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Exendin-4 antagonizes the metabolic action of acylated ghrelinergic signaling in the hypothalamic paraventricular nucleus

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ABSTRACT

In the current study we investigated the interaction of hypothalamic paraventricular nucleus (PVN) glucagon-like peptide-1 (GLP-1) and ghrelin signaling in the control of metabolic function. We first demonstrated that acylated ghrelin injected directly into the PVN reliably altered the respiratory exchange ratio (RER) of adult male Sprague Dawley rats. All testing was carried out during the initial 2 h of the nocturnal cycle using an indirect open circuit calorimeter. Results indicated that acylated ghrelin induced a robust increase in RER representing a shift toward enhanced carbohydrate oxidation and reduced lipid utilization. In contrast, treatment with comparable dosing of des-acyl ghrelin failed to significantly impact metabolic activity. In separate groups of rats we subsequently investigated the ability of exendin-4 (Ex-4), a GLP-1 analogue, to alter acylated ghrelin's metabolic effects. Rodents were treated with either systemic or direct PVN Ex-4 followed by acyl ghrelin microinjection. While our results showed that both systemic and PVN administration of Ex-4 significantly reduced RER, importantly, Ex-4 pretreatment itself reliably inhibited the impact of ghrelin on RER. Overall, these findings provide increasingly compelling evidence that GLP-1 and ghrelin signaling interact in the neural control of metabolic function within the PVN.

1. Introduction

Ghrelin, encoded by the *GHRL* gene, is an evolutionarily conserved neuroendocrine modulator critical for homeostatic energy balance and metabolism in a wide variety of species and first characterized in the rat (*Rattus norvegicus*) stomach by Kojima et al. (1999) (Abtahi et al., 2016, 2017; Blanco et al., 2017; Boswell and Dunn, 2017; Geelissen et al., 2006; Nakazato et al., 2001; Velasco et al., 2015). The acylated peptide functions as the endogenous ligand for the peripherally and centrally expressed growth hormone secretagogue 1a receptor (GHSR-1a) (Hosoda et al., 2003; Kojima et al., 1999; Zigman et al., 2006). A particularly unique feature of ghrelin is the requirement of octanoylation at the serine-3 residue by the enzyme ghrelin-o-acyl-transferase (GOAT) in order to render it acylated and therefore active at the GHSR-1a (Gutierrez et al., 2008; Mohan and Unniappan, 2013; Romero et al., 2010 Yang et al., 2008). A number of studies have demonstrated that acylated ghrelin not only stimulates food intake but also increases the respiratory exchange ratio (RER) in mammals and teleosts (Abtahi et al., 2016, 2017; Blanco et al., 2017; Currie et al., 2005; Currie et al., 2012; Fernandez et al., 2016; Thomas et al., 2016). RER is an index of the nutrient source that animals metabolize to generate energy and is measured using indirect calorimetry as the volume of carbon dioxide produced divided by the volume of oxygen consumed (VCO_2/VO_2). RER values typically range from 0.7 to 1.0 with the lower bound indicating lipid oxidation and the higher bound representing carbohydrate utilization. Values in the 0.8 range indicate a combined reliance on carbohydrate and fat utilization (Bray, 1989; Coscina et al., 1998; Kleiber, 1987).

Although ghrelin is orexigenic in mammalian species, in avians, the peptide is found to be anorexigenic and is observed to decrease RER (Geelissen et al., 2006; Oclon and Pietras, 2011). However, one report did demonstrate that low doses of ghrelin stimulated food intake in the Japanese Quail (*Coturnix japonica*) (Shousha et al., 2005). Indeed, the majority of circulating ghrelin consists largely of des-acyl ghrelin, with less than 10% of the acylated molecule available to bind to the GHSR-1a (Delporte, 2013). While the function of des-acyl ghrelin remains unclear, recent evidence suggests that it may play a role in thermoregulation and stress activation in mammals (Inoue et al., 2016; Mahbod et al., 2018). Unlike the acylated peptide, des-acyl ghrelin does not bind to the GHSR-1a (Chen et al., 2017; Delporte, 2013). In both

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goldfish (*Carassius auratus*) and rodents, high doses of des-acyl ghrelin may antagonize the effect of acylated ghrelin on food intake by preventing the binding of acyl ghrelin to the 1a receptor (Matsuda et al., 2006). Therefore, although des-acyl ghrelin may not bind directly to the GHSR-1a, it does appear to play a role in regulating acyl ghrelin signaling in these species. Further, it remains possible that des-acyl ghrelin could bind and elicit its actions through a separate, yet unidentified, receptor protein (Chen et al., 2017).

As indicated above, the orexigenic and metabolic activity of acylated ghrelin are purported to be exerted centrally via GHSR-1a, which has wide expression throughout the hypothalamus in teleosts, mammals, and avians (Chen et al., 2007; Sanchez-Bretano et al., 2015; Zigman et al., 2006). For example, in teleosts and mammals, ghrelin stimulates food intake via neuropeptide Y (NPY)/agouti related peptide (AgRP) immunoreactive neurons in the arcuate nucleus (ArcN) (Chen et al., 2017; Delgado et al., 2017; Guan et al., 2008; Kohno et al., 2003; van den Top et al., 2007). In avians however, ghrelin's feeding effects appear to be largely mediated through corticotropin releasing hormone (CRH), given that intracerebroventricular (ICV) ghrelin elevates plasma corticosterone, whereas the CRH antagonist astressin reduces ghrelin's anorexigenic effect (Saito et al., 2005). Despite the greater ratio of circulating des-acyl to acyl ghrelin, robust evidence demonstrates that the acylated molecule is responsible for the ghrelinergic control of appetite, energy balance, and metabolic function (Abtahi et al., 2016, 2017; Currie et al., 2005, 2011, 2012; Naleid et al., 2005; Nakazato et al., 2001; Tschöp et al., 2000). Other studies report that ghrelin injection into the ventral tegmental area (VTA) and nucleus accumbens (NAcc) of the mesolimbic reward pathway increases consumption of standard chow and palatable food, indicating that ghrelin influences both homeostatic and hedonic appetitive motivation (King et al., 2011; Naleid et al., 2005; Weinberg et al., 2011). Moreover, ghrelin administration into the CA1 of the hippocampus enhances social transmission of food preference, which is impaired by local viral knockdown of GHSR-1a (Hsu et al., 2018). These findings suggest a role for ghrelin in mediating food choice.

In addition to ghrelin, another critical homeostatic peptide involved in food intake and energy metabolism is glucagon-like peptide 1 (GLP-1), a peptide derived from the tissue specific post-translational processing of the proglucagon peptide (Alhadeff et al., 2012; Blanco et al., 2017; Hayes, 2012; Honda, 2016; Kanoski et al., 2011; Reiner et al., 2018). Encoded by the *GCG* gene, the predominant active form of GLP-1 in mammals upon cleaving is GLP-1(7–36) amide (Hayes et al., 2010). GLP-1 binds to the peripherally and centrally expressed GLP-1 receptor (GLP-1R) and is consistently anorexigenic across species (Kanoski et al., 2011; Silverstein et al., 2001; Tachibana et al., 2004). ICV treatment of exendin-4 (Ex-4), a GLP-1 analogue, enhances c-fos expression in ghrelin immunoreactive neurons in the arcuate and paraventricular (PVN) nuclei of the hypothalamus in mice (Dalvi et al., 2012). Systemic and ICV Ex-4 administration attenuates ghrelin plasma levels (Perez-Tilve et al., 2007). In gastrointestinal L-cells, ghrelin *in vitro* stimulates GLP-1 release (Gagnon et al., 2015), and circulating GLP-1 and proglucagon levels are higher in GHSR-1a null mice (Xu et al., 2015). Other work has shown that ghrelin decreases proglucagon mRNA in goldfish and that peripheral GLP-1 antagonizes the appetitive action of ghrelin (Blanco et al., 2017). Similarly, ghrelin's metabolic effects in rats are also suppressed by GLP-1 receptor antagonism (Abtahi et al., 2016).

GLP-1 has been well characterized with respect to its anorexigenic role in the nucleus of the solitary tract (NTS) in the hindbrain of mammals, however, emerging evidence now suggests that the peptide is integral to hypothalamic appetitive and metabolic circuitry (Alhadeff et al., 2012; Chen et al., 2017; Guan et al., 2008; Kohno et al., 2003; van den Top et al., 2007). For example, we have recently demonstrated that Ex-4 antagonizes the stimulatory effect of ArcN ghrelin on RER (Abtahi et al., 2016). In this same report, ArcN Ex-4 reliably suppressed the increase in RER found after co-infusion of ghrelin and NPY (Abtahi et al., 2016). As a result, and in order to further investigate the metabolic function of hypothalamic GLP-1, ghrelin, and NPY, in the present study we targeted the PVN, given the well-established role of this nucleus in regulating energy metabolism and substrate oxidation (Currie et al., 2010; Gao et al., 2017; Thomas et al., 2016; Vasselli et al., 2017). Specifically, the overall objective of the current report was to initially compare the impact of acyl and des-acyl ghrelin on energy substrate utilization. We then determined whether or not direct PVN treatment with the GLP-1 agonist Ex-4 would suppress the effect of PVN ghrelin or the combined effect of ghrelin and NPY on RER.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (N = 38; Envigo Laboratories, Madison, WI, USA) were housed individually in polypropylene cages equipped with an automated watering system. Rats weighing between 275 and 300 g were 10 weeks of age at the time of surgery. All animals had free access to standard rodent chow (LabDiet, St, Louis, MO, USA) and water. The colony facility was maintained on a 12 h light/dark cycle (lights off at 1500 h) and at a temperature of 22 ± 2 °C. The Institutional Animal Care and Use Committee of Reed College approved all experimental protocols (#A4425-01).

2.2. Stereotaxic surgery

Unilateral guide cannulae were implanted into the PVN. Stereotaxic coordinates relative to bregma were 1.8 mm posterior, ± 0.3 mm lateral, and 3.9 mm ventral (Paxinos and Watson, 2014). Rats were anesthetized with ketamine (100 mg/kg IP, Henry Schein, Melville, NY, USA) co-administered with xylazine (5 mg/kg IP, Sigma, St. Louis, MO, USA) and placed in a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA, USA), with the incisor bar set at −3.5 mm. Guide cannulae (22-gauge; Plastics One, Roanoke, VA, USA) were implanted 4 mm dorsal to target in order to minimize damage to PVN tissue. Each implant was secured with acrylic cement and a 28-gauge stainless-steel inner stylet was used to maintain cannula patency. RER testing was initiated after a two-week postoperative recovery period. During this time animals were handled daily and habituated with mock injections.

2.3. Measurement of RER

Oxygen (O_2) consumption and carbon dioxide (CO_2) production were measured using an open-circuit indirect calorimeter (Columbus Instruments, Columbus, OH, USA). Gas concentrations were sequentially derived for each test chamber using separate O_2 and CO_2 sensors. The flow rate was set at 2000 ml/min. Gases were assessed in ml/kg body weight/minute. Body weights for each rat were uploaded into the program immediately prior to testing. RER was expressed as the volume of CO_2 produced (VCO_2) divided by the volume of O_2 (VO_2) consumed. The analyzers were calibrated prior to each test using primary gas standards of high purity (Praxair, Vancouver, WA, USA).

In an initial study, rats with guide cannulae targeting the PVN, were administered acyl ghrelin (50–200 pmol; n = 8) or des-acyl ghrelin (50–200 pmol; n = 7) and then immediately placed into the Oxyscan calorimeter for 2 h. All rats were tested with each dose of the peptide and vehicle, with treatments presented on separate days and in randomized order. At least three non-injection days separated successive test days. In order to investigate the impact of Ex-4 on ghrelin-induced changes in RER, individual groups of rats were pretreated with systemic (0.1–1.0 μg/kg IP; n = 8) or PVN (0.01–0.05 μg; n = 8) Ex-4 and then immediately administered acyl ghrelin (50 pmol) into the PVN. We did not investigate the effects of Ex-4 paired with des-acyl ghrelin given that the preceding study demonstrated that des-acyl ghrelin alone failed to alter RER. In a final study (n = 7), low doses of ghrelin and NPY were co-administered in order to determine if Ex-4 pretreatment would

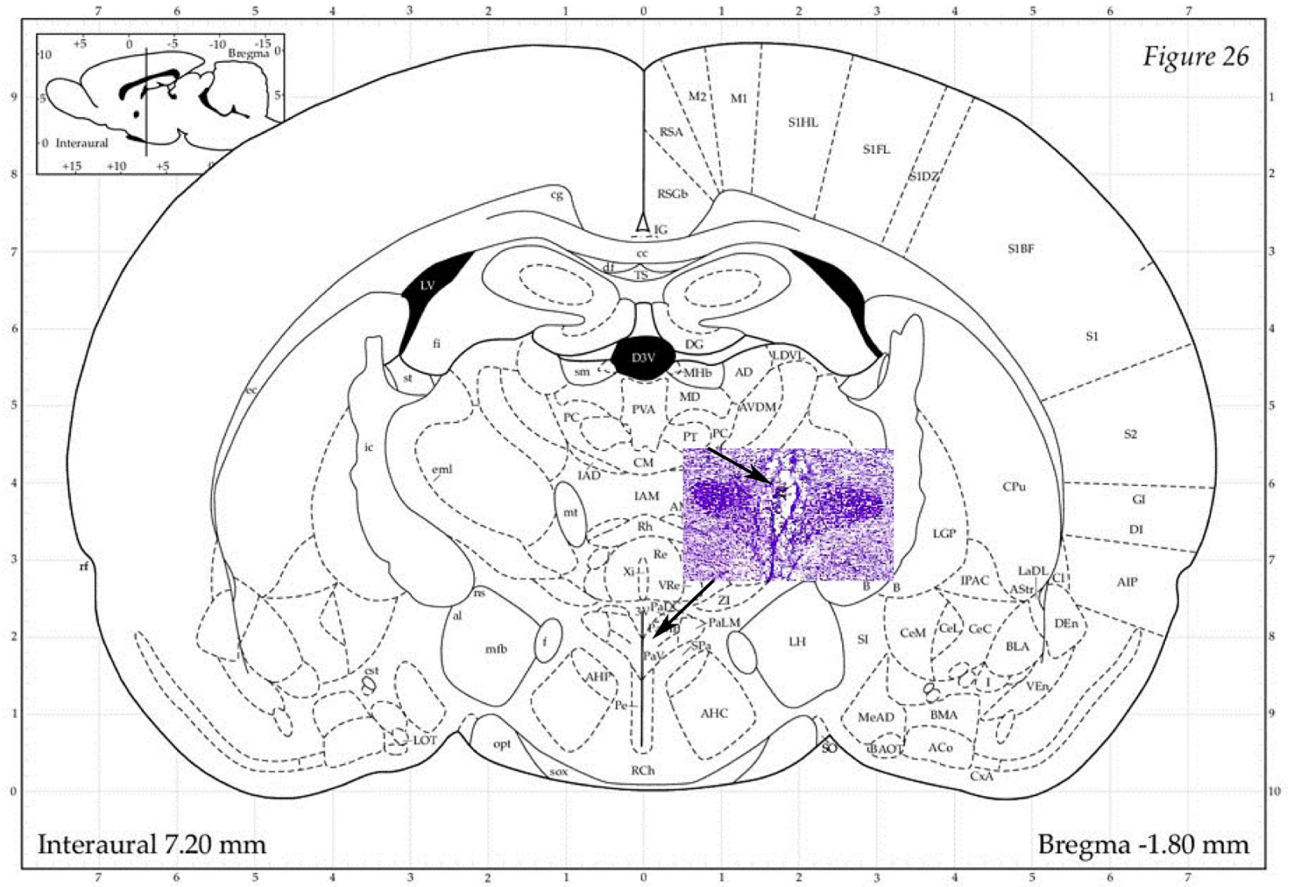

Fig. 1. Coronal section through the PVN of the hypothalamus illustrating the site of microinjection. One arrow targets black ink residue within the PVN, while the second arrow indicates PVN tissue as referenced by Paxinos and Watson (2014). Sections were cut at 20 µm, stained with Cresyl violet, and examined via light microscopy.

antagonize the combined effects of PVN ghrelin-NPY signaling. These very low doses of ghrelin and NPY were expected to be subthreshold when injected individually. In this experiment rats were treated with PVN ghrelin (10 pmol), NPY (15 pmol), ghrelin paired with NPY, or Ex-4 (0.01 µg) combined with ghrelin and NPY. Again, a repeated measures design was used with rats subjected to all treatments in randomized order.

Acyl ghrelin, des-acyl ghrelin, NPY, and Ex-4 were dissolved in sterile physiological saline and were each purchased from Tocris (Minneapolis, MN, USA). Doses were selected based on past work examining RER, body weight, and appetite (Abtahi et al., 2016; Alhadeff et al., 2012; López-Ferreras et al., 2017; Mietlicki-Baase et al., 2013,; Nakazato et al., 2001). PVN injections were administered via an injector cannula extending 4 mm beyond the indwelling guide cannula. The volume of each injection was 0.2 µl. Each microinjection was carried out over a 4-minute period. Intraperitoneal Ex-4 was delivered in a volume of 1 ml/kg. Metabolic testing began immediately postinjection. In order to avoid changes in metabolism associated with energy intake, food was removed from the home cage 1 h prior to testing and was not available in the metabolic chamber during RER testing. All experiments were carried out during the initial 2 h of the nocturnal active cycle.

2.4. Histological confirmation

Cannula placements were confirmed via histological examination as described previously (Abtahi et al., 2016, 2017). Immediately following CO_2 induced euthanasia, rats were injected with 0.4 µl of black ink to facilitate the identification of microinjector placement. Tissue sections were cut at 20 µm on a coronal plane using a cryostat (CryoStar NX50, Thermo Fisher, Waltham, MA, USA), mounted on slides, processed, stained with Cresyl violet (Sigma, St. Louis, MO, USA), and then coverslipped. Slides were examined via light microscopy and assessed using the stereotaxic atlas of Paxinos and Watson (2014). All animals reported here were found to have accurate placements in the PVN. A representative coronal section of the PVN is shown in Fig. 1.

2.5. Statistical analyses

Data were analyzed using one or two-way repeated measures analyses of variance (ANOVA) followed by post hoc Tukey tests of mean comparisons when justified. The criterion for statistical significance was $p < 0.05$. The statistical software used was TIBCO Statistica (Palo Alto, CA, USA).

3. Results

The RER effects of PVN acyl and des-acyl ghrelin are illustrated in Fig. 2. One-way repeated measures ANOVA revealed that acyl ghrelin administration evoked reliable, dose-dependent increases in RER ($F(3,21) = 66.91$, $p < 0.001$) whereas the des-acyl ghrelin molecule failed to alter RER at any of the doses tested ($F(3,18) = 0.23$, $p > 0.05$). In an additional group of rats, Ex-4 pretreatment was used to assess whether the GLP-1 receptor agonist could modify the robust effect of acyl ghrelin on substrate utilization. Two-way repeated measures ANOVA indicated that intraperitoneal Ex-4 injection elicited a significant reduction in the stimulatory impact of acyl ghrelin on RER ($F(2,14) = 4.22$, $p < 0.01$). The inhibitory effect of Ex-4 was dose-dependent and while both doses of Ex-4 suppressed ghrelin's effect,

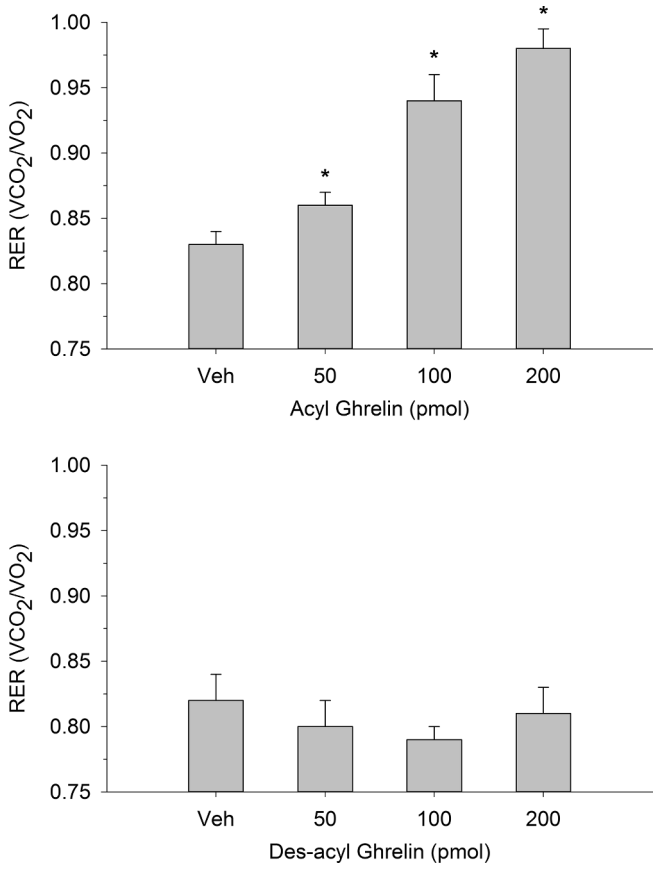

Fig. 2. The effect of acyl (n = 8) and des-acyl (n = 7) ghrelin microinjection on RER. Only PVN acyl ghrelin elicited significant increases in RER indicative of enhanced carbohydrate utilization and reduced lipid oxidation. PVN des-acyl ghrelin treatment was ineffective in altering RER. Values represent mean ± S.E.M.; $^{*}P < 0.05$ compared to vehicle (Veh).

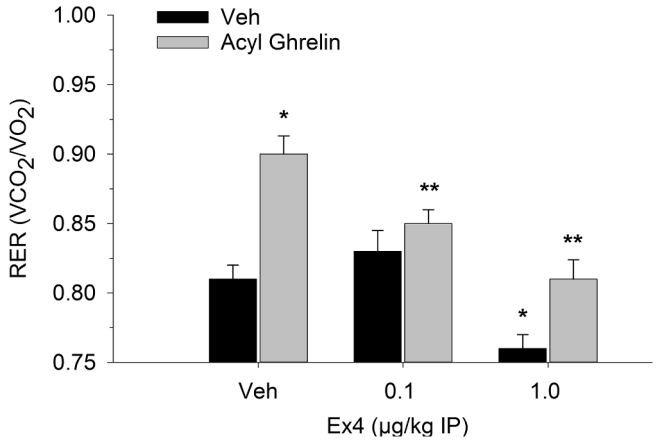

Fig. 3. Intraperitoneal injections of the GLP-1 agonist Ex-4 reduced RER and attenuated the stimulatory effect of ghrelin on substrate oxidation. Values are mean ± S.E.M.; n = 8. $^{*}P < 0.05$ compared to vehicle (Veh). $^{**}P < 0.05$ compared to ghrelin paired with Veh.

only the higher dose of 1.0 μg/kg significantly lowered RER on its own. (See Fig. 3). Comparable results were detected following direct hypothalamic PVN Ex-4 pretreatment ($F(2,14) = 9.97$, $p < 0.004$) with both subthreshold and threshold doses reliably inhibiting the stimulatory action of acyl ghrelin on RER (Fig. 4). Again, while the low dose of 0.01 μg did not significantly alter RER on its own, this contrasted with the higher dose of 0.05 μg Ex-4 which was found to attenuate RER in

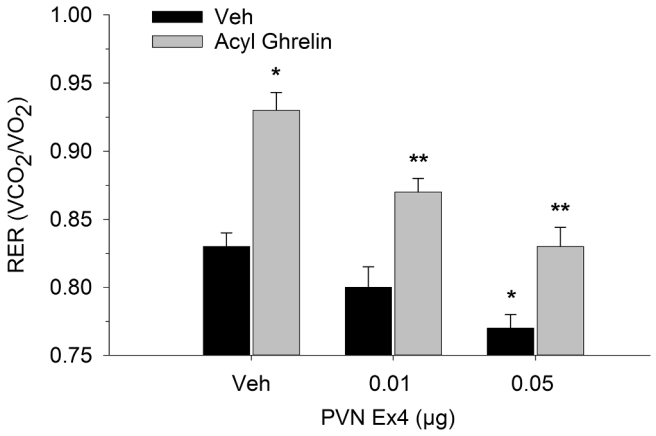

Fig. 4. PVN treatment of Ex-4 decreased RER and attenuated the stimulatory effect of ghrelin on substrate oxidation. Values represent mean ± S.E.M.; n = 8. $^{*}P < 0.05$ compared to vehicle (Veh). $^{**}P < 0.05$ compared to ghrelin paired with Veh.

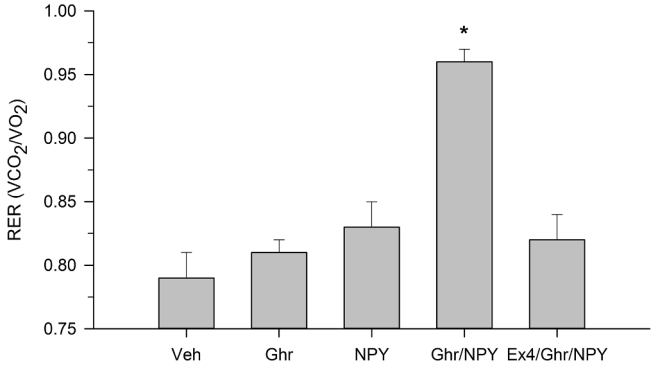

Fig. 5. PVN co-administration of subthreshold doses of ghrelin (10 pmol) and NPY (15 pmol) elicited significant increases in RER, an effect reversed by pretreatment with PVN Ex-4 (0.01 μg). Values represent mean ± S.E.M.; n = 6. $^{*}P < 0.05$ compared to vehicle (Veh).

comparison to vehicle control. Finally, as illustrated in Fig. 5, PVN co-administration of a low, subthreshold dose of acyl ghrelin paired with a subthreshold dose of the orexigenic molecule NPY, reliably increased RER values. This combined metabolic action was effectively reversed by PVN Ex-4 pretreatment ($F(4,24) = 7.35$, $p < 0.001$).

4. Discussion

In the present report we first demonstrated that acyl ghrelin, but not des acyl ghrelin, elicited robust increases in RER when administered into the hypothalamic PVN. The stimulatory effect of acyl ghrelin on RER was blocked by either systemic or PVN treatment with the GLP-1 agonist Ex-4. PVN Ex-4 injection further attenuated the potentiation of RER elicited by co-administration of ghrelin and NPY. Previous reports have found that ghrelin and NPY not only stimulate food intake, but also shift energy substrate utilization in favor of increased carbohydrate oxidation (Currie et al., 2005, 2010, 2011, 2012; Nakazato et al., 2001; Thomas et al., 2016; Wren et al., 2000). We have recently shown that Ex-4 antagonizes the metabolic effects of arcuate ghrelin (Abtahi et al., 2016) suggesting that GLP-1 may play a critical role in the control of metabolic regulation within distinct regions of the mammalian hypothalamus. Specifically, our findings indicate that GLP-1 modulates the appetitive and metabolic actions of ghrelin and NPY via medial hypothalamic neurons localized to the PVN and ArcN in rodents.

Prior evidence suggests that hypothalamic GLP-1 may regulate energy balance within other species as well. For example, ICV GLP-1

treatment decreases food intake in both chicks and catfish (*Ictalurus punctatus*) (Tachibana et al., 2004; Silverstein et al., 2001). Similarly, ICV microinjection of GLP-1 attenuates the orexigenic effect of NPY in neonatal chicks (Furuse et al., 1997) and increases c-fos expression and RER when administered into the ventromedial nucleus (VMN) and lateral hypothalamic (LH) area in chicks (Tachibana et al., 2004, Tachibana et al., 2007). In addition to suppressing appetite (Tachibana et al., 2004), ICV GLP-1 increases plasma corticosterone and this effect is inhibited by the CRH antagonist astressin (Tachibana et al., 2006). GLP-1 administration into the PVN of rats also elevates plasma corticosterone (Kinzig et al., 2003). Therefore, despite reports of distinct species differences with respect to ghrelin's impact on food intake, ghrelin-GLP-1 neurohormonal interactions appear to be conserved in both avians and mammals, and this may be particularly evident via CRH signaling.

In the mammalian hypothalamus, a critical appetitive circuit incorporates the anorexigenic POMC/CART and orexigenic NPY/AgRP neurons, both of which express GHSR-1a, in the ArcN with projections to the PVN (Kohno et al., 2008; Guan et al., 2008; van den Top et al., 2007). One report suggests that GLP-1R mRNA is coexpressed with 68% of POMC neurons in the ArcN of rats (Sandoval et al., 2008). The same study found that GLP-1 administration elicits a decrease in glucose uptake in the ArcN, but not the PVN, and reduces food intake when microinjected into the PVN, but not the ArcN (Sandoval et al., 2008). In addition, ICV Ex-4 treatment has been shown to enhance c-fos expression in ghrelin immunoreactive neurons in the ArcN and PVN (Dalvi et al., 2012). Ventricular Ex-4 has also been reported to decrease plasma ghrelin levels (Perez-Tilve et al., 2007), and in diet-induced obese mice and C57BL/6J mice, third ventricular injection of Ex-4 decreases gastric ghrelin protein expression, hypothalamic GHSR-1a mRNA, GOAT mRNA expression, and circulating ghrelin (Hong et al., 2016). With respect to the appetitive effects, a number of studies suggest that the anorexigenic action of PVN GLP-1 is dependent on glutamatergic signaling (Liu et al., 2017; Mietlicki-Baase et al., 2014). Overall, therefore, emerging evidence now indicates that GLP-1 plays an active role within the ArcN to PVN circuitry, impacting both food intake and metabolic function through its interactions with endogenous ghrelin receptor mechanisms. Specifically, increased ghrelin receptor activation within the ArcN and PVN could in turn promote GLP-1 expression and subsequent receptor stimulation. Elevated GLP-1 signaling could, as a result, suppress not only eating elicited by ghrelin, but also associated metabolic changes, including enhanced carbohydrate oxidation.

Finally, recent research has demonstrated that Ex-4 attenuates ethanol consumption when administered into the nucleus accumbens shell, and inhibits appetite when injected into the accumbens core. Both of these effects are augmented when rats are co-administered the GHSR-1a antagonist, JMV2959 (Abtahi et al., 2017). While ghrelin microinjection into the VTA and NAcc stimulates voluntary ethanol consumption and conditioned place preference (CPP) (Cepko et al., 2014; Jerlhag et al., 2009; Suchankova et al., 2013), in contrast, GLP-1 suppresses ethanol intake and CPP (Shirazi et al., 2013). VTA and accumbal ghrelin injections also increase palatable food consumption (King et al., 2011; Skibicka et al., 2011), and potentiate the stimulatory effect of cocaine on alcohol reward (Cepko et al., 2014), whereas Ex-4 treatment attenuates food intake and operant responding for cocaine (Hernandez et al., 2018; Mietlicki-Baase et al., 2013, 2014; Schmidt et al., 2016).

Given the above work, therefore, it has become increasingly clear that ghrelin and GLP-1 influence both homeostatic and hedonic behavior within both the hypothalamus and the mesolimbic reward pathway. While the present report focused on homeostatic ghrelinergic and GLP-1 signaling within the PVN, future research could be directed at investigating how ghrelin and GLP-1 interact with other endogenous signals. As an example, CRH is well integrated within ghrelin signaling pathways in both avians and mammals, despite the divergent effects on appetite induced by ghrelin in different species (Azzam et al., 2017; Kinzig et al., 2003; Khan et al., 2014; Tachibana et al., 2006). Recently, it has been demonstrated that GOAT KO increases PVN c-fos expression in response to stress (Stark et al., 2016) and GLP-1R knockdown in the PVN reduces anxiety-like behavior (Ghosal et al., 2017), indicating that both ghrelin and GLP-1 impact stress activation via the PVN. Ghrelin similarly activates CRH immunoreactive neurons within the PVN via gamma-aminobutyric acid (GABA) inhibition independently of both NPY and the ArcN, suggesting that PVN ghrelin may be part of a unique CRH circuit (Cabral et al., 2012, Cabral et al., 2016). Considering the relationship between ghrelin, stress, and appetite (Brockway et al., 2016; Spencer et al., 2015), it remains possible that the mechanisms through which ghrelin and GLP-1 interact in order to regulate metabolism within the PVN and ArcN could be partially independent. Finally, chronic peripheral Ex-4 treatment results in decreased hypothalamic NPY expression (Yang et al., 2014), suggesting that the interaction between GLP-1 and NPY should be further examined. These investigations would in turn elucidate how various regulatory peptides interact in the control of appetitive motivation, metabolism, and glucose homeostasis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.008.

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