduces cocaine seeking at doses that do not cause adverse side effects and acts on GLP-1Rs in the ventral tegmental area (VTA). Second, I show that the endogenous central GLP-1 system is dynamically changed by cocaine self-administration and abstinence. Chapter 3 demonstrates that GLP-1R activation in the nucleus accumbens (NAc) was sufficient to reduce cocaine seeking. To determine the effects of NAc GLP-1R activation on neuronal excitability in cocaine-experienced rats, electrophysiological studies were conducted and found that exendin-4 increased the intrinsic excitability of NAc medium spiny neurons. Chapter 4 continues to investigate the mechanism of exendin-4 to reduce cocaine seeking and the involvement of GLP-1R signaling in hindbrain nuclei. Administration of exendin-4

into the laterodorsal tegmental nucleus (LDTg) was found to decrease cocaine seeking by activating GLP-1Rs on GABAergic neurons that project to the VTA. Additionally, the ability of GLP-1-producing neurons in the nucleus tractus solitarius (NTS) to reduce cocaine seeking was examined. Activation of NTS-to-LDTg projections significantly attenuated cocaine seeking through a GLP-1-mediated mechanism without effecting food intake or body weight in cocaine-experienced animals. Collectively, this dissertation broadens our understanding of the neurobiological mechanisms that regulate cocaine seeking and also provides further evidence for the use of GLP-1 system-targeted therapeutics for treating cocaine use disorder.

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LIST OF ABBREVIATIONS

CNO	Clozapine-n-oxide (DREADD agonist)
GLP-1	Glucagon-like peptide-1
GLP-1R	GLP-1 receptor
Ex-4	Exendin-4 (GLP-1R agonist)
Ex-9	Exendin-(9-39) (GLP-1R antagonist)
LDTg	Laterodorsal tegmental nucleus
NAc	Nucleus accumbens
NTS	Nucleus tractus solitarius
PPG	Preproglucagon (GLP-1 precursor)
VTA	Ventral tegmental area

CHAPTER 1: Introduction

Cocaine: A Brief History

Cocaine is derived from the leaves of *Erythroxylum coca,* a plant that is native to South America. The leaves of the coca plant have been utilized for thousands of years by native peoples of the South American region. When the leaves are chewed or brewed for tea, the effects cause a reduction in appetite and create a mild stimulant effect that reverses fatigue. However, the power of the coca plant was left largely unexplored in the Western/European world until cocaine was isolated in 1860 by German scientist Albert Niemann (Gootenberg, 2008; Goldstein et al., 2009). By the late 1800's, cocaine was widely used in medical practice as an anesthetic and in commercial products such as wines and the famous soft drink, Coca-Cola. Due to an increased awareness of the addictive properties of cocaine and opioids, the U.S. passed the Harrison Narcotics Act in 1914 which restricted and regulated their distribution (Gootenberg, 2008; Goldstein et al., 2009). By the 1920's, the use of cocaine declined but with illegalization, the illicit cocaine market started to emerge. The demand for cocaine made a rapid resurgence in the 1970's with the help of the counterculture movement and the rise of large-scale illegal drug trafficking. Cocaine use continued to skyrocket into the 1980's and 1990's with Americans spending about 38 billion dollars on cocaine each year (Gootenberg, 2008; Goldstein et al., 2009). Although cocaine use is not at the same level today as it once was before the 2000's, cocaine remains the 3rd highest abused illicit drug after marijuana and prescription opioids and continues to be a significant public health concern (NSDUH, 2018).

Cocaine Use Disorder

In the United States, approximately 5.9 million Americans (2.2% of the population) aged 12 years or older reported having used cocaine in the past year (NSDUH, 2018). Recent epidemiological data indicate that the prevalence of cocaine use is increasing (Hughes, 2016). For example, ~2% of all young adults (18-25 years of age) were classified as current users of cocaine in 2017, the highest rate reported for this age group in the past 10 years (NSDUH, 2018). One hallmark of cocaine use disorder is the high rate of relapse following detoxification (O'Brien, 1997; Dackis and O'Brien, 2001). Unfortunately, there are no FDA-approved medications to treat cocaine craving-induced relapse despite decades of focused preclinical and clinical research that have advanced our understanding of the anatomical, neurochemical and molecular bases of drug addiction (O'Brien, 1997; Pierce et al., 2012). Thus, there is a clear need for innovative research aimed at identifying novel neurobiological mechanisms underlying cocaine addiction and new therapeutic drug targets to treat this disease.

Animal Models of Cocaine Use Disorder

Preclinical studies utilizing rodent models of addiction-like behaviors are critical towards identifying molecular substrates that could serve as targets for novel pharmacotherapies aimed at preventing or reducing relapse in human cocaine addicts. Cocaine craving and relapse of drug-taking behavior in abstinent human addicts are precipitated by three major stimuli: stressful life events, re-exposure to environmental cues previously associated with drug taking, and re-exposure to the previously self-administered drug itself (de Wit and Stewart, 1981; Jaffe et al., 1989; O'Brien et al., 1992; Sinha et al., 1999). Craving-induced relapse of drug taking/seeking in humans is typically modeled in laboratory animals as follows: after a period of drug self-administration and the subsequent extinction of the drug-reinforced operant behavior, the ability of stress exposure, drug-associated stimuli,

or re-exposure to the drug itself to reinstate drug-seeking behavior is assessed (Shalev et al., 2002; Shaham et al., 2003; Mantsch et al., 2016). For example, after extinction of cocaine self-administration, administration of relatively low doses of cocaine reinstate operant responding in the absence of drug reinforcement in both non-human primates and rodents (Gerber and Stretch, 1975; de Wit and Stewart, 1981; Spealman et al., 1999; Anderson et al., 2003). As the most commonly used animal model of relapse, the reinstatement model has proven invaluable for elucidating the neural circuits and neurobiological mechanisms underlying cocaine-seeking behavior (Schmidt et al., 2005; Schmidt and Pierce, 2010).

Neurocircuitry Mediating the Rewarding and Reinforcing Efficacy of Cocaine

Cocaine functions, in part, as a non-selective biogenic amine transporter inhibitor that binds to and inhibits dopamine, serotonin and norepinephrine transporters (Ritz et al., 1990). Biogenic amine transporters are directly coupled to transmembrane Na^+/Cl^- gradients and convey neurotransmitters from the extracellular space into the presynaptic nerve terminal (Elliott and Beveridge, 2005). Indeed, the primary mechanism by which dopamine, serotonin and norepinephrine signaling is inactivated in the brain is through high-affinity transporter-mediated uptake (Elliott and Beveridge, 2005). Thus, cocaine's main pharmacological mechanism of action in the brain is to increase extracellular concentrations of dopamine, norepinephrine and serotonin resulting in enhanced transmission of these biogenic amines.

Drugs of abuse including cocaine produce their reinforcing effects through actions in the mesolimbic reward system, a circuit of functionally and anatomically interconnected nuclei that are responsible for the influence of motivational, emotional, contextual and affective information on behavior (Pierce and Kumaresan, 2006; Schmidt and Pierce, 2010). Limbic

nuclei, including the amygdala, hippocampus, and medial prefrontal cortex (mPFC), send major glutamatergic projections to the nucleus accumbens (NAc), which functions as a hub integrating this information with midbrain dopamine innervation that encodes motivational information pertaining to the rewarding and reinforcing properties of drugs of abuse (Kelley, 2004). The NAc is broadly divided into two main subregions, the shell and core (Zahm, 1999). The NAc sends segregated efferent GABAergic projections to the ventral pallidum and ventral tegmental area (VTA)/substantia nigra (Groenewegen et al., 1999; Zahm, 1999). Both the ventral pallidum and VTA, in turn send GABAergic efferent projections to the medial dorsal thalamus. Glutamatergic projections from the medial dorsal thalamus to the mPFC close this limbic circuit (Alexander and Crutcher, 1990; Kalivas and Nakamura, 1999; Groenewegen and Uylings, 2000). Dopaminergic neurons in the VTA innervate the NAc, amygdala, hippocampus, mPFC, and ventral pallidum, and changes in dopaminergic transmission play a critical role in modulating the flow of information through the mesolimbic reward system (Wise, 2002; Jay, 2003; Feltenstein and See, 2008; Bromberg-Martin et al., 2010). Thus, excitatory input from cortical and subcortical structures to the NAc is filtered and integrated by dopamine-mediated mechanisms, thereby shaping information output to the basal ganglia (Schmidt and Pierce, 2010).

Midbrain dopamine neurons exhibit distinctive firing patterns including occasional, high-frequency trains of action potentials known as bursts (Grace and Bunney, 1984; Marinelli et al., 2006). Bursts of action potentials from midbrain dopamine neurons and their associated release of dopamine in target nuclei are collectively termed “phasic” dopamine signaling (Hsu et al., 2018). Phasic burst firing of midbrain dopamine neurons and subsequent phasic release of dopamine in the NAc are key neurophysiological mechanisms that implicate mesolimbic dopamine signaling as critical for goal-directed

behaviors including drug reinforcement (Roitman et al., 2004; Cheer et al., 2007; Day et al., 2007; Tsai et al., 2009; Schultz, 2013; Sulzer et al., 2016). Phasic mesolimbic dopamine signaling is thought to reinforce learned associations between predictive stimuli and primary reward (Schultz, 2007, 2013). Based on a large literature examining reward prediction error, phasic dopamine is commonly referred to as a "teaching" signal that functionally regulates motivational aspects of behavior (Hollerman and Schultz, 1998; Schultz, 2016; Hsu et al., 2018). Phasic dopamine signaling is also believed to play an important role in the incentive-sensitization theory of addiction (for further review, please see Robinson and Berridge, 1993; Berridge, 2012; Berridge and Robinson, 2016).

The reinforcing effects of cocaine are primarily dependent upon activation of the mesolimbic dopamine system (Wise, 1996; Pierce and Kumaresan, 2006; Schmidt and Pierce, 2006b). Dopaminergic projections from the VTA to limbic nuclei including the NAc play a critical role in cocaine self-administration and the reinstatement of cocaine-seeking behavior (Schmidt et al., 2005; Pierce and Kumaresan, 2006; Schmidt et al., 2006; Schmidt and Pierce, 2006b, a). Additionally, recent studies have begun to examine how the mesolimbic dopamine system is modulated by downstream nuclei. For example, regions such as the lateral septum, laterodorsal tegmental nucleus (LDTg), habenula, and lateral hypothalamus project to the VTA and regulate cocaine-mediated behaviors by modulating dopamine transmission in midbrain and forebrain nuclei (Schmidt et al., 2009; Aston-Jones et al., 2010; Sartor and Aston-Jones, 2012; Sotomayor-Zarate et al., 2013; Lammel et al., 2014; Zapata et al., 2017; Lopez et al., 2018).

In addition to regulating drug craving and relapse, the mesolimbic dopamine system also plays an important role in regulating the hedonic value of food and consequently behaviors directed towards the consumption of food (Wise, 2006; Narayanan et al., 2010; Kenny,

2011a; DiLeone et al., 2012). Moreover, the neurobiological mechanisms underlying food intake and drug seeking overlap to a degree (Kenny, 2011b). For example, neuropeptide-mediated signaling in the VTA plays a key role in modulating mesolimbic dopamine transmission and the rewarding properties of both food and drugs of abuse (Kenny, 2011b; DiLeone et al., 2012). These findings suggest that the same biochemical and molecular mechanisms that play a role in the control of food intake may also influence voluntary drug taking and seeking. Indeed, the idea that shared neural mechanisms regulating both food intake and drug seeking has informed basic science approaches toward identifying novel pharmacotherapies for drug addiction.

Metabolic Factors Regulate the Mesolimbic Reward System and Food Intake

Identifying endogenous modulators of the mesolimbic reward system may provide new targets for drug discovery programs aimed at developing novel medications to treat obesity and substance use disorders (DiLeone et al., 2012; Volkow et al., 2017). Over the last two decades, it has become clear that peripheral and central homeostatic regulators of hunger, satiety and body weight interact with and influence the mesolimbic reward system (Kenny, 2011b; Grill and Hayes, 2012; Williams and Elmquist, 2012). These metabolic factors regulate the mesolimbic reward system directly by stimulating or inhibiting VTA dopamine neurons through cognate receptors or indirectly through downstream nuclei projecting to the VTA (Figlewicz et al., 2003; Hommel et al., 2006; Skibicka et al., 2011; Alhadeff et al., 2012; Labouebe et al., 2013). Indeed, metabolic factors such as insulin, leptin, glucagon-like peptide-1, and amylin inhibit VTA dopamine neurons and decrease food intake (Wang et al., 2015; Volkow et al., 2017). Furthermore, central administration of both leptin and insulin are known to decrease striatal dopamine release as well as act directly on dopamine neurons to regulate food intake (McCaleb and Myers, 1979; Krugel et al., 2003; Hommel et al., 2006; Figlewicz et al., 2007). In contrast,

the feeding hormone ghrelin promotes *ad libitum* food intake through actions on ghrelin receptors expressed in the VTA and NAc (Naleid et al., 2005; Abizaid et al., 2006). The effects of ghrelin on mesolimbic dopamine signaling and food intake, however, are complex. For example, infusions of ghrelin directly into the VTA, but not the NAc, increase food motivated behaviors (i.e. operant responding for food) adding further complexity to how the mesoaccumbens dopamine system integrates feeding signals (Skibicka et al., 2011; Skibicka et al., 2012; Skibicka et al., 2013). These divergent effects may be due to poly-synaptic connections between feeding-relevant nuclei as infusions of ghrelin directly into the lateral hypothalamus, but not VTA, potentiate phasic dopamine release in the NAc in response to food reward and food-predictive cues (Cone et al., 2014; Cone et al., 2015). Based on their ability to modulate the mesolimbic reward system, it has been hypothesized that metabolic factors may regulate non-drug motivated behaviors including addiction-like phenotypes (Hayes and Schmidt, 2016; Jerlhag, 2018).

Glucagon-like Peptide-1 (GLP-1)

Glucagon-like peptide-1 (GLP-1) is an incretin hormone and satiation factor that is released predominantly from L cells of the small intestine and neurons in the nucleus tractus solitarius (NTS) of the caudal brainstem (Baggio and Drucker, 2007; Holst, 2007; Grill and Hayes, 2012). GLP-1 is the primary posttranslational product of the preproglucagon (PPG) gene (Han et al., 1986; Larsen et al., 1997). PPG-expressing neurons in the NTS are the primary central source of GLP-1 and these neurons project to many midbrain and forebrain areas including those implicated in goal-directed behaviors (e.g., VTA and NAc) (Merchenthaler et al., 1999; Rinaman, 2010; Alhadeff et al., 2012). Peripheral GLP-1 has a relatively short plasma half-life of ~2 minutes in both humans and rodents as it is rapidly degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV), which is highly expressed in tissue compartments throughout the body including the central

nervous system (Deacon et al., 1995a; Deacon et al., 1995b; Kieffer et al., 1995; Mentlein, 1999). The GLP-1 receptor (GLP-1R) is a G-protein coupled receptor that is expressed on both pre- and post-synaptic sites throughout the brain (Merchenthaler et al., 1999; Holst, 2007; Hayes et al., 2011b). The GLP-1R couples to different G proteins, including Gs, Gq, and Gi subunits, that stimulate or inhibit intra-cellular second messenger systems (Montrose-Rafizadeh et al., 1999; Hallbrink et al., 2001; Fletcher et al., 2016). However, most intra-cellular signaling is initiated predominately by Gs subunits (Fletcher et al., 2016). Studies have shown that activation of GLP-1Rs increases intracellular calcium levels and activity of downstream signaling molecules such as adenylate cyclase, phospholipase C (PLC), protein kinase A (PKA), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) (Holst, 2007; Hayes et al., 2011b; Rupprecht et al., 2013).

GLP-1 plays an important role in energy homeostasis and food intake in both humans (Gutzwiller et al., 1999a; Gutzwiller et al., 1999b) and animal models (Turton et al., 1996; Barrera et al., 2011). Activation of GLP-1Rs produces a number of physiological responses including increased insulin secretion from pancreatic β-cells, inhibition of gastric emptying and reduced food intake (Holst, 2007; Lovshin and Drucker, 2009; Ruttimann et al., 2009; Hayes et al., 2010; Kanoski et al., 2011). With regard to energy homeostasis, GLP-1R agonists reduce food intake, in part, by activating central GLP-1Rs (Kanoski et al., 2011; Secher et al., 2014; Sisley et al., 2014). Based on their ability to regulate blood glucose levels, food intake and body weight, GLP-1R agonists are FDA-approved for the treatment of type II diabetes mellitus (Lovshin and Drucker, 2009) and obesity (Shukla et al., 2015).

Of interest to the drug addiction field is a body of research showing that GLP-1R activation influences the hedonic value of palatable food by direct modulation of the mesolimbic reward system (Williams and Elmquist, 2012). GLP-1-producing neurons in the NTS send monosynaptic projections to the VTA and NAc and direct infusions of GLP-1R agonists into these brain regions have been shown to reduce consumption of palatable food (Schick et al., 2003; Dossat et al., 2011; Hayes et al., 2011b; Alhadeff et al., 2012; Williams and Elmquist, 2012; Mietlicki-Baase et al., 2013). Interestingly, some of these studies identified intra-cranial doses of GLP-1R agonists that selectively reduced intake of palatable food but not intake of normal chow (Alhadeff et al., 2012; Mietlicki-Baase et al., 2013). These findings indicate that activation of GLP-1Rs in the mesolimbic reward system selectively modulates the hedonic value of food and not homeostatic feeding (Hayes and Schmidt, 2016). Given that the reinforcing effects of drugs of abuse, including cocaine, are regulated by neural circuits that include the VTA and NAc, these findings suggest that central GLP-1R signaling may also reduce non-feeding motivated behaviors including drug taking and seeking.

GLP-1Rs in Animal Models of Cocaine Use Disorder

Emerging evidence indicates that GLP-1Rs play an important role in preclinical models of substance use disorder and that systemic administration of GLP-1R agonists reduces the rewarding and reinforcing effects of drugs of abuse, including cocaine (Skibicka, 2013; Engel and Jerlhag, 2014; Hernandez and Schmidt, 2019). For example, peripheral administration of the GLP-1R agonist exendin-4 (Ex-4) attenuates cocaine conditioned place preference (CPP) and self-administration in mice and decreases in cocaine-evoked dopamine release in the NAc (Egecioglu et al., 2013b; Graham et al., 2013; Sorensen et al., 2015). Additionally, work from our lab has shown that activation of GLP-1Rs in the VTA decreases cocaine self-administration in rats (Schmidt et al., 2016). However, the

central mechanisms that mediate the ability of Ex-4 to reduce cocaine-mediated behaviors are largely unknown. Furthermore, no studies have previously explored the role of the GLP-1 system to regulate animal models of relapse such as the reinstatement of cocaine-seeking behavior. Thus, this dissertation aims to investigate: 1) the ability of Ex-4 to attenuate the reinstatement of cocaine-seeking behavior and the potential mechanisms behind this effect; 2) the role of GLP-1Rs in mesolimbic reward nuclei and endogenous GLP-1 system to regulate cocaine seeking.

The Laterodorsal Tegmental Nucleus (LDTg)

Expanding our knowledge of the circuitry that encompasses the mesolimbic reward system is crucial for understanding the mechanisms that underly cocaine-mediated behaviors and how central GLP-1Rs play a role in this system. The LDTg is a hindbrain nucleus that is a part of the mesopontine tegmentum and is known for its role in arousal and motivated behaviors (Steriade et al., 1990; Maskos, 2008). The LDTg receives inputs from the prefrontal cortex, lateral hypothalamus, lateral habenula, interpeduncular nucleus, VTA, substantia nigra pars compacta, parabrachial nucleus, and NTS (Satoh and Fibiger, 1986; Cornwall et al., 1990). The LDTg sends projections to the prefrontal cortex, hippocampus, lateral septum, thalamic nuclei, lateral habenula, lateral hypothalamus, substantia nigra pars compacta, VTA, interpeduncular nucleus, dorsal and medial raphe, pedunculopontine tegmental nucleus, parabrachial nucleus, and the NTS (Satoh and Fibiger, 1986; Cornwall et al., 1990). The neurons in the LDTg are made up of cholinergic, glutamatergic and GABAergic subtypes. Studies quantifying these cell types in the LDTg have shown that 40-46% of LDTg neurons are GABAergic expressing glutamic acid decarboxylase (GAD), 31-38% are glutamatergic neurons expressing the vesicular glutamate transporter (vGlut2), and ~22% are cholinergic neurons expressing choline acetyltransferase (ChAT) (Wang and Morales, 2009; Luquin et al., 2018). These are

primarily distinct neuronal populations as studies have reported very minimal co-expression within these subtypes in the LDTg (Wang and Morales, 2009; Luquin et al., 2018).

Although GABAergic neurons are the major cell type in the LDTg, the literature regarding LDTg neurons in reward has focused primarily on the cholinergic and glutamatergic neurons. The LDTg along with the pedunculopontine tegmental nucleus is the primary source of cholinergic input into the VTA (Oakman et al., 1995; Mena-Segovia et al., 2008; Holmstrand and Sesack, 2011). LDTg cholinergic and glutamatergic neurons synapse directly onto VTA dopamine neurons and studies have shown that these projections are essential for dopamine burst firing (Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Dautan et al., 2016). Additionally, optogenetic stimulation of LDTg cholinergic or glutamatergic projections to the VTA induces conditioned place preference (CPP) providing further evidence that these pathways drive reward (Lammel et al., 2012; Xiao et al., 2016; Steidl et al., 2017). Moreover LDTg neurons synapse on to VTA-to-NAc projecting dopamine neurons and LDTg stimulation elicits dopamine release in the NAc which is blocked by intra-VTA acetylcholine receptor antagonists (Forster and Blaha, 2000; Lammel et al., 2012). Although it is clear that the excitatory LDTg projections to the VTA promotes reward-related mechanisms and behaviors, there is a paucity of studies examining LDTg GABAergic neurons in motivated behaviors.

Studies have shown that the excitatory neurons in the LDTg promote both drug-related and food-driven behaviors (Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Schmidt et al., 2009; Dickson et al., 2010; Shabani et al., 2010; Dickson et al., 2011; Jerlhag et al., 2012; Lammel et al., 2012; Shinohara et al., 2014; Steidl et al., 2015; Steidl et al., 2017). Lesion studies have shown that the LDTg is necessary for the motor

activating effects of drugs of abuse and increases the latency to self-administer cocaine (Blaha et al., 1996; Laviolette et al., 2000; Forster et al., 2002; Alderson et al., 2005; Dobbs and Cunningham, 2014; Steidl et al., 2015). In addition, cocaine exposure increases presynaptic glutamate release onto LDTg cholinergic neurons and facilitates LDTg stimulation-induced dopamine release to the NAc (Lester et al., 2010; Kurosawa et al., 2013). Moreover, pharmacological inhibition of excitatory signaling in the LDTg attenuates cocaine-mediated behaviors (Schmidt et al., 2009; Shabani et al., 2010; Shinohara et al., 2014). Specifically, our lab has shown that delivery of a glutamate receptor antagonist in the LDTg attenuates cocaine priming-induced reinstatement of drug seeking (Schmidt et al., 2009). While these findings clearly indicate that the LDTg plays an important role in cocaine-mediated behaviors, the neurobiological mechanisms in the LDTg that function to regulate drug-seeking behavior must be investigated.

The LDTg expresses receptors for a variety of metabolic hormones, including GLP-1, amylin, ghrelin, oxytocin, orexin and PYY (Sexton et al., 1994; Merchenthaler et al., 1999; Parker and Herzog, 1999; Greco and Shiromani, 2001; Gould and Zingg, 2003; Dickson et al., 2011; Cabral et al., 2013; Reiner et al., 2017; Reiner et al., 2018), some of which have been studied in the context of motivated behavior. For example, ghrelin administration into the LDTg increases acetylcholine release in the VTA, resulting in dopamine release in the NAc and consumption of food (Jerlhag et al., 2007; Dickson et al., 2010; Dickson et al., 2011; Jerlhag et al., 2012). Alternatively, amylin receptor activation in the LDTg reduces food intake and the motivation to self-administer highly palatable foods (Reiner et al., 2017). Additionally, amylin receptors are expressed on LDTg GABA neurons and the intake suppressive effects of amylin receptor activation are dependent on GABAergic signaling in the LDTg (Reiner et al., 2017). These findings suggest that LDTg GABA neurons may act to reduce motivated behaviors. GLP-1Rs in

the LDTg have also been shown to play an important role in energy balance regulation. Administration of Ex-4 into the LDTg causes a reduction in cumulative chow intake and body weight without any effects on nausea/malaise (Reiner et al., 2018). In addition to exogenously regulating food intake, endogenous GLP-1 signaling in the LDTg also acts to reduce feeding behaviors. GLP-1-producing neurons from the NTS project directly to the LDTg and delivery of the GLP-1R antagonist in the LDTg results in an increase in chow intake and body weight. However, the cell types in the LDTg that mediate the effects of GLP-1R activation on feeding are unknown. Overall, these data strongly suggest that GLP-1R signaling in the LDTg may also play an important role in cocaine-seeking behavior.

The Nucleus Tractus Solitarius (NTS)

The NTS is a brainstem region and an important nucleus in the control of energy balance. The NTS expresses receptors for a wide variety of peripherally- and centrally-derived peptides and neurotransmitters which process vagal nerve-mediated gastrointestinal (GI) satiation factors in addition to blood-borne energy status signals (Grill and Hayes, 2009, 2012). During the ingestion of food, the GI tract releases a number of gut peptides and neurotransmitters into the bloodstream and on vagal sensory afferents that synapse to the NTS (Grill and Hayes, 2009, 2012). In turn, the NTS sends projections to a variety of hindbrain and forebrain areas to control feeding behavior and other aspects of energy balance control (Grill and Hayes, 2009, 2012). Neurons from the caudal region of the NTS project to vagal efferent neurons of the dorsal motor nucleus to control for parasympathetic GI responses including insulin secretion and gastric emptying (Grill and Hayes, 2012). In addition, they send projections to nuclei within the hindbrain and hypothalamus to control neuroendocrine and sympathetic efferent responses related to energy expenditure. NTS neurons from the rostral region project to the parvocellular reticular formation to control

ingestive consummatory responses (i.e., licking, chewing, swallowing) and project to the parabrachial nucleus to regulate neuroendocrine responses (Grill and Hayes, 2012).

The caudal portion of the NTS has a heterogenous cell population which include PPG-expressing neurons that project locally within the NTS and throughout the brain to provide the primary source of central GLP-1 (Rinaman, 2010; Dossat et al., 2011; Alhadeff et al., 2012; Kanoski et al., 2016; Holt et al., 2019). More recent literature has examined the role of NTS GLP-1 signaling in regulating non-homeostatic feeding and motivated behaviors. Viral-mediated knockdown of PPG mRNA expression in the NTS produces hyperphagia and exacerbates high fat diet-induced obesity, suggesting that a reduction of GLP-1 in the NTS increases the rewarding value of food (Barrera et al., 2011). Additionally, NTS GLP-1R activation has been shown to decrease CPP and intake of palatable foods, and attenuate progressive ratio responding for sucrose (Alhadeff and Grill, 2014; Richard et al., 2015). Furthermore, chronic knockdown of NTS GLP-1Rs results in increased chow intake and meal size as well as increased operant responding for sucrose (Alhadeff et al., 2016). Moreover, NTS GLP-1-producing neurons have been shown to project to a wide variety of brain regions that are involved in reward (Figure 1.1) such as the VTA, NAc and LDTg (Rinaman, 2010; Alhadeff et al., 2012; Reiner et al., 2018). Overall, these studies suggest that the NTS may regulate motivated behaviors through its projections to the mesolimbic reward system. However, examination of these circuits in regulating drug-mediated behaviors needs to be established.

The NTS has been shown to be modulated by the effects of cocaine. Previous studies show that acute cocaine exposure increases neuronal activity in the NTS (Grabus et al., 2004; Zahm et al., 2010; Buffalari and Rinaman, 2014). Our lab expanded these findings to show that cocaine self-administration increases c-Fos expression in GLP-1-producing

neurons in the NTS (Schmidt et al., 2016). These data suggest that cocaine-induced activation of NTS GLP-1-producing neurons may result in increased GLP-1 release in midbrain and forebrain areas. The mechanisms by which cocaine activates NTS GLP-1-producing neurons are not clear but may involve increased corticosterone signaling in the hindbrain. Since cocaine taking increases plasma and central corticosterone levels (Goeders and Guerin, 1996; Galici et al., 2000; Mantsch et al., 2000; You et al., 2018) and peripheral administration of corticosterone increases activation of NTS GLP-1-producing neurons (Schmidt et al., 2016), it is possible and likely that cocaine self-administration increases activation of GLP-1-producing neurons in the NTS through a corticosterone-mediated mechanism of action. Indeed, administration of corticosterone directly into the 4th ventricle attenuates cocaine self-administration and these effects are blocked by pharmacological inhibition of GLP-1Rs in the VTA (Schmidt et al., 2016). Taken together, these findings suggest that the endogenous central GLP-1 system and NTS circuits may play an important role in regulating cocaine-seeking behavior.

Overview of Dissertation

The current literature thus far suggests that activation of central GLP-1Rs are an important mechanism to reduce cocaine-mediated behaviors. Therefore, the GLP-1 system may serve as a novel target for treating cocaine-induced craving and relapse. Due to this clinical relevance, it is important to characterize the specific circuits, nuclei and cell types that are activated by GLP-1 and its FDA-approved analogs to regulate addiction-like behaviors. Thus, this dissertation will investigate the role of GLP-1Rs in three mesolimbic reward nuclei, the VTA, NAc and LDTg, in cocaine seeking and the mechanisms by which the GLP-1R agonist Ex-4 decreases cocaine-seeking behavior. This dissertation also aims to understand how cocaine affects the central GLP-1 system and the role of GLP-1-producing NTS circuits in cocaine seeking.

Figures

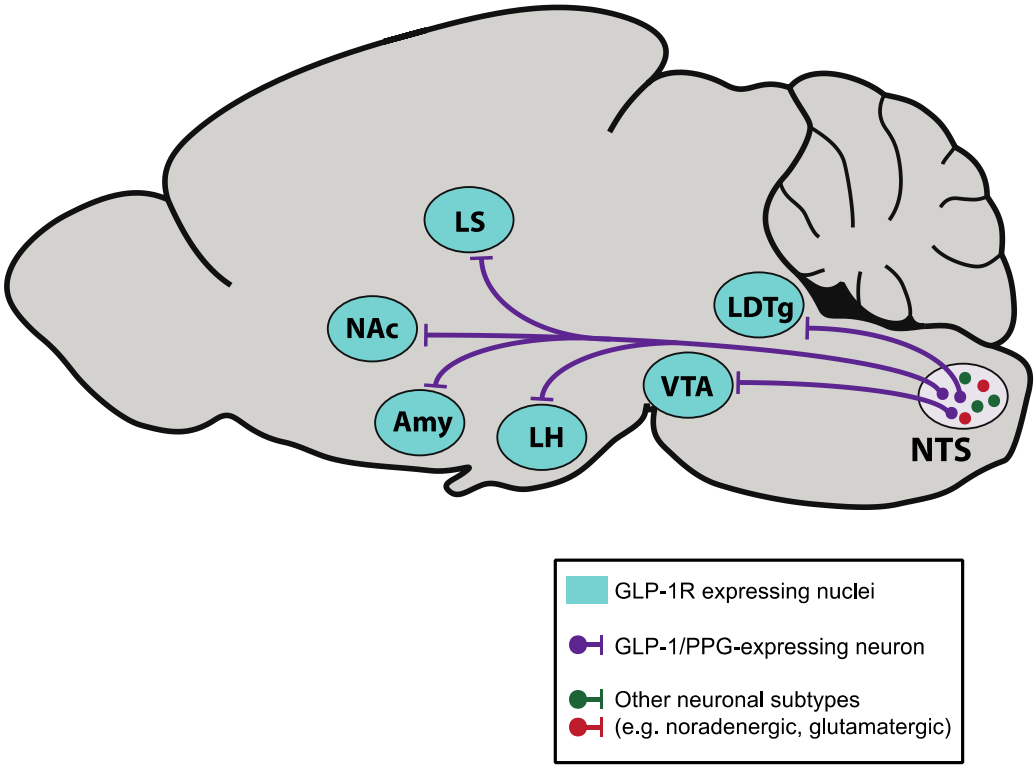

Figure 1.1 NTS PPG neurons innervate nuclei within the mesolimbic reward system PPG = preproglucagon, LDTg = laterodorsal tegmental nucleus, VTA = ventral tegmental area, LH = lateral hypothalamus, LS = lateral septum, Amy = amygdala, NAc = nucleus accumbens.

CHAPTER 2: Glucagon-like peptide-1 receptor activation in the ventral tegmental area attenuates cocaine seeking in rats

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Abstract

Novel molecular targets are needed to develop new medications for the treatment of cocaine addiction. Here, we investigated a role for glucagon-like peptide-1 (GLP-1) receptors in the reinstatement of cocaine-seeking behavior, an animal model of relapse. We showed that peripheral administration of the GLP-1 receptor (GLP-1R) agonist exendin-4 (Ex-4) dose-dependently reduced cocaine seeking in rats at doses that did not affect *ad libitum* food intake, meal patterns or body weight. We also demonstrated that systemic Ex-4 penetrated the brain where it putatively bound receptors on both neurons and astrocytes in the ventral tegmental area (VTA). The effects of systemic Ex-4 on cocaine reinstatement were attenuated in rats pretreated with intra-VTA infusions of the GLP-1R antagonist exendin-(9-39), indicating that the suppressive effects of systemic Ex-4 on cocaine seeking were due, in part, to activation of GLP-1Rs in the VTA. Consistent with these effects, infusions of Ex-4 directly into the VTA reduced cocaine seeking. Finally, extinction following cocaine self-administration was associated with decreased preproglucagon mRNA expression in the caudal brainstem. Thus, our study demonstrated a novel role for GLP-1Rs in the reinstatement of cocaine-seeking behavior and identified behaviorally-relevant doses of a GLP-1R agonist that selectively reduced cocaine seeking and did not produce adverse effects.

Introduction

One hallmark of cocaine addiction is the high rate of relapse to compulsive drug use during periods of abstinence (Leshner, 1997). Indeed, the most difficult aspect of treating cocaine addiction is preventing relapse (O'Brien, 1997). Unfortunately, there are currently no effective FDA-approved treatments for cocaine relapse, which continues to be a significant public health concern. Thus, there is a clear need to identify and develop novel pharmacotherapies for cocaine addiction.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone and neuropeptide that is produced peripherally by intestinal L cells and centrally by preproglucagon (PPG) neurons within the nucleus tractus solitarius (NTS) of the caudal brainstem (Holst, 2007; Grill and Hayes, 2012). GLP-1 receptor (GLP-1R) ligands are FDA-approved for treating type II diabetes mellitus and obesity based on their ability to increase insulin production and reduce food intake (Lovshin and Drucker, 2009; Pi-Sunyer et al., 2015; Shukla et al., 2015; Tella and Rendell, 2015). In addition, a growing literature indicates that peripheral administration of GLP-1R agonists attenuates drug-associated behavioral responses including cocaine-induced conditioned place preference (CPP) and the locomotor-activating effects of cocaine (Skibicka, 2013; Engel and Jerlhag, 2014; Hayes and Schmidt, 2016). However, no studies to date have examined the efficacy of GLP-1R agonists to reduce the reinstatement of cocaine-seeking behavior, an animal model of relapse (Shaham et al., 2003; Epstein et al., 2006).

GLP-1Rs are expressed throughout the brain including the ventral tegmental area (VTA), a nucleus known to play a critical role in cocaine-seeking behavior (Goke et al., 1995; Merchenthaler et al., 1999; Schmidt et al., 2005). Our lab recently identified a novel role for central GLP-1Rs in cocaine self-administration in rats (Schmidt et al., 2016).

Specifically, we found that activation of GLP-1Rs in the VTA attenuates cocaine self-administration (Schmidt et al., 2016). While these results suggest that GLP-1Rs may represent a novel target for cocaine addiction pharmacotherapies, the role of central GLP-1 signaling in cocaine seeking is unknown.

The present study had four main goals: 1) to assess the ability of systemic injections of the GLP-1R agonist exendin-4 (Ex-4) to attenuate the reinstatement of drug-seeking behavior elicited by an acute priming injection of cocaine or re-exposure to conditioned cues previously associated with cocaine self-administration; 2) to determine if the effects of systemic Ex-4 on cocaine seeking are due, in part, to activation of GLP-1Rs in the VTA; 3) to investigate the effects of direct activation of VTA GLP-1Rs on cocaine seeking; and 4) to characterize the effects of cocaine self-administration and subsequent extinction on GLP-1R mRNA expression in the VTA and PPG mRNA expression in the NTS. Our findings support the hypothesis that GLP-1R activation is sufficient to attenuate the reinstatement of cocaine-seeking behavior. Moreover, we identified doses of the GLP-1R agonist Ex-4 that significantly attenuated cocaine seeking and did not produce adverse malaise-like effects or reduce feeding behaviors. These results indicate a novel role for central GLP-1Rs in cocaine-seeking behavior and suggest that pharmacotherapies targeting GLP-1Rs may represent novel approaches for treating cocaine relapse.

Materials and Methods

Animals and housing

Male Sprague-Dawley rats (*Rattus norvegicus*) weighing 225-250 g were obtained from Taconic Laboratories. Rats were individually housed with food and water available *ad libitum* in their home cages. A 12/12 hr light/dark cycle was used with the lights on at 7:00 a.m. All experimental procedures were performed during the light cycle. The experimental protocols were consistent with the guidelines issued by the U.S. National Institutes of Health and were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee.

Drugs

Cocaine was obtained from the National Institute on Drug Abuse (Rockville, MD) and dissolved in bacteriostatic 0.9% saline. Ex-4 and exendin-(9-39) were purchased from the American Peptide Company (Sunnyvale, CA) and were dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA). Fluoro-Ex-4 was purchased from AnaSpec (Fremont, CA) and dissolved in bacteriostatic 0.9% saline. The doses and time course of administration for each of the aforementioned pharmacological compounds were based on the following systemic and intra-cranial microinjection experiments in rats: Ex-4 (Alhadeff et al., 2012; Schmidt et al., 2016), exendin-(9-39) (Alhadeff et al., 2012; Schmidt et al., 2016) and fluoro-Ex-4 (Kanoski et al., 2012; Reiner et al., 2016).

Surgery

Rats were handled daily and allowed one week to acclimate to their home cages upon arrival. Prior to surgery, rats were anesthetized with 80 mg/kg ketamine (Midwest Veterinary Supply, Valley Forge, PA) and 12 mg/kg xylazine (Sigma-Aldrich/RBI, St. Louis, MO). An indwelling catheter (SAI Infusion Technologies, Lake Villa, IL) was inserted into

the right jugular vein and sutured in place. The catheter was routed to a mesh backmount that was implanted subcutaneously above the shoulder blades. To prevent infection and maintain patency, catheters were flushed daily with 0.2 ml of the antibiotic Timentin (0.93 mg/ml; Fisher, Pittsburgh, PA) dissolved in heparinized 0.9% saline (Butler Schein, Dublin, OH). When not in use, catheters were sealed with plastic obturators.

After catheter insertion, some rats were then immediately mounted in a stereotaxic apparatus (Kopf Instruments, CA) and implanted with cannulae for intra-cranial microinjections. Bilateral guide cannulae (26 gauge; 16 mm; Plastics One, Roanoke, VA) were implanted 2mm dorsal to the VTA and cemented in place by affixing dental acrylic to stainless steel screws secured in the skull. The coordinates for the ventral ends of the guide cannulae, relative to bregma according to the atlas of Paxinos and Watson (1997), were as follows: -5.8 mm A/P, ±0.5 mm M/L, and -6.6 mm D/V. An obturator (33 gauge; Plastics One) was inserted into each guide cannula to prevent occlusion.

Cocaine self-administration, extinction and reinstatement of cocaine seeking

Rats were allowed 7 days to recover from surgery before behavioral testing commenced. Initially, rats were placed in operant conditioning chambers and allowed to lever-press for intravenous infusions of cocaine (0.25 mg/kg/infusion, infused over a 5 s period) on a fixed-ratio 1 (FR1) schedule of reinforcement. Rats were allowed to self-administer a maximum of 30 injections per 120 min operant session. Once a rat achieved at least 20 infusions of cocaine in a single daily operant session under the FR1 schedule, the subject was switched to a fixed-ratio 5 (FR5) schedule of reinforcement. The maximum number of injections was again limited to 30 per daily self-administration session under the FR5 schedule. For both FR1 and FR5 schedules, a 20 s time-out period followed each cocaine infusion, during which time active lever responses were tabulated but had no scheduled

consequences. Responses made on the inactive lever, which had no scheduled consequences, were also recorded during both the FR1 and FR5 training sessions. Following 21 days of daily cocaine self-administration sessions, drug-taking behavior was extinguished by replacing the cocaine solution with 0.9% saline. Daily extinction sessions continued until responding on the active lever was <15% of the total active lever responses completed on the last day of cocaine self-administration. Typically, it took ~7 days for rats to meet this criterion. Once cocaine self-administration was extinguished, rats entered the reinstatement phase of the experiment. During reinstatement test sessions, satisfaction of the response requirement (i.e., five presses on the active lever) resulted in an infusion of saline rather than cocaine. Using a between-sessions reinstatement procedure, each reinstatement test session was followed by extinction sessions until responding was again <15% of the total active lever responses completed on the last day of cocaine self-administration. Generally, 1–2 days of extinction were necessary to reach extinction criterion between reinstatement test sessions.

Cue-induced reinstatement of cocaine-seeking behavior was tested in a separate cohort of rats. The same self-administration procedure was used as described above with the addition of contingent light cues. Each cocaine infusion was associated with concurrent illumination of a cue light located directly above the active lever for 5 s. Following 21 days of cocaine self-administration sessions, drug-taking behavior was extinguished by replacing the cocaine solution with saline and turning off the drug-paired cue light. Once cocaine self-administration was extinguished (operationally defined as <15% of the total active lever responses completed on the last day of cocaine self-administration), rats entered the reinstatement phase of the experiment and the ability of the cue light to reinstate drug-seeking behavior was assessed. During reinstatement test sessions, every 5^{th} lever press resulted in an infusion of saline and illumination of the cue light previously

paired with cocaine taking during the self-administration phase of the experiment. The effects of systemic vehicle and fluoro-Ex-4 (0.2 µg/kg, i.p.) on cue-induced reinstatement of cocaine-seeking behavior were tested using a counterbalanced, within-subjects design.

Cocaine Reinstatement

To determine if systemic administration of a GLP-1R agonist reduces cocaine seeking and penetrates the brain, initial studies utilized the GLP-1R agonist Ex-4 tagged with fluorescein (fluoro-Ex-4). In addition to binding GLP-1Rs *in vitro* and *in vivo,* fluoro-Ex-4 produces behavioral responses identical to unlabeled Ex-4 (Rajan et al., 2015; Reiner et al., 2016). Once cocaine-taking behavior was extinguished, rats were pretreated with vehicle or 3.0 µg/kg fluoro-Ex-4 (i.p.) one hour prior to an acute priming injection of cocaine (10 mg/kg, i.p.). Rats were then placed immediately into the operant conditioning chambers and a two-hour reinstatement test session commenced. Separate groups of rats were used in the fluoro-Ex-4 dose-response study to identify doses that attenuate cocaine reinstatement, and are not associated with adverse malaise-like effects (Hayes et al., 2011a; Kanoski et al., 2012). In this experiment, rats were pretreated with vehicle, 0.01, 0.1, and 0.2 µg/kg fluoro-Ex-4 (i.p.) one hour prior to a 10 mg/kg priming injection of cocaine and subsequent reinstatement test sessions. Using a within-subjects design, each rat served as its own control and fluoro-Ex-4 doses were counterbalanced to avoid rank order effects of drug treatment. Twenty-four hours after each treatment, rats were weighed to confirm that 0.01, 0.1, and 0.2 µg/kg doses of fluoro-Ex-4 do not affect body weight.

To determine if the effects of peripherally administered Ex-4 on cocaine seeking are due, in part, to activation of GLP-1Rs in the brain, the GLP-1R antagonist exendin-(9-39) was microinjected into the VTA prior to systemic administration of fluoro-Ex-4. Obturators were removed from the guide cannulae and 33 gauge stainless steel microinjectors (18mm;

2mm projection, Plastics One) were inserted. Using a within-subjects design, rats were infused bilaterally with vehicle or 10 µg/100 nl exendin-(9-39) directly into the VTA. Microinjectors were left in place for an additional one minute following infusions in order to allow for diffusion of the drug solution away from the tips of the microinjectors. Rats were then placed back in their home cages. Thirty minutes later rats received a systemic injection of vehicle or 3.0 µg/kg fluoro-Ex-4 (i.p.)

Sucrose self-administration, extinction and reinstatement of sucrose seeking

Potential nonspecific rate-suppressing effects of intra-VTA Ex-4 were evaluated by assessing the influence of Ex-4 on the reinstatement of sucrose-seeking behavior. Separate cohorts of rats were trained initially to self-administer 45 mg sucrose pellets (Research Diets, New Brunswick, NJ) on a FR1 schedule of reinforcement during daily one hour operant sessions. Once rats achieved stable responding for sucrose (defined as <20% variation in responding over 3 consecutive days) on the FR1 schedule of reinforcement, the response requirement was increased to an FR5 schedule of reinforcement. Rats were limited to 30 sucrose pellets within each daily operant session and were restricted to ~20-25 g of lab chow (Harlan Teklad, Wilmington, DE) daily in their home cages for the duration of the experiment. Water was available *ad libitum* in the home cage.

After two weeks of sucrose-maintained responding on an FR5 schedule of reinforcement, rats underwent an extinction phase where active lever pressing no longer resulted in sucrose delivery. Once active lever responding decreased to <15% of the maximum number of responses completed on the last day of sucrose self-administration, rats proceeded to reinstatement testing. Ex-4 (0.005 and 0.05 µg/100 nl) and vehicle were microinjected into the VTA 10 min prior to the beginning of the reinstatement test sessions.

Using a within-subjects design, each animal served as its own control and doses were counterbalanced across test sessions. The experimenter remotely administered one sucrose pellet every two min for the first 10 min of the reinstatement session. A between-session procedure was used so that each daily reinstatement test session was followed by an extinction session the following day until responding was again <15% of the total active lever responses maintained by sucrose.

Ad libitum food intake

To assess the effects of fluoro-Ex-4 on chow intake in cocaine-experienced rats, a separate group of rats was housed in a custom-made automated feedometer during the extinction and reinstatement phases of the experiment. Rats were housed individually in hanging wire cages each with a small access hole leading to a food cup resting on an electronic scale as we have described previously (Alhadeff et al., 2016; Reiner et al., 2016). Each rat was pretreated with vehicle or fluoro-Ex-4 (0.1 or 0.2 μg/kg) one hour prior to a cocaine priming-induced reinstatement test session. Rats were returned to the feedometer immediately following the reinstatement session and given *ad libitum* access to normal chow (Purina LabDiet 5001, Purina, St. Louis, MO). Chow intake and meal patterns were quantified by measuring the weight of each food cup using computer software (LabView) every 10 s for 24 h. Feeding measurements were recorded 1, 3, 6, 12 and 24 h post session (4, 6, 9, 15 and 27 h post infusion). Total body weight and water intake were measured 24 h post session (27 h post infusion). Cumulative food intake and meal patterns were analyzed, with a meal defined as ingestion of at least 0.25 g of food with a minimum of 10 min between feeding bouts (Alhadeff et al., 2016; Reiner et al., 2016).

Verification of cannula placements

After completion of all VTA microinjection experiments, rats were given an overdose of pentobarbital (100 mg/kg, i.p.). Brains were removed and drop fixed in 10% formalin. Coronal sections (100 µm) were taken at the level of the VTA with a vibratome and mounted on gelatin-coated slides. An individual blinded to behavioral responses verified microinjection sites using light microscopy. Rats with cannula placements outside of the VTA and/or excessive mechanical damage were excluded from subsequent data analyses.

Immunohistochemistry

Rats pretreated with 0.2 or 3.0 µg/kg fluoro-Ex-4 (i.p.) were deeply anesthetized and transcardially perfused with 0.1 M PBS, pH 7.4, followed with 4% formalin in 0.1 M PBS immediately following their cocaine priming-induced reinstatement test session (i.e., three hours post infusion). Once brains were removed, they were postfixed overnight in 4% formalin in 0.1 M PBS and then cryoprotected in 20% sucrose in 0.1 M PBS at 4$_{o}$C for three days. Coronal sections (30 µm) were then taken at the level of the VTA using a cryostat (Leica 3050S; Leica Corp., Deerfield, IL). Brain sections were stored in 0.1 M PBS at 4$_{o}$C until processed.

Immunohistochemistry was performed on free-floating coronal sections containing the VTA according to modified procedures from previously published studies (Reiner et al., 2016; Schmidt et al., 2016). Briefly, sections were washed with 1% sodium borohydride followed by 0.1 M PBS. Sections were then blocked in 0.1 M PBS containing 5% normal donkey serum and 0.2% Triton-X for 1 h at room temperature. Sections were incubated in primary antibodies overnight, and then, following a PBS rinse, they were incubated in secondary antibodies for 2 h. The primary antibodies used were rabbit anti-NeuN (1:1000;

ab177487, Abcam, Cambridge, UK), goat anti-GFAP (1:1000; ab53554, Abcam, Cambridge, UK) and rabbit anti-tyrosine hydroxylase (1:1000; 2792; Cell Signaling, Danvers, MA). Secondary antibodies were donkey anti-goat Alexa Fluor 594 (1:500), donkey anti-rabbit Alexa Fluor 647 (1:500) and donkey anti-rabbit Alexa Fluor 594 (1:500) from Jackson ImmunoResearch (West Grove, PA). Sections were then washed and mounted onto glass slides and coverslipped using Vectashield (Vector Laboratories; Burlingame, CA). Sections were visualized with a Leica SP5 X confocal microscope using the 20x and 63x oil-immersion objectives along with 488, 594 and 633 nm laser lines. Image z-stacks were captured with a 2–3x optical zoom at the 63x oil-immersion objective with a step size of 0.5 µm.

Quantitative real-time PCR, cocaine self-administration and yoked saline controls

Separate rats underwent jugular catheterization as described above. Following a recovery period, the rats were randomly assigned to one of two groups: cocaine-experimental or yoked saline controls. Each rat allowed to respond for contingent cocaine infusions was paired with a yoked rat that received infusions of saline. While lever pressing for the saline-yoked rats had no scheduled consequences, these rats received the same number and temporal pattern of infusions as self-administered by their paired cocaine-experimental rat. Cocaine-experimental rats were allowed to lever press for intravenous cocaine infusions on a FR1 schedule as described above for a total of 21 days.

To assess the effects of cocaine self-administration and extinction on expression of VTA GLP-1Rs and PPG in the NTS, rats were sacrificed either immediately after the first extinction session (Ext1) or following 7 consecutive days of extinction (Ext7). Brains were collected and flash frozen in -20°C isopentane and stored at -80°C. Brains were subsequently mounted on a cryostat (Leica 3050S; Leica Corp., Deerfield, IL) and coronal

sections at the levels of the NTS and VTA were taken. Bilateral 1mm$_3$ micropunches of the VTA and NTS were collected for quantitative real-time PCR to determine GLP-1R and PPG expression, respectively. mRNA expression was quantified using Taqman gene expression kits (GLP-1R: Rn00562406_m1; PPG: Rn00562293_m1; GAPDH: Rn01775763_g1; ThermoFisher Scientific, Waltham, MA). qPCR was conducted using an Eppendorf Mastercycler ep realplex2 and the comparative threshold cycle method was used to quantify relative mRNA expression. Relative fold-expression of VTA GLP-1R and NTS PPG transcripts at Ext1 and Ext7 were normalized to yoked saline controls and GAPDH levels.

Statistics

For all cocaine and sucrose reinstatement experiments, the total mean active and inactive lever responses were analyzed with two-way ANOVAs. Analyses of body weight and feeding behaviors for the fluoro-Ex-4 dose-response experiment were conducted using repeated measures one-way ANOVAs. Pairwise analyses were made using Bonferroni post hoc tests ($p<0.05$). Changes in fold mRNA expression of VTA GLP-1Rs and NTS PPG were analyzed using unpaired t-tests.

Results

Systemic administration of a GLP-1R agonist dose-dependently attenuated cocaine seeking in rats.

Total active and inactive lever responses (mean ± SEM) in rats pretreated with systemic fluoro-Ex-4 (vehicle or 3.0 µg/kg, i.p.; n=10/treatment) prior to a cocaine priming-induced reinstatement test session are shown in Figure 2.1A. These data were analyzed with a two-way ANOVA, which revealed significant main effects of treatment [$F(1,36)=28.63$, $p<0.0001$] and lever [$F(1,36)=66.3$, $p<0.0001$] as well as a significant interaction between lever and treatment [$F(1,36)=29.01$, $p<0.0001$]. Subsequent pairwise analyses indicated that active lever responses were significantly different between vehicle and 3.0 µg/kg fluoro-Ex-4 (Bonferroni, $p<0.05$). No significant differences were found on inactive lever responding between treatments.

While peripheral administration of 3.0 µg/kg fluoro-Ex-4 significantly attenuated cocaine seeking, one could argue that the effects of fluoro-Ex-4 on drug seeking were due to malaise-like effects as previous studies have shown that an acute injection of 3.0 µg/kg Ex-4 produces a mild pica response and reduces food intake and body weight in rats (Hayes et al., 2011a; Kanoski et al., 2012). Therefore, we aimed to identify doses of systemic fluoro-Ex-4 that attenuated cocaine seeking and did not produce pica (Kanoski et al., 2012) or reduce body weight. We assessed the ability of lower doses of fluoro-Ex-4 (0, 0.01, 0.1, and 0.2 µg/kg, i.p.; n=16/treatment) to attenuate cocaine priming-induced reinstatement of drug seeking behavior. Total active and inactive lever responses (mean ± SEM) from the reinstatement test sessions are shown in Figure 2.1B. These data were analyzed with a two-way ANOVA, which revealed significant main effects of treatment [$F(3,120)=6.798$, $p<0.001$] and lever [$F(1,120)=98.85$, $p<0.0001$] as well as a significant lever x treatment interaction [$F(3,120)=6.585$, $p<0.001$]. Subsequent pairwise analyses

indicated significant decreases between vehicle and 0.1 and 0.2 µg/kg fluoro-Ex-4 on active lever responses (Bonferroni, $p<0.05$). No significant differences were found on inactive lever responding between treatments. Body weight was measured 24 h after each treatment to determine if peripheral fluoro-Ex-4 at these doses resulted in a reduction of body weight. Mean body weight changes for each treatment are shown in Figure 2.1C. There were no significant effects of fluoro-Ex-4 treatment on 24 h body weight in cocaine-experienced rats.

We also investigated the ability of low dose fluoro-Ex-4 to attenuate cue-induced reinstatement of cocaine seeking. Consistent with the effects on cocaine priming-induced reinstatement (Figure 2.1B), 0.2 µg/kg fluoro-Ex-4 pretreatment significantly reduced reinstatement of drug-seeking behavior elicited by re-exposure to conditioned cues previously associated with cocaine self-administration (Figure 2.1D).

Doses of systemic fluoro-Ex-4 that attenuated cocaine seeking had no effect on chow intake, number of meals or meal size in cocaine-experienced rats.

To further evaluate potential adverse effects of systemic fluoro-Ex-4 on feeding behaviors in cocaine-experienced rats, meal pattern analyses were performed on each reinstatement test day. Meal pattern analyses began immediately after reinstatement test sessions (i.e., three hours post fluoro-Ex-4 injection), a time point that coincides with decreased food intake in rats treated with higher doses of Ex-4 (Kanoski et al., 2012). Peripheral injections of fluoro-Ex-4 (0.1 and 0.2 µg/kg, i.p.) attenuated cocaine seeking in separate cohorts of rats (n=7/treatment; data not shown but consistent with Figure 2.1B) and had no effect on 24 h body weight gain (Figure 2.2A) similar to Figure 2.1C. Detailed analyses of feeding patterns revealed no effect of fluoro-Ex-4 treatment on cumulative chow intake (Figure 2.2B), number of meals (Figure 2.2C) and meal size (Figure 2.2D) at

any time point. Taken together, these data demonstrated that fluoro-Ex-4 attenuated cocaine seeking at doses (0.1 and 0.2 µg/kg) subthreshold for effects on *ad libitum* chow intake.

Peripherally administered fluoro-Ex-4 penetrated the brain and was visualized in the VTA. Immediately following the reinstatement test session, rats pretreated with a systemic injection of 3.0 µg/kg fluoro-Ex-4 (Figure 2.1A) or 0.2 µg/kg fluoro-Ex-4 (Figure 2.1B) were sacrificed and their brains removed to determine if fluoro-Ex-4 was present in the VTA (Figures 2.3A & 2.3D). Coronal sections of the VTA were immunohistochemically processed to label neurons and astroctyes. Confocal microscopy revealed co-localization of fluoro-Ex-4 with both GFAP-positive astrocytes and NeuN-positive neurons in the VTA (Figures 2.3B & 2.3E). Separate immunohistochemical analyses to evaluate if fluoro-Ex-4 bound putative GLP-1Rs expressed on dopamine neurons in the VTA revealed that fluoro-Ex-4 co-localized with tyrosine hydroxylase-positive neurons (Figures 2.3C & 2.3F).

Intra-VTA administration of the GLP-1R antagonist exendin-(9-39) prevented the ability of peripheral fluoro-Ex-4 to attenuate cocaine seeking in rats.

Our immunohistochemistry findings suggested that the effects of peripheral fluoro-Ex-4 on cocaine seeking are mediated, in part, by activation of GLP-1Rs in the VTA. To test this hypothesis, the GLP-1R antagonist exendin-(9-39) (Ex-9; 10 µg/100nl) was administered directly into the VTA prior to a systemic injection of fluoro-Ex-4 (3.0 µg/kg, i.p.) and a cocaine priming-induced reinstatement test session. Using a within-subjects design, there were 4 treatment conditions (intra-VTA vehicle/peripheral vehicle, intra-VTA vehicle/peripheral fluoro-Ex-4, intra-VTA Ex-9/peripheral vehicle, intra-VTA Ex-9/peripheral fluoro-Ex-4; n=13/treatment). Total active lever responses (mean ± SEM) are shown in Figure 2.3G. These data were analyzed with a two-way ANOVA which revealed

significant main effects of systemic treatment [$F(1,12)=18.06$, $p<0.01$] and intra-VTA treatment [$F(1,12)=10.94$, $p<0.01$] as well as a significant systemic treatment x intra-VTA treatment interaction [$F(1,12)=14.52$, $p<0.01$]. Subsequent pairwise analyses indicated a significant difference in total active lever responding between intra-VTA vehicle/peripheral vehicle, intra-VTA Ex-9/peripheral vehicle, and intra-VTA Ex-9/peripheral fluoro-Ex-4 versus intra-VTA vehicle/peripheral fluoro-Ex-4 treated rats (Bonferroni, $p<0.05$). There was no effect of treatment on total inactive lever responding (data not shown). Corresponding microinjection sites in the VTA are shown in Figure 2.3H. Taken together, these results suggested that the effects of peripheral fluoro-Ex-4 on the reinstatement of cocaine-seeking behavior were due, in part, to activation of GLP-1Rs in the VTA.

Extinction following cocaine self-administration was associated with decreased PPG mRNA expression in the NTS.

Using quantitative real-time PCR, we assessed the effects of cocaine self-administration and subsequent extinction sessions on expression of GLP-1R mRNA transcripts in the VTA and PPG mRNA transcripts in the NTS (Figure 2.4A). GLP-1R mRNA expression in the VTA was not changed in cocaine-experienced rats following one (Ext1) and seven (Ext7) days of extinction when compared to yoked saline controls (Figures 2.4B & 2.4C). While PPG mRNA expression in the NTS was not altered in cocaine-experienced rats following one day of extinction (Figure 2.4D), NTS PPG mRNA expression was significantly decreased following seven days of extinction [$t(14)=3.18$, $p<0.01$; n=8/treatment] (Figure 2.4E). These results indicated that extinction following cocaine self-administration is associated with decreased endogenous PPG mRNA expression in the NTS.

Administration of Ex-4 directly into the VTA dose-dependently attenuated cocaine seeking in rats.

To determine if increased activation of VTA GLP-1Rs during extinction is sufficient to attenuate cocaine seeking, Ex-4 was infused directly into the VTA 10 minutes prior to a cocaine priming-induced reinstatement test session. Total active and inactive lever responses (mean ± SEM) in animals pretreated with Ex-4 (vehicle, 0.005 or 0.05 µg/100nl, n=8/treatment) are shown in Figure 2.5A. These data were analyzed using a two-way ANOVA, which revealed significant main effects of treatment [$F(2,56)=6.735$, $p<0.01$] and lever [$F(1,56)=75.37$, $p<0.001$] as well as a significant treatment x lever interaction [$F(2,56)=6.828$, $p<0.01$]. Subsequent pairwise analyses indicated that active lever responses were significantly different between vehicle and 0.05 µg Ex-4 (Bonferroni, $p<0.05$). No significant differences were found on inactive lever responding between treatments. Microinjection sites corresponding to these experiments are shown in Figure 2.5B.

While intra-VTA administration of Ex-4 had no significant effect on inactive lever responding, one could argue that responses were too low to assess the potential rate-suppressant effects of intra-VTA Ex-4 treatment. Total active and inactive lever responses (mean ± SEM) are shown in Figure 2.5C for rats pretreated with intra-VTA Ex-4 prior to a sucrose reinstatement test session. There were no effects of intra-VTA Ex-4 on sucrose seeking. Infusion sites within the VTA for these experiments are depicted in Figure 2.5D. Taken together with the cocaine reinstatement studies, these data indicated that activation of VTA GLP-1Rs reduced cocaine seeking and that these effects were not due to deficits in operant responding.

Discussion

The present study identified a critical role for GLP-1Rs in drug-seeking behavior. Specifically, we showed that systemically administered Ex-4 attenuated the reinstatement of drug-seeking behavior elicited by both cocaine and conditioned cues. Importantly, we identified doses of Ex-4 that selectively reduced cocaine seeking and did not affect food intake or body weight. Moreover, these behaviorally-relevant doses of Ex-4 do not produce nausea/malaise-like adverse effects in rodents (Kanoski et al., 2012) further highlighting the selectivity of this behavioral response at these doses. We also showed that peripheral Ex-4 was able to cross the blood brain barrier and putatively bind receptors expressed on neurons and astrocytes in the VTA. Indeed, the suppressive effects of peripheral Ex-4 on cocaine seeking were due, in part, to activation of central GLP-1Rs in the VTA. Moreover, cocaine self-administration and subsequent extinction may reduce endogenous GLP-1 expression in the brain, as evidenced by a significant decrease in PPG mRNA expression in the NTS following seven days of extinction, a time point that coincides with drug-seeking behavior (Schmidt and Pierce, 2010; Schmidt et al., 2015b). Since the NTS sends direct monosynaptic GLP-1-expressing efferent projections to the VTA (Alhadeff et al., 2012), these results suggest that decreased endogenous GLP-1 release in midbrain areas including the VTA may facilitate cocaine seeking. This hypothesis is supported by our data indicating that infusions of Ex-4 directly into the VTA were sufficient to attenuate the reinstatement of cocaine-seeking behavior. Collectively, these findings identify a novel neural mechanism focused on central GLP-1Rs that could be targeted to prevent cocaine craving-induced relapse.

GLP-1R agonists and cocaine addiction

A growing literature indicates that GLP-1Rs play an important role in addiction-like behaviors and that systemic administration of GLP-1R agonists reduces the rewarding

and reinforcing effects of drugs of abuse (Skibicka, 2013; Engel and Jerlhag, 2014; Hayes and Schmidt, 2016). For example, peripheral administration of the GLP-1R agonist Ex-4 has been shown to attenuate cocaine-induced conditioned place preference (CPP) and cocaine self-administration in mice (Egecioglu et al., 2013b; Graham et al., 2013; Sorensen et al., 2015). Our findings expanded on these studies and identified an important role for GLP-1Rs in an animal model of cocaine craving-induced relapse.

The present study also identified doses of peripheral Ex-4 that selectively attenuated addiction-like behaviors and did not produce adverse effects commonly associated with higher doses in rodents. Indeed, a significant limitation to interpreting results from previous studies of peripheral Ex-4 in preclinical models of drug addiction is the exceedingly high doses of Ex-4 tested. While previous studies have laid the foundation for our understanding of the role of GLP-1 in drug addiction, the doses of peripheral Ex-4 used to pretreat mice in these studies ranged from 3.0 to 100.0 μg/kg (Egecioglu et al., 2013b; Graham et al., 2013; Sorensen et al., 2015). Doses of Ex-4 as low as 0.25 μg/kg have been shown to produce malaise-like adverse effects in rats, which can confound subsequent behavioral responses (Hayes et al., 2011a; Kanoski et al., 2012). Moreover, nausea and malaise are common adverse effects associated with high doses of peripherally administered GLP-1R agonists in humans (Buse et al., 2009). Since the doses of Ex-4 shown to reduce cocaine CPP and self-administration are likely producing malaise-like effects in mice, it is impossible to draw firm conclusions about the role of GLP-1Rs in addiction-like behaviors from these previous studies. In contrast, we identified systemic doses of Ex-4 as low as 0.1 and 0.2 μg/kg that were sufficient to attenuate cocaine seeking in rats. Importantly, these doses were subthreshold for effects on feeding behavior in cocaine-experienced rats (present findings) and do not produce malaise-like effects in rats (Hayes et al., 2011a; Kanoski et al., 2012). The translational implications of

these findings are profound in that they support potential therapeutic approaches toward the specific use of GLP-1R agonists for the treatment of cocaine craving and relapse.

In addition to causing nausea and malaise-like effects, peripheral administration of Ex-4 at doses as low as 0.25 µg/kg have been shown to significantly decrease food intake and body weight in rats (Hayes et al., 2011a; Kanoski et al., 2012). These findings further confound prior studies that examined the effects of Ex-4 on drug-mediated behaviors because they indicate that doses of Ex-4 greater than 0.25 µg/kg reduce motivated behaviors generally and not drug reinforcement specifically. Furthermore, reductions in *ad libitum* food intake and body weight limit the therapeutic potential of GLP-1R agonists for use in human addicts. Our novel findings clearly identified doses of Ex-4 (0.1 and 0.2 µg/kg) that selectively attenuated cocaine seeking and did not affect *ad libitum* food intake, meal patterns or body weight in cocaine-experienced rats. These data support selective effects of a peripherally administered GLP-1R agonist on drug reinforcement. Consistent with our peripheral Ex-4 dose-response study, we also showed that Ex-4 reduced cocaine seeking when infused directly into the VTA at a dose (0.05 µg) that does not affect *ad libitum* chow intake or promote malaise-like effects in rats (Alhadeff et al., 2012; Dickson et al., 2012; Mietlicki-Baase et al., 2013). Moreover, intra-VTA infusion of Ex-4 did not alter sucrose seeking further supporting the selectivity of lower doses to reduce cocaine seeking. There is some evidence that administration of 0.05 µg Ex-4 into the VTA reduces operant responding for palatable food (Dickson et al., 2012). However, these effects are transient and do not persist with more prolonged operant sessions (Dickson et al., 2012; Schmidt et al., 2016). Thus, the present study showed that a GLP-1R agonist reduced cocaine seeking at doses not associated with common adverse effects in rodents.

Central GLP-1Rs and cocaine seeking

Mutant mice lacking GLP-1Rs have enhanced cocaine CPP compared to wild-type controls (Harasta et al., 2015). These results suggest that activation of central GLP-1Rs may function to reduce the rewarding effects of cocaine. Consistent with this hypothesis, we recently showed that direct activation of GLP-1Rs in the VTA is sufficient to reduce cocaine self-administration in rats (Schmidt et al., 2016). We expanded upon these studies here and showed that the intake suppressive effects of peripheral Ex-4 on cocaine seeking were due to activation of GLP-1Rs in the VTA and that direct activation of VTA GLP-1Rs was sufficient to reduce cocaine seeking.

The current data clearly support a role for VTA GLP-1Rs in cocaine seeking, but it is possible and in fact likely that GLP-1Rs expressed in other brain regions also play an important role in cocaine seeking. GLP-1Rs are expressed ubiquitously throughout the rodent brain (Merchenthaler et al., 1999) including nuclei known to regulate drug-seeking behavior. Viral-mediated re-expression of GLP-1Rs in the lateral septum of constitutive GLP-1R knockout mice attenuates cocaine CPP (Harasta et al., 2015) indicating that the rewarding effects of cocaine are mediated, in part, by enhanced GLP-1 signaling in this nucleus. GLP-1Rs are also expressed in the NAc, hippocampus, habenula and amygdala (Merchenthaler et al., 1999; Tuesta et al., 2017) and future studies are required to define the exact role of GLP-1 signaling in these nuclei in addiction-like behaviors.

The exact mechanism(s) by which GLP-1R activation in the VTA attenuates cocaine seeking are unknown. Doses of peripheral Ex-4 that reduce cocaine CPP and self-administration attenuate cocaine-mediated increases in extracellular dopamine in the nucleus accumbens (NAc) (Egecioglu et al., 2013b; Sorensen et al., 2015). Since increased dopamine signaling in the NAc promotes cocaine seeking (Schmidt et al., 2005;

Schmidt et al., 2006; Schmidt and Pierce, 2006a), these results suggest that reduced cocaine seeking following peripheral Ex-4 administration may involve decreased extracellular dopamine levels in the NAc. The present findings also indicated that peripheral Ex-4 bound putative GLP-1Rs expressed on neurons and astrocytes in the VTA. However, it is not clear how exactly GLP-1R activation in the VTA may reduce dopamine cell firing. GLP-1Rs are predominantly Gs-coupled receptors (although they can activate Gq and Gi proteins as well) that are expressed on both pre- and post-synaptic sites in the VTA (Holst, 2007; Hayes et al., 2011b). While the exact phenotypes of GLP-1R-expressing cells in the VTA must be further characterized, there is some evidence that GLP-1R activation increases glutamate release in the VTA of drug-naïve rats (Mietlicki-Baase et al., 2013). GLP-1R activation also enhances GABAA receptor-mediated currents in the drug-naïve brain (Korol et al., 2015b; Korol et al., 2015a; Farkas et al., 2016), suggesting a possible GABA-mediated mechanism in the VTA that may underlie the suppressive effects of Ex-4 on cocaine seeking. Furthermore, a recent study showed that GLP-1R agonists increase activation of astrocytes in the hindbrain (Reiner et al., 2016). It is intriguing to think that increased activation of GLP-1Rs expressed on astrocytes in the VTA may regulate glutamate homeostasis and drug-seeking behavior. The effects of GLP-1R activation on dopamine cell firing in the VTA are likely to be complex and may differ between drug-naïve and cocaine-experienced brains. Therefore, future studies are required to determine the cellular and neurophysiological mechanisms through which activation of GLP-1Rs in the VTA suppresses cocaine-seeking behavior.

Extinction following cocaine self-administration dynamically regulated endogenous PPG mRNA expression in the NTS

To date no studies have examined the effects of cocaine self-administration and subsequent extinction on endogenous central GLP-1 signaling. We have previously

reported that cocaine activates GLP-1-expressing neurons in the NTS and that increased VTA GLP-1 signaling in the brain may function as a homeostatic response to reduce cocaine self-administration (Schmidt et al., 2016). To expand upon these data, we examined the effects of one day and seven days of extinction following 21 days of cocaine self-administration on GLP-1R mRNA expression in the VTA. We also quantified expression of PPG mRNA in the NTS. PPG is the protein precursor to GLP-1 in NTS neurons (Holst, 2007). Using quantitative real-time PCR, we found no effects of extinction on VTA GLP-1R mRNA expression. In contrast, we found a non-significant trend (P=0.09) toward increased PPG mRNA expression in the NTS during acute withdrawal from cocaine self-administration. These results are consistent with our previous study showing that cocaine increases activation of PPG-expressing neurons in the NTS (Schmidt et al., 2016) and together suggest that increased endogenous PPG mRNA expression in the NTS may represent a homeostatic compensatory response to cocaine self-administration that serves to reduce further drug taking. Interestingly, we observed a significant decrease in NTS PPG mRNA expression following seven days of extinction, a time point that coincides with maximal drug-seeking behavior (Schmidt and Pierce, 2010; Schmidt et al., 2015b). Since activation of central GLP-1Rs may serve as a 'brake' on cocaine self-administration, this decrease in endogenous PPG mRNA expression in the caudal brain may facilitate drug seeking during extinction. Support for this hypothesis comes from the present study showing that direct activation of VTA GLP-1Rs was sufficient to reduce cocaine priming-induced reinstatement of drug-seeking behavior. Our data are consistent with previous literature demonstrating that reduced PPG mRNA expression in the NTS increases the rewarding value of food. Viral-mediated knockdown of PPG mRNA expression in the NTS produces hyperphagia and exacerbates high fat diet-induced obesity (Barrera et al., 2011). Due to shared mechanisms mediating the rewarding

properties of food and drugs of abuse (DiLeone et al., 2012; Volkow et al., 2013), our data further support a role for central GLP-1 signaling in regulating motivated behaviors.

Conclusion

The present study identified a novel role for central GLP-1Rs in an animal model of relapse. Moreover, we have identified doses of the GLP-1R agonist Ex-4 that selectively reduced cocaine seeking and did not produce adverse effects in rats. Since GLP-1R agonists are FDA-approved for treating diabetes type II and obesity, these findings suggest that Ex-4 could be re-purposed as an anti-relapse medication. Additionally, we provided evidence that extinction following cocaine self-administration decreased endogenous PPG mRNA expression in the brain, which may facilitate drug-seeking behavior.

Figures

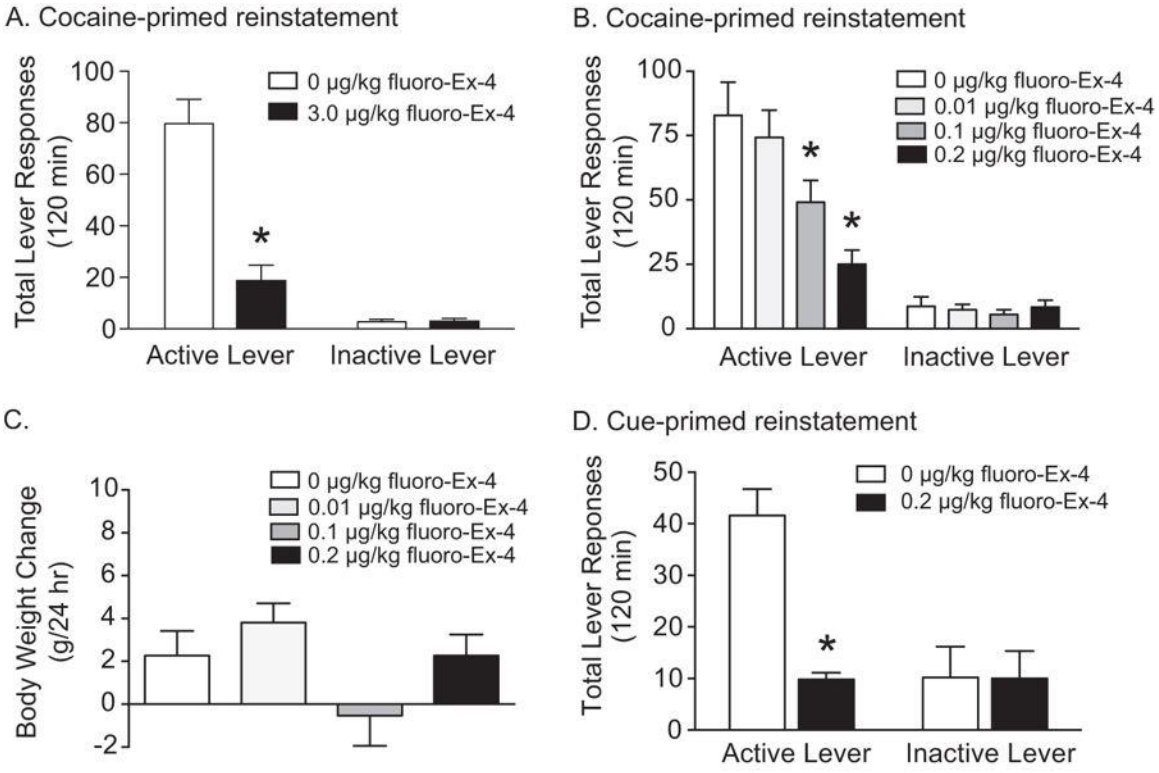

Figure 2.1 ***Systemic administration of fluoro-Ex-4 dose-dependently attenuated cocaine priming-induced reinstatement of drug-seeking behavior.*** **(A)** Cocaine seeking was significantly attenuated in rats pretreated with 3.0 µg/kg fluoro-Ex-4 compared to vehicle-treated controls (n=10/treatment). Since 3.0 µg/kg Ex-4 produces malaise-like effects in rats (Hayes et al., 2011a; Kanoski et al., 2012), lower doses of fluoro-Ex-4 were tested to identify doses that selectively reduce cocaine seeking. **(B)** Doses of fluoro-Ex-4 (0.1 and 0.2 µg/kg) subthreshold for producing malaise-like effects reduced cocaine seeking (n=16/treatment). **(C)** No effects of systemic fluoro-Ex-4 on 24 h body weight were found in rats pretreated with 0.01, 0.1 and 0.2 µg/kg fluoro-Ex-4 prior to a cocaine priming-induced reinstatement test session (n=16/treatment). **(D)** Cue-induced reinstatement of cocaine-seeking behavior was significantly attenuated in rats pretreated with 0.2 µg/kg fluoro-Ex-4 compared to vehicle-treated controls (n=5/treatment). *p<0.05, Bonferroni

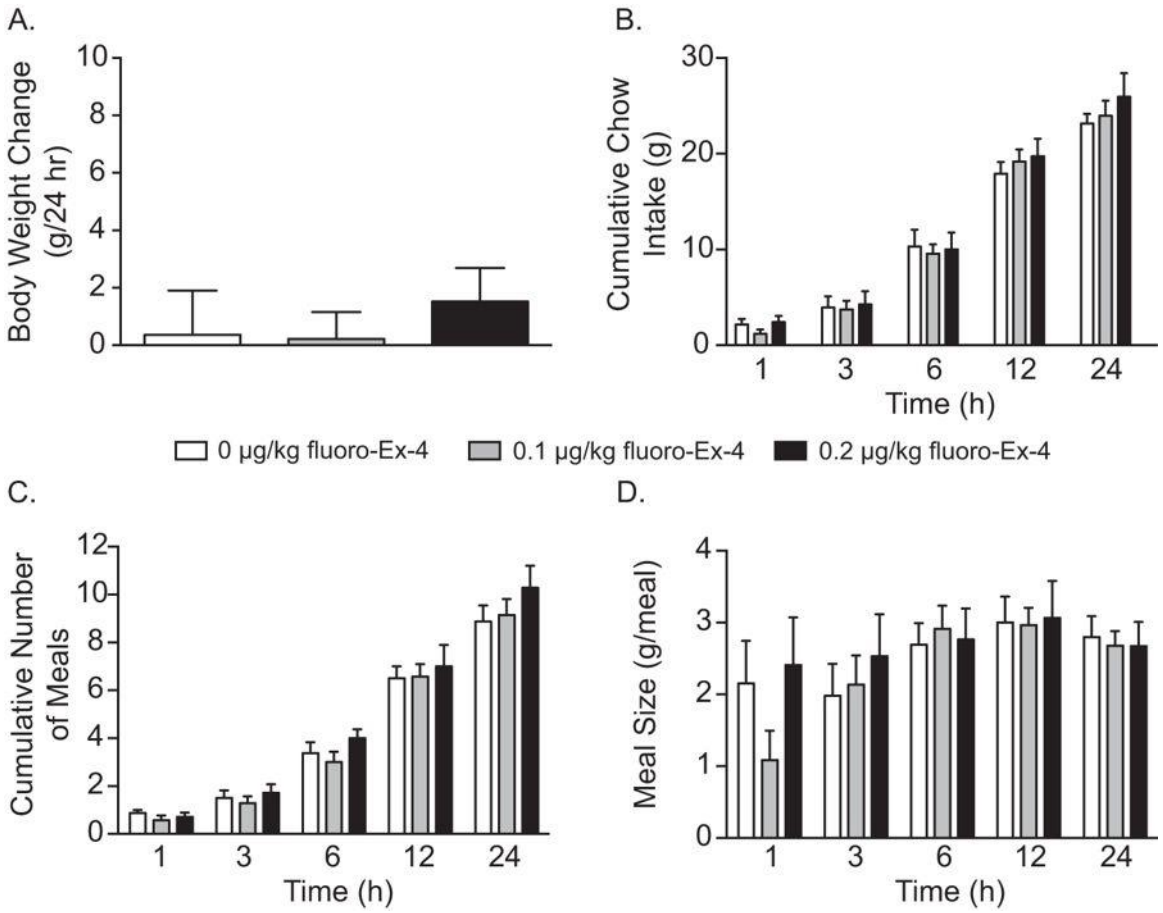

Figure 2.2 ***Systemic administration of fluoro-Ex-4 did not affect ad libitum feeding behavior in cocaine-experienced rats.*** There were no effects of 0.1 and 0.2 µg/kg fluoro-Ex-4 pretreatment on 24 h body weight **(A)** in cocaine-experienced rats. Moreover, fluoro-Ex-4 did not affect cumulative chow intake **(B)**, meal frequency **(C)** or meal size **(D)** at any time point following cocaine priming-induced reinstatement test sessions (n=7/treatment).

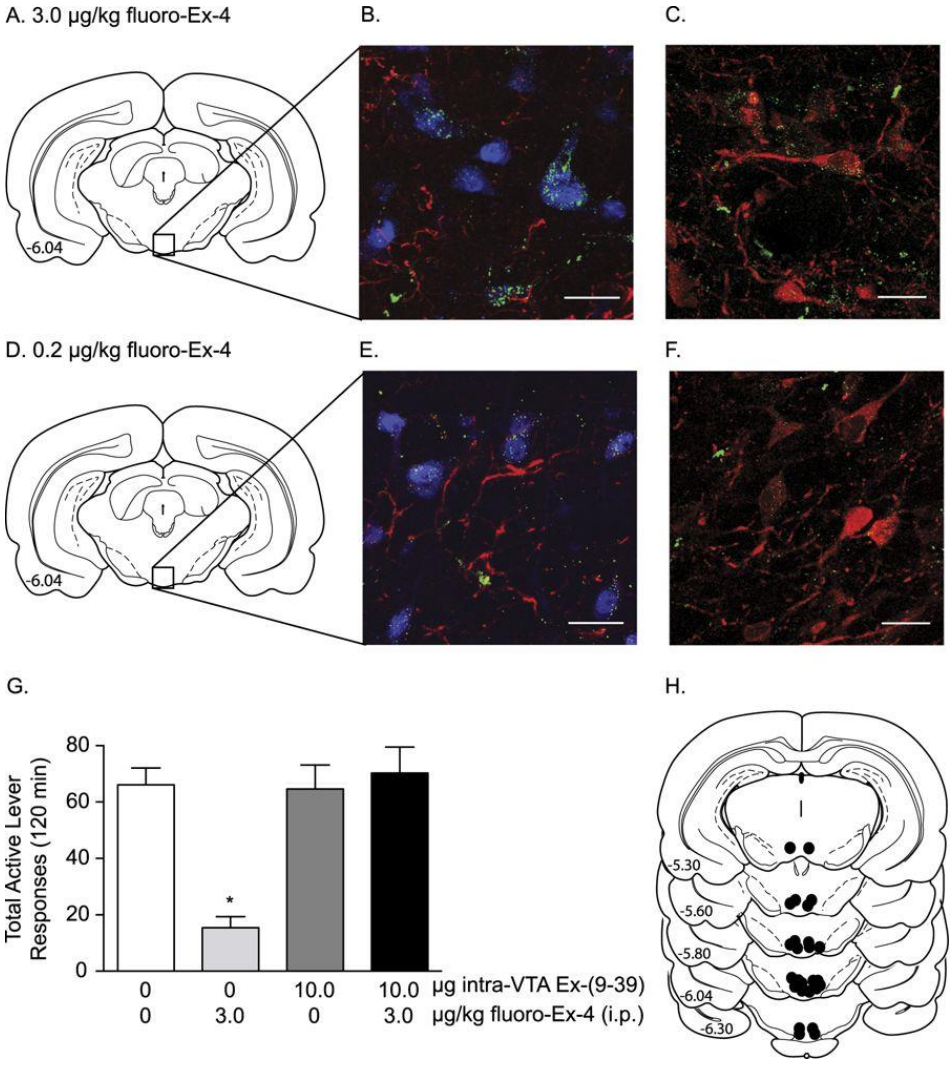

Figure 2.3 ***The suppressive effects of systemic fluoro-Ex-4 on cocaine seeking were blocked by antagonism of VTA GLP-1Rs.*** Rats pretreated with fluoro-Ex-4 (3.0 µg/kg, n=10) prior to a priming injection of cocaine in Figure 1A were perfused immediately after the reinstatement test session (3 h post fluoro-Ex-4 infusion). Coronal sections at the level of the midbrain **(A)** were used to determine if systemic fluoro-Ex-4 penetrates the brain and localizes in the VTA. **(B)** 3.0 µg/kg fluoro-Ex-4 (green fluorescence) co-localized with neurons labeled with NeuN (blue fluorescence) and astrocytes labeled with GFAP (red fluorescence) in the VTA. **(C)** 3.0 µg/kg fluoro-Ex-4 co-localized with tyrosine hydroxylase-labeled dopamine neurons (red fluorescence) in the VTA**.** The VTA **(D)** of separate rats treated with 0.2 µg/kg fluoro-Ex-4 were used to determine if lower doses of the GLP-1R agonist that selectively attenuated cocaine seeking also penetrated the brain and localized in the VTA **(E & F)**. Images are compressed z-stacks with a 0.5 µm step size (scale bar: 20 µm). All images shown at 63x magnification. **(G)** Intra-VTA infusions of the GLP-1R antagonist exendin-(9-39) (10 µg) prior to a systemic injection of 3.0 µg/kg fluoro-Ex-4 blocked fluoro-Ex-4-mediated reductions in total active lever responses during cocaine reinstatement test sessions (n=13/treatment). **(H)** Representative coronal sections at the level of the midbrain depict microinjection sites in the VTA. **p*<0.05, Bonferroni

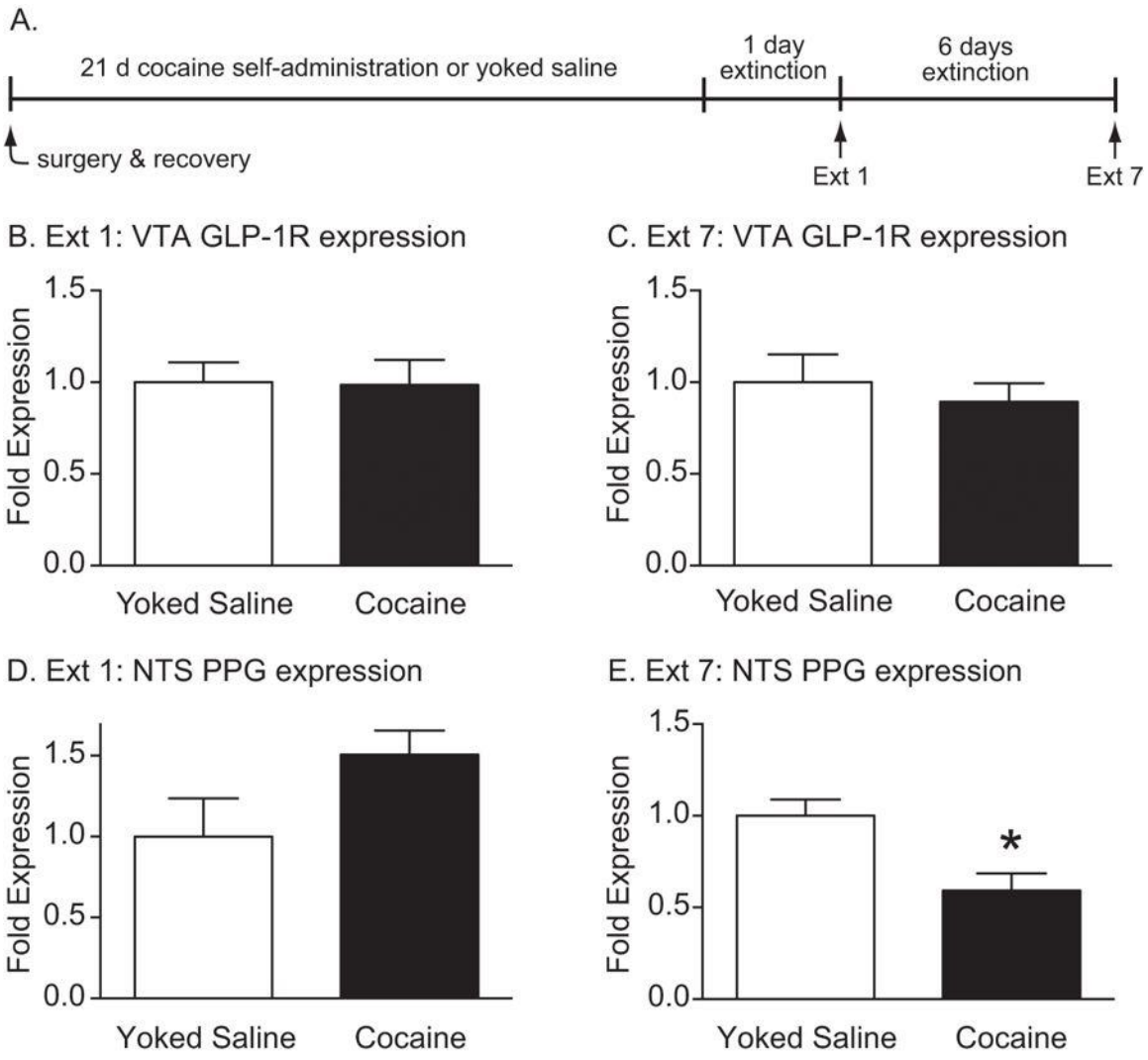

Figure 2.4 ***Cocaine self-administration and subsequent extinction reduced endogenous PPG mRNA expression in the NTS.*** **(A)** Expression of GLP-1R mRNA transcripts in the VTA and PPG mRNA transcripts in the NTS was quantified following one (Ext1) and seven (Ext7) days of extinction following cocaine self-administration. No differences in GLP-1R mRNA expression in the VTA were found after one day of extinction (n=8) **(B)** or seven days of extinction (n=7) **(C)** in cocaine-experienced rats compared to yoked saline controls. **(D)** While not statistically significant (p<0.09), there was a trend towards increased PPG mRNA expression in the NTS following one day of extinction (n=7) in cocaine-experienced rats compared to control rats. **(E)** In contrast, statistically significant decreases in NTS PPG mRNA expression were found following seven days of extinction in cocaine-experienced rats compared to yoked saline controls. *p<0.01, unpaired t-tests

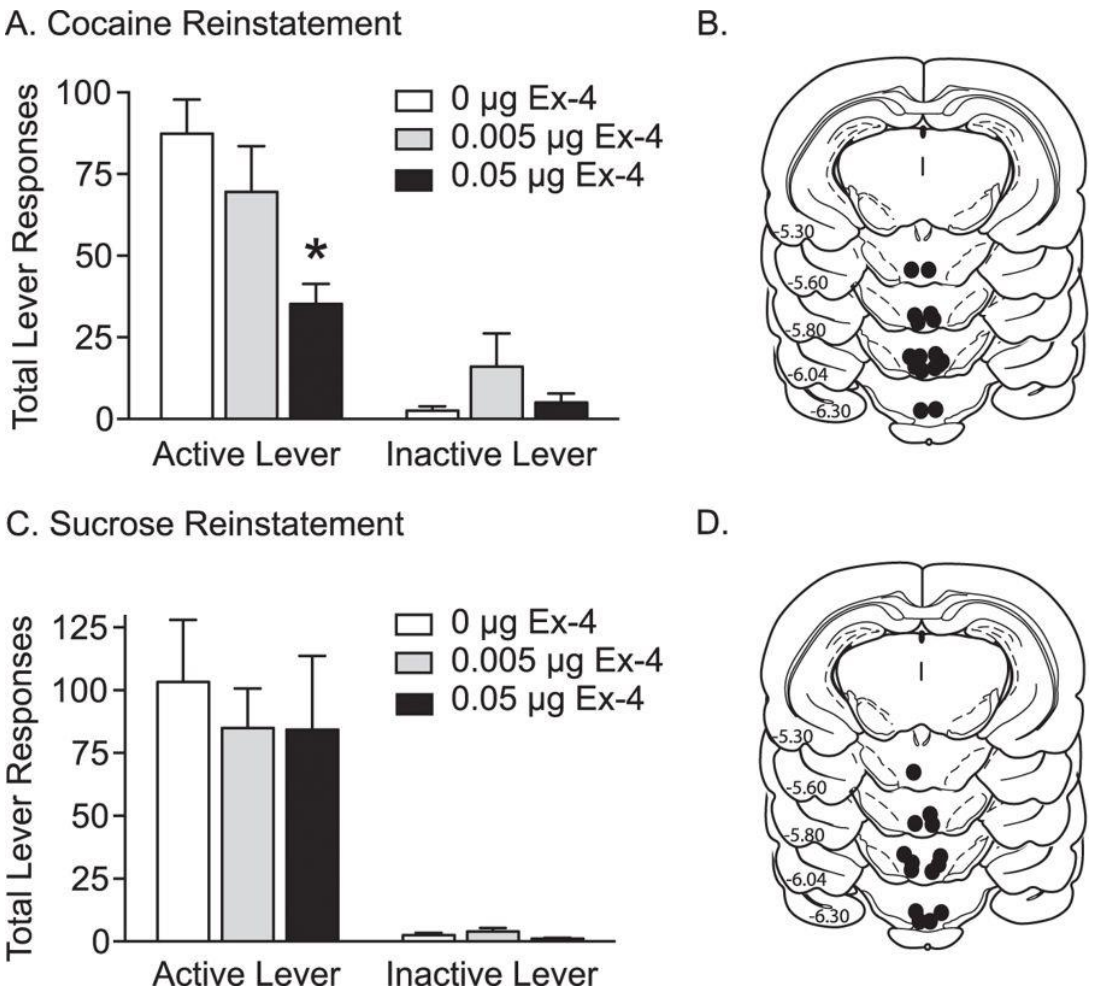

Figure 2.5 ***Administration of Ex-4 directly into the VTA dose-dependently attenuated cocaine priming-induced reinstatement of drug-seeking behavior.*** **(A)** Infusions of Ex-4 (0.05 µg) directly into the VTA prior to a cocaine priming injection reduced active lever responses during subsequent reinstatement test sessions (n=8). The asterisk indicates a significant difference in responding between rats pretreated with 0.05 µg Ex-4 and vehicle (*p<0.05, Bonferroni). Intra-VTA microinjection sites for the cocaine reinstatement study are shown in **B. (C)** Ex-4 administration into the VTA had no effect on sucrose seeking (n=7). Microinjection sites corresponding to intra-VTA infusions in sucrose reinstatement test sessions are shown in **D**.

CHAPTER 3: Activation of glucagon-like peptide-1 receptors in the nucleus accumbens attenuates cocaine seeking in rats

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Abstract

Recent evidence indicates that activation of glucagon-like peptide-1 (GLP-1) receptors reduces cocaine-mediated behaviors and cocaine-evoked dopamine release in the nucleus accumbens (NAc). However, no studies have examined the role of NAc GLP-1 receptors (GLP-1Rs) in the reinstatement of cocaine-seeking behavior, an animal model of relapse. Here, we show that systemic infusion of a behaviorally relevant dose of the GLP-1R agonist exendin-4 (Ex-4) penetrated the brain and localized with neurons and astrocytes in the NAc. Administration of Ex-4 directly into the NAc core and shell subregions significantly attenuated cocaine priming-induced reinstatement of drug-seeking behavior. These effects were not due to deficits in operant responding or suppression of locomotor activity as intra-NAc Ex-4 administration had no effect on sucrose-seeking behavior. To determine the effects of GLP-1R activation on neuronal excitability, Ex-4 was bath applied to *ex vivo* NAc slices from cocaine- and saline-experienced rats following extinction of cocaine-taking behavior. Ex-4 increased the frequency of action potential firing of NAc core and shell medium spiny neurons (MSNs) in cocaine-experienced rats while no effect was observed in saline controls. In contrast, Ex-4 did not affect the frequency or amplitude of spontaneous excitatory post-synaptic currents (sEPSCs) or alter the paired-pulse ratios (PPRs) of evoked EPSCs. These effects were not associated with altered expression of GLP-1Rs in the NAc following cocaine self-administration. Taken together, these findings indicate that increased activation of GLP-1Rs in the NAc during cocaine abstinence increases intrinsic, but not synaptic, excitability of MSNs and is sufficient to reduce cocaine-seeking behavior.

Introduction

Almost one million people aged 12 years or older initiated cocaine use in 2015, which represents a 61% increase compared to the total number of new users in 2013 (Hughes, 2016). Unfortunately, there are no FDA-approved medications to treat cocaine relapse, which continues to be a major public health concern (O'Brien, 1997; Pierce et al., 2012). Thus, novel molecular targets are needed to develop new medications for the treatment of cocaine addiction.

An emerging body of evidence indicates that metabolic regulators of hunger and satiety also modulate the rewarding and reinforcing properties of drugs of abuse (Kenny, 2011b; Engel and Jerlhag, 2014). For example, glucagon-like peptide-1 (GLP-1) is a satiation hormone and neuropeptide that has been shown to regulate cocaine-, alcohol-, nicotine- and amphetamine-related behavioral responses in rodents (Egecioglu et al., 2013b; Graham et al., 2013; Shirazi et al., 2013; Sorensen et al., 2015; Suchankova et al., 2015; Vallof et al., 2016; Tuesta et al., 2017). Since GLP-1 analogs are currently FDA-approved for treating type II diabetes and obesity (Lovshin and Drucker, 2009; Shukla et al., 2015), these pre-clinical findings suggest that GLP-1 receptor (GLP-1R) agonists could be repurposed for treating drug abuse in human addicts.

Studies have shown that systemic administration of GLP-1R agonists attenuates cocaine-induced locomotion, cocaine conditioned place preference (CPP) and cocaine self-administration (Egecioglu et al., 2013b; Graham et al., 2013; Sorensen et al., 2015). While systemic administration of GLP-1R agonists attenuates cocaine-mediated behaviors, the role of central GLP-1Rs in addiction-like behaviors is largely understudied (Hayes and Schmidt, 2016). GLP-1Rs are expressed throughout the brain including the mesolimbic reward system (Merchenthaler et al., 1999). GLP-1R activation in the nucleus accumbens

(NAc) and ventral tegmental area (VTA) has been shown to decrease food intake by reducing the rewarding value of food (Alhadeff et al., 2012; Dickson et al., 2012; Dossat et al., 2013; Mietlicki-Baase et al., 2013). Since there is significant overlap between the neural mechanisms and circuits mediating the reinforcing effects of palatable food and drugs of abuse (Narayanan et al., 2010; Kenny, 2011b; Volkow et al., 2013), it is likely that activation of central GLP-1Rs also reduces voluntary cocaine taking- and seeking-behaviors. Indeed, we recently showed that activation of GLP-1Rs in the VTA reduces the reinforcing efficacy of cocaine in rats self-administering cocaine (Schmidt et al., 2016). While these results clearly indicate a novel role for GLP-1Rs in voluntary cocaine taking, no studies to date have investigated central GLP-1Rs in the reinstatement of cocaine-seeking behavior, an animal model of relapse.

It is well established that the NAc core and shell subregions play critical roles in the reinstatement of cocaine-seeking behavior (Schmidt et al., 2005; Schmidt and Pierce, 2010). Here, we initially determined if systemic administration of the GLP-1R agonist exendin-4 (Ex-4) entered the brain, localized in the NAc and attenuated cocaine-seeking behavior. We then assessed the effects of direct activation of GLP-1Rs in the NAc core and shell on cocaine-seeking behavior. Previous studies have shown that GLP-1R agonists modulate neuronal activity in the brain by both pre-synaptic effects on neurotransmitter release and regulation of post-synaptic membrane excitability (Acuna-Goycolea and van den Pol, 2004; Mietlicki-Baase et al., 2014; Korol et al., 2015b). Therefore, we aimed to identify a potential neurophysiological mechanism underlying the effects of intra-NAc Ex-4 on cocaine seeking by examining *ex vivo* electrophysiological measures of neuronal activity and GLP-1R expression in the NAc of cocaine-experienced rats. Our results indicate that GLP-1R activation in the NAc following extinction of

voluntary cocaine taking increases the frequency of action potential firing in medium spiny neurons (MSNs) and attenuates the reinstatement of cocaine-seeking behavior.

Materials and Methods

Animals and housing

Male Sprague-Dawley rats (*Rattus norvegicus*) weighing 225-250 g were obtained from Taconic Laboratories. Rats were individually housed with food and water available *ad libitum* in their home cages. A 12/12 hr light/dark cycle was used with the lights on at 7:00 a.m. All experimental procedures were performed during the light cycle. The experimental protocols were consistent with the guidelines issued by the U.S. National Institutes of Health and were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee and the University of South Carolina School of Medicine's Institutional Animal Care and Use Committee.

Drugs

Cocaine was obtained from the National Institute on Drug Abuse (Rockville, MD) and dissolved in bacteriostatic 0.9% saline. Ex-4 was purchased from the American Peptide Company (Sunnyvale, CA) and dissolved in aCSF (Harvard Apparatus, Holliston, MA). Fluoro-Ex-4 was purchased from AnaSpec (Fremont, CA) and dissolved in bacteriostatic 0.9% saline. The doses and time course of administration for each of the aforementioned pharmacological compounds were based on the following systemic and intra-cranial microinjection experiments in rats: Ex-4 (Alhadeff et al., 2012; Schmidt et al., 2016) and fluoro-Ex-4 (Kanoski et al., 2012; Reiner et al., 2016).

Surgery

Rats were handled daily and allowed one week to acclimate to their home cages upon arrival. Prior to surgery, rats were anesthetized with 80 mg/kg ketamine (Midwest Veterinary Supply, Valley Forge, PA) and 12 mg/kg xylazine (Sigma-Aldrich/RBI, St. Louis, MO). An indwelling catheter (SAI Infusion Technologies, Lake Villa, IL) was inserted into

the right jugular vein and sutured in place as described in our previous publications (Schmidt et al., 2015b; Schmidt et al., 2016). The catheter was routed to a mesh backmount that was implanted subcutaneously above the shoulder blades. To prevent infection and maintain patency, catheters were flushed daily with 0.2 ml of the antibiotic Timentin (0.93 mg/ml; Fisher, Pittsburgh, PA) dissolved in heparinized 0.9% saline (Butler Schein, Dublin, OH). When not in use, catheters were sealed with plastic obturators.

After catheter insertion, some rats were then immediately mounted in a stereotaxic apparatus (Kopf Instruments, CA) and implanted with cannulae for intra-cranial microinjections similar to our previous studies (Schmidt et al., 2013; Schmidt et al., 2015a). Bilateral guide cannulae (26 gauge; 14mm; Plastics One, Roanoke, VA) were implanted 2mm dorsal to the NAc core and shell and cemented in place by affixing dental acrylic to stainless steel screws secured in the skull. The coordinates for the ventral ends of the guide cannulae, relative to bregma according to the atlas of Paxinos and Watson (1997), were as follows: NAc shell: +1.0 mm A/P, ±1.0 mm M/L, and -5.0 mm D/V; NAc core: +1.0 mm A/P, ±2.5 mm M/L, and -5.0 mm D/V. An obturator (14 mm, 33 gauge; Plastics One) was inserted into each guide cannula to prevent occlusion.

Cocaine self-administration, extinction and reinstatement of cocaine seeking

Rats were allowed 7 days to recover from surgery before behavioral testing commenced. Initially, rats were placed in operant conditioning chambers and allowed to lever-press for intravenous infusions of cocaine (0.25 mg cocaine/59 µl saline, infused over a 5 s period) on a fixed-ratio 1 (FR1) schedule of reinforcement. Rats were allowed to self-administer a maximum of 30 injections per 120 min operant session. Once a rat achieved at least 20 infusions of cocaine in a single daily operant session under the FR1 schedule, the subject was switched to a fixed-ratio 5 (FR5) schedule of reinforcement. The maximum number

of injections was again limited to 30 per daily self-administration session under the FR5 schedule. For both FR1 and FR5 schedules, a 20 s time-out period followed each cocaine infusion, during which time active lever responses were tabulated but had no scheduled consequences. Responses made on the inactive lever, which had no scheduled consequences, were also recorded during both the FR1 and FR5 training sessions. Following 21 days of daily cocaine self-administration sessions, drug-taking behavior was extinguished by replacing the cocaine solution with 0.9% saline. Daily extinction sessions continued until responding on the active lever was <15% of the total active lever responses completed on the last day of cocaine self-administration. Typically, it took ~7 days for rats to meet this criterion. Once cocaine taking was extinguished, rats entered the reinstatement phase of the experiment. During reinstatement test sessions, satisfaction of the response requirement (i.e., five presses on the active lever) resulted in an infusion of saline rather than cocaine. Using a between-sessions reinstatement paradigm, each reinstatement test session was followed by extinction sessions until responding was again <15% of the total active lever responses completed on the last day of cocaine self-administration. Generally, 1–2 days of extinction were necessary to reach extinction criterion between reinstatement test sessions.

Cocaine reinstatement

To determine if systemic administration of a GLP-1R agonist reduces cocaine seeking and penetrates the brain, initial studies utilized the GLP-1R agonist Ex-4 tagged with fluorescein (fluoro-Ex-4). In addition to binding GLP-1Rs *in vitro* and *in vivo,* fluoro-Ex-4 produces behavioral responses identical to unlabeled Ex-4 (Rajan et al., 2015; Reiner et al., 2016). Once cocaine-taking behavior was extinguished, rats were pretreated with vehicle, 0.1 and 0.2 µg/kg fluoro-Ex-4 (i.p.) one hour prior to a 10 mg/kg priming injection of cocaine and subsequent reinstatement test sessions. Using a within-subjects design,

each rat served as its own control and fluoro-Ex-4 doses were counterbalanced to avoid rank order effects of drug treatment. To determine if the suppressive effects of Ex-4 on cocaine seeking are mediated, in part, by activation of central GLP-1Rs, rats were deeply anesthetized and transcardially perfused with 0.1 M PBS, pH 7.4, followed with 4% formalin in 0.1 M PBS immediately following their cocaine priming-induced reinstatement test session (i.e., three hours post systemic fluoro-Ex-4 administration). Once brains were removed and post-fixed overnight in 4% formalin in 0.1 M PBS and then cryoprotected in 20% sucrose in 0.1 M PBS at 4$_{o}$C for three days. Coronal sections (30 µm) were then taken at the level of the striatum using a cryostat (Leica 3050S; Leica Corp., Deerfiled, IL). Brain sections were stored in 0.1 M PBS at 4$_{o}$C until processed using immunohistochemistry.

The effects of intra-NAc core and shell infusions of Ex-4 on cocaine priming-induced reinstatement of drug-seeking behavior were studied in separate cohorts of rats. Obturators were removed from the guide cannulae and 33 gauge stainless steel microinjectors (16 mm; Plastics One) were inserted. Ex-4 (0.005 and 0.05 µg/500 nl) and vehicle were microinjected into the core and shell 10 min prior to a priming injection of cocaine (10 mg/kg, i.p.). Microinjectors were left in place for an additional one min following infusions in order to allow for diffusion of the drug solution away from the tips of the microinjectors. Using a within-subjects design, each rat served as its own control. To control for potential rank order effects of drug and vehicle administrations, all treatments were counterbalanced across reinstatement test sessions.

Sucrose self-administration, extinction and reinstatement of sucrose seeking

Potential nonspecific rate-suppressing effects of intra-NAc Ex-4 were evaluated by assessing the influence of this compound on the reinstatement of sucrose-seeking

behavior. Separate cohorts of rats were trained initially to self-administer 45 mg sucrose pellets (Research Diets, New Brunswick, NJ) on a FR1 schedule of reinforcement. Once rats achieved stable responding for sucrose (defined as <20% variation in responding over 3 consecutive days) on the FR1 schedule of reinforcement, the response requirement was increased to an FR5 schedule of reinforcement. Rats were limited to 30 sucrose pellets within each daily operant session and were restricted to ~25 g of lab chow (Harlan Teklad, Wilmington, DE) daily in their home cages for the duration of the experiment. Water was available *ad libitum* in the home cage.

After two weeks of sucrose-maintained responding on an FR5 schedule of reinforcement, rats underwent an extinction phase where active lever pressing no longer resulted in sucrose delivery. Once active lever responding decreased to <15% of the maximum number of responses completed on the last day of sucrose self-administration, rats proceeded to reinstatement testing. Ex-4 (0.005 and 0.05 µg/500 nl) and vehicle were microinjected into the NAc core and shell 10 min prior to the beginning of the reinstatement test sessions. Using a within-subjects design, each rat served as its own control and doses were counterbalanced across test sessions. The experimenter remotely administered one sucrose pellet every two min for the first 10 min of the reinstatement session. A between-session paradigm was used so that each daily reinstatement test session was followed by an extinction session the following day until responding was again <15% of the total active lever responses maintained by sucrose.

Verification of cannula placements

After completion of all behavioral experiments, rats were given an overdose of pentobarbital (100 mg/kg, i.p.). Brains were removed and drop fixed in 10% formalin. Coronal sections (100 µm) were taken at the level of the striatum with a vibratome and

mounted on gelatin-coated slides. An individual blinded to behavioral responses verified microinjection sites using light microscopy. Rats with cannula placements outside of the targeted NAc subregion and/or excessive mechanical damage were excluded from subsequent data analyses.

Immunohistochemistry

Immunohistochemistry was performed on free-floating coronal sections containing the NAc according to modified procedures from previously published studies (Reiner et al., 2016; Schmidt et al., 2016). Briefly, sections were washed with 1% sodium borohydride followed by 0.1 M PBS. Sections were then blocked in 0.1 M PBS containing 5% normal donkey serum and 0.2% Triton-X for 1 hr at room temperature. Sections were incubated in primary antibodies overnight, and then, following a PBS rinse, they were incubated in secondary antibodies for 2 hrs. The primary antibodies used were rabbit anti-NeuN (1:1000; ab177487, Abcam), and goat anti-GFAP (1:1000; ab53554, Abcam). Secondary antibodies were donkey anti-goat Alexa Fluor 594 (1:500), and donkey anti-rabbit Alexa Fluor 647 (1:500) from Jackson ImmunoResearch. Sections were then washed and mounted onto glass slides and coverslipped using Vectashield with DAPI (Vector Laboratories; Burlingame, CA). Sections were visualized with a Leica SP5 X confocal microscope using the 20x and 63x oil-immersion objectives along with 405, 488, 594 and 633 nm laser lines. Image z-stacks with the 63x oil-immersion were collected with a step size of 1 µm, while 2–3x optical zoom z-stack images using the same objective were collected with a step size of 0.5 µm.

Cocaine self-administration and yoked saline controls for electrophysiology experiments

Rats underwent catheterization as described above and were allowed to recover for 7 days before cocaine self-administration commenced. Each rat trained to respond for

contingent cocaine infusions was paired with a yoked subject that received infusions of saline. Lever pressing for the saline-yoked rats had no scheduled consequences, but these animals received the same number and temporal pattern of infusions as self-administered by the paired cocaine-experimental rat. Cocaine-experimental rats were allowed to self-administer cocaine for 21 days. On day 22, cocaine was replaced with saline for the extinction phase, which lasted until total active lever responding decreased to <15% of the maximum number of responses completed on the last day of cocaine self-administration. Typically, it took ~7 days for rats to meet this criterion.

Electrophysiology

Rats were decapitated following isoflurane anesthesia 24 hrs after the last extinction session. Brains were rapidly removed and coronal slices (300 μm) containing the NAc were cut using a Vibratome (VT1000S; Leica Microsystems) in an ice-cold artificial cerebrospinal fluid (aCSF) solution in which NaCl was replaced with an equiosmolar concentration of sucrose. aCSF contained the following (in mM): 130 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 1 $MgCl_2$, and 2 $CaCl_2$, pH 7.2–7.4, when saturated with 95% O_2 and 5% CO_2. Slices were incubated in aCSF at 32–34°C for 45 min and kept at 22–25°C thereafter, until transfer to the recording chamber. All solutions had osmolarity between 305 and 315 mOsm. Slices were viewed under an upright microscope (Eclipse FN1; Nikon Instruments) with infrared differential interference contrast optics and a 40× water-immersion objective. For recordings, the chamber was continuously perfused at a rate of 1–2 ml/min with oxygenated aCSF heated to 32 ± 1°C using an automated temperature controller (Warner Instruments). Recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) to a resistance of 4–7 MΩ when filled with the intracellular solution. The intracellular solution contained the following (in mM): 145 potassium gluconate, 2 $MgCl_2$, 2.5 KCl, 2.5 NaCl, 0.1 BAPTA, 10 HEPES, 2

Mg-ATP, and 0.5 GTP-Tris, pH 7.2–7.3, with KOH, osmolarity 280–290 mOsm. NAc core and shell MSNs were identified by their morphology and low resting membrane potential (−70 to −85 mV) in current clamp. A current step protocol (from -500 to +500 pA; 20 pA increments; 500 ms step duration) was run to determine action potential frequency versus current (*f-I*) relationship. To evaluate spontaneous excitatory postsynaptic currents (sEPSCs) and paired-pulse ratio (PPR) of evoked EPSCs (eEPSCs), the cells were voltage-clamped at -70 mV. For PPR, evoked responses were triggered by 100 μs constant-current pulses with an inter-stimulus interval of 100 ms generated by an A310 Accupulser (World Precision Instruments) and delivered at 0.2 Hz via a bipolar tungsten stimulation electrode positioned within 100 μm of the recorded cell. Ex-4 (1 μM) was applied via the Y-tube perfusion system modified for optimal solution exchange in brain slices. Neurons from a total of four animals per group were analyzed. All Ex-4 data were collected after a minimum of 4 min of Ex-4 exposure. Currents were low-pass filtered at 2 kHz and digitized at 20 kHz using a Digidata 1440A acquisition board (Molecular Devices) and pClamp10 software (Molecular Devices). Access resistance (10–30 MΩ) was monitored during recordings by injection of 10 mV hyperpolarizing pulses; data were discarded if access resistance changed >25% over the course of data collection.

Cocaine self-administration and yoked saline controls for quantitative real-time PCR

A separate group of cocaine-experimental and yoked saline control rats was used to study the effects of voluntary cocaine taking and subsequent abstinence on GLP-1R expression in the NAc. Cocaine-experimental rats were allowed to lever press for intravenous cocaine infusions as described above for a total of 21 days. Rats were then sacrificed either immediately after the first extinction session (Ext1) or following 7 consecutive days of extinction (Ext7), a time point associated with robust cocaine-seeking behavior. Brains were collected and flash frozen in -20°C isopentane and stored at -80°C. Brains were

subsequently mounted on a cryostat (Leica 3050S; Leica Corp., Deerfiled, IL) and coronal sections at the level of the NAc were taken. Bilateral 1 mm_3 micropunches of the NAc core and shell were collected for quantitative real-time PCR analyses of GLP-1R expression. mRNA expression was quantified using Taqman gene expression kits (GLP-1R: Rn00562406_m1; GAPDH: Rn01775763_g1; ThermoFisher Scientific, Waltham, MA). qPCR was conducted using an Eppendorf Mastercycler ep realplex2 and the comparative threshold cycle method was used to quantify relative mRNA expression. Relative fold-expression of NAc GLP-1R transcripts at Ext1 and Ext7 were normalized to yoked saline controls and GAPDH levels.

Statistics

For all cocaine and sucrose reinstatement experiments, the total mean active lever responses, inactive lever responses, and infusions were analyzed with two-way repeated measures ANOVAs. Pairwise analyses were made using Bonferroni *post-hoc* tests ($p<0.05$). For electrophysiological studies, all analyses were completed using Clampfit 10 (Molecular Devices). Differences in action potential frequency were analyzed with two-way repeated measures ANOVAs with current injection step and drug treatment as repeated measures and action potential frequency as a dependent variable. Bonferroni *post-hoc* tests were used for pair-wise comparisons ($p<0.05$). Action potential amplitude was measured from threshold to peak. Action potential duration was measured as duration at 50% of peak amplitude (i.e. half-width). Mean sEPSC frequencies were analyzed from 20 s trace segments. Mean sEPSC amplitude and duration were quantified from an average trace of at least 50 individual events in each cell. sEPSC duration was calculated as the rate of a mono-exponential fitted to the decay phase of the mean sEPSC trace. PPRs were calculated by averaging 5–10 responses and dividing the peak amplitude of the second evoked EPSC by the peak amplitude of the first evoked EPSC. Statistical

comparisons of PPR and sEPSC data were done using two-tailed paired Student's t tests. Statistical significance thresholds were set at $p<0.05$.

Results

Peripheral administration of fluoro-Ex-4 penetrates the brain, localizes in the NAc and attenuates cocaine seeking in rats

Total active and inactive lever responses (mean ± SEM) in rats pretreated with systemic fluoro-Ex-4 (vehicle, 0.1 and 0.2 µg/kg, i.p.) prior to a cocaine priming-induced reinstatement test session are shown in Figure 1A. These data were analyzed with a two-way repeated measures ANOVA, which revealed significant main effects of treatment [$F(2,24)=7.681$, $p<0.01$] and lever [$F(1,12)=10.21$, $p<0.01$] as well as a significant interaction between lever and treatment [$F(2,24)=5.509$, $p<0.05$]. Subsequent pairwise analyses indicated that active lever responses were significantly different between vehicle and 0.1 and 0.2 µg/kg fluoro-Ex-4 (Bonferroni, $p<0.05$). No significant differences were found on inactive lever responding between treatments.

Immediately following the reinstatement test session, rats pretreated with systemic fluoro-Ex-4 were sacrificed and their brains were collected to determine if fluoro-Ex-4 localized in the NAc. Coronal sections of the striatum were processed using immunohistochemistry to label neurons and astrocytes. Confocal microscopy revealed colocalization of fluoro-Ex-4 with both GFAP-positive astrocytes and NeuN-positive neurons in both the NAc core (Figure 3.1B) and shell (Figure 3.1C). Overall, these data revealed that systemic administration of fluoro-Ex-4 prior to a cocaine reinstatement test session penetrates the brain and may activate GLP-1Rs in the NAc to reduce drug-seeking behavior.

Administration of Ex-4 directly into the NAc core and shell dose-dependently attenuates cocaine seeking in rats.

To determine if activation of NAc GLP-1Rs alone is sufficient to attenuate cocaine reinstatement, Ex-4 was infused directly into the core and shell subregions of the NAc 10

minutes prior to a cocaine priming-induced reinstatement test session. Total active and inactive lever responses (mean ± SEM) in rats pretreated with intra-NAc core Ex-4 (vehicle, 0.005 or 0.05 µg/500nl) are shown in Figure 3.2A. These data were analyzed using a two-way repeated measures ANOVA, which revealed significant main effects of treatment [$F(2,32)=5.004$, $p<0.05$] and lever [$F(1,16)=46.04$, $p<0.0001$] as well as a significant treatment x lever interaction [$F(2,32)=4.248$, $p<0.05$]. Subsequent pairwise analyses indicated that active lever responses were significantly different between vehicle and 0.005 and 0.05 µg Ex-4 (Bonferroni, $p<0.05$). No significant differences were found on inactive lever responding between treatments.

Total active and inactive lever responses (mean ± SEM) in rats pretreated with intra-NAc shell Ex-4 (vehicle, 0.005 or 0.05 µg/500nl) are shown in Figure 3.2B. These data were analyzed using a two-way repeated measures ANOVA, which revealed significant main effects of treatment [$F(2,28)=5.401$, $p<0.01$] and lever [$F(1,14)=100.3$, $p<0.0001$] as well as a significant treatment x lever interaction [$F(2,28)=5.435$, $p<0.05$]. Subsequent pairwise analyses indicated that active lever responses were significantly different between vehicle and 0.05 µg Ex-4 (Bonferroni, $p<0.05$). No significant differences were found on inactive lever responding between treatments. Microinjections sites in the NAc core and shell are illustrated in Figure 3.2C.

Administration of Ex-4 directly into the NAc core and shell had no effect on sucrose seeking in rats.

While intra-NAc administration of Ex-4 had no significant effect on inactive lever responding, one could argue that responses were too low to assess the potential rate-suppressing effects of intra-NAc Ex-4 treatment. Total active and inactive lever responses (mean ± SEM) are shown in Figures 3.3A and 3.3B for rats pretreated with intra-NAc core

and shell Ex-4, respectively, prior to a sucrose reinstatement test session. There were no effects of intra-NAc core or shell Ex-4 on sucrose seeking. Taken together with the cocaine reinstatement studies in Figure 3.2, these data indicate that activation of GLP-1Rs in the core and shell reduces cocaine seeking and that these effects are not due to general motor impairments.

Ex-4 increases action potential frequency in NAc MSNs of cocaine-experienced rats

Using whole-cell current clamp, we evaluated the effects of Ex-4 on action potential frequency of MSNs in the NAc of cocaine-experienced rats (Figure 3.4). Application of 1 μM Ex-4 increased the frequency of action potential firing in the NAc core (Figures 3.4A and 3.4B) and shell (Figures 3.4C and 3.4D) of cocaine-experienced rats. These data were analyzed with two-way repeated measures ANOVAs, which indicated no significant main effect of drug treatment [$F(1,6)=1.185$, $p=0.32$, partial $\eta^2=0.165$], a significant main effect of current injection [$F(10,60)=38.94$, $p<0.01$, partial $\eta^2=0.866$], and a significant drug by current interaction [$F(10,60)=3.18$, $p<0.01$, partial $\eta^2=0.346$] in the NAc core. Subsequent pairwise comparisons revealed significant differences between aCSF and Ex-4 at 100, 120, 140, 160, 180, 200, and 220 pA current steps (Bonferroni, $p<0.05$). In the NAc shell, two-way repeated measures ANOVAs revealed significant main effects of drug treatment [$F(1,5)=11.8$, $p<0.05$, partial $\eta^2=0.702$] and current injection [$F(10,50)=80.87$, $p<0.01$, partial $\eta^2=0.942$], and no drug by current interaction [$F(10,50)=0.864$, $p=0.57$, partial $\eta^2=0.147$]. Subsequent pairwise comparisons revealed significant differences between aCSF and Ex-4 at 160, 180, and 200 pA current steps (Bonferroni, $p<0.05$). When the core and shell data were combined, a two-way repeated measures ANOVA indicated a significant main effect of drug treatment [$F(1,12)=5.24$, $p<0.05$, partial $\eta^2=0.304$), a significant main effect of current [$F(10,120)=105.7$, $p<0.0001$, partial

η^2=0.898), and a significant drug by current interaction [F(10,120)=2.372, p<0.05, partial η^2=0.165). Subsequent pairwise comparisons indicated significant differences between aCSF and Ex-4 at 120, 140, 160, 180, 200 and 220 pA current steps (Bonferroni, p<0.05). Taken together, these results indicate that Ex-4 elicits broad changes in excitability of MSNs in the NAc core and shell of cocaine-experienced rats. Notably, the significant drug by current interaction in the core and combined core/shell datasets indicate that the effect of Ex-4 on intrinsic excitability may be sensitive to the depolarization state of the neuronal membrane (see Discussion).

Although we focused on electrophysiological correlates of behavioral effects in cocaine-experienced rats, we also collected data from MSNs in the NAc of yoked saline rats. We did not discriminate between NAc subregions in these experiments and our analyses of MSNs from these rats revealed no main effect of drug treatment [F(1,6)=0.004, p=0.95, partial η^2=0.001]; a significant main effect of current injection [F(10,60)=37.44, p<0.01, partial η^2=0.862] and no drug by current interaction [F(10,60)=1.253, p=0.27, partial η^2=0.173) (Figures 3.4E, 3.4F, and 3.6). These results indicate that, in contrast to our findings in the core/shell of cocaine-experienced rats, Ex-4 application has no effect on MSNs in the NAc of yoked saline rats. No other measures of membrane excitability were altered by Ex-4 application in cocaine-experienced or yoked saline control rats (Figure 3.6).

Previously, we reported presynaptic effects of GLP-1R activation in the VTA and NAc core of drug-naïve rats (Mietlicki-Baase et al., 2013; Mietlicki-Baase et al., 2014). To explore the role of presynaptic GLP-1R activation, we analyzed sEPSCs and PPR of eEPSCs of NAc core and shell MSNs in cocaine-experienced rats and yoked saline controls. In

contrast to our previous findings, Ex-4 application onto slices from cocaine-experienced rats had no effect on sEPSC frequency or PPR of eEPSCs (Figure 3.5). Ex-4 also had no effect on sEPSC amplitude or duration in MSNs from cocaine-experienced rats (Figure 3.7). Moreover, no effects of Ex-4 on PPR of eEPSCs or on sEPSC frequency, amplitude, and duration in MSNs from cocaine-naïve rats were observed (data not shown). Collectively, these findings indicate that stimulation of GLP-1Rs is associated with increased intrinsic, but not synaptic, excitability of MSNs in the NAc core and shell.

Abstinence following cocaine self-administration has no effect on GLP-1R expression in the NAc.

Using quantitative real-time PCR, we assessed the effects of cocaine self-administration and subsequent extinction sessions on expression of GLP-1Rs in the NAc core and shell to determine the potential effects of voluntary cocaine taking and subsequent abstinence on endogenous NAc GLP-1 signaling (Figure 3.7A). There were no changes in GLP-1R mRNA expression in the NAc core (Figure 3.7B) and shell (Figure 3.7C) of cocaine-experienced rats following one (Ext1) and seven (Ext7) days of extinction when compared to yoked saline controls.

Discussion

The present study represents the first investigation of the role of GLP-1Rs in the NAc core and shell in the reinstatement cocaine-seeking behavior. Here, we demonstrate for the first time that (1) systemically administered fluoro-Ex-4 localizes in the NAc and attenuates cocaine seeking; (2) direct activation of GLP-1Rs in the NAc core and shell is sufficient to reduce cocaine, but not sucrose, seeking; (3) activation of GLP-1Rs increases frequency of MSN action potential firing in the NAc core and shell following extinction of voluntary cocaine taking; and (4) abstinence following cocaine self-administration does not alter endogenous GLP-1R expression in the NAc. Taken together, these results suggest that increased excitability of NAc MSNs may contribute to the suppressive effects of a GLP-1R agonist on cocaine priming-induced reinstatement of drug-seeking behavior.

Our findings contribute to and expand upon previous studies demonstrating a role for GLP-1Rs in addiction-like behaviors. An emerging literature indicates that systemic administration of GLP-1R agonists reduces the rewarding and reinforcing effects of cocaine (Egecioglu et al., 2013b; Graham et al., 2013; Sorensen et al., 2015). However, the mechanisms underlying these effects are not clear. GLP-1Rs are expressed throughout the brain in nuclei known to mediate cocaine reinforcement and drug-seeking behavior, including the VTA and NAc (Merchenthaler et al., 1999; Schmidt et al., 2005; Schmidt and Pierce, 2010). We have shown previously that activation of GLP-1Rs in the VTA attenuates the reinforcing efficacy of cocaine (Schmidt et al., 2016). Here, we show that systemic fluoro-Ex-4 crosses the blood brain barrier, localizes in the NAc and attenuates cocaine priming-induced reinstatement of drug-seeking behavior. Fluoro-Ex-4 was identified in proximity to both neurons and astrocytes in the NAc core and shell suggesting that GLP-1R activation on these two cell populations mediates the effects of peripheral fluoro-Ex-4 on cocaine seeking. However, future studies are required to

investigate the exact role of GLP-1Rs on neurons and astrocytes in the NAc in cocaine seeking.

Infusions of Ex-4 directly into the NAc core and shell were sufficient to attenuate cocaine priming-induced reinstatement of drug-seeking behavior. These are the first data supporting a novel role for GLP-1Rs in the NAc in drug-seeking behavior, generally, and cocaine reinstatement, specifically. Interestingly, the effects of GLP-1R signaling on cocaine seeking differed between NAc subregions. Cocaine-seeking behavior was decreased in rats pretreated with 0.05 µg Ex-4 in both the NAc core and shell. In contrast, 0.005 µg Ex-4 significantly attenuated cocaine seeking when infused directly into the NAc core, but not shell. GLP-1 signaling in the NAc has also been shown to affect food intake (Dossat et al., 2013). The effects of GLP-1R activation on intake of palatable food are more pronounced when Ex-4 is infused into the NAc core compared to the shell (Alhadeff et al., 2012). The lowest effective dose of Ex-4 used in this study was 0.025 µg, which produced a transient effect on food intake following activation of GLP-1Rs in the NAc core and no effect on food intake when infused into the NAc shell (Alhadeff et al., 2012). Collectively, these findings suggest that GLP-1 signaling in the NAc core may play a more prominent role in regulating drug-seeking behavior and hedonic feeding. Moreover, results from the present study also identify doses of a GLP-1R agonist that selectively reduce cocaine seeking and do not affect *ad libitum* food intake (Alhadeff et al., 2012; Dickson et al., 2012; Mietlicki-Baase et al., 2014) or produce aversive malaise-like effects (Hayes et al., 2011a; Dickson et al., 2012; Kanoski et al., 2012).

Administration of Ex-4 directly into the NAc dose-dependently reduces sucrose self-administration on a progressive ratio schedule of reinforcement (Dickson et al., 2012). Doses of Ex-4 lower than 0.1 µg when infused into the NAc do not affect operant

responding for sucrose or chow intake (Dickson et al., 2012). Consistent with these effects, the behaviorally relevant dose of Ex-4 (0.05 µg) that attenuated cocaine seeking when infused into the NAc core and shell did not affect the reinstatement of sucrose-seeking behavior. Combined with our systemic administration study identifying doses of Ex-4 that reduce cocaine seeking and do not produce adverse feeding effects (Hayes et al., 2011a; Kanoski et al., 2012), these findings further strengthen evidence supporting the selectivity of lower doses of GLP-1R agonists in attenuating cocaine seeking versus non-drug motivated behaviors.

The neural mechanisms underlying the effects of GLP-1R agonists on drug-mediated behaviors are not clear. GLP-1R agonists have been shown to elicit increases in neuronal activity in various brain regions by both pre- and post-synaptic mechanisms. In hypocretin/orexin neurons of the lateral hypothalamus, Ex-4 increased action potential firing by post-synaptic sodium channel-dependent mechanisms and also increased pre-synaptic glutamate release (Acuna-Goycolea and van den Pol, 2004). In our study, the interaction between injected current level and Ex-4 suggests that Ex-4 may exert a preferential effect at specific subtypes of voltage-gated sodium channels or unidentified low voltage-gated conductances. Ex-4 has also been shown to enhance $GABA_A$ receptor-mediated synaptic and tonic currents in rat hippocampal pyramidal CA3 neurons (Korol et al., 2015b). Finally, we have previously reported that Ex-4 increases miniature EPSC frequency and decreases PPR of AMPA receptor-mediated currents in the VTA and NAc core of drug-naïve rats, effects consistent with increased release of pre-synaptic glutamate (Mietlicki-Baase et al., 2013; Mietlicki-Baase et al., 2014). Interestingly, while cell-to-cell variability in the NAc of yoked saline animals (Figure 4F, inset) may indicate discrete effects in the core and the shell, it also raises the possibility that regulation of action potential firing may depend on relative efficacy of Ex-4 at excitatory vs. inhibitory

inputs to NAc MSNs. Together, these observations paint a complex picture of the mechanisms by which GLP-1R activation regulates neuronal output. Here, we show that post-synaptic action potential frequency was increased by Ex-4 application in both the NAc core and shell of cocaine-experienced rats. However, pre-synaptic glutamate release mechanisms were not affected by GLP-1R activation in the NAc of cocaine-experienced or yoked saline rats. Our findings suggest that cocaine and/or operant chamber experience may facilitate a shift from a pre-synaptic to a post-synaptic site of Ex-4 action in the NAc. Notably, our qPCR results indicate that this dynamic switch is unlikely to result from an overall difference in GLP-1R mRNA expression in the NAc of cocaine-experienced rats, although a change in the ratio of pre-synaptic versus post-synaptic GLP-1R protein levels remains a possibility.

Our results show that GLP-1R activation in the NAc core and shell of cocaine-experienced rats facilitates MSN firing rates and attenuates cocaine-seeking behavior. Cocaine abstinence is associated with decreased NAc membrane excitability due to changes in conductance of sodium, calcium and potassium (Zhang et al., 1998; Zhang et al., 2002; Hu et al., 2004). Lower membrane excitability during cocaine abstinence may enhance action potential sensitivity to GLP-1R activation that we report here. Cocaine exposure is likely a critical component in this scenario, given the previous findings of minimal effects of Ex-4 on NAc MSN firing in drug-naïve rats (Mietlicki-Baase et al., 2014). Indeed, a recent study showed that Ex-4 reduces cocaine-evoked, but not electrically stimulated, phasic release of dopamine in the NAc core (Fortin and Roitman, 2017). Moreover, systemic Ex-4 pretreatment reduces cocaine-evoked dopamine release in the NAc (Egecioglu et al., 2013b; Sorensen et al., 2015) and lateral septum (Reddy et al., 2016). However, GLP-1 signaling has also been shown to regulate other neurotransmitter systems including glutamatergic (Mietlicki-Baase et al., 2014) and GABAergic (Korol et

al., 2015b) transmission in the brain. Understanding the mechanisms by which GLP-1R activation in the NAc reduces cocaine seeking will benefit from a comprehensive analysis of how Ex-4 regulates integration of synaptic and intrinsic MSN excitability in the context of drug priming-induced reinstatement. Our results indicate that intrinsic GLP-1R-coupled mechanisms may contribute to previous findings that a broad increase in NAc MSN action potential output is associated with attenuation of rewarding consummatory behaviors (Nicola et al., 2004; Wheeler and Carelli, 2009)

Previously, we showed that cocaine activates GLP-1-expressing neurons in the nucleus tractus solitarius (NTS) of the caudal brainstem (Schmidt et al., 2016). Since the NTS sends monosynaptic GLP-1-expressing efferent projections to the NAc (Alhadeff et al., 2012), these findings suggest that increased endogenous GLP-1 signaling in the brain may represent a homeostatic response to cocaine that functions to reduce on-going cocaine consumption (Schmidt et al., 2016). However, no studies to date have investigated the potential role of endogenous central GLP-1 signaling in cocaine seeking. Here, we show that GLP-1R mRNA expression in the NAc core and shell was not altered following one and seven days of extinction. Ex-4 binding has been shown to regulate trafficking and surface expression of GLP-1Rs (Roed et al., 2014). It is possible that Ex-4 infusions into the NAc increase trafficking of GLP-1Rs to the post-synaptic membrane resulting in enhanced firing of MSNs. Additionally, cocaine may affect endogenous GLP-1 release from the NTS, which could also result in altered GLP-1R trafficking and surface expression in the NAc. Future studies are required to further delineate the role of endogenous central GLP-1 signaling in cocaine seeking, including whether cocaine affects GLP-1R trafficking and/or the kinetics of ligand-mediated GLP-1R trafficking.

A growing literature supports the efficacy of GLP-1R agonists in reducing cocaine-mediated behaviors. Our findings expand this literature and are the first to indicate that exogenous activation of GLP-1Rs in the NAc core and shell is sufficient to reduce cocaine-seeking behavior. Importantly, the doses of Ex-4 shown to attenuate cocaine seeking do not produce adverse feeding and malaise-like effects. While future studies are needed to further delineate the molecular and neurophysiological mechanisms by which GLP-1R activation in the NAc reduces cocaine seeking, our data show that the suppressive effects of intra-NAc Ex-4 on cocaine seeking are associated with increased action potential firing of MSNs. These findings provide strong rationale for pilot translational studies screening the efficacy of GLP-1R agonists in human cocaine addicts.

Figures

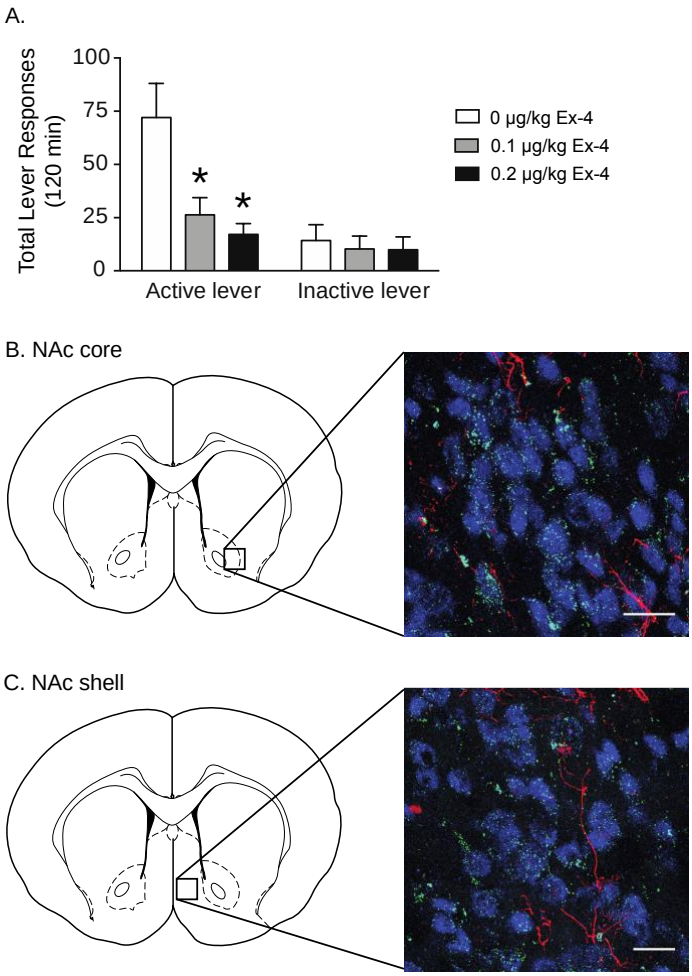

Figure 3.1 ***Systemic administration of the GLP-1R agonist Ex-4 attenuates cocaine seeking and localizes in the NAc.*** Rats were pretreated with intraperitoneal infusions of vehicle, 0.1 and 0.2 µg/kg fluoro-Ex-4 prior to a cocaine priming-induced reinstatement test session (*n*=7 per treatment). **(A)** Total numbers of active lever responses (mean ± SEM) were significantly different between rats pretreated with vehicle and 0.1 and 0.2 µg/kg fluoro-Ex-4. (**p*<0.05, Bonferroni). Immediately following the reinstatement test session, rats pretreated with systemic fluoro-Ex-4 were sacrificed and brains removed. Immunohistochemical analyses identified fluoro-Ex-4 (green fluorescence = fluoro-Ex-4) in the NAc core **(B)** and shell **(C)** where it localized with neurons (blue fluorescence = NeuN) and astrocytes (red fluorescence = GFAP).

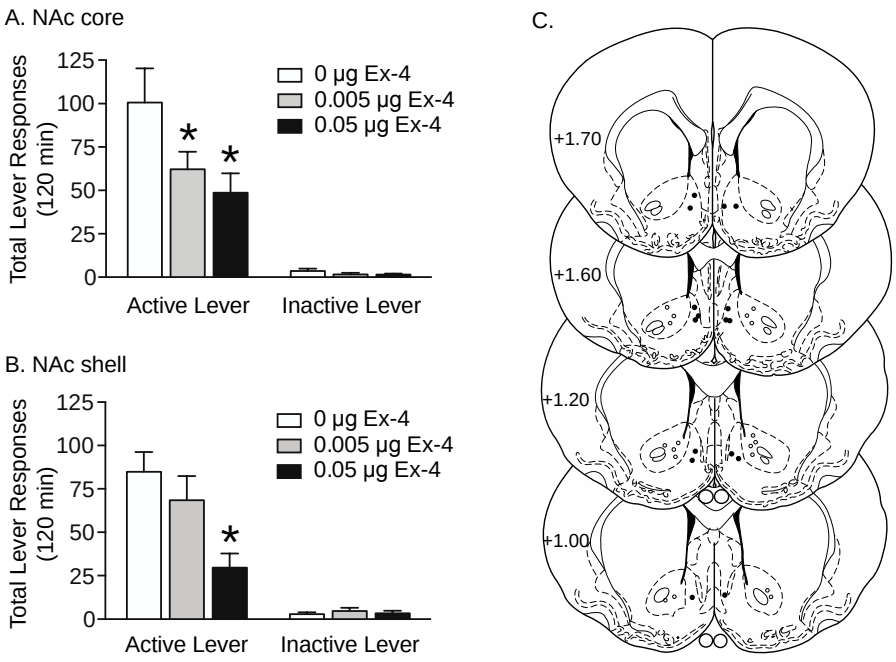

Figure 3.2 ***Administration of Ex-4 directly into the NAc dose-dependently attenuates cocaine-seeking behavior in rats***. Cocaine seeking was attenuated in rats pretreated with Ex-4 in the NAc core and shell prior to a cocaine priming-induced reinstatement test session. **(A)** Total numbers of active lever responses (mean ± SEM) were significantly different between rats pretreated with vehicle and 0.005 and 0.05 µg Ex-4 in the NAc core (*n*=9 per treatment). **(B)** Total numbers of active lever responses were also significantly different between rats pretreated with vehicle and 0.05 µg Ex-4 in the NAc shell (*n*=8 per treatment)**. (C)** Microinjection sites corresponding with infusions into the core (open circles) and shell (closed circles). (**p*<0.05, Bonferroni)

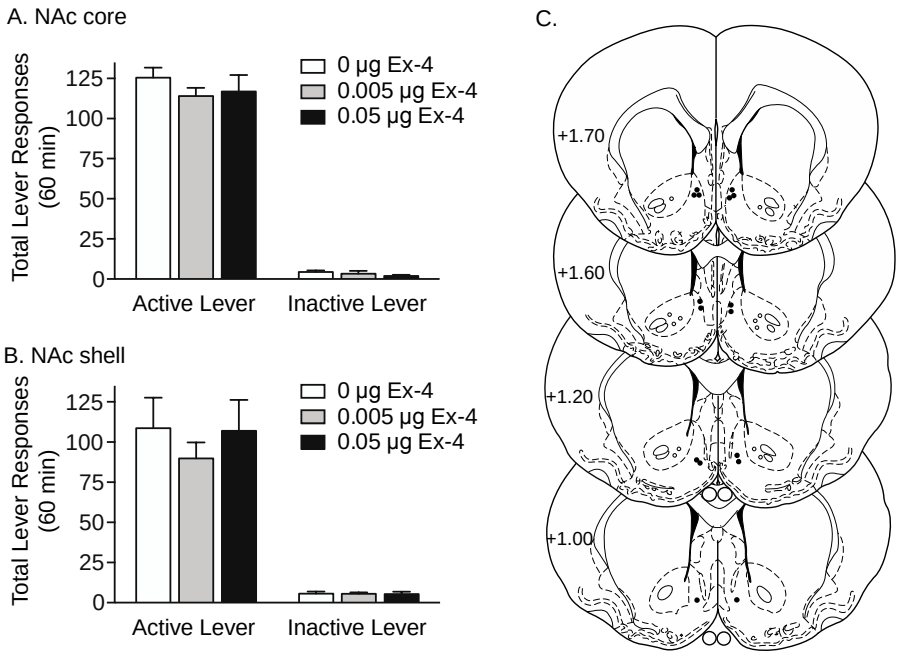

Figure 3.3 ***Intra-NAc administration of Ex-4 does not affect sucrose seeking in rats.*** Ex-4 (0.005 and 0.05 µg) administered directly into the NAc core **(A)** and shell **(B)** did not alter sucrose-seeking behavior in rats. Microinjection sites corresponding with infusions into the core (open circles) and shell (closed circles) are shown in **C**. (*n*=6 per treatment in the core; *n*=8 per treatment in the shell)

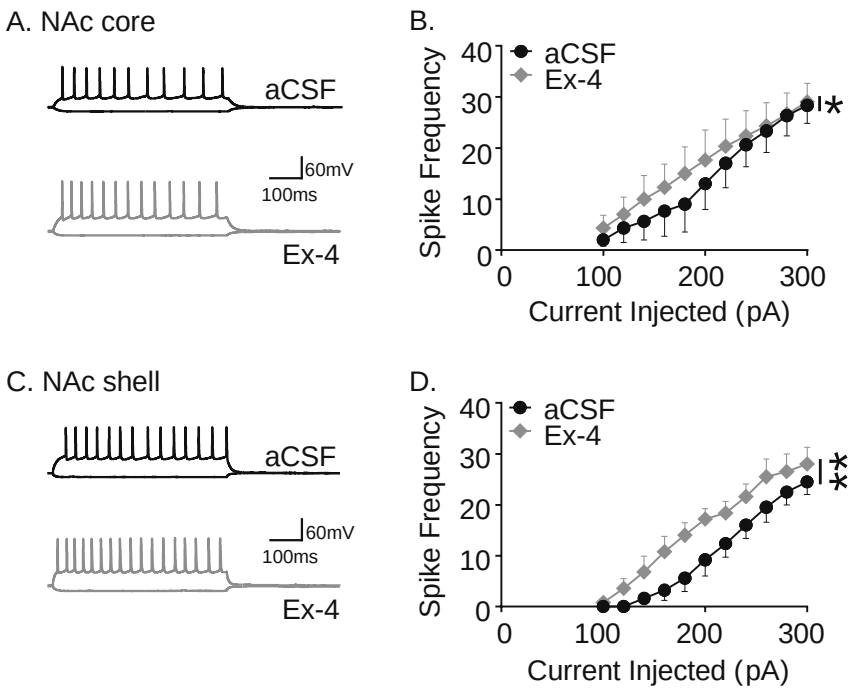

Figure 3.4 ***Ex-4 increases action potential frequency in NAc core and shell MSNs in cocaine-experienced rats.*** Representative membrane potential traces recorded before (aCSF) and after (Ex-4) application of Ex-4 (1μM) in response to a hyperpolarizing (-100 mV) and a depolarizing (+200 mV) current step in MSNs from cocaine-experienced and yoked saline rats (A, C, E). Corresponding plots of mean action potential frequency (+/- SEM) across a range of injected current steps are shown in B, D and F. Insets illustrate cell-to-cell variability of spike frequency at the +180 pA step for all recorded neurons before and after Ex-4 application. Note that cells with identical aCSF or Ex-4 values are not distinguishable. Thick black lines in the insets represent spike frequency means. (* $p<0.05$, for drug treatment by current interaction in Cocaine: NAc core and * $p<0.05$, for drug treatment in Cocaine: NAc shell, two-way repeated measures ANOVAs). Cocaine: NAc core, *n*=7 cells, N=4 rats; Cocaine NAc shell, *n*=6 cells, N=4 rats; Yoked Saline: NAc core/shell, *n*=7 cells, N=3 rats.

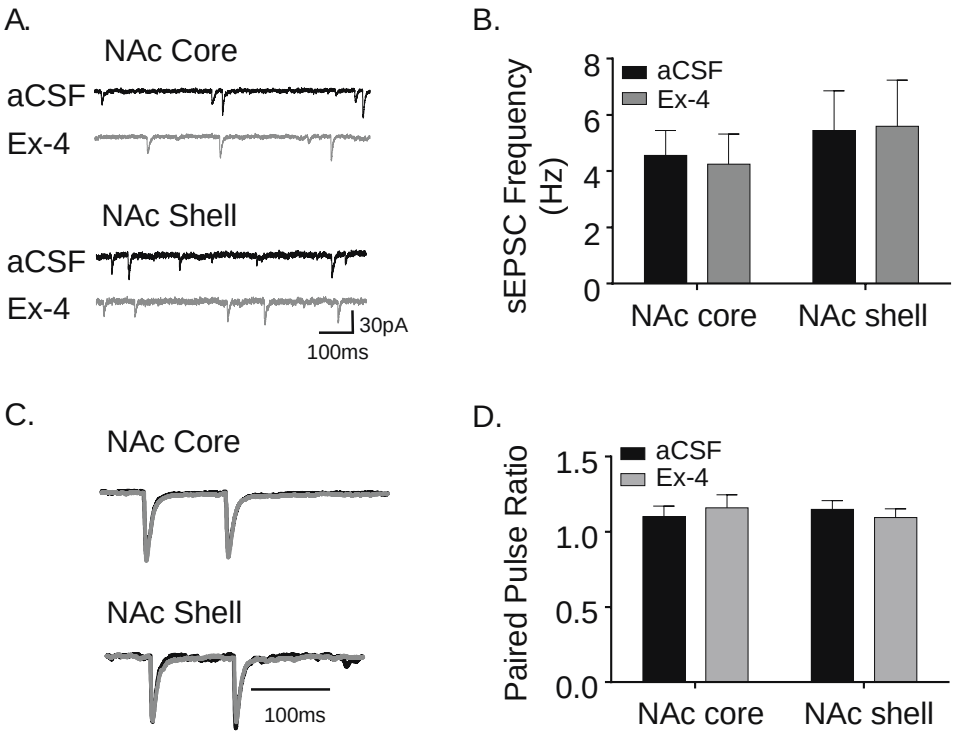

Figure 3.5 ***Ex-4 effects on action potential firing in NAc MSNs are not modulated by presynaptic mechanisms.***
Representative traces of sEPSCs from before (aCSF) and after (Ex-4) application of Ex-4 (1μM) in the NAc core and shell are shown in A. Ex-4 had no effect on sEPSC frequency in either subregion of the NAc (B). Representative traces showing paired pulse ratios (PPRs) before and after Ex-4 application are shown in C. Ex-4 had no effect on PPR in the core and shell (D). (*n*=8 cells, N=4 rats for Cocaine: NAc core; *n*=8 cells, N=4 rats for Cocaine: NAc shell).

	Cocaine: NAc Core		Cocaine: NAc Shell		Yoked Saline: NAc Core/Shell	
	aCSF	*exendin-4*	*aCSF*	*exendin-4*	*aCSF*	*exendin-4*
Resting membrane potential	76.0 ± 1.3 mV	71.0 ± 1.9 mV	73.5 ± 1.4 mV	72.0 ± 2.1 mV	78.0 ± 2.4 mV	77.0 ± 2.3 mV
Rheobase	160 ± 24 pA	132 ± 37 pA	151 ± 16 pA	140 ± 16 pA	190 ± 27 pA	176 ± 25 pA
Action potential amplitude	88 ± 1.9 mV	87 ± 2.6 mV	83 ± 1.5 mV	83 ± 3.2 mV	86 ± 2.8 mV	85 ± 3.3 mV
Action potential duration	1.1 ± 0.04 ms	1.2 ± 0.07 ms	1.1 ± 0.03 ms	1.1 ± 0.07 ms	1.02 ± 0.04 ms	1.05 ± 0.05 ms
sEPSC amplitude	24.58 ± 1.93 pA	24.09 ± 2.02 pA	25.88 ± 2.39 pA	24.45 ± 1.27 pA	24.79 ± 4.0 pA	23.93 ± 2.43 pA
sEPSC duration	4.52 ± 0.42 ms	4.86 ± 0.19 ms	4.21 ± 0.23 ms	4.05 ± 0.42 ms	4.75 ± 0.42 ms	4.86 ± 0.19 ms

Figure 3.6 ***Effects of Ex-4 on neuronal activity in the NAc***

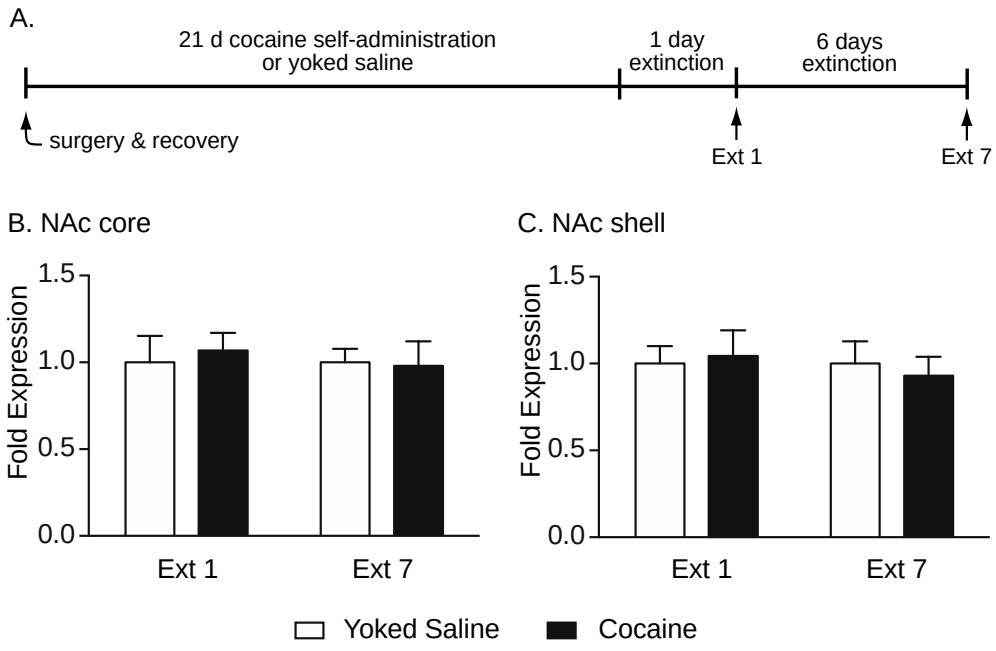

Figure 3.7 ***Cocaine self-administration and subsequent abstinence are not associated with altered expression of GLP-1Rs in the NAc.*** (A) Experimental design depicting the drug taking and extinction phases. Tissue was collected immediately after extinction sessions. GLP-1R mRNA expression in the NAc core (B) and shell (C) was not altered following one (Ext1; *n*=7) and seven (Ext7; *n*=9) days of abstinence compared to yoked saline controls. (p>0.05; un-paired t-tests)

CHAPTER 4: GLP-1 receptor signaling in the laterodorsal tegmental nucleus attenuates cocaine seeking through GABAergic projections to the VTA

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Abstract

Current preclinical literature demonstrates the promise of GLP-1 receptor (GLP-1R) agonists as therapeutics for cocaine use disorder. Our recently published studies showed that activation of central GLP-1Rs in the ventral tegmental area (VTA) attenuated the reinstatement of cocaine-seeking behavior, an animal model of relapse. The goal of this study was to expand our understanding of the central GLP-1R-expressing circuits and cell types that regulate cocaine seeking. The lateral dorsal tegmental nucleus (LDTg) is a brain region that expresses GLP-1Rs, receives direct projections from GLP-1-producing neurons in nucleus tractus solitarius (NTS), and plays a critical role in cocaine seeking. Here, we showed that administration of the GLP-1R agonist exendin-4 (Ex-4) into the LDTg significantly reduced cocaine seeking at a dose that does not affect food intake or body weight. We also demonstrated that GLP-1Rs are expressed primarily on GABAergic neurons in the LDTg and that Ex-4 decreased cocaine seeking, in part, through activation of LDTg GABA neurons that project to the VTA. Additionally, we investigated the role of endogenous hindbrain GLP-1-producing circuits in cocaine seeking by chemogenetically activating NTS afferents to the LDTg. We found that NTS-to-LDTg circuit activation decreased cocaine seeking via a GLP-1-dependent mechanism. Overall, these findings identified a central mechanism by which Ex-4 attenuates cocaine seeking and demonstrated that GLP-1R signaling in the LDTg is an important regulator of cocaine-craving induced relapse.

Introduction

Cocaine continues to be one of the highest used illicit drugs used in the United States and a major public health concern (NSDUH, 2018). Although there is still a lack of available FDA-approved pharmacotherapies to treat this disease, recent preclinical studies have highlighted the glucagon-like peptide-1 (GLP-1) system as an attractive potential target for treating cocaine use disorder [for review see: (Hernandez and Schmidt, 2019)].

GLP-1 is a satiation hormone and neuropeptide known to regulate the rewarding value of food as well as drugs of abuse through activation of central GLP-1 receptors (GLP-1Rs) in the mesolimbic reward system (Skibicka, 2013; Hayes and Schmidt, 2016). Our recently published findings show that central GLP-1Rs are a potential target for treating cocaine craving-induced relapse as administration of the GLP-1R agonist exendin-4 (Ex-4) into the ventral tegmental area (VTA) or nucleus accumbens (NAc) decreases the reinstatement of cocaine-seeking behavior, an animal model of relapse (Hernandez et al., 2018; Hernandez et al., 2019). Since GLP-1 analogs are currently FDA-approved for treating type II diabetes and obesity (Lovshin and Drucker, 2009; Shukla et al., 2015), these pre-clinical findings suggest that GLP-1R agonists could be repurposed for treating cocaine use disorder. However, the exact mechanisms by which Ex-4 attenuates cocaine seeking are unclear. Thus, in order further support the use of GLP-1R agonists in treating cocaine relapse, it is imperative to investigate the central mechanisms that underly the effects of Ex-4 on cocaine seeking and expand our knowledge of the GLP-1-expressing circuits and cell types that regulate cocaine-mediated behaviors.

The laterodorsal tegmental nucleus (LDTg), a region of the mesopontine tegmentum, receives input from limbic sites and sends direct projections to the VTA (Cornwall et al., 1990; Oakman et al., 1995; Omelchenko and Sesack, 2005). Thus, the LDTg is

anatomically positioned to influence the mesolimbic reward system and drug-mediated behaviors. Indeed, the LDTg is known to be critical for burst firing of dopamine neurons and dopamine release in the NAc through its connections to the VTA (Blaha et al., 1996; Forster and Blaha, 2000; Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Dautan et al., 2016). In addition, activation of excitatory LDTg-to-VTA projections elicits conditioned place preference (CPP) and reinforces operant responding in rodents (Lammel et al., 2012; Steidl and Veverka, 2015; Xiao et al., 2016; Steidl et al., 2017). With regard to cocaine-mediated behaviors, ablation or pharmacological inactivation of cholinergic neurons and inhibition of glutamate signaling in the LDTg increases the latency to self-administer cocaine, inhibits acquisition of cocaine CPP, and attenuates the reinstatement of cocaine-seeking behavior (Schmidt et al., 2009; Shinohara et al., 2014; Steidl et al., 2015). While these findings clearly indicate that excitatory LDTg signaling drives in reward and cocaine-mediated behaviors, the role of inhibitory signaling in the LDTg remains largely unexplored.

The LDTg expresses GLP-1Rs and LDTg GLP-1R activation via Ex-4 decreases feeding behaviors in drug-naïve rats (Goke et al., 1995; Merchenthaler et al., 1999; Reiner et al., 2018). In addition to expressing GLP-1Rs, the LDTg also receives direct projections from GLP-1-producing neurons in the nucleus tractus solitarius (NTS) of the brainstem (Cornwall et al., 1990; Rinaman, 2010; Grill and Hayes, 2012; Reiner et al., 2018). Studies show that cocaine increases neuronal activity in the NTS, including GLP-1-producing neurons (Zahm et al., 2010; Buffalari and Rinaman, 2014; Schmidt et al., 2016). Additionally, our previous studies show that cocaine taking and subsequent abstinence dynamically regulate expression of preproglucagon (PPG), the gene that encodes GLP-1, in the NTS (Hernandez et al., 2018). While these studies indicate that cocaine influences the NTS GLP-1 system, no studies to date have examined the endogenous relevance of

GLP-1 circuits in cocaine seeking. Taken together, we hypothesized that both exogenous activation of LDTg GLP-1Rs and endogenous GLP-1 signaling in the LDTg attenuates cocaine seeking, possibly through inhibitory mechanisms.

Overall, the present study aimed to: 1) determine if GLP-1R activation in the LDTg decreases cocaine seeking; 2) investigate the mechanisms by which Ex-4 acts in the LDTg to attenuate cocaine seeking; 3) examine the role of endogenous GLP-1 circuits that project to the LDTg in regulating cocaine-seeking behavior. Using behavioral pharmacology, fluorescent *in-situ* hybridization, transgenic rat models, viral-mediated knockdown of GLP-1Rs and chemogenetic manipulations of hindbrain circuits, our data establish that LDTg GLP-1Rs play a critical role in cocaine seeking. We show that GLP-1Rs are expressed primarily on GABA neurons in the LDTg and that Ex-4 activates GABAergic LDTg-to-VTA projections to reduce cocaine-seeking behavior. We also demonstrate that NTS-to-LDTg circuit activation is sufficient to decrease cocaine seeking through a GLP-1-dependent mechanism. Thus, these results highlight a novel role for hindbrain circuits and GLP-1R signaling in the regulation of cocaine-seeking behavior.

Materials and Methods

Animals and housing

Male Sprague-Dawley rats weighing 220-250g were obtained from Taconic Laboratories (Rensselaer, NY). Transgenic rats expressing Cre recombinase under the GAD1 promoter (LE-Tg(Gad1-iCre)3Ottc), were purchased from the Rat Resource and Research Center (RRRC P40OD011062; University of Missouri, Columbia, MO). Rats were housed individually on a 12/12 hr light/dark cycle and maintained on *ad libitum* food and water. The experimental protocols were consistent with the guidelines issued by the U.S. National Institutes of Health and were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee.

Drugs

Cocaine was obtained from the National Institute on Drug Abuse (Rockville, MD) and dissolved in bacteriostatic 0.9% saline. Ex-4 and exendin-(9-39) were purchased from Bachem (Torrance, CA) and were dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA). Fluoro-Ex-4 was purchased from AnaSpec (Fremont, CA) and dissolved in bacteriostatic 0.9% saline. Clozapine N-Oxide (CNO) was obtained from Toronto Research Chemicals (North York, ON) and dissolved in 1% DMSO in bacteriostatic 0.9% saline. The doses and time course of administration for each of the aforementioned pharmacological compounds were based on the following systemic and intra-cranial microinjection experiments in rats: Ex-4 (Alhadeff et al., 2012; Schmidt et al., 2016; Hernandez et al., 2018), exendin-(9-39) (Alhadeff et al., 2012; Schmidt et al., 2016; Hernandez et al., 2018), fluoro-Ex-4 (Kanoski et al., 2012; Reiner et al., 2016; Hernandez et al., 2018; Hernandez et al., 2019) and CNO (Boender et al., 2014; Scofield et al., 2015; Alhadeff et al., 2017a; McGlinchey and Aston-Jones, 2018).

Surgery

Rats were handled daily and allowed one week to acclimate to their home cages upon arrival. Prior to surgery, rats were anesthetized with 80 mg/kg ketamine (Midwest Veterinary Supply, Valley Forge, PA) and 12 mg/kg xylazine (Sigma-Aldrich/RBI, St. Louis, MO). An indwelling catheter (SAI Infusion Technologies, Lake Villa, IL) was inserted into the right jugular vein and sutured in place. The catheter was routed to a mesh backmount that was implanted subcutaneously above the shoulder blades. To prevent infection and maintain patency, catheters were flushed daily with 0.2 ml of the antibiotic Timentin (0.93 mg/ml; Fisher, Pittsburgh, PA) dissolved in heparinized 0.9% saline (Butler Schein, Dublin, OH). When not in use, catheters were sealed with plastic obturators.

After catheter insertion, some rats were then immediately mounted in a stereotaxic apparatus (Kopf Instruments, CA) and implanted with cannulae for intra-cranial microinjections. Bilateral guide cannulae (26 gauge; 16 mm; Plastics One, Roanoke, VA) were implanted 2.5mm dorsal to the LDTg (for LDTg injections) and 2 mm dorsal to the VTA (for VTA injections) and cemented in place by affixing dental acrylic to stainless steel screws secured in the skull. The coordinates for the ventral ends of the guide cannulae, relative to bregma according to the atlas of Paxinos and Watson (1997), were as follows: LDTg (-9.1 mm A/P, ±0.5 mm M/L, and -4.1 mm D/V), VTA (-6.0 mm A/P, ±0.5 mm M/L, and -6.6 mm D/V). An obturator (33 gauge; Plastics One) was inserted into each guide cannula to prevent occlusion.

Cocaine self-administration, extinction and reinstatement of cocaine seeking

Rats were allowed 7 days to recover from surgery before behavioral testing commenced. Initially, rats were placed in operant conditioning chambers and allowed to lever-press for intravenous infusions of cocaine (0.25 mg/kg/infusion, infused over a 5 s period) on a

fixed-ratio 1 (FR1) schedule of reinforcement. Rats were allowed to self-administer a maximum of 30 injections per 120 min operant session. Once a rat achieved at least 20 infusions of cocaine in a single daily operant session under the FR1 schedule, the subject was switched to a fixed-ratio 5 (FR5) schedule of reinforcement. The maximum number of injections was again limited to 30 per daily self-administration session under the FR5 schedule. For both FR1 and FR5 schedules, a 20 s time-out period followed each cocaine infusion, during which time active lever responses were tabulated but had no scheduled consequences. Responses made on the inactive lever, which had no scheduled consequences, were also recorded during both the FR1 and FR5 training sessions. Following 21 days of daily cocaine self-administration sessions, drug-taking behavior was extinguished by replacing the cocaine solution with 0.9% saline. Daily extinction sessions continued until responding on the active lever was <15% of the total active lever responses completed on the last day of cocaine self-administration. Typically, it took ~7 days for rats to meet this criterion. Once cocaine self-administration was extinguished, rats entered the reinstatement phase of the experiment. During reinstatement test sessions, satisfaction of the response requirement (i.e., five presses on the active lever) resulted in an infusion of saline rather than cocaine. Using a between-sessions reinstatement procedure, each reinstatement test session was followed by extinction sessions until responding was again <15% of the total active lever responses completed on the last day of cocaine self-administration. Generally, 1–2 days of extinction were necessary to reach extinction criterion between reinstatement test sessions.

The effects of intra-LDTg Ex-4 administration on cocaine reinstatement

Once cocaine-taking behavior was extinguished, obturators were removed from the guide cannulae and 33 gauge stainless steel microinjectors (18mm; 2.5mm projection, Plastics One) were inserted. Using a within-subjects counterbalanced design, rats were infused

bilaterally with 0, 0.005 or 0.025 µg/100 nl Ex-4 directly into the LDTg. Microinjectors were left in place for an additional one minute following infusions in order to allow for diffusion of the drug solution away from the tips of the microinjectors. Rats were then placed back in their home cages. Ten minutes later rats received a priming injection of cocaine (10mg/kg, i.p.) and then placed immediately into the operant conditioning chambers and a two-hour reinstatement test session commenced. Twenty-four hours after each treatment, rats were weighed to assess if Ex-4 affects body weight.

The effects of LDTg GABA neuron-specific GLP-1R knockdown on progressive ratio and cocaine reinstatement

We have previously used the short hairpin RNA (shRNA) sequence 5′-GATCGGGTTGCTGGTGGAAGGCGTGTATCTGTACTCAAGAGGTACAGATACACGC CTTCCACCAGCAACCTTTTTT-3′ to target GLP-1R transcripts (Schmidt et al., 2016; Alhadeff et al., 2017b). Our *in vitro* studies demonstrated ~ 88% knockdown of GLP-1R expression in a rat neuronal cell line transfected with this shRNA and ~50% knockdown *in vivo* (Schmidt et al., 2016; Alhadeff et al., 2017b). The shRNA sequence was cloned and packaged into an adeno-associated virus (AAV; serotype 1) downstream of the CB7 promotor and co-expressed with GFP. For Cre recombinase-dependent expression a FLEX switch was added to produce the following virus: AAV1.CB7.CI.FLEX.miR-GLP-1R-EGFP.WPRE.bGH, hereby referred to as AAV-FLEX-shRNA-GLP-1R. A GFP-expressing AAV1 (AAV1.CAG.FLEX.EGFP.WRPE.bGH) was used as a control and hereby referred to as AAV-FLEX-GFP. Viruses were obtained through the Viral Core at the University of Pennsylvania.

For cell type-specific knockdown of GLP-1R expression in GABA neurons, GAD1-Cre rats underwent catheter surgery and LDTg-directed guide cannulae insertion as described

above. Immediately after catheter and cannula implantation, rats received bilateral infusions of AAV-FLEX-shRNA-GLP-1R or AAV-FLEX-GFP ($2e_{12}$ gc/ml, 500nl over 90 sec) directly into the LDTg. Microinjectors were left in place for an additional 90 sec after infusion to allow for diffusion away from the injection site. After a one week recovery, rats underwent 14 days cocaine self-administration as described above. The effects of GLP-1R knockdown in LDTg GABA neurons on the motivation to self-administer cocaine was then assessed for 3 consecutive days on a progressive-ratio (PR) schedule of reinforcement as described previously (Schmidt et al., 2016). Briefly, under a PR schedule, the response requirement for each subsequent infusion increased exponentially until the rat failed to meet a requirement in 30 min. The response requirement for the i_{th} reinforcement was given by R(i)=[5e 0.2i -5]. The breakpoint was operationally defined as the last response requirement completed before the termination of the PR test session. Responding on the PR schedule were averaged across the three days of PR test sessions. After PR testing, rats underwent five days of FR5 responding to stabilize cocaine self-administration before extinction took place. Once cocaine-taking behavior was extinguished, the effect of GLP-1R knockdown in LDTg GABA neurons on the ability of intra-LDTg Ex-4 or systemic Ex-4 to reduce cocaine reinstatement was assessed. Rats were pretreated with vehicle or 0.005 µg Ex-4 in the LDTg 10 min prior to a priming injection of cocaine. Additional rats received vehicle or 0.2 µg/kg i.p. Ex-4 one hour prior to a priming injection of cocaine and cocaine reinstatement was assessed. These doses of intra-LDTg and systemic Ex-4 were used because of data showing that they are selective for decreasing cocaine seeking without having adverse side effects on feeding or body weight [Figure 4.1; (Hernandez et al., 2018)]. All treatments were given in a within-subjects counterbalanced design.

Following behavioral testing, brains were dissected and flash frozen in -20°C isopentane and stored at -80°C. 1 mm_3 bilateral micropunches of the LDTg were collected to assess GLP-1R expression using quantitative real-time PCR (qPCR). GLP-1R levels were quantified using Taqman gene expression kits (GLP-1R: Rn00562406_m1; GAPDH:Rn01775763_g1; Life Technologies) and PCR reagents (Applied Biosystems). qPCR was conducted using an Eppendorf Mastercycler ep realplex2 and the comparative threshold cycle method was used to quantify relative mRNA expression as described above. An LDTg-containing coronal section (30 µm) was collected from each brain to verify correct placement of AAV injections by visualizing GFP fluorescence. Only rats with correct placement and viral expression were included in data analysis.

The effects of chemogenetic inhibition of GABAergic LDTg-to-VTA projections on cocaine reinstatement

GAD1-Cre rats underwent catheter surgery and received bilateral infusions of an AAV expressing the Cre recombinase-dependent inhibitory DREADD (Designer Receptors Exclusively Activated by Designer Drugs) (AAV2-hSyn-DIO-hM4D(Gi)-mCherry) or control virus (AAV2-hSyn-DIO-mCherry) provided by Addgene (Watertown, MA) at the level of the LDTg (virus titer: $1e_{12}$ gc/ml, 500nl over 90 sec). Microinjectors were left in place for an additional 90 sec after infusion to allow for diffusion away from the injection site. After viral infusion, VTA-directed guide cannulae were implanted and cemented in place. After a one week recovery, rats underwent cocaine self-administration and extinction as described above. In order to determine if inhibiting LDTg GABA terminals in the VTA prevents the ability of Ex-4 to decrease cocaine seeking, prior to reinstatement test sessions, rats received bilateral microinjections of CNO (0, 1 mM; 100nl) in the VTA. After 10 min, rats received systemic injections of Ex-4 (0, 0.2 µg/kg, i.p) one hour prior to their

priming-injection of cocaine and reinstatement test sessions immediately commenced. All treatments were administered in a within-subjects counterbalanced design. After behavioral experiments, rats were perfused and brains collected. VTA- and LDTg-containing coronal sections (30 µm) were collected from each brain to verify correct placement of cannula and viral expression by visualizing mCherry fluorescence. Only rats with correct placement and viral expression were included in data analysis.

The effects of chemogenetic activation of NTS-to-LDTg projections on cocaine reinstatement

Sprague Dawley rats underwent catheter surgery and received bilateral infusions of the retrogradely infecting canine adeno virus-2 expressing Cre recombinase (CAV2-Cre; IGMM, Montpellier, FR) directly into the LDTg. In the same surgical session, an AAV (Addgene, Watertown, MA) expressing the activating DREADD (AAV2-hSyn-DIO-hM3D(Gq)-mCherry) or control virus (AAV2-hSyn-DIO-mCherry) was bilaterally infused directly into the caudal NTS (-1.0 mm to occipital A/P, ±0.5 mm M/L, -6.0 mm D/V) via microinjectors through guide cannula (2 mm projection). All viruses were infused at a titer of $1e_{12}$ gc/ml at a volume of 500 nl over 90 secs. Microinjectors were left in place for an additional 90 sec after infusion to allow for diffusion away from the injection site. This dual viral approach allowed for DREADD manipulation of NTS neurons that specifically project to the LDTg and was adapted from previously published studies (Boender et al., 2014; Alhadeff et al., 2017a). After a one week recovery, rats underwent cocaine self-administration, extinction and reinstatement as previously described above. In order to activate NTS to LDTg projections, using a within-subjects counterbalanced design, systemic injections of CNO (0, 0.1 or 1.0 mg/kg, i.p.) were given 30 min prior to a priming injection of cocaine and the ability of CNO to decrease cocaine reinstatement was assessed.

During surgery, a subset of rats had LDTg guide cannula cemented in place after viral infusion in order to administer the GLP-1R antagonist exendin-(9-39) (Ex-9) prior to cocaine reinstatement. The purpose of this experiment was to determine if the effects of activating NTS-to-LDTg projections on cocaine seeking are due to increased GLP-1R activation in the LDTg. Using a within-subjects counterbalanced design, prior to reinstatement test sessions, rats were pretreated with bilateral infusions of Ex-9 directly into the LDTg (10 µg/100nl) 10 min before a systemic injection of CNO (1.0 mg/kg, i.p.). A priming injection of cocaine was given 30 min later and the ability of intra-LDTg Ex-9 to block the effects of CNO on cocaine seeking was assessed.

After behavioral testing, brains were collected and immunohistochemistry was performed on coronal sections of the NTS to visualize viral expression, the ability of CNO to induce cFos in DREADD-expressing neurons, and co-localization of GLP-1 with AAV-DIO-hM3Dq-mCherry. mCherry and cFos co-localization following CNO treatment was quantified using 3 representative coronal sections of the NTS at the level of the obex for each brain (n=6/treatment). Any missed cannula placement or viral injection/expression resulted in animals being excluded from data analysis.

Sucrose self-administration, extinction and reinstatement of sucrose seeking

Separate cohorts of rats were trained initially to self-administer 45 mg sucrose pellets (Research Diets, New Brunswick, NJ) on a FR1 schedule of reinforcement during daily one-hour operant sessions. Once rats achieved stable responding for sucrose (defined as <20% variation in responding over 3 consecutive days) on the FR1 schedule of reinforcement, the response requirement was increased to an FR5 schedule of reinforcement. Rats were limited to 30 sucrose pellets within each daily operant session and were restricted to ~20-25 g of chow (Harlan Teklad, Wilmington, DE) daily in their

home cages for the duration of the experiment. Water was available *ad libitum* in the home cage.

After two weeks of sucrose-maintained responding on an FR5 schedule of reinforcement, rats underwent an extinction phase where active lever pressing no longer resulted in sucrose delivery. Once active lever responding decreased to <15% of the maximum number of responses completed on the last day of sucrose self-administration, rats proceeded to reinstatement testing. Ex-4 (0, 0.005 and 0.025µg/100 nl) was microinjected into the LDTg 10 min prior to reinstatement test sessions. A within-subjects counterbalanced design was used for treatments. The experimenter remotely administered one sucrose pellet every two min for the first 10 min of the reinstatement session. A between-session procedure was used so that each daily reinstatement test session was followed by an extinction session the following day until responding was again <15% of the total active lever responses maintained by sucrose.

Ad libitum food intake

We assessed if our manipulations affected *ad libitum* food intake after reinstatement test sessions in three different experiments: effects intra-LDTg 0.005 µg Ex-4 on cocaine seeking, effects of NTS-to-LDTg circuit activation on cocaine seeking or CNO itself on cocaine seeking. A subset of rats for each experiment were housed in hanging wire cages with *ad libitum* access to food and water as we have described previously (Reiner et al., 2016; Alhadeff et al., 2017b; Hernandez et al., 2018) during the extinction and reinstatement phases of the experiment. Rats were pretreated with vehicle, 0.005 µg Ex-4 or 1 mg/kg CNO prior to a cocaine priming-induced reinstatement test session. Rats were returned to the hanging wire cages immediately following the reinstatement session and given *ad libitum* access to normal chow. Food weights were measured every 1, 3, 6,

and 24 h post session (3, 5, 8, and 26 h post infusion) accounting for spillage. Body weight was measured 24 h post session (26 h post infusion).

Immunohistochemistry

Rats were deeply anesthetized and transcardially perfused with 0.1 M PBS, pH 7.4, followed with 4% formalin in 0.1 M PBS. Rats treated with Ex-4 or fluoro-Ex-4 (0.2 µg/kg, i.p.) were injected 90 min prior to perfusion. Once brains were removed, they were postfixed overnight in 4% formalin in 0.1 M PBS and then cryoprotected in 20% sucrose in 0.1 M PBS at 4$_{o}$C for three days. Coronal brain sections (30 µm) were taken using a cryostat (Leica 3050S; Leica Corp., Deerfield, IL). Brain sections were stored in 0.1 M PBS at 4$_{o}$C until processed. Immunohistochemistry was performed on free-floating coronal sections containing the LDTg, VTA or NTS according to modified procedures from previously published studies (Reiner et al., 2016; Schmidt et al., 2016; Hernandez et al., 2019). Briefly, sections were washed with 1% sodium borohydride followed by 0.1 M PBS. Sections were then blocked in 0.1 M PBS containing 5% normal donkey serum and 0.2% Triton-X for 1 h at room temperature. Sections were incubated in primary antibodies overnight, and then, following a PBS rinse, they were incubated in secondary antibodies for 2 h. The primary antibodies used were rabbit anti-NeuN (1:1000; ab177487, Abcam, Cambridge, UK), goat anti-GFAP (1:1000; ab53554, Abcam, Cambridge, UK), mouse anti-cFos (1:500, sc-271243, Santa Cruz Biotechnology, Inc., Dallas, TX), rabbit anti-GLP-1 (1:1000 T-4363, Peninsula Laboratories International, Inc., San Carlos, CA), rabbit anti-dsRed (1:1000; 632496, Takara, Kyoto, Japan), mouse anti-mCherry (1:1000 632543, Takara, Kyoto, Japan), and rabbit anti-tyrosine hydroxylase (1:1000; 2792; Cell Signaling, Danvers, MA). Secondary antibodies were donkey anti-rabbit Alexa Fluor 594 (1:500), donkey anti-goat Alexa Fluor 647 (1:500), donkey anti-mouse Alexa Fluor 488 (1:500), donkey anti-rabbit Alexa Fluor 488 (1:500), donkey anti-mouse Alexa Fluor 594 (1:500),

from Jackson ImmunoResearch (West Grove, PA). GABA neurons were labeled by infusing GAD1-Cre rats with AAV-hSyn-DIO-mCherry into the LDTg 2 weeks prior to perfusion. Sections were then washed and mounted onto glass slides and coverslipped using Vectashield (Vector Laboratories; Burlingame, CA). Sections were visualized with a Leica SP5 X confocal microscope using the 20x 63x oil-immersion objectives along with 488, 594 and 633 nm laser lines. Image z-stacks were captured at the 20x and 63x oil-immersion objective with a step size of 2 and 1 µm, respectively.

Fluorescent in situ hybridization (FISH)

Brains were collected and flash frozen in -20°C isopentane and stored at -80°C. Brains were subsequently mounted on a cryostat (Leica 3050S; Leica Corp., Deerfield, IL) and 18 µm coronal sections at the levels of the LDTg were taken and immediately mounted onto Superfrost Plus slides (Fisher Scientific). mRNA expression of GLP-1Rs, GAD1, vGlut2 and ChAT via FISH was carried out using RNAscope Multiplex Fluorescent Reagent Kit V2 (Cat. 320850; Advanced Cell Diagnostics (ACD), Hayward, CA) per manufacturer's protocol. In brief, slide mounted sections were rinsed in 4% paraformaldehyde for 15 min at room temperature (RT). Following two quick rinses in 0.1 M phosphate-buffered saline (PBS), the sections were dehydrated in ascending ethanol solutions (5 min washes in 50, 70, 100, 100% ethanol) followed by an overnight incubation in 100% ethanol at − 20 °C. Next, slides were air-dried and a hydrophobic barrier was made around all of the sections on the slide using a hydrophobic pen (Vector Labs). The sections were rinsed with PBS and treated with Protease IV for 30 min at RT (followed by 2×1 min rinses in PBS).

Pre-treated tissue sections were processed immediately using probes designed by ACD to detect GLP-1R mRNA (Rn-Glp1r; 315221), GAD1 mRNA (Rn-Gad1-C2; 316401-C2),

vGlut2 mRNA (Rn-Slc17a6-C3; 317011-C3), and ChAT mRNA (Rn-Chat-C2; 430111-C2). Sections were incubated in a cocktail containing probes (concentrations indicated by manufacturer) for 2 h at 40 °C in a HybEZTM oven (ACD). Slides were rinsed twice in RNAscope wash buffer followed by a series of amplification steps via Fluorescent Multiplex Detection Reagents (Cat. 320851, ACD) at 40 °C separated by two brief washes in RNAScope wash buffer (Cat. 310091, ACD): 30 min AMP 1-FL, 15 min AMP 2-FL, 30 min AMP 3-FL, and then 15 min AMP 4 Alt B-FL (C1, Atto 550 nm; C2 Alexa 488 nm; C3 Atto 647 nm). After the final wash buffer, slides were coverslipped using Fluorogel mounting medium with DAPI (Fisher Scientific). Sections were visualized with a Leica SP5 X confocal microscope using the 40x oil-immersion objectives and the 405, 488, 555, and 647 laser lines. Image z-stacks were collected with the 40x oil-immersion objective with a step size of 2 µm.

Quantitative real-time PCR, cocaine self-administration and yoked saline controls

Separate rats underwent jugular catheterization as described above. Following a recovery period, the rats were randomly assigned to one of two groups: cocaine-experimental or yoked saline controls. Each rat allowed to respond for contingent cocaine infusions was paired with a yoked rat that received infusions of saline. While lever pressing for the saline-yoked rats had no scheduled consequences, these rats received the same number and temporal pattern of infusions as self-administered by their paired cocaine-experimental rat. Cocaine-experimental rats were allowed to lever press for intravenous cocaine infusions on a FR1 schedule as described above for a total of 21 days.

To assess the effects of cocaine self-administration and extinction on expression of LDTg GLP-1Rs, rats were sacrificed either immediately after the first extinction session (Ext1) or following 7 consecutive days of extinction (Ext7). Brains were collected and flash frozen

in -20°C isopentane and stored at -80°C. Brains were subsequently mounted on a cryostat (Leica 3050S; Leica Corp., Deerfield, IL) and coronal sections at the levels of the LDTg were taken. Bilateral 1mm_3 micropunches of the LDTg were collected for quantitative real-time PCR. mRNA expression was quantified using Taqman gene expression kits (GLP-1R: Rn00562406_m1; GAPDH: Rn01775763_g1; ThermoFisher Scientific, Waltham, MA). qPCR was conducted using an Eppendorf Mastercycler ep realplex2 and the comparative threshold cycle method was used to quantify relative mRNA expression. Relative fold-expression of LDTg GLP-1Rs at Ext1 and Ext7 were normalized to yoked saline controls and GAPDH levels.

Electrophysiology experiments validating CNO-mediated DREADD activation

Rats expressing AAV2-hSyn-DIO-hM4D(Gi)-mCherry in the LDTg or AAV2-hSyn-DIO-hM3D(Gq)-mCherry in the NTS were deeply anesthetized with isoflurane then quickly transcardially perfused with ice-cold cutting solution containing (in mM): 92 N-methyl-d-glucamine (NMDG), 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4$, and 0.5 $CaCl_2$, saturated with carbogen (95% O2/5% CO2), pH 7.4 with HCl, osmolarity 305-315 mOsm (Ting et al., 2014). Following decapitation, brains were removed for dissection, and horizontal slices (250 μM thick) of target brain regions (either the NTS or LDTg) were obtained using a VT1000S vibratome (Leica, Weltzar, Germany). Slices were made at 4°C then transferred to a holding chamber of the same cutting solution, and incubated at 37°C for 10-12 min. Slices were then moved to a beaker of room temperature holding ACSF containing (in mM): 86 NaCl, 2.5 KCl, 1.2 NaH2PO4, 35 NaHCO3, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 1 MgCl2, and 2 CaCl2, saturated with carbogen, pH 7.3-7.4, osmolarity 305-315 mOsm. Slices were allowed to recover for at least 45 min before performing recordings.

Slices were transferred to a Nikon Eclipse FN1 upright microscope equipped for Differential Interference Contrast (DIC) infrared optics. The recording chamber was continuously perfused with oxygenated recording ACSF containing (in mM): NaCl 119, KCl 2.5, NaHCO3 26, NaH2PO4 1.2, glucose 12.5, HEPES 5, MgSO4 1, CaCl2 2, pH 7.3-7.4, osmolarity 305-315 mOsm. Solution was heated to 32 ± 1°C using an automatic temperature controller (Warner Instruments). Target brain regions were identified using a 4X objective and individual neurons were magnified with a 40X water immersion lens. DREADD-positive neurons were further identified by fluorescence with a DS-Red filter. Recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) to a resistance of 3.5-5.5 MΩ when filled with intracellular solution. The intracellular solution contained the following (in mM) potassium gluconate 145, KCl 2.5, NaCl 2.5, BAPTA 0.1, HEPES 10, L-glutathione 1.0, sodium phosphocreatine 7.5, Mg-ATP 2.0, and Tris-GTP 0.25, pH 7.2-7.3 with KOH, osmolarity 285-295 mOsm. All recordings were performed in whole-cell current-clamp mode using a MultiClamp 700B amplifier. Baseline spontaneous firing was recorded for ≥ 5 min prior to the activation or inhibition of DREADD-positive neurons by CNO (30 μM) dissolved in the recording ACSF. After ≥ 5 min CNO was washed out with regular recording ACSF. All recordings were low-pass filtered at 3kHz, amplified 5 times, and then digitized at 20kHz using a Digidata 1440A acquisition board and pClamp10 software (both from Molecular Devices). For all experiments, access resistance was 15–25 MΩ, uncompensated, and monitored continuously during recording. Cells with a change in access resistance >20% over the course of data acquisition were not accepted for data analysis.

Verification of cannula placements

After completion of all microinjection experiments, rats were given an overdose of pentobarbital (100 mg/kg, i.p.). Brains were removed and drop fixed in 10% formalin.

Coronal sections (100 µm) were taken at the level of the LDTg or VTA with a vibratome and mounted on gelatin-coated slides. An individual blinded to behavioral responses verified microinjection sites using light microscopy. Rats with cannula placements outside of the LDTg or VTA and/or excessive mechanical damage were excluded from subsequent data analyses.

Statistics

For all cocaine and sucrose reinstatement experiments, the total mean active lever responses, inactive lever responses, and food intake measurements were analyzed with two-way repeated measures mixed-factors ANOVAs. Pairwise analyses were made using Bonferroni post-hoc tests ($p<0.05$). LDTg GABA neuron GLP-1R knockdown virus versus control virus on PR responding and cocaine reinstatement, fold change in GLP-1R mRNA expression, and cFos quantification were analyzed using unpaired *t*-tests.

Results

Administration of Ex-4 directly into the LDTg significantly decreased cocaine priming-induced reinstatement of drug-seeking behavior at a dose that did not affect sucrose seeking, chow intake or body weight.

Our previously published studies identified systemic doses of the GLP-1R agonist fluoro-Ex-4 (0.2 µg/kg) that selectively decreased cocaine seeking without producing adverse feeding or malaise-like effects (Hernandez et al., 2018). Additionally we showed that peripheral fluoro-Ex-4 bound putative GLP-1Rs localized on neurons and astrocytes in the VTA and NAc (Hernandez et al., 2018; Hernandez et al., 2019). To determine if the effects of systemic fluoro-Ex-4 on cocaine seeking are also due, in part, to activation of GLP-1Rs in the LDTg, rats were pretreated with a systemic injection of 0.2 µg/kg fluoro-Ex-4. Sections of the LDTg were then immunohistochemically processed to label neurons and astrocytes, which revealed fluoro-Ex-4 binding to GLP-1Rs on both GFAP-positive astrocytes and NeuN-positive neurons in the LDTg (Figure 4.1A).

To directly test if activation of GLP-1Rs in the LDTg decreased cocaine seeking, rats were pretreated with the GLP-1R agonist Ex-4 directly into the LDTg 10 min prior to a cocaine priming-induced reinstatement test session. Total active and inactive lever responses in rats pretreated with Ex-4 (0, 0.005 or 0.025 µg/100nl, n=15) are shown in Figure 4.1B. These data were analyzed using a two-way repeated measures ANOVA, which revealed significant main effects of treatment [$F(2,28)=15.05$, $p<0.001$] and lever [$F(1,14)=76.23$, $p<0.001$] as well as a significant treatment x lever interaction [$F(2,28)=15.89$, $p<0.001$]. Subsequent pairwise analyses indicated that active lever responses were significantly different between vehicle treatment versus 0.005µg and 0.025 µg Ex-4 (Bonferroni, $p<0.01$). No significant differences were found on inactive lever responding between treatments. Microinjection sites corresponding to this experiment shown in Figure 4.1D&G.

Due to a previous study showing that intra-LDTg Ex-4 at 0.025 and 0.05 µg decreased food intake and body weight in drug-naïve rats (Reiner et al., 2018), we wanted to determine if the doses of intra-LDTg Ex-4 used in Figure 4.1B (0.005 and 0.025 µg) produced adverse feeding effects. Additionally, we wanted to control for any motor suppressing effects or deficits in operant responding following Ex-4 treatment in cocaine-exposed rats. Therefore, parallel studies of sucrose-seeking behavior were performed in a separate group of rats (Figure 4.1C). These data were analyzed using a two-way repeated measures ANOVA, which revealed significant main effects of treatment [$F(2,56)=6.735$, $p<0.01$] and lever [$F(1,56)=75.37$, $p<0.001$] as well as a significant treatment x lever interaction [$F(2,56)=6.828$, $p<0.01$]. Subsequent pairwise analyses indicated that active lever responses were significantly different between vehicle and 0.025 µg Ex-4 (Bonferroni, $p<0.05$). However, no significant effects between vehicle and 0.005 µg Ex-4 on active or inactive lever responding vehicle were observed. Microinjection sites corresponding to this experiment are shown in Figure 4.1H. Additionally, 24 hr body weight was assessed in rats that underwent cocaine reinstatement test sessions (Figure 4.1B). Mean 24 hr body weight change following intra-LDTg 0, 0,005 or 0.025 µg treatment and cocaine reinstatement are shown in Figure 4.1E (n=15). These data were analyzed using a one-way ANOVA which revealed significant main effects of treatment [$F(2,28)=6.10$, $p<0.01$]. Subsequent pairwise analyses indicated that 24 hr body weight change was significantly different between vehicle and 0.025 µg Ex-4 (Bonferroni, $p<0.05$). No differences were observed between vehicle and 0.005 Ex-4. Taken together, these data showed that although the 0.025 µg dose of Ex-4 decreased body weight and sucrose seeking, the 0.005 µg dose of Ex-4 selectively decreased cocaine seeking without causing any effects on 24 hr body weight or sucrose seeking. To further validate that 0.005 µg Ex-4 had no adverse effects on feeding, 24 hr cumulative chow intake was measured

in a subset of rats that underwent cocaine reinstatement test sessions (n=7). There were no significant effects of 0.005 µg Ex-4 treatment observed at any time point (Figure 4.1F). Microinjection sites corresponding to these experiments are shown in Figure 4.1D, G, & H. Taken together, these data demonstrate that intra-LDTg Ex-4 attenuates cocaine seeking at a dose (0.005 µg) subthreshold for effects on *ad libitum* chow intake, body weight and sucrose-seeking behavior.

Cocaine self-administration and subsequent abstinence do not affect GLP-1R expression in the LDTg.

To determine if cocaine exposure alters endogenous GLP-1 signaling in the LDTg, we assessed the effects of cocaine self-administration and subsequent extinction sessions on LDTg GLP-1R mRNA expression levels using quantitative real-time PCR (Figure 4.2A). There were no changes in GLP-1R mRNA expression in the LDTg of cocaine-experienced rats following one (Ext1) and seven (Ext7) days of extinction when compared to yoked saline controls (Figure 4.2B). These data indicated that the effects of GLP-1R activation on cocaine seeking are not due to altered GLP-1R mRNA expression in the LDTg.

GABA neurons are the primary cell-type in the LDTg that express GLP-1Rs and are activated by systemic Ex-4.

In order to investigate the mechanisms by which Ex-4 in the LDTg decreases cocaine seeking, we first characterized the neuronal cell types that express GLP-1Rs. Fluorescent *in situ* hybridization was conducted on LDTg coronal sections to determine the neuronal cell types that express GLP-1R mRNA. Colocalization of GLP-1R transcripts with GAD1, vGLUT2 or ChAT mRNA are shown in Figure 4.3A-C. Zoomed in representative images are shown in Figure 4.3D. LDTg GLP-1R-expressing neurons were found to co-express GAD1 and vGLUT2. However, there was no co-expression observed between GLP-1R

and ChAT. Quantification revealed that 70% of GLP-1R-expressing neurons are GABAergic, and 25% are glutamatergic, with 0% being cholinergic (Figure 4.3E). The 5% remaining GLP-1R-expressing cell types in the LDTg are most likely glial cells as GLP-1Rs are expressed on astrocytes and involved in energy balance control (Reiner et al., 2016).

Due to the majority of GLP-1Rs being expressed on GABAergic neurons in the LDTg, we hypothesized that activating GLP-1Rs on this cell population mediates Ex-4's suppressive effects on cocaine seeking. To further test this hypothesis, we determined if systemic fluoro-Ex-4 localized with GABAergic neurons in the LDTg. Indeed, we found that fluoro-Ex-4 colocalized with LDTg GABA neurons (Figure 4.3F). We then assessed if Ex-4 treatment increased activation of LDTg GABA neurons in cocaine-experienced rats. Ex-4 induced cFos expression in the LDTg as measured by immunohistochemistry to label cFos in rats treated with Ex-4 (0.2 µg/kg) when compared with saline treated controls (Figure 4.3G). Percentages of total LDTg GABAergic cells co-expressing cFos showed ~18% percent co-localization in Ex-4 treated rats compared to ~3% in saline treated controls [$t(6)=12.49$, $p<0.001$; n=4/treatment] (Figure 4.3H). Percentages of total cFos-positive cells co-expressing GABA showed ~62% co-localization in Ex-4 treated rats compared to ~31% in saline treated controls [$t(6)=4.57$, $p<0.01$; n=4/treatment] (Figure 4.3H). Taken together, these data support the hypothesis that Ex-4 activates GABA neurons in the LDTg to decrease cocaine-seeking behavior.

GLP-1Rs on LDTg GABA neurons are endogenously relevant for regulating cocaine taking and mediate the effects of systemic Ex-4 on cocaine seeking.

To directly test the functional relevance of LDTg GABA GLP-1Rs in cocaine taking and seeking, AAV-FLEX-shRNA-GLP-1R or AAV-FLEX-GFP were administered directly into

the LDTg of GAD1-Cre rats to knockdown (KD) GLP-1R expression exclusively in LDTg GABA neurons (Figure 4.4A&B). After viral infusion, rats were allowed to self-administer cocaine. There was no effect of LDTg GABA GLP-1R KD on FR5 responding for cocaine (data not shown), however when tested on a PR schedule of reinforcement, rats with reduced LDTg GABA GLP-1R expression had significantly greater total active lever responses [$t(20)=2.72$, $p<0.05$], infusions [$t(20)=3.30$, $p<0.01$], and breakpoints [$t(20)=3.11$, $p<0.01$] when compared to control rats (Figure 4.4C-E). These data indicate that endogenous GLP-1 signaling on LDTg GABA GLP-1Rs is functionally relevant for controlling cocaine self-administration.

After cocaine self-administration, rats underwent extinction and cocaine reinstatement tests to determine if endogenous LDTg GABA GLP-1Rs mediate the effects of intra-LDTg Ex-4 on cocaine seeking. Control rats and GLP-1R KD rats were treated with vehicle or 0.005 µg Ex-4 directly into the LDTg 10 min before cocaine priming-induced reinstatement test sessions. Total active lever responses for both groups and treatments are shown in Figure 4.4F with corresponding microinjection placements in Figure4.4H&I. These data were analyzed using a two-way repeated measures ANOVA, which revealed a significant treatment x group interaction [$F(1,6)=11.46$, $p<0.05$]. Subsequent pairwise analyses indicated that vehicle and Ex-4 treatment were significantly different in the control group (Bonferroni, $p<0.05$) but not for the GLP-1R KD group. These data show that LDTg GLP-1Rs on GABA neurons are necessary for the suppressive effects of intra-LDTg Ex-4 on cocaine seeking. We also wanted to determine the mechanisms of systemic Ex-4 to decrease cocaine seeking and hypothesized that these effects are mediated, in part, by activation of LDTg GABA GLP-1Rs. Therefore, control and GLP-1R KD rats were treated with vehicle or 0.2 µg/kg Ex-4 i.p. prior to a cocaine priming-induced reinstatement test session. Total active lever responses for both groups and treatments are shown in Figure

4.4G. These data were analyzed using a two-way repeated measures ANOVA, which revealed a significant treatment x group interaction [$F(1,17)=8.51$, $p<0.01$]. Subsequent pairwise analyses indicated that vehicle and Ex-4 treatment were significantly different in the control group (Bonferroni, $p<0.05$) but not for the GLP-1R KD group. Taken together, LDTg GABA GLP-1Rs play a role in the suppressive effects of Ex-4 on cocaine seeking.

Systemic Ex-4 decreases cocaine seeking by activating LDTg GABA neurons that project to the VTA.

To further investigate the circuits that are activated by systemic Ex-4 to attenuate cocaine seeking, we determined if the effects of Ex-4 are mediated through LDTg GABA neurons that project to the VTA. The role of the LDTg in motivated behaviors is largely attributed to its projections to the VTA and regulation of dopamine burst firing. (Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Lammel et al., 2012; Steidl et al., 2017). Thus, we hypothesized that Ex-4 reduces cocaine seeking by acting on LDTg GABA neurons that project to the VTA. We tested this hypothesis by using DREADD-mediated inhibition of GABAergic LDTg-to-VTA projections. A virus expressing the inhibitory DREADD (AAV-DIO-hM4D(Gi)-mCherry) was infused into the LDTg of GAD1-Cre rats and guide cannula were implanted at the level of the VTA (Figure 4.5A). Expression of hM4D(Gi) in the LDTg is shown in Figure 4.5B. GABAergic terminals expressing hM4D(Gi) were visualized in the VTA and localized with VTA dopaminergic neurons (Figure 4.5C). Representative electrophysiological traces validating the ability of the DREADD agonist clozapine-n-oxide (CNO) to inhibit activity of hM4D(Gi)-expressing neurons are shown in Figure 4.5E.

Prior to cocaine priming-induced reinstatement test sessions, CNO (1mM, 100 nl) was infused into the VTA to inhibit LDTg GABA terminals before an injection of Ex-4 (0.2 µg/kg, i.p.). Using a within-subjects design, there were 4 treatment conditions (intra-VTA

vehicle/i.p. vehicle, intra-VTA CNO/i.p. vehicle, intra-VTA vehicle/i.p. Ex-4, intra-VTA CNO/i.p. Ex-4; n=10/treatment). Total active lever responses are shown in Figure 4.5D. These data were analyzed with a two-way repeated measures ANOVA which revealed significant main effects of systemic treatment [$F(1,9)=11.62$, $p<0.01$] and intra-VTA treatment [$F(1,9)=7.22$, $p<0.05$] as well as a significant systemic treatment x intra-VTA treatment interaction [$F(1,9)=6.87$, $p<0.05$]. Subsequent pairwise analyses indicated a significant difference in total active lever responding between intra-VTA vehicle/i.p. vehicle, intra-VTA CNO/i.p. vehicle, and intra-VTA CNO/i.p. Ex-4 versus intra-VTA vehicle/i.p. Ex-4 treated rats (Bonferroni, $p<0.05$). There was no effect of treatment on total inactive lever responding (data not shown). Corresponding microinjection sites in the VTA are shown in Figure 4.5G. These data demonstrate that Ex-4 decreases cocaine seeking, in part, by activating LDTg GABAergic projections to the VTA.

Although CNO is often used as the inert ligand for DREADDs, recent studies show minimal, yet significant, reverse metabolism to clozapine, which may cause off-target effects on behavior (Manvich et al., 2018). Therefore, we validated the effects we observed from intra-VTA CNO treatment on cocaine seeking were due to inhibiting hM4D(Gi)-expressing LDTg terminals and not due to any off-target effects of CNO. Thus, a separate group of rats was infused with control virus AAV-DIO-mCherry in the LDTg and the same experimental procedures were performed as described above. Total active lever responses are shown in Figure 4.5F (n=10/treatment). These data were analyzed with a two-way repeated measures ANOVA which revealed significant main effects of systemic treatment [$F(1,9)=43.50$, $p<0.01$] with no effects on intra-VTA treatment [$F(1,9)=0.74$, $p=0.41$] or treatment x intra-VTA treatment interaction [$F(1,9)=0.89$, $p=.37$]. Subsequent pairwise analyses indicated a significant difference in total active lever responding between intra-VTA vehicle/i.p. vehicle and intra-VTA CNO/i.p. vehicle versus intra-VTA

vehicle/i.p. Ex-4 and intra-VTA CNO/i.p. Ex-4 treated rats (Bonferroni, $p<0.05$). There was no effect of treatment on total inactive lever responding (data not shown). Corresponding microinjection sites in the VTA are shown in Figure 4.5H.

Activation of the endogenous NTS-to-LDTg pathway attenuates cocaine seeking

Thus far, our data identified important mechanisms that underly the role of exogenous LDTg GLP-1R activation on cocaine-seeking behavior. However, the role of endogenous GLP-1 signaling from the NTS to the LDTg in regulating cocaine seeking needs to be explored. The LDTg receives direct projections from the NTS (Cornwall et al., 1990; Rinaman, 2010; Reiner et al., 2018), however, the role of the NTS-to-LDTg pathway in addiction-like behaviors is not clear. Therefore, we determined if activation of NTS projections to the LDTg regulates cocaine seeking. In order to selectively activate the NTS-to-LDTg pathway, a retrogradely infecting virus CAV2 expressing Cre recombinase (CAV2-Cre) was infused in the LDTg and a Cre-dependent virus expressing the neural activating DREADD (AAV-DIO-hM4D(Gi)-mCherry) was infused into the NTS (Figure 4.6A). A representative image of hM3D(Gq)-expressing NTS-to-LDTg neurons is shown in Figure 4.6B. To validate that CNO activates hM3D(Gq)-expressing NTS neurons, immunohistochemistry was performed on NTS sections to label cFos after CNO injection in both hM3D(Gq)- and mCherry control-expressing rats (Figure 4.6C). Quantification revealed that CNO treatment significantly induced cFos in NTS hM3D(Gq)-expressing neurons (n=5) compared to mCherry-expressing neurons (n=6) [$t(9)=3.50$, $p<0.01$] (Figure 4.6C). Additionally, representative electrophysiological traces validating the ability of CNO to increase neuronal activity of NTS hM3D(Gq)-expressing neurons are shown in Figure 4.6D.

To asses if the NTS-to-LDTg pathway regulates cocaine seeking, CNO (0, 0.1, 1 mg/kg, i.p) was administered 30 min prior to a cocaine priming-induced reinstatement test session. Total active and inactive lever responses are shown in Figure 4.6E (n=8). These data were analyzed using a two-way repeated measures ANOVA, which revealed significant main effects of treatment [$F(2,14)=11.93$, $p<0.01$] and lever [$F(1,7)=21.21$, $p<0.01$] as well as a significant treatment x lever interaction [$F(2,14)=11.87$, $p<0.01$]. Subsequent pairwise analyses indicated that active lever responses were significantly different between vehicle and 1 mg/kg CNO (Bonferroni, $p<0.05$). These results indicate that activation of the NTS-to-LDTg circuit is sufficient to reduce cocaine-seeking behavior.

To validate that the effects of systemic CNO treatment on cocaine seeking were due to activating NTS hM3D(Gq)-expressing neurons and not due any off-target effects, a separate group of rats was infused with the control virus AAV-DIO-mCherry in the NTS and the same experimental procedures were performed as described above. Total active and inactive lever responses are shown in Figure 4.5F (n=7). These data were analyzed using a two-way repeated measures ANOVA, which revealed significant main effects of lever [$F(1,6)=17.70$, $p<0.01$] and no effect of treatment [$F(1,6)=0.24$, $p=0.64$] or treatment x lever interaction [$F(1,6)=0.44$, $p=0.53$]. Subsequent pairwise analyses indicated that there were no differences between vehicle and 1 mg/kg CNO on total active lever responses.

Activation of the NTS-to-LDTg circuit decreases cocaine seeking through a GLP-1-dependent manner.

The NTS is a heterogeneous population of neurons (Rinaman, 2010; Buffalari and Rinaman, 2014; Zheng et al., 2015), thus, it is unclear if GLP-1-producing neurons mediate the effects of NTS-to-LDTg activation on cocaine seeking. Through

immunohistochemical analysis, hM3D(Gq)-expressing NTS neurons that project to LDTg were found to colocalize with GLP-1 (Figure 4.6G). To test if reduced cocaine-seeking behavior following NTS-to-LDTg activation was GLP-1-mediated, rats were infused with CAV2-Cre in the LDTg, AAV-DIO-hM4D(Gi)-mCherry in the NTS, and implanted with guide cannula at the level of the LDTg. Prior to reinstatement test sessions, the GLP-1R antagonist Ex-9 (10 µg/100nl) was infused directly into the LDTg before administration of CNO (1 mg/kg, i.p.) to determine if pharmacological inhibition of LDTg GLP-1Rs blocked the ability of NTS-to-LDTg activation to decrease cocaine seeking. Using a within-subjects design, there were 4 treatment conditions (intra-LDTg vehicle/i.p. vehicle, intra-LDTg Ex-9/i.p. vehicle, intra-LDTg vehicle/i.p. CNO, intra-LDTg Ex-9/i.p. CNO; n=9/treatment). Total active lever responses are shown in Figure 4.6H. These data were analyzed with a two-way repeated measures ANOVA which revealed a significant systemic treatment x intra-LDTg treatment interaction [$F(1,8)=7.69$, $p<0.05$]. Subsequent pairwise analyses indicated a significant difference in total active lever responding between intra-LDTg vehicle/i.p. vehicle, intra-LDTg Ex-9/i.p. vehicle, and intra-LDTg Ex-9/i.p. CNO versus intra-LDTg vehicle/i.p. CNO treated rats (Bonferroni, $p<0.05$). There was no effect of treatment on total inactive lever responding (data not shown). Corresponding microinjection sites in the LDTg are shown in Figure 4.6I. Taken together, these data demonstrate that NTS GLP-1-producing neurons that project to the LDTg regulate cocaine-seeking behavior.

NTS-to-LDTg activation has no effect on food intake or body weight in cocaine-experienced rats.

Previous studies have shown that endogenous GLP-1R signaling in the LDTg and DREADD manipulation of NTS GLP-1-producing neurons alters feeding behavior in rats and mice (Gaykema et al., 2017; Liu et al., 2017; Shi et al., 2017; Reiner et al., 2018; Holt

et al., 2019). Therefore, we assessed if activation of NTS to LDTg projections caused any adverse effects on feeding and body weight in rats that underwent cocaine reinstatement. Cumulative chow intake and body weight were measured in rats that received vehicle or 1 mg/kg CNO treatment during cocaine reinstatement test sessions (Figure 4.7A&B; n=9). No effects from CNO treatment were observed at any time point. We also determined if CNO itself had any off-target effects on feeding by assessing 24 hr cumulative chow intake and body weight in rats that expressed control virus AAV-DIO-mCherry and underwent cocaine reinstatement. No effects from CNO treatment were observed (Figure 4.7C&D; n=7).

Discussion

The present study identified the GLP-1 system as a critical mechanism by which the LDTg acts to reduce cocaine seeking behavior. Specifically, we found that activation of LDTg GLP-1Rs via Ex-4 decreased cocaine seeking at a dose that had no effect on food intake or body weight in cocaine-experienced animals or sucrose seeking behavior in drug-naïve rats. Next, we found that GLP-1Rs are primarily expressed on GABA neurons in the LDTg. In addition, Ex-4 treatment significantly activated LDTg GABA neurons. Using GAD1-Cre rats, we selectively manipulated GLP-1Rs on LDTg GABAergic neurons to specifically test the role of this receptor population in cocaine self-administration and reinstatement. Knockdown of GLP-1R expression on LDTg GABA neurons augmented cocaine self-administration and prevented the ability of Ex-4 to decrease cocaine seeking. To investigate the GABAergic LDTg circuits that mediate Ex-4's effects on cocaine seeking, we chemogenetically inhibited LDTg-to-VTA GABAergic neurons which blocked the ability of Ex-4 to decrease cocaine seeking. These results highlight, for the first time, the role of the LDTg GABAergic system in regulating cocaine-seeking behavior. To continue to investigate the mechanisms by which GLP-1 signaling in the LDTg decreases cocaine seeking, we investigated the endogenous GLP-1 circuits that project to the LDTg. Using a dual virus approach, we selectively activated NTS afferents that project to the LDTg and assessed this circuit in controlling cocaine-seeking behavior. Our results showed that activation of the NTS-to-LDTg pathway attenuated cocaine seeking, was mediated by increased GLP-1R activation in the LDTg, and did not cause adverse feeding effects. Overall, these findings contribute to and expand upon previous studies demonstrating a role for central GLP-1Rs in addiction-like behaviors and highlight a novel role for hindbrain circuits in the regulation of cocaine-seeking behavior.

GLP-1R activation in the LDTg decreases cocaine seeking behavior

Within the mesolimbic reward system, the LDTg receives glutamatergic projections from the prefrontal cortex and sends direct projections to the VTA which are essential for the burst firing of dopamine neurons (Satoh and Fibiger, 1986; Sesack et al., 1989; Cornwall et al., 1990; Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Schmidt et al., 2009; Dautan et al., 2016). Thus, the LDTg is a key nucleus to study in the context of cocaine seeking. Indeed, studies have shown that cocaine exposure increases presynaptic glutamate release onto LDTg cholinergic neurons and facilitates LDTg stimulation-induced dopamine release to the NAc (Lester et al., 2010; Kurosawa et al., 2013). In addition, pharmacological blockade of excitatory signaling LDTg attenuates cocaine-mediated behaviors including cocaine seeking (Schmidt et al., 2009; Shabani et al., 2010; Shinohara et al., 2014). Therefore, studies examining mechanisms that can regulate the effects of excitatory transmission in the LDTg to promote cocaine-mediated behaviors may be critical in order to find potential targets for treating cocaine use disorder.

Here, we showed that activation of LDTg GLP-1Rs via intra-LDTg administration of Ex-4 (0.005 µg and 0.025 µg) significantly attenuated cocaine seeking. Due to the known effects of Ex-4 to cause reductions in food intake and nausea/malaise (Hayes et al., 2011a; Kanoski et al., 2012), it is important to control for any adverse effects in order to support the therapeutic efficacy of GLP-1R agonists in humans with cocaine use disorder. Previous studies have shown that intra-LDTg 0.025 µg Ex-4 decreases food intake and body weight in drug-naïve rats without causing any effects on nausea/malaise (Reiner et al., 2018). However, the effects of lower doses of Ex-4 on food intake and body weight have not been assessed. Consistent with previous findings, we observed that 0.025 µg Ex-4 in the LDTg decreased body weight in rats that underwent cocaine reinstatement. Additionally, we showed for the first time, that GLP-1R signaling in the LDTg reduces

responding for palatable foods as intra-LDTg Ex-4 (0.025 µg) attenuated sucrose seeking in drug-naïve rats. Conversely, we found that the 0.005 µg dose of intra-LDTg Ex-4 significantly decreased cocaine seeking without affecting body weight or sucrose seeking. Additional food intake experiments were conducted and revealed that 0.005 µg Ex-4 had no effects on cumulative chow intake in cocaine-experienced rats. Therefore, these findings identified a dose of intra-LDTg Ex-4 (0.005 µg) that selectively attenuated cocaine seeking without causing adverse feeding effects. These data are consistent with our previous studies showing that lower doses of systemically administered Ex-4 (0.1 and 0.2 µg/kg) are sufficient to decrease cocaine seeking and do not affect *ad libitum* food intake (cumulative chow intake, meal size or meal frequency) or body weight in cocaine-experienced rats (Hernandez et al., 2018). Similarly, our previous studies examining the effects of GLP-1R activation in the NAc and VTA show that intra-VTA and intra-NAc Ex-4 (0.005 µg and 0.05 µg) reduce cocaine seeking at doses that do not affect *ad libitum* chow intake or promote malaise-like effects in rats (Alhadeff et al., 2012; Dickson et al., 2012; Mietlicki-Baase et al., 2013; Hernandez et al., 2018; Hernandez et al., 2019). Moreover, intra-VTA or intra-NAc infusions of Ex-4 (0.05 µg) do not alter the reinstatement of sucrose seeking, which further support the selectivity of lower doses to reduce cocaine seeking (Hernandez et al., 2018; Hernandez et al., 2019). The translational implications of these findings are profound in that they support potential therapeutic approaches toward the specific use of GLP-1R agonists for the treatment of cocaine craving and relapse. These findings are also provocative in that they suggest that drug and non-drug motivated behaviors can be differentially modulated by GLP-1R activation in a cocaine-experienced animal.

Cocaine seeking is reduced by activation of GLP-1Rs on LDTg GABA neurons

The LDTg contains distinct populations of cholinergic, glutamatergic and GABAergic neurons (Wang and Morales, 2009; Luquin et al., 2018). However, the role of the LDTg in addiction has been largely attributed to the excitatory cholinergic projections and their ability to promote reward-related behaviors (Oakman et al., 1995; Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Mena-Segovia et al., 2008; Holmstrand and Sesack, 2011; Dautan et al., 2016; Xiao et al., 2016; Kaneda, 2018). The role of the other cell types in the LDTg to regulate addiction-like behaviors is largely unexplored. Therefore, we investigated the exact cell types that express GLP-1Rs in the LDTg in order to determine the mechanisms by which LDTg GLP-1R activation decreases cocaine seeking. Using FISH, we characterized the GLP-1R-expressing neuronal cell types in the LDTg and found that 70% were GABAergic neurons, 25% were glutamatergic neurons and 0% were cholinergic neurons. The 5% remaining GLP-1R-expressing cell types in the LDTg are most likely glial as there are no other neuronal cell-types in the LDTg and GLP-1Rs are known to be expressed on astrocytes and involved in energy balance control (Reiner et al., 2016). These findings, to our knowledge, are the first to characterize GLP-1R-expressing neurons in the LDTg and provide important insights on the mechanisms that underly the effects of GLP-1R activation on cocaine seeking. The GLP-1R is a G-protein coupled receptor known to interact predominately through the Gs subunit (Fletcher et al., 2016). Studies have shown that activation of GLP-1Rs increases intracellular calcium and cAMP, PKA and MAPK activation (Perfetti and Merkel, 2000; Gomez et al., 2002; Hayes et al., 2011b). Indeed, previous electrophysiological studies show that GLP-1R agonists enhances neuronal activity in the brain by both pre-synaptic effects on neurotransmitter release and of post-synaptic membrane excitability (Acuna-Goycolea and van den Pol, 2004; Mietlicki-Baase et al., 2013; Mietlicki-Baase et al., 2014; Korol et al., 2015b; Hernandez et al., 2019). Therefore, the effects of LDTg GLP-1R activation to attenuate

cocaine seeking are unlikely due to glutamatergic or cholinergic neurons as activation of these excitatory neurons in the LDTg to the VTA are rewarding and promote cocaine-mediated behaviors (Schmidt et al., 2009; Shabani et al., 2010; Lammel et al., 2012; Shinohara et al., 2014; Xiao et al., 2016; Steidl et al., 2017). Furthermore, although we saw a 25% colocalization of GLP-1Rs on LDTg glutamate neurons, glutamate release in the mesolimbic reward system is well established to promote cocaine-seeking behavior (Schmidt and Pierce, 2010), and since administration of Ex-4 in the LDTg reduced cocaine seeking, it is unlikely that these effects are due to glutamatergic neurons.

Due to this knowledge and the fact that we found the majority of GLP-1Rs expressed on LDTg GABAergic neurons, we hypothesized that the suppressive effects of LDTg GLP-1R activation on cocaine seeking are mediated by this cell type. Indeed, we showed that Ex-4 activated LDTg GABA neurons and that selectively knocking down GLP-1Rs in this neuronal population blocked the ability of both intra-LDTg Ex-4 and systemic Ex-4 to decrease cocaine seeking. These data show that GLP-1Rs on LDTg GABA neurons are necessary for Ex-4's effects on cocaine seeking. Additionally, we found that LDTg GABA GLP-1R knockdown augmented cocaine-self administration, indicating the endogenous relevance of GLP-1 signaling to LDTg GABA neurons in controlling cocaine-taking behavior. However, GLP-1R knockdown had no effect on cocaine-seeking behavior in vehicle-treated rats compared to controls. These findings may be due to a potential ceiling effect from the 10 mg/kg priming-dose of cocaine. Thus, lower priming-doses of cocaine may be needed to visualize differences in cocaine seeking compared to controls. Overall, our results show for the first time that LDTg GABA neurons play critical role in animal models of cocaine addiction and that both exogenous and endogenous GABAergic GLP-1R activation in the LDTg regulates cocaine-mediated behaviors.

The efficacy of Ex-4 in reducing cocaine seeking depends upon activation of GABAergic projections from the LDTg to the VTA.

In addition to establishing a role for GABAergic GLP-1Rs in the LDTg, we also investigated the cell type-specific circuits in the LDTg that that mediate the suppressive effects of Ex-4 on cocaine seeking. We chemogenetically inhibited LDTg GABA projections to the VTA upon administration of Ex-4 and found that the suppressive effects of Ex-4 on cocaine seeking were reversed when this pathway was inhibited. These data reveal an important mechanism of action of Ex-4 to decrease cocaine seeking but also highlight the relevance of LDTg-to-VTA GABA projections. Although 40-45% of neurons in the LDTg are GABAergic (Wang and Morales, 2009; Luquin et al., 2018), very little is known about the role of LDTg GABAergic circuits in motivated behaviors as the majority of studies focus solely on LDTg cholinergic neurons. To date, the existing literature consists of a study that identified presumed GABAergic LDTg synapses on VTA neurons (Omelchenko and Sesack, 2005) and a study who found that non-cell type-specific stimulation of LDTg neurons in rats resulted in a ~30% inhibition of VTA neuronal activity which included putative dopaminergic and GABAergic VTA neurons (Coimbra et al., 2017). Here, we showed that LDTg GABAergic fibers are localized in the VTA and associated with dopamine neurons. Our findings suggest that Ex-4 decreases cocaine seeking by inducing LDTg GABA-mediated inhibition of dopamine neurons. Support for this hypothesis comes from previous studies showing that systemic and ICV infusions of Ex-4 decreases cocaine-evoked dopamine release in the NAc (Egecioglu et al., 2013b; Sorensen et al., 2015; Fortin and Roitman, 2017). However, future studies are needed to directly assess if LDTg GABAergic neurons make synaptic contacts on to VTA dopamine neurons. Additionally, studies must continue to investigate the neural circuits activated by Ex-4 as other brain regions are also known to be involved in Ex-4's effects on cocaine seeking (Hernandez et al., 2018; Hernandez et al., 2019). Overall, we highlighted that the LDTg GABA system is

an important mechanism of action by which Ex-4 decreases cocaine seeking and the first to establish the relevance of LDTg-to-VTA GABAergic projections in regulating cocaine-seeking behavior.

Endogenous GLP-1-expressing NTS-to-LDTg circuits regulate cocaine-seeking behavior

While the current study revealed no effects of cocaine self-administration and abstinence on LDTg GLP-1R expression, our previously published findings have shown that cocaine taking and subsequent abstinence dynamically regulate expression of preproglucagon (PPG), the gene that encodes GLP-1, in the NTS. Specifically, cocaine self-administration increased endogenous PPG expression in the NTS (Hernandez et al., 2018). These data are consistent with findings that support the hypothesis that increased central GLP-1 signaling may serve as a 'brake' to reduce further cocaine consumption (Schmidt et al., 2016). In contrast, PPG expression in the NTS was significantly decreased following seven days of abstinence (Hernandez et al., 2018), a time point that coincides with robust drug-seeking behavior (Anderson et al., 2008; Schmidt et al., 2015b; Hernandez et al., 2019). These results suggest that decreased endogenous PPG expression in the NTS and reduced GLP-1 signaling in target nuclei during abstinence may facilitate cocaine craving and relapse. Therefore, we tested if chemogenetically activating NTS-to-LDTg circuits would attenuate cocaine-seeking behavior. Indeed, NTS-to-LDTg activation significantly decreased cocaine seeking and these effects were dependent on GLP-1R activation in the LDTg. Therefore, our data indicates that endogenous NTS GLP-1R signaling to the LDTg regulates cocaine-seeking behavior. It is important to note that NTS GLP-1-producing neurons are also known to co-release glutamate (Zheng et al., 2015). However, the observed suppressive effects on cocaine seeking following NTS-to-LDTg activation are unlikely due to increased glutamate release as blocking glutamate transmission in the LDTg attenuates cocaine-seeking behavior (Schmidt et al., 2009). Our

data also found that not all hM3D(Gq)-expressing NTS-to-LDTg neurons were GLP-1 positive. Thus, future studies should investigate if other NTS cell types, such as noradrenergic neurons, also play a role in regulating cocaine-seeking behavior as cocaine exposure has been shown to activate noradrenergic neurons in the NTS (Buffalari and Rinaman, 2014).

In addition to assessing NTS-to-LDTg activation on cocaine seeking, our data showed that activating NTS-to-LDTg circuits in cocaine-experienced rats had no effect on cumulative chow intake or body weight when measured after cocaine reinstatement test sessions. These data are consistent with previous studies that chemogenetically activated GLP-1-producing neurons in the NTS using glucagon (*Gcg*) promoter-driven Cre mice. These studies show that both global chemogenetic activation of NTS GLP-1-producing neurons and selective activation of NTS to the paraventricular hypothalamus circuit reduces chow intake in only the first 2-4 hrs with no effects on body weight or food intake at longer time points (Gaykema et al., 2017; Liu et al., 2017; Shi et al., 2017; Holt et al., 2019). Taken together, these findings show that the chow intake-suppressive effects of NTS circuit activation are transient and do not cause no long-term effects on feeding or body weight. Overall, to our knowledge, this study is the first to demonstrate that NTS GLP-1 circuits not only play a role in energy balance control but also function to regulate cocaine-mediated behaviors.

Conclusion

We report that the LDTg is a critical nucleus in regulating cocaine seeking through activation of the GLP-1 system. Our results show, for the first time, that GLP-1Rs on LDTg GABA neurons and LDTg-to-VTA GABAergic projections mediate the effects of Ex-4 on cocaine seeking. Additionally, we demonstrate that endogenous GLP-1 circuits from the

NTS are important regulators of cocaine craving-induced relapse. Thus, our findings support the ever growing literature that central GLP-1 system may be an essential target for treating cocaine abuse disorder.

Figures

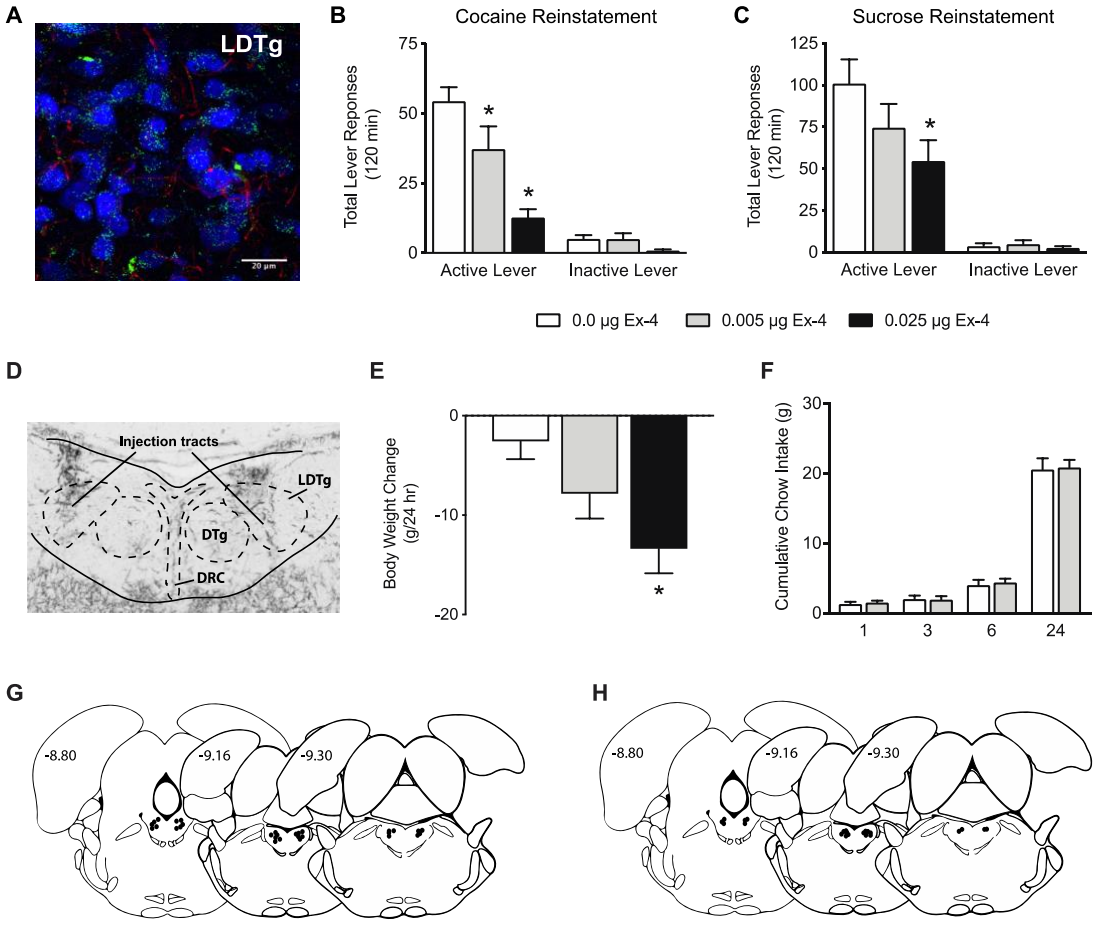

Figure 4.1 ***Intra-LDTg Ex-4 attenuates cocaine seeking at a dose that does not affect sucrose seeking, food intake or body weight.*** **(A)** 0.2 µg/kg fluoro-Ex-4 (green) colocalized with neurons (blue) and astrocytes (red) in the LDTg (63x). **(B)** Bilateral infusions of intra-LDTg Ex-4 (0.005 and 0.025 µg) significantly attenuated cocaine seeking (n=15). **(C)** Bilateral infusions of intra-LDTg Ex-4 at 0.025 µg significantly decreased sucrose seeking in drug naïve rats while 0.005 µg had no effect on sucrose seeking (n=11). **(D)** Representative histological section showing verification of LDTg cannula placement. **(E)** Intra-LDTg Ex-4 (0.005 µg) had no effect on body weight while 0.025 µg Ex-4 significantly reduced body weight in rats that underwent cocaine reinstatement (n=15). **(F)** 0.005 µg intra-LDTg Ex-4 had no effect on chow intake in rats that underwent cocaine reinstatement (n=7). **(G)** Microinjection sites corresponding to the cocaine reinstatement group in B. **(H)** Microinjection sites corresponding to the sucrose reinstatement group in C. Data are mean ± SEM. **p*<0.05

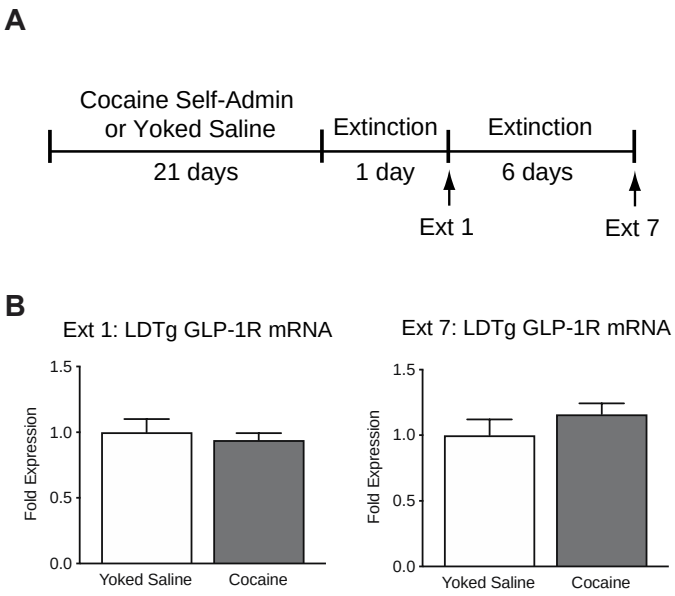

Figure 4.2 ***Intra-LDTg Ex-4 attenuates cocaine seeking at a dose that does not affect sucrose seeking, food intake or body weight***
(A) Expression of GLP-1R mRNA transcripts in the LDTg were quantified following one (Ext1) and seven (Ext7) days of extinction following cocaine self-administration. **(B)** There were no differences in LDTg GLP-1R mRNA expression after one (n=7) or 7 days (n=9) of extinction compared to controls. Data are mean ± SEM

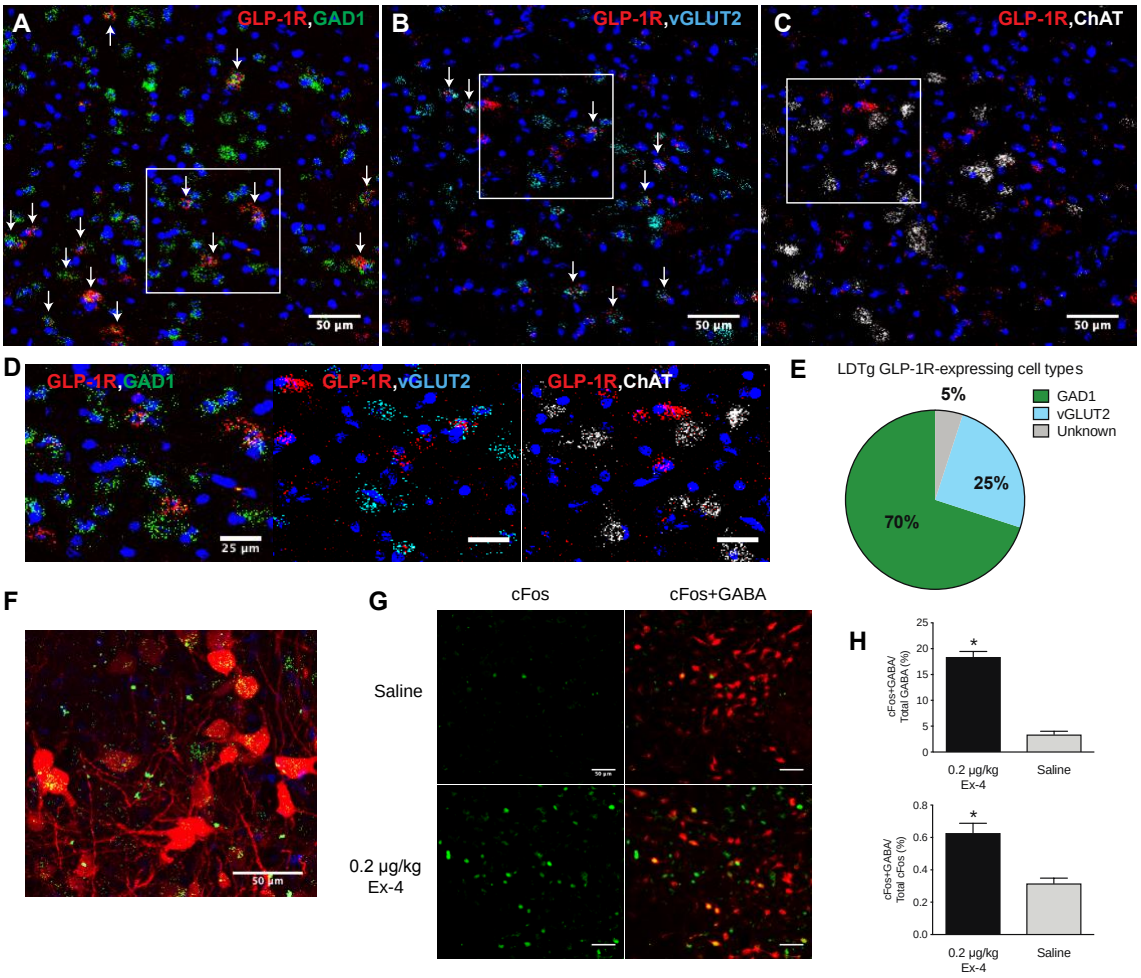

Figure 4.3 ***GLP-1Rs are primarily expressed on GABA neurons in the LDTg.*** Representative 40x images of FISH in the LDTg with GLP1R in red and DAPI in blue with GAD1 **(A),** vGLUT2 **(B)**, and ChAT **(C)**. White arrows indicate colocalization with GLP-1R. White boxes indicate zoomed-in images shown in **(D). (E)** Quantification of GLP-1R colocalization with different cell types. **(F)** 63x image of 0.2 µg/kg fluoro-Ex-4 (green) colocalized with GABA neurons (red). **(G)** 0.2 µg/kg Ex-4 induces cFos (green) in LDTg GABA neurons (red), taken at 20x and quantified in **(H)** (n=4/treatment). Data are mean ± SEM. **p*<0.05

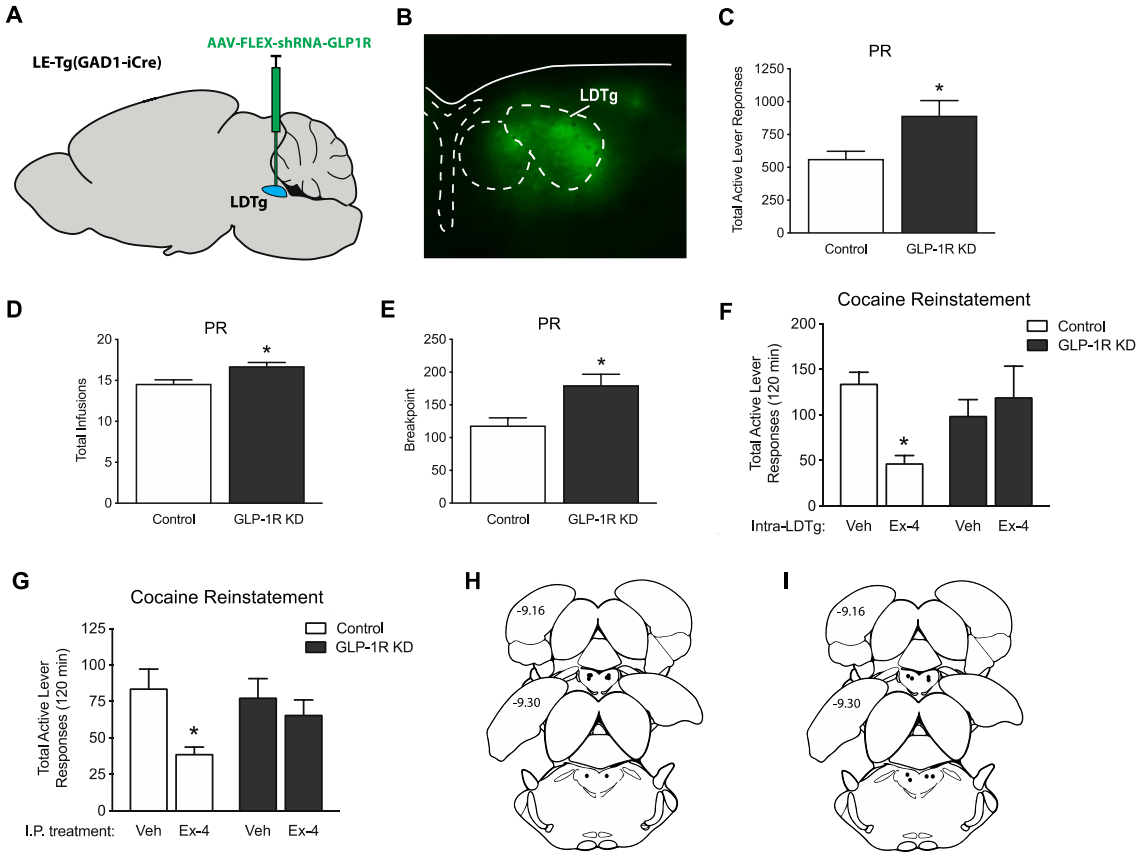

Figure 4.4 ***Knockdown of GABA GLP-1 receptors in the LDTg augments cocaine self-administration and prevents the ability of Ex-4 to reduce cocaine seeking.*** **(A)** Bilateral infusions of a Cre-dependent AAV1 expressing shRNA against the GLP-1R or control virus was administered in the LDTg of GAD1-Cre rats. **(B)** Visualization of virus expression in the LDTg at 10x. Knockdown (KD) of LDTg GABA GLP-1Rs significantly increased total active lever responses **(C),** total infusions **(D),** and breakpoint **(E)** (n=10) for cocaine a progressive ratio (PR) schedule of reinforcement compared to rats infused with control virus (n=12). Knockdown of GABA GLP-1Rs blocked the ability of intra-LDTg 0.005 µg Ex-4 (n=4/group) **(F)** and 0.2 µg/kg i.p. Ex-4 (control: n=8; GLP-1R KD: n=11) **(G)** to reduce cocaine seeking. Microinjection sights corresponding to Control **(H)** and GLP-1R KD **(I)** groups shown in F. Data are mean ± SEM. **p*<0.05

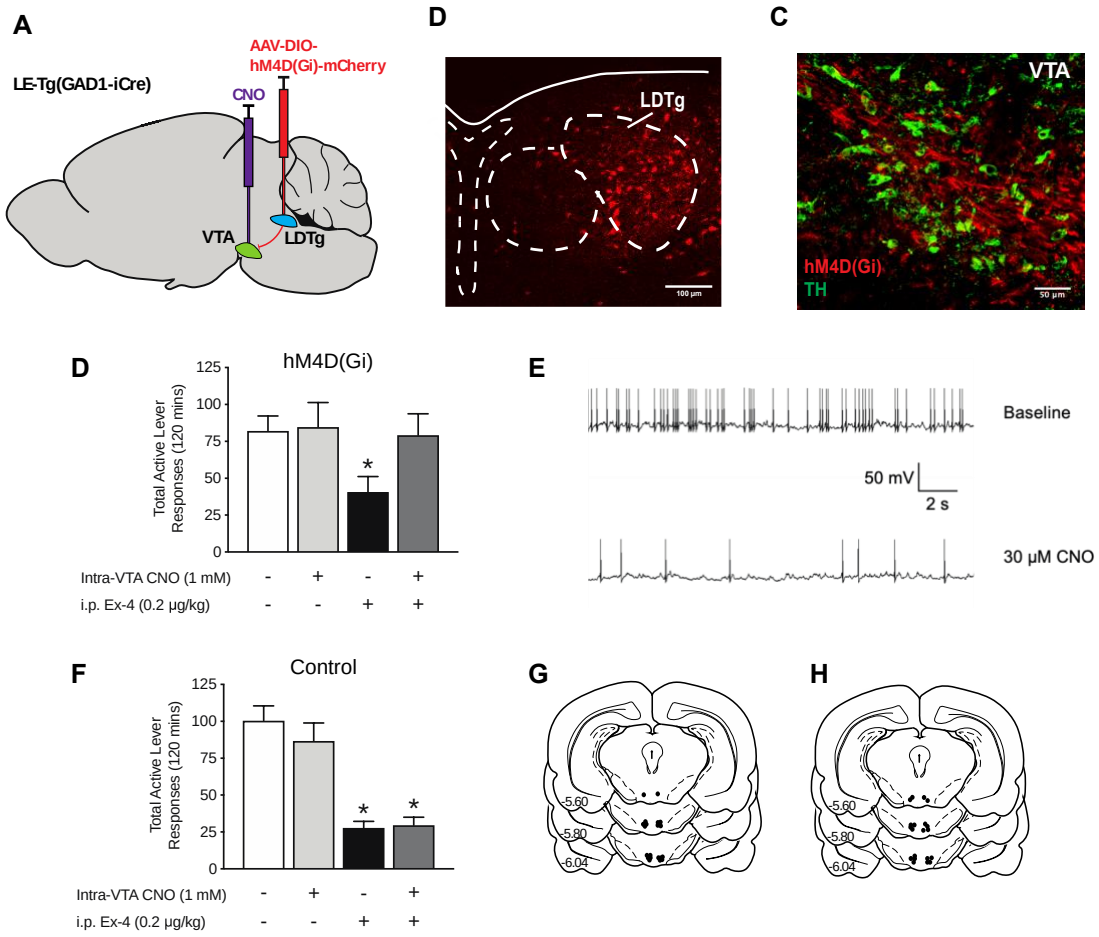

Figure 4.5 ***LDTg GABA neurons projecting to the VTA mediate the ability of Ex-4 to reduce cocaine seeking***. **(A)** GAD1-Cre rats received bilateral infusions of a Cre-dependent AAV2 expressing the inhibitory DREADD hM4D(Gi) or control virus in the LDTg. Guide cannula were inserted at the level of the VTA for CNO administration. **(B)** Visualization of hM4D(Gi) expression in LDTg GABA neurons at 10x. **(C)** LDTg GABA hM4D(Gi)-expressing terminals (red) localized in the VTA with dopamine neurons (green). **(D)** Inhibition of LDTg GABA terminals in the VTA via intra-VTA CNO infusions prevented the ability of Ex-4 (0.2 µg/kg, i.p.) to reduce cocaine seeking (n=10). **(E)** Electrophysiological recordings validating the ability of CNO to decrease neuronal activity in LDTg hM4D(Gi)-expressing neurons. **(F)** There was no effect of intra-VTA CNO on the attenuation of cocaine seeking from Ex-4 treatment in rats that were infused with control virus (n=10). **(G)** Microinjection sites corresponding to rats in D. **(H)** Microinjection sites corresponding to the rats in F. Data are mean ± SEM. *p<0.05

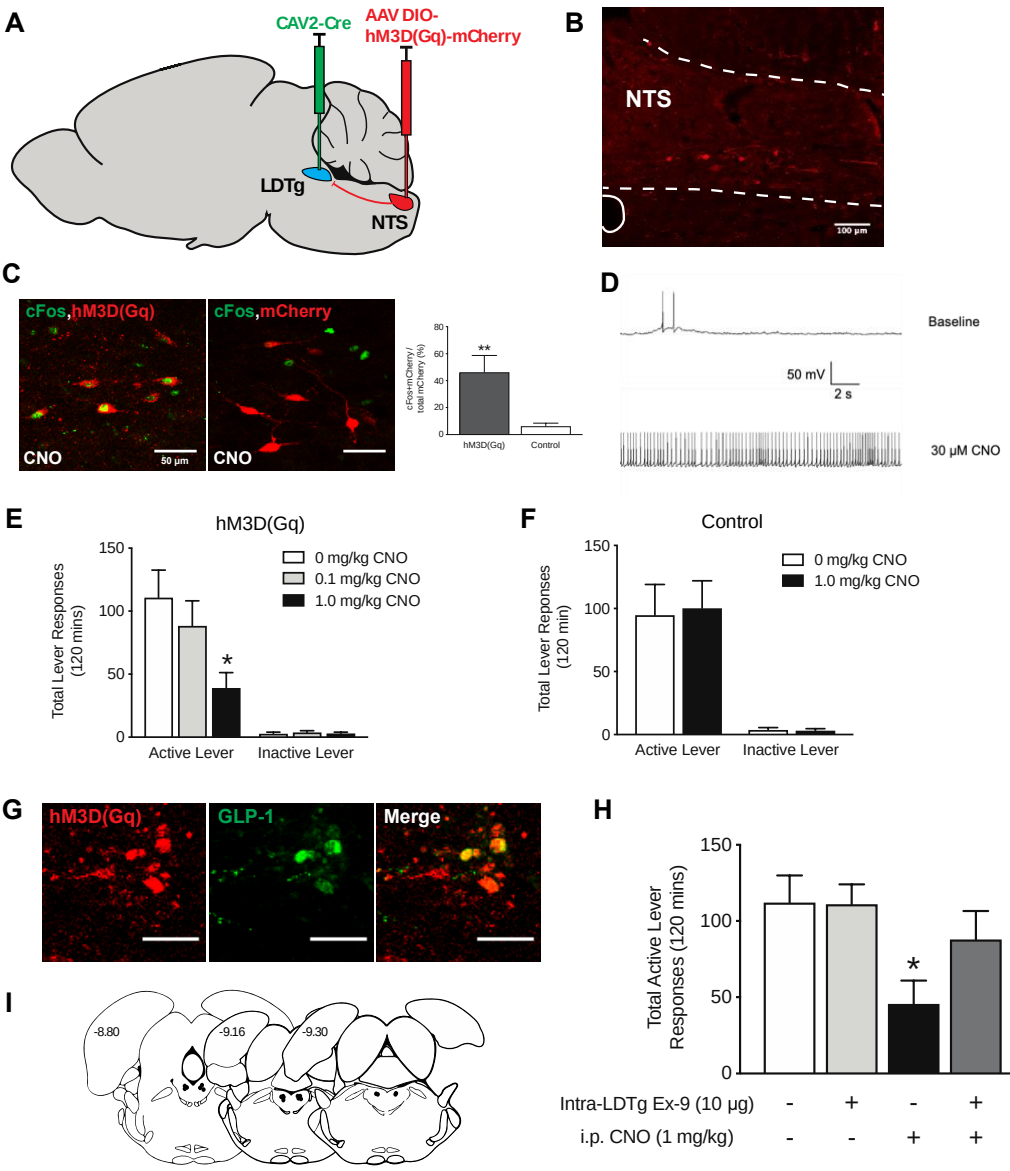

Figure 4.6 ***NTS to LDTg circuit activation attenuates cocaine seeking.*** **(A)** Bilateral infusions of CAV2-Cre and Cre-dependent AAV2 expressing the activating DREADD hM3D(Gq) were administered in the LDTg and NTS, respectively **(B)** Visualization of hM3D(Gq) expression in NTS neurons that project to the LDTg taken at 20x. **(C)** Representative images and quantification of CNO inducing cFos in NTS hM3D(Gq)-expressing neurons compared to rats expressing control virus (hM3D(Gq): n=5; control: n=6). **(D)** Electrophysiological recordings validating the ability of CNO to increase neuronal firing in NTS hM3D(Gq)-expressing neurons. **(E)** 1 mg/kg, i.p. CNO administration in rats to activate NTS to LDTg circuits significantly attenuated cocaine seeking (n=8). **(F)** CNO treatment had no effect on cocaine seeking in rats infused with control virus (n=7). **(G)** Representative image of NTS hM3D(Gq)-expressing neurons co-expressing GLP-1. **(H)** Pharmacologically inhibiting LDTg GLP-1Rs via Ex-9 blocked the effect of NTS to LDTg circuit activation on cocaine seeking (n=9). **(I)** Microinjection sights in the LDTg corresponding to H. Data are mean ± SEM. **p*<0.05

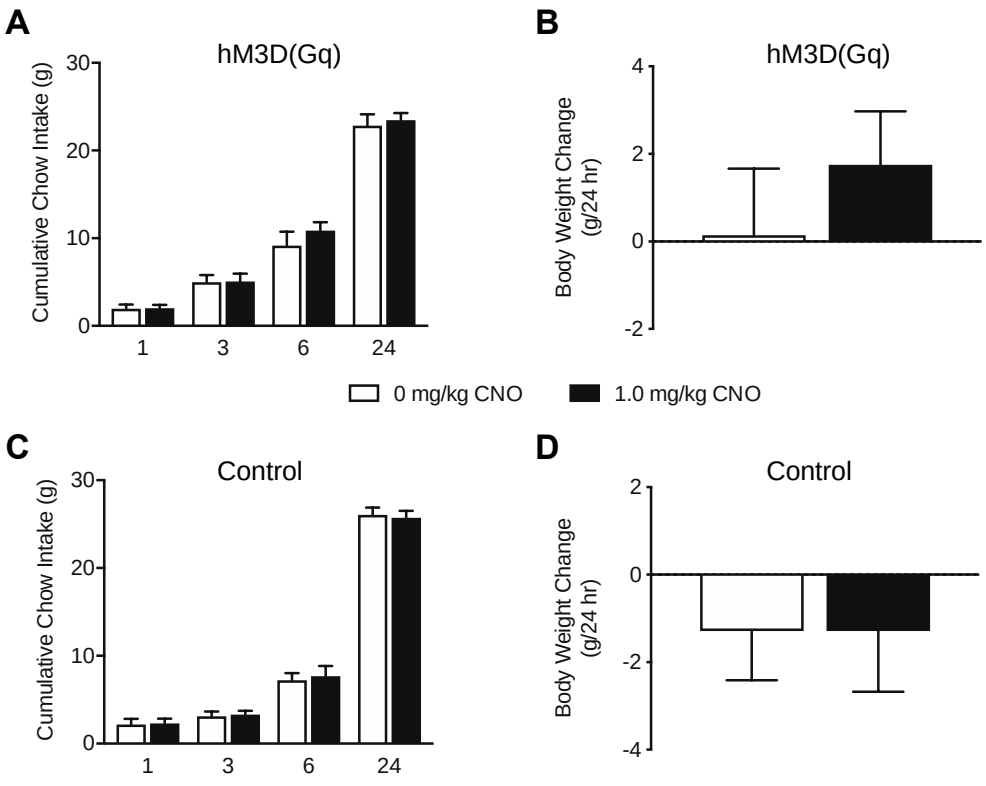

Figure 4.7 ***NTS to LDTg circuit activation has no effect on chow intake or body weight in cocaine-experienced rats.*** There was no effect of CNO treatment on cumulative chow intake **(A)** or 24 hr body weight **(B)** in rats expressing hM3D(Gq) (n=9) or control virus **(C&D)** (n=6) that underwent cocaine reinstatement test sessions. Data are mean ± SEM.

CHAPTER 5: General Discussion and Conclusion

There is a critical need for preclinical research to identify key neurobiological systems that can be targeted by novel pharmacotherapies for treating cocaine use disorder. A promising target is the central GLP-1 system and identifying the mechanisms and sites of action of GLP-1 and its clinically relevant receptor agonists is paramount in approving GLP-1 analogs for treating cocaine use disorder. The data presented in this dissertation provides evidence that GLP-1Rs expressed in nuclei of the mesolimbic reward system, VTA, NAc and LDTg, are important for regulating cocaine-seeking behavior. Additionally, this dissertation shows that endogenous GLP-1 circuits from the NTS of the brainstem function to reduce cocaine seeking. As a whole these data broaden our view of the central GLP-1 system as well as the neural circuits that regulate addiction-like behaviors. In this chapter, I summarize and discuss key findings and propose future avenues of research.

The Role of Systemic Ex-4 on Cocaine Seeking

Previous studies have shown that systemic administration of Ex-4 (2.4 – 100.0 µg/kg) reduces cocaine CPP and self-administration in mice (Egecioglu et al., 2013b; Graham et al., 2013; Sorensen et al., 2015). However, a notable caveat to these studies is the exceedingly high doses of Ex-4 used, which have been shown to produce nausea and malaise-like symptoms in addition to suppressing locomotion. Doses of Ex-4 as low as 0.3 µg/kg suppress locomotor activity in both mice and rats (Talsania et al., 2005; Mack et al., 2006; Sorensen et al., 2015) and doses of Ex-4 as low as 0.25 µg/kg produce nausea/malaise in rodents (Kanoski et al., 2012). These findings clearly indicate that doses of Ex-4 greater than 0.25 µg/kg produce malaise-like adverse effects. Additionally, nausea is associated with high doses of GLP-1R agonists in humans (Buse et al., 2009). Since the high doses of Ex-4 (2.4 – 100.0 µg/kg) are likely producing malaise-like effects

in rodents, it is impossible to draw firm conclusions about the exact role of GLP-1Rs in addiction-like behaviors from the previous studies

The data in Chapter 2 found that systemic administration of Ex-4 (0.1 and 0.2 µg/kg, i.p.) significantly attenuated the reinstatement of drug-seeking behavior elicited by both a priming injection of cocaine and re-exposure to conditioned cues previously paired with cocaine taking. Importantly, these doses of Ex-4 did not affect *ad libitum* food intake (cumulative chow intake, meal size or meal frequency) or body weight in cocaine-experienced rats. Moreover, these behaviorally-relevant doses of Ex-4 do not produce nausea/malaise-like adverse effects in rodents (Kanoski et al., 2012) further highlighting the selectivity of this behavioral response at these doses. This is the first study to our knowledge identifying systemic doses of a GLP-1R agonist that selectively attenuate cocaine-mediated behaviors and do not produce adverse effects commonly associated with higher doses in rodents. The translational implications of these findings are profound in that they support potential therapeutic approaches toward the specific use of GLP-1R agonists for the treatment of cocaine craving and relapse. However, future studies are also needed to assess the efficacy of repeated administration of GLP-1R agonists on voluntary cocaine taking and seeking as studies to date have only assessed the acute effects of Ex-4 on these behaviors. It will be important to know if tolerance and/or adverse effects develop to repeated administration of GLP-1R agonists. Furthermore, no studies to date have examined potential sex differences in the efficacy of GLP-1R agonists to reduce cocaine-mediated behaviors. Recent evidence indicates that females are more sensitive to the effects of a GLP-1R agonist infused directly into the brain on food intake (Richard et al., 2016). It is provocative to think that the potency of GLP-1R agonists may differ between sexes in preclinical models of addiction. In addition to Ex-4, preclinical studies should also test the efficacy of other GLP-1 system-targeted drugs with different

pharmacokinetic profiles (e.g., liraglutide) and pharmacological mechanisms of action (e.g., sitagliptin) in reducing cocaine-taking and -seeking behaviors. Overall, more research is still needed to prove the therapeutic efficacy of GLP-1R agonists to treat cocaine use disorder.

The Role of VTA and NAc GLP-1Rs in Cocaine Seeking

The data in Chapter 2 showed that VTA GLP-1Rs play an important role in the reinstatement of cocaine-seeking behavior. Systemic doses of Ex-4 that selectively attenuated cocaine reinstatement crossed the blood brain barrier and bound putative GLP-1Rs located on neurons and astrocytes throughout the brain including the VTA, which suggested that the suppressive effects of peripheral Ex-4 on cocaine seeking are due, in part, to activation of VTA GLP-1Rs. Pharmacological inhibition of GLP-1Rs in the VTA was sufficient to attenuate the efficacy of systemic Ex-4 to reduce cocaine reinstatement providing further support for this hypothesis. More direct evidence supporting a critical role of central GLP-1Rs in cocaine seeking was provided in Chapter 2 by showing that infusions of Ex-4 (0.05 µg) directly into the VTA significantly decreased cocaine seeking. Overall, these findings clearly indicate that activation of VTA GLP-1Rs is sufficient to reduce cocaine-seeking behavior. Moreover, intra-VTA infusion of Ex-4 (0.05 µg) did not alter the reinstatement of sucrose seeking supporting the selectivity of this dose to reduce cocaine seeking. The dose of intra-VTA Ex-4 (0.05 µg) used in this study reduced cocaine seeking and has been shown to have no effects on *ad libitum* chow intake or promote malaise-like effects in rats (Alhadeff et al., 2012; Dickson et al., 2012; Mietlicki-Baase et al., 2013). There is some evidence that administration of 0.05 µg Ex-4 into the VTA reduced operant responding for palatable food (Dickson et al., 2012). However, these effects were transient and did not persist with more prolonged operant sessions (Dickson et al., 2012; Schmidt et al., 2016). Overall, Chapter 2 identified an intra-VTA dose of Ex-4

that selectively reduce seeking-behaviors in rats and not produce adverse feeding or malaise-like effects.

While activation of GLP-1Rs in the VTA attenuates cocaine seeking, the neurophysiological mechanisms underlying these behavioral responses are not clear. Previous studies have shown that GLP-1R agonists modulate neuronal activity in the brain by both pre-synaptic effects on neurotransmitter release and regulation of post-synaptic membrane excitability (Acuna-Goycolea and van den Pol, 2004; Mietlicki-Baase et al., 2014; Korol et al., 2015b). In drug-naïve mice, Ex-4 reduces AMPA receptor-mediated EPSCs in VTA dopamine neurons that project to the NAc shell (Wang et al., 2015). AMPA/NMDA EPSC ratio in the VTA is also decreased following Ex-4 application suggesting a postsynaptic modification that functions to reduce excitatory synaptic strength via removal of AMPA receptors from the postsynaptic membrane (Wang et al., 2015). Interestingly, Ex-4 also increases amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) in VTA dopamine neurons (Wang et al., 2015). These results indicate that GLP-1R activation modulates both excitatory and inhibitory transmission in VTA dopamine neurons that project to the NAc. It is interesting to think that similar mechanisms (i.e., decreased excitatory synaptic strength and/or facilitation of inhibitory synaptic inputs in VTA dopamine neurons) may mediate the effects of GLP-1R activation in the VTA on cocaine taking and seeking. Indeed, data in Chapter 4 demonstrated the relevance of VTA-projecting GABAergic terminals from the LDTg in mediating the effects of Ex-4 on cocaine seeking. Thus, future electrophysiological studies in the VTA are needed to investigate the pre- and post-synaptic mechanisms induced by Ex-4 in cocaine-experienced animals. Additional studies investigating the cell types in the VTA that express GLP-1Rs are also necessary to determine the mechanisms that underly VTA GLP-1R activation on cocaine-mediated behaviors. As data in Chapter 4 showed the

importance of GABAergic neurons in mediating the effects of LDTg GLP-1R activation on cocaine seeking, future experiments should investigate if VTA GABAergic interneurons express GLP-1Rs. A potential hypothesis is that Ex-4 decreases cocaine-mediated behaviors through activation GLP-1Rs on VTA GABAergic interneurons to reduce dopamine cell firing and subsequent dopamine release to forebrain areas.

Previous studies have begun to investigate the effects of Ex-4 on dopamine release, particularly in the NAc. Initial studies show that systemic infusions of Ex-4 reduces cocaine-evoked dopamine release in the NAc (Egecioglu et al., 2013b; Sorensen et al., 2015). However, the relative contributions of peripheral and central GLP-1Rs to these neurochemical changes is not clear. A recent study investigated the role of central GLP-1Rs in attenuating cocaine-evoked dopamine release in the NAc core and shell subregions using *in vivo* fast-scan cyclic voltammetry. Intracerebroventricular (ICV) infusion of Ex-4 attenuated phasic dopamine release in the NAc core, but not the shell, of rats receiving non-contingent infusions of cocaine (Fortin and Roitman, 2017). To investigate the mechanisms by which Ex-4 reduces cocaine-evoked dopamine release in the NAc core, electrical stimulation of the VTA was performed in rats receiving non-contingent infusions of cocaine. Ex-4 did not affect the ability of cocaine to attenuate dopamine uptake, which suggests that GLP-1R activation exerts its suppressive effects on cocaine-induced dopamine release in the NAc core by reducing the excitability of midbrain dopamine neurons (Fortin and Roitman, 2017). While these findings are the first to highlight a role for central GLP-1Rs in suppression of cocaine-evoked dopamine release in the NAc (Fortin and Roitman, 2017), one limitation of this study is that ICV delivery of Ex-4 causes broad GLP-1R activation throughout the brain. Therefore, it is difficult to delineate the exact brain regions mediating the neurochemical and neurophysiological effects of Ex-4. Also, while Ex-4 attenuates cocaine self-administration (Sorensen et al.,

2015; Schmidt et al., 2016), it is not clear whether Ex-4 suppresses conditioned phasic dopamine release aligned with cocaine-predictive cues and operant responding observed during voluntary cocaine taking (Phillips et al., 2003; Stuber et al., 2005). Additionally, very little is known about how activation of central GLP-1Rs affects phasic versus tonic dopamine cell firing. Thus, our understanding of how central GLP-1R activation influences the mesolimbic dopamine system to reduce addiction-like behaviors would benefit from future electrophysiological studies delineating these effects.

Chapter 3 directly tests the role of GLP-1Rs in the NAc to regulate cocaine-seeking behavior. The data in Chapter 3 show that, in addition to binding putative GLP-1Rs in the VTA, doses of systemic Ex-4 that attenuate cocaine seeking also distribute to the core and shell subregions of the NAc. These functionally distinct subregions are known to play differential roles in motivated behaviors including drug addiction (Di Chiara, 2002). The NAc shell, which is classified as part of the limbic system, is implicated in the primary rewarding effects of drugs of abuse as well as regulating instrumental responding in the presence of motivationally relevant stimuli (Schmidt and Pierce, 2010). Alternatively, the NAc core, which is considered part of the basal ganglia, mediates the incentive value of reward-conditioned stimuli and contributes to drug-associated, cue-induced cocaine seeking (Schmidt and Pierce, 2010). In Chapter 3, infusions of Ex-4 directly into the NAc core and shell were sufficient to attenuate cocaine seeking. Interestingly, the potency of Ex-4 differed between subregions. Cocaine-seeking behavior was decreased in rats pretreated with 0.05 µg Ex-4 in both the NAc core and shell. In contrast, 0.005 µg Ex-4 significantly attenuated cocaine seeking when infused directly into the NAc core, but not shell. Previous studies have also demonstrated differential effects of GLP-1R agonist doses in the shell and core subregions in decreasing food intake (Dossat et al., 2011; Alhadeff et al., 2012). Lower doses of Ex-4 reduced intake of palatable food when infused

into the NAc core compared to the NAc shell (Alhadeff et al., 2012). The lowest effective dose of Ex-4 used in this study was 0.025 µg, which produced a transient effect on food intake following activation of GLP-1Rs in the NAc core and no effect on food intake when infused into the NAc shell (Alhadeff et al., 2012). Collectively, these findings suggest that GLP-1 signaling in the NAc core may play a more prominent role in regulating drug-seeking behavior and hedonic feeding. A potential explanation may be that there is differential expression of GLP-1Rs in the shell vs core, with a possibility of more GLP-1Rs in the NAc core. Thus, quantification of GLP-1R-expressing cells in these two sub-regions should be conducted.

In contrast to the effects intra-NAc Ex-4 on cocaine seeking, 0.05 µg Ex-4 in the NAc core and shell had no effects on sucrose seeking. Although administration of Ex-4 directly into the NAc shell reduced sucrose self-administration on a progressive ratio schedule of reinforcement (Dickson et al., 2012), doses of Ex-4 lower than 0.1 µg when infused into the NAc shell or core do not affect operant responding for sucrose, *ad libitum* chow intake, or produce adverse malaise-like effects (Alhadeff et al., 2012; Dickson et al., 2012; Mietlicki-Baase et al., 2014). Therefore, Ex-4 infusions into the NAc core (0.05 and 0.005 µg) and shell (0.05 µg) of cocaine-experienced rats selectively reduced cocaine seeking and did not affect food intake.

In order to investigate the neurophysiological mechanisms by which GLP-1R activation in the NAc attenuates cocaine-mediated behaviors, Chapter 3 employed *ex vivo* electrophysiological studies to elucidate the effects of GLP-1R activation on neuronal activity in a cocaine-exposed brain. Specifically, we assessed the effects of Ex-4 on activity of medium spiny neurons (MSNs) in the NAc of cocaine-experienced rats. Following extinction of cocaine self-administration, application of 1 µM exendin-4

increased the frequency of MSN action potential firing in the NAc core and shell. No other measures of membrane excitability (e.g., resting membrane potential, rheobase, action potential amplitude and action potential duration) were altered by GLP-1R activation in *ex vivo* striatal slices from cocaine-experienced rats. In contrast to previous studies showing presynaptic effects of GLP-1R activation in the VTA and NAc core of drug-naïve rats (Mietlicki-Baase et al., 2013; Mietlicki-Baase et al., 2014), Ex-4 had no effects on spontaneous excitatory post-synaptic currents (sEPSCs) frequency and paired pulse ratio (PPR) of evoked EPSCs in NAc core and shell MSNs from cocaine-experienced rats. Taken together, the results in Chapter 3 indicate that increased activation of GLP-1Rs in the NAc during cocaine abstinence increases intrinsic, but not synaptic, excitability of MSNs and is sufficient to reduce the reinstatement of cocaine-seeking behavior. It should be noted that these results are limited to activity exclusively within the NAc, as these studies were conducted in NAc slices without input from VTA cell bodies. The effects of GLP-1R activation on NAc MSN activity should be further explored in an *in vivo* model, as it has been shown that input from the VTA to the NAc impacts dopamine signaling dynamics. Specifically, phasic dopamine release in the NAc is dependent on burst firing of VTA dopamine neurons (Sombers et al., 2009). Moreover, studies utilizing amphetamine have highlighted the importance of VTA input on NAc dopamine release patterns. While dopamine efflux is preserved in *ex vivo* slices treated with amphetamine (Jones et al., 1998), phasic dopamine release is only observed in *in vivo* studies with preserved VTA inputs to the NAc (Covey et al., 2016). Thus, active VTA input into the NAc may be required for a more accurate picture of how GLP-1R activation modulates NAc MSN activity in cocaine-experienced animals.

An interesting alternative hypothesis is that VTA input may not be necessary to stimulate dopamine release in the NAc. Recent studies have revealed that cholinergic interneurons

in the NAc are capable of stimulating dopamine release and that these effects are regulated, in part, by the endocannabinoid system and prefrontal cortical afferents to the NAc (Cachope et al., 2012; Cachope and Cheer, 2014; Mateo et al., 2017). Since GLP-1R activation has been shown to modulate both endocannabinoid signaling and presynaptic glutamate release (Mietlicki-Baase et al., 2014; Reddy et al., 2016), it is possible that Ex-4 may influence cholinergic interneuron signaling in the NAc via these systems or by direct action on interneurons themselves. Further support for cholinergic interneurons playing a role in modulating the effects of metabolic factors on striatal dopamine levels and reward comes from a recent study showing that insulin increases striatal dopamine release by activating cholinergic interneurons in the NAc (Stouffer et al., 2015). Thus, future studies are needed to determine if GLP-1Rs are expressed on NAc interneurons and if the effects of Ex-4 on MSN activity are due to activation of cholinergic interneurons alone or require VTA input as discussed above.

GLP-1R activation increases MSN intrinsic excitability in cocaine-experienced rats

Cocaine abstinence is associated with decreased NAc MSN membrane excitability due to changes in conductance of sodium, calcium and potassium (Zhang et al., 1998; Zhang et al., 2002; Hu et al., 2004). Lower membrane excitability during cocaine abstinence may enhance action potential sensitivity to GLP-1R activation in the NAc. Cocaine exposure is likely a critical component in this scenario, given the previous findings of minimal effects of Ex-4 on NAc MSN firing in drug-naïve rats (Mietlicki-Baase et al., 2014) combined with data in Chapter 3 showing that increased frequency of MSN action potential firing in the NAc core and shell in cocaine-experienced rats. In line with this hypothesis, a recent study showed that Ex-4 suppressed cocaine-evoked phasic release of dopamine in the NAc core, effects not due to changes in dopamine reuptake (Fortin and Roitman, 2017). These neurophysiological changes, however, may not be specific to the NAc as Ex-4

administration was also associated with reduced cocaine-evoked dopamine release in the lateral septum (Harasta et al., 2015; Reddy et al., 2016). Understanding the mechanisms by which GLP-1R activation in the NAc reduces cocaine seeking will benefit from a comprehensive analysis of how Ex-4 regulates integration of synaptic and intrinsic MSN excitability in the context of drug priming-induced reinstatement. Thus, intrinsic GLP-1R-coupled mechanisms may contribute to previous findings that a broad increase in NAc MSN action potential output is associated with attenuation of rewarding consummatory behaviors (Nicola et al., 2004; Wheeler and Carelli, 2009). Additionally, studies show that decreased NAc MSN membrane excitability from cocaine withdrawal induces synaptic accumulation of calcium-permeable AMPA receptors and upregulation of excitatory synaptic strength which promotes cocaine seeking after abstinence (Wang et al., 2018). Indeed, disruption of this synaptic dysregulation after cocaine exposure prevents incubation of cocaine craving (Wang et al., 2018). Thus, increased membrane excitability in NAc MSNs following Ex-4 treatment may attenuate cocaine seeking by blocking enhanced excitatory synaptic drive onto NAc MSNs following a priming injection of cocaine.

The Role of LDTg GLP-1Rs in Cocaine Seeking

The data in Chapter 4 showed that the LDTg is an important nucleus in which GLP-1R activation regulates cocaine-seeking behavior. Systemic Ex-4 bound to putative GLP-1Rs located on neurons and astrocytes in the LDTg, which suggested that the suppressive effects of peripheral Ex-4 on cocaine seeking are due, in part, to activation of LDTg GLP-1Rs. Indeed, infusions of Ex-4 (0.005 and 0.025 µg) directly into the LDTg significantly decreased cocaine seeking. Overall, these findings indicate that activation of LDTg GLP-1Rs is sufficient to reduce cocaine-seeking behavior. Moreover, intra-LDTg infusion of Ex-4 (0.005 µg) did not alter *ad libitum* feeding or body weight in rats that underwent cocaine

reinstatement nor altered the reinstatement of sucrose seeking supporting the selectivity of this dose to reduce cocaine seeking. Taken together with data in Chapter 2 and 3 that identified behaviorally-selective systemic, intra-VTA and intra-NAc doses of Ex-4 that reduced cocaine seeking, these findings further strengthen evidence supporting the efficacy of lower doses of GLP-1R agonists to selectively attenuate cocaine seeking versus non-drug motivated behaviors.

The experiments in Chapter 4 set out to determine the mechanisms by which LDTg GLP-1R activation decreased cocaine-seeking behavior, first, by characterizing the exact cell types that express GLP-1Rs in the LDTg. The GLP-1R-expressing neuronal cell types in the LDTg were found to be 70% GABAergic, 25% glutamatergic and 0% cholinergic. The 5% remaining GLP-1R-expressing cell types in the LDTg are most likely glial cells as there are no other neuronal cell-types in the LDTg and GLP-1Rs are known to be expressed on astrocytes and involved in energy balance control (Reiner et al., 2016). It is unlikely that the cocaine seeking suppressive effects of intra-LDTg Ex-4 were due to activation of GLP-1Rs on LDTg glutamate neurons, as glutamate release in the mesolimbic reward system such as the VTA is well established to promote cocaine-seeking behavior (Sun et al., 2005; Schmidt et al., 2009; Schmidt and Pierce, 2010). Similarly, for GLP-1R-expressing astrocytes in the LDTg, GLP-1R activation increases PKA (Hayes et al., 2011b; Rupprecht et al., 2013), and PKA is known to contribute to the decrease of glutamate transporters on astrocytes after morphine exposure (Lim et al., 2005). Thus, a GLP-1R-mediated decrease in glutamate transporter expression would result in an increase in synaptic glutamate and promote cocaine seeking. Support for this hypothesis comes from studies showing that cocaine exposure alters glutamate reuptake in astrocytes to increase synaptic glutamate levels that act to promote cocaine-mediated behaviors (Kalivas, 2009). Additionally, delivery of a glutamate receptor antagonist into the LDTg attenuates cocaine

seeking in rats (Schmidt et al., 2009). Therefore, it makes sense that LDTg GLP-1R activation on inhibitory GABAergic neurons would mediate the suppressive effects of Ex-4 on cocaine seeking. Indeed, data in Chapter 4 show that LDTg GLP-1Rs on GABA neurons mediate the effects of both intra-LDTg Ex-4 and systemic Ex-4 to decrease cocaine seeking.

There is an extreme lack of literature examining the role of GABA neurons in the LDTg to control motivated behaviors. The existing literature on the role of the LDTg in reward and addiction focus almost exclusively on LDTg cholinergic neurons and their ability to drive cocaine-mediated behaviors (Kaneda, 2018). The current knowledge thus far on LDTg GABA neurons influencing the mesolimbic reward system are that a subset of LDTg projections to the VTA are presumed to be GABAergic (Omelchenko and Sesack, 2005) and that stimulation of LDTg neurons results in a ~30% inhibition of putative dopaminergic and GABAergic neurons in the VTA (Coimbra et al., 2017). However, one study has assessed the role of LDTg GABA neurons in mediating the effects of the metabolic factor amylin on decreasing food intake. Similar to GLP-1Rs, amylin receptors were not expressed on cholinergic neurons but were expressed on GABAergic neurons in the LDTg. Moreover, pharmacologically inhibiting GABA receptor transmission in the LDTg blocked the effects amylin to decrease food intake and body weight (Reiner et al., 2017). Overall, this study showed a role for LDTg GABA neurons in regulating feeding behaviors and combined with the data in Chapter 4, suggests that GABA-mediated decreases in motivated behavior may be a conserved mechanism across different metabolic hormones. Additionally, it is possible that combination therapy of GLP-1 and amylin receptor agonists may be more effective at attenuating cocaine-mediated behaviors and could serve as better therapeutics for cocaine use disorder. Thus, future studies are needed to explore this possibility. Overall, more studies are needed to characterize the cell types that

express different metabolic factor receptors in nuclei that are known to regulate motivated behaviors.

Future studies are needed to further delineate the types of GABAergic neurons in the LDTg that mediate the effects of GLP-1R activation on cocaine seeking. In Chapter 4, inhibition of LDTg GABAergic terminals that project to the VTA blocked the effects of Ex-4 on cocaine seeking, showing for the first time the relevance of VTA-projecting LDTg GABA neurons in cocaine-mediated behaviors. Additionally, LDTg GABAergic fibers were visualized in the VTA and associated with dopamine neurons. Our hypothesis is that Ex-4 decreases cocaine seeking by inducing LDTg GABA-mediated inhibition of dopamine neurons. However, future studies are needed to directly assess if LDTg GABAergic neurons make synaptic contacts onto VTA dopamine neurons and if these neurons express GLP-1Rs. Furthermore, chemogenetic or optogenetic studies directly activating GABAergic LDTg-to-VTA projections are needed to further show the significance of this circuit in reducing reward-mediated behaviors independent of Ex-4. It is also possible that GLP-1Rs are expressed on LDTg GABAergic interneurons that may decrease cocaine seeking through local inhibition of cholinergic neurons. One study showed that chronic cocaine exposure induced a noradrenergic-mediated reduction of inhibitory synaptic transmission to LDTg cholinergic neurons which is thought to contribute to the development of addiction-like behavior (Taoka et al., 2016). However, these findings did not determine if the decrease in inhibitory transmission was from presynaptic GABAergic terminals or local GABAergic interneurons. A previous study has examined the role of LDTg GABAergic interneurons in the context of innate fear and showed differential roles of parvalbumin vs somatostatin interneurons in promoting or decreasing olfactory cue–induced innate fear responses (Yang et al., 2016). It would be an interesting hypothesis if only a subset of LDTg interneurons mediate the effects of Ex-4 on cocaine seeking. Thus,

further characterization of the types of LDTg GABAergic neurons that express GLP-1Rs (i.e. projection neurons vs interneurons; parvalbumin vs somatostatin) are needed to directly test each of these populations in reducing cocaine seeking behavior. Overall, the role of the LDTg GABAergic system in motivated behaviors is largely unexplored and future studies are needed to further delve into this exciting new avenue of research.

The Role of the Endogenous GLP-1 System in Cocaine Seeking

In Chapters 2, 3, and 4, the effects of cocaine self-administration and subsequent abstinence on GLP-1R mRNA expression in the VTA, NAc and LDTg were examined. Collectively, cocaine self-administration and extinction had no effect on endogenous VTA, NAc or LDTg GLP-1R expression. However, Ex-4 binding has been shown to regulate trafficking and surface expression of GLP-1Rs (Roed et al., 2014). Therefore, it is possible that cocaine taking and subsequent abstinence may alter GLP-1R trafficking and/or surface expression in nuclei known to regulate drug-taking and -seeking behaviors. Future studies are required to further delineate the role of endogenous central GLP-1 signaling in cocaine-mediated behaviors, including if cocaine affects GLP-1R trafficking and/or the kinetics of ligand-mediated GLP-1R trafficking. Additionally, the intra-cellular signaling pathways that are activated by GLP1 receptors to reduce cocaine taking and seeking are not known. The GLP-1R is known to exhibit signal bias (Koole et al., 2013), and thus chronic cocaine exposure may change the known downstream signaling cascades of the GLP-1R such as PKA, PKC, MAPK and AKT (Holst, 2007; Hayes et al., 2011b; Rupprecht et al., 2013). Thus, the exact signal transduction pathways activated following GLP-1R activation after repeated cocaine exposure should be examined.

In Chapter 2, cocaine taking and subsequent abstinence were shown to dynamically regulate mRNA expression of PPG (gene that encodes GLP-1) in the NTS. Specifically,

cocaine self-administration increased endogenous PPG expression in the NTS. These data are consistent with findings that support the hypothesis that increased central GLP-1 signaling may serve as a 'brake' to reduce further cocaine consumption (Schmidt et al., 2016). In contrast, PPG expression in the NTS was significantly decreased following seven days of abstinence, a time point that coincides with robust drug-seeking behavior (Anderson et al., 2008; Schmidt et al., 2015b). These results suggest that decreased endogenous NTS PPG expression and presumed GLP-1 tone in the brain during abstinence may facilitate cocaine craving and relapse. It is possible that that decreased NTS PPG expression may result in a 'hunger-like' state that promotes cocaine seeking, as hunger is a potent driver of reward seeking and motivated behaviors (Hsu et al., 2018; Maniscalco and Rinaman, 2018). For example, previous studies have shown that chronic food restriction results in enhanced burst firing of substantia nigra dopamine neurons, augmentation of cocaine-induced burst firing, and persistence of increased burst firing after animals are refed (Branch et al., 2013). Consistent with these neurophysiological effects, food-restriction enhances cocaine-induced conditioned place preference indicating an important modulatory role for energy balance in drug reward (Zheng et al., 2012). Further evidence supporting the hypothesis that decreased NTS PPG expression produces a hunger-like behavioral state that promotes/facilitates drug seeking comes from a previous study showing that decreased PPG expression in the NTS produces hyperphagia and exacerbates high fat diet-induced obesity (Barrera et al., 2011).

In Chapter 4, the effects of increased endogenous GLP-1 signaling to the LDTg on cocaine-seeking behavior were examined. Chemogenetic activation of NTS-to-LDTg circuits significantly attenuated cocaine seeking. These effects were shown to be dependent on GLP-1 as pharmacologically inhibiting GLP-1Rs in the LDTg prevented the ability of NTS-to-LDTg activation to decrease cocaine seeking. Although the data in

Chapter 4 show the importance of NTS-to-LDTg projections in cocaine seeking, NTS GLP-1-producing neurons project to many midbrain and forebrain areas relevant in addiction-like behaviors such as the VTA and NAc (Alhadeff et al., 2012). Thus, a circuit-wide assessment of NTS GLP-1 projections and the functional relevance of each circuit to regulate cocaine seeking should be examined. However, Chapter 4 demonstrated, for the first time, that NTS GLP-1 circuits function to regulate cocaine-seeking behavior and supported our hypothesis that the endogenous central GLP-1 system is a homeostatic regulator of drug reward. Further support for this hypothesis comes from previous studies that have selectively manipulated NTS GLP-1-producing neurons in mice and found that these neurons have no significant effect on daily chow intake, body weight or glucose tolerance (Holt et al., 2019). Only after bigger challenges to homeostasis were GLP-1-producing neurons necessary for food intake control such as large meals and stressed-induced hypophagia (Holt et al., 2019). Cocaine exposure is well known to alter homeostasis in both stress and reward responses, which along with our previous studies and data in this dissertation, provides strong evidence that NTS GLP-1-producing neurons play a critical role in regulating homeostatic responses to cocaine.

The NTS is comprised of heterogeneous cell populations (Rinaman, 2010; Buffalari and Rinaman, 2014; Zheng et al., 2015), and in addition to GLP-1, investigating the exact role of each of the different neuronal populations in cocaine-mediated behaviors should be investigated. For example, the noradrenergic system is involved in cocaine-seeking behaviors and noradrenergic neurons in the NTS are known to be the primary source of noradrenaline in the NAc shell (Delfs et al., 1998; Zhang and Kosten, 2005; Brown et al., 2011; Schroeder et al., 2013). Additionally, cocaine exposure has been shown to activate noradrenergic neurons in the NTS (Buffalari and Rinaman, 2014). Thus, it would be interesting to determine if NTS noradrenergic neurons function to promote or reduce

cocaine-seeking behaviors and would highlight the brainstem as a whole as a critical nucleus in the regulation of addiction-like behaviors.

An important characteristic of the endogenous GLP-1 system to consider is that NTS GLP-1-producing neurons also co-express glutamate (Zheng et al., 2015). Thus, there are two important future directions: 1) examine the relationship between GLP-1 and glutamate release and 2) how GLP-1 may influence glutamatergic signaling in the mesolimbic reward system. Electrophysiological studies should assess the time course of release of glutamate vs. GLP-1 in GLP-1/glutamate-releasing neurons in the NTS and determine the overall effects of this co-release on the neuronal activity of postsynaptic targets. Additionally, previous studies in drug-naïve rats have shown that GLP-1R activation can increase presynaptic glutamate signaling in the VTA and NAc (Mietlicki-Baase et al., 2013; Mietlicki-Baase et al., 2014). However, the data in Chapter 2 showed that in cocaine-experienced rats GLP-1R activation had no effect on presynaptic mechanisms in the NAc but increased postsynaptic intrinsic excitability in MSNs. These data provide strong evidence that cocaine exposure changes how GLP-1R activation influences presynaptic vs postsynaptic mechanisms. Future electrophysiological studies should assess how GLP-1R activation effects presynaptic and postsynaptic glutamatergic signaling in the VTA and LDTg in cocaine experienced rats. The glutamate system is well known to be altered by chronic cocaine exposure and critical in promoting drug-seeking behaviors (Schmidt and Pierce, 2010), thus characterizing the mechanisms by which GLP-1 influences this system is an important future direction to investigate how the GLP-1R signaling reduces cocaine-seeking behavior.

Circuitry mediating the effects of GLP-1R activation on cocaine seeking

The data in Chapter 2, 3, and 4 show the VTA, NAc and LDTg as important sites of action for Ex-4 to attenuate cocaine seeking. Additionally, data in Chapter 4 show the importance of NTS-to-LDTg GLP-1 projections and LDTg-to-VTA GABAergic projections in regulating cocaine-seeking behavior. Furthermore, the data in Chapters 2 and 4 showed that pharmacological inhibition of GLP-1Rs in the VTA or blocking LDTg GABAergic signaling in the VTA completely reversed the effects of systemic Ex-4 on cocaine seeking. These findings highlight the VTA as a critical nucleus in mediating the effects of GLP-1R activation on cocaine-seeking behavior. Overall the data in this dissertation suggests that GLP-1 release from the NTS activates GLP-1Rs on LDTg GABAergic neurons that project to the VTA which may result in a decreased dopamine release in the NAc to reduce cocaine seeking (Figure 5.1).

Much remains to be discovered about the central GLP-1 circuits activated by Ex-4 that function to reduce cocaine-mediated behaviors. GLP-1Rs are expressed in many other brain regions involved in motivated behaviors such as the lateral hypothalamus, lateral septum, hippocampus, and amygdala (Goke et al., 1995; Merchenthaler et al., 1999; Hayes and Schmidt, 2016). Therefore, research must be dedicated to assessing the potential mechanisms of Ex-4 to act in these brain regions and others to control for cocaine-mediated behaviors. For example, previous studies have shown that GLP-1Rs in the ventral hippocampus play a role in food intake control and spatial learning (During et al., 2003; Hsu et al., 2015). Thus, it is possible that hippocampal GLP-1Rs regulate cocaine-associated contexts/memories that contribute to addiction-like behaviors. In addition, due to the importance of the amygdala in regulating drug seeking elicited by cocaine-associated cues (Berglind et al., 2006; Arguello et al., 2017), the role of GLP-1Rs

in this nucleus should be investigated as Chapter 2 showed that systemic Ex-4 significantly decreased cue-induced cocaine seeking.

Clinical Potential of GLP-1R Agonists to Treat Cocaine Use Disorder

Although strong preclinical evidence indicates that various metabolic hormones regulate addiction-like phenotypes in animal models (Engel and Jerlhag, 2014; Jerlhag, 2018; Hernandez and Schmidt, 2019), the relationship between endogenous levels of metabolic factors and substance abuse disorders in humans is not clear. A recent study investigated the effects of cocaine on plasma levels of metabolic factors [ghrelin (total and acly-ghrelin), amylin, GLP-1, insulin, leptin and peptide YY (PYY)] in human cocaine addicts (Bouhlal et al., 2017). Acute cocaine (25 mg) significantly decreased serum GLP-1 and PYY concentrations in cocaine users with trends towards decreased insulin and amylin concentrations (Bouhlal et al., 2017). However, these effects were seen in a relatively small sample size (n=8). Thus, future studies with larger sample sizes and healthy controls are needed to further validate and determine the specificity of these effects in human cocaine addicts. Additionally, future studies would benefit from correlating metabolic hormone serum concentrations with drug craving to further support the hypothesis that GLP-1, and possibly PYY, may serve as potential targets to treat cocaine craving and relapse. Consistent with these effects, other clinical studies have shown that crack cocaine use is associated with lower serum levels of leptin (Escobar et al., 2018). Preclinical studies have also assessed the correlation between circulating levels of metabolic hormones and cocaine taking and seeking in rats. Serum ghrelin levels have been shown to be positively correlated with increased reinstatement of cocaine-seeking behavior (Tessari et al., 2007). Interestingly, a recent study has shown that 14 days of cocaine self-administration in rats was associated with increased ghrelin and GLP-1 plasma levels while leptin and insulin levels were decreased (You et al., 2018). While these preclinical

findings appear to contrast findings from human GLP-1 studies, they are consistent with the working hypothesis that cocaine-induced increases in central GLP-1 signaling may represent a homeostatic response to reduce further cocaine consumption (Schmidt et al., 2016). Thus, larger clinical trials are needed to determine the exact effects of cocaine taking and withdrawal on plasma GLP-1 levels and validate findings from preclinical models quantifying drug-induced changes in circulating metabolic factors.

The mechanisms by which cocaine decreases GLP-1 serum concentrations in human cocaine addicts and the functional significance of decreased circulating GLP-1 remains to be determined. However, taken together with preclinical studies showing that increased GLP-1R activation decreases cocaine taking- and seeking-behaviors, these results suggest that decreased GLP-1 may promote cocaine use. This hypothesis is consistent with data presented in Chapter 2 demonstrating that cocaine taking and subsequent abstinence decreased PPG mRNA expression in the NTS, effects likely associated with reduced GLP-1 signaling in the mesolimbic reward system, which may promote cocaine seeking. It is also interesting to speculate that decreased serum GLP-1 levels observed in human addicts may serve as a biomarker for cocaine use disorder and/or treatment response (Bough et al., 2014). Moreover, there are several known single nucleotide polymorphisms (SNPs) in the human GLP-1R (Beinborn et al., 2005). One SNP in particular, the T149M variant, displayed reduced GLP-1 binding and cAMP signaling as well as differences in the extent of reduced functional responses to various GLP-1R ligands (Beinborn et al., 2005; Koole et al., 2011). It is possible that expression of the T149M variant and/or decreased baseline circulating GLP-1 levels in human cocaine addicts may serve as predictors of treatment response and/or relapse, as these phenotypes are associated with decreased endogenous GLP-1 signaling in the body. If increased endogenous GLP-1 signaling functions as a ‘brake’ to reduce cocaine

consumption (Schmidt et al., 2016), cocaine users with the T149M variant and/or reduced blood GLP-1 levels may be more susceptible to cocaine craving and relapse. This hypothesis is supported by clinical studies showing that another GLP-1R SNP variant (168Ser) is associated with alcohol use disorder and increased alcohol consumption in humans with alcohol use disorder (Suchankova et al., 2015). Thus, it is provocative to think that GLP-1R variants may be predictors of relapse and/or treatment response in humans with cocaine use disorders.

From a translational perspective, previous studies and data shown in Chapters 2, 3, and 4 have identified systemic and intra-cranial doses of Ex-4 that attenuate cocaine taking and seeking (Schmidt et al., 2016) and do not produce notable metabolic or adverse effects associated with GLP-1R agonists in humans and rodents (Buse et al., 2009; Hayes et al., 2011a; Kanoski et al., 2012). Since GLP-1 analogs are currently FDA-approved for treating type II diabetes and obesity (Lovshin and Drucker, 2009; Shukla et al., 2015), these preclinical findings suggest that GLP-1R agonists could be re-purposed at low doses to treat cocaine use disorder. However, clinical studies are needed to validate the efficacy of GLP-1R agonists in reducing cocaine craving-induced relapse and to determine if circulating GLP-1 could be used as a biomarker for craving and/or treatment response.

There are currently many variations of GLP-1R agonists available on the market to treat type II diabetes (Gentilella et al., 2019). The elimination half-lives of GLP-1R agonists differ depending upon their chemical structures, that include, but are not limited to, specific amino acid sequences that are resistant to DPP-IV degradation, as well as conjugations (i.e., pegylation, acetylations, etc.) to the peptide sequence (Hayes et al., 2014). These important pharmacokinetic properties are likely to influence brain penetrance, distribution to nuclei known to regulate drug reinforcement, and the therapeutic utility of GLP-1R

agonists for treating cocaine use disorder. Thus, it is important for future studies to directly compare the efficacy of different GLP-1R agonists in attenuating drug-mediated behaviors. It will also be important for future clinical trials to determine the efficacy of recently developed oral formulations of GLP-1 analogs in treating cocaine use disorder as the safety and pharmacokinetics of these medications may differ from GLP-1R agonists administered subcutaneously (Granhall et al., 2018).

In addition to causing nausea and malaise in humans, GLP-1 analogs have also been shown to reduce blood glucose concentrations and food intake in healthy subjects (Edwards et al., 2001; Thazhath et al., 2016). However, GLP-1R agonists cause fewer hypoglycemic events compared to insulin in patients with type II diabetes and no reports of hypoglycemia or severe weight loss have been observed in healthy subjects (Edwards et al., 2001; Diamant et al., 2010; Giorgino et al., 2015; Thazhath et al., 2016). Therefore, it is unlikely that GLP-1R agonist treatment will result in glycemic dysregulation in otherwise healthy patients (Holst, 2007; Drucker, 2018). Moreover, there is no scientific evidence supporting development of pancreatic toxicity as initially suggested in patients treated with GLP-1R agonists (Drucker, 2018). Thus, the benefit/risk profile of GLP-1R agonists supports their clinical use and the translational relevance of preclinical drug addiction studies.

Potential Efficacy of GLP-1R Agonists in Treating Other Substance Use Disorders

In addition to reducing cocaine-mediated behaviors, the efficacy of GLP-1R agonists in regulating behaviors associated with other drugs of abuse has been investigated. Specifically, GLP-1R agonists have been shown to reduce ethanol-, amphetamine-, and nicotine-mediated behaviors in rodents (Egecioglu et al., 2013b, a; Egecioglu et al., 2013c; Shirazi et al., 2013; Vallof et al., 2016; Tuesta et al., 2017). Systemic administration of Ex-

4 (2.4 µg/kg) decreased ethanol-induced CPP, the locomotor-activating effects of ethanol, and ethanol self-administration (Egecioglu et al., 2013c). These behavioral responses were associated with reduced ethanol-induced dopamine release in the striatum (Egecioglu et al., 2013c). Other studies have replicated these findings and shown that higher doses of Ex-4 (20 µg/kg) decreased ethanol-induced CPP in mice while 0.3 and 1.0 µg/kg Ex-4 decreased voluntary ethanol intake in rats (Shirazi et al., 2013). These effects are mediated, in part, by central GLP-1Rs as infusions of Ex-4 directly into the VTA were sufficient to reduce ethanol self-administration in rats (Shirazi et al., 2013). Analogous findings have been shown in studies investigating the efficacy of GLP-1R agonists in reducing amphetamine-mediated behaviors. Specifically, administration of Ex-4 (2.4 µg/kg) attenuated amphetamine-induced locomotor activity, CPP and accumbal dopamine release (Egecioglu et al., 2013b). With regard to nicotine use disorder, Ex-4 (2.4 µg/kg) pretreatment reduced nicotine-induced locomotion, sensitization, CPP and accumbal dopamine release (Egecioglu et al., 2013a). Another study has expanded these findings and shown that nicotine activates endogenous GLP-1-producing neurons in the NTS (Tuesta et al., 2017). Moreover, systemic administration of Ex-4 (10 µg/kg) and the DPP-IV inhibitor sitagliptin (10 mg/kg) decreased nicotine consumption in mice (Tuesta et al., 2017). Consistent with these effects, GLP-1R knockout mice self-administer more nicotine than wild-type controls (Tuesta et al., 2017). To investigate the endogenous circuits mediating these behavioral responses, optogenetic studies revealed that GLP-1 excites medial habenula projections to the interpeduncular nucleus and that activation of GLP-1Rs in this circuit is sufficient to attenuate nicotine consumption (Tuesta et al., 2017). The authors conclude that GLP-1 acts as a ‘satiety sensor’ to limit nicotine intake by stimulating the medial habenula to interpeduncular nucleus circuit to promote nicotine avoidance before its aversive effects are encountered (Tuesta et al., 2017). One study examined the ability of Ex-4 to regulate animal models of opioid use disorder and found

that systemic treatment of Ex-4 had no effects on self-administration of remifentanil in mice (Bornebusch et al., 2019). However, more studies are needed to validate these findings. Collectively, these studies indicate that systemic administration of a GLP-1R agonist decreases the reinforcing and rewarding efficacy of multiple drugs of abuse and that these effects are associated with decreased drug-evoked dopamine release in the NAc. It is important to note, however, that relatively high doses of systemic GLP-1R agonists were used in these studies and that these doses have been shown previously to produce nausea/malaise and decrease food intake and body weight in rats (Hayes et al., 2011a; Kanoski et al., 2012). Achieving behavioral selectivity through proper dosing of GLP-1R agonists is critical for drawing firm conclusions about the efficacy of GLP-1R agonists to reduce addiction-like phenotypes in preclinical models. These foundational studies do, however, provide preliminary data supporting the potential efficacy of GLP-1R agonists in treating alcohol, nicotine, and amphetamine use disorders.

Conclusion

Collectively, the data presented in this dissertation expands our understanding of the central GLP-1 circuits that regulate cocaine-seeking behavior, as well as the cellular, molecular and neurophysiological mechanisms by which GLP-1R agonists attenuate cocaine seeking. This dissertation takes the critical first steps toward characterizing central GLP-1 circuits and phenotyping GLP-1R-expressing cells in brain nuclei know to regulate drug-taking and -seeking behaviors as well as their downstream targets. Additionally, this dissertation shows that the endogenous central GLP-1 system, particularly GLP-1-producing neurons in the NTS, are novel neurobiological mechanisms that regulate cocaine seeking. In conclusion, the data in this dissertation supports clinical studies examining the efficacy of GLP-1R agonists in treating cocaine use disorder, as

well as the development of pharmacotherapies aimed at increasing GLP-1R signaling in the brain to prevent cocaine craving-induced relapse.

Figures

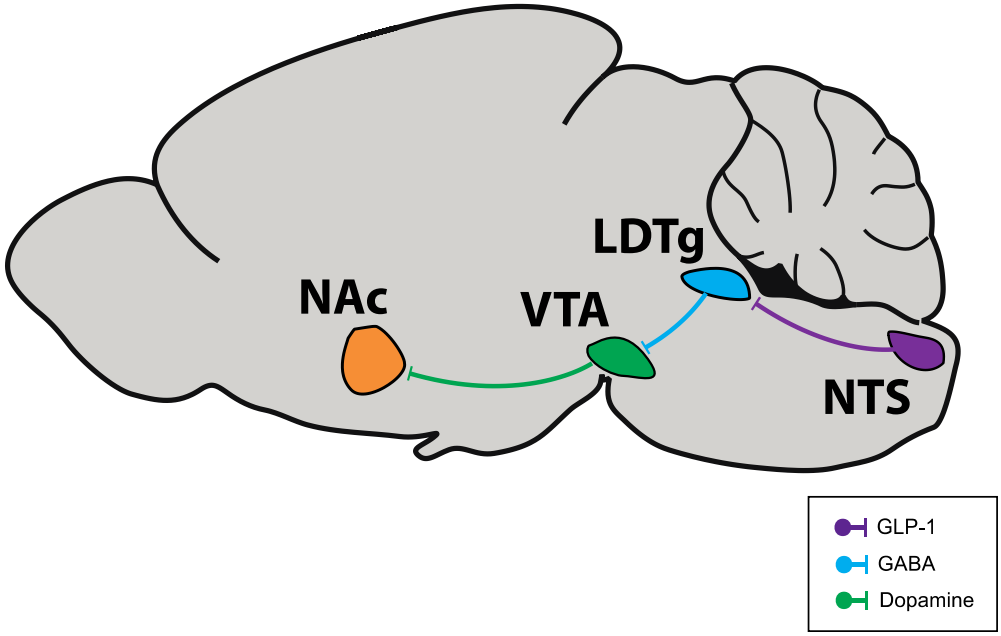

Figure 5.1 Circuitry influenced by the GLP-1 system to regulate cocaine-seeking behavior

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