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ASSESSMENT OF GLUCAGON-LIKE PEPTIDE-1 ANALOGS AS POTENTIAL TREATMENTS FOR OPIOID USE DISORDER

A Dissertation in

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by

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ABSTRACT

Addiction is the most severe stage of substance use disorder (SUD) in which compulsive taking of the drug leads to further loss of self-control. This disease is particularly difficult to treat because, due to its chronic and relapsing nature, individuals that suffer from SUD can be abstinent for long periods of time and still relapse. During this period people are more susceptible to overdose, making it imperative that we find new treatments to prevent craving and relapse. The classic view suggests that drugs of abuse hijack the reward system. However, we proposed that addiction, in addition to affecting the normal functioning of reward-associated areas, also produces a dysregulation in the homeostatic system, generating a state of 'need' for the drug. In a normal animal, motivated behaviors are guided, in large part, by its internal state and persist until the goal is obtained and homeostasis is reached. In this way, the behavior switches from one motivated state to another, depending on the needs of the system at any particular time. However, in the case of addiction, drugs of abuse engage one motivated behavior (i.e., drug-seeking and drug taking) to the exclusion of others. In this context, the physiological state associated with craving drug is similar to that observed in animals that seek food when starved, water when thirsty and salt when sodium deficient. When such a state is reached, there is one goal and there can be no substitute. If this is true, then, it is possible that the need state associated with craving and/or withdrawal, just like that associated with hunger or thirst, can be placated by satiety signals.

The goal of the present dissertation is to test the need state hypothesis described above, by studying the effects of different analogs of the satiety agent glucagon-like peptide-1 (GLP-1) in different components of reward, and on behaviors associated with heroin addiction. To begin, in **Chapter 2,** we used intake and taste reactivity studies and showed that Exendin-4 (Ex-4; 2.4

μg/kg ip), a short-acting GLP-1 analog known to reduce approach towards a rewarding taste stimulus, does not alter the palatability of such stimulus. Moreover, we observed that when the taste stimulus is naturally aversive, Ex-4 reduced both approach and perceived aversiveness. However, when the aversion was learned, such as in a lithium chloride conditioned taste aversion (CTA), Ex-4 did not alter either approach or perceived palatability towards the taste stimulus. This GLP-1R analog, then, reduces motivation for innately rewarding and aversive gustatory stimuli. **In Chapter 3**, we used our model of reward devaluation and showed that chronic treatment with Ex-4 (2.4 μg/kg ip) during abstinence and at test reduced both cue-induced heroin seeking and drug-induced reinstatement of heroin seeking, without affecting body weight in heroin-experienced rats. Moreover, we observed that those rats that belong to the most susceptible population and most greatly avoided intake of the heroin-paired saccharin cue (i.e., the large suppressors) reinitiated responding for the natural saccharin reward after Ex-4 treatment was discontinued. As such, this relatively short-acting GLP-1R analog can rescue vulnerable rats from cue- and drug-induced heroin seeking and can restore responding for natural rewards. In **Chapter 4**, we extended these findings by showing that chronic treatment with the long-acting GLP-1 analog, liraglutide (LIR, 0.1 mg/kg sc), reduced heroin self-administration and prevented escalation of heroin taking over time. Treatment during abstinence, also, was effective in reducing drug-induced reinstatement without affecting body weight or plasma glucose. Cue-induced seeking, however, was not reduced by 0.1 mg/kg LIR, at least not when assessed only one hour after LIR administration. In **Chapter 4**, we also studied side effects associated with LIR treatment. We showed that while different doses (0.1, 0.3, 0.6, and 1.0 mg/kg sc) supported the development of a CTA to a saccharin-paired cue, none of these doses triggered the ingestion of kaolin, suggesting that the reduction in intake of the saccharin-paired cue was not due to

nausea/malaise. Finally, in **Chapter 5**, we showed that acute treatment with LIR (0.3 mg/kg sc) was effective in reducing heroin-seeking whether elicited by cues, drug, or stress. This is an important finding, as no other non-opioid intervention has been found to be so uniformly effective in reducing drug seeking behavior. Overall, the data in this dissertation provide the foundation for the safe and effective use of GLP-1 analogs as a potential treatment for substance use disorder in humans and suggest, importantly, that addiction may be driven, at least in part, by a potent state of physiological need.

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LIST OF ABBREVIATIONS

AGRP	Agouti-related peptide
ANOVA	Analysis of variance
ARC	Arcuate Nucleus
CART	Cocaine-and amphetamine-regulated transcript
CIN	Cholinergic interneuron
CPP	Conditioned place preference
CTA	Conditioned taste aversion/avoidance
D2R	Dopamine receptor 2
DA	Dopamine
DNA	Desoxyribonucleic acid
DPP-4	Dipeptidyl peptidase-4
DSMV	Diagnostic and statistical manual of mental disorders, 5th edition.
Ex-4	Exendin-4
Ex-9	Exendin-9
FDA	Food and Drug Administration
FR	Fixed-ratio
g	Gram
GABA	gamma-aminobutyric acid
GIN	GABAergic interneuron
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GLP-2	Glucagon-like peptide-2

Glu	Glutamate
GPCR	G protein-coupled receptor
ILI	Inter-lick interval
im	Intramuscular
ip	Intraperitoneal
IP3	Inositol triphosphate
iv	Intravenous
Kg	Kilogram
LepR1	Leptin receptor-1
LH	Lateral hypothalamus
LiCl	Lithium Chloride
LIR	Liraglutide
LS	Large suppressor
M	Molar
MAT	Medication-assisted treatment
MCH	Melanin-concentrating hormone
mg	milligram
min	minute
mL	milliliter
mRNA	messenger ribonucleic acid
Nac	Nucleus accumbens
NAcS	Nucleus accumbens shell
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract

OUD	Opioid use disorder
OX1	Orexin receptor 1
PFC	Prefrontal cortex
POMC	proopiomelanocortin
ppg	pre-proglucagon
PVT	Paraventricular thalamus
QHCl	Quinine
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
Sac-sal	Saccharin-saline
sc	Subcutaneous
sec	Second
SS	Small suppressor
SUD	Substance use disorder
μg	Microgram
Veh	Vehicle
VTA	Ventral tegmental area

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Chapter 1: Introduction

Substance Use Disorder: Costs and Consequences

Substance-use disorder (SUD) is a combination of behavioral, cognitive and physiological symptoms associated with recurrent use of drugs despite significant clinical or functional impairment (health problems, disability, failure to meet responsibilities, etc.) [1]. Addiction is the most severe stage of SUD, a progressive, chronic, relapsing disorder in which compulsive taking of the drug leads to further loss of self-control [2]. Both substance use disorder and drug addiction represent a major economic and health issue in the United States [3]. In 2019, for example, 20.8% of Americans older than 12 years of age (57.2 million) used illicit drugs at some time, and approximately 8.1 million of these people had developed substance use disorder [3]. In the United States in 2018, costs related to crime, health and lost productivity due to the opioid epidemic was estimated to be above $631 billion dollars [4]. Specifically, the biggest costs incurred by America due to substance abuse are premature death (40%), health care expenses (33%), and loss of productivity (15%) [4].

Substance abuse takes a major toll on the economy of the United States, but the most devastating consequence of this disease is death due to accidental drug overdose. Overdose related deaths tripled in the last two decades, taking almost 72,000 lives in 2019 [5]. In 2018, there was a decline in overdose deaths largely due to the reduction of prescription opioid medication [6, 7]. However, in 2019, misuse of prescription pain relievers, heroin, and synthetic opioids caused the death of more than 137 people a day - close to 70% of the total overdose deaths in the United States, and the number of deaths is expected to increase by at least a 20% due to the stress associated with COVID-19 in 2020 [5, 8, 9]. These numbers suggest that the opioid crisis is a tragedy of epidemic proportion, as opioid overdose deaths are higher than the yearly peak deaths ever recorded for deaths related to guns, car accidents or even AIDS [10]. It is

imperative, then, to explore different approaches to understand substance abuse and drug addiction that will, in turn, allow for the development of new and more effective treatments to mitigate the craving and withdrawal that precipitates relapse and, thereby, increases vulnerability to opioid overdose [11].

Addiction: The Problem with the Current Interpretation and an Alternate Approach

Drug addiction can be defined by two major characteristics: loss of self-control with regard to intake, and a compulsion to seek and take drug that narrows the behavioral range only towards excessive drug intake [1, 12]. The most important challenge in the field is to understand the molecular, cellular and systemic changes that occur during the transition from controlled drug use to loss of control, which will eventually lead to addiction [13]. The descriptive and observational nature in which the field of biology is rooted has led numerous investigators to a teleological and reductionist interpretation of addiction as the mere disruption of the reward system. In other words, these investigators argue that drugs of abuse act exclusively on the reward system, altering its function and re-directing behavior towards drug-seeking. Although this is partly true, an understanding of the complexity of the processes that underlie addiction requires an approach based on ontological and epistemological principles. In other words, understanding the context in which the reward system has evolved and its ultimate purpose, along with the basis in which our current understanding of addiction lies, will allow us to better understand the nature of this disease in a more integral manner, and possibly provide new insights into the development of improved strategies to treat addiction.

Drug addiction, as other behavioral dysregulations (e.g., binge eating, compulsive gambling, etc.), has been described as a dynamic phenomenon comprising several components

that constitute a cycle of increasing pathology [14]. The main components of this cycle are: 1. Preoccupation-anticipation, 2. Binge-intoxication, and 3. Withdrawal-negative affect (see Figure 1-1) [13]. These components are understood as sources of failure of self-regulation that can lead to emotional distress, generating additional negative affect that will reinitiate the cycle [14]. At the early stages, this cycle is dominated by impulsivity, which is characterized by an increasing sense of arousal prior to taking the drug, and gratification and relief while committing the act. As the cycle develops, compulsivity develops, in which anxiety and stress are present before committing the act, and relief of stress at the time of the act. As impulsivity shifts towards compulsivity, the behavioral drive switches from positive reinforcement to negative reinforcement [1, 15]. In this way, the different components within this cycle can be modeled as cycles of conditioned positive reinforcement (binge-intoxication) and conditioned negative reinforcement (negative affect/withdrawal) - it is believed that both kinds of reinforcement contribute to the preoccupation/anticipation phase [16, 17]. However, the compulsive use of drug that exacerbates the cycle and leads towards loss of control cannot be explained as components of reinforcement. As a consequence, there is a transition between the controlled use and compulsive use of drugs that leads to addiction that has not been extensively modeled [17].

The increasing distress that occurs with repeated cycles also has been associated with progressive dysregulation of the brain reward system. It was hypothesized that drug addiction represents an allostatic state in the reward system, expressed as changes in reward set points via the dysregulation of not only the reward system, but also via recruitment of the hormonal stress response [17]. As allostasis is the process by which a system responds to change in order to regain homeostasis, it is possible then that addiction can be interpreted as a chronic and pathological deviation from homeostasis, not only at the level of the reward system, but at the level of the whole organism [18]. In this context, then, we will next discuss the ways in which the

reward system and the homeostatic system work together to modulate behavior, and how a disruption in these systems can lead to biobehavioral dysregulations that ultimately are expressed in compulsive behaviors.

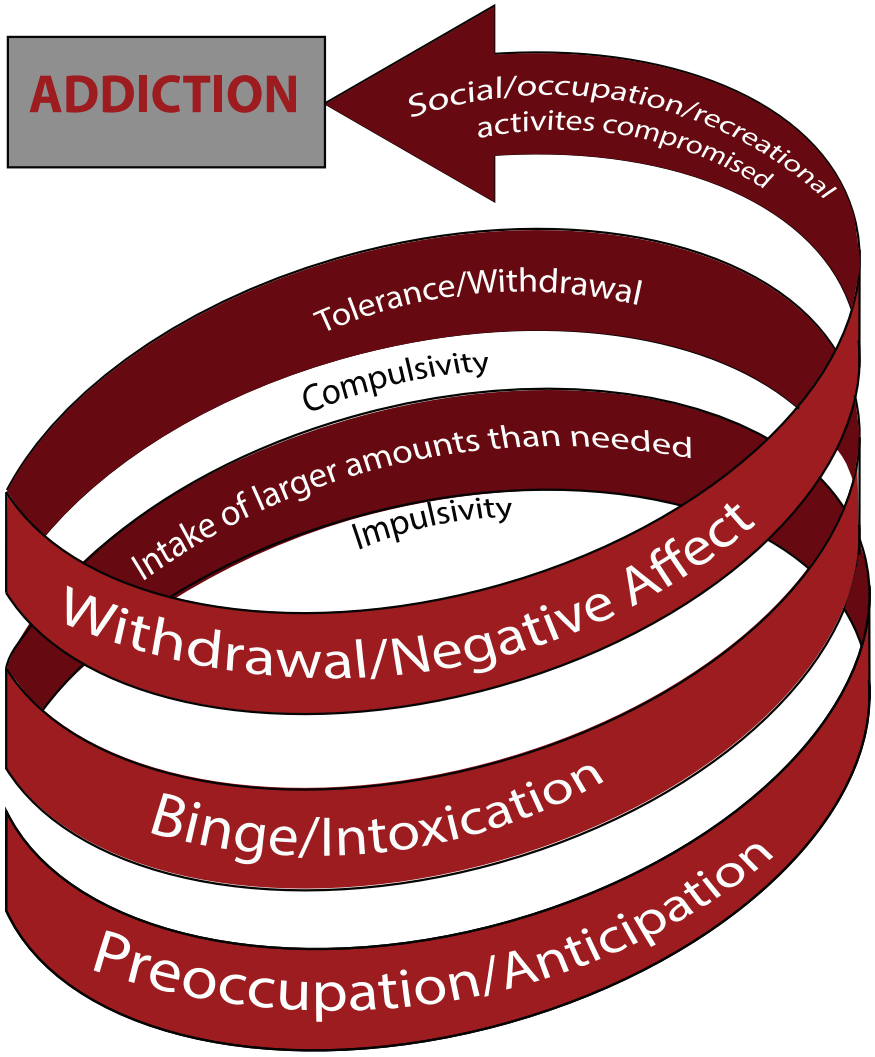

Figure 1-1. The cycle of addiction. From a psychiatric perspective, addiction is a cycle of increase distress. The diagram describes the spiraling, increasing pathology, incorporating different criteria for substance dependence from the Diagnostic and Statistical Manual of Mental Disorders. The cycle is composed of three major components: preoccupation/anticipation, binge-intoxication and withdrawal/negative affect. These components are thought to be associated with different sources of reinforcement. For example, the rewarding properties of the drug produce a positive reinforcement that leads towards the binge/intoxication phase. On the other hand, the aversive state associated with withdrawal/negative affect serve as negative reinforcement. Experimentally, it is thought that both positive and negative reinforcement are involved in the preoccupation/anticipation phase. It is also thought that, at early stages of the cycle, the arousal in anticipation and the gratification after taking the drug leads to an impulsive behavior. However, as the cycle develops, anxiety and stress precede the act, while actually taking the drug produces a relief from stress, leading to a compulsive behavior. Even though most of the cycle can be modeled as cycles of positive and negative reinforcements, the compulsive intake that leads to uncontrolled intake and eventually addiction cannot be explained as components of reinforcement. Figure adapted from Koob and Le Moal, 1997 [13].

Evolution of Motivated Behavior

Motivated behaviors are a fundamental component of life. The concept of motivation allows us to understand the ways in which the interaction between the past history of an organism and its current state modulate goal-directed behaviors. As such, in Hull's theory of motivation [19], motivated behaviors were performed in order to fulfill biological needs, optimizing, in this way, survival. If this is true, and the structures that regulate motivated behaviors are tightly linked to survival of the organism, it is crucial then, to analyze the phylogenetic and ontogenetic history of the systems involved in modulating both reward (i.e., reward system) and need (i.e., homeostatic system).

During the Cambrian explosion, around 540 million years ago, the very first free-moving animals that appeared in the ocean needed to perform behaviors that would allow them to obtain food, seek shelter, and reproduce in order to survive. Even then, several millions of years ago, these ancient organisms had incorporated regulatory structures and mechanisms within their primitive nervous systems that modulated their behavior (Figure 1-2). These structures induced the appropriate set of behaviors that would let them most successfully achieve the goals needed in each particular circumstance, and that were essential for their survival. Indeed, if we look at our own history, the forebrain of the first chordates (e.g., lancelet) contained a hypothalamus-like structure (i.e., infundibulum) associated with a structure similar to the current pituitary gland (i.e., Hatschek'spit) [20].

In the ancient organisms described above, the brainstem contained a locomotor control center, comparable to the tegmental and reticulospinal system of modern chordates [21]. Although the presence of a proper striatum was first observed in Hagfish, later in evolution, it is believed that the dopaminergic system has an even longer history, dating back more than 600

million years ago to a common ancestor of Cnidaria and Bilateria [22, 23]. Throughout evolution, dopamine has remained as an integral neurotransmitter acting as a motor signal and modulating motor activity [24]. As observed in this brief recount, all these structures that are still crucial to regulate behavior, have primitive counterparts that were present since the origin of free-moving animals, and have evolved together to guide the organism towards homeostasis. Their presence from the very beginning suggests that they are of vital importance, as homeostasis is the property that allows a system to regulate its variables and it is the process by which the internal state of an organism maintains stability. The survival of every living organism depends on maintaining its internal conditions within controlled limits, and these limits will provide a reference point for adaptations in response to the changing environment. From the single cell to complex multi-cellular organisms, homeostasis is the very foundation of life, and also, it is its' main evolutionary force [25].

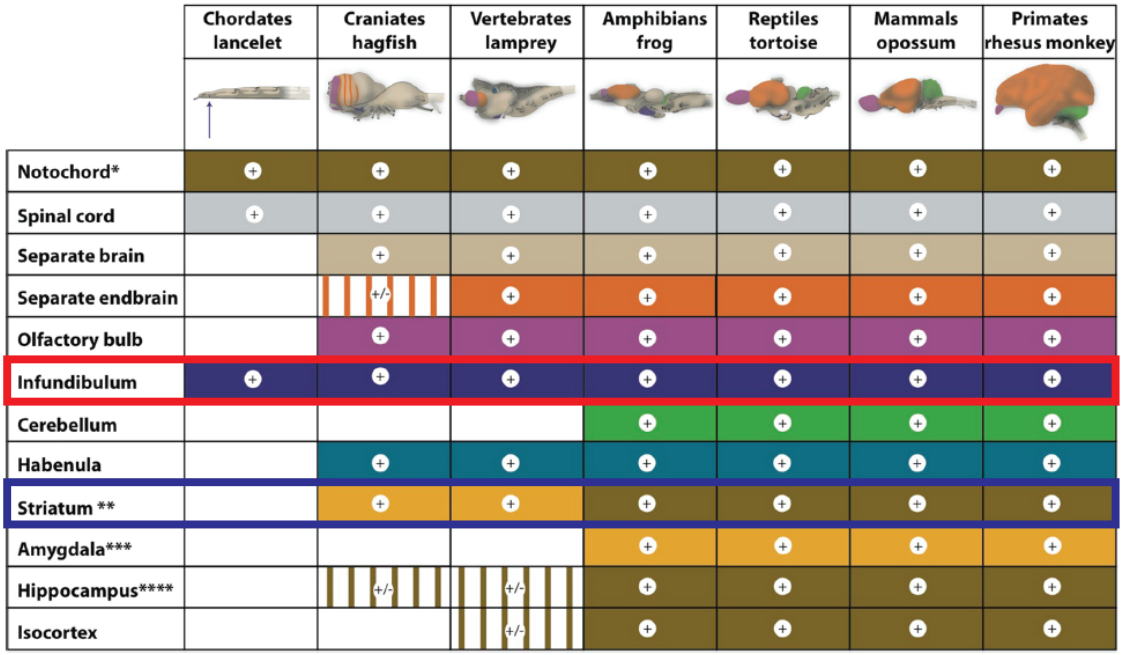

Figure 1-2. Evolution of the central nervous system. Comparison of the brain of different clades within the phylum Chordata. The lancelet is believed to be closely related to Pikaia, the first representative from the phylum. By comparing the structures across the different clades, it is possible to assess which brain structures may have developed earlier. For example, in the red box, we can observe that the infundibulum, a structure similar to the present-day hypothalamus, was present as early as the origin of chordates during the Cambrian explosion (541 million years ago). In addition, in the blue box, we can observe that a structure similar to the striatum is common for all vertebrates, appearing around 500 million years ago. Figure adapted from Loonen and Ivanova, 2015 [26]

Homeostatic and Hedonic Integration

Energy acquisition is vital for living organisms to perform physiological functions and behaviors. Eating is a complex behavior mediated by several factors, and these factors comprise homeostatic signals such as those related to body fat stores, energy availability (glucose, fatty acids), as well as non-homeostatic information related to stress, hedonics and experience. Food can serve both as an energy source and/or a reward, and this is probably the reason why food intake has been the most studied behavior in which the teleological understanding of the reward system (i.e., the reward system exists just to modulate rewards) generates a clash between homeostatic-driven and hedonic-driven behaviors. For this reason, we will analyze the modulation of motivated behavior through feeding, and then translate into the field of addiction everything we learnt, in order to, hopefully, have a better understanding of such complex behavior.

Most animals that live in an environment with abundant resources will consume nutrients in a way that roughly matches energy expenditure, maintaining body weight at a specific set point [27]. When energy intake is less than energy expenditure (i.e., hunger), the animals will seek and work harder to obtain food in order to reverse the energy deficit [28, 29]. This is the need-based homeostatic motivation [30-32]. Non-homeostatic feeding, on the other hand, is driven by the rewarding properties of specific palatable foods (e.g., high-fat foods, sweets, etc.), and food-associated stimuli that can directly affect the reward system and can override homeostatic signals [33]. For a long time, it was considered that the brain processes these signals separately through different structures, integrating them closer to the behavioral outcome, in hindbrain motor control areas [34]. However, in recent years, evidence of overlapping structures and cross talk between

the systems that regulate homeostatic and non-homeostatic feeding has been observed (Figure 1-3) [35-40] .

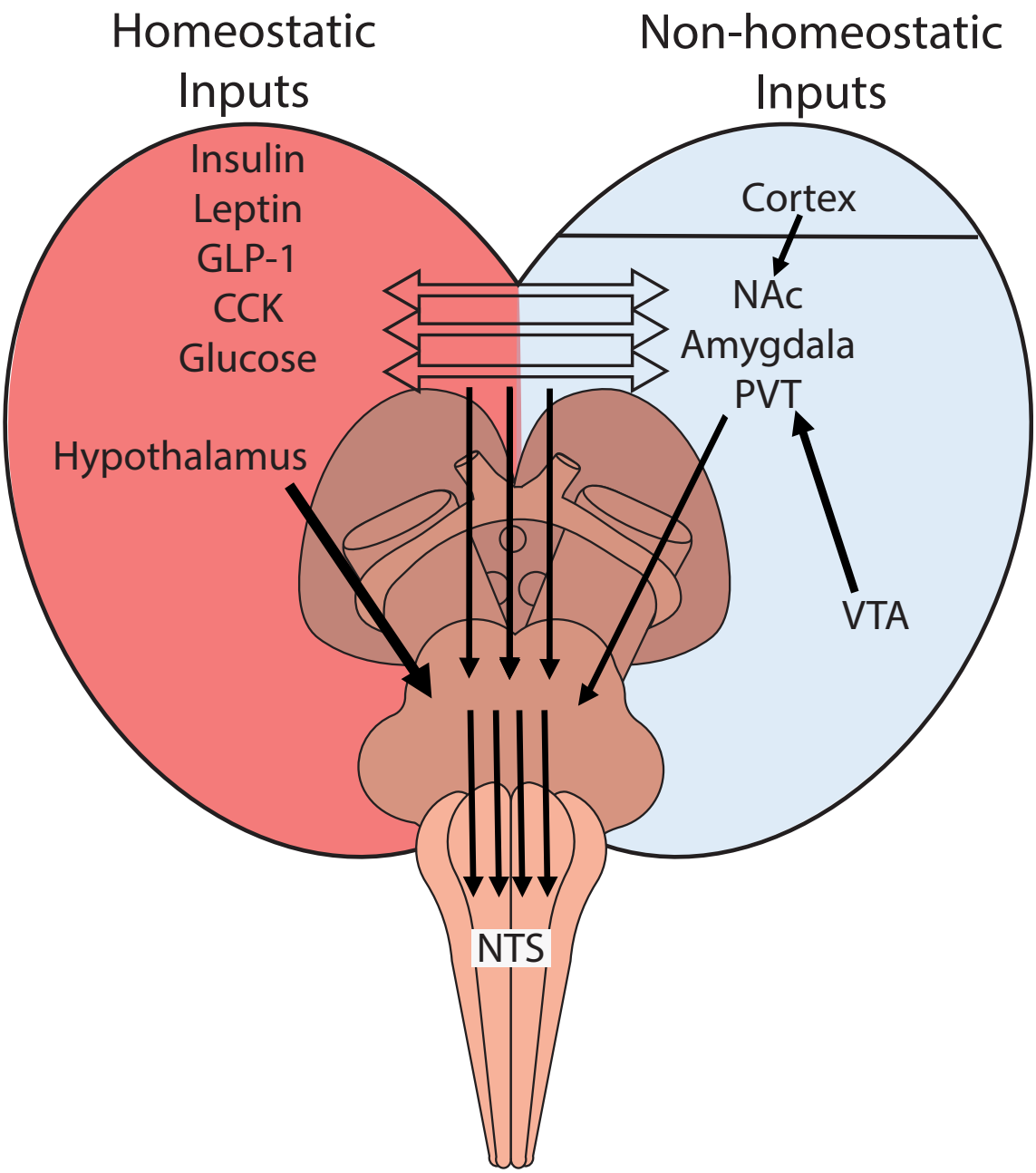

Figure 1-3. Homeostatic and Hedonic interaction. Both homeostatic and non-homeostatic inputs influence motivated behavior. The information about the homeostatic state (energy availability) and non-homeostatic state (stress, previous experience, affect) of the organism are integrated throughout the brain at several levels in order to elicit the most appropriate behavior. NTS: Nucleus of the Solitary Tract; VTA: Ventral Tegmental Area. NAc; Nucleus Accumbens; PVT: Paraventricular Thalamus

Motivated Behaviors and the Reward System

Non-homeostatic feeding behavior is driven by the perception of the food stimulus and is modulated mainly by the reward system. However, how the brain perceives and responds to these palatable rewards is not yet fully understood. The first experiments showing that intracranial self-stimulation increased operant behavior led to a breakthrough: there are areas in the brain that modulate pleasure and reward [41, 42]. Since then, several areas have been identified as part of the reward system, such as lateral hypothalamus (LH), the medial forebrain bundle - which contains the ascending dopaminergic pathways from the ventral tegmental area (VTA) to the nucleus accumbens (NAc)-, the amygdala, the septal nuclei and the raphe nuclei [43].

Even though a diverse number of brain regions had been associated with reward modulation, the first theories about motivation led to a reductionist understanding of the reward system, focused exclusively on the ascending mesocorticolimbic dopamine (DA) system that connects the VTA to the ventral NAc and dorsal striatum [44]. This dopaminergic system has been shown to be critical for processing information and to learn approach behaviors towards a reward. In the context of addiction, all drugs of abuse have been shown to trigger dopamine release, and blocking dopamine receptors, or lesions in dopaminergic neurons, reduce the rewarding effects of these drugs and reduce the rewarding impact of intracranial self-stimulation [44, 45]. However, reward behavior is the result of a much larger integrative process that involves, in addition to the internal physiological state, the emotional state, and the associative history of the individual [46].

In this view, reward is not a single, simple process that can be reduced to one neurotransmitter or one system. Instead, reward comprises several separate, but yet interconnected, neurobiological and psychological components. Behaviorally, reward can be

divided into two components. On the one hand, the motivational component related to the willingness of the animal to approach and execute consummatory behaviors is termed “wanting”. On the other hand, the affective component related to the perceived value of the reward is termed “liking” [46]. In general, these two components correlate with each other and rewards that are wanted also are liked. However, by eliminating the operant component of the task (i.e., approach) using intraoral delivery and assessment of taste reactivity, researchers have shown that this is not always accurate, that these components (“wanting” and “liking”) are dissociable, and are under the control of different neural systems [46] that are not dependent exclusively on the dopaminergic system. For example, such experiments showed that appetitive orofacial expressions to sucrose, as well as the aversive reactions to quinine, persisted even after a 99% depletion of dopamine neuron fibers in the NAc and neostriatum [47]. On the other hand, depletion of dopamine did reduce the motivation of the animal to seek and approach the palatable solution [48]. In addition, lesions in the NAc using the neurotoxin 6-OHDA induced aphagia but did not block food preference [49]. These observations evidence the complexity of reward, both at the neuronal and the behavioral level, and show that, although crucial, the dopamine system is not the sole modulator of reward behavior.

A more comprehensive understanding of the function of dopamine is described in the incentive salience hypothesis [50, 51]. In this hypothesis, the role of dopamine is to convert the sensory information associated with rewards (i.e., cues) into an attractive and desirable incentive that will promote and stimulate specific goal-directed behaviors. Hence, dopamine is more strongly related to the anticipatory and reward seeking phases than to the consummatory phase of ingestive behavior [47, 50, 52]. However, the strength with which DA will respond to the cues associated with rewards, ultimately will be dependent on the internal homeostatic state and, hence, the need of the animal [53, 54].

Homeostatic and Hedonic Interaction in the Modulation of Motivated Behaviors

As mentioned, food intake, just as any other motivated behavior, is a complex behavior that is, in principle, modulated by energy signals [35, 55-58]. For example, in order to maintain homeostasis, when energy is available in excess, hormones such as leptin, insulin, cholecystokinin or GLP-1 can act centrally to promote energy expenditure and suppress food intake [59-63]. Conversely, other hormones such as ghrelin can promote food intake when there is an energy deficit [64, 65]. These energy signals can also act directly on the reward system to control behavior (Figure 1-4). Insulin receptors, for example, are present in DA neurons, and its activation can reduce food anticipation and conditioned place preference (CPP) for palatable food [66, 67]. Moreover, while leptin reduces DA neuron activity and decreases food intake and willingness to work for food [68, 69], ghrelin increases DA activity and produces the opposite behavioral effect [70-73]. Likewise, the appetite suppressant glucagon-like peptide-1 (GLP-1) can also act in the VTA to reduce intake of high-fat diet, and similar modulation by these hormones can be observed in the nucleus accumbens [74-78]. As mentioned, the mesocorticolimbic system is critically involved in modulating motivated behaviors (including feeding). However, we have also observed that this system does not act alone, but interacts with others, including those in charge of regulating homeostasis. As such, it is possible that drugs of abuse, by producing an abnormal and artificial increase in dopamine transmission [79], also can produce the dysregulation of other systems, closely connected with the reward system, but involved in a more systemic control of the organism. As a consequence, drugs of abuse would not only hijack the reward system to focus the behavior towards drug-seeking and taking, but also the homeostatic system, producing a state of psychological and physiological need for the drug.

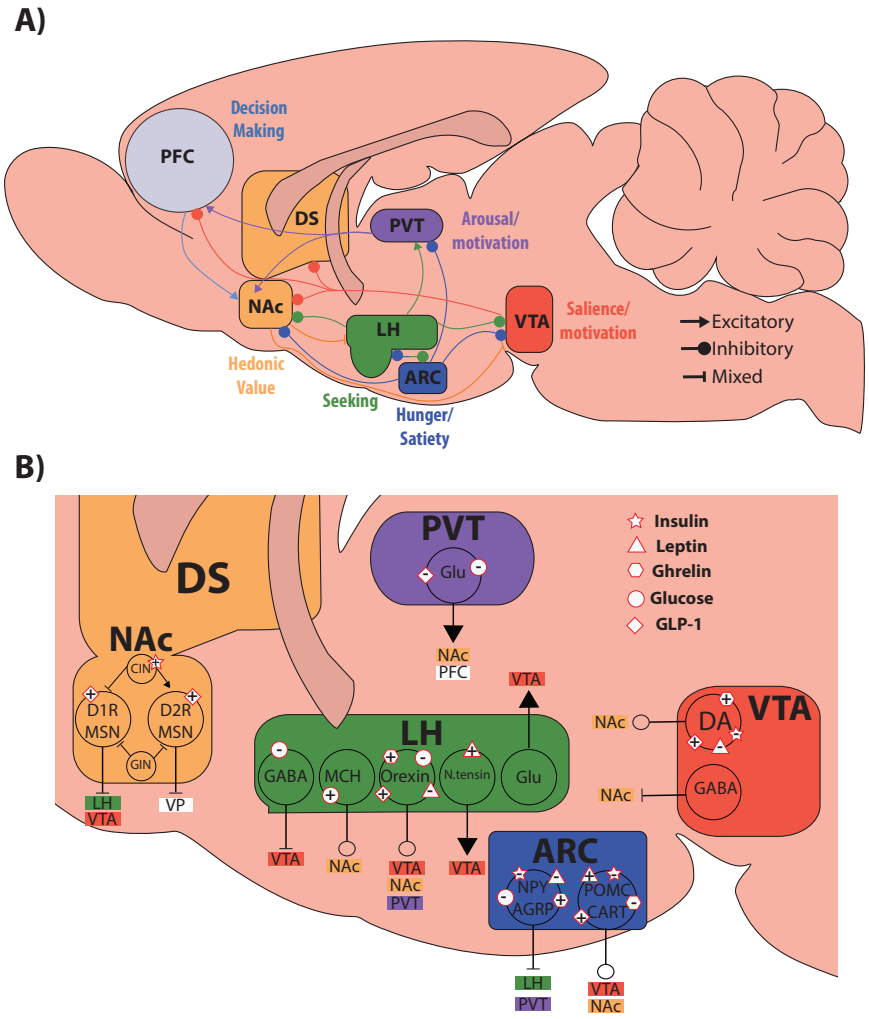

Figure 1-4. Major structures involved in the control of feeding behavior. A. Different areas of the brain are involved and interact in order to elicit behaviors associated with food consumption. For example, areas associated with hunger and satiety (ARC) interact with areas associated with arousal and seeking (PVT, LH). There is also modulation by areas of the reward system involved in providing hedonic value and salience to the stimuli (DS, NAc and VTA), and top down modulation from areas associated with decision making (PFC). These structures interact with each other via stimulating, inhibiting or mixed-modulating projections. **B**. Detailed microcircuitry between the nuclei. There is extensive cross-talk between the structures involved in modulating motivated behavior. Also, metabolic signals such as insulin, leptin, ghrelin, glucose and GLP-1 can act directly on receptors located withing these structures in order to further modulate the response. The illustration is a simplification and only some interconnections and brain regions are depicted. ARC: arcuate nucleus; DS: dorsal striatum, NAc: nucleus accumbens, LH: lateral hypothalamus; PFC: prefrontal cortex; PVT: paraventricular thalamus; VTA: ventral tegmental area; CART: cocaine- and amphetamine-regulated transcript; GIN: GABAergic interneuron; CIN: cholinergic interneuron; Glu: glutamate; MCH: melanin-concentrating hormone; NPY: neuropeptide Y, DA: dopamine; POMC: proopiomelanocortin; AGRP agouti-related peptide.

Addiction: Homeostatic Deficit and Need for Drug

Addiction has been described as the pathological usurpation of neural systems associated with reward-related learning [80, 81]. In other words, by artificially and excessively stimulating the release of dopamine, drugs of abuse hijack the reward system, directing its focus exclusively towards drug-seeking and drug-taking [79, 82]. As a consequence, drugs become the main goal to the detriment of other natural rewards such as food, sex, money or work. This phenomenon has been called devaluation of natural rewards, and has devastating consequences for the individuals and the society. For example, human studies have demonstrated that addicted individuals will invest more time and resources in seeking and taking drugs than in other activities. Moreover, individuals that suffer from addiction exhibit decreased efficiency and loss of productivity in the workplace [83], show a decreased sensitivity to monetary rewards [84, 85], and often provide insufficient care to their children [86]. In addition to the behavioral manifestation of drug addiction, a deviation of normal physiology is also observed in this population. For example, individuals that show substance abuse frequently are anorexic, under-nourished, and show wasting of muscle mass along with immunosuppression [87].

The phenomenon of reward devaluation has also been observed in animals. For example, in a conditioned place preference test female rats showed greater preference for the box associated with cocaine than the one associated with their own pups [88]. Similarly, hungry and thirsty rats avoid intake of a palatable saccharin solution that predicts the availability of a drug of abuse [89]. This phenomenon was used to model reward devaluation in rodents. In this model, the saccharin cue predicts availability of the drug and is avoided whether the drug of abuse is a depressant or stimulant of the central nervous system. [90-94]. However, the shift in motivation to consume the drug-paired cue is not the only phenotype observed. When the drug-paired

saccharin cue is infused directly into the oral cavity, rats will emit aversive stereotypic orofacial expressions called gapes (i.e., aversive taste reactivity behaviors) [95]. Rats, then, demonstrate both a decrease in "wanting" and "liking" of the drug-paired cue. In fact, as described, intraoral delivery of the drug-paired cue elicits not only a reduction in ingestion, but an increase in rejection (i.e., conditioned aversion). Importantly, the aversive response emitted by the rats depends upon there being a delay between presentation of the saccharin cue and drug availability [96]. In this context, it is hypothesized that rats reject the drug-paired saccharin cue because, in addition to predicting drug availability in the near future, cue availability also is associated with the waiting period, and as such it elicits the onset of an aversive state of conditioned withdrawal [95] .

Similar to conditioned withdrawal [97-100], avoidance of the drug-paired taste cue is also associated with blunted levels of dopamine [101], an increase in levels of the stress hormone corticosterone [102], the emission of aversive, 22 kHz ultrasonic vocalizations (unpublished data) and loss of body weight [103]. Moreover, greater avoidance of the taste cue and greater aversive taste reactivity behaviors are associated with increased drug-taking and load-up on drug, greater drug-seeking during periods of signaled drug non-availability, more willingness to work for drug, and greater susceptibility to drug-induced reinstatement of drug-seeking behavior [93, 95, 104, 105]. We hypothesize, then, that the aversive state of conditioned withdrawal elicited by the drug-associated cue, is similar to a state of physiological need.

The Need-State Hypothesis

Previously, our laboratory has hypothesized that the physiological state associated with craving a drug is akin the state observed in individuals under physiological need, who seek food

when starved, seek water when thirsty and salt when sodium deficient [106]. When such a state of need is reached, there is one goal and there is no substitute. In a normal animal, the internal physiological state guides the motivated behaviors in order reach homeostasis, and the behavior switches from one motivated state to another, depending on the needs of the system at any given time. However, as mentioned, drugs of abuse engage one motivated behavior (i.e., drug-seeking and drug taking) to the detriment of others, preventing the switch to other needed behaviors (see Figure 1-5). If the physiological state of an individual when craving drug is similar to the physiological need state of hunger, a known satiety signal that reduces seeking for food, can then potentially reverse such state and, in turn, reduce addiction-like behaviors such as drug-seeking and drug taking. In the last decade, a great number of scientists have taken advantage of the cross-talk between the reward and the energy homeostasis system in order to study addiction. In particular, the modulation of drug-associated behaviors by endocrine signals from the gut, such as GLP-1 and ghrelin, has shown exciting results. In this context, in this dissertation, we tested the need state hypothesis by studying the effects of different GLP-1 analogs on different aspects of heroin addiction in a rodent model.

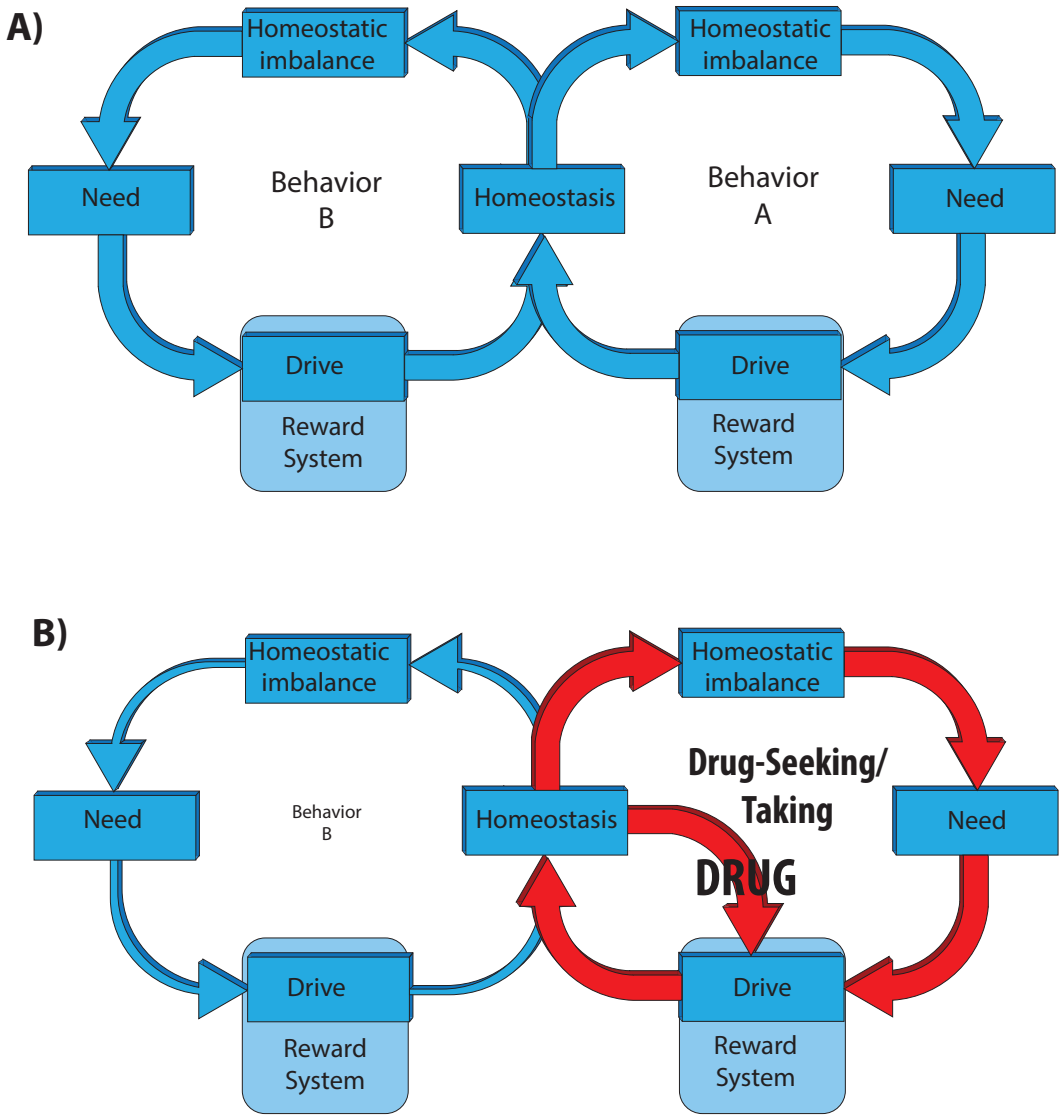

Figure 1-5. Homeostatic cycle and the need state hypothesis of addiction. A. In a healthy individual, the internal state (hemostatic, stress, hedonic) of the organism guide the motivated behavior. When the organism deviates from homeostasis, a state of need is generated. This will elicit the performance of a specific set of behaviors directed towards the goal that is needed, and such behavior will persist until the need is fulfilled and homeostasis is reached. Hence, in a normal individual, the behavior switches from one motivated state to another depending on the need of the system. **B**. Drugs of abuse artificially engage the reward system, generating impulsivity towards drug-taking. When addiction develops, a persistent homeostatic imbalance generates a state of need for the drug. Such a state of need narrows the behavior towards compulsive drug-seeking and drug-taking. Under this hypothesis, drugs of abuse not only hijack reward-associated areas, but also the homeostatic system. Then, if the state of need for the drug observed in individuals experiencing withdrawal is a state similar to that experienced during hunger and thirst, a 'satiety' agent that can reduce food intake in hungry animals, may be also effective on reducing craving for the drug.

Glucagon-like Peptide-1

Glucagon-like peptide-1 (GLP-1) was first described as an incretin hormone, secreted by enteroendocrine cells in response to nutrient ingestion that stimulates glucose-induced insulin secretion from the β-cells in the pancreas [107, 108]. GLP-1, like glucagon, is derived from pre-proglucagon, a peptide that is translated from the proglucagon gene [109] and can be processed into several other peptides such as glucagon, GLP-2, glicentin and oxyntomodulin [107, 109-111]. The main synthesis and release of GLP-1 occurs in the L-cells in the intestine, but it is also produced, to a lesser extent, in pancreatic α-cells and centrally within neurons in the nucleus of the solitary tract (NTS) [112-115]. In the intestine, the apical surface of the L-cells faces the lumen and has direct contact with luminal nutrients [116]. These nutrients interact with different receptors to either promote sodium influx and stop potassium efflux which will lead to the depolarization of the cell (Figure 1-6). The depolarization will produce an increase in intracellular calcium that will trigger vesicular exocytosis and, in consequence, secretion of GLP-1 into circulation [117]. There are several nutrients that stimulate GLP-1 secretions. For example, metabolizable monosaccharides, such as glucose, fructose and galactose, or non-metabolized monosaccharides, fatty acids, proteins and certain amino acids [118-125]. However, in addition to nutrients, GLP-1 release also can be directly regulated neurally and hormonally via direct stimulation or inhibition of L-cells, and/or indirectly, via modulation of gut motility [126-129].

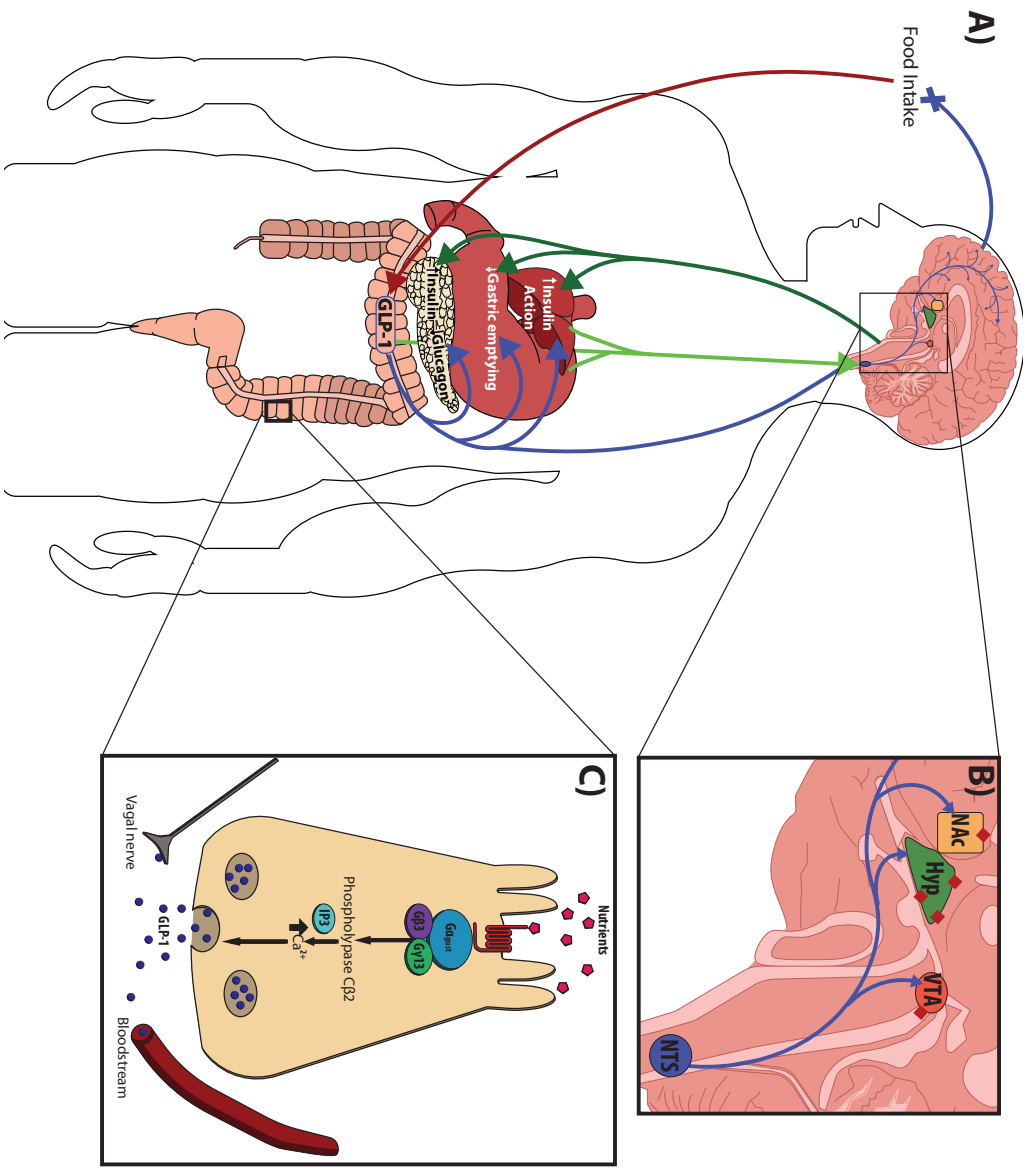

Figure 1-6. Central and peripheral effects of GLP-1. A. Food intake stimulates the release of GLP-1 by L-cells from the intestine. Gut GLP-1 can act directly in the pancreas to increase insulin and prevent glucagon release, reduce gastric emptying and increase insulin action in the liver. GLP-1 release can also stimulate the vagal nerve that will activate pre-proglucagon neurons in the NTS, promoting GLP-1 transmission in the brain. From the brainstem, efferent neurons signal to peripheral organs to increment these effects. **B**. Neurons that synthesize GLP-1 are found in the NTS. From there, these neurons project throughout the brain, sepcifically to areas associated with reward (nucleus accumbens and VTA) and homeostasis (Hypothalamus). Centrally, GLP-1 promotes satiety and reduces food intake. **C**. Peripherally, L-cells from the intestine produce GLP-1. Although intestinal cells can sense nutrient via different receptors, here the process is simplified using a G-protein coupled taste receptor. Upon nuetrient sensing in the cell surface, the signal cascade will elicit an increase in intracellular calcium that will trigger GLP-1 exocytosis. As mentioned, GLP-1 can act on vagal afferents or travel through the bloodstream to target organs.

GLP-1 Metabolism and Analogs

Endogenous GLP-1 has a short-half life, as it is quickly metabolized. GLP-1 is degraded by the enzyme dipeptidylpeptidase-4 (DPP-4), which cleaves GLP-1 at the alanine residue in the second position in the N-terminus. This process, generates GLP-1 (9-36) amide and GLP-1 (9-37), both peptides with lower affinity for the GLP-1 receptor [130-132]. In addition, circulating bioactive GLP-1, and its metabolites, are rapidly cleared by the kidneys. DPP-4 is both present in the cell surface and soluble in circulation [133], and has a high presence in the enterocyte brush border and endothelial cells lining the capillaries of the lamina propia [134]. As a consequence, most GLP-1 is degraded in the capillaries of the distal gut, only 25% reaches the liver and, finally, only a 10-15% reaches general circulation [134-136]. The effectiveness of this enzyme gives endogenous GLP-1 a half-life of 1-2 minutes. Exogenous GLP-1, on the other hand, can achieve a half-life of up to 13 minutes if administered intraperitoneally (ip), 7.1 minutes if administered subcutaneously (sc), and 4.7 minutes if administered intravenously (iv) [137].

Due to the incretin and food-reduction effects of GLP-1, several GLP-1 receptor agonists have been developed and are FDA-approved for the treatment of type 2 diabetes and obesity (Figure 1-7)[138, 139]. The first GLP-1R agonist, Exenatide, was approved for use in humans in 2005, and since then, 5 more agonists reached the market, providing extensive evidence on safety use in humans. Exenatide is a synthetic version of Exendin-4 (Ex-4), a natural GLP-1 analog derived from the saliva of the Gila Monster (*Heloderma suspectum*)[140]. Ex-4 has 53% homology to human GLP-1 and is resistant to degradation by DPP-4, by having a glycine as a second amino acid residue in the N-terminal region instead of the alanine, extending its half-life to 2.4 hours in rats and humans [137, 141]. Of the other 5 analogs, the second most used in animal research after Ex-4 has been liraglutide (LIR). This analog is a direct modification of

endogenous GLP-1, sharing a 97% homology with it [142]. These modifications consist of the addition of a C16 free fatty acid in the place of a lysine at position 26, and the replacement of arginine at position 34 that allows for a non-covalent binding to albumin [143, 144]. This interaction with proteins in the blood prevents LIR from being rapidly degraded by DPP-4 and cleared by the kidneys, and allows the GLP-1 portion to be released from albumin at a slow and consistent rate. As a consequence, the half-life of LIR can reach up to 13 hours in humans and 6-8 hours in rats [143, 145, 146].

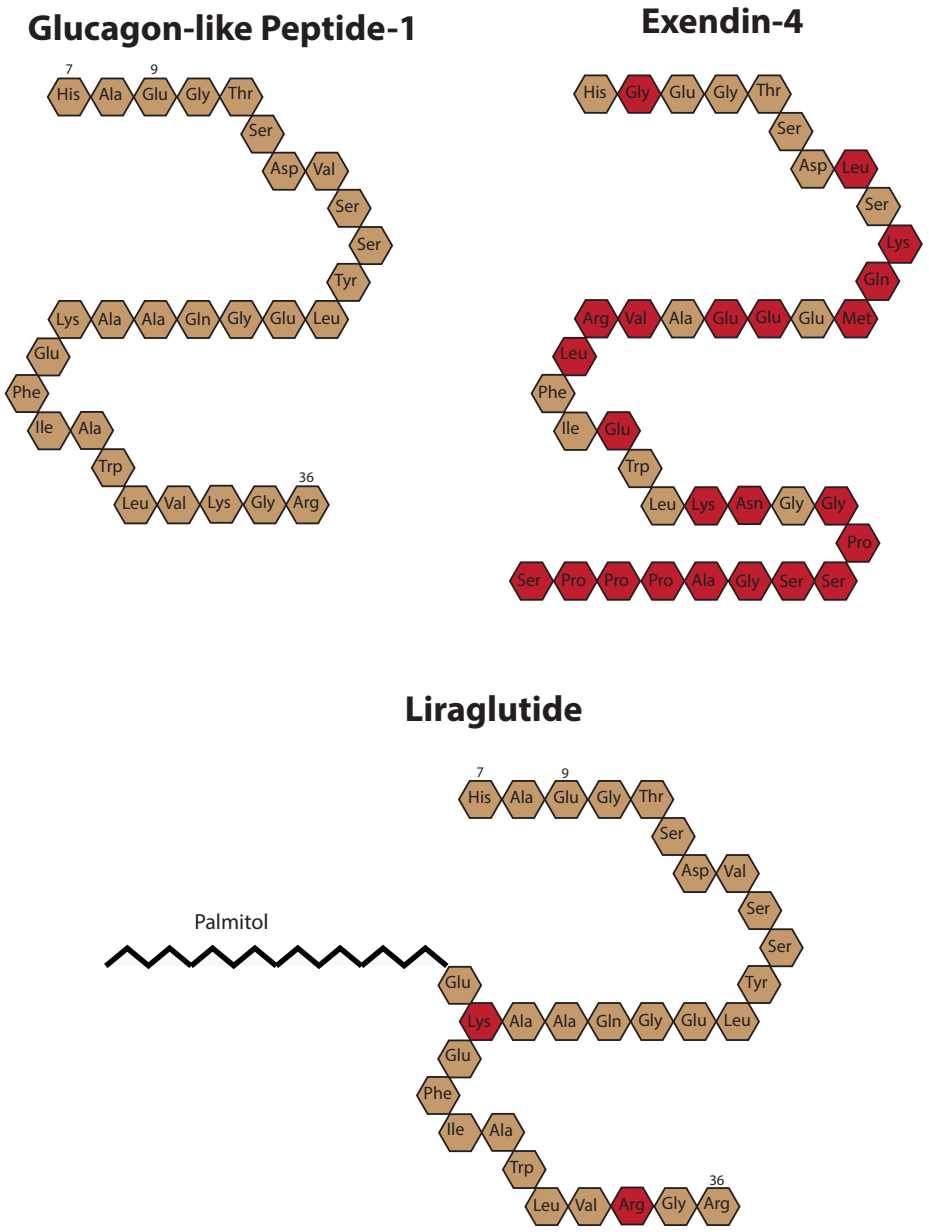

Figure 1-7. Glucagon-like Peptide-1 and its analogs. GLP-1 is a peptide comprised of 36 or 37 amino acids. GLP-1 (1-37 or 1-36) is further truncated on the N-terminus generating GLP-1 (7-37 or 7-36). Exendin-4 is a natural analog of GLP-1, sharing 53% homology with GLP-1. Liraglutide is a synthetic lipopeptide that shares 97% homology with GLP-1. Indeed, liraglutide is a modified GLP-1 molecule, with the addition of a C16 fatty acid chain (palmitoyl) in the position 26 in the place of a lysine, and the addition of an arginine at position 34 that allows for albumin binding. The amino acid sequence of endogenous GLP-1 is depicted in brown. In the exendin-4 and liraglutide, the residues that differ from endogenous GLP-1 are depicted in red.

Glucagon-Like Peptide-1 Acts Centrally to Modulate Food Intake

As mentioned, GLP-1 is also produced in a discrete set of pre-proglucagon (ppg) neurons in the caudal portion of NTS that serves as the primary source of endogenous GLP-1 in the brain [147-151]. From the NTS, ppg neurons project throughout the brain, where GLP-1 is released and acts upon the local GLP-1 receptors (GLP-1Rs). GLP1-Rs belong to the class B family of G-protein-coupled receptors (GPCRs) and as such, it is a seven transmembrane Gαs-coupled receptor that increases cAMP levels through adenylate cyclase activation [152, 153]. GLP-1Rs are widely expressed in the brain, especially in feeding-associated areas such as the hypothalamic nuclei, thalamus, hippocampus, lateral septum, and subfornical organ [151, 154, 155]. The expression of GLP-1Rs in these areas lead to the notion that GLP-1 has extra-pancreatic effects, probably related to the regulation of food intake, body weight, satiety and appetite. Indeed, suppression of food intake by GLP-1R activation has been described in numerous species [63, 156-162]. Although both centrally and peripherally administered GLP-1 analogs can reduce food intake, central GLP-1Rs are fundamental to elicit its anorectic effects [163, 164]. Specifically, the afferent projection of the vagal nerve transmits the information from the gut to the brain. This is evidenced by the attenuated effects of GLP-1 on food intake after a total subdiaphragmatic vagotomy [165]. In addition, GLP-1R can be found in the nodose ganglion, a region that shows c-fos activation following peripheral administration of GLP-1 [166]. From the nodose ganglion, the information is transmitted to the NTS, and from there to the rest of the brain [167]. Finally, it is also possible that GLP-1 secreted by the gut can cross the blood brain barrier to reach the central nervous system, but due to rapid degradation of GLP-1, it is not expected to act beyond proximal structures under physiological conditions (e.g., area postrema, subfornical organ) [168].

Glucagon-like Peptide-1 and Reward Behavior

The expression of GLP-1R in areas associated with reward such as the VTA, NAc, lateral septum and paraventricular thalamus (PVT) [169-173] has led scientists to think that central GLP-1 has a role beyond homeostatic feeding. For example, peripheral administration of Ex-4 has been shown to increase c-fos expression in the NAc [174]. In addition, direct activation of GLP-1Rs in this region produces hypophagia, while blockade of the receptors with Exendin (9-39) amide (Ex-9), a GLP-1R antagonist, increases food intake [171]. Moreover, both peripheral and central administration of a GLP-1R agonist in the NAc shell reduces operant responding for sucrose pellets in a progressive ratio test [169]. It also has been observed that Ex-4 reduces consumption of high-fat diet in sated rats given the choice between regular chow and the more palatable high-fat diet [170]. In addition, blockade of GLP-1Rs in the NAc alters licking microstructure for sucrose, increasing meal size and the frequency of licking during the first meal [175]. The authors of these last reports concluded that the GLP-1R agonist-mediated reduction in food intake is the consequence of a reduction in perceived palatability. However, there is evidence that this notion is not entirely accurate and this problem will be directly addressed in **Chapter 2** of this dissertation.

Glucagon-like Peptide-1 and Drugs of Abuse

Besides its effect on reward-driven feeding, GLP-1 has been shown to modulate drug-associated behaviors. The first experiment using GLP-1 in the context of drugs of abuse showed that doses as small as 0.1 μg/kg reduced cocaine-elicited CPP in mice [176]. Later, it was shown, also in mice, that a systemic injection of a dose of 2.4 μg/kg ip of Ex-4 could attenuate CPP,

locomotor activity and dopamine release elicited by both amphetamine and cocaine [177]. In addition, systemic Ex-4 reduced cocaine self-administration in mice [178] and both cue and drug-induced reinstatement of cocaine seeking behavior in rats [179]. Specifically, fluorescent-labeled Ex-4 injected peripherally was found in the NAc and the VTA, and injection directly into these areas reduced cocaine seeking [179, 180]. The effects of GLP-1R agonists on drug-associated behaviors also can be extended to other drugs, as they can reduce nicotine self-administration alcohol intake, and relapse-like drinking in mice [181-184]. However, little research has been done using GLP-1 analogs in the context of opioid addiction, and the results observed were contradictory. In one study, Ex-4 had no effect on CPP elicited by morphine, and failed to reduce remifentanil self-administration in mice [185]. On the contrary, in another study, both peripheral and intra-NAc Ex-4 reduced oxycodone self-administration and reinstatement of oxycodone-seeking behavior in rats [186]. In an effort to further understand the effects of GLP-1 analogs on opioid addiction, in the present study we used different formulations of the commercially available GLP-1R agonists and studied their safety and efficacy in the context of heroin addiction in rats.

Summary

The opioid epidemic has devastating consequences, including the death of more than 130 people per day in the United States. It is clear, then, that the focus must be on the development of new and more effective treatments in order to prevent more deaths. New avenues for treatment have been identified following a novel insight into our understanding of the disease. Specifically, by viewing drug-craving as a physiological need state, similar to that observed in individuals who crave food when hungry, we have identified new systems to target in order to prevent drug-

seeking and potentially drug-taking. Indeed, by understanding feeding regulating hormones as "seeking" or "stop-seeking" signals that potentially affect a wide variety of motivated behaviors, rather than as "feeding" or "satiety" signals linked only to food intake, new pathways and new treatments have been revealed. Glucagon-like peptide-1 and its analogs serve as a great example for this, and have shown excellent potential to reduce addiction-like behaviors associated with several drugs of abuse. However, little research has been done using GLP-1 analogs in the context of opioid addiction. In the present study, then, **Chapter 2** explores the effects of Exendin-4 in the different components of reward and aversion behavior, and the ways in which a GLP-1 analog can modulate the liking and wanting of stimuli with different hedonic valences in rats. In the context of heroin addiction, **Chapter 3** tests whether administration of the short-acting Ex-4 during abstinence and at test can reduce both cue- and drug-induced reinstatement of heroin seeking behavior. In addition, **Chapter 4** tests whether treatment with the longer-acting GLP-1R agonist, LIR, can reduce not only cue- and drug-induced reinstatement of heroin-seeking behavior, but heroin self-administration behavior as well. Finally, given the clinical need for a non-opioid treatment to reduce acute withdrawal, **Chapter 5** tests whether LIR can be used, acutely, to prevent heroin seeking in different models including cue-, drug-, and stress-induced reinstatement of heroin-seeking behavior in rats. Overall, the data in this dissertation show that GLP-1 analogs are safe and effective to treat opioid addiction, and as such they can be used as a potential treatment to help people that suffer from OUD.

Chapter 2

Effects of a glucagon-like peptide-1 analog on appetitive and consummatory behavior for rewarding and aversive gustatory stimuli in rats

Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by the gut [187] and a neurohormone released by neurons located in the nucleus tractus solitarius (NTS) [151, 154, 188, 189]. GLP-1 is essential for the regulation of food intake, as both central and peripheral administration [63, 158, 165] reduce food consumption. Consequently, GLP-1 is thought to act as a 'satiety' agent, and GLP-1 analogs have been approved for the treatment of type 2 diabetes and obesity [139, 190, 191]. More recently, in addition to its homeostatic effects, GLP-1 and its analogs (e.g., Liraglutide, Exendin-4) have been investigated for their ability to modulate the reward system. In this context, the 'satiety' effects of GLP-1 on reward-driven eating [169] are extended to inhibition of reward-driven behaviors for drugs of abuse as well [176, 178, 179, 181, 182, 192].

How GLP-1 modulates reward-associated intake behaviors is not fully understood. It was first thought that the anorectic effects of GLP-1 were mediated by visceral illness, as doses that reduced food intake in animals also supported conditioned-taste aversion/avoidance (CTA) [193, 194]. It was later discovered, however, that the aversive and anorectic effects of GLP-1 are dissociable [195]. Indeed, while GLP-1 receptor activation can itself support CTA [193, 194], it does not always induce pica (i.e., the eating of clay or kaolin, which has been observed to have antiemetic effects in humans) as would be expected if the agonist caused gastrointestinal malaise [169, 196]. These data imply more nuanced mechanisms by which GLP-1 modulates both reward and aversion.

Reward-associated intake behavior has two components: the motivational component inferred from the willingness of the animal to approach and initiate consumption; and the affective component inferred from the behavior driven by the sensory feedback of the stimulus

[46, 197]. Evidence suggests that the reduction in food intake induced by GLP-1 or its analogs is due, at least in part, to a reduction in the motivation to obtain food [198, 199]. Thus, it has been observed that Ex-4 can reduce self-administration and conditioned place preference for sugar pellets [169]. A microstructural analysis showed that central Ex-4 decreases licking of sucrose by sham feeding rats, and blockade of GLP-1 receptors in the nucleus accumbens shell increases sucrose consumption [175, 200]. Importantly, each of these protocols (operant conditioning, conditioned place preference, and intake) requires that the rat approach and initiate consumption, suggesting that GLP-1 and its analogs reduce the motivational/consummatory aspect of reward.

That said, it is not at all clear how GLP-1 receptor agonists modulate the affective component of food reward. This question pertains to both rewarding and aversive gustatory stimuli. Thus, while GLP-1 and its analogs reduce intake of a sweet, it is not known whether the same GLP-1 analog will reduce ingestive taste reactivity behavior (i.e., licking) when the sweet solution is infused directly into the oral cavity. This question is addressed in Experiment 1 of the present study. We predict that treatment with the GLP-1 receptor agonist, exendin-4 (Ex-4), will reduce ingestive responding following the intraoral delivery of sucrose indicating a corresponding reduction in the perceived palatability of the stimulus. Previous results support the hypothesis that GLP-1 affects not only satiety, but perceived palatability as well [200].

In contrast to the effects of GLP-1 and its analogs on responding to rewarding stimuli, little data addresses the impact of a GLP-1 agonist on responding for aversive stimuli. As a result, Experiment 1 also tests whether Ex-4 will alter the aversive taste reactivity behavior (i.e., gapes) emitted following the intraoral delivery of a quinine (QHCl) solution. Experiment 2 examines the effect of Ex-4 on the motivation to approach and ingest QHCl in water restricted rats. We predict that Ex-4 will increase the perceived aversive properties of QHCl leading to an increase in gaping behavior following intraoral delivery in Experiment 1 and a probable reduction in QHCl intake by

thirsty rats in Experiment 2. Finally, Experiment 3 tests the effect of Ex-4 on the conditioned aversive taste reactivity behavior (i.e., gapes) elicited by intraoral delivery of a Lithium Chloride (LiCl)-paired saccharin cue. Given that GLP-1 and its analogs can, themselves, induce a CTA [193, 194], we predict that treatment with the GLP-1 analog, Ex-4, will increase aversive taste reactivity behavior following intraoral delivery of the LiCl-paired taste cue. Together, this set of experiments explores the role of a GLP-1 receptor agonist on the different aspects of reward behavior (consummatory and appetitive) to innate and learned rewarding and aversive stimuli.

Materials and Methods

Subjects

The subjects were 43 naive, male Sprague-Dawley rats delivered from Charles River Laboratories (Wilmington, MA) weighing between 300 – 400 g at the start of the experiment. All subjects were housed individually in standard, suspended, stainless steel cages. The environment in the animal care facility had controlled humidity and temperature (21 °C), with a 12/12 hour light/dark cycle, with lights on at 7:00 am. All experimental manipulations were conducted 2 hours into the light phase of the cycle. After one week of acclimation to their home cages, rats were habituated to experimenter handling by daily weighing. Food and water were available ad-libitum except where noted otherwise.

Experiment 1:

Taste Reactivity Test

The taste reactivity (TR) test takes advantage of the fact that affective responses (ingestion and rejection responses) to sweet and bitter solutions are conserved across species. Humans, chimpanzees, and even rats demonstrate immediate stereotypic oromotor responses when a tastant is placed in the oral cavity [201, 202]. In such experiments, facial expressions are recorded and analyzed during and after the taste is infused into the oral cavity in an effort to measure ingestion and rejection responses [202, 203]. To do so here, two intraoral cannulas were custom-made [104] and, under ketamine (70mg/kg, im) and xylazine (14mg/kg, im) anesthesia,

surgically implanted on both sides between the cheek and the jaw, lateral to the first maxillary molar, as describe previously [95, 96, 202, 203]. Once recovered from surgery, all rats were adapted to 15 min intraoral infusions (0.2 ml delivered over 3.5 s once/min) of either 0.5M sucrose or 0.003M QHCl in operant chambers with transparent Plexiglas sides and floors (MED Associates, St. Albans, VT) for seven consecutive days. On the eighth day, rats received an intraperitoneal (ip) injection of vehicle (saline), and 15 min later, were placed in the chamber where they received a 15-min intraoral infusion of either 0.5M sucrose (n=8) or 0.003M QHCl (n=9) while a video camera recorded their orofacial behavior as described [104]. On the ninth day, the same procedure was performed but a 2.4 μg/kg ip injection of Ex-4 was administered 15 minutes prior to the intraoral delivery of the tastants.

An angled mirror was located below the floor, allowing for a view of the ventral surface of the rat. A high-speed camera was positioned below the floor of the chamber facing the mirror to record the behavior. Taste reactivity behaviors were manually scored after the conclusion of the experiment. The responses were scored according to the categories described by Grill and Norgren [202]. Appetitive responses were scored as the frequency of bouts, separated by 4 seconds, of appetitive ingestive behaviors including tongue protrusions and rhythmic mouth movements. Aversive responses were scored as the total number of gapes, the latency in sec to emit the first gape, and the latency or number of passive drippings.

Experiment 2:

Quinine intake

This experiment was performed in behavioral chambers (MED Associates, Inc., St. Albans, VT) and QHCl intake was measured using a lickometer. Since rats will not voluntarily drink strong concentrations of QHCl, a different set of 12 naïve male Sprague-Dawley rats underwent a water restriction protocol in which they had 15 min access to fluid during the daily session and to 20 mL of water overnight. On the first two training days the rats drank water for 15 min daily from the spout in the chamber. The availability of the spout was signaled by a cue light located above. The rats then had 4 daily sessions of 15 min access to a lower concentration of quinine (0.0003M). On the test day, rats were injected ip with either 2.4 μg/kg Ex-4 (n=6) or vehicle (n=6). Fifteen min later, they were placed in the chamber, were given 15 min access to a more concentrated 0.003M QHCl solution, and latency to lick, number of licks, and inter lick intervals were measured.

Experiment 3:

Conditioned Taste Aversion/Avoidance

Intraoral cannulas were placed as described above in a naïve set of 14 adult male Sprague Dawley rats. After recovering from surgery, the rats were subjected to a water restriction protocol in which they had 15 min access to water each morning and 20 mL overnight. The rats had 2 consecutive days of habituation to the taste reactivity chamber where they received intraoral

infusions of water for 5 minutes. Then, they were subjected to two saccharin-LiCl pairings. On pairing days, two hours into the light cycle, rats were given 15 min access to 0.15% saccharin via an inverted graduated cylinder with a spout affixed to the front of the home cage. Immediately after, all rats received an ip injection of 0.15M LiCl (1.33 mL/100 g body weight). The pairings were followed by a single test day, with 48 hours between each conditioning and test trial. On the test day, rats were injected ip with either 2.4 μg/kg Ex-4 (n=7) or vehicle (n=7) fifteen minutes prior being placed in the Taste Reactivity Chamber. Once in the chamber, all rats received intraoral infusions of 0.15% saccharin (0.2 mL delivered over 3.5 s once/min) and their oromotor behavior was recorded as previously described across a 15 min test period. Once the session was over, rats were returned to their home cage where they were presented with 0.15% saccharin and water in a 24-hour, two-bottle test. The left/right placement of water and saccharin was counterbalanced across rats, and total intake was analyzed 24 hours later.

Data Analysis

All data were analyzed using Student's t-test or mixed factorial ANOVA. The Newman-Keuls post-hoc test, with $\alpha=0.05$, was used when appropriate.

Results

Experiment 1:

Sucrose. Figure 2-1 shows the responses emitted during the Taste Reactivity Test for vehicle (Veh) or Ex-4 treated rats during the intraoral infusions of sucrose. The Veh-treated group showed a high frequency of appetitive bouts in the taste reactivity test following the infusion of sucrose (Figure 2-1A). In addition, these rats showed a low number of passive drips (Figure 2-1B), and no gaping behavior (Figure 2-1D). The latency to gape was, consequently, quite long (Figure 2-1C). Pretreatment with Ex-4 fifteen min prior the intraoral delivery of sucrose had no effect on these behaviors and did not reduce appetitive responding for sucrose (Figure 2-1A).

Quinine. Figure 2-2 shows the orofacial responses in the Taste Reactivity Test for Veh or Ex-4 treated rats during the intraoral delivery of 0.003M QHCl. As expected, this group showed a low frequency of appetitive bouts during the session, whether treated with Veh or Ex-4 (Figure 2-2A). Intraoral delivery of quinine was associated with a relatively high number of passive drips (Figure 2-2B), but passive dripping was not significantly affected by treatment with Ex-4, $p > 0.05$. Veh treated rats infused with QHCl showed a short latency to gape (Figure 2-2C) and a high number of gapes throughout the session (Figure 2-2D). Contrary to our prediction, treatment with Ex-4 fifteen minutes prior to infusion of the tastant, however, significantly increased the latency to initiate the 1st gape [$t(8)=2.58$; $p<0.05$; Figure 2-2C] and significantly reduced the number of gapes elicited by 0.003M QHCl compared with the number emitted by the Veh-treated QHCl infused rats [$t(8)=2.6$; $p<0.005$; Figure 2-2D].

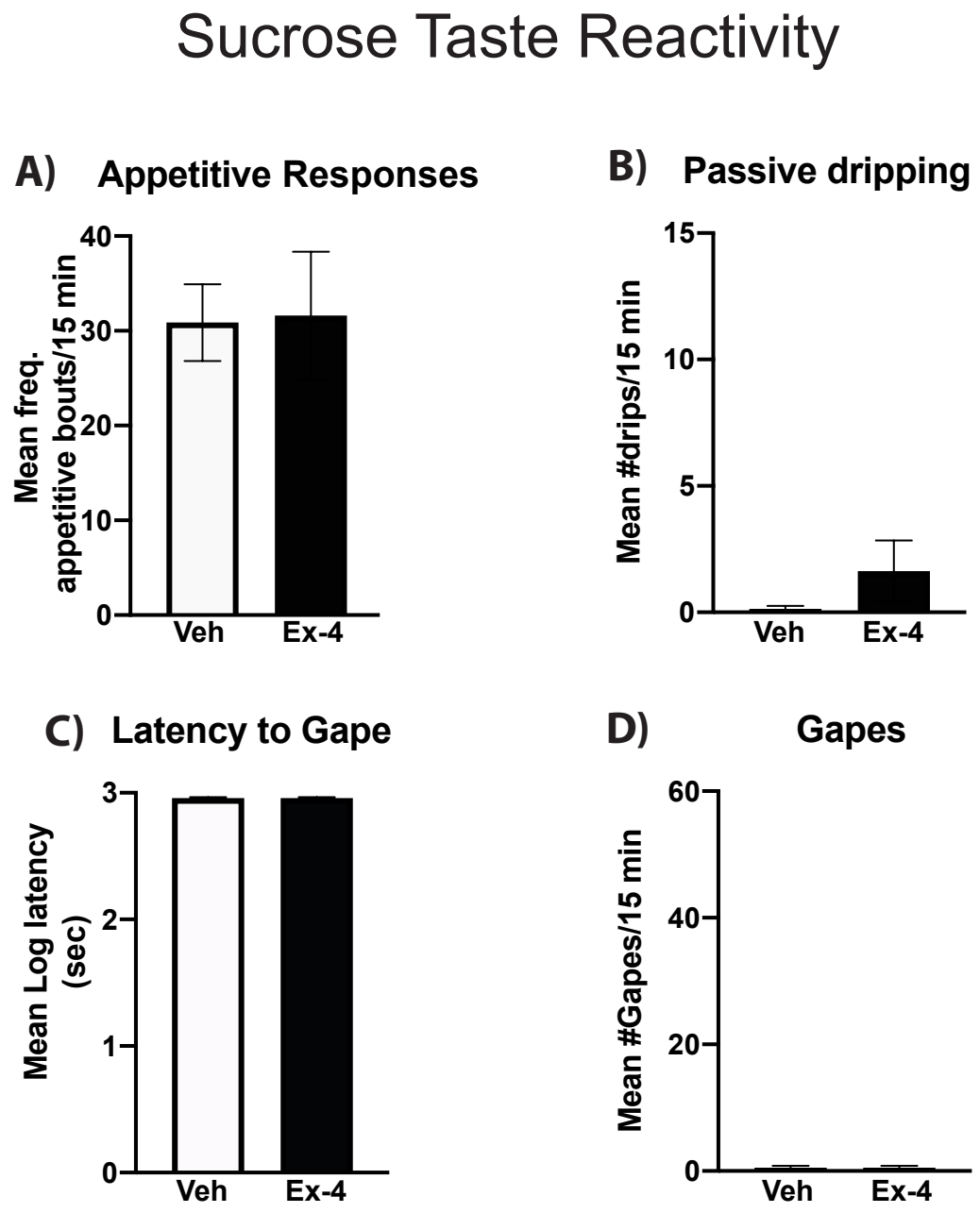

Figure 2-1. Sucrose Taste Reactivity Test. Rats (n=8) were subjected to this test across two days. On the first day they were treated with vehicle (Veh) and the second day with 2.4μg/kg Ex-4. **A**. Mean (± SEM) frequency of appetitive bouts during the 15 min during the intraoral (IO) delivery of 0.5 M sucrose in rats treated with vehicle or exendin 4 (Ex-4). **B**. Mean (± SEM) number of passive drips emitted during the 15 min IO delivery of 0.5 M sucrose for Veh and Ex-4 treated rats. **C**. Mean (± SEM) log 10 latency (in sec) to elicit the 1st gape to sucrose for Veh and Ex-4 treated rats. **D**. Mean (± SEM) number of aversive responses (gapes) elicited by intraoral infusion of 0.5M sucrose in Veh vs. Ex-4 treated rats.

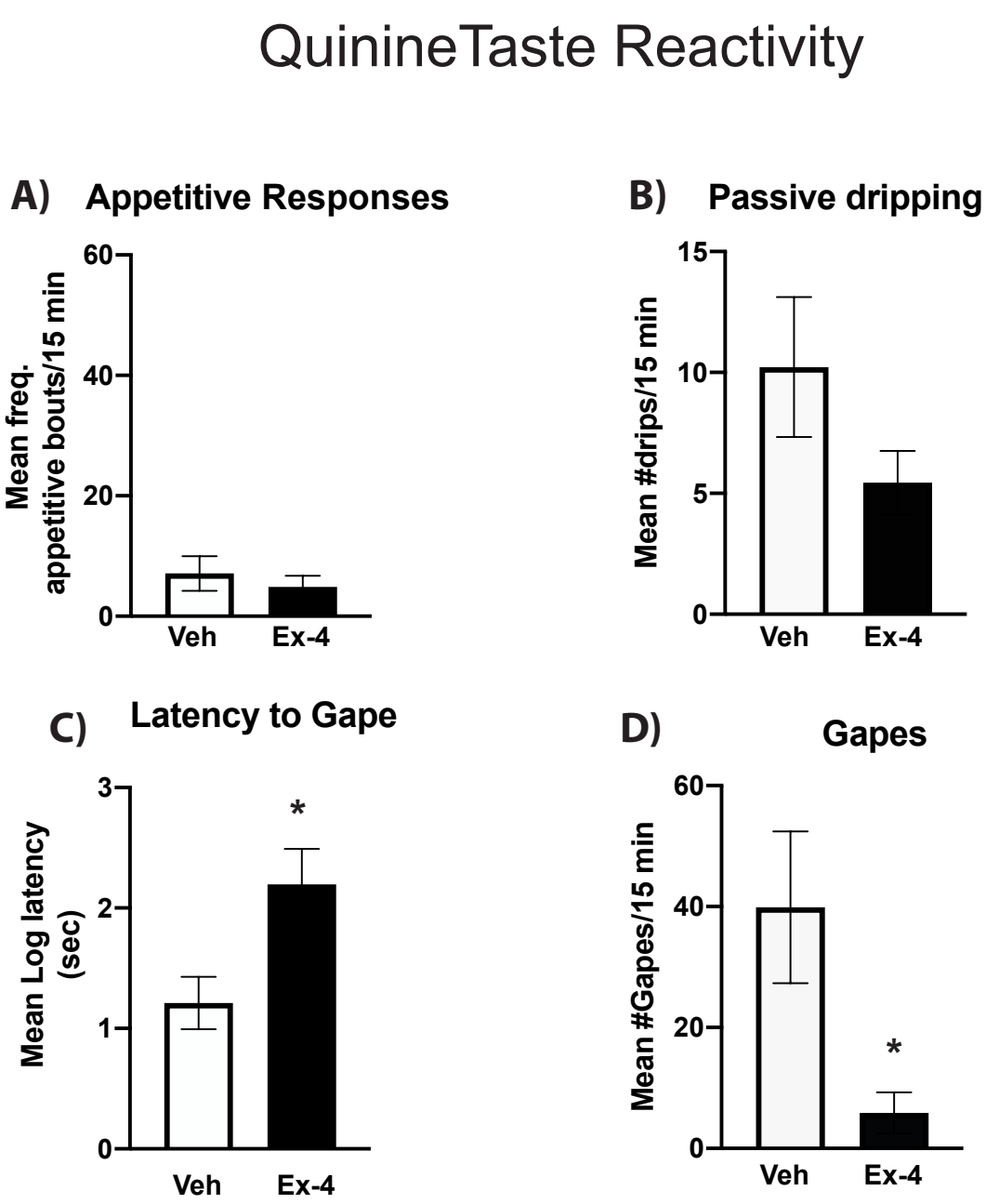

Figure 2-2. Quinine Taste Reactivity Test. Rats (n=9) were subjected to this test in two consecutive days. On the first day they were treated with vehicle (Veh) and the second day with 2.4 µg/kg Ex-4. **A**. Mean (± SEM) frequency of appetitive responses/15 min during the IO infusion of 0.003 M quinine in rats treated with vehicle or exendin-4 (Ex-4). **B**. Mean (± SEM) number of passive drips/15 during the IO infusion of 0.003 M quinine in rats treated with Veh or Ex-4. **C**. Mean (± SEM) log 10 latency (sec) to elicit the 1st gape following the IO infusion of quinine in Veh and Ex-4 treated rats. **D**. Mean (± SEM) number of aversive responses (gapes)/15 min IO infusion of quinine in Veh and Ex-4 treated rats. *p<0.005

Experiment 2:

Figure 2-3 shows behaviors associated with QHCl intake in water deprived rats. There was no significant difference in the latency to initiate the 1st lick of QHCl between Ex-4 and Veh-treated rats, [$p>0.05$; Figure 2-3A]. Nevertheless, intake of the aversive solution was different between the groups. While Veh-treated rats made over 200 licks/15 min for the 0.003 M QHCl solution, rats treated with Ex-4 made less than 50 licks/15 min for the same solution [Figure 2-3B; $t(10)=3.6$; $p<0.001$]. In addition, the reduced number of licks emitted by the Ex-4-treated group for the 0.003 M QHCl solution was associated with a significantly longer inter-lick interval (ILI)[$t(10)=4.35$, $p=0.001$; Figure 2-3C].

Quinine Intake

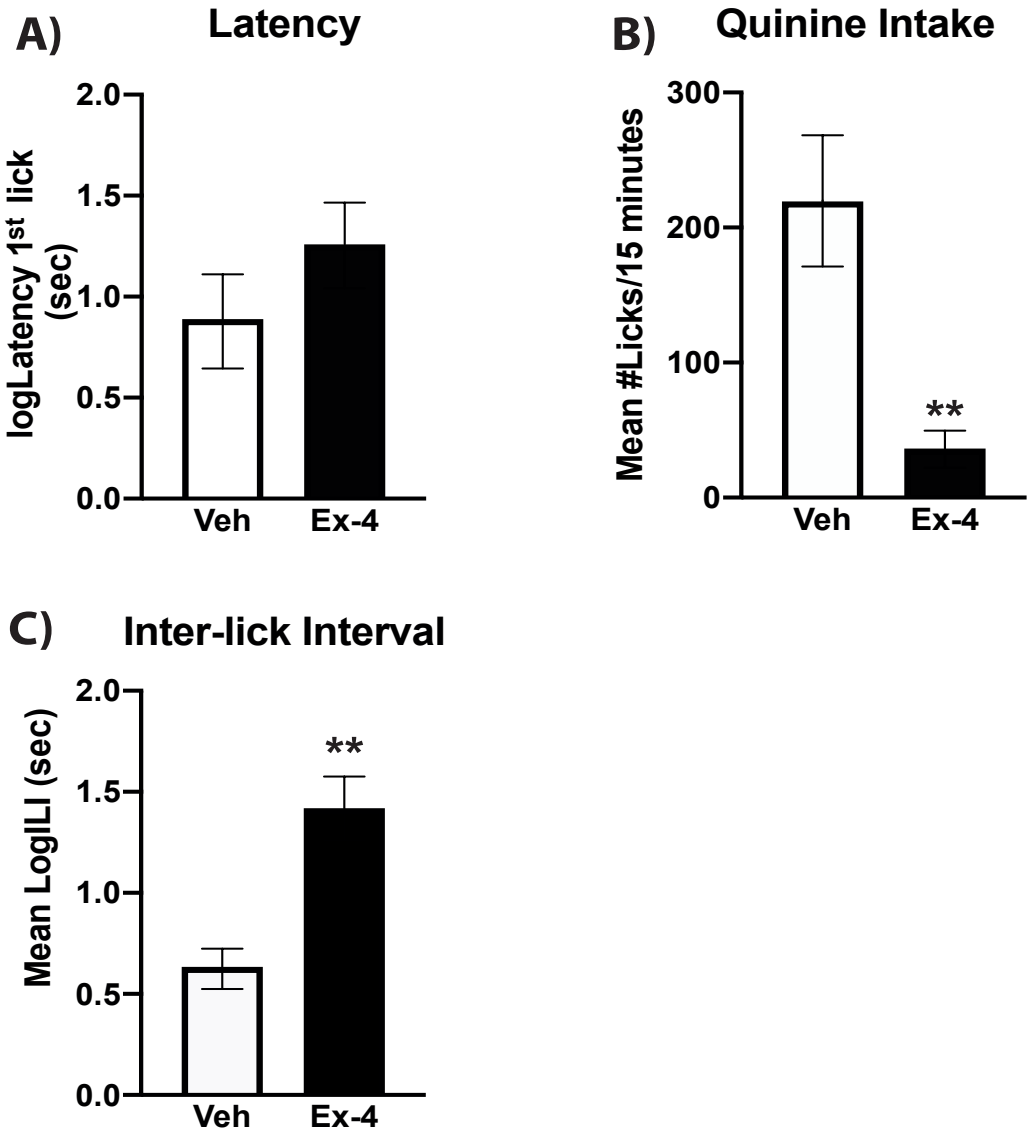

Figure 2-3. Intake of quinine. Water deprived rats were trained to drink 0.0003M QHCl. On the test day they were treated with either vehicle (Veh; n=6) or 2.4 μg/kg Ex-4 (n=6) 15 min before being placed in the chamber where they had access to 0.003M QHCl. **A.** Mean (± SEM) log 10 latency (sec) to emit the 1st lick for 0.003M quinine. **B**. Mean (± SEM) number of licks/15 min emitted for 0.003 M Quinine. **C**. Mean (± SEM) log 10 Inter-lick interval. Vehicle: white bars, n=6. Ex-4: black bars, n=6. **p<0.001.

Experiment 3:

In Experiment 3 we tested the effect of Ex-4 on the aversive taste reactivity behavior elicited by a LiCl-paired saccharin cue, followed by a 24-hour two-bottle test. The results of the Taste Reactivity Test confirmed that treatment with Ex-4 had no effect on either the latency to passive drip or on the latency to gape following intraoral infusion of the LiCl-paired saccharin cue, [$p>0.05$; see Figures 2-4A and 2-4B]. Moreover, rats that had the saccharin paired with LiCl and were treated with Veh on the test day showed a high number of gapes during the session. While numerically lower (Figure 2-4C), treatment with Ex-4 did not significantly reduce the number of gapes emitted following intraoral infusion of the LiCl-paired saccharin cue (35.5 gapes/15min) relative to the Veh-treated controls (54.8 gapes/15 min) [$p>0.05$]. Finally, in the Two-bottle Test no difference was observed. All rats, Veh and Ex-4 treated, showed high 24-h water consumption, while none of the rats consumed the LiCl-paired saccharin cue (Figure 2-4D).

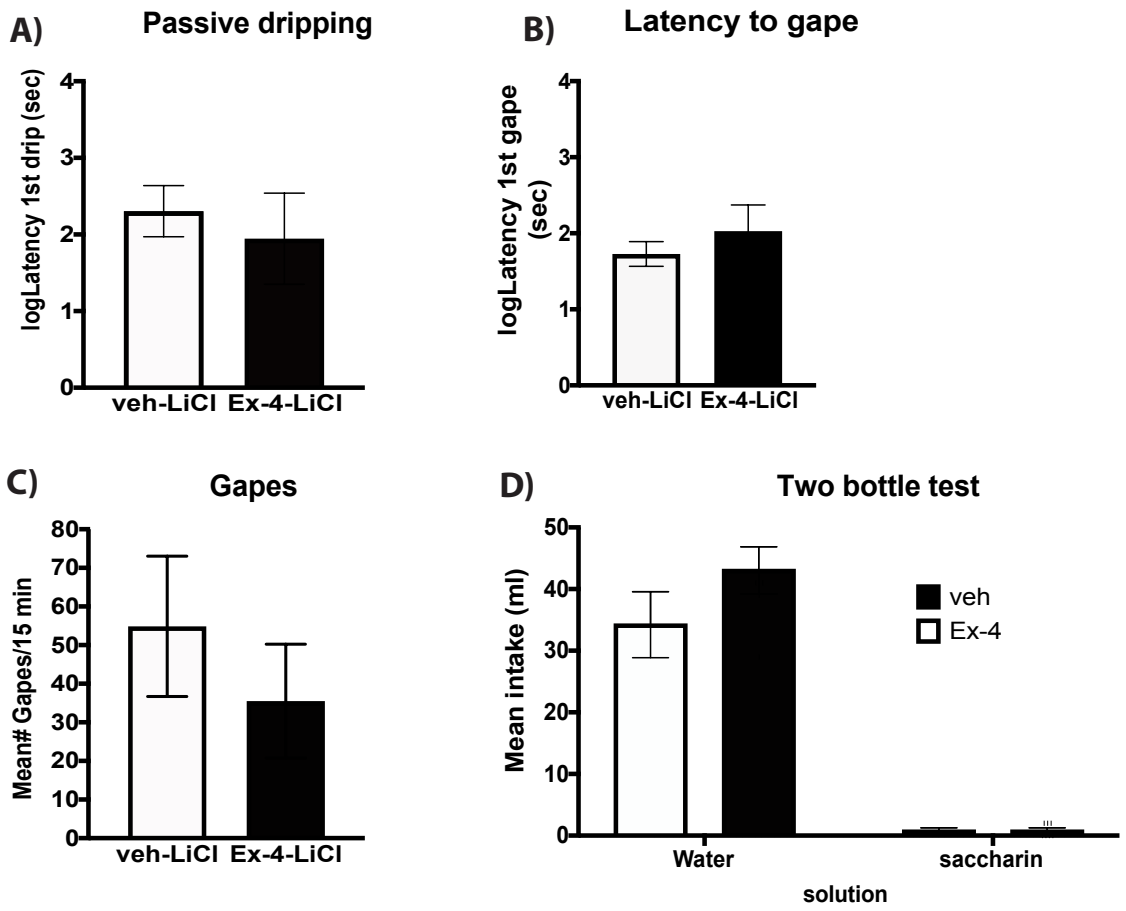

Figure 2-4. Conditioned Taste Aversion/Avoidance. All rats had 2 pairings of 0.15% saccharin with LiCl. On the test day, rats were treated with either vehicle (Veh; n=7) or 2.4 μg/kg Ex-4 (n=7) 15 minutes prior test. Conditioned taste aversion was measured using the Taste Reactivity Test and conditioned taste avoidance was assessed using a Two-bottle Test. Taste Reactivity Test: **A**. Mean (± SEM) log 10 latency (sec) to 1st passive drip following intraoral delivery of the LiCl-paired saccharin cue in Veh and Ex-4 treated rats. **B**. Mean (± SEM) log 10 latency (sec) to elicit the 1st gape following the IO infusion of the LiCl-paired saccharin cue in Veh and Ex-4 treated rats. **C**. Mean (± SEM) number of aversive responses (gapes/15 min) during the IO delivery of the LiCl-paired saccharin cue in Veh or Ex-4 treated rats. **D**. Two-bottle Test. Mean (± SEM) intake (ml/24 h) of water vs. saccharin in rats treated with Veh or Ex-4. Vehicle: white bars, n=7. Ex-4: black bars, n=7.

Discussion

The Taste Reactivity Test is a reliable method to detect ingestion and rejection responses to sapid stimuli when administered directly into the oral cavity [202, 204]. Mouth movements following intraoral delivery of a sweet match those made during normal licking behavior, occurring at a rate about 7/sec [205], and they increase in frequency monotonically with increasing concentrations of the stimulus [202]. Rejection behaviors, such as gapes, are important, particularly for non-emetic animals like rats, as gapes are part of a complex set of behaviors that allow the animal to expel any potentially harmful substance from the oral cavity [202]. Like mouth movements, the number of gapes emitted also increases as a function of the concentration of the bitter substance [206]. Collectively, these behaviors are thought to reflect the valence of the stimulus (i.e., whether the stimulus is perceived as rewarding or aversive) and, in some cases, to reflect the "affective" response of the organism [46, 197].

In the present study, we showed that treatment with Ex-4 had no effect on taste reactivity responses following intraoral delivery of 0.5M sucrose. Thus, while GLP-1R agonists reduce conditioned place preference for a context paired with a sweet and intake of a sweet [169, 170, 198, 207], in this study Ex-4 did not reduce the frequency of mouth movements or tongue protrusions elicited by the palatable stimulus when placed directly into the oral cavity. Altogether, these data suggest that GLP-1 analogs reduce the motivation to seek and consume sucrose. Once the solution is in the mouth, however, Ex-4 does not alter the apparent perceived intensity or palatability of the stimulus. This finding is consistent with an earlier report showing that, while treatment with Ex-4 reduced intake of sucrose in a two-bottle test, it did not alter the concentration-response function for sucrose in a brief-lick test, a test that is more sensitive to the sensory properties of the stimulus but relatively insensitive to post-ingestive feedback [208]. That

said, it has been observed that Ex-4 reduced sham intake of sucrose by decreasing the lick rate, burst size, and cluster size [200]. Given that sham-feeding rats lack most post-ingestive feedback, this reduction in licking behavior by Ex-4 was thought to reflect a disruption in the perceived orosensory value of sucrose rather than an increase in satiety. Even so, the rather long test duration used in the sham feeding study (45 min) may not allow for a separate assessment of the motivational vs. the affective components of food intake because longer test periods require the rats to approach and initiate consumption repeatedly. The advantage afforded by the taste reactivity test is that it by-passes approach and hence the motivational aspect, allowing for an independent assessment of the affective component. As such, we conclude that Ex-4 reduces the willingness to seek and consume the sweet, without affecting its perceived palatability.

We also found that Ex-4 treatment can delay the onset of gaping behavior and reduce the number of gapes emitted following an intraoral infusion of 0.003M QHCl. When placed directly into the oral cavity, rats treated with Ex-4 exhibited an 84% reduction in gapes to QHCl and a 1-fold delay in the start of this usually robust rejection behavior. These results are remarkable as 0.003M QHCl typically is perceived as a potent aversive stimulus, and few substances are known to reduce the apparent aversion by rats when a bitter solution is introduced into the oral cavity [209, 210]. In two studies, pretreatment with either morphine (2 mg/kg sc) or low doses of d-amphetamine (0.25-0.5 mg/kg ip), both 30 min prior, reduced gaping behavior elicited by 0.005% or 0.05% QHCl. It was suggested that these drugs reduced QHCl palatability via an increase in dopaminergic transmission. Nevertheless, amphetamine infused directly into the nucleus accumbens shell [211] failed to alter taste reactivity to either sucrose or QHCl, complicating the simpler interpretation. Further research is needed to identify the structure(s), and the mechanism(s), by which the GLP-1R agonist so effectively reduces the apparent perceived aversiveness of QHCl.

These data raised the question as to whether the Ex-4-induced reduction in aversive taste reactivity behavior to QHCl would be accompanied by an increase in intake of the stimulus. This hypothesis was tested in Experiment 2 and the results showed that ip administration of Ex-4 reduced, rather than increased, intake of the aversive concentration of QHCl in thirsty rats. These results mimic those observed with intake of other substances such as salt, water, sweets, and chow [63, 169, 212, 213]. Thus, our data suggest that the GLP-1R agonist reduces overall motivation, not only for rewarding, but also for aversive gustatory stimuli. Rats, then, are less likely to approach and consume the QHCl solution with Ex-4 on board but, if placed directly in the oral cavity, exhibit markedly less aversive TR behavior.

Along with gaping following intraoral delivery of an innately aversive gustatory stimulus, such as QHCl, rats also gape following the intraoral delivery of an otherwise palatable gustatory cue following pairings with the illness-inducing agent, LiCl [214, 215]. Consequently, we tested whether the GLP-1R agonist also would block a LiCl-induced CTA using both taste reactivity to study the affective response, and a two-bottle test to study motivation. The results verified that systemic administration of Ex-4 does not affect the expression of a conditioned taste aversion elicited by LiCl. Specifically, compared with the Veh-treated controls, Ex-4 did not delay passive dripping, the onset of gaping behavior, or significantly reduce the number of gapes to the LiCl-paired cue. In addition, Ex-4 did not affect consumption of the LiCl-paired saccharin solution (which was nil) in a subsequent 24-hour two-bottle test. A GLP-1 agonist, then, can reduce rejection of an innately aversive gustatory stimulus, without affecting learned aversion. Further research is needed to assess the neural substrates to better understand how Ex-4 differentially modulates motivated vs. affective behaviors and innate vs. learned aversion.

Taken together, these findings shed light on how GLP-1 modulates both reward and aversion, motivated and affective behavior, and innate and learned aversion. Specifically, while

GLP-1 receptor activation reduces the motivation to seek and ingest a palatable foodstuff [169, 175], it does not reduce the perceived hedonic-value of that stimulus, at least in the case of sustained appetitive taste reactivity for 0.5M sucrose. GLP-1 receptor activation also reduces the motivation to seek and ingest a normally rejected stimulus, such as QHCl, but it does reduce gaping to that stimulus once placed in the oral cavity. Finally, peripheral administration of Ex-4 had no effect on either the motivation to consume or the perceived aversiveness of a LiCl-paired saccharin solution. If these data provide insight into the function of endogenous GLP-1, then the satiety mediated by this peptide reduces intake without reducing the perceived value of an otherwise palatable foodstuff and without supporting the establishment of a new conditioned aversion. If this relationship generalizes across other food stimuli, the adaptive value of such a function could be the following: Satiety is a necessary but temporary state. During this period the animal should sustain the value of the sated food even while it refrains from consuming it.

Manuscript: Effects of a glucagon-like peptide-1 analog on appetitive and consummatory behavior for rewarding and aversive gustatory stimuli in rats. (*Under Review*)

Authors: Joaquin E. Douton, Ralph Norgren, and Patricia Sue Grigson

Respective contributions: J.E. Douton: Experimental design and execution, data analysis and interpretation and manuscript preparation. R. Norgren: Data interpretation and manuscript revision. P.S. Grigson: Experimental design, data interpretation and manuscript revision.

Chapter 3

Glucagon-like peptide-1 receptor agonist, exendin-4, reduces reinstatement of heroin seeking behavior in rats

Introduction

Drug addiction is a chronic disease that is difficult to treat due to its relapsing nature [2]. Deaths related to drug overdose tripled from 1999 to 2014 and, in 2017, around 70% of the drug overdose deaths involved opioid use [216]. Indeed, misuse of prescription pain relievers, heroin, and synthetic opioids causes the death of more than 130 people a day in the United States alone, a tragedy of epidemic proportion and a major concern for the Centers for Disease Control and Prevention (CDC) [10]. As a consequence, it is imperative that we find effective treatments for drug addiction that can mitigate the craving and withdrawal that precipitates relapse and, thereby, increases vulnerability to opioid overdose [11].

Addiction is recognized as the pathological usurpation of neural systems associated with reward-related learning [80, 81]. In this context, the drug becomes the prominent goal, and in turn, natural rewards such as sex, food, work and money are devalued. This shift in motivation can be observed in individuals with a substance use disorder who fail to provide sufficient care to their children [86], show loss of productivity in the workplace [83], and decreased sensitivity to monetary rewards [84]. Reward devaluation also has been observed in animal models, as female rats exposed to cocaine show greater preference for drug-associated stimuli than for their own pups [88] and hungry and thirsty rats will avoid intake of a palatable solution when it predicts the availability of a drug of abuse [89, 93, 105].

In this latter case, we have hypothesized that rats avoid intake of a drug-paired saccharin cue because the taste cue elicits the onset of an aversive state of conditioned withdrawal and conditioned withdrawal invokes the ‘need’ for drug [105]. In accordance, like conditioned withdrawal [97-100], avoidance of a drug-paired taste cue also is associated with blunted levels of dopamine in the nucleus accumbens [101][103]. Further, greater avoidance of (or aversion to)

the drug-paired cue also predicts greater drug-seeking, greater drug-taking, a strong willingness to work for the drug, and greater susceptibility to cue- and drug-induced relapse [93, 95, 104, 105, 217, 218]. Responding for drug, then, may be driven by not only liking and wanting [197], but also by needing.

In recent years, scientists have shown that hormones that modulate hunger and satiety also can modulate responding for drugs of abuse [219, 220]. One of the most studied has been Glucagon-like peptide-1 (GLP-1), an incretin hormone [187] essential for regulation of food intake in both animals and humans. GLP-1 also is a neurohormone released by neurons located in the nucleus tractus solitarius (NTS) that project to different brain regions, including key structures in the reward pathway such as the ventral tegmental area (VTA) and the nucleus accumbens (NAc) and hypothalamic nuclei involved in homeostasis and motivated behavior [151]. Exendin-4 (Ex-4) is a natural GLP-1 receptor (GLP-1R) agonist [140] and, due to its incretin and appetite suppressant action, has been used to treat type 2 diabetes mellitus and obesity [139, 190]. Moreover, treatment with Ex-4 also has been reported to reduce conditioned place preference for a drug of abuse, accumbens dopamine release induced by nicotine, cocaine and amphetamine, cocaine self-administration and seeking in rats [177-179, 181], and relapse to ethanol drinking in mice [182]. Thus far, one study found Ex-4 not effective in reducing abuse-related effects of remifentanil in mice [185]; while a second study reported that Ex-4 was effective in reducing oxycodone seeking and self-administration in rats [221].

The present study sought to further our understanding of the effects of Ex-4 on opioid addiction, using the taste-drug model described above. Specifically, we tested the effect of treatment with Ex-4 on acceptance of the drug-paired saccharin cue and on both cue-induced heroin seeking and drug-induced reinstatement of heroin seeking in rats. Here, Ex-4 was administered daily throughout a 16-day abstinence period and prior to test in an effort to model

such a treatment regimen in humans. We also analyzed mRNA expression in the NAc shell (NAcS), a crucial structure in reward modulation, to assess long-lasting changes in receptors associated with homeostatic regulation and reward. We hypothesized that daily treatment with Ex-4 during abstinence and at test will facilitate recovery of responding for the natural reward cue and will reduce cue- and drug-induced heroin-seeking following a period of abstinence in rats.

Materials and Methods

The subjects were 55 outbred male Sprague-Dawley rats delivered from Charles River (Wilmington, MA) at approximately 90 days of age, weighing between 300 – 400 g at the start of the experiment. Because prior access to a sweet can be more protective against drug self-administration in female rats [222], we performed the present study in male rats. All subjects were housed individually in standard, suspended, stainless steel cages. The environment in the animal colony room had controlled humidity and temperature (21 °C), with a 12/12-hour light/dark cycle, and lights on at 7:00 am. All experimental manipulations were conducted starting 2 hours into the light phase of the cycle. Following one week of acclimation to their home cages, rats were habituated to experimenter handling by daily weighing. Food and water were available ad libitum, except where noted otherwise. All studies were approved by the Pennsylvania State University College of Medicine, Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health specifications outlined in their Guide for the Care and Use of Laboratory Animals.

Self-Administration Catheter

Jugular Catheter Implantation Surgery

Rats were anesthetized with intramuscular (im) ketamine (70 mg/kg) and xylazine (14 mg/kg) and then implanted with custom-made intravenous jugular catheters as described previously [93]. Following surgery, rats received two days of post-op care consisting of a daily subcutaneous (sc) injection of the NSAID, carprofen, and a full week to recover. Carprofen

treatment was continued when indicated. Maintenance of catheter patency included daily flushing of catheters using heparinized saline (0.2 mL of 30 IU/mL heparin). Catheter patency was verified at the end of each week of drug self-administration and a day before each test day using 0.3 mL of propofol (Diprivan 1%).

Phase 1: Acquisition of Heroin Self-administration Behavior

Habituation

The animals experienced three days of habituation to the self-administration chamber to learn the behavioral task (spout licking). One day before the first habituation session, ad-libitum water was removed. The animals then had a 5 min habituation session on each of 3 days, starting 2 hours into the light phase. During this 5 min period, water was available in one of the three spouts, varying the location each day (left, middle, right). The center spout was the future "inactive" spout on which responding will lead to no consequence, and the rightmost spout was the future "active" spout where completion of a given number of contacts will lead to an intravenous (iv) infusion of drug or saline. In order to maintain proper hydration, each day rats had overnight access to 20 mL filtered water at the front of the home cage beginning at 5pm. After the third day of habituation ad-libitum water was restored.

Taste-Drug Pairings

Two hours into the light cycle, rats were placed in the self-administration chambers (MED Associates, Inc., St. Albans, VT) where they had 5 min access to 0.15% saccharin via the

leftmost spout. Thereafter, the saccharin spout retracted, the house light was turned off and the middle and right empty spouts advanced. Licking on the inactive spout (middle spout) had no consequence. The availability of the empty active, rightmost spout was signaled by a cue light located above, and completion of a fixed ratio of 10 (FR=10) contacts with this spout led to a 6-second iv infusion of either saline (n=15) or 0.06 mg/infusion heroin (n=40) for a period of 6 hours. Each infusion was followed by a 20-second time-out period in which the cue light turned off, the house light turned on, the empty spouts retracted and the sound of a tone signaled the time out period. Rats were trained using this protocol 5 days a week for 15 trials (Figure 3-1). Due to lack of catheter patency, seven animals in the saccharin-heroin group were excluded from the experiment after Phase 1.

Behavioral Stratification

It has been established that, in this model, rats in the saccharin-drug group show individual differences when responding for saccharin. Some rats greatly avoid the drug-paired saccharin cue and greater avoidance is associated with greater drug-seeking and taking [93]. Consequently, terminal saccharin intake (i.e., average saccharin licks emitted on trials 14 and 15) was used to separate the heroin self-administration group into large and small suppressors [93, 218]. Specifically, using 200 licks/5min as the cutoff, Large suppressors (LS) emitted <200 licks/5min of the saccharin cue (n=19), while small suppressors (SS) emitted >200 licks/5min (n=21) of the saccharin cue. Saccharin-saline (Sac-sal) controls were not stratified.

Abstinence

A 16-day home cage abstinence period followed immediately after the last taste-drug pairing. During this abstinence period, rats remained in their home cage with ad-libitum access to water and food and were injected intraperitoneally (ip) once daily with either vehicle (n=23) or 2.4 µg/kg of Ex-4 (n=25) one hour into the light cycle.

Cue-induced Seeking and Drug-induced Reinstatement Test 1

Twenty-four hours later, rats received their daily injection of vehicle (Veh) or Ex-4 one hour prior to placement in the self-administration chamber. They were then given 5 min access to saccharin followed by a 5-hour extinction test during which all cues associated with the drug (cue light, tone, etc.) were presented as usual, but contacts on the active spout did not deliver an infusion of saline or drug. Immediately after the fifth hour, a computer-controlled non-contingent iv infusion of saline or heroin (0.06 mg/infusion) was delivered and subsequent contacts with the spouts were recorded to assess drug-induced reinstatement of heroin seeking behavior. Due to lack of catheter patency, five additional rats in the saccharin-heroin group were excluded from the experiment after Phase 2. In addition, one rat in the Sac-sal control group developed an infection and was euthanized.

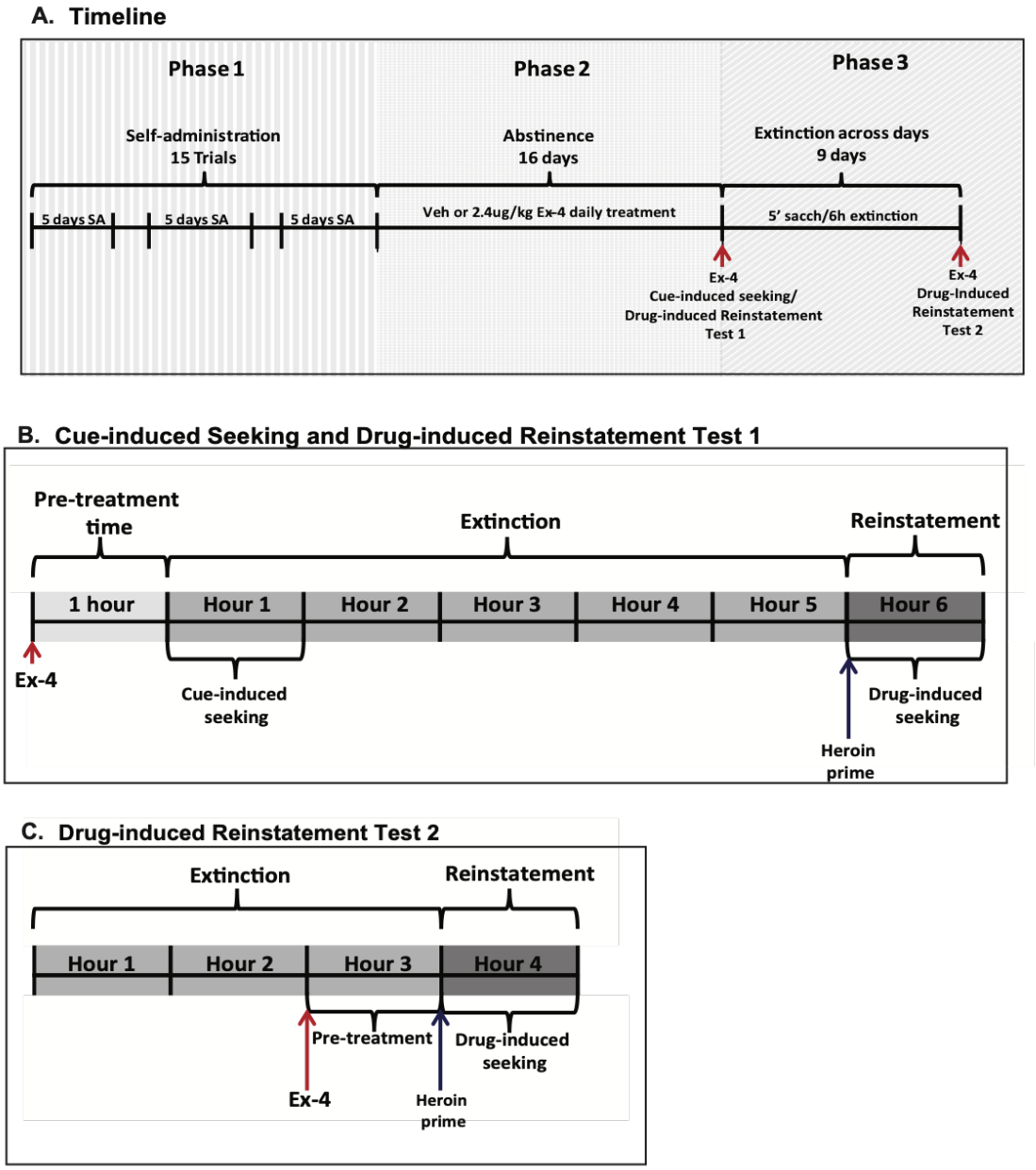

Figure 3-1. Timeline of the study and test design. A. The study consisted of 3 phases. During Phase 1, rats had 5 min access to a saccharin cue followed by the opportunity to self-administer either heroin or saline 5 days a week for 15 trials. Phase 2 consisted of a 16-day period of home cage abstinence followed by Cue-induced seeking/Drug-induced Reinstatement Test 1. During this period rats were treated with a daily ip injection of either Veh (saline) or 2.4 µg/kg Exendin-4 (Ex-4). In phase 3, rats were subjected to a daily pairing of saccharin with a 6-h extinction period for 9 days followed by Drug-induced Reinstatement Test 2. **B.** During Cue-induced Seeking and Drug-induced Reinstatement Test 1 rats received an injection of either Veh or Ex-4. One hour later, they were given 5-min access to saccharin followed by a 5-hour period of extinction. After the 5th hour, an infusion of either heroin or saline was automatically delivered and reinstatement of heroin seeking behavior (Drug-induced seeking) was recorded for one hour. **C.** During Drug-induced Reinstatement Test 2, the rats were given four hours of extinction. Then, they were injected with Veh or Ex-4 at the end of hour 2, the iv heroin prime injection was infused at the end of hour 3, and reinstatement of heroin seeking behavior was examined for 1 hour thereafter.

Phase 2: Abstinence, Treatment and Drug-Seeking Tests

Phase 3: Extinction across Days and Drug-induced Reinstatement Test 2

As described, in Phase 2, our standard protocol led to a 6-hour delay between Ex-4 treatment and the drug prime. Since Ex-4 has a half-life of approximately 150 minutes [137], by the time the drug prime was delivered the levels of Ex-4 would have been low. In order to more effectively assess the impact of Ex-4 on drug-induced reinstatement, the same rats were subjected to a protocol of extinction as described below to reduce cue-induced seeking, followed by a 2nd drug-induced reinstatement test with a 1 hour Ex-4 pretreatment.

Extinction across Days

Two hours into the light cycle rats were placed in the chamber and given 5 minutes access to saccharin, followed by a 6-hour extinction test in which all the cues were present as usual, but contacts with the active spout were without consequence. During this 9-day period, no Ex-4 or Veh was administered.

Drug-induced Reinstatement Test 2

On day 10, and two hours into the light phase, rats had 5 minutes access to saccharin. Because cue-induced seeking was not fully extinguished across the 9-day period, rats were exposed to 3 hours of within session extinction on Day 10. At the beginning of hour 2, rats were injected ip with either Veh or Ex-4. One hour thereafter, rats received a non-contingent iv

infusion of saline or heroin (0.06 mg/infusion) and reinstatement of heroin seeking was examined across the next hour.

Sacrifice, Dissection and Molecular Analysis

One hour after the last test, all rats were sacrificed by live decapitation, the skull was removed and the brain dissected. The harvested right NAcS was flash frozen and stored at −80 °C until mRNA was extracted and analyzed by real time quantitative polymerase chain reaction (RT-qPCR). Briefly, NAc shell was homogenized and RNA was isolated using the Allprep DNA/RNA kit (Qiagen). Complimentary DNA (cDNA) was generated from the isolated RNA using Random hexamer primers (Invitrogen) and Superscript III Reverse Transcriptase (Invitrogen). Messenger RNA expression was analyzed using real time quantitative polymerase chain reaction (RT-qPCR) Quantstudio 102k flex. The $2^{-\Delta\Delta CT}$ method was employed to assess relative gene quantification with beta actin as the endogenous control. Quantitative PCR analysis of targets of interest was performed using standard laboratory methods, with 384-well optical plates, TaqMan Assay-On-Demand (Applied Biosystems, Foster City, CA, USA) gene-specific primers/probe assays and a 7900HT Sequence Detection System (Applied Biosystems). Gene expression assays included Orexin Receptor 1 (Hcrtr1, Rn0056995_m1), dopamine D2 receptor (d2r, Rn00561126_m1), Leptin receptor (Lepr, Rn00565158_m1) and GLP-1 receptor (glp-1r, Rn00562406_m).

Data Analysis

Subjects that lost catheter patency were removed from the subsequent phase of the experiment. All data were analyzed using Prism version 8.00, GraphPad Software (La Jolla

California USA). Mixed factorial Analysis of Variance (ANOVAs), followed by Tukey's post hoc tests, were used to compare different groups. In addition, Student-t tests were used when comparing only two means, with alpha set at 0.05.

Results

Phase 1: Acquisition of Heroin Self-administration Behavior

Saccharin Intake

As shown in Figure 3-2A, the number of saccharin licks/5min increased across trials in both Sac-sal and SS, but not in LS. Support for this conclusion was provided by post-hoc (Tukey's) assessment of a significant interaction of a 3 x 15 mixed factorial ANOVA varying group (Sac-sal, SS and LS) and trials (1-15) ($F_{28,728}$=10.52, p<0.0001) showing a significant reduction in intake of the saccharin cue on trials 5 - 15 for the LS vs. both the SS and Sac-sal (ps<0.05).

Heroin Self-administration

There were no differences in the mean number of heroin infusions/6 hours across the 15 acquisition trials between SS and LS, and both groups self-administered more infusions than Sac-sal (Figure 3-2B). This conclusion was supported by a 3 x 15 mixed factorial ANOVA showing a significant group x trial interaction ($F_{28,728}$=4.53, p<0.0001). Post-hoc tests revealed a greater number of infusions by both SS and LS compared to Sac-sal beginning on trial 6 (ps<0.05). When averaged across trials 14 and 15, LS evidenced a shorter terminal latency to obtain the first infusion compared with SS and Sac-sal (see Figure 3-2C). This was confirmed by post-hoc analysis of a significant one-way ANOVA ($F_{2,51}$=5.78, p<.005, p<0.05). Finally, post hoc tests of a significant interaction of a two-way ANOVA ($F_{1,30}$=8.13, p<0.01, ps<0.01) showed, on the last

trial, that LS obtained more infusions during the 1st hour than SS (Figure 3-2D). Moreover, 1st hour intake was found to increase significantly from trial 1 to trial 15 (i.e., to escalate across trials) for the LS ($p<0.05$), but not for the SS ($p>0.05$) (Figure 3-2D).

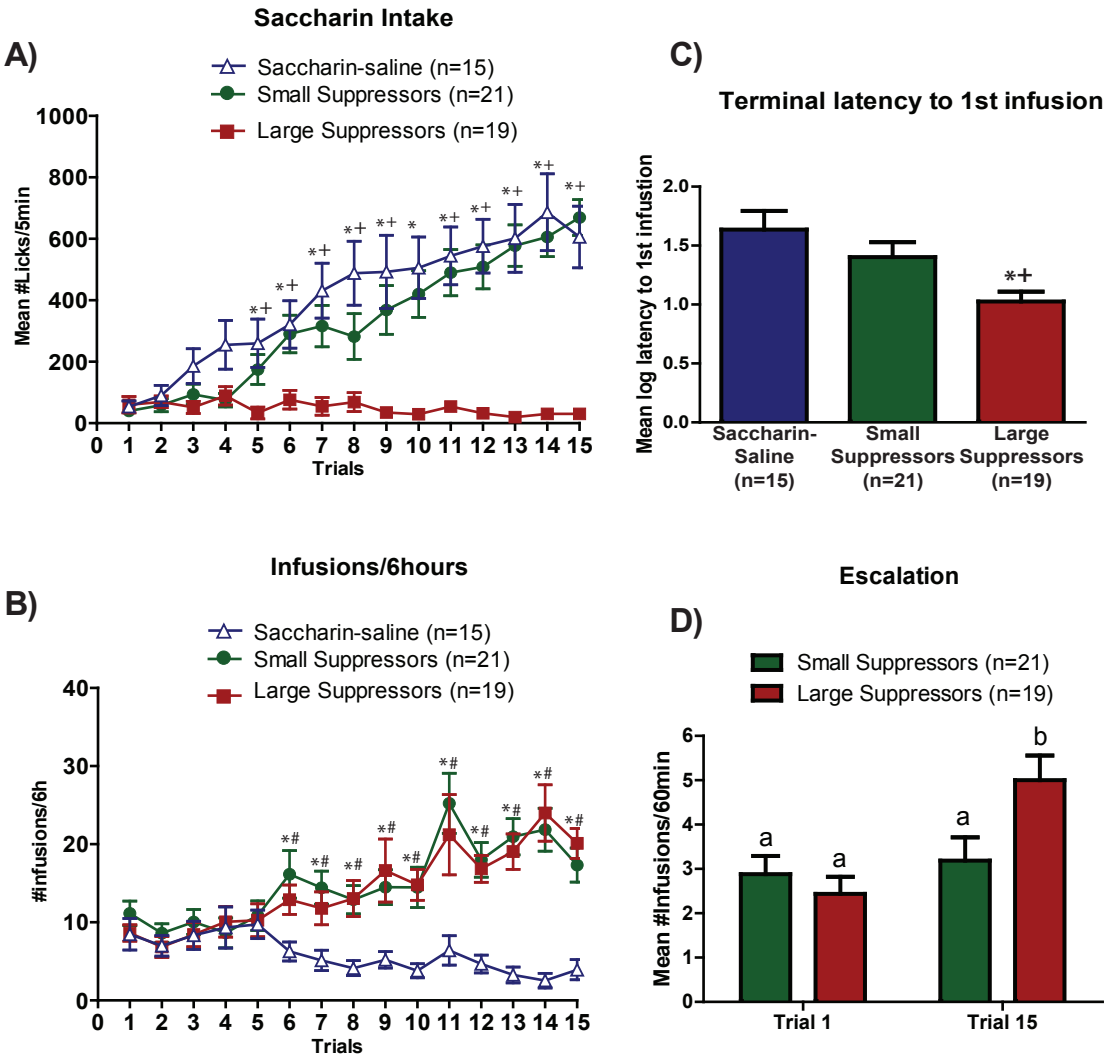

Figure 3-2 Acquisition of drug-taking behavior. A. Saccharin intake. Mean (± SEM) number of licks/5min of 0.15% saccharin across trials 1 – 15 for rats in the saccharin-saline, small suppressor or large suppressor group. **B**. Self-administration. Mean (± SEM) number of saline or heroin infusions/6h across trials 1 – 15 for rats in the saccharin-saline, small suppressor, or large suppressor groups. **C.** Latency to 1st infusion. Mean (± SEM) terminal log latency to 1st heroin infusion for saccharin-saline, small suppressor, or large suppressor groups. **D.** Escalation. Mean (± SEM) infusions during the 1st hour of self-administration in trials 1 and 15 (saccharin-saline controls: n=15, small suppressors: n=21, large suppressors: n=19). +Significant difference between large suppressors and small suppressors; *Significant difference between large suppressors and saccharin-saline controls. # Significant difference between small suppressors and saccharin-saline controls. Different letters indicate significant differences.

Phase 2: Abstinence, Treatment, and Drug-seeking Test 1

Saccharin Intake after Abstinence

Ex-4 treatment during abstinence did not affect body weight (see Appendix B) or saccharin intake during Test 1 (Figure 3-3A). Thus, the number of saccharin licks/5min during Test 1 was analyzed using a 3 x 2 ANOVA varying group (Sac-sal, SS and LS) and treatment (Veh vs. Ex-4). The results revealed a significant main effect of group ($F_{2,22}$=26.98, p<0.0001), with the LS continuing to emit fewer saccharin licks/5min than SS or Sac-sal overall (p<0.0001). Neither the main effect of treatment ($F_{1,18}$=1.3, p=0.27) nor the group x treatment interaction (F<1.0) was significant.

Cue-induced Seeking

Figure 3-3B shows that treatment with Ex-4 increased the latency to the first contact with the active spout overall. In support, the 3 x 2 ANOVA varying group and treatment found a significant main effect of treatment ($F_{1,42}$=29.3, p<0.0001) and group ($F_{2,42}$=10.42, p<0.001), but not group x treatment interaction ($F_{2,42}$=1.935, p=0.16). Post-hoc analysis of the significant main effect of group (ps<0.005) confirmed that groups SS and LS exhibited a shorter latency to make first contact with the active spout than group Sac-sal overall (ps<0.05).

When examined during the 1st hour of the 5-hour extinction session, treatment with Ex-4 significantly reduced the number of contacts with the active spout in both SS and LS (Figure 3-3C). Support for this conclusion was provided by a mixed factorial ANOVA showing a significant group x treatment x spout interaction ($F_{2,40}$=5.9, p<0.01) and confirmed by post-hoc

analyses (ps<0.05). No effect on inactive spout responding was observed. Finally, when examined across the 5-hour extinction trial, the data show that active spout contacts extinguished after the first hour for all groups (Figure 3-3D). Thus, post hoc analysis of a significant group x treatment x hour interaction ($F_{8,160}$=8.072, p<0.0001) confirmed a reduction in contacts with the active spout following the 1st hour for both the Veh-treated SS (Figure 3-3D, middle panel) and the Veh-treated LS (Figure 3-3D, bottom panel; ps<0.05). Cue-induced heroin seeking by the Ex-4 treated rats, on the other hand, was low in the 1st hour and remained so thereafter (ps<0.05).

Drug-induced Reinstatement Test 1

Treatment with Ex-4 six hours earlier had no effect on drug-induced heroin seeking (Figure 3-3E). While both Sac-sal groups did not interact with the active spout, Veh- and Ex-4-treated SS and LS showed a higher number of contacts than Sac-sal in the hour following the drug prime. In support, a 3 x 2 x 2 ANOVA varying group (Sac-sal, SS, and LS), treatment (Veh vs. Ex-4), and spout (Active vs. Inactive) revealed a significant group x spout interaction ($F_{2,40}$=7.56, p<0.001) and a post-hoc analysis showed significantly greater active spout contacts by both SS and LS vs. Sac-sal (p<0.05). Neither the treatment x spout (F<1.0) nor the group x treatment x spout (F<1.0) interaction was significant.

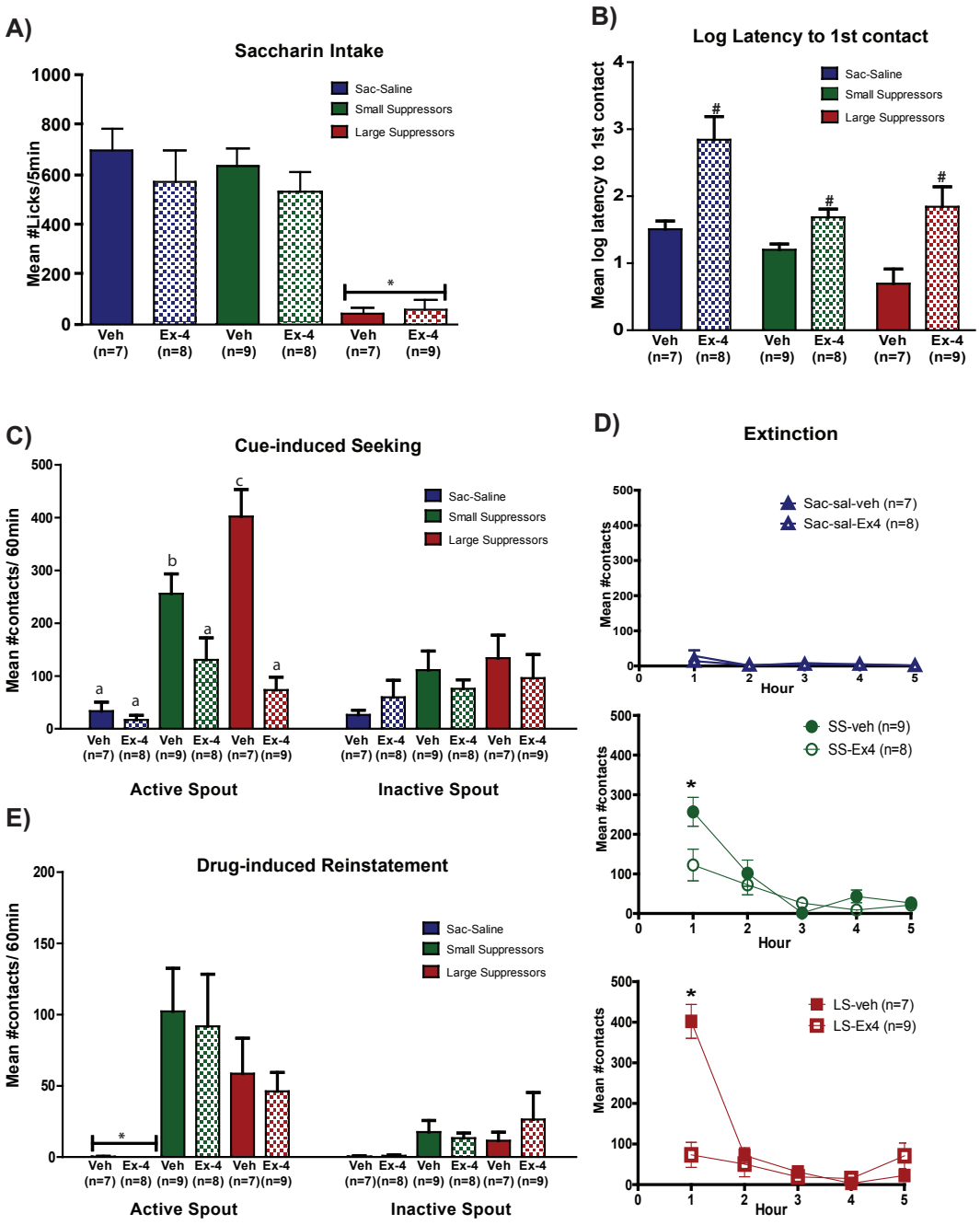

Figure 3-3. Effect of Ex-4 treatment on cue- and drug-induced reinstatement. A. Saccharin intake on test day. Mean (± SEM) number of licks/5min of 0.15% saccharin for rats in the saccharin-saline controls, small suppressors and large suppressors groups treated with Veh or Ex-4 on cue- and drug-induced reinstatement test 1. **B.** Latency to 1st contact. Mean (± SEM) log latency to 1st contact with the active spout for saccharin-saline, small and large suppressor groups treated with Veh or Ex-4. **C.** Cue-induced seeking. Mean (± SEM) number active (left) and inactive (right) spout contacts/60min for saccharin-saline, small and large suppressors groups treated with Veh or Ex-4. **D.** Extinction. Mean (± SEM) number of contacts with active and inactive spouts/6h of extinction for saccharin-saline (top panel), small suppressors (middle panel) and large suppressors (bottom panel) treated with Veh or Ex-4. **E.** Drug-induced reinstatement test 1. Mean (± SEM) number of active (left) and inactive (right) spouts contacts during the 60 minutes after the drug prime for saccharin-saline, small and large suppressors groups treated with Veh or Ex-4 six hours prior to the drug prime (Sac-sal-veh: n=7, Sac-sal-Ex-4: n=8, SS-veh: n=9, SS-Ex-4: n=8, LS-veh: n=7, LS-Ex-4: n=9). *Significant difference between groups. #Significant difference between Veh and Ex-4 treatment. Different letters indicate significant differences.

Phase 3: Extinction across Days and Drug-induced Reinstatement Test 2

Saccharin Intake across Extinction Days

A history of treatment with Ex-4 during abstinence significantly increased the average number of saccharin licks/5min emitted across the 9 days of extinction training by the LS (Figure 3-4A). In support, a 2 x 3 ANOVA revealed a significant group x treatment interaction ($F_{2,44}$=5.33, p<0.0001) and post-hoc tests confirmed a significant increase in saccharin intake by the LS rats with a history of Ex-4 treatment (ps<0.05), and a smaller, but still significant, increase in saccharin intake by the Sac-sal rats with a history of Ex-4 treatment (ps<0.05). No effect of prior Ex-4 treatment was observed on saccharin intake in the SS group (ps>0.05).

Drug Seeking across Extinction Days

Small suppressors and LS exhibited greater seeking (i.e., average number of contacts with active spout) during extinction training than Sac-sal and, while a history of Ex-4 treatment tended to reduce seeking in SS, this trend did not attain statistical significance (Figure 3-4B). This conclusion is confirmed by a 3 x 2 ANOVA showing a significant main effect of group ($F_{2,44}$=37.15, p<0.0001) and no significant main effect of treatment ($F_{1.44}$=2.39, p=0.13) or group x treatment interaction ($F_{2,44}$=1.05, p=0.36).

Drug-induced Reinstatement Test 2

Figure 3-4C shows that acute pretreatment with Ex-4 one hour prior the drug prime significantly increased the latency to make the 1st contact with the active spout in both the SS and the LS. This conclusion was supported by a 3 x 2 ANOVA that revealed a significant main effect of treatment ($F_{1,24}$=10.36, $p<0.005$, $ps<0.05$), mainly carried by SS and LS. Group Sac-sal emitted no contacts with the active spout. Figure 3-4D shows that acute-pretreatment with Ex-4 completely abolished heroin seeking during Drug-induced Reinstatement Test 2. Veh-treated SS and LS showed an increased number of contacts with the active spout, while Ex-4-treated SS and LS groups did not contact the active spout at all. This conclusion was supported by a mixed factorial 3 x 2 x 2 ANOVA varying group, treatment, and spout showing a significant main effect of treatment ($F_{1,27}$=5.21, $p<0.05$) and a significant treatment x spout interaction ($F_{1,27}$=5.19, $p<0.05$). Neither the group x spout ($F_{2,27}$=1.73, $p=0.19$), nor the group x treatment x spout interaction ($F_{2,27}$=1.47, $p=0.25$) was significant.

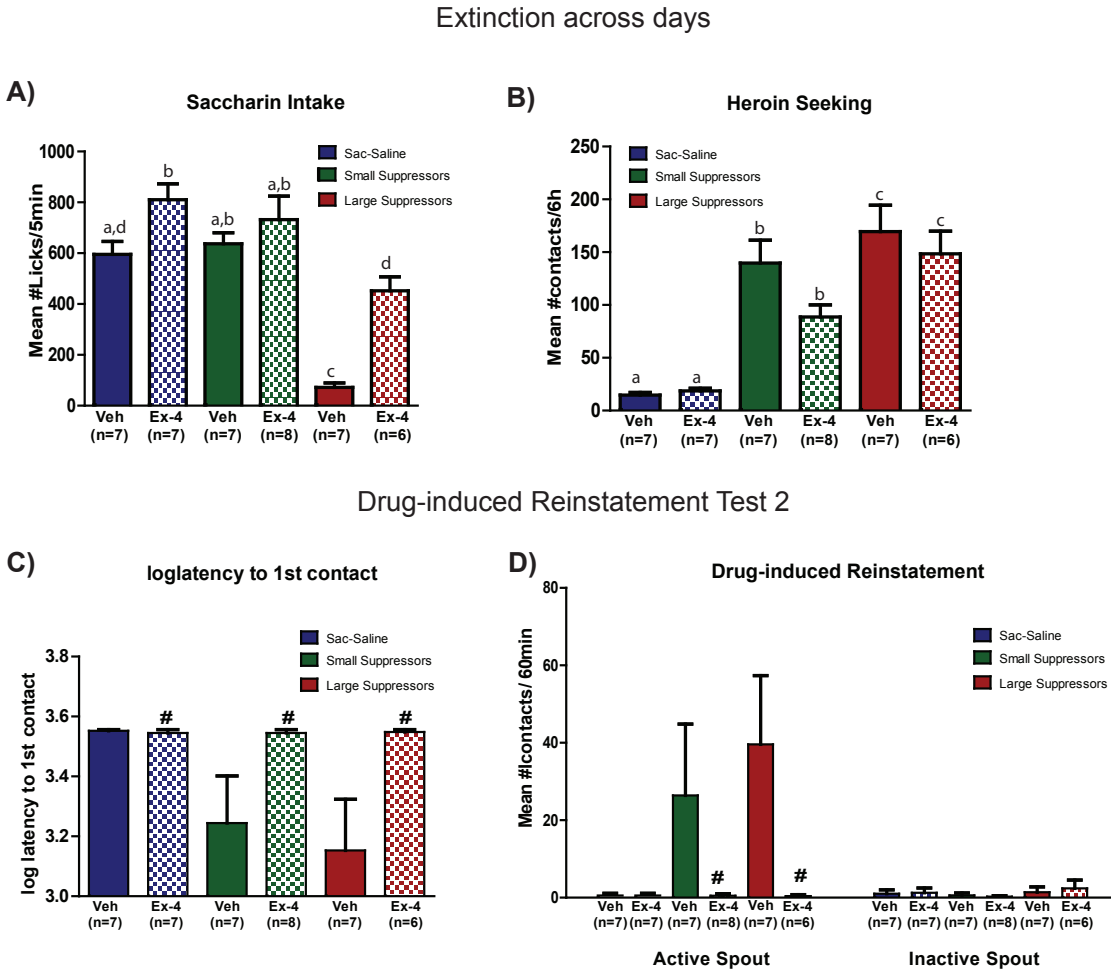

Figure 3-4. Extinction across days and drug-induced reinstatement Test 2. **A**. Saccharin intake during extinction. Mean (± SEM) number of licks/5min of 0.15% saccharin averaged across the 9 days of extinction training for rats in the saccharin-saline, small suppressors or large suppressors groups with history of treatment with Veh or Ex-4. **B.** Heroin seeking during extinction. Mean (± SEM) number of infusion attempts/6h averaged across 9 days of extinction testing for saccharin-saline, small or large suppressors with a history of treatment with Veh or Ex-4. **C.** Latency to 1st contact. Mean (± SEM) log latency in sec to 1st contact with the active spout during drug-induced reinstatement Test 2 for saccharin-saline, small and large suppressor groups with a history of treatment with Veh or Ex-4. **D.** Drug-induced reinstatement Test 2. Mean (± SEM) number of active (left) and inactive (right) spout contacts/60min for saccharin-saline, small and large suppressors treated with Veh or Ex-4 one hour prior to the drug prime (Sac-sal-veh: n=7, Sac-sal-Ex-4: n=7, SS-veh: n=7, SS-Ex-4: n=8, LS-veh: n=7, LS-Ex-4: n=6). #Significant difference between Veh and Ex-4 treatment. Different letters indicate significant differences.

Molecular Analysis

To further explore the effects of Ex-4, mRNA expression of select genes known to be associated with reward and feeding behaviors was examined in the NAcS. Figure 3-5 shows the relative expression of 4 such genes in rats that self-administered heroin and were treated with Veh or Ex-4. Compared to Veh-treated controls, no significant difference in fold change expression was observed in GLP-1R (Figure 3-5A), D2R (Figure 3-5B), or leptin receptors (Figure 3-5C). There was, however, a significant increase in the expression of Orexin receptor 1 (OX1) in heroin self-administering rats with a history of Ex-4 treatment compared to vehicle-treated controls ($t=3.16$; $p=0.006$, Figure 3-5D).

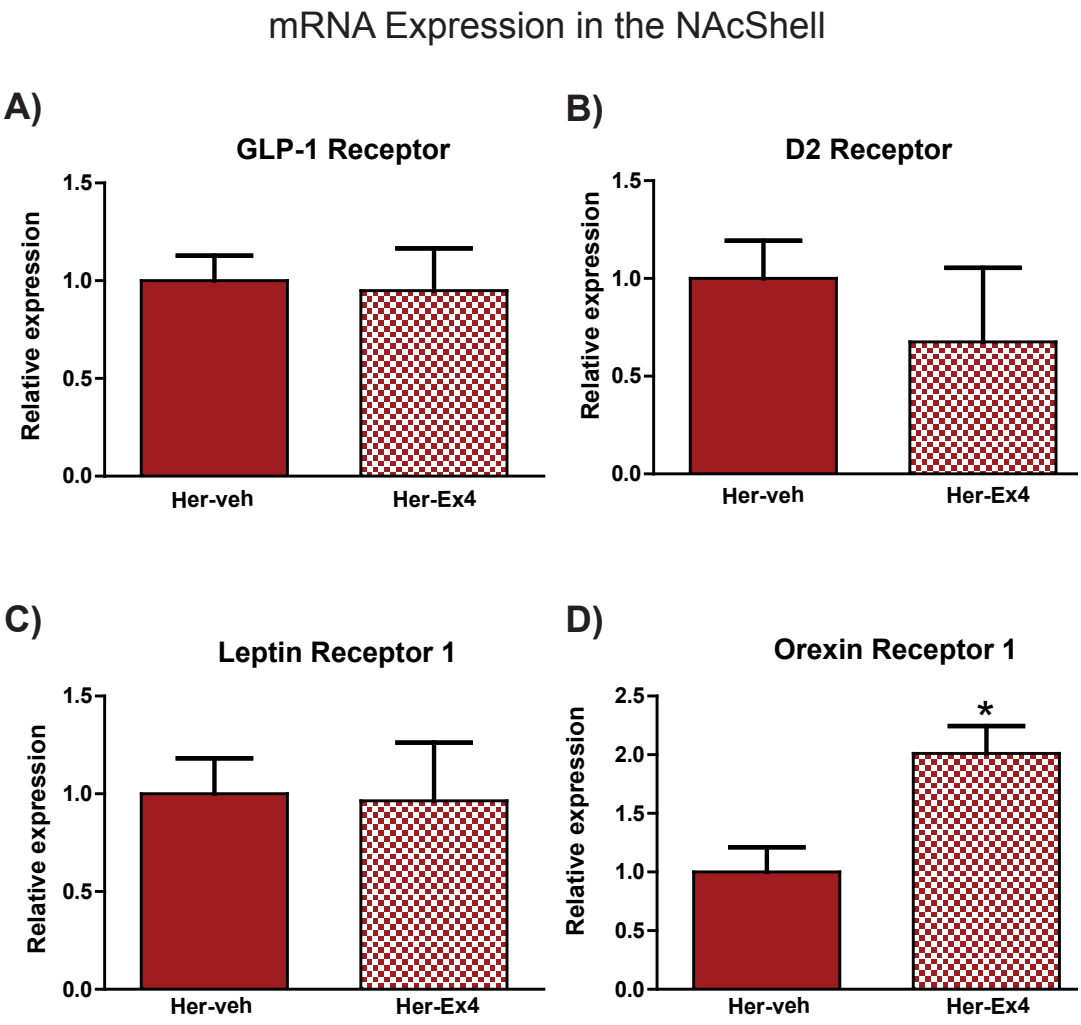

Figure3-5. Relative mRNA expression of GLP-1 receptor (A), dopamine D2 receptor (B) leptin receptor 1 (C) and orexin receptor 1 (D) in rats with a history of heroin self-administration and treatment with Veh or Ex-4 throughout the abstinence period and test (Heroin-veh: n=10, herion-Ex-4: n=9). *Significant difference between groups.

Discussion

This study examined the effects of Ex-4 on heroin seeking in rats. Specifically, we showed that systemic treatment with Ex-4 significantly attenuated heroin seeking when the rats were re-exposed to the heroin-associated cues, without affecting body weight. Importantly, treatment with Ex-4 also abolished drug-induced reinstatement of heroin seeking behavior when administered 1 hour, but not 6 hours, prior to the drug challenge. As such, we can conclude that the protective effects of this dose of Ex-4 on cue- and drug-induced reinstatement are related to the acute, rather than the chronic, effects of this relatively short-acting GLP-1R agonist. Although treatment with Ex-4 did not attenuate suppression of intake of the heroin-paired saccharin cue, a history of treatment with Ex-4 was associated with increased acceptance of the heroin-paired saccharin cue, particularly in the most vulnerable LS population. Finally, reduced reinstatement of heroin seeking by Ex-4 treated rats was accompanied by an increase in the expression of the OX1 mRNA in the NAcS.

Drug-induced Suppression of Saccharin Intake

In Phase 1, we observed two subpopulations within the heroin group. Using terminal saccharin intake and a median split [93], we identified a group of rats, referred to as LS, that greatly avoided intake of the saccharin cue compared to the other group, referred to as the SS, that did not. Such avoidance of the drug-paired taste cue has been interpreted as a conditioned taste aversion (CTA), akin to a lithium chloride CTA [223]. This phenomenon was later re-interpreted as a reward comparison effect, reflecting drug-induced devaluation of the lesser valued saccharin solution [224]. More recent data, however, suggest that devaluation of the

saccharin cue (i.e., reduced intake) may be indicative of anhedonia, a symptom associated with the onset of a conditioned aversive state of withdrawal [95, 103].

Effects of Ex-4 on Drug-paired Saccharin Intake

As stated in the Introduction of this chapter, part of our hypothesis was that treatment with Ex-4 would increase responding for the natural reward cue. This hypothesis was partially correct. Treatment with Ex-4 did not alter intake of the saccharin cue in SS or LS during the 1st cue-induced seeking test – i.e., when Ex-4 was on board, rats ingested (SS), or avoided (LS), the drug-paired saccharin cue as they did during acquisition. That said, a history of daily treatment with Ex-4 during the abstinence period was associated with an increase in acceptance of the saccharin solution by the LS in later extinction training. The overall increase in responding for saccharin in the LS is a promising finding, as natural rewards can be highly protective against addiction [225, 226]. Future studies will need to determine whether extended treatment with Ex-4 increases intake of the saccharin cue due to an increase in the perceived palatability of the saccharin cue, consequent to a dissociation of the saccharin cue from the conditioned aversive state of withdrawal, or by a direct reduction in withdrawal itself. GLP-1 analogs have been shown to reduce ethanol withdrawal-induced anxiety [227] and aversive taste reactivity behavior to a gustatory cue paired with naloxone-induced withdrawal [Olsen et al., in preparation].

Avoidance of the Drug-paired Taste Cue and Drug-taking

It has been shown that rats that most greatly avoid the taste cue take less time to obtain the first infusion, take more drug, work hard to get the drug, and exhibit the greatest cue-induced

drug seeking and drug-induced reinstatement of drug seeking behavior [93, 105, 218]. That said, here, during acquisition, both the SS and LS actually self-administered the same amount of heroin across trials. Greater saccharin suppression in the LS group, however, was associated with a shorter latency to obtain the first infusion of heroin, with greater 1st hour drug taking, and with escalation of drug-taking across trials – a cardinal feature of addiction [81]. This pattern of behavior is consistent with a greater conditioned-withdrawal state elicited by the saccharin cue in the LS subpopulation.

The Effect of Ex-4 on Cue-induced seeking and Drug-induced Reinstatement

In Phase 2 of this study, vehicle-treated rats exhibited clear cue-induced heroin seeking. Treatment with 2.4 μg/kg of Ex-4, on the other hand, increased the latency to seek heroin and reduced cue-induced heroin seeking during the 1st hour of the extinction test in both the SS and LS groups. That said, Ex-4 treatment also led to an increase in the latency to 'seek' saline in the Sac-Sal controls. This finding raises the possibility that Ex-4 may have an inhibitory effect on motivated responding per se, rather than a specific effect on heroin seeking. There are, however, a number of arguments against this interpretation. First, while Ex-4 can lead to locomotor depression [178], the use of spout-licking as the operant task presents an advantage over lever-pressing, as it is a more natural behavior for the rats that does not require high locomotor effort. Second, if locomotor depression were to have mediated these effects, the rats should have begun seeking the drug once Ex-4 was cleared from the system (i.e., during hours 3, 4 and 5 of extinction testing). This, however, was not the case. Third, sedative effects, or a general lack of motivation, are unlikely to account for the data because this dose of Ex-4 failed to diminish saccharin licking on the test day in either the SS or the Sac-Sal controls. Indeed, these Ex-4-

treated rats made approximately 600 licks of saccharin/5 min (the equivalent of 60 infusions on a FR 10 schedule of reinforcement) and, importantly, the latency to initiate the first contact with the saccharin spout was quite short for both Ex-4 treated Sac-sal controls and SS (0.087 sec and 0.64 sec respectively). Finally, this finding may be an anomaly, as Ex-4 failed to increase the latency to 'seek' saline compared to the vehicle treated Sac-sal controls during Drug-induced Reinstatement Test 2. A general disruption in motivation, per se, and/or increased locomotor depression, then, are unlikely to account for the decrease in heroin seeking by the Ex-4 treated groups.

In contrast to the Ex-4-induced reduction in cue-induced heroin seeking, Ex-4 did not reduce heroin-seeking during drug-induced reinstatement of heroin seeking in Test 1. As mentioned, in this case, Ex-4 was administered 1 hour before rats were placed in the chamber. As such, there was a 6-hour (i.e., more than twice the half-life of the drug) delay between Ex-4 administration and the heroin prime. Thus, while we intended a chronic treatment regimen, the protective properties of this dose of the shorter acting GLP-1R agonist clearly did not carry across even the entire 6-hour test session. For this reason, in Phase 3, the effects of Ex-4 were tested directly on drug-induced reinstatement by reducing the pretreatment time to one hour. Using this regimen, Ex-4 completely abolished heroin-induced reinstatement of heroin-seeking behavior (i.e., 'relapse') in both SS and LS rats. It should be noted, however, that the reinstatement behavior observed in the vehicle treated rats was not as robust as expected (this group, showed an average of 20 to 40 active spout responses, approximately 2 – 4 infusion attempts over the 60 min test period). Even so, confidence is gained by observing nearly zero contacts with the inactive spout, showing clear goal-directed behavior. Thus, when using an appropriate pretreatment time, the short-acting GLP-1R agonist, Ex-4, effectively reduced both cue- and drug-induced heroin seeking in rats.

The effects of Ex-4 on the rewarding properties of opioids have only been studied twice, yielding opposite results. Bornebusch et al. studied several opioid-associated behaviors in mice and showed that Ex-4 had no effect on morphine-induced conditioned place preference, naloxone-precipitated morphine withdrawal, or locomotor activity [185]. The effect of Ex-4 on opioid-seeking was not tested, but Bornebusch et al. did show a tendency for Ex-4 to increase remifentanil intake. Here, a firm conclusion was hampered by large variability and a small sample size. In contrast, Zhang et al. used rats as a model and showed that Ex-4 can robustly and dose-dependently reduce oxycodone self-administration [186] . In the present study we did not examine the effects of Ex-4 on heroin self-administration, but our model is more akin to that used by Zhang et al. Similar to that study, we found that Ex-4 also can reduce cue- and drug-induced heroin seeking. Further research is needed to be done to elucidate the discrepancies across these reports, but differences in species (i.e., rats vs. mice) and drug (oxycodone and heroin vs. remifentanil) should be explored.

Molecular Data

In the molecular analysis, treatment with Ex-4 increased mRNA expression for OX1 in the NAcS, but did not change the expression for GLP-1R, D2R or LepR1. The integrative nature of NAc, receiving inputs from different parts of the brain, including regions that control reward and feeding-motivated behaviors [228, 229], makes it a candidate area to look for long-term changes produced by Ex-4 treatment. Indeed, GLP-1 producing neurons in the NTS project to the NAc [170]. Moreover, systemic administration of Ex-4 reaches the NAc, and infusion of a GLP-1R agonist directly into the NAcS dose-dependently reduces cocaine seeking [180]. It was

therefore expected that prolonged, daily treatment with Ex-4 may affect the expression of GLP-1R in the NAcS. This, however, was not observed by qPCR analysis.

Another important source of input to the NAc is via dopaminergic projections from the VTA. GLP-1Rs are found in this area [151] and intra-VTA injections of Ex-4 have been shown to reduce drug-primed reinstatement of cocaine-seeking behavior [179]. In addition, systemic injections of Ex-4 were found to attenuate amphetamine-, cocaine-, and nicotine-induced accumbal dopamine release [177], showing that activation of GLP-1R can modulate drug-associated dopaminergic release in the NAc. Moreover, the protective effects of systemic Ex-4 on cocaine-seeking are blocked by infusion of a GLP-1R antagonist directly into the VTA [179, 181]. That said, we did not find changes in the expression of D2 receptors in the NAcS associated with Ex-4 treatment.

Finally, we focused on the expression of receptors associated with the homeostasis-related hormones leptin and orexin. Leptin is an adipose-derived peptide hormone that can attenuate drug-related behaviors [230]. However, in this experiment, Ex-4 did not have an effect on LepR1 mRNA expression in the NAcS. On the other hand, we did see a marked increase in the expression of OX1 in the NAcS. This receptor is exclusively activated by Orexin A, which is produced mainly by neurons within the lateral hypothalamus, and has an important role in arousal and feeding motivated behavior [231-233]. Orexins increase food intake, but its modulatory role also has been extended to non-feeding reward related processes such as addiction [231, 233-235]. Indeed, it has been observed that opioids can increase the number of orexin-producing neurons [236]. In this context, our results may suggest that heroin self-administration increases the number of orexin neurons and its transmission. Treatment with Ex-4, then, may have reversed this effect, reducing levels of orexin in the NAcS, and leading to a compensatory increase in the expression of the OX1. Although further testing is required, this hypothesis is consistent with

previous research showing that GLP-1Rs in the lateral hypothalamus are critical for the control of ingestive behaviors, body weight, and food reinforcement in rats [237]. In addition, blocking the effects of OX1 with systemic administration of the OX1 antagonist, SB-334867, dose-dependently blocked reinstatement of seeking for cocaine [238], alcohol [239, 240], nicotine [241] and remifentanil [242]. As such, the molecular results raise the specter that drugs of abuse hijack not only reward substrates, but also substrates engaged by a potent state of physiological 'need' – in this case the 'need' for drug. Future studies, then, must be conducted to test the merits of this hypothesis and to parse the roles of the GLP-1R and the OX1 in the Ex-4-induced eventual acceptance of the natural reward cue and in the reduction of both cue- and drug-induced reinstatement of heroin-seeking behavior.

Conclusion

Our results demonstrate that treatment with the GLP-1R agonist, Ex-4, attenuates cue-induced heroin seeking and drug-induced reinstatement of heroin seeking in rats. This observation is consistent with other published data showing that treatment with GLP-1R agonists can reduce cue and/or drug-induced seeking for other drugs of abuse. Currently, there are only two opioid medications (methadone and buprenorphine), and one non-opioid medication (naloxone), approved for the treatment of OUD and both classes present problems for the patients. While medicated assisted treatment (MAT) using opioids is effective, and buprenorphine has been found effective in the extended access model [243, 244], MAT has the associated stigma of replacing one opioid with another and treatment with the opioid blocker naloxone often shows poor compliance from patients. For this reason, while MATs are very effective in both animal models and in humans, the findings in this study are important as they reveal a novel non-opioid

for the potential treatment of OUD. Fortunately, the extensive research done with GLP-1R agonists in the context of type 2 diabetes and obesity [139, 190] provides several formulations with differing half-lives (e.g., Exenatide, Liraglutide, Semaglutide), allowing researchers to further their understanding of the role of GLP-1 in addiction, and to develop new treatment regimens that may prevent relapse. Indeed, such studies are underway to test whether treatment with a GLP-1R agonist can reduce craving, withdrawal, and risk of relapse in humans in treatment for an OUD [245].

Manuscript: Glucagon-like peptide-1 receptor agonist, exendin-4, reduces reinstatement of heroin seeking behavior in rats. (*Accepted*)

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Respective contributions: J.E. Douton: Experimental design and execution, data analysis and interpretation and manuscript preparation. C. Augusto and B. Stoltzfus: Behavioral experimental execution. N Carkaci-Salli: Molecular experimental execution. K.E. Vrana: Molecular data interpretation. P.S. Grigson: Experimental design, data interpretation and manuscript revision.

Chapter 4

Glucagon-like peptide-1 receptor agonist, liraglutide, reduces heroin self-administration and drug-induced reinstatement of heroin seeking behavior in rats

Introduction

Drug addiction is a brain disease characterized by the uncontrolled use of a substance, and is defined as the most severe stage of substance use disorder [1]. Addiction is difficult to treat as it is a relapsing disorder and individuals are highly susceptible to overdose after having been abstinent, even for long periods of time [2, 11]. The opioid epidemic has taken a serious toll on individuals, their families, and on society as a whole over the last decade [10]. Moreover, while 2019 saw the first downturn in overdose deaths, due likely to the increased availability of Narcan, 2020 has been met with a steep upturn in opioid overdose deaths in the wake of the COVID-19 pandemic [8, 246, 247]. Consequently, it remains imperative that we explore novel avenues for treatment.

Current pharmacological treatments, while effective, are associated with the stigma of replacing one opioid with another (e.g., methadone, buprenorphine/suboxone) and/or support poor compliance (e.g., naloxone) [248-250]. Glucagon-like peptide-1 (GLP-1) analogs, in contrast, may serve as an effective non-opioid treatment for opioid use disorder. GLP-1 is an incretin hormone secreted by the gut and a neurohormone synthetized in the brainstem by neurons in the nucleus tractus solitarius [187]. Both peripheral and central GLP-1 are involved in regulating blood glucose levels and food intake, and GLP-1 receptor ligand pharmacotherapies are used as current treatments for obesity and type 2 diabetes in humans [139, 190]. Additionally, it has been observed that GLP-1 analogs not only produce satiety [63], but also reduce motivation to consume palatable food [169], and the motivation to seek and consume drugs of abuse [178, 179, 181, 182, 186].

The most studied GLP-1R agonist in the field of addiction has been the short-acting Exendin-4 (Ex-4), which has been observed to reduce motivated behaviors associated with

amphetamines, cocaine, nicotine, alcohol, and oxycodone [178, 179, 181, 182, 186]. In our hands, treatment with Ex-4 significantly reduced both cue-induced seeking and drug-induced reinstatement of heroin seeking in rats (**see Chapter 3**). That said, while Ex-4 has been shown to be effective in reducing drug-associated behaviors in rats, its short half-life requires it to be administered at least twice a day in humans. Other longer-acting analogs such as liraglutide (LIR) and semaglutide have not been fully explored in this context, but LIR has been observed to reduce alcohol-related behaviors in both rats and mice [183]. Given its longer half-life, LIR is administered only once/day in humans. Reducing the number of injections required per day is particularly relevant for this patient population as the injection of the GLP-1 analog might serve as a cue that can trigger craving or even relapse. The potential effect of the longer-acting LIR on opioid taking and seeking, however, has not yet been evaluated in animal models. As a consequence, in this study, rats were treated with vehicle or LIR starting mid-way through the acquisition phase, making it possible to assess the effect of LIR on, not only heroin-seeking, but on heroin taking as well. Finally, it should be noted that the primary aversive side effect of GLP-1 based therapies is nausea and malaise [138, 251]. Thus, while a small percentage of the patient population – around 10% - discontinues use of GLP-1R agonists due to this side effect [138, 251-254]. In rats, this can be assessed with the conditioned avoidance test, in which rats avoid intake of a palatable solution that is paired with a malaise-producing agent such as lithium chloride or x-radiation [215, 255-258], and by analyzing the consumption of kaolin, which has been observed to have anti-emetic effects in humans. Follow up studies here examined the effect of LIR on body weight, food, water and kaolin intake, and the development of a conditioned taste avoidance to LIR in rats.

Materials and Methods

The subjects were 77 outbred male Sprague-Dawley rats delivered from Charles River (Wilmington, MA) at approximately 90 days of age, weighing between 300 – 400 g at the start of the experiment. All subjects were housed individually in standard, suspended, stainless steel cages. The environment in the animal colony room had controlled humidity and temperature (21 °C), with a 12/12-hour light/dark cycle, and lights on at 7:00 am. All experimental manipulations were conducted starting 2 hours into the light phase of the cycle. Following one week of acclimation to their home cages, rats were habituated to experimenter handling by daily weighing. Food and water were available ad libitum, except where noted otherwise. All studies were approved by the Pennsylvania State University College of Medicine, Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health specifications outlined in their Guide for the Care and Use of Laboratory Animals.

Experiment 1:

Habituation

The animals experienced two days of habituation to the self-administration chamber to learn the behavioral task (spout licking). On the evening before the first habituation session, ad-libitum water was removed. The rats then had a 5 min habituation session on each of the 2 days, starting 2 hours into the light phase. During this 5 min period, water was available in one of the two spouts, varying the location each day (center and right). The center empty spout was the future “inactive” spout on which responding would lead to no consequence, and the rightmost

empty spout was the future “active” spout where completion of a given number of contacts will lead to an intravenous (iv) infusion of drug. In order to maintain proper hydration, rats also were given overnight access to 20 mL filtered water at the front of the home cage beginning at 5pm. After the third day of habituation, ad-libitum water was restored.

Self-administration

Two hours into the light cycle, rats were placed in the operant chambers where they had 6 hours access to heroin. In the beginning of the session, the middle and right empty spouts advanced. Licking on the inactive spout (middle spout) had no consequence. The availability of the empty active spout (rightmost spout) was signaled by a cue light located above, and completion of a fixed ratio of 10 (FR=10) contacts with this spout led to onset of a tone and a 6-second intravenous (iv) infusion of 0.06 mg/0.2 mL heroin (n=16). Each infusion was followed by a 20-second time-out period in which the cue light turned off, the house light turned on, the empty spouts retracted and the sound of the tone signaled the time out period. Rats were trained using this protocol, for a period of 6 hours per trial, 5 days a week for 22 trials.

Treatment and Abstinence

On day 11 of acquisition rats started receiving a daily subcutaneous (sc) injection of either 0.1 mg/kg of liraglutide (LIR, Novo Nordisk, Bagsvaerd, Denmark) or saline one hour before being place in the operant chamber. Following the 1st test day (which occurred 24 hours after the final acquisition trial), a 19-day forced abstinence period was begun. During this period, rats remained in their home cage with ad-libitum access to water and food, and were injected sc

once daily with either vehicle (Veh) or 0.1 mg/kg of LIR one hour into the light cycle. Treatment with LIR continued until the 2nd test day.

Extinction/Reinstatement Tests

There were two extinction/reinstatement tests. The first test (Test 1) was performed the day after the 22nd self-administration trial, while the second test (Test 2) was performed after 19 days of forced abstinence. During these tests, rats received their daily injection of Veh or LIR one-hour prior being placed in the operant chamber. Then, rats were subjected to a 5-hour extinction period during which all cues associated with the drug (cue light, tone, etc.) were presented, but contacts on the active spout did not result in delivery of drug. Immediately after the fifth hour, a non-contingent iv infusion heroin (0.06 mg/infusion) was automatically delivered by the computer and subsequent contacts with the spouts were recorded to assess drug-induced reinstatement of heroin seeking behavior.

Blood glucose.

During the 6th day of abstinence, 10 µl of blood was extracted from the catheter 1 hour before and 1 and 6 hours after the LIR injection. Then, blood glucose concentration was measured using a glucometer (Prodigy AutoCode, Charlotte, North Carolina, United States).

Experiment 2:

Conditioned Taste Avoidance

Forty-nine naïve male Sprague-Dawley rats had 15 min access to water two hours into the light cycle and 20 mL overnight. To habituate the rats to the protocol, water restriction (15 min am/20 mL overnight) started one week before the taste-drug pairing. Starting on the second week, the rats had 15 min access to 0.15% saccharin in the front of the home cage immediately followed by a sc injection of either saline (n=10) or LIR (0.1 mg/kg, n=10; 0.3 mg/kg, n=10; 0.6 mg/kg, n=10; or 1.0 mg/kg, n=9). There was a total of 5 pairings, followed by one 15 min saccharin only test, with 4 water days (15 min am/20 mL overnight) elapsing between each. Daily measurements included 15 min saccharin intake, food intake, water intake and body weight.

Kaolin Intake Test (Pica Response)

Ten rats from the saline group of the conditioned taste aversion study were maintained in the water restriction protocol, injected with increasing doses of LIR and the ingestions of the non-nutritive kaolin (i.e., clay) intake was assessed. Immediately after the 15 min access period to water in the morning, rats were injected with LIR at three-day intervals, increasing the dosage from 0.06 mg/kg to 0.1 mg/kg to 0.3 mg/kg to 0.6 mg/kg to 1.0 mg/kg. Six additional naïve rats were injected with 0.3M LiCl (12.72 mol/kg). Kaolin was placed on the floor of the home cage along with standard chow and was weighed daily to calculate intake.

Data Analysis

All data were analyzed using Prism version 8.00, GraphPad Software (La Jolla California USA). Student-t tests were used when comparing only two means, with alpha set at 0.05. Mixed factorial Analysis of Variance (ANOVAs), followed by Tukey's post hoc tests, were used to compare differences across groups.

Results

Experiment 1:

Heroin Self-administration

Rats self-administered heroin over 22 days, with daily veh or LIR treatment beginning on trial 11. To assess the effect of LIR on heroin self-administration, the number of heroin infusions was first averaged across trials 1 – 10 (pre) and trials 11 – 22 (post) and analyzed using a 2 x 2 mixed factorial ANOVA varying treatment (veh-LIR) and time (pre-post). Results showed a significant main effect of treatment ($F_{1,10}$=17.19, p=0.0020) and a significant treatment by time interaction ($F_{1, 10}$=11.94, p=0.0062). A post-hoc analysis confirmed that, after treatment, the Her-LIR group self-administered significantly fewer infusions than the Her-veh group, overall (Figure 4-1A) (ps<0.005). In addition, when escalation of heroin self-administration was analyzed using a 2 x 2 mixed factorial ANOVA to compare the number of infusions emitted on the first and the last trial for both groups, post hoc tests of a significant interaction ($F_{1,14}$=5.6, p<0.05), confirmed that group Her-Veh escalated heroin intake (ps < 0.05), while group Her-LIR did not, (Figure 4-1B; ps > 0.05). Finally, treatment with LIR significantly increased the latency to initiate contacts with the active spout (Figure 4-1C). A Students t-test (p<0.05) confirmed that group Her-LIR took a longer time to contact the active spout than group Her-Veh, which immediately approached the spout after being placed in the chamber.

Liraglutide is a synthetic GLP-1 analog that, due to some structural changes, binds to albumin which prevents LIR from being rapidly degraded, allows for a steady and slower release into the blood stream, and delays its actions in the central nervous system. As a consequence, we

then analyzed heroin self-administration by hour across treatment days to assess the time (hour 1 – hour 6) at which LIR was most effective. Figure 4-2 shows the mean number of heroin infusions as a function of trial for each of the six hours of self-administration. Even though LIR-treated rats consistently self-administered less heroin than the Veh control group during every hour, the main effect of treatment was significant only during hour 5 ($F_{1, 14}$=10.73, p=0.0055) and hour 6 ($F_{1,14}$=9.570, p=0.0079). The treatment by trial interaction was not significant, however, during either hour 5 (F<1) or hour 6 ($F_{11,154}$=1.35, p=0.2).

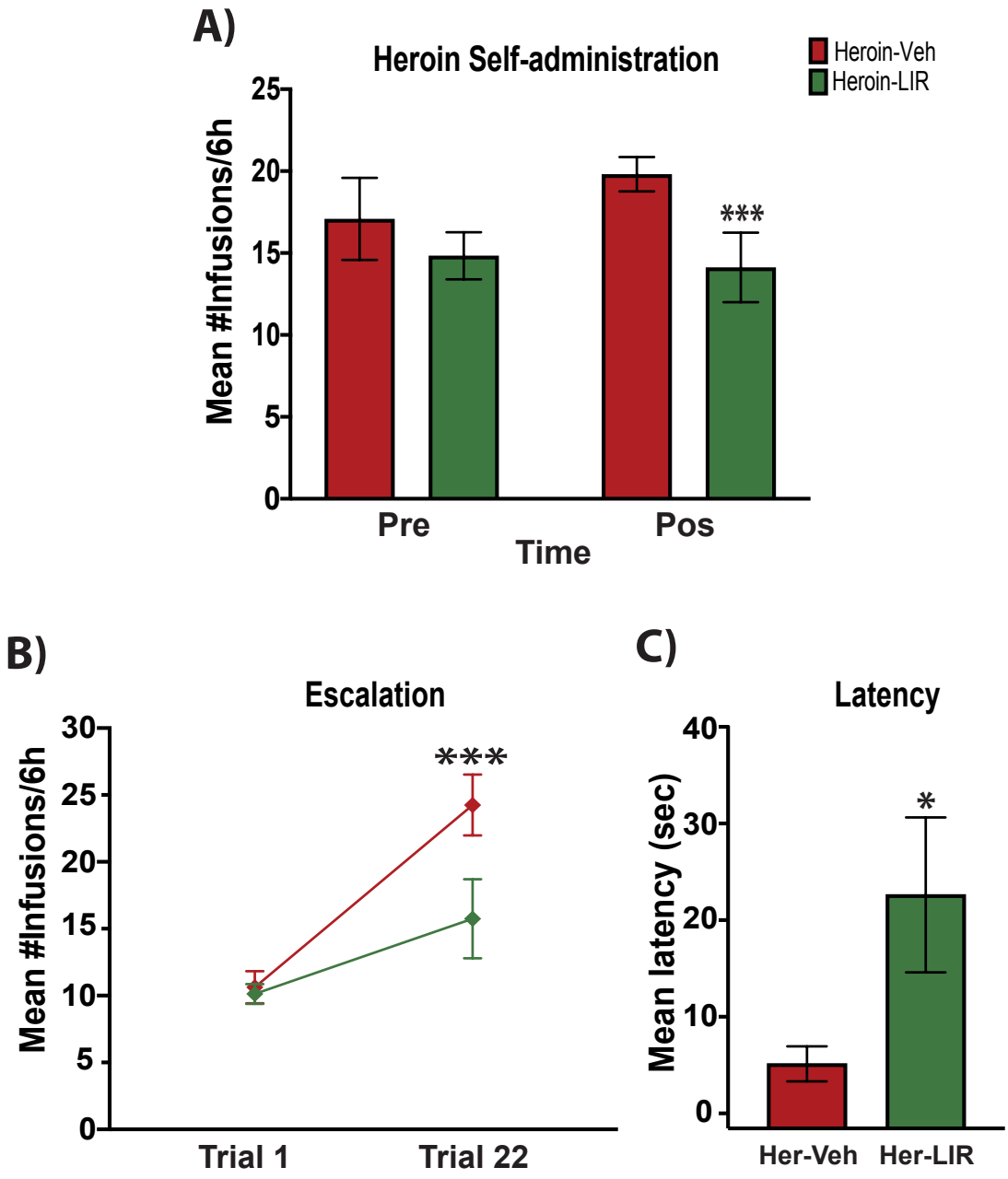

Figure 4-1. Effect of Liraglutide on heroin self-administration. A. Self-administration. Mean (± SEM) number of heroin infusions/6h both before (pre trials 1 – 11) and after (post trials 11 – 22) daily treatment with saline (Veh) or liraglutide (LIR). **B**. Escalation. Mean (± SEM) number of heroin infusions/6h on trials 1 and 22 for rats treated with Veh or LIR. **C**. Latency. Mean (± SEM) latency (in seconds) to initiate contacts with the active spout for rats treated with Veh or LIR. Her-Veh (n=8) or Her-LIR (n=8). Symbols denote significant differences. *:p<0.5; ***:p <0.001.

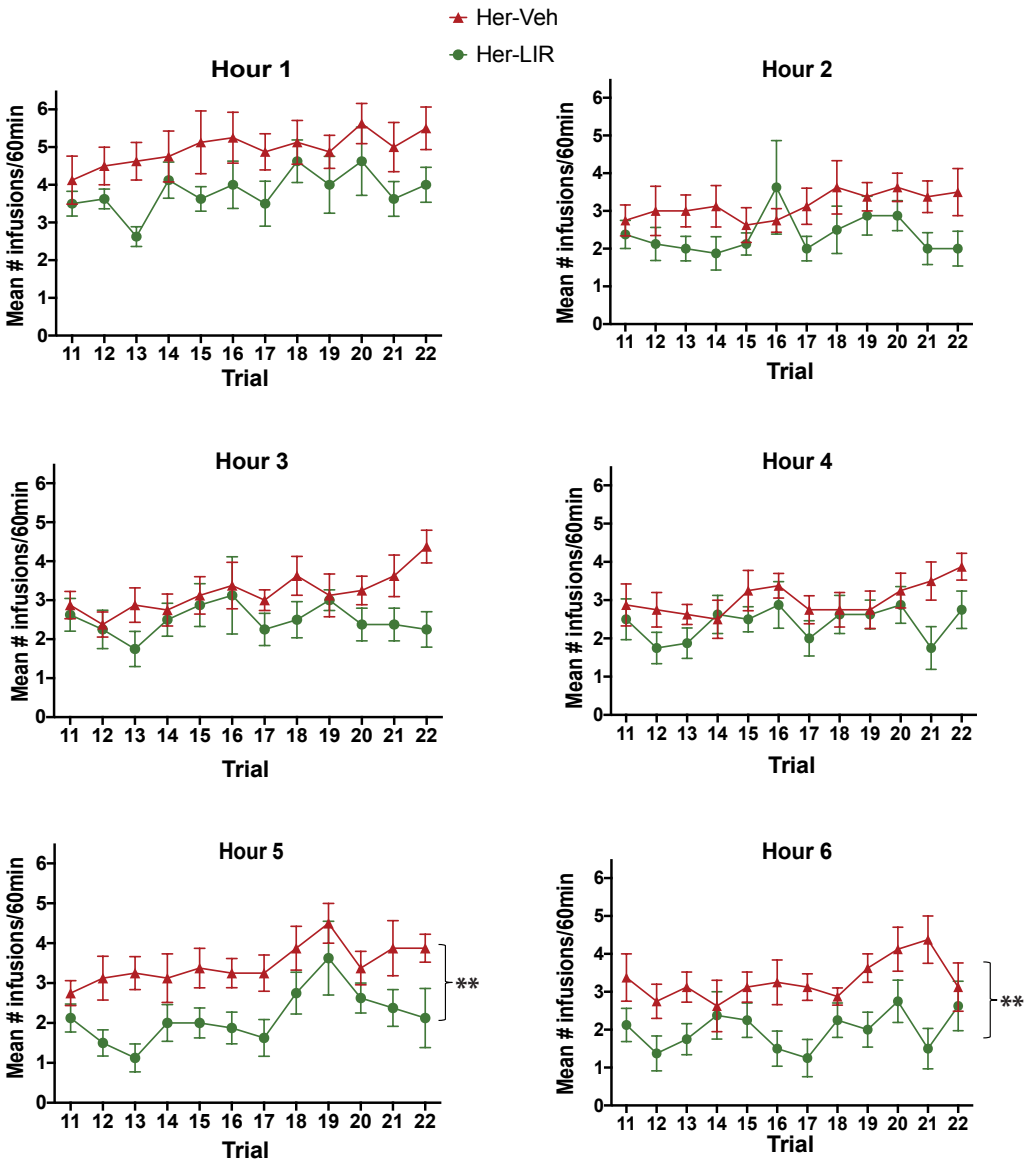

Figure 4-2. Effect of Liraglutide on heroin self-administration by hour across treatment trials. Mean (± SEM) number of heroin infusions during each of the 6-h session across trials 11 – 22 for Veh (n=8) or LIR (n=8) treated rats.

Heroin seeking

Heroin seeking was examined in two extinction/reinstatement tests. Figure 4-3A shows heroin seeking during the 1st test, performed 24 hours after the last self-administration session. During this test, both vehicle and LIR-treated rats show a high number of responses on the active spout during the first hour of extinction (i.e., during cue-induced heroin seeking). However, during hour 6, after the automated infusion of heroin (i.e., drug-induced reinstatement of heroin seeking), rats treated with vehicle showed a significantly higher number of active spout contacts than LIR-treated rats. This conclusion was supported by post hoc tests (ps < 0.05) of a significant 2 x 2 mixed factorial ANOVA varying treatment and time ($F_{1,13}$=4.68; p<0.05). Figure 4-3B shows heroin seeking during the 2nd extinction/reinstatement test which followed the 19-day abstinence period. During this test, both groups again showed a high number of active spout responses during cue-induced seeking. However, during drug-induced reinstatement of heroin seeking, vehicle treated rats showed a significantly higher number of contacts with the active spout compared with LIR-treated rats. This conclusion was confirmed by a 2 x 2 mixed factorial ANOVA showing a significant main effect of hour ($F_{1,10}$=9.9; p<0.05) and a significant hour by treatment interaction ($F_{1,10}$=5.531; p<0.05).

The data shown in Figure 4-3 also demonstrate that prolonged treatment with 0.1 mg/kg LIR did not affect body weight gain across the experimental weeks (Figure 4-3C). This conclusion was supported by a 2 x 8 mixed factorial ANOVA showing a significant main effect of time ($F_{2.329,30.6}$=86.19, p<0.0001) and time by treatment interaction ($F_{7,92}$=2.57, p=0.03), but no significant main effect of treatment ($F_{1, 14}$=2.19, p>0.05). Post hoc tests of the significant interaction did not reveal any significant differences between Her-veh and Her-LIR at any week. In addition, treatment with LIR did not affect plasma glucose at 1 and 6 hours post injection

(Figure 4-3D), as there was no significant main effect of time ($F<1$), main effect of treatment ($F<1$) or time by treatment interaction ($F<1$).

Experiment 2:

Conditioned taste aversion and pica response

Figure 4-4A shows saccharin consumption during the five saccharin-LIR pairings across the CTA conditioning period. Rats that received saccharin-saline pairings increased intake of saccharin across trials. On the other hand, pairings with all doses of LIR produced robust avoidance of the saccharin cue. These results were supported by a 5 x 5 mixed factorial ANOVA varying treatment and trials, showing a significant main effect of treatment ($F_{4,44}=647.34$, $p<0.00001$), a significant main effect of trials ($F_{4,176}=18.5$, $p<0.00001$), and a significant treatment by trial interaction ($F_{16,176}=40.3$, $p<0.0001$) and confirmed by a post-hoc analysis ($ps<0.05$).

Mean intake of the saccharin cue (mL/15 min) on the test day is shown in Figure 4-4B. The control group that received saccharin-saline pairings showed high consumption of the saccharin solution on the test day, while rats in all saccharin-LIR treatment groups avoided intake of the solution. A One-way ANOVA and follow-up post-hoc analysis showed a significant difference between the control group and groups treated with all doses of LIR ($F_{4, 44}=180.6$, $P<0.0001$).

Figure 4-4C shows the pica response as kaolin consumption across groups. Control rats that received an injection of LiCl showed high kaolin consumption over 24 hours. On the other hand, rats treated with saline or increasing doses of LIR differed from the LiCl treated controls as

they did not consume kaolin. This was confirmed by a one-way ANOVA ($F_{6,149}$=10.18, $p<0.0001$) and post-hoc analysis (ps<0.05).

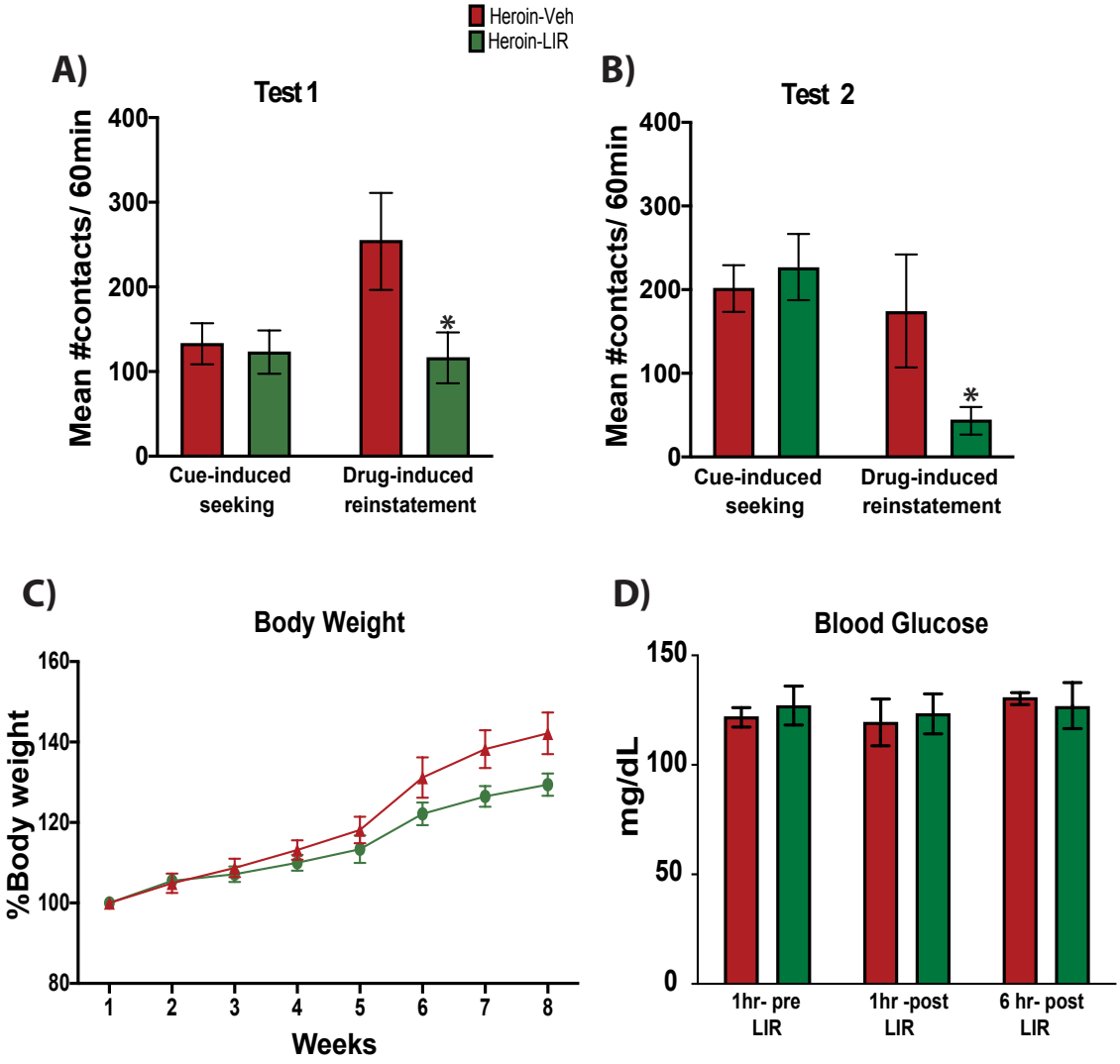

Figure 4-3. Effects of Liraglutide on heroin seeking. **A**. Extinction/Reinstatemnt Test 1. Mean (± SEM) number of contacts with the active spout during 1st hour cue-induced seeking and during 6th hour drug-induced reinstatement of heroin seeking. **B**. Extinction/Reinstatement Test 2. Mean (± SEM) number of contacts with the active spout during 1st hour cue-induced seeking and during 6th hour drug-induced reinstatement of heroin seeking. **C**. Body weight change across experimental weeks. Mean (± SEM) percentage change in body weight from day 1 across 8 weeks of testing for rats treated with Veh or LIR beginning during week 3. **D**. Blood glucose. Mean (± SEM) glucose in plasma (mg/dl) 1 hour before, 1 hour after, and 6 hours after treatment with Veh or LIR on Abstinence day 6. Her-Veh (n=8) or Her-LIR (n=8). *:p<0.5.

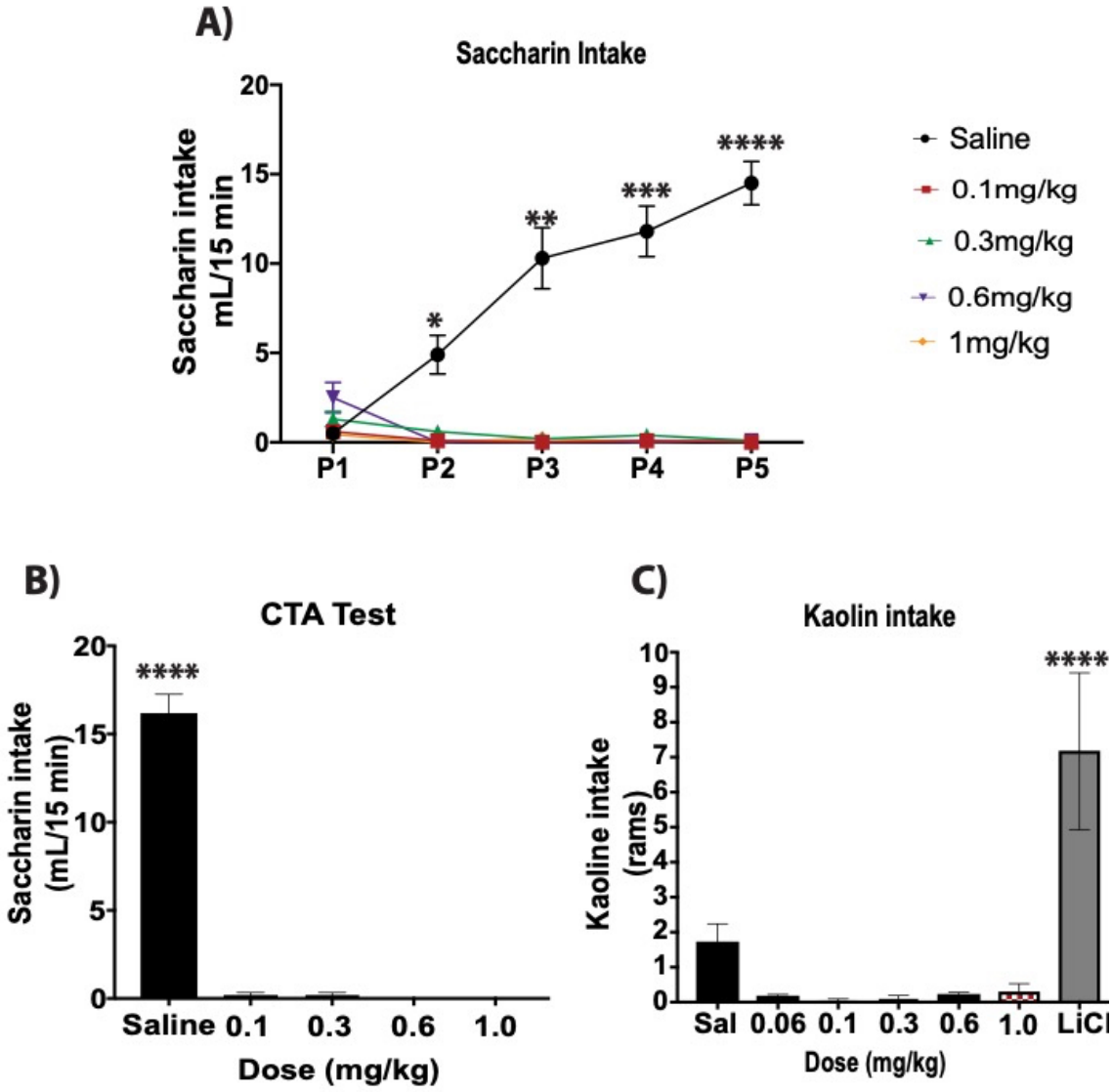

Figure 4-4. Conditioned taste avoidance and pica response. A. Mean (± SEM) saccharin intake (mL/15min) across five pairings with Saline or 0.1, 0.3, 0.6, or 1.0 mg/kg LIR (n=9-10/cell). **B**. Mean (± SEM) saccharin intake (mL/15min) on the saccharin only CTA test day (n=9-10/cell). **C**. Mean (± SEM) kaolin intake (grams/24 h) in rats (n=10) injected with increasing doses of LIR (saline, 0.06, 0.1, 0.3, 0.6, 1.0 mg/kg) or with Lithium Chloride (n=6). Symbols denote significant differences. *:p<0.5; **:p <0.01; ***:p <0.001; ****:p <0.0001.

Body weight, food intake and water intake

Glucagon-like peptide-1 and its analogs can affect normal food and water intake and as a consequence body weight in rats. For this reason, all of these parameters were assessed during the CTA experiment. All rats showed an overall increase in body weight across experimental days (Figure 4-5A). However, saline treated rats gained weight at a higher rate than other groups, except rats treated with 0.1 mg/kg LIR. In addition, rats treated with 0.3 mg/kg LIR showed an intermediate rate of increase in body weight, similar to rats treated with 0.1 mg/kg, but different from the control group. Finally, rats treated with both 0.6 mg/kg LIR and 1.0 mg/kg LIR showed the slowest rate of increase in body weight and a greater fluctuation between injection days. This conclusion was supported by a 5 x 22 mixed factorial ANOVA varying dose and day, showing a significant main effect of treatment ($F_{4,44}$=17.08, p<0.0001), a significant main effect of day ($F_{20,800}$=673.43, p< 0.00001) and a significant treatment x day interaction ($F_{80,880}$=28, p<0.00001), and confirmed by post-hoc analysis (ps<0.05).

Figure 4-5B shows cumulative food intake across experimental days. Rats treated with 0.1mg/ LIR did not show differences in food intake when compared with the vehicle-treated controls. On the other hand, rats treated with 0.3 mg/kg, 0.6 mg/kg, and 1.0 mg/kg LIR did consume less food than the controls. These results were supported by a 5 x 21 mix factorial ANOVA varying dose and trials and revealing a significant main effect of treatment ($F_{4,44}$=5.9, p<0.00001), a significant main effect of day ($F_{20,880}$=657.3, p<0.00001) and post hoc tests of a significant day by treatment interaction ($F_{80,880}$=1.76, p<0.00001).

Cumulative water intake (mL/24 h) is shown in Figure 4-5C for rats treated with saline or increasing doses of LIR across trials 1 – 22. A mixed factorial ANOVA did not show a significant effect of treatment ($F_{4, 44}$ =1.356; ps>0.05) or treatment x day interaction ($F_{60,660}$=1.025, ps>0.05)

but it did reveal a significant main effect of day ($F_{15,660}$=466.25). These results show that, while cumulative water intake increased across trials, it did not differ as a function of treatment group.

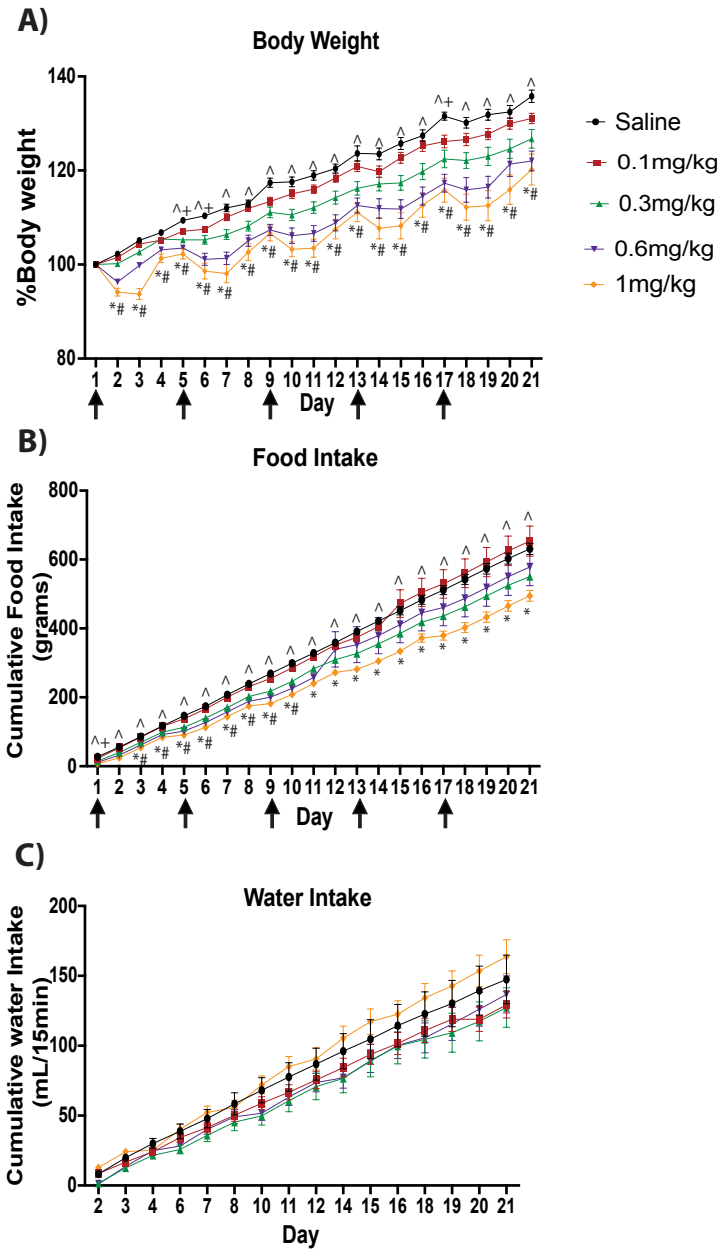

Figure 4-5. Effects of LIR treatment on body weight, food and water intake during the conditioned-avoidance experiment. A. Body weight change across experimental days. Mean (± SEM) percentage change in body weight from day 1 to day 21 for rats treated with Saline or 0.1, 0.3, 0.6 or 1.0 mg/kg LIR. **B.** Food intake. Mean (± SEM) cumulative food intake from day 1 to day 21 for rats treated with Saline or 0.1, 0.3, 0.6 or 1.0 mg/kg LIR **C.** Water intake. Mean (± SEM) cumulative water intake from day 1 to day 21 Saline or 0.1, 0.3, 0.6 or 1.0 mg/kg LIR. (n=9-10/cell). Symbols represent significant differences between saline and LIR doses. +:0.1; ^0.3; #:0.6; *:1.0. Arrows indicate the saccharin-LIR pairing days.

Discussion

The most important finding in this study is that treatment with the long-acting GLP-1R agonist, liraglutide, not only reduced drug-induced heroin seeking, but also reduced ongoing heroin self-administration. GLP-1R analogs are promising as a potential treatment for opioid use disorder. We have demonstrated in **Chapter 3** that the short-acting GLP-1R agonist, Exendin-4, was effective in reducing cue-induced and drug-induced heroin seeking. In here, in Experiment 1, we analyze the effects of LIR in both heroin-seeking and heroin taking. Previous research using this drug showed that a dose of 0.1 mg/kg of LIR attenuates the reinforcing properties of alcohol as indicated by a reduction in alcohol-associated dopamine release, conditioned place preference, alcohol intake and alcohol preference [183]. Using this information, we studied the effect of chronic treatment with 0.1 mg/kg LIR on both heroin-taking and heroin-seeking. Results showed that LIR treated rats self-administered significantly less heroin than their Veh treated counterparts. Additionally, while Veh treated rats escalated heroin self-administration over trials 1 – 22, rats treated with LIR failed to so. Finally, rats treated with LIR exhibited a longer latency to initiate contacts with the active spout when compared with Veh treated controls. These results are consistent with a general reduced motivation to seek and consume the drug. Further, the potential for LIR to control escalation of drug self-administration is promising, as escalation has been described as a hallmark of addiction that is associated with other addiction-like behaviors including decreased sensitivity to negative consequences, increased motivation for drug use, difficulty maintaining abstinence, and a more rapid transition from recreational to uncontrolled use of a substance [81, 259].

When analyzing heroin seeking, treatment with 0.1 mg/kg LIR reduced drug-induced heroin seeking by around 66%. However, no effects were observed in this study on cue-induced

seeking. This finding is in contrast to that obtained in **Chapter 3** following treatment with the short-acting GLP-1R agonist, Ex-4, which significantly reduced both cue- and drug-induced heroin seeking. The kinetics of the drug and the design of the experiment might provide an explanation for these contrasting effects. Specifically, LIR is a synthetic analog, made by direct modification of endogenous GLP-1. These modifications prevent the molecule from degradation by allowing it to bind with albumin and, as a consequence, to be released at a slower and more consistent rate. As such, the slower kinetics may have prevented the drug from reaching the brain in timely manner to affect cue-induced seeking just one hour after LIR administration. On the contrary, the 6-hour period elapsing between LIR administration and the drug-induced reinstatement test is enough, apparently, to allow LIR to reach the brain and hence to reduce drug-induced seeking. This interpretation also is consistent with the results obtained during heroin self-administration testing, where the most robust effects of LIR treatment were observed during hours 5 and 6. This finding suggests that, while LIR must be in the system to produce its effects, an even greater reduction in heroin self-administration may be obtained by adjusting the pretreatment time or the dose of LIR. If this is true, we may prevent not only escalation of heroin intake, but may considerably reduce heroin intake, cue-induced seeking, and drug-induced reinstatement of heroin seeking behavior. Further research is needed to assess the optimal pretreatment time and the ideal treatment regimen to maximize the effect and to produce long-term, steady protection against relapse.

As mentioned, another aspect to consider when analyzing GLP-1 analogs as a potential treatment for substance use disorder relates to known side effects such as nausea and malaise. In this study we used two tests (CTA and pica response) to assess the effects of different doses of LIR on malaise associated behaviors. We observed that all doses of LIR caused a significant conditioned-taste avoidance when paired with a saccharin cue, similar to that described using

high doses of LiCl [224, 255, 256]. Besides producing CTA, LiCl and other emetic agents also are known to produce pica (i.e., the ingestion of a non-nutritive foodstuff). In our experiment we observed that rats treated with LiCl consumed around 7 gr of kaolin in 24 hours. However, no dose of LIR induced pica, as evidenced by the lack of kaolin consumption in rats treated for 3 consecutive days with increasing doses of LIR. These results are consistent with previous research showing that 0.05 mg/kg LIR did not elicit kaolin intake [196]. Moreover, in this study even higher doses such as 0.6 mg/kg and 1.0 mg/kg LIR, doses that produced a reduction in body weight and food intake along with a CTA, did not elicit consumption of non-nutritive kaolin. Conditioned taste avoidance is assumed to be the result of the association between a novel gustatory stimulus and illness [255, 257, 258], as demonstrated by pairing tastants with known toxic, emetic agents [256]. Hence, the aversive properties of a specific agent are inferred by the reduction in the amount of the tastant consumed. This measure, however, requires the rat to perform certain motivated behaviors such as approaching and consuming the solution [260]. GLP-1 analogs have been shown to reduce motivated behaviors for food and other rewarding and even aversive substances [178, 179, 181, 182, 186]. In this context, it is possible that LIR is not producing illness, per se, but, as observed in **Chapter 2,** is reducing the overall motivation of the rat to approach and consume. The reduction in intake of the LIR-paired solution, then, would reflect a conditioned reduction in motivation.

When we analyzed the effects of chronic treatment with LIR, we found that LIR, dose-dependently reduced body weight gain. There was no sustained weight loss, and all rats gained weight throughout the experiment. At the lowest dose (0.1 mg/kg LIR) rats showed weight gain similar to that of controls. At higher doses, both 0.6 mg/kg LIR and 1.0 mg/kg LIR, rats gained less weight than controls and exhibited greater fluctuation in body weight over time. Specifically, after the LIR injection, a reduction in body weight was observed for two consecutive days,

followed by two days of weight gain, after which, the cycle repeated itself. This is consistent with previous research showing that LIR can reduce body weight 24 hours after treatment and daily doses can produce a sustained reduction in the rate of body weight gain [163, 261]. However, with the regimen used in the present study, we found that the doses that elicit such an effect on body weight are higher than the lowest dose (0.05 mg/kg) previously reported [196, 261]. Additionally, and contrary to previous results, treatment with any of the doses of LIR did not affect water intake on the days in between pairings [212, 213]. It is possible, that the discrepancy of the results may be due to the water restriction protocol utilized in the present study. Furthermore, similar to the body weight gain, LIR treatment reduced food intake dose-dependently, particularly at the higher 0.6 mg/kg and 1.0 mg/kg doses. With these doses, there is an evident lack of food intake accumulation on the days after treatment that match the fluctuations in body weight. This is consistent with previous reports showing that the most robust effect of LIR (0.05 mg/kg) on food intake occurs during initiation of treatment and lasts for 3 days following the injection. After this period, consumption of food normalizes [196]. In addition, it has been observed that doses as low as 0.01 mg/kg LIR can produce a reduction in food intake 24 hours post injection [261]. However, this was not observed in this study when the injections were spaced 4 days apart. On the other hand, when the injections were daily during both self-administration and abstinence in Experiment 1, 0.1 mg/kg LIR tended to reduce the rate of body weight gain, but the effect was not significant, at least for a period of 8 weeks. Acute treatment with 0.1 mg/kg LIR also did not affect blood glucose. Taken together, these data suggest that it is safe to use low doses daily without expecting considerable side effects. However, if dosage needs to be increased, spacing injections at 4-day intervals may produce a bigger fluctuation in food intake and body weight, but will allow the organism to return to baseline before the next injection is scheduled. How the injection schedule affects body weight

and food intake is important when considering a potential treatment for opioid use disorder, as people that suffer OUD often are undernourished [87] and a sustained reduction in food intake may be dangerous.

The results of this study show that the long-acting GLP-1R agonist, liraglutide, is a promising compound to treat opioid use disorder. As we observed here, LIR did not produce a robust or sustained physiological disruption, since all LIR treated rats increased body weight and food intake, and water intake was not significantly affected. In addition, while all rats avoided intake of the saccharin cue paired with all doses of LIR, this avoidance was not accompanied by an increase in intake of kaolin – which has been observed to have antiemetic effects in humans, suggesting that the reduction in intake was not due to malaise. Importantly, we observed that treatment with even the lowest dose used in the CTA study (0.1 mg/kg) reduced heroin self-administration by about 25%, prevented escalation of heroin self-administration over time - a 'hallmark' of addiction-, and reduced drug-induced reinstatement of heroin seeking behavior. That said, when using a 1-hour pretreatment time, this same dose of LIR was not sufficient to prevent cue-induced seeking. Further studies are required to verify that LIR, like Ex-4 in **Chapter 3**, can reduce not only drug-, but also cue-induced heroin seeking. Overall, the present results add to our Ex-4 data to suggest that GLP-1R agonists may serve as a promising, non-opioid treatment for opioid use disorder in humans. A clinical trial designed to test the safety and efficacy of LIR in humans with an opioid use disorder is underway [245].

Manuscript: Glucagon-like peptide-1 receptor agonist, liraglutide, reduces heroin self-administration and drug-induced reinstatement of heroin seeking behavior in rats.(*In Preparation*)

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Respective contributions: J.E. Douton: Experimental design and execution, data analysis and interpretation, and manuscript preparation. N. Horvath: Experiment 2 execution. A. Hajnal: Experimental design, data interpretation and manuscript revision. P.S. Grigson: Experimental design, data interpretation and manuscript revision.

Chapter 5

Acute glucagon-like peptide-1 receptor agonist liraglutide prevents drug-induced heroin seeking in rats

Introduction

The chronic and relapsing nature of substance use disorder (SUD) makes it particularly difficult to treat because relapse can occur even after prolonged periods of abstinence [2]. It is during this abstinence period that people are more susceptible to overdose [11]. Accordingly, opioid use disorder (OUD) is a serious threat to public health and has been declared an epidemic by the Centers for Disease Control and Prevention [10]. This is particularly tragic considering there are effective treatments to help to maintain abstinence and to prevent relapse. Medication assisted treatment (MAT) has been proven to reduce relapse in patients with SUD [248-250]; however, current therapeutic options are not ideal. For example, both methadone and buprenorphine carry the stigma of 'replacing one opioid with another.' Further, the opioid receptor antagonist naloxone requires several days of abstinence prior to use and does not alleviate symptoms of withdrawal, resulting in poor compliance [250]. Most importantly, there are no current treatment options that alleviate craving – a major risk factor for relapse. It is therefore imperative to find alternative treatments to assist patients with OUD to maintain abstinence.

The incretin hormone glucagon-like peptide-1 (GLP-1), best known for its regulation of homeostatic feeding, also has been observed to modulate reward-motivated behavior. Specifically, the short acting GLP-1 receptor agonist, Exendin-4 (Ex-4), has been shown to reduce responding for normally rewarding stimuli such as food, cocaine, alcohol, nicotine, oxycodone and, as observed in **Chapter 3**, heroin [169, 178, 179, 181, 182, 186]. Many GLP-1 analogs already have been approved for the treatment of type 2 diabetes and obesity, and, if found effective in reducing responding for drugs, can be re-purposed to treat SUD [139, 190]. Of the numerous formulations of GLP-1R agonists, Ex-4 has been the main focus of study in the context

of addiction. Its short half-life, however, will require patients to receive at least two injections throughout the day for continuous prophylactic protection [137]. This would not be a practical solution as it would lead to poor compliance, particularly when the reported side-effects associated with such therapies are taken into account [251]. Less research, though, has focused on the effects of GLP-1 agonist formulations with longer half-lives such as liraglutide (LIR) or semaglutide on drug seeking behavior. So far, LIR has been found effective in reducing the rewarding properites of alcohol and, as observed in **Chapter 4**, in reducing heroin self-administration and drug-induced reinstatement of heroin seeking behavior in rats [183]. The longer half-life of LIR makes it a better candidate as a potential treatment to relieve craving and/or withdrawal and to prevent relapse in opioid use disorder.

In the present study, we examined the bioavailability of increasing doses of liraglutide over time in a pharmacokinetics (PK) study. In addition, we assessed the protective effects of LIR against the three major precipitating factors of relapse: the exposure to drug-paired cues (i.e., cue-induced seeking), exposure to the drug itself (i.e., drug-induced reinstatement) and exposure to stress (i.e., stress-induced reinstatement). Specifically, we tested whether acute treatment with LIR could reduce heroin seeking in these different models of relapse in heroin-experienced rats.

Materials and Methods

The subjects were 44 outbred male Sprague-Dawley rats from Charles River (Wilmington, MA) delivered at approximately 90 days of age, weighing between 300 – 400 g at the beginning of the experiment. All subjects were housed individually in standard, suspended, stainless steel cages. The environment in the animal colony room had controlled temperature (21 °C) and humidity, a 12/12 light/dark cycle, with the light phase starting at 7:00 am. All experimental manipulations were conducted beginning 2 hours into the light phase of the cycle. Following a one-week acclimation period to their home cages, rats were habituated to experimenter handling by daily weighing. Water and food were available ad libitum, except where otherwise noted. All studies were approved by the Pennsylvania State University College of Medicine, Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health specifications outlined in their Guide for the Care and Use of Laboratory Animals.

Self-Administration and arterial Catheters

Jugular and Arterial Catheter Implantation Surgery

Rats were anesthetized with isoflurane (4% induction; 2–3% maintenance) and implanted with either arterial catheters in the carotid artery for repeated blood collection (n=3) or intravenous jugular catheters (n=41) (Instech Laboratories, Inc., Playmouth Meeting, PA) for drug self-administration as described previously [93]. Following surgery, rats received subcutaneous (sc) injection of the NSAID, carprofen as post-operative care for at least two days,

and were given a full week to recover. Maintenance of catheter patency included flushing catheters using heparinized saline (0.2 mL of 30 IU/mL heparin) every four days. Catheter patency was verified at the end of each week of drug self-administration and the day before each test day using 0.3 mL of propofol (Diprivan 1%).

Blood Collection

Three rats implanted with arterial catheters were injected daily with increasing doses of LIR. Treatment started with 0.06 mg/kg LIR sc every 24 hours for three days. On the fourth day, the dose was increased to 0.3 mg/kg for another three days. On the seventh day, the dose was increased to 1.0 mg/kg for another three-day period. Blood was extracted from the arterial catheter on the 1st, 3rd and 7th day, coinciding with the first day of each dose. On these days, the catheters were infused with 50 μL of heparinized saline, immediately followed by extraction of 200 μL of blood. Then, another 200 μL of blood was extracted and placed in an EDTA-lined blood collection tube placed in ice. The 200 μL of blood that was extracted first was then reinfused through the catheter, followed by 200 μL of heparinized saline. Using this procedure, blood was collected prior to LIR administration, and then every two hours for a total of 10 hours. Samples where centrifuged at 1000 x g for 10 minutes at 4 °C, and the plasma (supernatant) was transferred to another tube and stored at -80 °C until analysis.

High Performance Liquid Chromatography and Tandem Mass Spectrometry

Materials

Liraglutide was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Formic acid was purchased from J. T. Baker (New Jersey, USA). Optima LC-MS grade water, acetonitrile and methanol and other chemicals were purchased from Fisher Scientific (New Jersey, USA).

Sample Preparation Procedure

A standard stock solution of liraglutide (1 mg/mL) was prepared in methanol with 2% NH_4OH. Liraglutide standard working solutions from 50 ng/mL to 5,000 ng/mL were prepared by serial dilution of the liraglutide stock solution by acetonitrile/water/formic acid (80/20/0.1). All stock and working solutions were stored at -20 °C until use. Standard curves were prepared by adding 10 μL of the standard working solution into 5 μL of untreated plasma, followed by the addition of 85 μL acetonitrile/methanol (50/50) to final concentrations of 5 - 500 ng/mL. Experimental plasma samples were treated in the same manner as the standards: 95 μL acetonitrile/methanol (50/50) was added to 5 μL plasma to precipitate the proteins and clean up the matrix. The samples were vortexed and centrifuged at 4 °C for 10 min at 8,765 x *g*. The supernatant was loaded onto the UPLC/MS/MS system.

HPLC-MS/MS Analysis

Liraglutide concentrations in the plasma samples were analyzed using a Sciex QTrap 6500+ mass spectrometer coupled with an Exion HPLC separation system. A 1.8 μm Acquity UPLC HSS T3 analytical column (2.1 x 100 mm, Waters, Ireland) was used to separate liraglutide from the plasma, and a gradient elution was conducted using a flow rate of 0.4 mL/min with the following conditions: Initiated in 45% mobile phase B (0.1% formic acid in acetonitrile) and 55% solvent A (0.1 % formic acid in water) for 0.5 min, followed by a linear gradient to 56% mobile phase B over the course of 2.5 minutes, after which there was a final gradient to 95% mobile phase B over 1 minute, and then maintained at 95% mobile phase B for 1 minute to flush the column before going back to initial condition. The autosampler was maintained at 4 °C, and the column temperature was maintained at 40 °C. The Sciex QTrap 6500+ mass spectrometer was equipped with an electrospray ionization probe operated in positive mode. The decluster potential (DP) was 70 V, the entrance potential (EP) was 10 V, the collision energy (CE) for the compound was 35 V, and the collision cell exit potential (CXP) was 10 V. The curtain gas (CUR) was 35 L/h, the collision gas (CAD) was medium, the ionSpray voltage was 5500 V, the temperature was 450 °C, the ion source gas 1 was 30 L/h and the ion source gas 2 was 20 L/h. Multiple reaction monitoring mode (MRM) was used to analyze and quantify the compound, the transition is m/z 938.2 > 1128 for triple charged liraglutide. All peaks were integrated and quantified by Sciex OS 1.5 version software.

Behavioral Procedures

Apparatus

The experiments were conducted in 24 self-administration chambers (MED Associates, Inc., St. Albans, VT) as previously described [262]. Each chamber measured 30.5 cm in length, 24.0 cm in width, 29.0 cm in height, and was equipped with two retractable sipper tubes that entered the chamber through two holes. A stimulus light was located above each hole, and a lickometer circuit was used to monitor licking on all two spouts. In addition, each chamber was equipped with a house light (25 W), a speaker for white noise and a tone generator. Collection of data and events in the chamber were controlled on-line with a Pentium computer using programs written in the Medstate notation language (MED Associates, Inc., St. Albans, VT).

Habituation

Rats experienced two days of habituation to the self-administration chambers. On the evening prior to the first habituation session, ad libitum water was removed overnight. The rats then underwent one 5-min habituation session per day for 2 days. Starting 2 hours into the light phase, rats were placed in the self-administration chambers for 5 min. During this 5-min period, water was available in one of the two spouts, starting with the center spout (future ‘inactive’ spout) on the first day and the rightmost spout (future ‘active’ spout) on the second day. In order to maintain proper hydration during habituation, rats received overnight access to 20 mL filtered water at the front of the home cage beginning at 5pm. Ad libitum access to water was resumed after the second habituation session.

Heroin Self-administration

Two hours into the light cycle, rats were placed in the self-administration chambers (MED Associates, Inc., St. Albans, VT). At the start of each session, two empty spouts advanced: the rightmost empty spout served as the 'active' spout, while the centrally located empty spout served as the 'inactive' spout. A cue light above the rightmost spout indicated its availability. Completion of a fixed ratio of 10 (FR=10) contacts with this empty active spout led to the onset of a tone and a 6-second intravenous (iv) infusion of either saline (n=20) or 0.06 mg/infusion heroin (n=21). Each infusion was accompanied by a 20-second time-out period in which the cue light turned off, the house light turned on, the empty spouts retracted, and the sound of the tone signaled the time out period. After each timeout period, the active and inactive spouts were again advanced. Licking the inactive (center) spout had no consequence. Rats were allowed the opportunity to self-administer heroin or saline for 6 hours each day for 5 days per week until 11 trials were completed.

Cue-induced Seeking and Drug-induced Reinstatement 4 Hours after Liraglutide Pretreatment

Twenty-four hours after the last self-administration trial, 11 rats from the heroin and 8 rats from the saline group were randomly divided into vehicle (n=9) or LIR (n=10) pretreatment groups. At the beginning of the light cycle, rats received a single subcutaneous (sc) injection of vehicle (saline) or 0.3 mg/kg LIR. Four hours later, rats were placed in the self-administration chamber and subjected to a 3-hour extinction test during which all cues associated with the drug (cue light, tone, etc.) were presented as usual, but contacts with the active spout did not deliver an infusion of saline or drug. Immediately after the third hour, when seeking behavior extinguished,

rats received a single computer-controlled non-contingent iv infusion of saline or heroin (0.06 mg/infusion, depending on their initial group assignment) and reinstatement of heroin seeking behavior was assessed across another hour. The number of contacts with the active spout during the first hour of the three-hour extinction period was interpreted as cue-induced seeking, while the number of contacts during the hour after the single, non-contingent infusion was interpreted as drug-induced reinstatement of heroin seeking.

Cue-induced Seeking, Drug-induced Reinstatement, and Stress-induced Reinstatement 6 Hours after Liraglutide Pretreatment

An additional group of rats was tested using a six-hour pretreatment on the test day. All other procedures were identical to those described for the four-hour pretreatment group, including the 6-hour self-administration sessions for a total of 11 trials, with the exception of an additional test for stress-induced reinstatement following 14 days of abstinence. Twenty-four hours after the last self-administration trial, rats received a sc injection of vehicle (n=10) or 0.3 mg/kg LIR (n=12) six hours before the beginning of the first extinction/drug-induced reinstatement test. Then, the rats were given 14 days of home cage abstinence before being subjected to the second test. As with the first test, the rats received a sc injection of vehicle or LIR (according to their previous group) six hours before the test. Thereafter, rats were re-exposed to a 3-hour period of extinction. At the end of the third hour, all rats were injected ip with the alpha-2 adrenergic receptor antagonist, yohimbine (0.5 mg/kg), which has been observed to induce anxiety- and stress-like responses in laboratory animals and humans, and to induce reinstatement of drug seeking behavior [263-265]. Following yohimbine administration, seeking behavior was monitored for two hours.

Data Analysis

Differences between groups were analyzed using two-way and mixed factorial Analysis of Variance (ANOVAs) varying group (saline, heroin), pretreatment (vehicle, LIR), and trial or time where applicable. Post hoc analyses were conducted using Tukey's post hoc tests. All data, including area under the curve (AUC) were analyzed using Prism version 8.00, GraphPad Software (La Jolla California USA).

Results

Liraglutide Pharmacokinetic Analysis

Figure 5-1A shows the mean plasma concentration of LIR over time following the first daily sc injection of each of the following escalating doses: 0.06 mg/kg, 0.3 mg/kg and 1.0 mg/kg. Rats were injected daily, and the dose was increased after every 3rd day. The lowest dose (0.06 mg/kg) showed a maximum concentration (Cmax) of 3,393 ng/mL at 6 hours post injection; the intermediate dose (0.3 mg/kg) had a Cmax of 33,056 ng/mL at 8 hours post injection; and the highest dose (1.0 mg/kg) had a Cmax of 90,808 ng/mL at 4 hours post LIR injection. In addition, the area under the curve ($AUC_{0\text{-}10h}$) dose dependently increased from 24,437 ngh/mL for the 0.06 mg/kg dose, to 211,575 ngh/mL for the 0.3 mg/kg dose, and 500,686 ngh/mL for the 1.0 mg/kg dose. For each dose, the AUC at its peak time (Tmax) was 11,978 ngh/mL for 0.06 mg/kg, 158,664 ngh/mL for 0.3 mg/kg and 124,821 ngh/mL for 1.0 mg/kg (see Table 5-1). Importantly, LIR is not completely cleared after 24 hours, as the baseline observed for the 0.3 mg/kg and 1.0 mg/kg doses is increased, reflecting a carry-over from the final injection of the previous dose. During the first day, the baseline concentration for the 0.06 mg/kg dose, prior to any LIR injection, was 0 ng/mL. The first administration of the 0.3 mg/kg dose was given 24 hours after the last day of the three daily injections of the 0.06 mg/kg dose. As a consequence, at the time of the first 0.3 mg/kg injection, the plasma concentration was 4,532 ng/mL. Similarly, the first administration of the 1.0 mg/kg dose was given 24 hours after the last day of the three daily injections of the 0.3 mg/kg dose, and as a consequence the LIR concentration prior to the first injection of the 1.0 mg/kg dose was 17,160 ng/mL.

	0.06 mg/kg	0.3 mg/kg	1.0 mg/kg
Baseline concentration (ng/mL)	0	4,532	17,160
AUC $_{0\text{-}10}$ (ngh/mL)	24,437	211,575	500,686
Tmax (hours)	6	8	4
Cmax (ng/mL)	3,393	33,056	90,808
AUC $_{0\text{-}peak}$ (ngh/mL)	11,978	158,664	124,821

Table 5-1. Pharmacokinetic parameters for different doses of liraglutide. AUC: Area Under the Curve. Tmax: Time of maximum concentration. Cmax: Maximum concentration

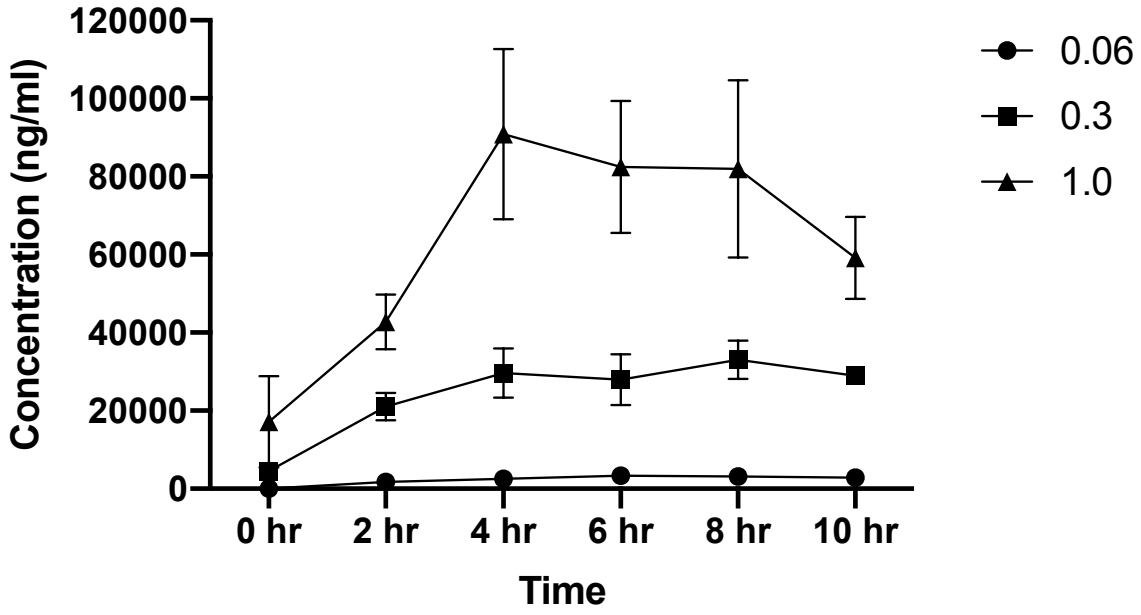

Figure 5-1. Liraglutide plasma concentration. Rats (n=3) were treated daily with increasing doses of LIR. Blood samples were collected on days 1, 4 and 7 before injection (0h), and at 2, 4, 6, 8 and 10 hours post injection. Figure 5-1 shows Mean (± SEM) liraglutide concentration (ng/mL) in plasma over ten hours following the first sc injection of 0.06 mg/kg, 0.3 mg/kg, and 1.0 mg/kg doses of liraglutide.

Heroin Self-Administration

Rats had the opportunity to self-administer either heroin or saline for a period of six hours per session, five days per week, over a total of 11 trials (Figure 5-2). A mixed factorial ANOVA was conducted to identify statistical differences in the number of infusions between rats self-administering heroin or saline. The results found a significant group x trial interaction, ($F_{10,420}$=11.78, $p < 0.0001$), and significant main effects of trial ($F_{3.933, 165.2}$=5.127; p=0.0007) and group ($F_{1,42}$=8.843, p=0.0049). Post hoc analyses of the significant interaction indicated that rats in the heroin group took increasingly more infusions over time compared with the saline group, and this reached significance for trials 5 through 11 (ps<0.05).

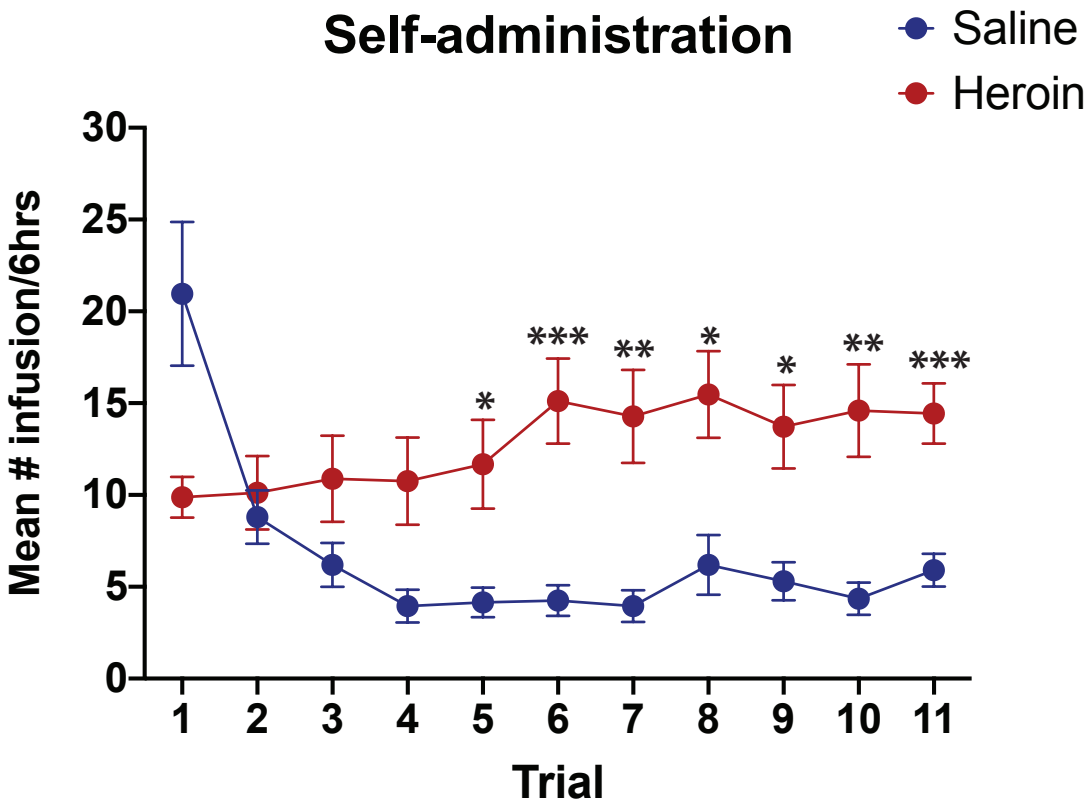

Figure 5-2. Heroin self-administration. Mean (± SEM) number of infusions/ 6h across 11 trials for rats that self-administered saline (n=19; blue) or 0.06 mg/infusion heroin (n=21; red). Significant difference between groups. *p<0.05; **p<0.01; ***p<0.00112

Extinction/Reinstatement Test with 4-hour Pretreatment

During the extinction/reinstatement test, rats were subjected to 3 hours of extinction followed by a single non-contingent infusion of saline or heroin and one additional hour to assess drug-induced reinstatement of heroin seeking behavior. The effect of LIR (0.3 mg/kg, sc) pretreatment on heroin seeking was assessed in rats treated with either vehicle or LIR 4 hours before the beginning of the test (see Figure 5-3A). When treated 4 hours prior to testing (Figure 5-3B), there was no effect of LIR on cue-induced seeking during the first hour of extinction as indicated by lack of a significant group x treatment interaction ($F<1$) nor significant main effects of group ($F_{1, 15}= 2.673$, $p=0.1229$) or treatment ($F_{1,15}=3.064$, $p=0.1004$). However, during the drug-induced reinstatement test (Figure 5-3C), which occurred 7 hours after pretreatment, the rats in the LIR-heroin group (n=6) made significantly fewer contacts with the previously active spout than rats in the vehicle-heroin group (n=5). In fact, seeking was completely abolished, as no contacts were observed with the active spout. In contrast, there was no difference in seeking for rats in the saline group treated with vehicle (n=4) or LIR (n=4). Results of a mixed factorial ANOVA varying group (saline vs. heroin) and treatment (vehicle vs. LIR) supported this conclusion as indicated by a significant group x treatment interaction ($F_{1,15}=15.51$, $p=0.0013$) and confirmed by post hoc analyses ($p<0.05$).

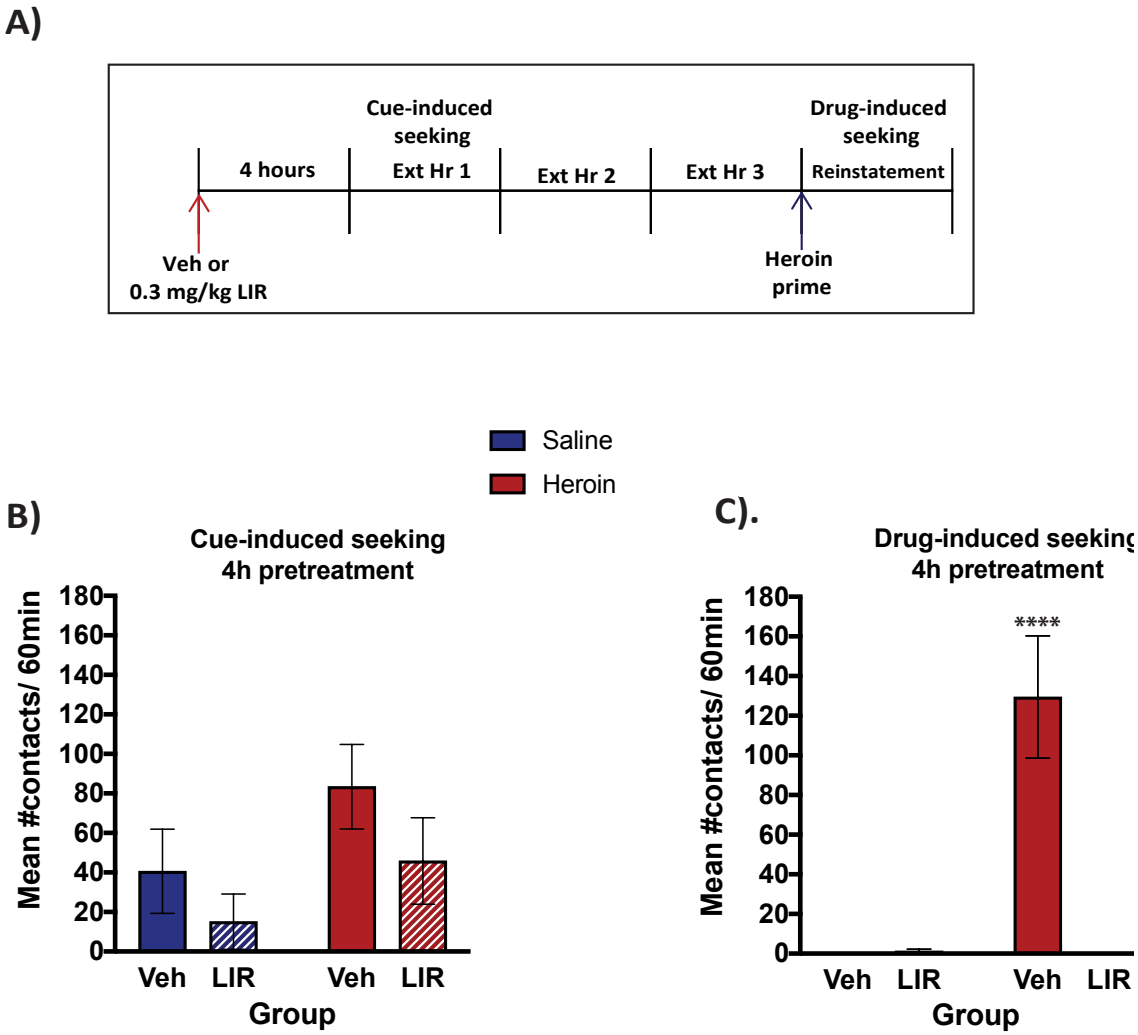

Figure 5-3. Cue-induced seeking and drug-induced seeking with 4h pretreatment. A. Extinction/Reinstatement protocol with 4h pretreatment. Rats were pretreated 4h before the beginning of the test. At test, rats had 3h of extinction in which all the cues associated with the drug were present but contacts with the active spout did not deliver any drug. At the end of hour 3, an iv infusion of saline or heroin was automatically delivered in order to reinstate seeking behavior over the next hour. Cue-induced seeking was evaluated during the first hour of extinction and drug-induced seeking was evaluated during the 1-hour reinstatement test. **B**. Mean (± SEM) number of active spout contacts during the first hour of extinction (cue-induced seeking) for rats with a history of saline or heroin self-administration pretreated with vehicle (saline) or LIR 4 hours prior the start of the test. **C**. Mean (± SEM) number of active spout contacts during reinstatement (drug-induced seeking) for rats with a history of saline or heroin self-administration pretreated with vehicle (saline) or LIR 4 hours prior the start of the test. Saline-veh (n=4), saline-LIR (n=4), heroin-veh (n=5), heroin-LIR (n=6). Significant difference between LIR and Veh. ****p<0.0001

Extinction/Reinstatement Test with 6-hour Pretreatment

In contrast to rats that received a 4-hour pretreatment, rats that received pretreatment 6 hours prior to the start of the extinction/reinstatement test (see Figure 5-4A) showed a significant reduction in both cue-induced and drug-induced seeking (Figure 5-4B and C). During cue-induced seeking, rats in both the saline and heroin groups treated with LIR made significantly fewer contacts with the previously active spout compared with rats pretreated with vehicle. This conclusion was supported by a lack of a significant group x treatment interaction ($F_{1,9}$=2.829, p=0.1269) and significant main effects of both treatment ($F_{1,9}$=8.560, p=0.0169) and group ($F_{1,10}$=13.13, p=0.0047), indicating greater cue-induced seeking by rats with a history of heroin self-administration and reduced seeking in LIR treated rats overall.

Results of the drug-induced seeking test, which occurred 9 hours after treatment for this group, were similar to those observed with the shorter pretreatment time (Figure 5-4D). As with the 4-hour pretreatment, rats with history of saline self-administration, regardless of pretreatment condition, made few contacts with the active spout. Rats with a history of heroin self-administration exhibited greater seeking following the iv heroin challenge, and this seeking behavior was significantly reduced by LIR. In support, post hoc analysis of a significant group x treatment interaction ($F_{1,9}$=13.31, p=0.0053) confirmed that LIR-treated rats made fewer contacts with the active spout than vehicle-treated rats within the heroin group (ps <0.05), and no difference due to treatment was observed within the saline group (ps >0.05).

Stress-induced seeking

A subset of rats from the 6-hour pretreatment group were given an additional 14-day period of abstinence after the extinction/reinstatement test. Following abstinence, cue-induced seeking was re-analyzed along with stress-induced reinstatement. Similar to the previous test, the sc administration of 0.3 mg/kg LIR, with a 6-hour pretreatment time, reduced cue-induced seeking during the first hour of extinction testing in rats with history of heroin self-administration (Figure 5-4D). This was supported by a significant group x treatment interaction ($F_{1,18}$=11.02, p=0.0038) and a significant main effect of treatment ($F_{1,18}$=34.11, p<0.0001). Post hoc analyses of the two-way interaction confirmed that the heroin-vehicle group (n=4) made significantly more contacts on the formerly active spout compared with the other three groups during the first hour of the test (ps<0.5). In addition, 0.3 mg/kg LIR also reduced stress-induced reinstatement of heroin seeking behavior. Following yohimbine administration, both groups with a history of saline self-administration made a low number of contacts on the previously active spout (Figure 5-4E). Rats in the heroin group treated with vehicle, on the other hand, showed robust reinstatement of drug-seeking behavior following yohimbine administration. This effect was eliminated in the heroin group treated with LIR. These findings were supported by a significant main effect of group ($F_{1,18}$=4.99; p=0.38), main effect of treatment ($F_{1,18}$=12.76; p=0.002) and by post hoc assessment of a significant group x treatment interaction ($F_{1,18}$=5.713, p=0.028; ps<0.005).

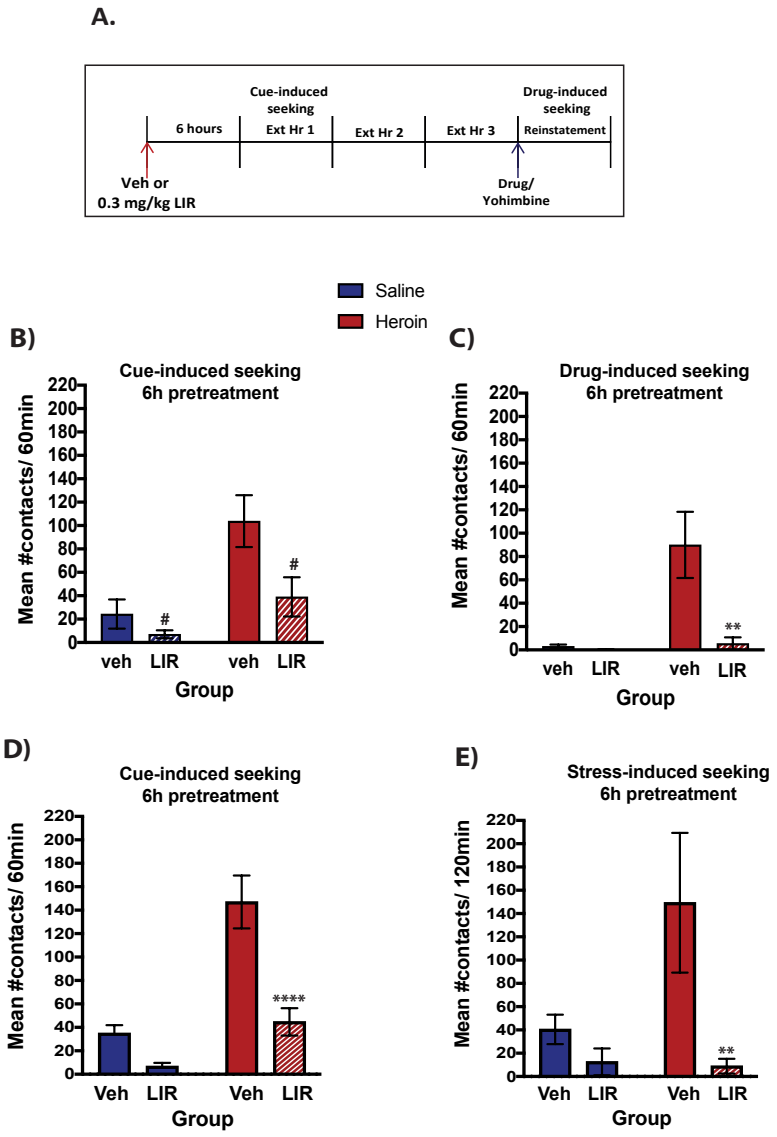

Figure 5-4. Cue-induced, drug-induced, and stress-induced seeking with 6h pretreatment. A. Extinction/Reinstatement protocol with 6h pretreatment. Rats were pretreated 6h before the beginning of the test. At test, rats had 3h of extinction in which all the cues associated with the drug were present but contacts with the active spout did not deliver any drug. At the end of hour 3, an iv infusion of saline or heroin was automatically delivered in order to reinstate seeking behavior. Cue-induced seeking was evaluated during the first hour of extinction and drug-induced seeking was evaluated during reinstatement. **B**. Mean (±SEM) number of active spout contacts during the first hour of extinction (cue-induced seeking) for rats with a history of saline or heroin self-administration pretreated with vehicle (saline) or LIR 6 hours prior the start of the test. **C**. Mean (±SEM) number of active spout contacts during reinstatement (drug-induced seeking) for rats with a history of saline or heroin self-administration pretreated with vehicle (saline) or LIR 6 hours prior the start of the test. **D**. Mean (±SEM) number of active spout contacts during the first hour of extinction (cue-induced seeking) following 14 days of abstinence for rats with a history of saline or heroin self-administration pretreated with vehicle (saline) or LIR 6 hours prior the start of the test . **E**. Mean (±SEM) number of active spout contacts after yohimbine (0.5 mg/kg) administration (stress-induced seeking) for rats with a history of saline or heroin self-administration pretreated with vehicle (saline) or LIR 6 hours prior the start of the test. Saline-veh (n=6), saline-LIR (n=6), heroin-veh (n=4), heroin-LIR (n=6). #Significant main effect of treatment. Significant difference between LIR and Veh. **p<0.01; ****p<0.0001

Discussion

In the present study, we showed that acute administration of a 0.3 mg/kg sc dose of LIR reduced drug-induced seeking at both seven hours and nine hours post injection. The acute administration of the 0.3 mg/kg sc dose of LIR also reduced cue-induced heroin seeking, but only when assessed 6 hours, but not a 4 hours, post injection. Finally, this acute LIR dose also reduced stress-induced reinstatement when administered 9 hours prior to the yohimbine challenge.

As mentioned, research over the last decade has provided evidence that GLP-1R agonists, particularly Exendin-4, can reduce the rewarding properties of drugs [177-182, 186]. In the present study, we added to the findings from **Chapter 4** by showing that the longer-acting GLP-1R agonist, liraglutide (0.3 mg/kg, ip), can reduce cue-induced seeking and completely abolish both drug-induced and stress-induced reinstatement of heroin seeking behavior in rats. However, we observed that the effects of LIR on cue-induced seeking were effective after six hours, but not four hours post injection. In the first test, using a 4-hour pretreatment, LIR exerted no effect on cue-induced seeking, but drug-induced reinstatement was markedly reduced when assessed seven hours post injection. This finding suggests that a minimum effective pretreatment time for 0.3 mg/kg LIR is between four and six hours in rats. Indeed, in the follow-up tests, rats were injected with the same dose of LIR six hours before the cue-induced test, and both cue-induced seeking and drug-induced reinstatement of heroin seeking were significantly reduced. It is difficult to parse out whether the effects observed in these experiments are due to the timing between injection and test, or to the LIR concentration achieved during that time. If we revisit the pharmacokinetic parameters of the drug, the Tmax for this dose in the plasma concentration curve, was eight hours post-injection. However, while the drug was first effective at six hours post-injection, the concentration obtained by a 0.3 mg/kg dose at hour 4 (29,618.7 ng/mL) was

higher than the one obtained at hour 6 (27,984 ng/mL). On the other hand, if the AUC is proportional to bioavailability, the AUC is higher at hour 6, making it possible that an AUC of at least 106,687 ngh/mL will be required to reduce heroin-seeking. In addition, LIR is a modified GLP-1 molecule, attached to a fatty acid chain that allows it to bind to albumin to prevent degradation and to be released at a slower rate. Because the effects of GLP-1 analogs on reward-associated behaviors are centrally mediated, it is possible that, due the slower cleavage of LIR into its active form, LIR Cmax may not correlate with the drug reaching its targets in the brain [143, 144]. So far, only one study has shown the presence of fluorescently labeled LIR (120 nmol/kg) in the hypothalamus four hours after a single iv injection [266]. Even so, the timing remains unclear regarding the interaction of LIR with GLP-1R in the brain, particularly in nuclei associated with the reward system such as the nucleus accumbens and the ventral tegmental area, in which Ex-4 has proven to be effective on reducing drug-associated behaviors [179, 180].

The pharmacological induction of stress-like behaviors using the alpha-2 adrenergic receptor antagonist has been widely described in both humans and laboratory animals [263, 264]; indicating that injection of yohimbine is a translatable method to study stress-induced reinstatement of drug-seeking behavior. Indeed, years of research have shown that yohimbine can reinstate seeking of alcohol, methamphetamine, cocaine and nicotine in rats [267-270]. However, some discrepancies regarding pharmacological and behavioral effects related to this method and natural stressors (e.g., foot-shock), have brought the validity of this model into question [271]. Having said that, even if the effects of LIR on stress-induced reinstatement must be validated using a different model - either a natural stressor or a different pharmacological manipulation such as injections of corticotropin releasing factor [272] - the effectiveness of LIR to reduce drug-seeking across all three models of ‘relapse’ (cue-, drug- and stress-induced reinstatement) is remarkable.

While the results observed in this experiment are promising, further research needs to assess the possibility of achieving long-lasting protection. In the present experiment, we showed that a single, acute dose of 0.3 mg/kg LIR can reduce seeking across several models of reinstatement, but only after a specific period of time following injection. We showed also that daily LIR treatment produced an increase in baseline plasma concentration of liraglutide, suggesting that LIR is not completely metabolized after 24 hours. This increase in baseline LIR produced by daily treatment, then, may be helpful in reducing the effective pretreatment time in subsequent days. In this context, if LIR were to be administered chronically, and the levels of LIR in the system were to be maintained at a specific concentration, it may be possible to achieve prolonged protection both within the day as well as across days, regardless of treatment time. Indeed, to this end, once weekly formulations of GLP-1 receptor agonists have been developed [273]. It may be possible, also, to increase the dose to 1.0 mg/kg, in which the 'effective concentration' - and effective AUC - was reached by the fourth hour after injection, to allow for a shorter pretreatment time. However, gastrointestinal side effects in humans warrant titrating the dose in increasing steps. Future research is needed to further elucidate the effects of chronic LIR treatment on heroin-seeking and potential side effects during abstinence in rats with history of heroin self-administration.

The results found in this study are promising as they show that the long-acting GLP-1R agonist liraglutide can not only reduce cue-induced seeking, but also drug-induced and stress-induced reinstatement of heroin-seeking behavior. It is worth noting that the different models of reinstatement are regulated by different neural mechanisms (see [274]). Hence, not all compounds that reduce one are necessarily effective on reducing another. For example, current drugs used as MAT to treat OUD, buprenorphine and methadone, can reduce cue- and drug-induced seeking, but do not have an effect on stress-induced seeking [243, 275]. On the other hand, orexin

antagonists, for example, have been shown to reduce stress and cue-induced seeking, but they are not effective in reducing drug-induced reinstatement [276]. In addition, the effectiveness of LIR in reducing heroin seeking across the different models of reinstatement suggests that all of these models share a common feature. This feature may be the onset of withdrawal, and it appears to be blocked or reduced by acute treatment with GLP-1R agonists. Since, GLP-1R agonists already are approved for the treatment of type 2 diabetes and obesity, the effectiveness of liraglutide across a wide variety of reinstatement models is a promising finding that may lead to the development of more effective treatments for OUDs in humans [139, 190]

Manuscript: Acute glucagon-like peptide-1 receptor agonist liraglutide prevents drug-induced heroin seeking in rats. (*in preparation*)

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Respective contributions: J.E. Douton: Experimental design, data analysis and interpretation and manuscript preparation. N. Acharya: Experimental design and execution. B. Stoltzfus: experimental execution. D. Sun: HPLC/tandem MS method development and execution. P.S. Grigson: Experimental design, data interpretation and manuscript revision. JE Nyland: Experimental design, data interpretation and manuscript revision.

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Chapter 6

General Discussion

The goal of this dissertation was to use rodent models to study the effects of glucagon-like peptide-1 receptor agonists on heroin addiction, and to determine their potential for the safe and effective treatment of opioid use disorder. Addiction is an all-consuming, complex disease that changes not only reward-associated structures in the brain, but also those involved in homeostasis of the organism. The fundamental **hypothesis** here is that addiction to a drug of abuse engages homeostatic substrates like those engaged when an animal experiences physiological need states such as hunger and thirst, and that this potent artificial 'need' state can be placated by known satiety signals. In the current dissertation, we focused exclusively on GLP-1 and its analogs, as previous research in the context of drug-addiction showed a reduction in seeking and taking across several classes of drugs including cocaine, amphetamine, nicotine and alcohol [176-178, 181-183]. Further, extensive research in the areas of type 2 diabetes and obesity provides several formulations of GLP-1R agonists with different half-lives and pharmacokinetic parameters currently available as FDA-approved treatments for these diseases [253, 254, 277]. Then, we first studied the ways in which GLP-1 analogs modulate different aspects of motivated behavior. Thereafter, we assessed the effects of different formulations of GLP-1R agonists in different models of heroin taking, seeking, and 'relapse'. Finally, we analyzed the potential side effects of GLP-1R agonists and the safety of its chronic administration on gastrointestinal malaise, food intake, water intake, and body weight.

GLP-1 Reward and Aversion

Having as a goal for this dissertation to study the effects of GLP-1 analogs on different aspects of heroin addiction such as heroin-taking and heroin-seeking, we first need to understand the ways in which GLP-1 can modulate non-homeostatic motivated behaviors. After all, drug

addiction produces an alteration in the behavioral repertoire that narrows its focus away from natural rewards and towards drug-seeking and taking [1, 12]. To understand the effects of GLP-1 during disease, then, we first need to understand the effects of GLP-1 under normal circumstances. To this end, in **Chapter 2** we sought to study GLP-1 modulation of different components of both reward and aversion (see Table 6.1). As mentioned before, motivated behavior comprises two separate components, the motivational component associated with approach and consumption of the reward; and the affective component associated with the perceived value/palatability of stimulus [46]. Although it was first described to have anorectic effects, GLP-1 was later discover to not just be a 'satiety' signal, but also to be involved in modulating motivated behaviors [157-159, 169, 170]. Mainly using studies analyzing licking microstructures, researchers concluded that GLP-1 receptor activation was involved in altering the perceived palatability of food [175]. However, in this chapter, by using intraoral infusions and the taste reactivity test to circumvent approach behavior, we showed that the short-acting GLP-1 analog Ex-4 does not affect the affective component (i.e., perceived palatability) of a rewarding stimulus such as sucrose. It did, however, alter the perceived palatability of the aversive gustatory stimulus, QHCl, by reducing the number gapes emitted following intraoral infusion.

The differential modulation of the affective response to stimuli of different valences was surprising. In this context, and because the reduced approach towards palatable food elicited by GLP-1 analogs has been widely described, we studied the same behavior but to an aversive stimulus. We found that, although the effects on affective responses were opposite depending on the hedonic valence (i.e., positive taste reactivity behavior was not altered to the sweet; while aversive taste reactivity behavior was reduced to the bitter), the effect on the motivational component was the same, suggesting a general inhibitory effect of GLP-1R agonists on approach behavior irrespective of the nature of the stimulus (see Table 6.1).

Consequently, we can conclude that GLP-1 is more than a mere satiety signal. As mentioned in **Chapter 1**, there is a complex interplay between homeostatic signals and reward. In this context, GLP-1 serves not only as a satiety signal, but also as a signal to stop ongoing consummatory behavior. Under normal conditions, endogenous GLP-1 reduces the motivation to perform and stops the behavior that was physiologically needed, allowing for the transition to another behavior that might be required at the present. If so, GLP-1 may be the switch that allows an organism to move from one motivated behavior to another, depending on the needs of the organism at the time. As discussed in **Appendix A**, GLP-1 may be the modulator that prevents a motivated behavior from becoming uncontrolled. For this reason, when the stimulus is rewarding, it inhibits further approach in order to prevent compulsive behaviors. On the other hand, when the stimulus is aversive, a GLP-1R agonist reduces approach and dampens perceived aversiveness if the stimulus is encountered (e.g. intraoral infusion), in order to prevent the development of a long-lasting aversion. As mentioned, satiety is necessary but temporary. In this context, it is critical that normal response to stimuli remain within a normal range, allowing for a resumption of consummatory behavior when next needed.

Behavior	Affective		Motivational
Stimulus	Appetitive	Aversive	Licks
0.5M Sucrose	-	-	N/A
0.03M Quinine	-	↓	↓
0.15%Saccharin-LiCl	-	-	-
0.15%Saccharin-Heroin	N/A	-	↑*

Table 6-1. Effects of Exendin-4 on reward and aversion. The effects of treatment with 2.4 μg/kg Ex-4 were assessed on the motivational (i.e., licks) and affective (i.e., aversive or rewarding taste reactivity behaviors) components of motivated behavior in response to rewarding and aversive taste stimuli. *: Increase intake of the heroin-paired saccharin solution was observed after discontinuation of Ex-4 treatment. – indicates no change, N/A indicates not assessed here, down arrow indicates a reduction in responding.

GLP-1 and Natural Rewards

If GLP-1 is the switch that allows the organism to alternate between motivated behaviors, then it could potentially be useful to treat addiction. As mentioned, if drugs of abuse and addiction hijack the reward system and lock the homeostatic system towards drug-seeking and drug-taking without allowing a switch to other needed behaviors, then stimulating that 'switch' may help unlock the behavioral repertoire and allow for engagement in other motivated behaviors.

With this in mind, in **Chapter 3** we used our model of reward devaluation in order to study the effects of the short-acting GLP-1 analog, Ex-4, on both heroin-seeking and acceptance of a devalued natural reward. In particular, since Ex-4 was shown in **Chapter 2** to reduce negative affective responses towards an aversive stimulus, we explored the possibility that this compound may recover normal responding for the devalued natural reward. The results observed in this study were encouraging, as a history of Ex-4 treatment indeed recovered responding for a natural reward such as saccharin in rats that previously showed avoidance towards it (see Table 6.1). The puzzling finding, though, is that these effects were only observed after the Ex-4 treatment was discontinued. Previous treatment with Ex-4, then, may facilitate the learning process that allows these subjects to dissociate the taste cue from drug availability during future extinction sessions, which was not observed in high suppressors that were treated with vehicle [278, 279]. It would be interesting, though, to assess whether treatment with Ex-4 would have increased acceptance to the saccharin cue if treatment was not discontinued. Alternatively, it is possible to further study whether Ex-4 treatment during early taste-drug pairings will prevent the development of reward devaluation altogether. Interestingly, the fact that some behavioral effects along with changes in the brain (see discussion below) were observed after discontinuation of the

Ex-4 treatment, suggest that chronic treatment with Ex-4 has sustained effects that may produce a re-wiring, allowing the brain to respond in a way that increases the behavioral repertoire and produces a resumption of responding to other needs and for natural rewards.

Avoidance of a taste cue that predicts the availability of a drug of abuse was first described as conditioned taste aversion [90, 92, 280]. If this were so, just as we observed in **Chapter 2** when analyzing learned aversion, we should not expect Ex-4 to not alter saccharin intake behavior in our reward devaluation model (see Table 6.1). However, the fact that we do see an increase in responding for the saccharin cue, and this effect is accompanied by a reduction in heroin seeking, supports the theory that the avoidance of the taste cue is not due to a CTA, but probably a state of conditioned-withdrawal [95, 103]. This conditioned state is similar to a state of 'need' for the drug, and as any state of need, can be reduced by satiety signals, such as GLP-1, allowing for a switch towards other kinds of behavior that are not exclusively related to drug-seeking or drug-taking. Indeed, preliminary data in our laboratory has shown that Ex-4 can also reduce a withdrawal-associated aversive state, as shown by a reduction in aversive taste reactivity to a taste paired with naloxone-elicited heroin withdrawal [Olsen et al., in preparation].

GLP-1, Drug-seeking and Drug-taking

In **Chapters 3, 4** and **5** we extended the findings related to the effects of GLP-1 analogs on drug-associated behaviors. We showed that, besides modulating behaviors associated with cocaine, amphetamine, nicotine and alcohol, GLP-1 analogs can also modulate behaviors associated with heroin (See Table 6.2) [177, 181-183]. First, we showed that treatment with the short-acting GLP-1R agonist, Ex-4, reduced both cue-induced and drug-induced heroin seeking. Then, we showed that treatment with the longer-acting GLP-1R agonist, LIR, reduced ongoing

heroin self-administration as well as seeking in several models of relapse, including cue-induced, drug-induced, and stress-induced seeking. Given that GLP-1 did produce a reduction in heroin seeking that extended across several reinstatement models, there is a possibility that the effects observed are non-specific, producing a generalized behavioral suppression. Indeed, it has been observed that doses as small as 0.3 μg/kg of Ex-4 can reduce locomotor activity in mice [178]. However, locomotor depression does not appear to be the reason for the reduction on heroin-seeking, since both Sac-sal controls and SS rats treated with Ex-4 in **Chapter 3** did perform the same operant behavior (i.e., spout licking) that was required to obtain the drug, but only in order to drink the saccharin solution. Moreover, it has been observed, also, that liraglutide treatment (0.5 mg/kg ip) does not produce a reduction of locomotor activity in rats [281]. Overall, the effects of GLP-1 analogs observed in this dissertation appear to be specific to heroin seeking, since even Ex-4, that has been shown to reduce locomotor activity, did not affect the specific behavior required to seek the drug.

The results observed in **Chapters 3, 4** and **5** also are consistent with a reduced state of need in rats treated with GLP-1 analogs. For example, it has been observed that animals in a state of need, such as food deprived rats, will exhibit a shorter latency to seek and consume food [29]. Accordingly, we observed that treatment with both Ex-4 and LIR increased the latency to seek or take drug. Moreover, we showed that chronic treatment with LIR also prevented escalation of heroin self-administration over trials. Escalation of drug self-administration is a critical symptom of uncontrolled use of a substance, usually associated with neuronal and behavioral changes that correlate with the transition from controlled to uncontrolled drug use [81, 259]. GLP-1 analogs, then, not only reduced the latency to seek and take drug, evidence of a reduced state of need, but also prevented exposed subjects from escalating intake of the drug over time. These two findings are consistent with the hypothesis that GLP-1 may be the switch that allows a behavior to be

performed to a certain extent, but prevents impulsive and compulsive behaviors that may drive the organism away from homeostasis. It is expected, then, that subjects treated early with GLP-1 analogs will not develop a behavioral narrowing focused exclusively on drug-associated behaviors. For example, even though the rats in **Chapter 4** did take the drug, LIR treatment prevented them from 'needing' the drug, leading to prevention of escalation of drug taking over time, and as a consequence, GLP-1-treated rats may be able to stop such behavior at will, if another relevant goal is need.

Behavior		Drug Taking	Reinstatement model		
Drug	Dose		Cue-induced seeking	Drug-induced seeking	stress-induced seeking
Ex-4	2.4 ug/kg	N/A	↓	↓	N/A
Liraglutide	0.1 mg/kg	↓	-	↓	N/A
	0.3mg/kg	N/A	↓	↓	↓

Table 6-2. Effects of GLP-1 analogs on heroin-associated behaviors. The table shows the effects of treatment with either 2.4 μg/kg Ex-4 or LIR (0.1 mg/kg or 0.3 mg/kg) on heroin taking and seeking in different models of reinstatement. – indicates no change, N/A indicates not assessed here, down arrow indicates a reduction in the behavior.

Conditioned Taste Aversion and Body Weight Reduction as Potential Side Effects

Research in the context of drug-addiction has made GLP-1 analogs a promising alternative to treat patients that suffer from SUDs. The extensive research done for the treatment of type 2 diabetes and obesity has led to the development of several formulations with different pharmacokinetic parameters that are safe and already approved for use in humans [253, 254, 277]. However, different aspects of treatment must to be studied in order to guarantee that patients will comply with a prolonged treatment regimen.

Given that nausea and malaise are main side effects of GLP-1 receptor agonist-based pharmacotherapies, and an estimated 10% of patients discontinued the treatment due to adverse effects, we studied the development of conditioned-taste aversion and consumption of the anti-emetic kaolin (i.e., pica response) in rats treated chronically with different doses of LIR (see Table 6.3) in **Chapter 4** [282]. In so doing, we observed that intake of the LIR-paired saccharin solution was avoided at all doses of LIR, suggesting the development of CTA. However, no dose of LIR elicited kaolin intake. While LIR side effects in humans include nausea, and GLP-1 was first described to produce its anorectic effects through visceral illness, we do not see an indication of that in our results [138, 251]. As mentioned earlier, CTA is a common test to analyze aversion, however, it is indirectly measured as a performance that requires motivated behaviors, such as approach and consumption [260]. This makes development of CTA to GLP-1 analogs particularly hard to assess, since we showed that these compounds do affect motivated behaviors in general, particularly the motivational component associated with approach and consumption. In addition, although we did observe that Ex-4 can reduce innate aversiveness to a taste stimulus, Ex-4 did not reduce the LiCl-induced conditioned taste aversion (**Chapter 2**). However, whether GLP-1 analogs themselves generate aversion is harder to parse out. The lack of a kaolin consumption

elicited by LIR at all doses, even those that reduce food intake, suggests that GLP-1 does not generate potent gastrointestinal malaise in these rats. Indeed, we did see that Ex-4 can actually prevent innate aversive responses and these, as shown in **Appendix A**, were associated with a reduction in histamine, which is usually released in response to aversive stimuli. That said, the mechanism by which LIR induces a conditioned reduction in saccharin intake is still unknown. It is possible that a learned association is formed with GLP-1R agonist-induced satiety and/or amotivation.

GLP-1 analogs are used for the treatment of type 2 diabetes and obesity and, as such, it is expected for them to reduce blood glucose concentration and to affect body weight. In **Chapter 3** and **Chapter 4** we showed that chronic treatment with a dose of 2.4 μg/kg Ex-4 and 0.1 mg/kg LIR respectively, did not affect body weight. However, also in **Chapter 4**, we studied the effects of different doses of LIR administered every 4 days on food intake, body weight and water intake. Here, we showed that, even when not administered daily, a dose as low as 0.3 mg/kg LIR can produce a sustained reduction in body weight gain and food intake. Even though body weight gain was not prevented, higher doses such as 0.6 mg/kg and 1.0 mg/kg produced a big fluctuation comprising cycles of body weight loss in the subsequent days after the injection and body weight gain thereafter (see Table 6.3). This can be concerning, as patients who suffer from OUD usually already show reduced body weight [283]. However, animal research has shown that the most robust effects of GLP-1 analogs on food intake and body weight are observed 24 to 72 hours after the first injection [163, 261]. After that, as we observed in our data, body weight gain does occur, but at a slower rate than in control rats. Hence, as long as the treatment is daily, a marked reduction in body weight is expected within the first three days of the treatment, but no further negative impact as the treatment extends. In non-obese humans, for example, prolonged, daily treatment with a dose similar to 0.6 mg/kg in rats (1.8 mg/kg in humans) produced a reduction in

body weight of about only 3% during the 26 weeks of the experiment [254]. Finally, none of these doses tested here produced a significant change in water intake.

Drug	Dose	Food intake	Body weight	water intake	CTA	Pica Response
Liraglutide	0.06 mg/kg	N/A	N/A	N/A	+	-
	0.1 mg/kg	-	-	-	+	-
	0.3 mg/kg	↓	↓	-	+	-
	0.6mg/kg	↓ *	↓	-	+	-
	1.0mg/kg	↓	↓	-	+	-

Table 6-3. Side effects. The table shows the effects on food intake, body weight, water intake and aversion associated with treatment with different doses of the GLP-1 receptor agonist, liraglutide. – indicates no change, N/A indicates not assessed here, down arrow indicates a reduction. * Reduction in body weight was not significant after the 10th trial. + indicates the development of conditioned-taste avoidance (CTA).

Effective Doses and Pretreatment Times

By analyzing the data, 0.3 mg/kg appears to be the most effective dose of LIR with less side effects. This dose, as mentioned, did produce CTA, but it did not induce kaolin intake. Further, while it did elicit a reduction in food intake and body weight gain when compared to controls, the dose of 0.3 mg/kg did not produce the significant fluctuation between weight loss and weight gain seen with 0.6 mg/kg and 1.0 mg/kg LIR. Most importantly, so far, when administered acutely this dose was very effective in reducing heroin seeking across different models of 'relapse' (e.g., cue-induced seeking and drug- and stress-induced reinstatement). However, the most puzzling result is that, while effective, all doses and formulations used showed successful results only within a specific time-frame after treatment (see Table 6.4). For example, a dose of 2.4 μg /kg of Ex-4 reduced cue-induced and drug-induced seeking, but only when tested 1 hour post-injection and not 6 hours post injection. When administered acutely, the 0.3 mg/kg dose of LIR was only effective in a specific time-frame between 6 and 9 hours post injection. On the other hand, the lower dose of 0.1 mg/kg, when administered chronically, was effective in reducing drug-induced seeking 6 hours post injection and heroin self-administration 5 and 6 hours post injection. Specifically, although rats chronically treated with 0.1 mg/kg consistently self-administered less heroin than the vehicle treated rats starting at hour one, the effects did not attain statistical significance until later, in the last two hours of the session. This finding suggests that, at least for this low dose, carry over effects from one day to the next are not sufficient to mitigate drug taking or cue-induced heroin seeking, even after 30 days of daily treatment. The only evident carry over effects observed here using different GLP-1 analogs, are those seen in body weight and food intake for both 0.6 mg/kg and 1.0 mg/kg in **Chapter 4**, and the increase in acceptance of the saccharin solution in **Chapter 3**. In summary, we do see that

acute LIR can reduce heroin seeking, however, even a prolonged treatment such as that used in **Chapter 4**, does not have carry over effects, suggesting that there has to be at least a certain level of LIR in the system for the treatment to be effective.

In an effort to better identify effective pretreatment times, in **Chapter 5** we analyzed the pharmacokinetic parameters of different doses of LIR. Importantly, we found that LIR was still present in plasma 24 hours post injection, as evidenced by increased baseline LIR levels on blood collection days. These levels, however, may not be in the amounts needed to exert protective effects against drug seeking. It could be assumed that the effective concentration of LIR in plasma - that associated with a reduction in heroin seeking- would be the one observed with the administration of 0.3 mg/kg at 6 hours. Still, this does not appear to be the case, since the levels of plasma with the same dose, observed two hours earlier, are even higher. Consequently, we must conclude that it is not blood levels of LIR, per se, that determine the effectiveness of the drug, but the time taken to impact the brain. As mentioned, the release of the active GLP-1 from the LIR compound is, in general, slow and it has been observed that GLP-1 analogs modulate reward behaviors by interaction with receptors located centrally [169, 170, 179, 180]. Although it has been shown that LIR reaches the brain within four hours, particularly in hypothalamic nuclei, the time it takes to reach areas in the reward system, in which infusion of Ex-4 has been shown to reduce reward-related behaviors associated with drugs of abuse, such as the NAc or the VTA has not been studied [179, 180, 266]. However, further analysis on liraglutide metabolism needs to be done in order to understand its central target and the timing between its peripheral administration and its central effects.

Drug	Dose	Pre-treatment time					
		1h	4h	5h	6h	7h	9h
Ex-4	2.4 ug/kg	+	N/A	N/A	-	-	N/A
Liraglutide	0.1 mg/kg	-	N/A	+	+	N/A	N/A
	0.3 mg/kg	N/A	-	N/A	+	+	+

Table 6-4. Effective pretreatment times. The table shows the times at which the GLP-1 agonists Exendin-4 and liraglutide were effective on reducing either drug taking or drug seeking. + Indicates that the dose was effective on reducing either heroin seeking or heroin taking. – indicates that the dose was not effective on reducing heroin seeking or heroin taking. N/A indicates not assessed here.

Potential Use of GLP-1 to Treat Different Aspects of Heroin Addiction

Although it has not experimentally been proven, the general understanding in the field is that, in rats, the liraglutide dose of 0.6 mg/kg is akin 1.8 mg - the maximum dose used for the treatment of type 2 diabetes in humans - and 1.0 mg/kg is akin 3.0 mg - maximum dose used for the treatment of obesity in humans. In this context, we can conclude that, in rats, the doses of liraglutide that are effective on reducing heroin seeking and taking are lower than the maximum doses used in humans. After analyzing the data presented here carefully, we also can conclude that different kinds of treatments can be studied using GLP-1 receptor agonists. First, GLP-1 analogs may be used by people that consume opioids recreationally in order to prevent intake escalation that can lead to the development of substance use disorder, as observed in **Chapter 4**. The most suitable option for this kind of treatment would be a daily injection - or even an acute injection, when the individuals know they will be exposed to the drug - with a small dose of LIR (i.e., similar to the 0.1 mg/kg dose in rats). Second, GLP-1 analogs may be used acutely in patients that are undergoing acute withdrawal in order to reduce the aversive state and prevent the symptoms associated with it [227][Olsen et al., in preparation]. In this way, GLP-1 can help the patient to remain abstinent and prevent relapse. In such a case, the cost/benefits of the treatment must be assessed and a high dose of LIR, similar to 1.0 mg/kg in rats or even higher may be used in order to alleviate the aversive state in the patient. Finally, a chronic, long term intervention may be used for patients that are abstinent and undergoing treatment for OUD. In such a case, the dose most likely will have to be increased to at least 1.0 mg/kg, a dose for which the 'effective' bioavailability and concentration were observed at four hours. To prevent the side effects associated with such a dose, the treatment will have to be titrated and increased by steps. Further research will need to determine if chronic, sustained treatments with higher doses of LIR can

produce a long-lasting effect that will protect the individuals across days, in between injections, and following exposure to drug-paired cues, stress, and to the drug itself – the three main precipitators of relapse. If the LIR kinetics does not allow for this to happen, or the doses that achieve this are big enough to produce side effects that will reduce compliance, there is one available option. Both LIR and Ex-4 prevented heroin-seeking and both formulations worked only during a specific time frame. While Ex-4 effects extended only for near two hours, LIR only started to be effective after five to six hours (see Table 6.4). As such, it also is possible that a mixture of LIR/Ex-4 can be used in order to reduce the window in which the individual is susceptible to relapse. However, further research will need to be conducted to determine if this combination is safe, whether it will enhance side effects or not, and to identify the ideal dose to produce full protection.

Molecular Pathways

The nucleus accumbens is a structure within the ventral striatum that serves a vital function in the reward system in the brain. The NAc is key to modulate motivated behaviors due to its role in giving salience to stimuli associated with motivationally relevant goals, in order to increase the probability of executing behaviors directed towards those goals [44]. The NAc, then, integrates all this information from a wide variety of systems, allowing for the determination of the best course of action at any given time [44]. For this reason, in **Chapter 3**, in order to study long-lasting changes in the brain associated with Ex-4 treatment, we assessed the expression of messenger RNA of receptors involved in reward and homeostasis modulation. Even though it has been shown that GLP-1 analogs can reduce drug-related behaviors by acting upon the NAc and the VTA, chronic treatment with Ex-4 did not produce long-lasting changes in the expression of

D2 or GLP-1 receptors [179, 180]. Moreover, GLP-1 did not appear to produce an effect indirectly via another energy signal, leptin. However, chronic treatment with Ex-4 and subsequent reduction in heroin-seeking were associated with an increase in the in the expression of Orexin 1 receptor (OX1) in the NAc. Although interesting, these findings are not surprising. Orexin-producing neurons are mainly located within the lateral hypothalamus (LH) [232, 233]. The lateral hypothalamus is another nucleus that serves as a link between homeostasis and motivation. For example, the seminal work of Olds and Milner using brain stimulation showed that rats would press a lever just to stimulate neurons within the LH, suggesting that activation of such neurons is rewarding in and of itself [41]. Furthermore, the LH also serves an important role in the modulation of feeding behavior, arousal, regulation of body temperature and blood pressure among other functions [234, 276, 284, 285]. Similar to GLP-1, soon after researchers described orexin as a feeding stimulatory neurotransmitter, its effects were extended towards non-homeostatic motivated behaviors [286, 287]. In particular, it has been observed that orexin neurons are engaged in situations that require high motivation [238, 288-290]. In the context of opioid addiction, it has been observed that individuals exposed to opioids show an increased number of orexin-producing neurons , and orexin antagonists can reduce conditioned place preference to morphine and reinstatement of morphine seeking [236, 291, 292]. As a consequence, in **Chapter 3** we hypothesized that exposure to heroin during the self-administration period must have produced an increase in orexin neurons and orexin transmission resulting in a compensatory reduction on OX1 expression in the NAcS (see Figure 6-1). On the contrary, treatment with Ex-4 appears to either reduce the number of orexin neurons or orexin transmission, leading to a compensatory increase in OX1 transcription in the NAcS. Thus, the increased orexin transmission during the self-administration period would lead to high heroin seeking of the drug in the control rats during future tests. However, Ex-4 would reduce orexin

transmission - inferred by the elevated levels of OX1 expression - and, as a consequence, reduce heroin seeking (see figure 6-1). These results identify the LH as a novel target of GLP-1in the context of addiction, and supports the notion that it is not only the reward system, but the homeostatic system as well, that is involved in modulating these aberrant motivated behaviors. Consequently, and due to its ability to promote arousal, feeding, etc., orexin can be labeled as a 'seeking' signal, particularly in context of high motivation. If GLP-1, as mentioned earlier, is the 'stop' signal that allows the organism to switch between different behaviors and opposes orexin effects, it is plausible that it acts on the LH in order to reduce orexin transmission and hence reduce motivation and performance of the behavior. This is consistent with findings showing that knock-down of GLP-1 receptors within the LH increases food intake, sucrose self-administration and food-seeking [237].

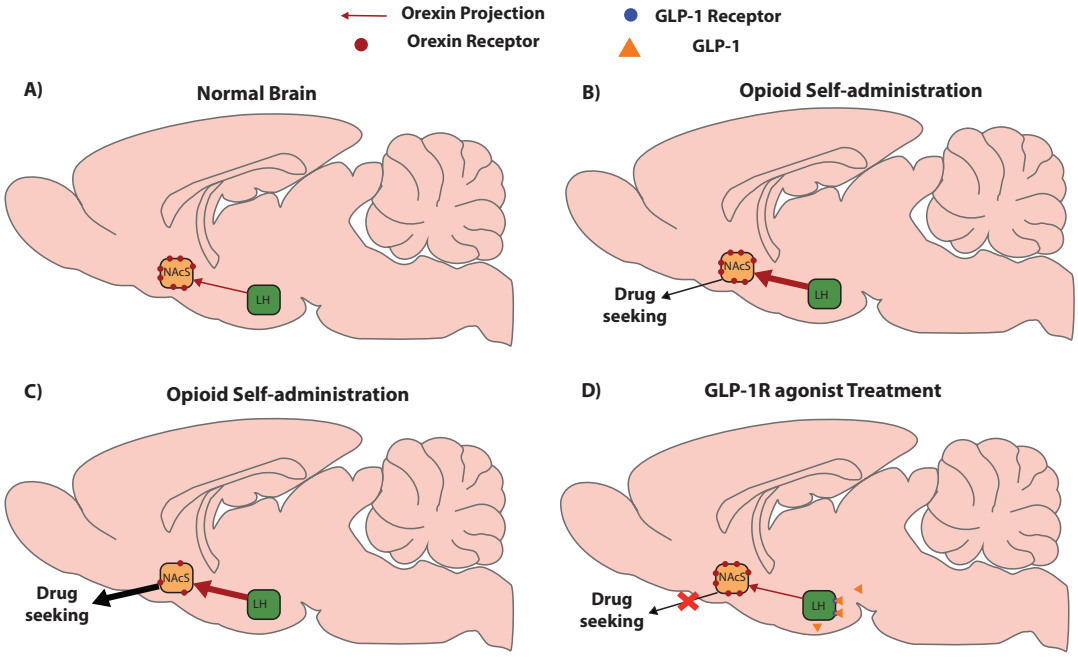

Figure 6-1. Proposed mechanism of GLP-1 on orexin transmission and heroin seeking. A. In a normal brain, orexin projections from the lateral hypothalamus (LH) act via the activation of orexin receptors in the nucleus accumbens shell (NAcS). **B**. Upon opioid self-administration, the number of orexin neurons increase and, in consequence, the orexin transmission to the NAcS intensifies. **C**. The exaggerated transmission that promotes opioid seeking, generates compensatory changes that lead to a reduction in the expression of the orexin receptor in the NAcS. **D**. GLP-1 analogs, then, may act on GLP-1 receptors located within the LH in order to reduce orexin transmission, that will translate into a reduced drug seeking and increased expression of orexin receptors.13

Summary and conclusion

In order to investigate new approaches to treat opioid use disorder, in the present dissertation we argued that drugs of abuse not only hijack the reward system, but also produce a homeostatic dysregulation that leads the organism to a state of 'need' for the drug. Under this notion, we explored the possibility that, akin any other need state, craving for drug can be reduce by hormones typically described as satiety agents. Specifically, we studied the effects of GLP-1 receptor agonists and the ways in which they can modulate heroin-associated behaviors. The results are promising as they show that different GLP-1 analogs are effective on reducing the impact of heroin addiction. From the recovery of natural reward responding after reward devaluation to the reduction of heroin self-administration; and from prevention of escalation to the reduction of heroin seeking in different models of reinstatement, GLP-1 analogs have been found very effective in the rat model. This is the first step towards developing new treatments to reduce intake or stop craving and seeking of drug, and hence towards helping people that suffer from OUD. Indeed, potential treatments with GLP-1 analogs may be beneficial beyond just its effect on opioid-associated behaviors, as it has also been observed that GLP-1 receptor agonist-pharmacotherapies can reduce depression and anxiety [227, 293]. Future research will need to parse out the brain structures in which GLP-1 is acting upon when exerting its effects, but previous research in the fields of type 2 diabetes and obesity has shown that they are safe to use in humans. The use of GLP-1R agonists may help to control a problem that has reached epidemic proportions and has increased, tragically, with the COVID-19 pandemic. Indeed, the data presented in this dissertation set the basis for the first clinical trials. Our findings have, with the necessary translations, the potential to help thousands of people that suffer from OUD, their families and their communities.

Appendix A

Effects of glucagon-like peptide-1 receptor agonist, exendin-4, on the neurochemical profile in the nucleus accumbens shell elicited by aversive and rewarding stimuli

Introduction

The mesocorticolimbic dopamine system, comprised of the dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and dorsal striatum is the major component of the reward system [44]. As such, the NAc has a critical role in processing information that is relevant to learn and elicit motivated behaviors [44, 45]. In this structure, the medial shell portion has been described as a hedonic hotspot, as rats exposed to an appetitive solution such as sucrose, showed increased dopaminergic release and metabolism in this area [294]. This area is also capable of tracking changes in the hedonic perception of a naturally rewarding solution [101, 295, 296]. For example, pairing a sucrose solution with morphine produces a devaluation of its perceived value. This devaluation translates into a decreased consumption of the solution that was associated with the elimination of the observed release of DA in this area [101]. Moreover, other studies using electrophysiology showed that, during such taste-drug pairings, there is an inversion of the phasic DA profile from a rewarding-like to an aversive-like profile [95]. However, the integrative nature of the NAc requires a more complex processing that it is not solely dependent on DA, but on an interplay of excitatory, inhibitory and modulatory inputs from different systems and areas of the brain. Indeed, the NAc receives cholinergic inputs from the caudate-putamen, glutamatergic inputs from the hippocampus, insular

cortex, prefrontal cortex and amygdala, GABAergic inputs from the globus pallidus, and serotonergic inputs from the raphe nucleus [229]. Further, manipulating these neurocheimcals in the NAc can produce changes in taste reactivity behaviors, feeding behavior or even elicit a conditioned-taste aversion [295, 297, 298]. For this reason, the purpose of this experiment was to assess the neurochemical response in the NAc shell, associated with subjective rewarding and aversive responses to solutions of different hedonic valences that are intraorally infused. Specifically, in order to further understand the modulation of such behaviors by glucagon-like peptide-1 (GLP-1), we studied the effects of the GLP-1 receptor agonist, Exendin-4, on the neurochemical profile associated with the behavioral changes observed in **Chapter 2**. To do so, the rats from **Chapter 2**, Experiment 1, were implanted with microdialysis cannulas and samples were collected during the test session.

Materials and Methods

Subjects

The subjects where the 17 rats from Chapter 2, Experiment 1.

Intraoral Cannulas and Placements

Surgeries were performed as described in Chapter 2, Experiment1.

Microdialysis Guide Cannula Implant

Rats were anesthetized with intramuscular (im) ketamine (70 mg/kg) and xylazine (14 mg/kg). Their heads were shaved and the skull exposed. Three anchor screws were attached to the skull and fixed with dental cement (C&B-Metabond®, Parkell Inc.). One screw was located rostral to the location where the cannula was inserted, while the other two were located caudally. Then, a 12-gauge stainless steel guide cannula was implanted stereotaxically above the right posterior medial NAc (A 1.2 mm, 1.2 mm from midline, and V 5.0 from the surface of the skull) as described by Hajnal et al. (2004) and Grigson and Hajnal (2007) [101, 296]. Finally, a metal protector was located around the cannula and a head cap was made with dental acrylic in order to fix all the components in place.

Apparatus and Behavioral Procedures

The behavioral procedures where the one described in Chapter two, Experiment 1 and hence performed in the same chambers.

Microdialysis

Microdialysis probes (CMA/Microdialysis AB, Stockholm, PAES membrane, 3 mm membrane length, 0.5 mm membrane OD, 20 kDa cut-off, 14 mm shaft length) were perfused with artificial cerebrospinal fluid (145 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl2, 1.0 mM MgCl2, 2.0 mM Na2HPO4, pH 7.4) at a flow rate of 1.0 μl/min through a 2-channel counterbalanced swivel (Instech Laboratories, Plymouth Meeting, PA). The microdialysis probe was connected to

a syringe pump on the side of the chamber via the swivel. PE50 tubing (Becton Dickinson and Co., Sparks, MD) connected the pump to the swivel and the swivel to the microdialysis probe on the head of the animal. FEP tubing connected the outlet arm of the microdialysis probe with an Eppendorf tube where the samples were collected (SciPro Inc., Sanborn, NY). Both the PE-50 and FEP tubing inside the chamber were protected by a metal spring tethered with a clip and secured with a plastic collar to the metal protector on the head of the animal.

Microdialysis Collection

The night before the test, rats were briefly anesthetized with an intramuscular injection of ketamine (70 mg/kg) and xylazine (14 mg/kg) and unilateral microdialysis sampling probes were inserted through the guide cannula to reach the target area. Probes were continuously perfused with an artificial cerebrospinal fluid (ACSF) solution at a rate of 1µl/min throughout the study using microsyringe pumps (model R99E; Razel Scientific Instruments, Stamford, CT). On the subsequent test day (Day 8), eight microdialysis samples were collected, one every 15 minutes. The pretreatment samples (samples 1-4) were averaged and used to create a baseline (BL). All data were normalized to each rat's baseline and transformed to percent change. During the fifth sample (sample 5), rats had 20 min access to either sucrose or QHCl. Three additional 20 min samples (samples 6-8) were taken thereafter in order to assess recovery.

Histology

In order to evaluate the correct placement of the probes in the NAc shell, rats were deeply anesthetized with 0.5 ml ketamine and perfused transcardially. The brain was removed form the skull and sectioned at 50 μm with a microtome, mounted onto slides, and stained with cresyl violet for examination under a light microscope to corroborate the placement of the cannulas.

Results

Solution of different hedonic valences elicited different neurochemical profiles (Figure A-1). Even though both sucrose and quinine produce and increase in DA and both affected glycine, quinine produced an increase in NE, 5-HT, GABA, glutamate and histamine. On the contrary, sucrose produced a small reduction, relative to baseline, in these neurochemicals.

As observed in **Chapter 2**, treatment with Ex-4 did not reduce affective responses to intraorally delivered sucrose. Likewise, here, treatment with Ex-4 did not affect the rewarding profile elicited in response to sucrose (figure A-2). While there appears to be an increase in NE after the initial reduction, and a small increase in glutamatergic release after sucrose infusion in the rats when treated with Ex-4, the rest of the neurochemicals showed similar profiles to vehicle-treated rats.

In **Chapter 2** we also showed that treatment with Ex-4 reduced aversive taste reactivity behaviors (i.e., gapes) following the intraoral infusion of QHCl. Similarly, here, Exendin-4 substantially altered the neurochemical aversive profile elicited in response to QHCl (Figure A-3). Specifically, Ex-4 produced an increase in dopamine release in response to the solution, an increase usually associated with reward. In addition, glutamatergic transmission also appears to

be increase when the rats were treated with Ex-4. More importantly, the behavioral effects of Ex-4 observed in **Chapter 2** that showed a reduction in aversive taste reactivity to quinine, were accompanied by the reduction of GABA and histamine levels in the NAc shell, neurotransmitters usually associated with aversion.

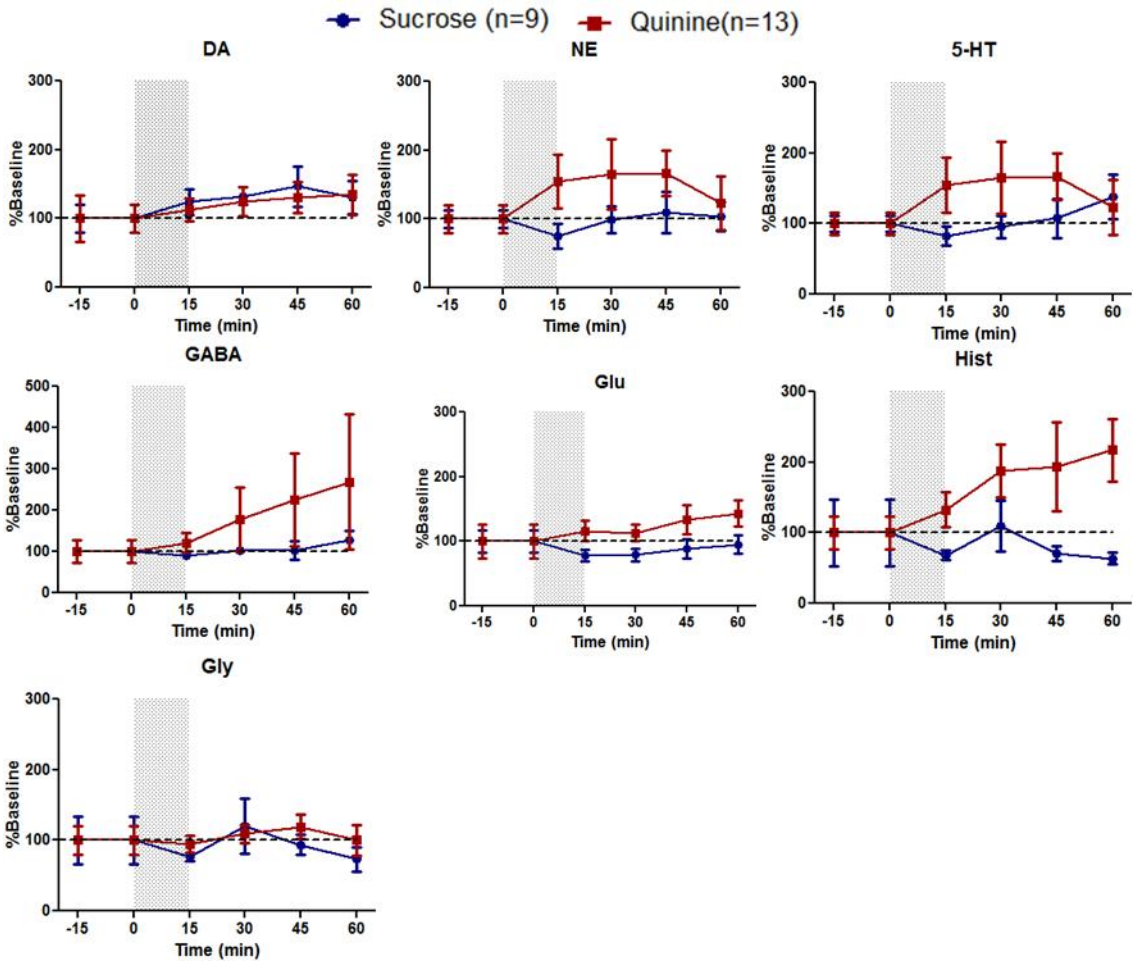

Figure A-1. Neurochemical profile of rewarding and aversive taste stimuli. Rats were intraorally infused by either 0.03M quinine (n=8) or 0.5M sucrose (n=9) and cerebrospinal fluid samples were collected via microdialysis every 15 minutes. Two samples were collected as baseline before, on during and three after the intraoral infusion of the tastant. Both quinine and sucrose elicited an increase in dopamine (DA). While sucrose elicited a decrease in norepinephrine, serotonin, glutamate, and histamine, and did not change GABA levels, quinine triggered an increase in the concentration of all these neurochemicals. DA: dopamine; NE: norepinephrine; 5-HT: serotonin; Glu: glutamate; Hist: histamine. Gly: glycine.

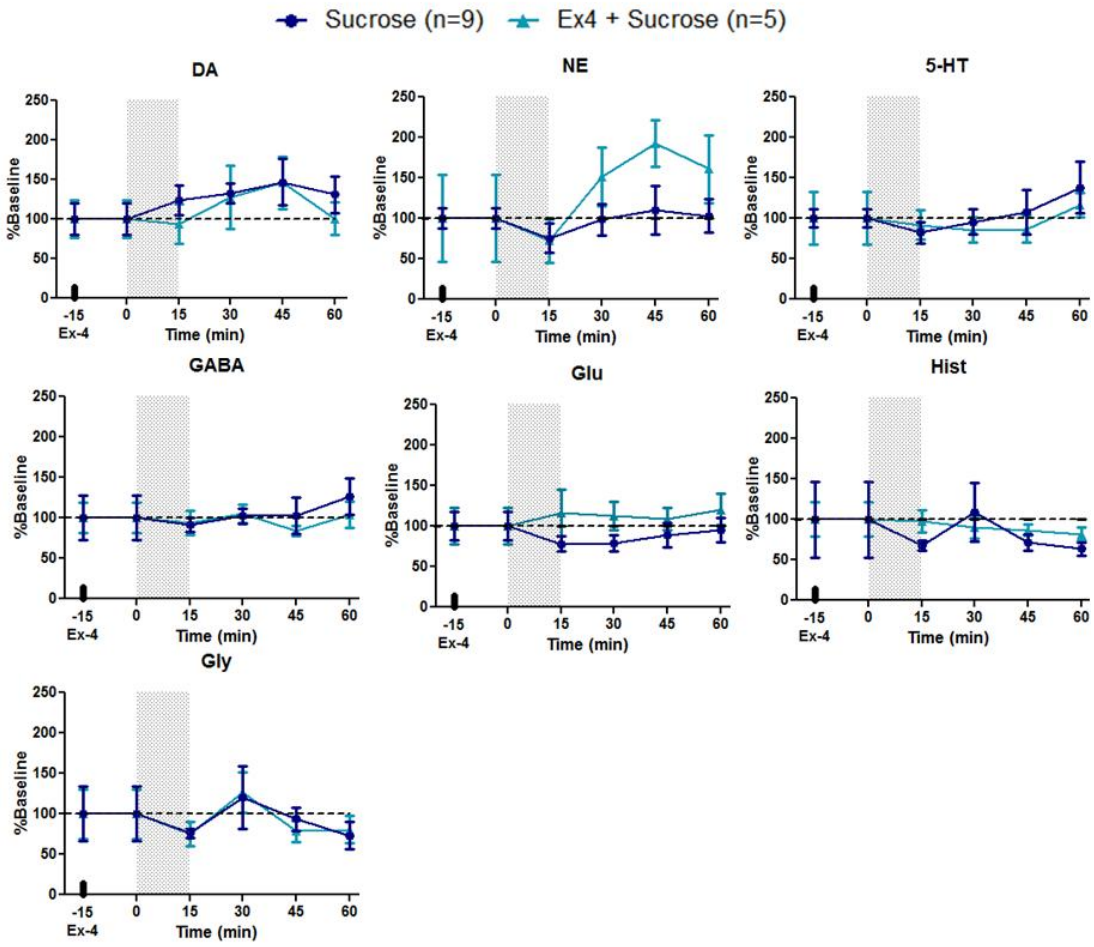

Figure A-2. Exendin-4 did not affect the rewarding profile. Following the first collection day, rats underwent another test day in which they were pretreated with Ex-4 15 minutes prior the intraoral infusion. While exendin-4 did not considerably affect the neurochemical profile elicited in response to the intraoral infusion of 0.5M sucrose, an increase is observed in norepinephrine and glutamate. DA: dopamine; NE: norepinephrine; 5-HT: serotonin; Glu: glutamate; Hist: histamine. Gly: glycine.

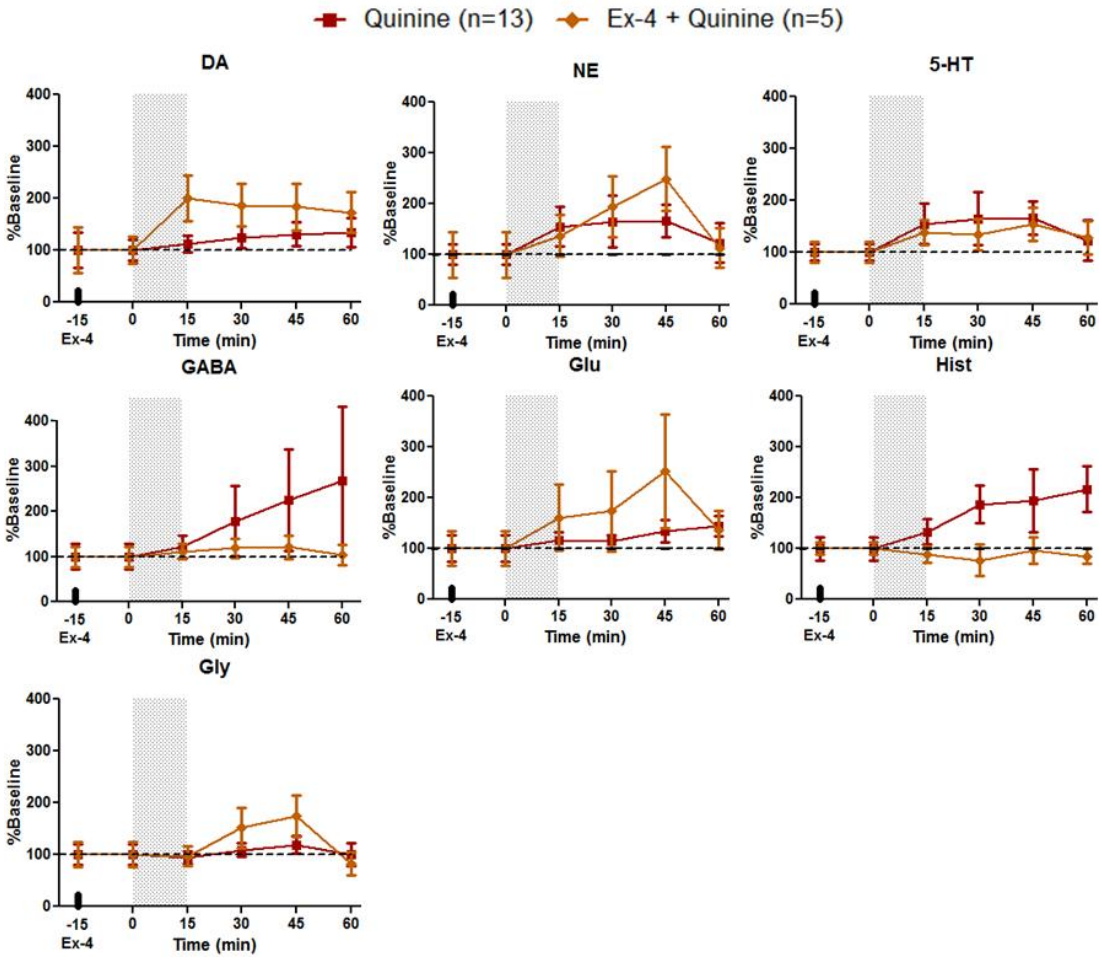

Figure A-3. Exendin-4 shifts the aversive neurochemical profile. Following the first collection day, rats underwent another test day in which they were pretreated with Ex-4 15 minutes prior the intraoral infusion. Pretreatment with Ex-4 produced an increase of dopamine DA and glutamate, and a reduction in GABA and histamine in response to the intraoral infusions of 0.003M quinine. DA: dopamine; NE: norepinephrine; 5-HT: serotonin; Glu: glutamate; Hist: histamine. Gly: glycine.

Discussion

The purpose of this experiment was to elucidate the neurochemical profiles elicited following intraoral delivery of a putatively rewarding and putatively aversive taste stimuli, and to determine the ways in which the GLP-1R agonist, Ex-4, alters that profile. As previously shown, the neurochemical profiles in the NAc shell differ for taste stimuli of different hedonic valences [299]. Here, intraoral delivery of both the sweet and the bitter was associated with a slight increase in DA and no change in glycine, while intraoral delivery of the bitter, alone, was associated with higher levels of norepinephrine, serotonin, GABA, glutamate, and histamine compared to intraoral delivery of sucrose. These findings are of great interest, but require replication. The trend, however, suggests a distinct, and complex, neurochemical profile for stimuli perceived as rewarding and aversive. Here, we will focus on the underlying neurochemistry that accompanies GLP-1-induced modulation of the affective response, particularly that associated with a reduction in the apparent perceived aversiveness of quinine and the accompanying reversal of GABA and histamine.

GABAergic transmission has been observed to have a role in the expression of affective behaviors towards reward. For example, stimulating the GABA A receptors across the rostro-caudal axis of the NAc shell alters the affective responses towards sweet and bitter taste stimuli. In response to a bitter-sweet solution (i.e., a mixture of sucrose and quinine), a microinfusion of the GABA A agonist, muscimol, into the rostral NAc shell increased appetitive reactions, while infusions into the caudal portion increased aversive reactions. In addition, the stimulation of rostral GABA receptors increased food intake, and the stimulation of the caudal receptors was associated with fearful treading. Interestingly, while the caudal portion of the NAc shell was associated with increased aversive behaviors, activation of these receptors even in the rostral

portion of the NAc shell elicited distress calls [295]. In the medial NAc shell, from which the samples have been taken in this experiment, activation of GABA A receptors via muscimol elicits a combination of aversive behaviors including distress calls, escape attempts and bite attempts [295]. As a consequence, GABAergic transmission in the NAcS has been associated with aversion. This conclusion is consistent with QHCl-induced elevation of GABA in the present study, and with its reversal by treatment with Ex-4.

Within the brain, the tuberomammillary nucleus is the main source of neuronal histamine [300]. From there, histamine neurons project throughout the brain and are involved in many physiological and behavioral functions such as arousal, feeding behavior and learning [301]. In particular, the reward inhibitory hypothesis of histamine states that histamine opposes the effects of dopamine, playing a negative role in reward. This, is evidenced in experiments showing that lesions in the tuberomammillary nucleus facilitates hypothalamic self-stimulation and increase learning in various negatively reinforced tasks [302]. In addition, microdialysis experiments have shown that blocking the histamine receptor 1 (H1R) produces an increase in acetylcholine in the cortex and DA in the NAc, suggesting a modulatory role of such receptors in different areas of the brain [303, 304]. Behaviorally, it has been observed that conditioned place preference to morphine was attenuated by injection of the histamine precursor, histidine. On the contrary, histamine depletion facilitated this behavior [305, 306]. Taken together, these data suggest that histamine reduces the rewarding value of reinforcers. These data are consistent with the high number of gapes and elevated extracellular histamine concentration elicited by QHCl, possibly associated with the perceived low value of the stimulus, and the reversal of both by treatment with Ex-4.

Taken together, the data shows that modulation of reward in the NAc shell is a complex process that involves several actors. As observed here, such modulation is not only dependent on

the mesocorticolimbic dopamine pathway. Indeed, we observed that both the aversive and the rewarding stimuli produced an increment on extracellular dopamine in the NAc shell. Given that both stimuli were not novel, the increase in dopamine may be involved to provide salience to the stimuli in order to elicit the correct response (e.g., an approach behavior towards sucrose and an escape response towards the aversive quinine). However, this DA response may be modulated by other neurochemicals. It has been observed that the NAc can be inhibited by histamine through the stimulation of GABA interneurons via H2R [307]. The infusion of the aversive QHCl, then, generates an increase in histaminergic transmission that results in the activation of such interneurons, generating an increase in extracellular GABA concentration. Moreover, Ex-4 may reduce the perceived aversiveness of QHCl by reducing histamine levels and, in consequence, halting NAc inhibition by GABA. This phenomenon, then may contribute to the robust DA release observed in response to QHCl in Ex-4-treated rats. It is possible, also, that as the perceived palatability of QHCl changes with Ex-4 treatment, the stimulus is now sensed as novel for the rat, producing DA release greater than that observed for the already known sucrose or the unshifted quinine. GLP-1 then, may serve as a modulator that allows for responses to not go awry. For example, by elevating NE in response to sucrose after the infusion, Ex-4 may counteract, but not block, the rewarding properties of the stimulus in order to allow the system to return to baseline [308]. On the other hand, Ex-4 may reduce the aversiveness perceived in response to quinine in order to prevent an excessive response that will not allow the rat to resume normal feeding behaviors.

Appendix B

Effects of chronic treatment with exendin-4 on body weight across abstinence days

Body weights are shown in Figure B1 from the start of the experiment (time 0) through abstinence days 1–16. Results show that, while heroin self-administration was associated with lower body weight overall, it was not significantly impacted by daily treatment with 1.4 μg/kg Ex-4 throughout the abstinence period. Support for these conclusions was provided by conducting a 2 x 2 x 16 mixed factorial ANOVA varying drug (saline or heroin), treatment (vehicle or Ex-4), and abstinence days 1–16. Results revealed a significant main effect of drug ($F_{1,47}$=22.96, $p<0.0001$), with rats that self-administered heroin weighing significantly less than rats that self-administered saline. Additionally, while there was a trend for Ex-4-treated rats to weigh less, neither the main effect of treatment ($F_{1,47}$=1.47, $p=0.23$), the group x treatment ($F<1.0$), or the group x treatment x day ($F< 1.0$) interactions were statistically significant. Hence, treatment with the present dose of Ex-4, then, did not have an effect on body weight in either the saccharin-heroin group or in the saccharin-saline controls.

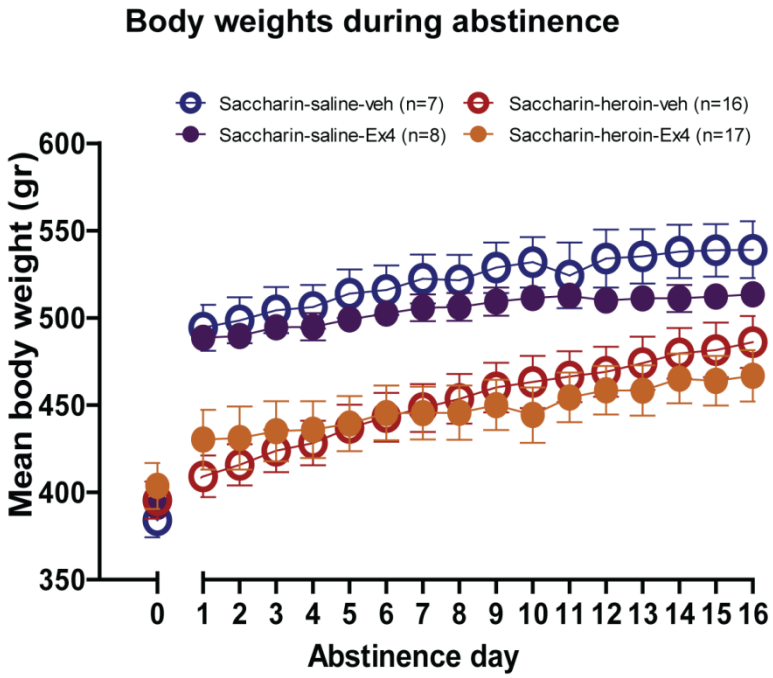

Figure B-1. Body weight across experiment. Mean (± SEM) body weight in grams for saccharin-saline rats treated with Veh (n=7) or Ex-4 (n=8) and saccharin-heroin rats treated with Veh (n=16) or Ex-4 (n=17). Day 0 represents average body weight at the beginning of acquisition and days 1 to 16 represent abstinence days.

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Selected Publications

JE Douton, C Augusto, B Stoltzfus, N Carkaci-Salli, KE Vrana, PS Grigson. "Glucagon-like peptide-1 receptor agonist, exendin-4, reduces cue- and drug-induced reinstatement of heroin seeking behavior and increases orexin receptor 1 expression in the nucleus accumbens shell of rats". (Preprint DOI: https://doi.org/10.1101/730408). *Behavioural Pharmacology (accepted)*.

JE Douton, R Norgren, PS Grigson. "Effects of Glucagon-like peptide-1 receptor agonist, exendin-4, on reward behavior of aversive and rewarding stimuli". *Under review.*

JE Douton, N Horvath, A Hajnal, PS Grigson. "Long acting Glucagon-like peptide-1 receptor agonist, liraglutide, reduces heroin self-administration and drug-induced reinstatement of heroin seeking behavior in rats". *In preparation.*

JE Douton, NK Acharya, B Stoltzfus, PS Grigson, JE Nyland. "Acute Glucagon-Like Peptide-1 Receptor Agonist Liraglutide Prevents Drug-Induced Heroin Seeking in Rats". *In preparation.*

Awards

BEC.AR Fellowship 2014. 2014 - 2016
Argentine Fulbright Commission and Argentine President's Cabinet

Selected Presentations

JE Douton, C Augusto, B Stoltzfus, N Carkaci-Salli, KE Vrana, PS Grigson (2019). "Glucagon-like peptide-1-induced reduction in heroin taking and seeking is accompanied by changes in the expression of 'satiety' genes in 'reward' nuclei in the rat". Annual Meeting of the Society for the Study of Ingestive Behavior (SSIB). Utrecht, Netherlands. (Poster).

JE Douton, CG Imperio, AJ McFalls, KE Vrana, WM Freeman, PS Grigson (2018). "Distinct mRNA profiles for reward devaluation, heroin addiction-like behaviors and heroin-induced 'relapse' in the nucleus accumbens of vulnerable and resilient rats". Society for Neuroscience 2018. San Diego, CA, USA. (Poster)

JE Douton, C Augusto, B Stoltzfus, PS Grigson (2018). "Glucagon-like peptide-1 receptor agonist, exendin-4, reduces heroin seeking during extinction and drug-induced reinstatement". Annual Meeting of the Society for the Study of Ingestive Behavior (SSIB). Bonita Springs, Florida, USA. (Oral Presentation)