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# Endogenous Glucagon-like Peptide-1 Receptor Signaling in the Nucleus Tractus Solitarius is Required for Food Intake Control

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Alhough the glucagon-like peptide-1 (GLP-1) system is critical to energy balance control and is a target for obesity pharmacotherapies, the receptor-population-mediating effects of endogenous GLP-1 signaling are not fully understood. To address this, we developed a novel adeno-associated virus (AAV-GLP-1R) that utilizes short hairpin RNA to chronically knock down GLP-1 receptors (GLP-1R) in rats. As pharmacological studies highlight the hindbrain nucleus tractus solitarius (NTS) as a brain region important for GLP-1R-mediated effects on energy balance, AAV-GLP-1R was injected into the NTS to examine the role of endogenous NTS GLP-1R signaling in energy balance control. Chow intake and meal size were significantly increased following chronic NTS GLP-1R knockdown. In addition, NTS GLP-1R knockdown significantly increased self-administration of palatable food under both fixed and progressive ratio schedules of reinforcement. Collectively, these data demonstrate that endogenous NTS GLP-1R signaling is required for the control of food intake and motivation to feed, and provide a new strategy to investigate the importance of distinct GLP-1R populations in the control of a variety of functions.
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## INTRODUCTION

The neuropeptide glucagon-like peptide-1 (GLP-1), produced by preproglucagon-expressing L cells of the intestine and neurons in the hindbrain nucleus tractus solitarius (NTS), is important for the control of energy balance and glycemia (see (Hayes *et al*, 2010a; Holst, 2007) for review). In fact, current FDA-approved pharmacotherapies targeting the GLP-1 system are efficacious for the treatment of type-2 diabetes mellitus and obesity (Blonde *et al*, 2006; Buse *et al*, 2009; Tella and Rendell, 2015). GLP-1 receptors (GLP-1R) expressed in both the periphery and central nervous system (CNS) are involved in mediating the metabolic effects of these exogenous GLP-1R agonists (Kanoski *et al*, 2011; Secher *et al*, 2014; Sisley *et al*, 2014) and thus it is important to understand the contributions of discrete GLP-1R populations to energy balance control.

Exogenous activation of GLP-1R in many nuclei distributed throughout the brain (eg, NTS, nuclei of the hypothalamus, ventral tegmental area, nucleus accumbens, hippocampus, and lateral parabrachial nucleus; Merchenthaler *et al*, 1999) can trigger reductions in food intake and body weight loss (Alhadeff *et al*, 2012, 2014a; Hayes *et al*, 2011; Hsu *et al*, 2015; McMahon and Wellman, 1998; Schick *et al*, 2003; Secher *et al*, 2014). However, aside from a recent report demonstrating the requirement of vagal GLP-1R for food intake control (Krieger *et al*, 2016), the endogenous role of distinct GLP-1R subpopulations in the daily control of energy balance has not been explored. GLP-1R in the NTS are of particular interest with regard to food intake control, given the importance of NTS neurotransmitter/neuroendocrine processing for energy balance regulation (Grill and Hayes, 2012). Indeed, exogenous stimulation of NTS GLP-1R results in potent reductions in food intake (Hayes *et al*, 2011) and more recently has been shown to reduce motivated and appetitive aspects of feeding (Alhadeff and Grill, 2014b; Richard *et al*, 2015). In addition, acute pharmacological blockade of NTS GLP-1R attenuates the intake-suppressive effects of a gastric preload (Hayes *et al*, 2009), suggesting a role for endogenous NTS GLP-1R in the control of food intake and satiation.

Here to assess the role of endogenous NTS GLP-1R signaling in the daily control of food intake and body weight regulation, we developed a novel adeno-associated virus (AAV) that utilizes short hairpin RNA to chronically knock down GLP-1R (AAV-GLP-1R). After injecting AAV-GLP-1R or a control virus (AAV-CONTROL) into the NTS of rats, food intake, body weight, meal patterns, and operant responding for food were analyzed. Our data suggest that endogenous NTS GLP-1R signaling is required for the

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control of food intake, meal size, and the motivation to work for palatable food.

## MATERIALS AND METHODS

### Subjects

Adult male Sprague-Dawley rats (250–265 g upon arrival, Charles River Laboratories, Wilmington, MA; Taconic Laboratories, Hudson, NY) were individually housed in hanging, wire-bottom metal cages and had *ad libitum* access to pelleted chow (Purina Rodent Chow, 5001) and water unless otherwise noted on a 12 h light/12 h dark cycle. All procedures conformed to and received approval from the institutional standards of the University of Pennsylvania Animal Care and Use Committee.

### Viral Production

RNA sequences were screened *in vitro* (OriGene Technologies, Rockland, MD) for their ability to reduce GLP-1R expression according to previously published methods (Mietlicki-Baase *et al*, 2015) to create a short hairpin RNA (shRNA) targeting the GLP-1R transcript. Briefly, a plasmid designed to express GLP-1Rs (NM_053816; OriGene) was transiently transfected alone or in combination with an shRNA to reduce GLP-1R expression in a rat immortalized hypothalamic neuronal cell line (R-19; Cedarlane Laboratories, Burlington, NC). The most robust knockdown of GLP-1Rs following transient transfection was obtained using the following shRNA sequence: 5′-GATCGGGTTGCTG GTGGAAGGCGTGTATCTGTACTCAAGAGGTACAGTA-CACGCCTTCCACCAGCAACCTTTTTT-3′. Our *in vitro* studies demonstrated an 88.9% knockdown of GLP-1R expression following a 3-day incubation with this AAV-shRNA in the R19 rat neuronal cell line transfected to overexpress the GLP-1R (Figure 1a). To knockdown GLP-1R expression *in vivo*, this shRNA sequence was cloned and packaged into an adeno-associated virus (AAV) downstream of the U6 promoter and co-expressing GFP downstream of the CB7 promoter (AAV1.U6.shRGlp1r07.CB7.EGFP.SV40; here referred to as: AAV-GLP-1R; serotype 1; titer = 5.22e12) by the Viral Core at the University of Pennsylvania. A GFP-expressing AAV1 downstream of the Cb7 promoter (AAV-CONTROL; titer = 5.22e12) was used as a control.

### AAV-GLP-1R and AAV-CONTROL Delivery to the NTS

Rats received intramuscular anesthesia (ketamine (90 mg/kg; Butler Animal Health Supply, Dublin, OH), xylazine (2.7 mg/kg; Anased, Shenandoah, IA), and acepromazine (0.64 mg/kg; Butler Animal Health Supply), and subcutaneous analgesia (2.0 mg/kg Metacam; Boehringer Ingelheim Vetmedica, St Joseph, MO)). Rats were subsequently positioned in a stereotaxic device and bilateral cannulae were positioned above the caudomedial NTS according to the following coordinates based off of a rat atlas (Paxinos and Watson, 2005) and preliminary studies: ±0.5 mm lateral from midline, 0.9 mm anterior to occipital, and 6.8 mm ventral from skull surface using a 15° angle (negative slope in the anterior to posterior direction), with the injector aimed 2.0 mm below the guide cannula. Animals (matched for body weight and food intake) received 200 nl bilateral injections of AAV-GLP-1R or AAV-CONTROL in the NTS via a micropump (PHD 2000; Harvard Apparatus, Holliston, MA) with a Hamilton syringe connected to an injector (Plastics One, Roanoke, VA). Injectors were left in place for 1 min and then removed. Guide cannulae were removed from the brain and the head incisions were sutured.

### Tissue Collection

At the completion of experiments rats were anesthetized, killed by decapitation and brains were rapidly removed, flash-frozen in isopentane, and stored at −80 °C until processing. Coronal sections at the level of the NTS were slide-mounted and viewed under a fluorescence microscope (Nikon 80i) until GFP-expressing cells were visualized in the caudomedial NTS (at the level of the area postrema). Bilateral micropunches (1 × 1 × 1 mm) were taken from this region and kept frozen for qPCR analysis. Postmortem analyses indicated that the viral spread was predominately contained within the dorsal vagal complex (DVC), between −14.6 and −13.2 mm posterior from bregma. Importantly, within the DVC, the GLP-1R expressed in the NTS (and not the area postrema or dorsal motor nucleus of the vagus) are the functionally relevant GLP-1R population for feeding behavior (Hayes *et al*, 2011). Data from rats with targeted virus injections outside of the NTS were excluded from all analyses.

### RNA Isolation and Real-Time PCR

Total RNA was extracted from micropunches using TRIzol (Invitrogen, Life Technologies, Grant Island, NY) and the RNeasy kit (Qiagen, Germantown, MD). cDNA was synthesized from using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies, Grant Island, NY). TaqMan gene expression kits and PCR reagents (Applied Biosystems) were used to quantify relative mRNA levels of GLP-1R (Glp1r, Rn00562406) relative to rat GAPDH (Gapdh, Rn01775763_g1). Relative mRNA expression was calculated using the comparative Ct method as previously described (Hayes *et al*, 2010b).

### Behavioral Analyses

*Food intake and body weight.* Beginning 1 week prior to AAV delivery, rats ($n = 9$ AAV-GLP-1R, $n = 10$ AAV-CONTROL) were monitored daily for chow intake (accounting for spillage) and body weight throughout the duration of the experiment.

*Meal patterns.* A separate cohort of rats ($n = 8$ AAV-GLP-1R, $n = 8$ AAV-CONTROL) was housed in a custom automated feedometer consisting of hanging wire-bottom cages with an access hole to a food cup with powdered chow that rested on an electronic scale. The feedometer was connected to software (LabView) that records the weight of the food cup every 10 s. Cumulative food intake, meal size and meal number data were subsequently analyzed at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, and 24 h relative to dark-cycle onset over 3 days immediately before virus injection, as well as over 3 days 2 weeks post-virus injections. Data are expressed

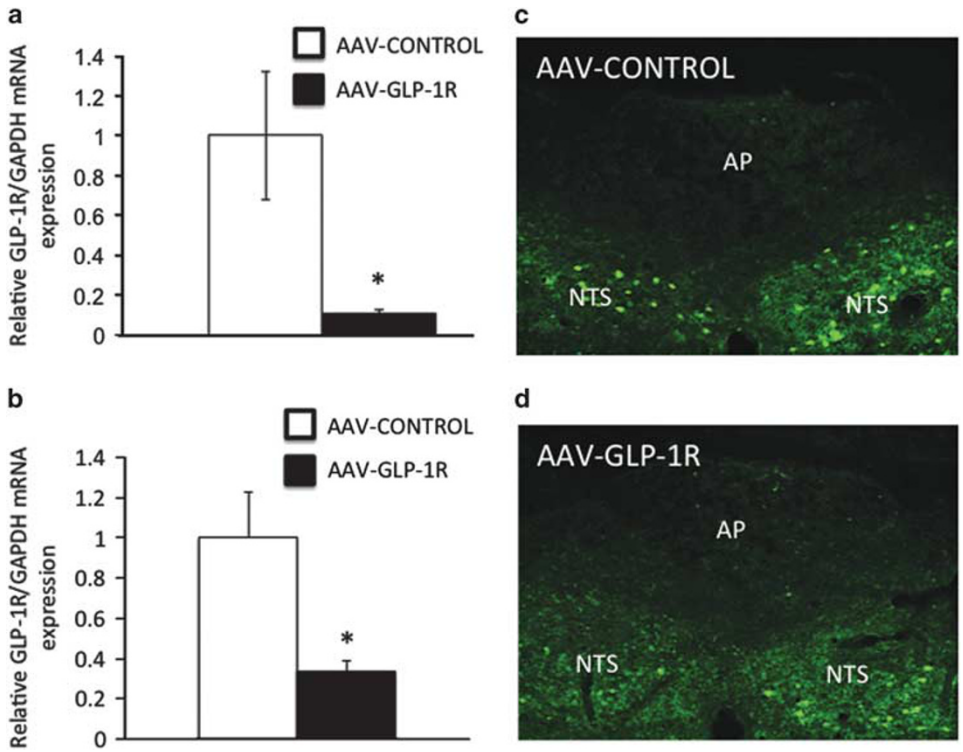


**Figure 1** (a) rtPCR from *in vitro* studies demonstrated ~88% knockdown of GLP-1R expression in rat R19 neurons overexpressing the GLP-1R following 3-day transfection with AAV-GLP-1R compared with AAV-CONTROL. (b) Representative real-time PCR (rtPCR) reveals ~66% suppression of GLP-1R mRNA in micropunched NTS tissue in AAV-GLP-1R- *vs* AAV-CONTROL-treated rats. (c) Representative image of GFP tagged-AAV-CONTROL injection placement in the NTS. (d) Representative image of GFP tagged-AAV-GLP-1R injection placement in the NTS. AP, area postrema. Data expressed as means ± SEM, $^{*}p < 0.05$.

as average cumulative intakes, meal sizes, and meal numbers over the 3-day test period. A meal was defined as any intake $\geqslant 0.25$ g; $\geqslant 10$ min must have elapsed for feeding bouts to be considered two separate meals (Alhadeff *et al*, 2014c; Mietlicki-Baase *et al*, 2013).

*Operant responding*. A third cohort of rats ($n = 8$) maintained *ad libitum* on chow and injected with AAV-GLP-1R was trained to press a lever to receive a 45 mg sucrose pellet as follows. Rats received 1 h daily operant sessions: five sessions under a fixed ratio (FR)-1 schedule of reinforcement (1 lever press required for one sucrose pellet (reinforcer)), three sessions of FR-3 (3 lever presses required for 1 reinforcer), and three sessions of FR-5 (5 lever presses required for 1 reinforcer). Next, rats underwent 5 consecutive days of progressive ratio (PR) sessions, where the effort (number of lever presses) required to obtain each reinforcer increased exponentially throughout the session as previously described (Alhadeff *et al*, 2014a; Kanoski *et al*, 2014), using the formula $F(i) = 5e^{0.2i} - 5$, where $F(i)$ is the number of lever presses required to obtain the next reinforcer at $i =$ pellet number. Under this function, the number of presses required to obtain each pellet is according to the following sequence: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 693, 737, 901 (…). The PR session ended when a 20 min period elapsed without the rat earning a pellet. The numbers of lever presses and/or numbers of reinforcers earned were averaged for each rat across days within each training condition (ie, average FR-1 responding, average FR-3 responding, average FR-5 responding, and average PR responding). These values were correlated with the mRNA expression of NTS GLP-1R expression relative to GAPDH (rtPCR methods described above).

A final cohort of rats ($n = 17$) was restricted to 20–25 g of lab chow per rat per day in their home cages for the pre-surgery training phase. Initially, rats were allowed to self-administer 45 mg sucrose pellets on a FR-1 schedule of reinforcement. Once a rat achieved at least 20 sucrose pellets in a single operant session, the response requirement was increased to an FR-5 schedule of reinforcement. Following 20 daily sucrose self-administration sessions, rats matched for FR-5 responding received NTS injections of AAV-GLP-1R ($n = 9$) or AAV-CONTROL ($n = 8$). Immediately after surgery, rats were returned to their home cages with food and water available *ad libitum* for the remainder of the experiment. Following 7 days of recovery, rats were placed back into the operant conditioning chambers and allowed to self-administer sucrose on an FR-1 schedule of reinforcement. After three days of responding on an FR-1 schedule, the response requirement was increased to FR-5. Rats were allowed to respond for sucrose on an FR-5 schedule for 12 days before they were tested on a PR schedule of reinforcement as described above. Number of lever presses and reinforcers earned from the final day of FR-5 as well as the PR test were analyzed.

### Statistical Analyses

Data for each experiment were analyzed separately with unpaired *t*-tests, one-way ANOVA with *post hoc* Newman–Keuls analyses, or Pearson's correlation using Statistica (version 7; StatSoft, Tulsa, OK) and expressed as means ± SEM. Alpha levels were set to $\alpha = 0.05$ for all analyses.

## RESULTS

### *In vivo* Quantification and Histological Confirmation of NTS GLP-1R Viral Infection

Real-time rtPCR performed on NTS-enriched micropunches of AAV-transfected tissue revealed a 66.5% reduction in GLP-1R mRNA (relative to GAPDH) in NTS tissue transfected by AAV-GLP-1R compared with tissue transfected by AAV-CONTROL (Figure 1b). Figure 1c (AAV-GLP-1R) and Figure 1d (AAV-CONTROL) are representative images showing NTS cells expressing the GFP-tagged AAV-GLP-1R and GFP-tagged AAV-CONTROL transfection in the NTS. GAPDH expression was not significantly different between AAV-GLP-1R- and AAV-CONTROL-treated rats (data not shown).

### NTS GLP-1R Knockdown Increases Chow Intake But Not Body Weight

NTS AAV-GLP-1R rats maintained on standard chow showed a significant increase in daily food intake on some, but not all days post-virus injection (Figure 2a), and showed a significant increase in cumulative food intake post-virus injection (Figure 2b), compared with AAV-CONTROL rats. Average daily body weights of rats treated with AAV-GLP-1R and AAV-CONTROL in the NTS were not significantly different (Figure 3a), although there was a non-significant trend ($p = 0.095$) toward an increase in cumulative body weight gain in AAV-GLP-1R animals (Figure 3b).

### NTS GLP-1R KD Increases Dark Cycle Cumulative Food Intake and Meal Size but Not Meal Number

Prior to virus delivery, there were no significant differences in cumulative chow intake, meal size, or meal number between AAV-GLP-1R and AAV-CONTROL groups at any time point analyzed (3-day averages, data not shown). Two weeks post-virus delivery, AAV-GLP-1R rats had significantly elevated cumulative chow intake at 1.5, 2, 3, 4, and 5 h, and a nonsignificant trend for elevated intake at 1 and 6 h, after onset of the dark cycle (Figure 4). These increases in cumulative chow intake were mediated by increases in average meal size (Figure 5a) with no alteration in average meal number (Figure 5b). During the light cycle, there were no significant differences in cumulative chow intake or average meal number, but there was a non-significant trend for an increase in meal size ($p = 0.09$, data not shown).

### Knockdown of NTS GLP-1R is Correlated with an Increase in Operant Responding and the Motivation to Work for Palatable Food: Fixed and Progressive Ratio Responding

In rats that received NTS AAV-GLP-1R, the relative NTS GLP-1R mRNA expression negatively correlated with average FR-1 responding ($r^2 = 0.30$, $p = 0.16$, $p = $ NS, Figure 6a), FR-3 responding ($r^2 = 0.88$, $p < 0.001$, Figure 6b), and FR-5 responding ($r^2 = 0.83$, $p < 0.01$, Figure 6c), suggesting that reduced endogenous NTS GLP-1R signaling is associated with higher fixed ratio responding for sucrose reinforcers. Furthermore, relative NTS GLP-1R mRNA expression negatively correlated with average PR responding [lever presses ($r^2 = 0.63$, $p < 0.05$, Figure 6d) and reinforcers earned ($r^2 = 0.85$, $p < 0.01$, Figure 6e)], suggesting that reduced endogenous NTS GLP-1R signaling is associated with a heightened motivation to work for sucrose reinforcers.

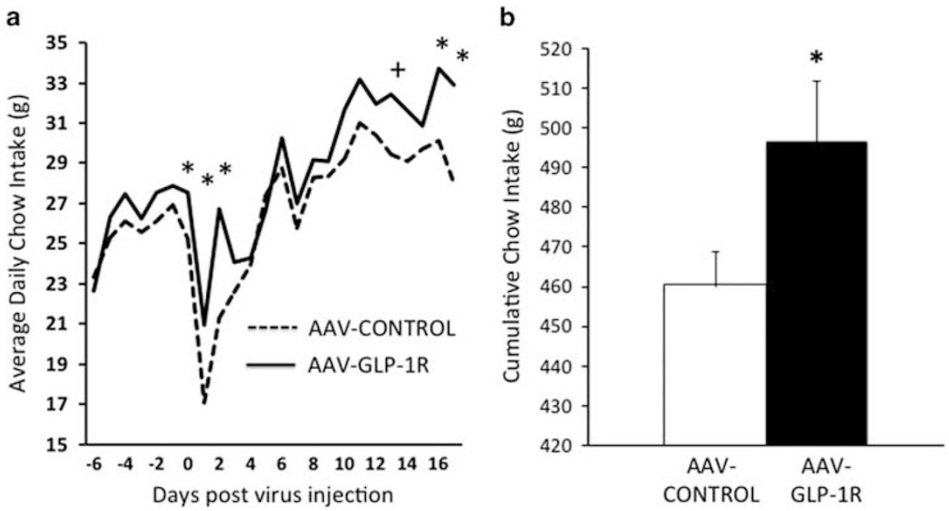


**Figure 2** (a) Average daily chow intake for NTS AAV-GLP-1R and AAV-CONTROL rats. (b) Cumulative chow intake (from day of virus injection) in NTS AAV-GLP-1R and AAV-CONTROL rats. Data expressed as means ± SEM, $^{+}p < 0.10$, $^{*}p < 0.05$.

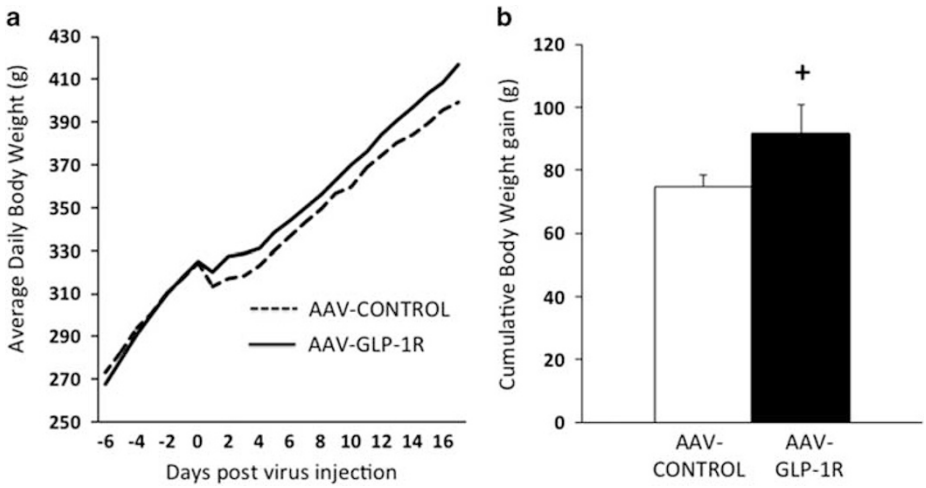


**Figure 3** (a) Average daily body weight for NTS AAV-GLP-1R and AAV-CONTROL rats. (b) Cumulative body weight gain (from day of virus injection) in NTS AAV-GLP-1R and AAV-CONTROL rats. Data expressed as means ± SEM, $^{+}p<0.10$.

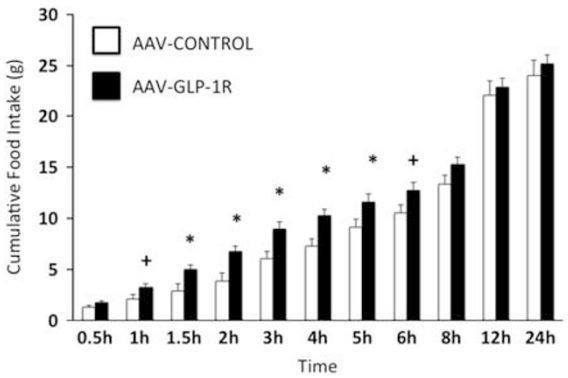


**Figure 4** Average (3-day) cumulative food intake in NTS AAV-GLP-1R and AAV-CONTROL rats, dark cycle begins at 0 h. Data expressed as means ± SEM, $^{+}p<0.10$, $^{*}p<0.05$.

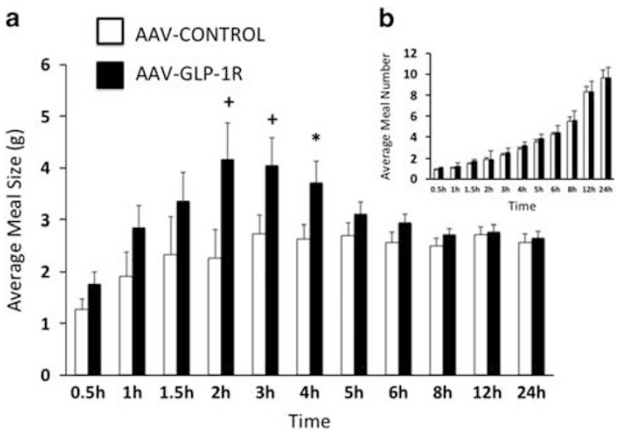


**Figure 5** (a) Average (3-day) meal size in NTS AAV-GLP-1R and AAV-CONTROL rats, dark cycle begins at 0 h. (b) Average (3-day) meal number in NTS AAV-GLP-1R and AAV-CONTROL rats. Data expressed as means ± SEM, $^{+}p<0.06$, $^{*}p<0.05$.

### NTS GLP-1R KD Increases Fixed and Progressive Ratio Responding for Sucrose

NTS AAV-GLP-1R rats maintained *ad libitum* on chow had significantly increased FR-5 responding for sucrose as they performed more lever presses (Figures 7a, $p=0.06$) and earned more reinforcers (Figure 7b, $p<0.05$) compared with AAV-CONTROL rats. Similarly, the same NTS AAV-GLP-1R rats demonstrated increased PR responding for sucrose as they performed more lever presses (Figure 7c, $p=0.10$) and earned more reinforcers (Figure 7d, $p<0.05$) compared with AAV-CONTROL rats. Altogether, these data demonstrate a role for endogenous NTS GLP-1R signaling in the motivation to work for palatable food.

## DISCUSSION

The GLP-1 system is a well-established target for obesity and type II diabetes pharmacotherapies, yet the receptor populations mediating the energy balance effects of endogenous GLP-1R signaling are unknown. To address this, we created a novel adeno-associated virus (AAV-GLP-1R) that utilizes short-hairpin RNA to chronically knockdown the GLP-1R. We injected the AAV-GLP-1R into the NTS of rats, as pharmacological data highlight the NTS as a brain region important for GLP-1R-mediated effects on food intake (Alhadeff and Grill, 2014b; Hayes *et al*, 2009, 2011). NTS GLP-1R knockdown significantly increased chow intake, meal size, and operant responding for sucrose reinforcers, demonstraing that endogenous NTS GLP-1R signaling is required for the normal control of food intake and motivation to feed.

AAV-mediated knockdown of GLP-1R restricted to the caudomedial NTS increased daily and cumulative chow intake, suggesting that NTS GLP-1R signaling is required for food intake control. These data complement and extend results from pharmacological studies showing that exogenous NTS GLP-1R stimulation reduces food intake (Hayes *et al*, 2011), and acute blockade of NTS GLP-1R attenuates the intake-suppressive effects of a gastric preload (Hayes *et al*, 2009). Unlike previous studies that utilized food-deprivation and preload paradigms, we showed here that chronic reduction of NTS GLP-1R signaling was sufficient to increase food intake under normal *ad libitum*-fed conditions. It is interesting to note that increases in food intake were observed despite no significant differences in average body weight or body weight gain in NTS AAV-GLP-1R *vs*

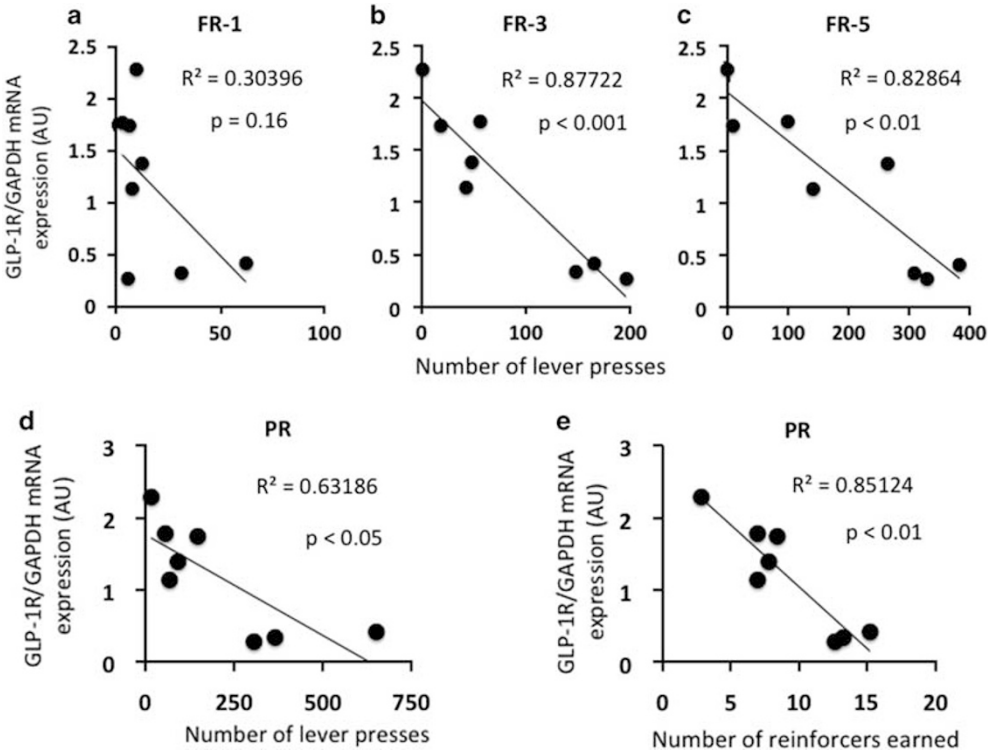


**Figure 6** Relative NTS GLP-1R mRNA expression negatively correlates with (a) FR-1 operant responding ($p = NS$), (b) FR-3 operant responding and (c) FR-5 operant responding. In addition, relative NTS GLP-1R mRNA expression negatively correlates with (d) number of lever presses and (e) number of reinforcers earned on a progressive ratio schedule of reinforcement.

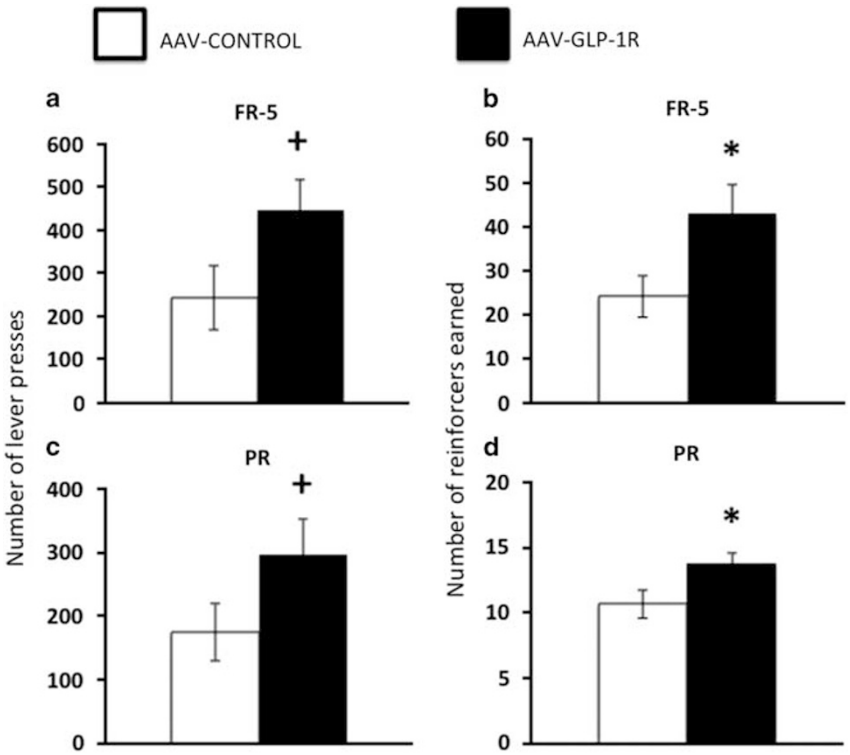


**Figure 7** Number of lever presses (a) and reinforcers earned (b) in AAV-GLP-1R and AAV-CONTROL rats under an FR-5 schedule of reinforcement. Number of lever presses (c) and reinforcers earned (d) in AAV-GLP-1R and AAV-CONTROL rats under PR schedule of reinforcement. Data expressed as means ± SEM, $^{+}p \leqslant 0.1$, $*p < 0.05$.

AAV-CONTROL-treated rats. It is possible that changes in energy expenditure account for the lack of body weight phenotype, especially given that exogenous hindbrain (ie, fourth ICV) GLP-1R agonist injection decreases core temperature and activity (Hayes *et al*, 2008); however, until explicitly investigated this remains speculative. In addition, it is possible that maintenance on the standard, low-fat chow diet precluded excess body weight gain, and/or that the magnitude of GLP-1R knockdown was not sufficient to achieve a body weight phenotype. Thus, future studies are warranted to investigate the effects of NTS GLP-1R knockdown in animals maintained on calorie-dense palatable diets and with various magnitudes and time-course durations of NTS GLP-1R knockdown.

Meal-pattern analyses revealed that NTS GLP-1R knockdown increased mid-dark cycle food intake, even in the absence of increases in 24 h food intake. These significant increases in food intake during the rats' normal feeding period likely contributed to the significant increases in daily and cumulative food intake that were observed over time. Our data also indicate that NTS GLP-1R knockdown increased food intake by increasing meal size, with no effect on meal number. These results are in line with a large literature demonstrating that the NTS (Grill, 2010; Grill and Hayes, 2012) and GLP-1 (Dossat *et al*, 2013; Mietlicki-Baase *et al*, 2013; Punjabi *et al*, 2011; Scott and Moran, 2007; Williams *et al*, 2009) both have well-established roles in the control of satiation and meal size. However, previous data show that pharmacological activation of NTS GLP-1R reduces food intake specifically via a reduction in meal number (Hayes *et al*, 2011). This apparent discrepancy is likely due to methodological differences (eg, acute knockdown virus injection *vs* repeated pharmacological injections in awake animals), and/or differences in pharmacokinetics or pharmacodynamics between exogenous GLP-1R ligands and endogenous GLP-1R signaling. Given that the virus we used in the current studies produces a chronic knockdown of GLP-1R, the animals remain undisturbed (eg, no agonist/antagonist injection that may cause off-target effects; eg, stress) through the automatic measurements of meal patterns. Thus, we argue that the results presented here are the best representation of the effects of endogenous NTS GLP-1R signaling on meal patterns to date. That NTS knockdown of endogenous GLP-1R produced a specific increase in meal size suggests that effects on meal size are physiologically relevant and involve a potential interaction between NTS GLP-1R signaling and the processing of gastrointestinal satiation signaling.

GLP-1R signaling has received increasing attention for its role in food reward and the motivation to feed (Alhadeff *et al*, 2012; Dickson *et al*, 2012; Dossat *et al*, 2011). Recent pharmacological studies have extended the sites of action for GLP-1R-mediated effects on food reward beyond the mesolimbic system to include the NTS (Alhadeff and Grill, 2014b; Richard *et al*, 2015). However, until now the role of endogenous GLP-1R signaling in the control of food reward has remained unexplored. Thus, we measured the motivation to work for palatable food by analyzing FR and PR operant responding in animals injected with AAV-GLP-1R or AAV-CONTROL in the NTS. Rats receiving NTS delivery of AAV-GLP-1R showed a significantly increased FR and PR responding for palatable food compared with AAV-CONTROL-injected rats. NTS GLP-1R mRNA levels were inversely correlated with FR-3, FR-5, and PR responding for sucrose reward, further demonstrating that endogenous NTS GLP-1R signaling contributes to the motivation to work for food under *ad libitum*-fed conditions. These data are the first to highlight the NTS as a region where endogenous GLP-1R signaling affects food reward. It would be useful for future studies to further explore this hypothesis by determining whether the effects of reduced endogenous NTS GLP-1R signaling extend to other food reward paradigms such as conditioned place preference and reinstatement of operant responding for palatable foods.

Endogenous GLP-1 is produced by cells in the intestine and in the NTS and is released upon food ingestion (Holst, 2007). Given the very short half-life of intestinally derived GLP-1 (Vilsboll *et al*, 2003), we speculate that the observed phenotypic effects following NTS GLP-1R KD are mediated by reduced GLP-1R binding of GLP-1 synthesized by and released from NTS neurons. Although in the current studies, AAV-GLP-1R knockdown was targeted to the NTS and was sufficient to affect chow intake, meal size, and food reward, it is likely that NTS GLP-1R-expressing neurons engage other brain regions to exert effects on these feeding behaviors. Importantly, as AAV-1 serotypes are able to be transported to neuronal processes (Castle *et al*, 2014), we cannot rule out the possible contribution of GLP-1R knockdown in distal CNS nuclei innervated by NTS neurons. However, there is no convincing evidence that AAV1 serotypes are transsynaptically transfecting distant cell types (Aschauer *et al*, 2013; Castle *et al*, 2014). Thus, the effects observed here are likely attributed to knockdown of GLP-1R on NTS cells. Indeed, although it is well known that NTS neurons project to a variety of regions throughout the hindbrain, midbrain, and forebrain (Norgren, 1978; Rinaman, 2010), the target projection sites of NTS GLP-1R-expressing neurons and the downstream circuits mediating the effects described here remain unknown. In addition, it is entirely possible that our observed effects on food intake and motivation are not directly due to reduced NTS GLP-1R signaling, but rather are mediated by interactions between NTS GLP-1R and other feeding-related peptides and signaling pathways. GLP-1 signaling pathways are known to interact with other hormonal systems such as leptin (Kanoski *et al*, 2015; Williams *et al*, 2006; Zhao *et al*, 2012), ghrelin (Chelikani *et al*, 2006), and amylin (Bello *et al*, 2010) to exert effects on food intake and energy balance control. Disruption of these peptide interactions by our NTS GLP-1R KD manipulation may in part mediate the food intake phenotypes in the current study. These mechanistic questions are critical to understanding how endogenous NTS GLP-1R signaling mediates food intake and motivation and should be investigated in future studies.

As previously mentioned, GLP-1R agonists are currently used to treat obesity and type-II diabetes. As these agonists enter the brain (Goke *et al*, 1995; Secher *et al*, 2014) and act on central GLP-1R to affect food intake (Kanoski *et al*, 2011; Sisley *et al*, 2014), the current data may have clinical implications with regard to the central sites of action involved in mediating therapeutic effects of these drugs. In future research, our strategy for GLP-1R knockdown could be applied to a variety of brain and/or peripheral sites to determine the receptor populations necessary for

GLP-1R-mediated effects on food intake, energy balance, and glycemia. As new potential applications for GLP-1 pharmacotherapies emerge in the literature (eg, addiction (Egecioglu *et al*, 2013; Shirazi *et al*, 2013; Skibicka, 2013; Sorensen *et al*, 2015; Suchankova *et al*, 2015), neurodegenerative diseases (Bao *et al*, 2015; Holscher, 2014)), it is our hope that this tool can be broadly used to elucidate the relevant receptor populations involved in a wide variety of GLP-1R-mediated physiological functions.

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