Differential effects of intra-VTA ghrelin and glucagon-like peptide-1 on the stimulatory action of amphetamine and cocaine-induced alcohol intake

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Data available upon request from the authors

Short title: GLP-1, Ghrelin, and Alcohol

Abstract

In order to further elucidate the role of mesolimbic peptides in the expression of alcohol reward, the present study investigated the effects of ghrelin and glucagon-like peptide-1 (GLP-1) on alcohol intake, in addition to alcohol intake stimulated by systemic d-amphetamine or cocaine treatment. All rats were initially habituated to a 6% alcohol solution. We then demonstrated that intraperitoneal injections of d-amphetamine and cocaine increased alcohol compared to the vehicle condition. In subsequent testing we examined the effects of ventral tegmental area (VTA) ghrelin or vehicle paired with a fixed dose of d-amphetamine or vehicle. In separate rats we then investigated the impact of the GLP-1 agonist exendin-4 (Ex-4), injected into the VTA, on alcohol intake alone, or when Ex-4 was co-administered with d-amphetamine or cocaine. Our results indicated that VTA ghrelin significantly increased alcohol intake, and most importantly, potentiated the effect of d-amphetamine and cocaine on alcohol consumption. Conversely, VTA Ex-4 inhibited alcohol intake and antagonized the stimulatory effect of d-amphetamine and cocaine on alcohol consumption. In a final study we further demonstrated that VTA Ex-4 treatment significantly inhibited the combined stimulatory effects of ghrelin paired with d-amphetamine or ghrelin paired with cocaine. Overall our findings are consistent with a critical role for both ghrelin and GLP-1 receptor mechanisms in mesolimbic alcohol reward circuitry. Moreover, our results further suggest that ghrelin and GLP-1 modulate the stimulatory effect of psychostimulants on alcohol intake.

Key words: Cocaine, D-Amphetamine, Ethanol Intake, Ghrelin, Glucagon-like-peptide-1, Mesolimbic, Ventral Tegmental Area, Voluntary Alcohol Intake, Reward

1. Introduction

Cocaine use disorder (CUD) remains an issue in the United States with over 1 million Americans (0.4% of the population) aged 12 and older currently suffering from CUD during 2019 (Substance Abuse and Mental Health Services Administration, 2020). Similarly, over 4.8 million Americans over the age of 12 (1.8% of the population) consumed amphetamines during 2015 (Hughes et al., 2015). The use of cocaine and amphetamines remains more prevalent in European countries, and as of 2017, it was estimated that approximately 5.6% of European adults (17.5 million people between the ages of 15 and 64) have used cocaine and approximately 3.8% of European adults (12.5 million people of the same age group) have used amphetamine at some point in their lives (European Monitoring Centre for Drugs and Drug Addiction, 2017). Since nearly 14.5 million American adults (5.3% of the population) currently suffer from alcohol use disorder (AUD), and nearly 20% of Europeans over the age of 15 report heavy episodic drinking (consuming five or more drinks on an occasion) at least once a week, there exists immense overlap for polysubstance use (Substance Abuse and Mental Health Services Administration, 2020; WHO Expert Committee on Drug Dependence & World Health Organization, 2019).

In particular, both cocaine and amphetamines are regularly consumed with alcohol (Brache et al., 2012; Lawyer et al., 2010; Willner et al., 2005) and more than 50% of cocaine users report simultaneous alcohol consumption (Leyrer-Jackson & Olive, 2020). The concurrent consumption of alcohol and cocaine produces the metabolite cocaethylene, a cardiotoxic byproduct which is more rewarding than either alcohol or cocaine alone (Blanco-Presas et al., 2018; Graziani et al., 2014; McGrath et al., 2019). To better understand how these psychostimulants interact with alcohol, recent rodent models have been utilized to elucidate the underlying neurobiological mechanisms (Cepko et al., 2014; Stennett & Knackstedt, 2020). It remains to be explored, however, how cocaine or amphetamine interact with alcohol in the

mesolimbic reward circuit and how gut-derived peptides, such as ghrelin and glucagon-like peptide-1 (GLP-1), may mediate concurrent alcohol and psychostimulant use.

The mesolimbic reward pathway, which is comprised of dopaminergic neurons in the ventral tegmental area (VTA) that innervate the nucleus accumbens (NAc), modulates the rewarding effects of drugs of abuse, including alcohol and psychostimulants (Cooper et al., 2017; Koob & Weiss, 1992; Koob & Volkow, 2016). For instance, ethanol acts directly on dopaminergic neurons within the VTA by increasing neuronal firing rate, sensitivity, and somatodendritic and downstream dopamine (DA) release in the NAc (Boileau et al., 2003; Brodie et al., 1990; Campbell et al., 1996: Ding et al., 2009; Foddai et al., 2004; Gessa et al., 1985; Juarez et al., 2017; Liu et al., 2020; Yim & Gonzales, 2000). Furthermore, DA D1 receptor antagonism as well as DA D1 and D2 receptor agonism decreases alcohol intake and self-administration in rodents, further emphasizing the importance of mesolimbic DA signaling in the maintenance of alcohol reward (Ng & George, 1994; Price & Middaugh, 2004; Rodd et al., 2004; Sneddon et al., 2021).

Cocaine affects the central nervous system (CNS) by inhibiting the dopamine transporter (DAT), which in turn dysregulates mesolimbic dopamine signaling, specifically the VTA to NAc projections, in acute and chronic cocaine use (Addy et al., 2010; Kalivas & Duffy, 1990; Ritz et al., 1987; Siciliano et al., 2015). Despite slight differences in mechanisms of action between d-amphetamine and cocaine, d-amphetamine also elevates extracellular DA in the NAc, and this effect is inhibited by central injection of 6-hydroxydopamine (6-OHDA) (Albarrán-Bravo et al., 2019; Koski et al., 2020; Lyness et al., 1979; Ranaldi et al., 1999).

Taken together, these previous findings suggest that the rewarding effects of alcohol, cocaine, and d-amphetamine are mediated by mesolimbic DA signaling. Previous research has demonstrated that ghrelin and GLP-1, both gut-derived peptides, are critically involved in mediating the rewarding effects of alcohol and psychostimulants, such that ghrelin potentiates,

and GLP-1 attenuates, the rewarding effects of alcohol, cocaine, and amphetamine (Chaves Filho et al., 2020; Colvin et al., 2020; Dunn et al., 2019; Egecioglu et al., 2013; Hernandez & Schmidt, 2019; Koopmann et al., 2018; Wellman et al., 2013). While many of these studies have investigated the peripheral effects of ghrelin and GLP-1's mediation of reward, fewer studies have investigated the direct action of ghrelin and GLP-1 signaling within the mesolimbic system. Moreover, no study has yet examined the effects of ghrelin or GLP-1 agonism on alcohol intake stimulated by amphetamine or cocaine administration.

Ghrelin is a gastric derived, 28 amino-acid peptide that regulates various processes related to homeostatic and hedonic food intake within the CNS and the periphery (Ct et al., 2010; Seidel et al., 2021). Acylated ghrelin binds to the growth hormone secretagogue receptor 1a (GHS-R1a), more recently referred to as the ghrelin-1a receptor, which is localized in brain areas including hypothalamic nuclei, the VTA, hippocampus, NAc, and the PFC (Skibicka et al., 2011; Zigman et al., 2006). Activation of ghrelin-1a receptors via systemic and central injections of ghrelin elicits orexigenic effects including increased appetite, food intake, and increased carbohydrate oxidation (Ct et al., 2010; Currie et al., 2012; Massadi et al., 2015; Skibicka et al., 2011). While ghrelin signaling influences homeostatic behaviors, especially within the hypothalamus, extensive research has implicated the peptide in appetitive motivation. Studies using operant responding and conditioned place preference (CPP) for palatable food in a rodent model demonstrate that peripheral and intra-VTA ghrelin administration enhances the incentive motivation for food and the use of ghrelin-1a receptor antagonists attenuates these effects (D'Cunha et al., 2020; Egecioglu et al., 2010; Perello et al., 2010; Skibicka et al., 2012). The relationship between ghrelin activity and food reward has been elucidated by the finding that ghrelin-1a receptors and D1R are co-expressed in mesolimbic DA neurons (Jiang et al., 2006; Wellman & Abizaid, 2015). Research has shown that ghrelin-1a receptor activation in the VTA significantly increases action potentials, impacting the organization of synaptic inputs in DA

neurons as well as increasing extracellular DA concentrations in the NAc (Abizaid et al., 2006; Cone et al., 2015; Jerlhag et al., 2007; Weinberg et al., 2011). Furthermore, intra-VTA 6-OHDA administration attenuates ghrelin-induced increased rodent operant responding for palatable food (Weinberg et al., 2011). Thus, previous studies have provided substantial evidence that ghrelin activates mesolimbic DA neurons to elicit increases in hedonic feeding behaviors.

Glucagon-like-peptide-1 (GLP-1) is another gastric derived peptide that is critical in mediating food intake, albeit in a way that contrasts ghrelin signaling. GLP-1 is endogenously produced in intestinal L-cells and in hindbrain nuclei, namely in the nucleus of the solitary tract (NTS) (Górska & Arciszewski, 2020; Larsen et al., 1997; Lovshin & Drucker, 2009). The GLP-1 receptor, GLP-1R, is expressed in areas throughout the CNS, including hypothalamic regions, as well as other midbrain and forebrain structures like the VTA and NAc, where GLP-1R activation is known to suppress food intake in rats and humans (Chelikani et al., 2005; Cork et al., 2015; Farkas et al., 2021; Flint et al., 1998; Gu et al., 2013; Heppner et al., 2015; Merchenthaler et al., 1999; Tang-Christensen et al., 1996). This anorexigenic peptide has not only been implicated in homeostatic functions, as more recent work has demonstrated that peripheral and central injections of the GLP-1 analogue, exendin-4 (Ex-4) decreases motivation for intake of palatable food in operant responding and CPP paradigms (Alhadeff et al., 2012; Dickson et al., 2012; Dossat et al., 2011; Eren-Yazicioglu et al., 2021; Howell et al., 2019; Richard et al., 2015; Wang et al., 2015). Of particular importance are the findings that GLP-1 neurons in the NTS have significant projections to the VTA and NAc (Dossat et al., 2011; Richard et al., 2015) and that GLP-1R activation results in a downregulation of VTA-to-NAc DA signaling (Wang et al., 2015) as well as altered expression of DA-related genes in the VTA (Richard et al., 2015). These findings support the hypothesis that, like ghrelin, GLP-1 signaling acts directly on the mesolimbic reward circuit to mediate the rewarding effects of appetitive behaviors.

Since ghrelin and GLP-1 receptor activation modulate mesolimbic DA signaling, the effects of these peptides on drug intake and drug-motivation have been examined over the past decade. Systemic injections of ghrelin have been shown to augment the rewarding effects of cocaine in rodents via increasing cocaine-induced locomotor activity (Davis et al., 2007) and the threshold dose at which cocaine results in CPP (Wellman et al., 2008). Furthermore, prior work in our lab has extended these findings by showing that VTA-microinjections of ghrelin also enhance cocaine-induced CPP (Schuette et al., 2013). The notion that expression of reward-related behaviors is ghrelin-dependent has been further supported by studies using ghrelin-1a receptor antagonists and ghrelin-1a receptor knockout (KO) models which show that cocaine-induced and amphetamine-induced locomotor stimulation, accumbal dopamine release, and CPP are attenuated when ghrelin signaling is reduced or absent (Clifford et al., 2012; Jerlhag et al., 2010; Suchankova et al., 2016). GLP-1 signaling has also been shown to influence cocaine and amphetamine reward. For instance, peripheral Ex-4 treatment decreases cocaine-induced and amphetamine-induced locomotor stimulation, accumbal dopamine release, and CPP (Egecioglu et al., 2013; Erreger et al., 2012). In addition, systemic Ex-4 attenuates acute and chronic cocaine self-administration as well as phasic striatal DA release (Sørensen et al., 2015). Central GLP-1 receptor agonism within the VTA and lateral ventricle has also been shown to attenuate various rewarding effects of cocaine, while intra-VTA GLP-1 receptor antagonism and knock-down (KD) augments cocaine self-administration in rats. (Fortin & Roitman, 2017; Hernandez et al., 2018; Schmidt et al., 2016).

In regards to alcohol reward, intra-VTA or intra-laterodorsal tegmental area (LDTg) ghrelin administration increases voluntary alcohol consumption (Jerlhag et al., 2009) and the use of the ghrelin antagonist JMV2959 or ghrelin KO attenuates voluntary alcohol intake (Bahi et al., 2013; Gomez & Ryabinin, 2014; Jerlhag et al., 2014). Various studies have also demonstrated that peripheral treatment with GLP-1 agonists, such as Ex-4 or liraglutide, reduces behaviors related

to alcohol reward such as alcohol intake, CPP, and accumbal DA release (Egecioglu et al., 2013; Shirazi et al., 2013; Sørensen et al., 2016; Vallöf et al., 2016). Microinjections of Ex-4 into the NAc, VTA, LDTg, dorsomedial hippocampus (DMHipp), and the lateral hypothalamus (LH) attenuate alcohol intake (Colvin et al., 2020; Shirazi et al., 2013; Vallöf et al., 2019). Furthermore, intra-NAc shell JMV2959 combined with intra-NAc shell Ex-4 treatment decreased voluntary alcohol intake in male and female rats, demonstrating potential overlap between the mechanisms of action between ghrelin and GLP-1 (Abtahi et al., 2018). Given that both ghrelin and GLP-1 receptor signaling have been shown to mediate the acquisition and maintenance of alcohol and psychostimulant reward, emerging data has begun to analyze how these peptides can affect concurrent alcohol and psychostimulant use.

Previous work in our lab has revealed that systemic and intra-VTA ghrelin administration augments cocaine-induced alcohol intake in a two-bottle choice paradigm (Cepko et al., 2014). Furthermore, a recent publication has shown that co-expression of GLP-1 and the human butyrylcholinesterase (hBChE) in mice reduces toxicity and drug-taking for alcohol and cocaine co-administration (Kong et al., 2021). Given the above findings, the present study sought to investigate the effects of direct VTA injection of ghrelin and Ex-4 on alcohol intake, and most importantly, the impact of these gut-derived peptides on amphetamine and cocaine-stimulated alcohol intake. Moreover, we further examined the effect of Ex-4 and ghrelin co-administration to determine if ghrelin and GLP-1 systems functionally interact within this region of the mesolimbic reward pathway.

2. Methods

2.1. Animals

Adult Male Sprague-Dawley rats (N=64; *n*=8 per experiment) were obtained from the Reed College Animal Care Facility. The pups were progeny of male and female breeders purchased

from Envigo Laboratories (South Kent, WA). Adult rats were housed individually in polypropylene cages with ad libitum access to standard rodent chow pellets (LabDiet, St. Louis, MO) and water. The animal colony room was maintained on a 12 hour light-dark cycle (with lights off at 15:00 h) with a temperature of 22±2^{o}C. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC, A4425-01) of Reed College.

2.2. Drug and Peptides

D-Amphetamine sulfate (Sigma, St. Louis, MO), cocaine hydrochloride (Sigma), acylated rat ghrelin (Tocris, Bristol, United Kingdom) and the GLP-1 agonist exendin-4 (Sigma) were dissolved in sterile physiological saline vehicle. Central injections were delivered in a volume of 0.2 µl into the VTA using a microinjector that extended 4 mm beyond the indwelling guide cannula. Intraperitoneal (IP) d-amphetamine and cocaine injections were administered in a volume of 0.1 ml/100 g.

2.3. Stereotaxic Surgery

Permanent indwelling guide cannulae were surgically implanted into the brains of adult male rats weighing 275-295 g. Animals were anesthetized with ketamine (100 mg/kg IP, Covetrus, Portland, ME), and xylazine (5 mg/kg IP, Sigma). After showing evidence of anesthesia, rats were then placed in a Kopf stereotaxic frame with the incisor bar set at 3.5 mm below the interaural line. Stereotaxic coordinates for the VTA were determined relative to bregma. The actual coordinates were posterior 5.7 mm, lateral +/-0.5 mm, and ventral 4.1 mm (Paxinos & Watson, 2014). Unilateral guide cannulae (22-gauge; P1 Technologies, Roanoke, VA) were implanted 4 mm dorsal to the VTA and secured with acrylic cement. After the guide cannula was cemented, the rodent was placed on a heating pad until recovery from the anesthesia was evident. In order to minimize discomfort associated with the surgery, rats were treated with carprofen (5 mg/kg subcutaneously, Covetrus) at the start of the surgical procedure and again

at 24 h post-surgery. Cannula patency was maintained using a 28-gauge stainless-steel inner stylet. Rats were provided a two-week postoperative recovery period where food and water intakes and overall health were closely monitored.

2.4. Design and procedure

All rats were initially habituated to alcohol prior to any testing. During this period of alcohol exposure, we adopted a home cage two-bottle choice and limited access paradigm (Abtahi et al., 2018; Cepko et al., 2014; Egecioglu et al., 2013). On day one, a bottle containing 2% alcohol by volume was placed in the home cage along with a separate bottle containing water only. Both bottles were available to the rat for 24 h. Alcohol was presented on every alternate day (along with a water bottle) while water alone was provided on intervening days. Again, to be clear, on alcohol exposure days rats also had free access to water. The concentration of alcohol was increased by 1% for the next exposure period until intakes of 6% alcohol showed stability across 6 consecutive alcohol days. The placement of the alcohol bottles was alternated for each session in order to control for side preference. After habituation was established, rats were assigned to the various experimental paradigms outlined below.

2.4.1 Systemic D-Amphetamine and Cocaine Treatment

Two separate systemic injection studies were employed. In the first study we investigated the effect of d-amphetamine treatment on alcohol intake using a repeated measures design. Rats (*n*=8) were administered varying doses of d-amphetamine (0.5, 1.0, and 2.0 mg/kg IP) or saline vehicle at the onset of the dark cycle. After injections, animals were returned to their home cage where they had free access to a bottle containing 6% alcohol. Food and water were also freely available at this time. Alcohol intakes were measured 6 h later. Rats were tested in each condition and treatment conditions were presented in random order. Testing was separated by 4 intervening days. In a separate group of animals (*n*=8) a similar protocol was followed. However

these rats were injected with cocaine (1.25, 2.5, and 5.0 mg/kg IP) or vehicle using a repeated measures design. Again, alcohol intakes were measured 6 h post-injection.

2.4.2 Effects of VTA ghrelin and Ex-4 treatment

In an additional group of rats we examined the effects of VTA ghrelin paired with d-amphetamine on alcohol intake. Specifically, rats (*n*=8) were administered vehicle or d-amphetamine (1.0 mg/kg IP) followed by a VTA ghrelin (50 and 100 pmol) or saline injection. A repeated measures design was used with 4 days separating the various treatment conditions. This resulted in a total of 6 treatment conditions for each rat. Treatments were administered in randomized order. In a subsequent test and in separate animals d-amphetamine was replaced with a fixed dose of cocaine (2.5 mg/kg IP, *n*=8). All other experimental conditions were similar with intakes assessed 6 h postinjection.

In subsequent groups of rats we wanted to determine whether or not VTA Ex-4 injection would impact the potential stimulatory effects of d-amphetamine (*n*=8) or cocaine (*n*=8) on alcohol consumption. Ex-4 was administered at doses of (0, 0.05, and 0.5 μg) into the VTA with d-amphetamine (1 mg/kg) or cocaine (2.5 mg/kg) injected IP. Further, two final studies investigated the ability of Ex-4 (0.05 μg) to attenuate the effect of ghrelin (100 pmol) on either d-amphetamine (1 mg/kg, *n*=8) or cocaine (2.5 mg/kg, *n*=8) stimulated alcohol intake. In both experiments, rodents were injected IP with either d-amphetamine or cocaine immediately followed by an injection of Ex-4 followed by an injection of ghrelin. All three injections were administered at the onset of the dark cycle. All other experimental conditions, including alcohol exposure training, food, and water availability were as described above.

2.5. Statistical and histological analyses

Data were analyzed using one or two-way analyses of variance (ANOVA). Intakes were expressed as grams of alcohol ingested per kg body weight over 6 hours post-injection. Direct

comparisons between means were examined using post hoc Tukey tests. The criterion for statistical significance was determined as $p<0.05$. Histological analysis was performed to confirm accurate cannula placement. After euthanizing the rat, an injection of black ink was administered directly into the VTA to facilitate identification of this structure. Brains were processed as described previously (Abtahi et al., 2018; Colvin et al., 2020). Coronal sections (40 um) were cut through the VTA. Sections were examined using light microscopy and viewed relative to the stereotaxic atlas of Paxinos and Watson (2014). A schematic of coronal sections of the rat brain illustrating representative placements of injector cannulae within the VTA is shown in Figure 1.

3. Results

3.1 Systemic injection of d-amphetamine and cocaine increased alcohol consumption

As shown in Figures 2 and 3, using a one-way repeated measures ANOVA we observed that systemic injection of d-amphetamine and cocaine stimulated alcohol intake. Post hoc testing revealed that both the 1 mg/kg and 2 mg/kg doses of d-amphetamine were effective in stimulating intake, although the two doses did not differ statistically from one another ($F(3,21)=53.3$, $p<0.00001$). With respect to cocaine, both the 2.5 mg/kg and 5 mg/kg doses increased alcohol intake compared to control in a dose-dependent manner ($F(3,21)=90.3$, $p<0.00001$).

3.2 Injection of ghrelin into the VTA potentiated d-amphetamine and cocaine-induced increases in alcohol consumption

In Figure 4, d-amphetamine (1 mg/kg IP) injection co-administered with 100 pmol of VTA ghrelin potentiated alcohol intake compared to either the vehicle-vehicle condition or the ghrelin paired with vehicle condition. While we did not observe a stimulatory effect at the 50 pmol ghrelin dose when paired with vehicle, this dose of ghrelin did potentiate the effect of d-amphetamine on

alcohol intake when the two were co-administered. Further, 100 pmol of ghrelin did reliably increase intake, and again, this effect was potentiated when ghrelin was co-administered with d-amphetamine. These findings were confirmed by a significant two-way repeated measures ANOVA ($F(2,14)=7.93$, $p<0.005$) and post hoc analysis. In subsequent testing with cocaine, and as shown in Figure 4, we found that 2.5 mg/kg IP of cocaine stimulated alcohol intake and that this effect was potentiated by both the 50 pmol and 100 pmol doses of ghrelin administered into the VTA ($F(2,14)=5.0$, $p<0.02$). Again, it is interesting to note that while the 50 pmol dose of ghrelin appeared to be subthreshold in that it alone did not increase alcohol intake, when paired with cocaine, we observed a significant increase in alcohol consumption. With respect to the 100 pmol ghrelin dose, VTA injection did increase alcohol intake and effectively potentiated the effect of cocaine on alcohol consumption. In short, therefore, the increase in alcohol intake was greater in magnitude when ghrelin and cocaine were co-administered compared to when ghrelin or cocaine were paired with vehicle.

3.3 Injection of Ex-4 into the VTA attenuated d-amphetamine and cocaine-induced increases in alcohol consumption

In Figure 5 the effects of Ex-4 on d-amphetamine or cocaine-stimulated alcohol intake are reported. Two-way repeated measures ANOVA indicated that while Ex-4 alone reliably decreased alcohol consumption, it also effectively blunted intakes in rats treated with either d-amphetamine ($F2,14)=96.4$, $p<0.00001$ or cocaine ($F(2,14)=192.6$, $p<0.00001$). In fact, the higher dose of Ex-4 (0.5 µg) completely reversed the stimulatory effects of either d-amphetamine or cocaine on alcohol consumption.

3.4 Intra-VTA Ex-4 suppressed the effects of ghrelin on d-amphetamine and cocaine-induced increases in alcohol consumption

In two final studies (see Figure 6), we wanted to determine if Ex-4 (0.05 μg) would attenuate the stimulatory effect of VTA-ghrelin (100 pmol) on d-amphetamine (1 mg/kg IP) and cocaine (2.5 mg/kg IP) induced alcohol consumption. One-way repeated measures ANOVA indeed confirmed that combined injections of ghrelin paired with d-amphetamine ($F(2,14)=43.4$, $p<0.00001$) or ghrelin co-administered with cocaine ($F(2,14)=80.4$, $p<0.00001$) enhanced intakes compared to vehicle and these effects were in fact entirely blocked by Ex-4. These rodents were still consuming alcohol but at levels now comparable to control.

4. Discussion

The present study investigated the role of mesolimbic ghrelin and GLP-1 in the expression of voluntary alcohol intake. Our findings demonstrated that direct ghrelin injection into the VTA stimulated alcohol intake while Ex-4 inhibited alcohol consumption. We additionally demonstrated that low doses of d-amphetamine and cocaine also stimulated alcohol intake. Moreover, the stimulatory effects of d-amphetamine and cocaine on alcohol intake were potentiated by VTA ghrelin co-administration but inhibited by VTA Ex-4 microinjection. We provided further evidence for a possible interaction between mesolimbic dopamine, ghrelin, and GLP-1, by showing that VTA Ex-4 administration significantly suppressed the combined stimulatory effect of ghrelin paired with d-amphetamine or ghrelin paired with cocaine. Specifically, when rats were co-administered ghrelin with d-amphetamine or ghrelin with cocaine, they exhibited a reliable increase in alcohol intake compared to control. However, when these same rats were microinjected with Ex-4, this GLP-1 analogue effectively suppressed intakes to those comparable to consumption levels in the control condition. Overall, the present findings suggest that VTA ghrelin and GLP-1 play critical roles in mediating alcohol reward. Our findings further demonstrate that the stimulatory effects of psychostimulants, via mesolimbic dopamine signaling, on alcohol intake are additionally modulated by VTA ghrelin and GLP-1 neuronal function.

Despite the reported voluntary concurrent use of psychostimulants and alcohol in humans, studies examining the effects of co-administration of these drugs in rodent models have often presented mixed findings (Brache et al., 2012; Lawyer et al., 2010; Willner et al., 2005). Cocaine and alcohol concurrent consumption produces the metabolite cocaethylene which is more rewarding than either substance individually (James et al., 2021; Raven et al., 2000). The rewarding effects of cocaethylene, like cocaine, are mediated by mesolimbic dopaminergic neurons and cocaethylene increases postsynaptic neuronal activity via blocking the reuptake of dopamine (Wolf, 2016). Since the plasma elimination half-life of cocaethylene is longer than cocaine, the associated pleasurable effects are accentuated (Jones, 2019; McCance-Katz et al., 1998).

In rat models, acute administration of cocaine has been shown to potentiate alcohol intake (McMillan et al., 1991) and preference over water (Knackstedt et al., 2006). Rodents have been shown to possess species-specific differences in cocaine reward salience as Katner et al. (2011) demonstrated that alcohol preferring rats self-infused cocaine starting at lower doses than Wistar rats and infused more often, suggesting an increased sensitivity to the rewarding properties of cocaine. Additionally, cocaine has been shown to increase alcohol seeking and relapse behavior in alcohol-preferring rats (Hauser et al., 2014). In contrast to the reported findings that cocaine potentiates alcohol consumption, several studies have observed decreases in alcohol consumption following administration of cocaine; however, these studies all used significantly higher doses of cocaine. For example, Hammad et al. (2017), demonstrated that repeated cocaine exposure at a high dose (20 mg/kg IP) decreased alcohol intake across a period of several days. This suggests that while chronic, high dose cocaine exposure may decrease alcohol's rewarding properties, low dose, acute cocaine exposure may potentiate alcohol reward. Another plausible explanation may be that high doses of psychostimulants induce behavioral disruption and stereotypic behaviors in rodents, which

would inhibit consumption and activity globally (Bhattacharyya & Pradhan, 1979; Blanchard et al., 1998; Schlussman et al., 2005; Tang et al., 2008; Vanderschuren et al., 1999).

Consistent with previously reported dose-dependent differential effects of psychostimulants on alcohol intake, Busse et al. (2004) demonstrated a bidirectional effect of alcohol pretreatment on cocaine-induced CPP, such that at high doses of cocaine (30 and 40 mg/kg IP), alcohol attenuated cocaine-induced CPP, while at low doses of cocaine (5 mg/kg), alcohol potentiated cocaine-induced CPP. Furthermore, pre-treatment with high, but not low doses of alcohol has been shown to inhibit cocaine-induced CPP and reconsolidation of cocaine reward (Zhu et al., 2020). Previous work in our lab has demonstrated an increase in alcohol consumption following administration of low doses of cocaine (Cepko et al., 2014). The present findings add to the growing body of research on this subject by illustrating that low doses of cocaine increase alcohol consumption in a two-bottle choice paradigm and ghrelin potentiates, while EX-4 attenuates, this effect.

The results of the present study are also consistent with previous findings that d-amphetamine increases alcohol intake, although larger doses of d-amphetamine were employed in these studies (Potthof et al., 1983, Ruiz et al., 2018). Unlike cocaine, however, the modulatory effect of d-amphetamine on alcohol consumption has been less comprehensively explored. Despite the lack of extensive research, alcohol has been shown to increase the absorption and distribution of amphetamine in the brain of rats (Liang et al., 2012) as well as decrease amphetamine metabolism in humans, resulting in higher blood concentrations (Shimosato, 1988). Interestingly, Pohorecky and Sweeny (2012) found that in psychosocially stressed male rats, alcohol consumption decreased following administration of amphetamines. This result, as speculated above, may in fact be due to psychostimulant-induced stereotypic behaviors. Although there are discrepancies within the data overall, previous studies provide compelling

evidence that cocaine and amphetamine administration alter alcohol reward, consumption, and relapse behaviors.

Extensive research has confirmed ghrelin's critical involvement in the regulation of dopaminergic reward processing in the VTA. Jerlhag and colleagues found that systemic ghrelin antagonism with JMV2959 reduced cocaine and amphetamine-induced locomotor stimulation, CPP, and accumbal dopamine release (Jerlhag et al., 2010). They also showed in a prior study that ghrelin administered centrally into the VTA increased locomotion and accumbal dopamine release measured by *in vivo* microdialysis (Jerlhag et al., 2007). Our present findings, which demonstrate that VTA ghrelin injections increase amphetamine and cocaine-induced alcohol consumption, suggest that ghrelinergic neurons within the VTA mediate the rewarding effects of cocaine, amphetamine, and alcohol. Indeed, previous research has begun to elucidate the mechanism by which ghrelin and ghrelin-1a receptor signaling regulates dopamine activity. For example, prior research has demonstrated D1/D2-ghrelin-1a receptor dimerization in mesolimbic regions (Damian et al., 2018; Jiang et al., 2006; Kern et al., 2012) and that ghrelin administration (IP or centrally to tegmental areas) increases dopamine activity and turnover in the NAc (Abizaid et al., 2006; Jerlhag, 2008; Jerlhag et al., 2006, 2007; Quarta et al., 2009). Further, Damian and colleagues found that ghrelin-1a receptors form tetramers with D2 receptors which more effectively recruit G proteins and increase the rate of GTP binding through changes in the α-subunit (Damian et al., 2018). Taken together, this emerging research strongly suggests ghrelin’s key involvement in mesolimbic reward processing.

Similar to ghrelin, emerging research suggests that GLP-1 is critically involved in the regulation of mesolimbic reward and motivated behavior, specifically in the context of drugs of abuse. Past work has demonstrated that injections of Ex-4 into the VTA decreases motivation for self-administration of cocaine (Hernandez et al., 2018; Schmidt et al., 2016) and operant responding for food (Howell et al., 2019). Peripheral injections of Ex-4 has been shown to reduce d-

amphetamine and cocaine-induced locomotor stimulation (Egecioglu et al., 2013), and striatal dopamine levels (Sørensen et al., 2016). Recent studies have also shown that infusion of Ex-4 directly into the VTA decreases voluntary alcohol consumption in two-bottle choice and intermittent access protocols (Colvin et al., 2020; Dixon et al., 2020). Vallöf et al. (2020) demonstrated that long term systemic injection of dulaglutide, a GLP-1 agonist, decreased alcohol intake in rats, providing further evidence for GLP-1's putative role in reward-related behaviors. In addition, acute injection of Ex-4 into the NAc shell, a region which receives dopaminergic projections from the VTA, attenuates alcohol mediated behaviors (Breton et al., 2019; Vallöf et al., 2019). Thus, in the context of previous research, the present findings demonstrate for the first time that Ex-4 reduces d-amphetamine and cocaine-stimulated alcohol consumption.

The mechanisms by which GLP-1 receptor activity within the mesolimbic reward pathway modulate food and drug reward have recently begun to be elucidated. GLP-1 receptors are densely expressed in the NTS, a brain area with significant projections to the VTA and NAc (Dossat et al., 2011; Richard et al., 2015). Prior studies in rodents have found that intra-NTS injection of Ex-4 decreases intake of palatable food, attenuates excitatory synaptic inputs from VTA DA neurons projecting to the NAc, and upregulates tyrosine hydroxylase and DR2 gene expression within the VTA (Richard et al., 2015; Wang et al., 2015). Furthermore, Konanur and colleagues (2020) recently demonstrated that treatment with Ex-4 into the lateral ventricle reduced sucrose directed behavior and inhibited cue-evoked phasic dopamine activity within the VTA. Taking the prior research into account, we propose that our current finding that Ex-4 administration into the VTA attenuates cocaine and d-amphetamine-induced alcohol intake likely occurrs through suppressions in VTA DA activity and excitatory synaptic inputs.

Our present finding that Ex-4, when microinjected into the VTA, suppresses ghrelin-potentiated, d-amphetamine and cocaine-induced increases in alcohol intake align with previous studies

detailing the inhibitory effect of GLP-1 receptor activation on ghrelin activity. For example, peripheral and lateral ventricle injection of Ex-4 has been shown to significantly reduce circulating levels of ghrelin (Pérez-Tilve et al., 2007). Work from our lab has revealed that when administered peripherally, into the arcuate nucleus of the hypothalamus, or the paraventricular nucleus of the hypothalamus, Ex-4 antagonizes ghrelin-dependent increases in respiratory exchange ratio (Abtahi et al., 2016, 2019). Additionally, we have shown that peripheral and intra-VTA Ex-4 treatment attenuates intra-VTA ghrelin-induced increases in operant responding for palatable food, providing further evidence that centrally and peripherally stimulated GLP-1 receptor activity impacts mesolimbic ghrelinergic signaling (Howell et al., 2019). Given the fact that ghrelin-1a receptors form tetramers with D2 receptors and Ex-4 treatment has demonstrated inhibitory action on VTA-to-NAc DA excitation, it is likely that Ex-4 interferes with ghrelin-1a receptor activity through interactions of VTA DA neurons (Damian et al., 2018; Kern et al., 2012; Richard et al., 2015; Wang et al., 2015). The presenting findings, as well as previous findings from our lab, suggest that GLP-1 receptors may be located on dopaminergic neurons in the VTA, where dopamine activity could be regulated through opposing action of the ghrelin-1a-D2R tetromer complexes. Further research is needed to fully understand the interactions between ghrelin and GLP-1 signaling during the acquisition and maintenance of drug reward.

Author Contributions: AIF, KJC, PJC, MBD, LE, MCH, JGH, MJK, HSK carried out data acquisition, data analysis and visualization, as well as writing and editing of the manuscript.

Conflicts of Interest: The authors declare no conflict of interests.

Funding: This research was supported by the Stillman-Drake Fund of Reed College to PJC and the Reed College Science Research Fellowship to MCH, MJK and JGH as well as the Reed College Neuroscience Fellowship to HSK.

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Figure Captions

Figure 1. Schematic coronal sections representative of microinjector placements at the level of the VTA.

Figure 2. Systemic d-amphetamine administration increased alcohol intake compared to vehicle control. Values represent mean alcohol intake ± SEM. Injections were administered at the start of the dark cycle and intakes were measured 6 h postinjection (n=8). *P<0.05 compared to control.

Figure 3. Intraperitoneal injection of cocaine stimulated alcohol intake compared to saline vehicle control. Values represent mean alcohol intake ± SEM with intakes assessed at 6 h postijnection (*n*=8). *P<0.05 compared to control.

Figure 4. Direct ghrelin injection into the VTA stimulated alcohol consumption and potentiated the effect of both d-amphetamine (n=8) and cocaine (n=8) on alcohol intake. Values represent mean alcohol intake ± SEM with intakes assessed at 6 h postinjection.. *P<0.05 compared to control. **P<0.05 compared to d-amphetamine or cocaine paired with vehicle. ***P<0.05 compared to ghrelin paired with vehicle.

Figure 5. VTA administration of Ex-4 decreased alcohol consumption and inhibited the stimulatory effect of d-amphetamine (n=8) and cocaine (=8) on alcohol intake. Values represent mean alcohol intake ± SEM. Intakes were determined at 6 h postinjection. *P<0.05 compared to control. **P<0.05 compared to d-amphetamine or cocaine paired with vehicle.

Figure 6. Combined injections of d-amphetamine (1 mg/kg IP) with VTA ghrelin (100 pmol, n=8) or administration of cocaine (2.5 mg/kg IP) paired with VTA ghrelin (100 pmol, n=8) elicited an increase in alcohol intake compared to control. This effect was completely reversed by pretreatment with VTA Ex-4 (0.05 μg). Values represent mean alcohol intake ± SEM. Measurements were taken at 6 h postinjection. *P<0.05 compared to control.