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Dose titration with the glucagon-like peptide-1 agonist, liraglutide, reduces cue- and drug-induced heroin seeking in high drug-taking rats

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ABSTRACT

Opioid use disorder (OUD), like other substance use disorders (SUDs), is widely understood to be a disorder of persistent relapse. Despite the use of three FDA-approved medications for OUD, typically in conjunction with behavioral treatments, relapse rates remain unacceptably high. Whereas medication assisted therapy (MAT) reduces the risk of opioid overdose mortality, the benefits of MAT are negated when people discontinue the medications. Currently approved medications present barriers to efficient use, including daily visits to a treatment center or work restrictions. With spiking increases in opioid relapse and death, it is imperative to identify new treatments that can reduce the risk of relapse. Recent evidence suggests that glucagon-like peptide-1 receptor agonists (GLP-1RAs), currently FDA-approved to treat obesity and type two diabetes, may be promising candidates to reduce relapse. GLP-1RAs have been shown to reduce relapse in rats, whether elicited by cues, drug, and/or stress. However, GLP-1RAs also can cause gastrointestinal malaise, and therefore, in humans, the medication typically is titrated up to full dose when initiating treatment. Here, we used a rodent model to test whether cue- and drug-induced heroin seeking can be reduced by the GLP-1RA, liraglutide, when the dose is titrated across the abstinence period and prior to test. The results show this titration regimen is effective in reducing both cue-induced heroin seeking and drug-induced reinstatement of heroin seeking, particularly in rats with a history of high drug-taking. Importantly, this treatment regimen had no effect on either circulating glucose or insulin. GLP-1RAs, then, appear strong candidates for the non-opioid prevention of relapse to opioids.

1. Introduction

During the first ten months of 2020, i.e., during the rapid growth of the COVID-19 pandemic, overdose deaths increased in almost every state in the nation (Pandemic and Policy Options to Move Forward, 2021). This increase can be attributed largely to a spike in deaths related to the use of opioids (Pandemic and Policy Options to Move Forward, 2021). Currently, there are three medication assisted treatments (MATs) for opioid use disorder (OUD), naltrexone, buprenorphine/buprenorphine + naloxone (Suboxone), and methadone, yet relapse rates remain high (Hser et al., 2014; Pierce et al., 2016; Lee et al., 2018). Compliance is low with extended-release naltrexone, (Lee et al., 2018) treatment with Suboxone can elicit potent withdrawal in patients with fentanyl experience, (Bhatia and Sarkar, 2020) and the full μ-agonist, methadone, requires daily visits to a treatment center and may pose work restrictions in certain industries (i.e. airlines, hospitals, work with heavy equipment, etc.). Given these limitations, and spiking opioid overdose deaths, it is imperative that we better understand OUD to identify new and effective avenues for treatment.

A great deal of attention in the substance use disorder (SUD) literature has focused on the reward pathway and the view that drugs of abuse hijack natural reward substrates (Koob and Bloom, 1988; Bejerot, 1971). In 2008, (Grigson, 2008) we stated that "Drug-seeking animals and humans behave as though they *need* the drug. They seek to satisfy this need state much as they seek food when hungry, water when thirsty, and salt when sodium deficient. When these biological drives are activated, there is a single goal and there can be no substitute. This is the state of the [addicted individual] when actively engaged in drug-seeking." If this need state hypothesis is correct, then treatment with a known 'satiety' signal, glucagon-like peptide-1 (GLP-1), should reduce drug seeking and

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taking in rats. GLP-1 is a hormone produced by L cells in the small intestine that reduces food intake, in part, by increasing insulin release, decreasing glucagon release, and decreasing gastric emptying (Prasad-Reddy and Isaacs, 2015). GLP-1 also is produced by neurons in the nucleus of the solitary tract (NST), and these neurons project widely throughout the brain, including to reward and feeding circuitry (Alhadeff et al., 2012; Merchenthaler et al., 1999). In accordance, treatment with GLP-1 receptor agonists (GLP-1RA) inhibits the ingestion of sweets, water, and salt even when made more palatable by need (Alhadeff et al., 2012; Lopez-Ferreras et al., 2018; Turton et al., 1996; McKay and Daniels, 2013; Dickson et al., 2012; Mietlicki-Baase et al., 2013). Treatment with the GLP-1RA, Exendin-4 (Ex-4), also reduces a conditioned place preference for abused substances, blunts the resulting spike in dopamine release within the nucleus accumbens (NAc) from nicotine, cocaine, and amphetamine, and reduces cocaine self-administration in rodents (Egecioglu et al., 2013a, 2013b; Sorensen et al., 2015; Reddy et al., 2016; Graham et al., 2013). Likewise, treatment with Ex-4, in a dose that does not affect responding for sucrose, decreases fixed and progressive ratio responding for cocaine (Schmidt et al., 2016) and, when administered centrally, cocaine-induced reinstatement of cocaine-seeking behavior (Hernandez et al., 2018).

Regarding responding for opioids, one study conducted in mice failed to find Ex-4 effective in reducing remifentanil self-administration and other opioid-related behaviors (Bornebusch et al., 2019). Since this report, however, supportive data have been obtained in rats. Ex-4 was found effective in reducing oxycodone self-administration and seeking in rats; (Zhang et al., 2020) we found Ex-4 [2.4 μg/kg intraperitoneal (i.p.)] effective in reducing cue-induced heroin seeking and drug-induced reinstatement of heroin seeking in rats (Douton et al., 2021). In addition, Ex-4 recently was reported to reduce fentanyl self-administration and fentanyl seeking in rats following a drug/cue challenge. This dose of Ex-4, however, also increased intake of the anti-emetic, kaolin, suggesting Ex-4 may cause gastrointestinal malaise (Zhang et al., 2021).

In a follow up study, we tested the effectiveness of a 0.1 mg/kg subcutaneous (sc) dose of the longer-acting GLP-1RA, liraglutide, administered beginning on the 11th day of a 22-day heroin self-administration regimen, throughout a subsequent two-week abstinence period, and 1 h prior to extinction testing. Results showed: (1) reduced heroin self-administration, (2) reduced escalation of heroin self-administration, and (3) a significant reduction of drug-induced reinstatement of heroin seeking behavior assessed 6 h later (Douton et al., 2022b). Cue-induced heroin seeking, however, was not reduced when assessed 1 h following the administration of liraglutide. This likely was due to slower onset of the long-acting GLP-1RA compared with Ex-4. Indeed, the acute administration of a 0.3 mg/kg dose of liraglutide was highly effective in reducing both cue-induced heroin and fentanyl seeking and drug-induced reinstatement of heroin and fentanyl seeking when administered at least 6 h prior to test (Urbanik et.al., this issue) (Douton et al., 2022a; Urbanik et al., 2022). Importantly, we also demonstrated that the 0.3 mg/kg dose of liraglutide did not impair movement on a rotarod (Douton et al., 2022a) and, while liraglutide across a range of doses supported conditioned avoidance of a saccharin-paired cue, liraglutide, from the lowest to the highest dose tested, did not increase intake of the anti-emetic clay, kaolin (Douton et al., 2022b).

As such, GLP-1RAs are promising as a non-opioid treatment for OUD in humans. Importantly, GLP-1RAs can be readily applied for this new indication, as various formulations already are approved for the treatment of obesity and type 2 diabetes mellitus (T2DM) in humans (Prasad-Reddy and Isaacs, 2015; Blonde et al., 2006). That said, the primary side effect of GLP-1RA treatment is gastrointestinal malaise (Lundgren et al., 2021). In an effort to reduce the negative impact of this unpleasant side effect on treatment, the dose of GLP-1RAs is titrated over a number of weeks in humans to reach the target dose. Here, we use an animal model to test whether such a treatment regimen (i.e., dose titration of liraglutide) will be effective in reducing both cue-induced heroin seeking and drug-induced reinstatement of heroin seeking in rats. In Experiment 1, the dose increased every three days from 0.06 mg/kg to 0.1 mg/kg, 0.3 mg/kg and finally 0.6 mg/kg. In Experiment 2, the dose increased similarly but did not exceed the 0.3 mg/kg dose. Additionally, as an incretin hormone, GLP-1 increases the secretion of insulin which leads to a reduction of blood glucose and to an increase in satiety. Whereas this is excellent for the treatment of T2DM, (Prasad-Reddy and Isaacs, 2015; Blonde et al., 2006) sudden onset of hypoglycemia could lead to a severe adverse event in otherwise normoglycemic individuals in treatment for OUD. Consequently, we also tested whether liraglutide, across a range of doses, alters levels of either plasma glucose or plasma insulin (Experiment 3).

2. Methods

Fifty-two naïve male outbred Sprague-Dawley rats delivered from Charles River (Wilmington, MA) at approximately 90 days of age, weighing between 300 and 400 g at the start of the experiment served as subjects. 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 h light/dark cycle, and lights on at 7:00 am. All experimental manipulations began 2 h 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.

2.1. Jugular catheter implantation surgery

Rats in Experiment 1 (n = 24) and Experiment 2 (n = 24) were anesthetized with isoflurane (4% for induction and 2–3% for maintenance) and implanted with an intravenous (iv) jugular catheter (Instech Laboratories, Inc., Plymouth Meeting, PA) for drug self-administration as described previously (Grigson and Twining, 2002). Rats in Experiment 3 (n = 4) went through a similar surgery as rats in Experiments 1 and 2, but they also had a catheter placed in the carotid artery. Following surgery, rats received a sc injection of the non-steroidal anti-inflammatory drug (NSAID), carprofen, and the antibiotic, enrofloxacin, as post-operative care for at least two days, and were given a week to recover. Maintenance of jugular and carotid catheter patency included flushing catheters using heparinized saline (0.2 mL of 30 IU/mL heparin) once every four days. Catheter patency was verified at the end of each week of drug self-administration and the day before each test using 0.3 mL of propofol (Diprivan 1%).

2.2. Habituation

Rats in Experiments 1 and 2 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. During this 5-min period, rats were placed in the self-administration chambers and water was available in one of the two spouts (center future 'inactive' spout and rightmost future 'active' spout), counterbalanced across the first and second day. In order to maintain proper hydration during habituation, rats also received overnight access to 20 mL of filtered water at the front of the home cage beginning at 5 PM. Ad libitum access to water was resumed following the second habituation session.

2.3. Self-Administration (SA)

2.3.1. Apparatus

Twenty-four drug self-administration chambers (MED Associates, Inc., St. Albans, VT) were used as previously described (Puhl et al., 2013). Each 30.5 cm × 24.0 cm × 29.0 cm chamber was equipped with a 25 W light, tone generator, white noise speaker, and two empty, retractable spouts. A lickometer circuit was used to record contacts on both spouts. All recorded data from the lickometers and all events in the chamber (e.g., lights, tones, spout advancement and retraction) are controlled, measured and stored by a Pentium computer using the MED Associates programing language (MED Associates, Inc., St. Albans, VT).

2.3.2. Acquisition

After habituation, rats were placed in the self-administration chambers daily around 9 AM. They were then given 6 h to self-administer heroin (n = 32) or saline (n = 16) in Experiments 1 and 2. At the start of the session, the middle and rightmost empty spouts advanced, with the spouts centered in the aperture and flush with the Plexiglas wall. Contacts on the inactive empty spout (middle) led to no consequence. The availability of the rightmost active empty spout was signaled by a cue light located above and completion of a fixed ratio of 10 (FR10) contacts with this spout led to a 6 s iv infusion of 0.06 mg/0.2 mL of heroin, previously shown to produce robust heroin self-administration and seeking in rats (Douton et al., 2021, 2022a; Kuntz et al., 2008). Each infusion was accompanied by a 20 s time-out period during which the cue light turned off, the house light turned on, the empty spouts retracted and was signaled by the sound of a tone. Self-administration occurred as described 5 days a week for 11 days. This paradigm was run for both Experiment 1 and Experiment 2.

2.3.3. Cue/drug-induced reinstatement test 1

On the 12th day, the rats from both Experiments 1 and 2 were placed in their self-administration chambers beginning at 1PM, but no heroin was delivered across a 3 h extinction period. The number of contacts made on the right empty spout during the first h was an indication of cue-induced heroin seeking, with the behavior typically extinguishing across the 2nd and 3rd h of extinction. At the end of Hour 3, rats were given a computer-controlled, non-contingent iv administration of saline or heroin (as per predetermined group assignment) and drug-induced reinstatement of heroin seeking behavior was measured across an additional 60-min extinction session (i.e., in h 4). **Home cage abstinence.** Thereafter, all rats experienced 2 weeks of abstinence in their home cages, which can serve to augment heroin-seeking behavior (Zhou et al., 2009).

2.3.4. Liraglutide or vehicle treatment

Rats with a history of saline or heroin self-administration were matched for heroin taking and seeking behavior after Test Day 1 and injected sc daily (beginning at 7AM) with either saline vehicle (n = 8/experiment) or liraglutide (n = 16/experiment). The dose of liraglutide started at 0.06 mg/kg and increased every 3 days. For Experiment 1, the dose increased from 0.06 mg/kg to 0.1 mg/kg, 0.3 mg/kg and finally 0.6 mg/kg. For Experiment 2, the dose increased similarly but did not exceed the 0.3 mg/kg dose.

2.3.5. Cue/drug-induced reinstatement test 2

After 2 weeks of abstinence and daily vehicle or liraglutide treatment, the rats in both Experiment 1 and Experiment 2 underwent a second extinction test to measure the effect of daily treatment with vehicle or increasing doses of liraglutide (rats also received their daily sc administration of vehicle or liraglutide 6 h prior to Test 2) on cue-induced heroin seeking and drug-induced reinstatement of heroin seeking as described in Test 1 above.

2.3.6. Abstinence/Re-test 0.3 mg/kg liraglutide (test 3: experiment 1 only)

Given data suggesting that the 0.6 mg/kg dose of liraglutide may be too high (see below), rats in Experiment 1 were re-tested with a 0.3 mg/kg liraglutide challenge. Specifically, rats in Experiment 1 (i.e., those titrated to the 0.6 mg/kg dose of liraglutide), were given 3 additional days of home cage abstinence with daily vehicle or 0.3 mg/kg liraglutide injections. On the 4th day, the rats were injected sc with vehicle or 0.3 mg/kg liraglutide and returned 6 h later to the test chambers under extinction conditions to examine the effect of the lower dose on cue-induced heroin seeking and drug-induced reinstatement of heroin seeking as described in Test 1 above.

2.4. Experiment 3: glucose and insulin levels

To assess the safety of liraglutide in maintaining glucose homeostasis, glucose and insulin levels were measured using a within subject design where rats (n = 4) received a daily sc injection of liraglutide across increasing doses including 0.06, 0.1, 0.3, 0.6, and 1.0 mg/kg and compared to baseline (BL). The rats were ad libitum-fed overnight, but food was withheld following the 7 A.M. liraglutide injection and throughout sampling. Glucose was measured by a glucometer (Prodigy AutoCode, Charlotte, NC, USA) from arterial plasma samples taken at baseline and at 6, 8, and 10 h post-liraglutide administration. We examined only 6–10 h post-liraglutide since peak plasma concentrations of liraglutide occur in this time frame (Agersø et al., 2002). Arterial plasma insulin concentrations were measured at these same time points using a rat ultrasensitive insulin ELISA kit (80-INSRTU-E01; Alpco Diagnostics, Salem, NH, USA).

2.5. Data analysis

The data from Experiments 1 and 2 were analyzed using Statistica Version 13.5.0.17, TIBCO Software Inc. (Palo Alto, CA, USA). Significant mixed factorial Analysis of Variance (ANOVAs) were followed by Newman-Keuls post hoc tests to identify group differences. The data from Experiment 3 were analyzed using Prism Version 9.2.0, GraphPad Software (La Jolla, CA, USA). For glucose and insulin levels, data are presented as means ± standard error of the mean (SEM). Raw values for glucose and insulin levels are shown at each time point and an area under the curve (AUC) was calculated to summarize changes over time. Raw data were analyzed using a two-way repeated measures ANOVA to account for main effects of liraglutide dose, time, and their interaction. The data for the AUC were analyzed using a one-way repeated measures ANOVA. Fig. 1.

3. Results

3.1. Experiment 1

3.1.1. Acquisition and division of high and low heroin takers

To identify high and low heroin takers, the number of infusions self-administered during terminal Trials 10 and 11 were averaged for all rats in the heroin condition (n = 16). The median number of terminal infusions of heroin was 14.5. Thus, high drug-takers were identified as rats that self-administered more than the median (n = 8) and low drug-takers, rats that self-administered less than the median (n = 8).

3.1.2. Acquisition

Fig. 2 **A** shows the mean number of infusions per 6 h across 11 acquisition trials for low and high drug takers and for saline self-administering controls. The data were analyzed using a 3 × 11 mixed factorial ANOVA varying group x trials. Due to missing data from saline controls, 3 data points were added for 3 subjects via interpolation in order for Statistica to analyze the data.

The results yielded a significant main effect of group, ($F_{2,19}$ =14.16, p = 0.0002), with post hoc Newman-Keuls tests showing that high drug

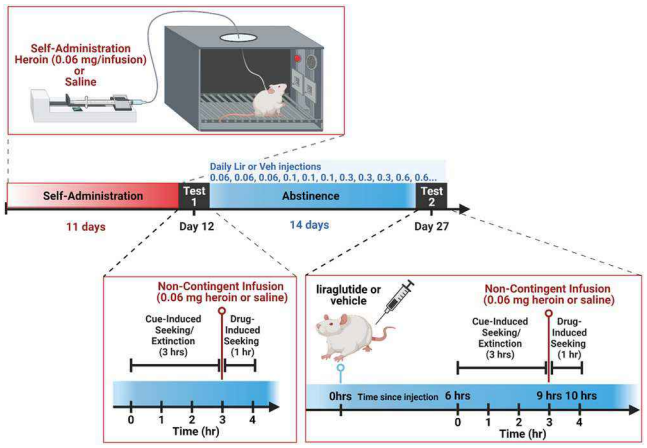

Fig. 1. Outline for Experiments 1 and 2. After jugular catheter implantation surgery, recovery, and habituation, the rats had 11 days of saline or heroin self-administration (SA). On the 12th day, the rats had a first test day (Test Day 1) to assess cue-induced seeking during extinction and drug-induced reinstatement. After Test Day 1, the rats had 2 weeks of abstinence in their home cage and were treated with the titrated doses of liraglutide or saline. The dose starts at 0.06 mg/kg and goes up every three days until it reaches the maximum (0.6 mg/kg for Experiment 1 and 0.3 mg/kg for Experiment 2). Thereafter, that dose is given for the remaining days of abstinence. After two weeks of abstinence and titrated dosing with liraglutide or saline, the rats received a second test day (Test Day 2) to test the effect of the titrated dose of liraglutide on cue-induced seeking and drug-induced reinstatement of heroin seeking. For Experiment 1, the rats had an additional three days of abstinence and daily saline or 0.3 mg/kg of liraglutide treatment before going through a similarly conducted Test Day 3. Figure created with BioRender.com.

takers (her/high) took more infusions of heroin than low drug takers (her/low), $p < 0.05$, and group her/low took more than the saline self-administering controls, $p < 0.05$, overall. The group x trials interaction also was significant ($F_{20,200} = 3.23$, $p < 0.0001$) and post hoc Newman-Keuls tests revealed that high drug takers took more infusions of heroin than controls took of saline from Trial 5 through Trial 11, p-values (ps) < 0.05. Otherwise, there were no significant differences between groups across trials. Such individual differences in drug self-administration behavior are consistent with our previous reports (Colechio et al., 2014; Twining et al., 2009; Kuntz et al., 2008; Grigson and Twining, 2002) involving cocaine and heroin self-administration.

3.1.3. Cue/drug-induced reinstatement test 1

As described, on the 12th day, the rats were placed in the test chambers as usual, but under extinction conditions (i.e., with no drug delivered) and cue-induced seeking was measured in Hour 1 and drug-induced reinstatement of heroin seeking in Hour 4 (Fig. 2**B**). Infusion attempts were analyzed using a 3 x 4 mixed factorial ANOVA varying group (high heroin-taker, low heroin-taker, or saline) and hour (1 − 4). The results showed a significant group x time interaction ($F_{6,57} = 4.34$, $p = 0.0011$). Post hoc Newman-Keuls tests confirmed that, in Hour 1, greater cue-induced heroin seeking was evidenced by the high drug takers vs. the low drug takers and the saline self-administering controls, ps< 0.05, which did not differ one from the other, $p > 0.05$ (see Fig. 2, panel B). The high drug takers also evidenced greater drug-induced reinstatement of heroin-seeking behavior in Hour 4 than did the low drug takers, which exhibited greater seeking than did rats with a history of saline self-administration, ps < 0.05.

3.1.4. Cue/drug-induced reinstatement test 2

Following 2 weeks of home cage abstinence and daily sc injections of vehicle or increasing doses of liraglutide, rats were returned to the test chamber for a second cue/drug-induced reinstatement test. The data from this test are shown in Fig. 3**A and B.** These data were analyzed using a 3 x 2 x 4 mixed factorial ANOVA varying group (high heroin-taker, low heroin-taker, or saline), drug (liraglutide or vehicle), and hour (1 − 4). The results of this ANOVA revealed a significant main effect of group, ($F_{2,16} = 27.02$, $p < 0.0001$), and group x time interaction ($F_{6,48} = 8.56$, $p < 0.0001$). Neither the drug x time interaction ($F_{3,48} = 1.77$, $p = 0.1662$) nor the group x drug x time interaction was statistically significant ($F_{6,48} = 2.02$, $p = 0.0816$). Given the significant main effect of group, the data from the low takers and the high takers were analyzed separately utilizing 2 x 2 x 4 mixed factorial ANOVAs. **Low drug takers.** Results yielded a significant group x time interaction ($F_{3,30} = 20.70$, $p < 0.0001$), but no significant drug x time ($F < 1$) or group x drug x time interaction ($F_{3,30} = 1.39$, $p = 0.2652$). **High drug takers.** There was a significant group x time interaction ($F_{3,33} = 11.99$, $p < 0.0001$), but again no significant drug x time ($F_{3,33} = 2.65$, $p = 0.0650$) or group x drug x time interaction ($F_{3,33} = 1.64$, $p = 0.1981$). Post hoc tests on the significant group x time interactions revealed greater seeking in Hour 1 and Hour 4 by rats with a history of heroin self-administration vs. the saline self-administration, regardless of drug treatment (i.e., vehicle or liraglutide), ps < 0.05. Given the effectiveness of the 0.3 mg/kg dose of liraglutide in our acute studies (Douton et al., 2022a; Urbanik et al., 2022) the same experimental subjects were challenged following a brief period of abstinence with this lower dose of liraglutide.

3.1.5. Abstinence/Re-test 0.3 mg/kg liraglutide (test 3: experiment 1 only)

After three days of home cage abstinence and daily sc injections with vehicle or 0.3 mg/kg liraglutide, rats were injected sc with vehicle or

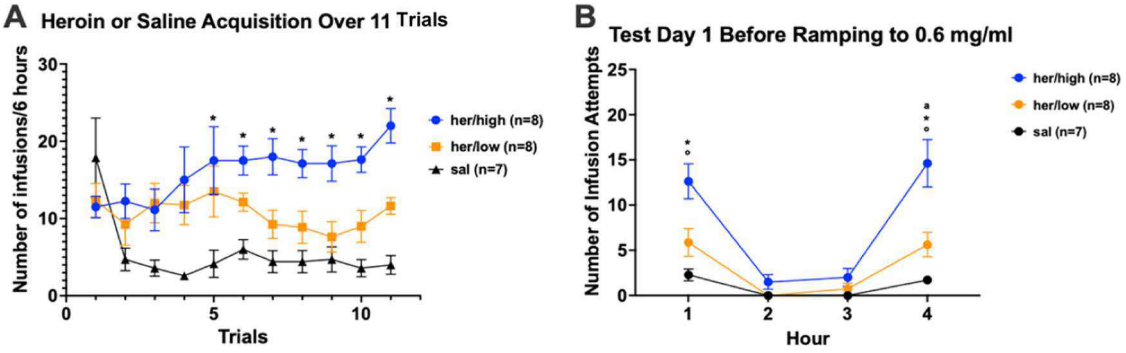

Fig. 2. A. Heroin or Saline Acquisition Over 11 Trials; B. Cue-induced Seeking and Drug-induced Reinstatement Test 1. A) Acquisition: Mean (+/- SEM) number of infusions/6 h across 11 daily trials for heroin high drug takers (blue), heroin low drug takers (orange), and saline controls. **B) Test Day 1:** Mean (+/- SEM) number of infusion attempts across Hours 1 – 4 of extinction for heroin high takers (blue), heroin low takers (orange) and saline controls (black). A single iv infusion of heroin was administered at the end of Hour 3. Cue-induced heroin seeking was assessed in Hour 1; Drug-induced reinstatement of heroin seeking was assessed in Hour 4. Symbols denote significance between groups (*=$p < 0.05$ for her/high vs. sal; [a]= $p < 0.05$ for her/low vs. sal; °=$p < 0.05$ for her/high vs. her/low).

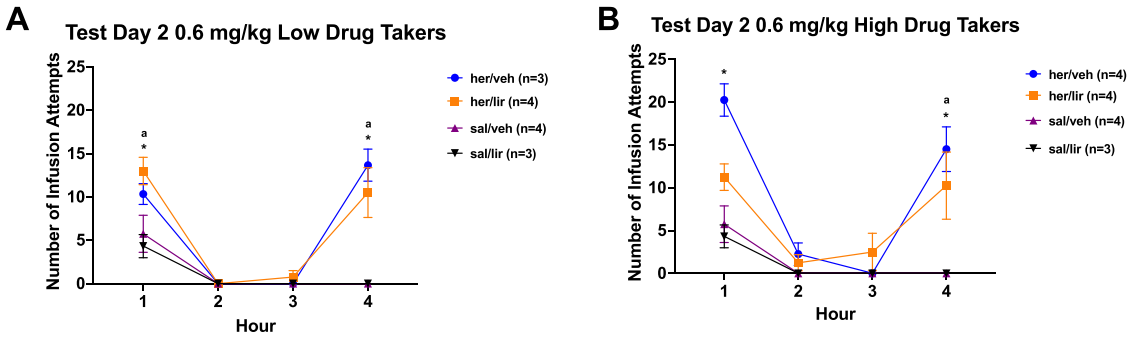

Fig. 3. Cue-induced Seeking and Drug-induced Reinstatement After Chronic Liraglutide Titrated to 0.6 mg/kg. A) Low Drug Takers: Mean (+/- SEM) number of infusion attempts across Hours 1 – 4 for low heroin takers and saline self-administering controls treated with vehicle (saline) or 0.6 mg/kg liraglutide during abstinence and prior to test. **B) High Drug Takers:** Mean (+/- SEM) number of infusion attempts across Hours 1 – 4 for high heroin takers and saline self-administering controls treated with vehicle (saline) or 0.6 mg/kg liraglutide during abstinence and prior to test (*=$p < 0.05$ for her/veh vs. sal/veh; [a]=$p < 0.05$ for her/lir vs. sal/lir).

liraglutide (0.3 mg/kg) and, 6 h later, placed back into the test chamber for Cue/Drug-Induced Reinstatement Test 3. The data for this test are shown in Fig. 4**A and B**.

Once again, the data were analyzed using a 3 x 2 x 4 ANOVA varying group, drug, and time. The results revealed a significant group x time interaction ($F_{6,48}$ =4.52, p = 0.0011) and significant drug x time interaction ($F_{3,48}$ =6.90, p = 0.0006), but only trending significance with group x drug x time interaction ($F_{6,48}$ = 2.20, p = 0.0591). Given a significant main effect of group, ($F_{2,16}$ =11.51, p = 0.0008), the data from the low takers and the high takers were once again analyzed separately utilizing 2 x 2 x 4 mixed factorial ANOVAs. **Low drug takers.** There was a significant group x time ($F_{3,30}$ =9.62, p = 0.0001), drug x time ($F_{3,30}$ =6.09, p = 0.0023), and group x drug x time interaction ($F_{3,30}$ =7.30, p = 0.0008). Post hoc Newman-Keuls tests of this significant 3-way ANOVA confirmed higher cue-induced seeking and drug induced reinstatement in her/veh (heroin rats treated with vehicle) compared to sal/veh (saline rats treated with vehicle) rats, $p < 0.05$. There also was reduced cue-induced seeking in Hour 1 and drug-induced reinstatement in her/lir (heroin rats treated with liraglutide) in Hour 4 compared to her/veh treated rats, $p < 0.05$. **High drug takers.** For high drug takers, the group x time interaction attained statistical significance ($F_{3,33}$ =6.12, p = 0.0020), but this was not the case for either the drug x time ($F_{3,33}$ =2.42, p = 0.0835) or group x drug x time ($F_{3,33}$ =2.42, p = 0.0837) interaction. Overall, high drug takers had higher cue-induced seeking and drug-induced reinstatement of heroin seeking compared to rats with a history of saline self-administration, $p < 0.05$. When the data from Hour 1 (cue-seeking) was analyzed alone, there was a significant main effect of group ($F_{1,11}$ =10.85, p = 0.0072), but not drug ($F_{1,11}$ =2.89, p = 0.1169) nor group x drug interaction (F<1). Thus, there was greater cue-induced seeking and drug-induced reinstatement for group her/veh compared with group sal/veh. An analysis of the data from Hour 4 showed a significant group x drug interaction ($F_{1,11}$ =7.08, p = 0.0221). Post hoc Newman-Keuls tests on the group x drug interaction demonstrated reduced heroin seeking in group her/lir vs. group her/veh, $p < 0.05$.

3.2. *Experiment 2*

As discussed, in a separate set of studies, the acute administration of 0.3 mg/kg liraglutide robustly reduced cue-induced heroin seeking and drug- and stress-induced reinstatement of heroin seeking (Douton et al., 2022a). Similar findings were reported with fentanyl (Urbanik et al., this issue) (Urbanik et al., 2022). Based on the results from Experiment 1, and the now completed series of studies investigating the effectiveness of the lower 0.3 mg/kg dose of liraglutide, (Douton et al., 2022a) we replicated the above study, but titrated the dose only to 0.3 mg/kg liraglutide. The data from one high heroin taker treated with liraglutide was removed due to equipment failure.

3.2.1. *Acquisition and division of high and low heroin takers*

To identify high and low heroin takers, the number of infusions self-administered during terminal Trials 10 and 11 were averaged for all rats in the heroin condition (n = 15). The median number of terminal infusions of heroin was 16.5. Thus, high drug-takers were identified as rats that self-administered more than the median (n = 7) and low drug-takers, rats that self-administered less than the median (n = 8).

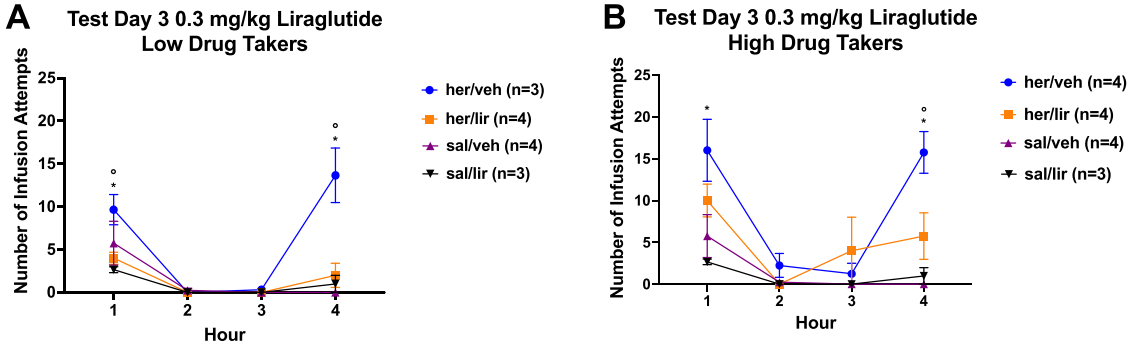

Fig. 4. Cue-induced Seeking and Drug-induced Reinstatement after 3 days of Vehicle or 0.3 mg/kg Liraglutide Treatment. A) Low Drug Takers: Mean (+/- SEM) number of infusion attempts across Hours 1 – 4 for low heroin takers and saline self-administering controls treated with vehicle (saline) or 0.3 mg/kg liraglutide during 3 days of abstinence and 6 h prior to test. **B) High Drug Takers:** Mean (+/- SEM) number of infusion attempts across Hours 1 – 4 for high heroin takers and saline self-administering controls treated with vehicle (saline) or 0.3 mg/kg liraglutide during 3 days of abstinence and 6 h prior to test. Symbols denote significance between groups (*=$p < 0.05$ for her/veh vs. sal/veh; °=$p < 0.05$ for her/veh high (or low) vs. her/lir high (or low)).

3.2.2. Acquisition

Fig. 5 A shows the mean number of infusions per trial for high drug takers, low drug takers, and saline controls. Conduct of a 3 × 11 ANOVA varying group and trial found a significant main effect of group ($F_{2,19}$ =22.03, $p < 0.0001$) and a significant group x trial interaction ($F_{20,190}$ =4.05, $p < 0.0001$). Post hoc Newman-Keuls tests revealed a significant split between high and low drug takers on acquisition Trial 10, ps < 0.05, with high drug takers self-administering a higher number of infusions. High drug takers also took significantly more infusions of heroin than saline self-administering controls across Trials 2 through 11, ps < 0.05. Post hoc tests did not find any significant differences in infusions between low drug takers and saline self-administering controls on a trial-by-trial basis.

3.2.3. Cue/drug-induced reinstatement test 1

Fig. 5B shows the results of Test Day 1. Infusion attempts were analyzed using 3 × 4 ANOVA which showed a significant group x time interaction ($F_{6,60}$ =6.17, $p < 0.0001$). For cue-induced seeking in Hour 1, there were significantly more infusion attempts emitted by high drug takers vs. low drug takers, high drug takers vs. saline controls, and low drug takers vs. saline controls, ps < 0.05. For drug-induced reinstatement in Hour 4, there was a significantly higher number of infusion attempts made by high drug takers vs. saline controls and high drug takers vs. low drug takers, ps< 0.05.

3.2.4. Cue/drug-induced reinstatement test 2

Fig. 6A and B show the results of Cue/Drug-Induced Reinstatement Test 2, i.e., the extinction test that followed 2 weeks of home cage abstinence and daily treatment with saline or increasing doses of liraglutide up to 0.3 mg/kg. Again, the data were analyzed using a 3 × 2 x 4 mixed factorial ANOVA varying group, drug, and time and results found a significant group x drug x time interaction ($F_{6,51}$ =3.44, $p = 0.0062$). For the low drug takers, post hoc Newman-Keuls tests confirmed that in Hour 1, rats in the her/veh group exhibited significantly more infusion attempts than rats in the sal/veh group, $p < 0.05$. Liraglutide was not effective in reducing cue-induced heroin seeking in the low drug takers. During Hour 4, drug-induced reinstatement of heroin seeking was greater in the her/veh group compared with the sal/veh group and when compared with the her/lir group, ps < 0.05. For high drug takers, post hoc tests of the significant 3-way ANOVA found significantly more infusion attempts in the her/veh group compared with the sal/veh group for cue-induced seeking (Hour 1) and for drug-induced reinstatement of heroin seeking (Hour 4), ps < 0.05. For high drug takers, treatment with the titrated dose of liraglutide during abstinence and prior to test significantly reduced both cue-induced heroin seeking in Hour 1 and drug-induced reinstatement of heroin seeking in Hour 4 compared to the her/veh treated controls, ps < 0.05. These results demonstrate that titrating the dose of liraglutide to 0.3 mg/kg significantly attenuates cue-induced heroin seeking in high drug takers and drug-induced reinstatement of heroin seeking in both high and low drug takers.

3.3. Experiment 3

Liraglutide, across a range of doses, does not significantly alter levels of circulating glucose or insulin when measured as change from baseline using a within-subjects design. This conclusion was supported by a non-significant two-way repeated measures ANOVA varying dose and time, F< 1, for plasma glucose levels (Fig. 7**A**) and a non-significant two-way repeated measures ANOVA, F < 1, for insulin (Fig. 7**B**). When evaluated for the area under the curve, one-way ANOVAs again failed to find any effect of liraglutide, across a range of doses, on either plasma glucose ($F_{4,12}$ =1.30, $p = 0.32$) or plasma insulin ($F_{4,12}$ =1.03, $p = 0.43$) Fig. 7**C and D.** Taken together, these findings show that, liraglutide, at these doses does not cause hypoglycemia in rats, which is consistent with that previously reported (Douton et al., 2022b).

4. Discussion

The results demonstrate that chronic treatment with increasing doses of liraglutide across a 2-week abstinence period and prior to test in rats reduces cue-induced heroin seeking for high drug takers and drug-induced reinstatement of heroin seeking for both high and low drug takers. Thus, titrating to the 0.3 mg/kg dose of liraglutide was effective in reducing cue-induced heroin seeking in high drug takers and drug-induced reinstatement of heroin seeking in high and low drug takers; while titrating to the 0.6 mg/kg dose of liraglutide was not. Further, the protective effect of liraglutide treatment was greater in high vs. low drug takers. Finally, across a wide range of doses of liraglutide, there was no significant effect on plasma glucose or insulin levels in rats. Taken together, these data suggest that the GLP-1RA, liraglutide, can effectively reduce both cue-induced heroin seeking and drug-induced reinstatement of heroin seeking in rats - even when the dose is gradually titrated to the 0.3 mg/kg dose of the drug.

The present data add to a now growing literature suggesting that GLP-1RAs show promise as a new treatment for substance use disorders. As alluded to, a large number of reports suggest that GLP-1RAs may be useful in the treatment of other SUDs, (Egecioglu et al., 2013a, 2013b; Sorensen et al., 2015; Schmidt et al., 2016; Graham et al., 2013; Hernandez et al., 2018) while our lab and others have provided evidence that GLP-1RAs also are effective in animal models of OUD (Douton et al., 2021, 2022a,b, Zhang et al., 2020, 2021). In the present paper we found

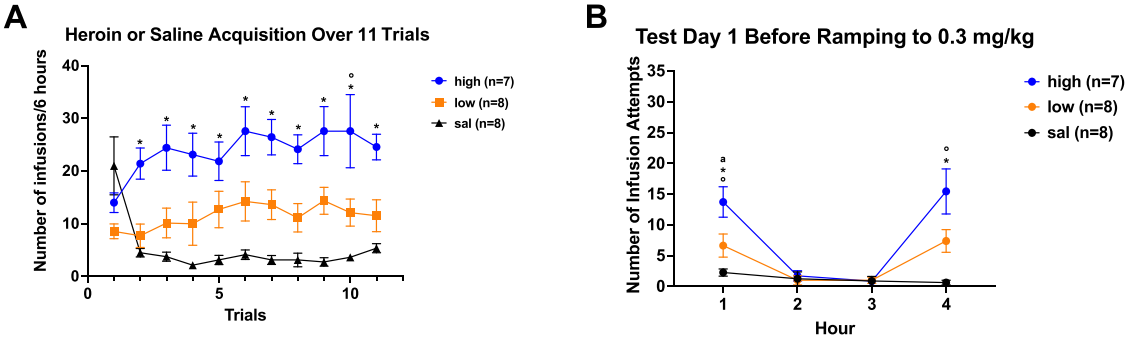

Fig. 5. A. Heroin or Saline Acquisition Over 11 Trials; B. Cue-induced Seeking and Drug-induced Reinstatement Test 1 for Experiment 2. A) Acquisition: Mean (+/- SEM) number of infusions/6 h across 11 daily trials for heroin high drug takers (blue), heroin low drug takers (orange), and saline controls (black). **B) Test Day 1:** Mean (+/- SEM) number of infusion attempts across Hours 1 – 4 of extinction for heroin high takers (blue), heroin low takers (orange) and saline controls (black). A single iv infusion of heroin was administered at the end of Hour 3. Cue-induced heroin seeking was assessed in Hour 1; Drug-induced reinstatement of heroin seeking was assessed in Hour 4. Symbols denote significance between groups (*=p < 0.05 for her/high vs. sal; [a]= p < 0.05 for her/low vs. sal; °=p < 0.05 for her/high vs. her/low).

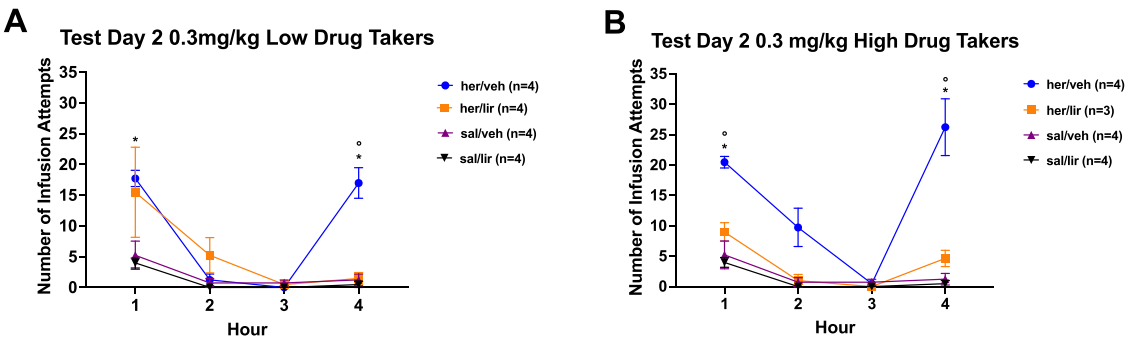

Fig. 6. Cue-induced Seeking and Drug-induced Reinstatement After Chronic Liraglutide Titrated to 0.3 mg/kg. A) Low Drug Takers: Mean (+/- SEM) number of infusion attempts across Hours 1 – 4 for low heroin takers and saline self-administering controls treated with vehicle (saline) or 0.3 mg/kg liraglutide during abstinence and prior to test. **B) High Drug Takers:** Mean (+/- SEM) number of infusion attempts across Hours 1 – 4 for high heroin takers and saline self-administering controls treated with vehicle (saline) or 0.3 mg/kg liraglutide during abstinence and prior to test (*=$p < 0.05$ for her/veh vs. sal/veh; °=$p < 0.05$ for her/veh high (or low) vs. her/lir high (or low); [a]=$p < 0.05$ for her/lir vs. sal/lir).

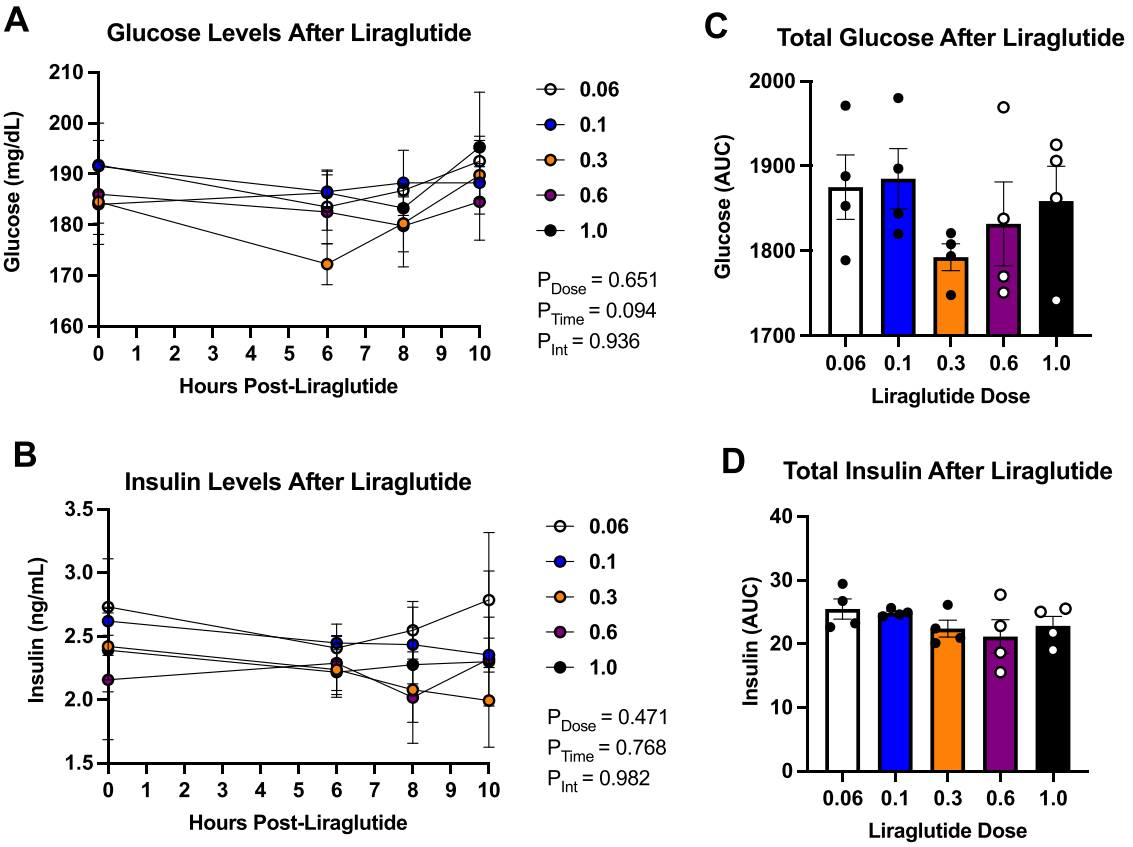

Fig. 7. Glucose and Insulin Levels After Liraglutide Treatment. There is no significant decrease or increase in **(A)** glucose, **(B)** insulin, **(C)** total glucose ($p = 0.324$ one-way repeated measures ANOVA), and **(D)** total insulin levels ($p = 0.431$ one-way repeated measures ANOVA) following the administration of a range of doses of liraglutide (0.06, 0.1, 0.3, 0.6, and 1.0 mg/kg). AUC = Area Under the Curve.

that titrating the dose to 0.3 mg/kg liraglutide was most effective in reducing heroin-seeking behaviors, particularly in rats with a history of high heroin self-administration. This is consistent with our findings that the acute injection of 0.3 mg/kg of liraglutide significantly reduced cue-induced heroin seeking and drug- and stress-induced reinstatement of heroin seeking in rats, (Douton et al., 2022a) and with similar findings with cue-induced fentanyl seeking and drug-induced reinstatement of fentanyl seeking (Urbanik et al., 2022). As discussed, the present data also are consistent with a previously published report showing protective effects of chronic daily treatment with 0.1 mg/kg liraglutide. In that case, however, chronic daily treatment with 0.1 mg/kg liraglutide reduced drug self-administration and drug-induced reinstatement of heroin seeking behavior, but not cue-induced heroin seeking (Douton et al., 2022b). The failure of the 0.1 mg/kg dose of liraglutide to reduce cue-induced heroin seeking in Douton et al. likely was due to the use of a short 1 h pretreatment time. Interestingly, titrating to the higher 0.6 mg/kg dose of liraglutide, even with the 6 h pretreatment time, was not more effective than was titrating to the 0.3 mg/kg dose. Such a finding suggests the development of tolerance following chronic administration of higher doses of the drug. Depending on the cell or tissue type, GLP-1Rs can be desensitized and internalized with chronic exposure of GLP-1RA (Fletcher et al., 2016). Thus, with higher doses there may be a greater likelihood for desensitization or internalization – i.e., tolerance (see below for further discussion).

Cues associated with drug taking can lead to onset of withdrawal symptoms in humans (Childress et al., 1984; O'Brien et al., 1977) and rats (Zhou et al., 2009; Ahmed et al., 2000). Cue-induced seeking likely is an index of cue-induced withdrawal since rats are placed into an environment with drug-associated cues (e.g., the self-administration chambers, the light cue, white noise, sounds of spouts advancing). Further, a fairly recent report showed that greater withdrawal in humans with an OUD was associated with greater activation of the NAc to drug-paired cues (Shi et al., 2021). Because GLP-1RAs are able to reduce cue-induced seeking in rats, as demonstrated in our previous papers, (Douton et al., 2021, 2022a,b, Zhang et al., 2020, 2021) and in Fig. 6 of this report (see high drug takers), we speculate that GLP-1RAs are able to reduce cue-induced withdrawal. In accordance, in an in preparation manuscript, treatment with Ex-4 also greatly reduces the conditioned aversive taste reactivity behavior (i.e., gapes) associated with naloxone induced withdrawal (Olsen). Like exposure to drug-related cues, re-exposure to the drug itself also can prompt withdrawal symptoms in people with a history of fentanyl taking that are treated with buprenorphine, for example, (Varshneya et al., 2021) and drug-induced reinstatement can sensitize an individual to the rewarding properties of the drug (De Vries et al., 1998). Thus, because GLP-1RAs also are able to reduce drug-induced reinstatement as demonstrated in our previous papers, (Douton et al., 2021, 2022a,b, Zhang et al., 2020, 2021) and in Figs. 4 and 6 of this manuscript, we speculate that GLP-1RAs also may reduce sensitization to the rewarding properties of the drug. Finally, our data showing that liraglutide does not alter glucose homeostasis is consistent with published preclinical and clinical data indicating that GLP-1RAs do not induce hypoglycemia in the absence of resting hyperglycemia (Ja'arah et al., 2021; Zhang et al., 2019).

In several studies, we focused on acute treatment of liraglutide and its effect on opioid seeking and taking behavior. While we have found that acute treatment of liraglutide is able to reduce cue-induced heroin seeking, drug- and stress-induced reinstatement of heroin seeking (Douton et al., 2022b), cue-induced fentanyl seeking, and drug-induced reinstatement of fentanyl seeking (Urbanik et al., 2022), long term treatment of OUD will require chronic, rather than acute, administration of the drug. As such, for the present report, we modeled the titration regimen used to treat obesity and T2DM in humans where the dose of the drug is titrated over time to prevent side effects (e.g., nausea) (Lundgren et al., 2021). Here, we found that chronic treatment with liraglutide titrated to the 0.3 mg/kg dose across a 2-week abstinence period and prior to test was able to reduce cue-induced heroin seeking in high drug takers and drug-induced reinstatement of heroin seeking. While we cannot be certain if liraglutide-reduced seeking at test is due to chronic or acute administration of the drug (as liraglutide was administered chronically, but also 6 h prior to test), it appears to the best of our knowledge, that the drug needs to be administered daily – i.e., the drug needs to be on board for the drug to be effective. Further, higher doses of the drug may not be more effective, as higher doses may be more likely to support the development of tolerance. Here, titrating to the 0.6 mg/kg dose of liraglutide was not effective in reducing drug seeking; and this effect of apparent tolerance was moderated when the dose of the drug was reduced by half to 0.3 mg/kg sc. Of course, this 0.3 mg/kg dose was most effective in Experiment 2, when the rats had no experience with the higher 0.6 mg/kg dose of the drug. In rodents, tolerance develops to the glucose lowering effects and to the gastric emptying effect of liraglutide (Sedman et al., 2020; Jelsing et al., 2012). However, there may not be tolerance development to glucose lowering effects in humans (Sedman et al., 2017).

4.1. Mechanism of action

As alluded to, in 2008, we hypothesized that withdrawal is a need state like starvation or dehydration (Grigson, 2008). Thus, withdrawal would drive individuals to seek drug like one would pursue food when starved or water when severely dehydrated. In line with this hypothesis, we demonstrated that treatment with a GLP-1RA, a known satiety agent, reduced evidence of this 'need' state (i.e., reduced cue- and drug-induced heroin seeking). That being said, while we have obtained evidence that treatment with GLP-1RAs reduces heroin taking and seeking behaviors in rats, the underlying mechanism of action remains unknown. There are GLP-1Rs in brain areas associated with reward including the ventral tegmental area (VTA) and the NAc (van Bloemendaal et al., 2014). There also are GLP-1 producing neurons that project directly from the NST to the VTA and NAc (Alhadeff et al., 2012; Rinaman, 2010) and activation of GLP-1Rs in the NTS leads to a reduction in the expression of dopamine-related genes in the VTA (Richard et al., 2015). As such, it is possible that GLP-1R activation modulates dopamine production and release in mesolimbic reward areas, thereby blunting the rewarding effects of the drug. This conclusion is consistent with the hypothesis that GLP-1RAs may have a general inhibitory effect on motivation, per se. While this may be so, and GLP-1RAs do reduce a great deal of motivated behavior and NAc dopamine as discussed, (Egecioglu et al., 2013a, 2013b; Sorensen et al., 2015; Reddy et al., 2016; Graham et al., 2013) it is interesting to note that the GLP-1RA in the present report reduced responding more in the high heroin takers than in the low heroin takers – a dissociation that is not necessarily consistent with a general motivation deficit. Further, humans treated with GLP-1RAs for T2DM report a decrease, rather than an increase, in depression (Wium-Andersen et al., 2022). Finally, GLP-1 also is implicated in stress. It is possible that GLP-1RAs are able to modulate the stress system and reduce withdrawal symptoms associated with drug-seeking (Schmidt et al., 2016). For example, there is evidence that stimulation of GLP-1Rs increases stress hormones when in a fed state (Kinzig et al., 2003). Thus, when one is sated, GLP-1R activation increases stress to prevent the organism from taking risks to search for food. When an organism is in a fasted state, decreased activation of GLP-1 receptors attenuates the stress response (Maniscalco et al., 2015). Accordingly, when starved, there is decreased GLP-1R activation and a decrease in perceived stress, allowing for engagement in higher-risk behaviors in an effort to successfully satisfy one's needs.

4.2. Individual differences

There is evidence that only 20% of people that take heroin develop an OUD (National Institute on Drug Abuse, 2014). This demonstrates that some people are more susceptible than others to develop OUD or other SUDs. There also is evidence that OUD has a hereditary component, (Tsuang et al., 1998; Wilens et al., 2002) which further demonstrates that genes are important for developing OUD. Through our previous research, and as shown in Figs. 2 and 5, we have discovered that about half of the rats become high drug takers and the other half become low drug takers (Colechio et al., 2014; Twining et al., 2009; Kuntz et al., 2008; Grigson and Twining, 2002). In this paradigm, we do find a higher percentage (50%) of rats that we categorize as high drug takers compared to humans who take heroin and develop OUD (20%). This simply may be due to the use of the median split where, by definition, 50% of the subjects are denoted as high drug takers. A second consideration is that our rats, unlike many humans, live in an unenriched environment. In our hands, rats with environmental enrichment self-administer less cocaine than their non-enriched counterparts, (Puhl et al., 2012) work less for heroin on a progressive ratio schedule of reinforcement, exhibit less cue-induced seeking for heroin, and demonstrate less drug-induced reinstatement of heroin seeking behavior (Imperio et al., 2018). Thus, while biology is important to consider for risks of developing OUD, social factors also play a prominent role (Webster, Nov, 2017). That being said, and regardless of the percentages, treatment with the GLP-1RA throughout abstinence and prior to test clearly reduced heroin seeking, and this effect was most robust in the most vulnerable high drug-taking/seeking rats.

4.3. Glucose and insulin (and GLP-1RA side effects)

Glucose and insulin levels were examined across a range of doses of liraglutide. While GLP-1RAs are good for treating diabetes and obesity due to the fact that they help increase insulin levels and decrease glucose levels, there is a risk of hypoglycemia in those receiving GLP-1RA treatment for OUD (Edwards et al., 2001). The results shown in Fig. 7, however, demonstrate that liraglutide treatment at 0.06, 0.1, 0.3, 0.6, and 1.0 mg/kg did not lead to any significant changes in glucose and insulin levels. While there was a numerical reduction in plasma glucose at the critical 0.3 mg/kg dose of liraglutide, this trend did not approach statistical significance, it did not lead to a plasma glucose level below 170 mg/dL, which remains high, and the results of a power analysis indicated that 10 – 12 subjects would be needed to attain statistical significance at the 6 h time point and, even then, would result in only a 6% reduction in plasma glucose relative to time zero (i.e., BL). It is well established that there is a circadian rhythm of insulin secretion and blood glucose concentrations in rats (Kalsbeek and Strubbe, 1998), with these hormones peaking at night during the active phase and during feeding behavior (Qian et al., 2013). Despite this, recent studies using continuous blood glucose monitoring show very small differences in mean night versus day blood glucose concentrations (<10 mg/dL) in healthy rats in the absence of obesity or diabetes (King et al., 2017). Further, for our experiments, rats were fasted throughout blood sample collection, which would have minimized circadian fluctuation of glucose and insulin levels (Bizot-Espiard et al., 1998). Taken together, the data suggest that, while a saline control should be included in the future, treatment with liraglutide likely did not block the naturally occurring circadian rhythm of these hormones and it did not result in hypoglycemia or abnormal insulin levels when assessed using a within subjects design. Finally, baseline glucose levels (time point zero) in these rats were high. This likely reflects the fact that glucose was measured from arterial plasma. It is known that whole blood arterial glucose is ~5% higher than whole blood venous glucose, and glucose measured from plasma can be 10–20% higher than when measured in whole blood (Kim, 2016). This may explain why resting glucose values were ~180 mg/dL in these rats, which would correspond to 140–150 mg/dL in whole blood. Thus, in addition to our previous research showing that various doses of liraglutide did not lead to nausea, (Douton et al., 2022b) we conclude at this juncture that liraglutide, at doses effective in treating OUD in the rodent model, does not precipitate the adverse side effects of either nausea or hypoglycemia in the rat. This finding expands the null effect on plasma glucose as previously reported (Douton et al., 2022b). Together, these findings suggest that the GLP-1RA may be not only effective, but also safe for the treatment of OUD in humans.

4.4. Limitations/promise

Whereas this paper further demonstrates the promise of GLP-1RAs for the treatment of OUD, and provides important information on its use, there are limitations. This study was conducted in male rats only. Future studies will need to repeat these experiments in female rats. Additionally, differences have been found between rats and mice, where a study of GLP-1RA treatment for OUD in mice did not demonstrate reduction of opioid seeking behaviors as has been found in rat studies (Bornebusch et al., 2019). Future studies will need to more thoroughly test the effectiveness of GLP-1RA treatment in mice. That being said, and despite these limitations, the present data combine with a growing number of published reports to suggest that treatment with a GLP-1RA can reduce cue-induced heroin seeking and drug-induced reinstatement of heroin seeking in rats. Clinical trials in humans with an opioid use disorder are underway (Bunce, 2021)

CRediT authorship contribution statement

Evans, B: Data collection, data analysis, and manuscript preparation and revision; Stoltzfus, B.: Data collection; Acharya, N.: Surgery, data collection, manuscript revision; Nyland, J.E.: Study design and manuscript revision; Arnold, A.C.: Analysis of plasma and manuscript revision, Freet, C.S.: Manuscript revision, Bunce, S.: Manuscript revision, Grigson, P.S.: Design of the study, data analysis, manuscript preparation and revision.

Conflict of Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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