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Paraventricular Thalamic Control of Food Intake and Reward: Role of Glucagon-Like Peptide-1 Receptor Signaling

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Paraventricular thalamic nucleus (PVT) neurons receive hindbrain and hypothalamic inputs, and project to forebrain sites involved in reward and motivation function. The role of PVT in energy balance and reward control is however understudied. Given that PVT neurons express glucagon-like peptide-1 receptors (GLP-1R), which are critical to feeding and body weight control, we tested the hypothesis that PVT GLP-1R signaling contributes to food intake and reward inhibition. To assess the hypothesis, behavioral tests including chow and high-fat diet intake, meal patterns, conditioned place preference for high-fat food, cue-induced reinstatement of sucrose-seeking, and motivation to work for sucrose were employed following intra-PVT delivery of either GLP-1R agonist, exendin-4 (Ex4), or GLP-1R antagonist, exendin-9–39 (Ex9). Anatomical and electrophysiological experiments were conducted to examine the neural connections and cellular mechanisms of GLP-1R signaling on PVT-to-nucleus accumbens (NAc) projecting neurons. PVT GLP-1R agonism reduced food intake, food-motivation, and food-seeking, while blocking endogenous PVT GLP-1R signaling increased meal size and food intake. PVT neurons receive GLP-1 innervation from nucleus tractus solitarius preproglucagon neurons that were activated by food intake; these GLP-1 fibers formed close appositions to putative GLP-1R-expressing PVT cells that project to the NAc. Electrophysiological recordings of PVT-to-NAc neurons revealed that GLP-1R activation reduced their excitability, mediated in part via suppression of excitatory synaptic drive. Collectively, these behavioral, electrophysiological and anatomical data illuminate a novel function for PVT GLP-1R signaling in food intake control and suggest a role for the PVT-to-NAc pathway in mediating the effects of PVT GLP-1R activation.
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INTRODUCTION

Neurons of the paraventricular thalamic nucleus (PVT) receive neurochemically diverse projections from neurons in the caudal brainstem (eg, glucagon-like peptide-1 (GLP-1)), hypothalamus (eg, hypocretin/orexin), and other forebrain areas (eg, corticotrophin releasing factor from amygdala), and transmit their efferent outflow to nucleus accumbens (NAc), bed nucleus of the stria terminalis, central nucleus of the amygdala and associated cortical regions (Kirouac, 2015). These homeostatic, visceral, and arousal-related inputs from hindbrain/hypothalamic neurons and outputs to NAc neurons associated with motivation and reward function suggest important roles for PVT neurons in energy balance control, particularly on feeding behavior. However, to date, there are insufficient data to adequately evaluate this suggestion.

In considering a role for PVT neurons in neural control of feeding behavior, the functional effect of its GLP-1 receptor (GLP-1R) signaling is unexplored. GLP-1 is a hormone with anorectic effects that are mediated in part through its receptors in the brain (Kanoski *et al*, 2011). GLP-1R-expressing cells (Cork *et al*, 2015; Merchenthaler *et al*, 1999) and GLP-1 immunopositive fibers are located within the PVT, which suggests possible GLP-1 innervation from preproglucagon (PPG) neurons in the caudal nucleus tractus solitarius (NTS; Gu *et al*, 2013; Llewellyn-Smith *et al*, 2011; Rinaman, 2010). NTS PPG neurons are activated by vagal afferent stimulation resulting from ingested food (eg, gastric distension; Kreisler *et al*, 2014; Vrang *et al*, 2003), which in turn promotes GLP-1 release at the projection targets of PPG neurons, that may include the PVT. On the basis of these findings, we hypothesized that GLP-1R signaling in PVT contributes to the inhibitory control of feeding behaviors.

To address this hypothesis, we assessed the effects of PVT GLP-1R activation by conducting a series of pharmaco-behavioral, anatomical, and electrophysiological experiments. Collectively, results obtained support the hypothesis that PVT GLP-1R signaling reduces food -intake, -seeking, and -motivation, inhibits PVT output to NAc, and highlights the PVT-to-NAc projecting neurons as possible targets of PVT GLP-1R activation.

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MATERIALS AND METHODS

Animals

All procedures conformed to the institutional standards of the University of Pennsylvania and Rutgers Robert Wood Johnson Medical School Institutional Animal Care and Use Committee (IACUC). Adult male Sprague Dawley rats (250–265 g on arrival, Charles River Laboratories, Wilmington, MA) were individually housed in metal hanging cages under a 12 h light/12 h dark cycle. C57BL/6J mice (Jackson Laboratory) were bred in-house, and animals aged 5–8 weeks were used for electrophysiological analyses. Rats or mice had *ad libitum* access to pelleted chow (Purina 5001, St Louis, MO) and water, unless otherwise stated.

Rats or mice were implanted with cannula directed to 2 mm above either the lateral ventricle (bregma − 0.9 mm, lateral ± 1.6 mm, and ventral − 2.8 mm) or PVT (bregma − 2.8 mm, on midline, and ventral − 4.2 mm). All experiments measuring food intake were conducted using within-subjects, counterbalanced designs, with at least 48 h intervening between experimental conditions, unless otherwise specified. See Supplementary Information for drugs and infusion methods.

Chow and High-Fat Diet Intake

Naïve rats maintained on chow ($n = 7$) or high-fat diet (HFD; $n = 10$; 45% kcal/fat, Research Diets, New Brunswick, NJ) received intra-PVT injections of Veh or Ex4 (12.5 ng, 25 ng, and 50 ng). Chow and HFD intake (accounting for spillage) were measured 1, 2, 4, 6, and 24 h post-injection.

Cumulative Chow Intake and Meal Pattern Effects of PVT GLP-1R Antagonism

Naïve rats ($n = 13$) were housed in modified hanging wire cages equipped with an automated food intake measuring system (DiaLog instruments) that makes continuous measurements of food intake as well as meal parameters including meal size and meal number (Alhadeff *et al*, 2014a). Cumulative chow intake and meal patterns were analyzed following PVT injections of Veh or Ex9 (5 and 10 μg).

Conditioned Place Preference for HFD

Conditioned place preference (CPP) was performed as described previously (Alhadeff and Grill, 2014b). Naïve rats ($n = 17$) were trained to associate a context with HFD intake in a CPP apparatus. Rats were injected with Veh or Ex4 (25 ng) to the PVT 4 h prior to test and the amount of time spent in each compartment of the CPP apparatus was calculated to determine the percent shift in preference (from baseline) to the HFD-paired side. See Supplementary Information for experimental details.

Cue-Induced Reinstatement of Sucrose-Seeking Behavior

Naïve rats ($n = 16$) with PVT cannulae were trained to associate tone and light cues with the delivery of a 45 mg sucrose pellet (Bio-Serv, Frenchtown, NJ). During reinstatement test day, rats were injected with either Veh or 25 ng Ex4 to the PVT 4 h prior to test and the number of lever presses emitted was recorded. See Supplementary Information for experimental details.

Progressive Ratio Responding for Sucrose Reward

To assess food-motivated behaviors, Progressive ratio (PR) responding for sucrose reward was conducted as previously described (Alhadeff and Grill, 2014b), in rats ($n = 14$) that were previously tested on CPP. On PR test days, rats received intra-PVT injections of either Veh or Ex4 (25 and 50 ng) and 4 h later tested for their PR responding for sucrose reward. See Supplementary Information for experimental details.

Pica (Model for Nausea/Malaise in Rodents)

To insure that the intake inhibitory effect of PVT GLP-1R signaling is not secondary to nausea and malaise in rodents, pica (ingestion of non-nutritive substance) was assessed by measuring 24 h kaolin clay intake following intra-PVT delivery of Veh or Ex4 (50 ng) immediately prior to dark onset.

Characterization of PVT-Projecting NTS PPG Neurons that are Activated by Food Intake

To examine whether PVT neurons receive monosynaptic GLP-1 inputs from NTS PPG neurons that are sensitive to afferent signals driven by food intake, rats with fluorogold (FG; a retrograde tracer) injected to the PVT were trained to self-ingest Ensure (1.42 kcal/ml) as previously described (Ong *et al*, 2015). Three days post-surgery, rats were fasted for 24 h and given either *ad lib* access to Ensure ($n = 5$) until sated or no Ensure ($n = 2$) the next day. Rats were perfused 90 min after Ensure access. Caudal NTS sections were analyzed for GLP-1, c-Fos immunoreactivity, FG-labeled cells, and their colocalization using fluorescence microscopy (Nikon 80i; NIS Elements AR 3.0; see Supplementary Information for retrograde tracing and immunohistochemistry methods).

Identification of NAc-Projecting PVT GLP-1R-Expressing Cells

To identify whether NAc-projecting PVT cells that express GLP-1R closely appose GLP-1 fibers, rats ($n = 2$) were injected with FG to the NAc (lateral ± 1.9 mm, bregma +1.5 mm, and ventral − 5.5 mm) 4 days before receiving 10 μg/kg (i.p.) fluorescein-labeled Ex4 (FLEx; which binds to GLP-1R, is internalized into cells and serves as a marker of GLP-1R-expressing cell (Reiner *et al*, 2016)). Rats were perfused 90 min post-injection and PVT sections analyzed for GLP-1 immunoreactivity, FG-labeled cells, FLEx, and their colocalization using a Leica SP5 X confocal microscope.

Electrophysiological Recordings in Brain Slices

Brain slice physiology was performed in retrolabeled PVT-to-NAc projecting neurons as described previously (Pang *et al*, 2002) with modifications noted in Supplementary Information.

Statistics

Results are shown as mean ± SEM. Experiments on food intake, meal patterns, and food-motivated behaviors were

conducted in a within-subjects design and data were analyzed using repeated measures one-way or two-way ANOVA. When ANOVA identified a significant effect, Neuman-Keuls test was conducted. Food-seeking behavior experiments and immunohistochemistry were conducted between subjects and data were analyzed using Student's unpaired *t*-test. Electrophysiological data were analyzed using paired *t*-test (before and after the application of Ex4 or Ex9). All statistical analyses were conducted using Statistica software (Statsoft) and statistical significance was defined as $P<0.05$.

RESULTS

PVT GLP-1R Agonism Reduced Food Intake Independent of Malaise; Blocking GLP-1R Signaling Increased Food Intake and Average Meal Size

There was a significant main effect of Ex4 treatment (chow: $F_{3,18}=10.9$, HFD: $F_{3,30}=5.6$; $P<0.01$), time (chow: $F_{4,24}=190.2$, HFD: $F_{4,40}=122.2$; $P<0.001$) and treatment × time interaction (chow: $F_{12,72}=13.5$, HFD: $F_{12,120}=9.1$; $P<0.001$) on chow intake and HFD intake. PVT Ex4 significantly reduced chow intake at 6 h ($F_{3,18}=4.6$, $P<0.05$) and at 24 h ($F_{3,18}=27.8$, $P<0.001$) and HFD intake at 6 h ($F_{3,30}=5.9$, $P<0.01$) and 24 h ($F_{3,30}=12.5$, $P<0.001$), where PVT targeted delivery of 25 and 50 ng Ex4 reduced chow and HFD intake at 6 h ($P<0.05$) and 24 h ($P<0.01$) and 12.5 ng Ex4 only at 24 h ($P<0.05$; Figure 1a and b). Blocking PVT GLP-1R signaling with Ex9 (5 and 10 μg) increased cumulative chow intake at 3 h ($F_{2,24}=8.3$, $P<0.01$), 4 h ($F_{2,24}=4.9$, $P<0.05$), and 5 h ($F_{2,24}=4.0$, $P<0.05$); an effect mediated by an increase in average meal size at 3 h ($F_{2,24}=3.6$, $P<0.05$), 4 h ($F_{2,24}=4.2$, $P<0.05$), and 5 h ($F_{2,24}=4.2$, $P<0.05$) post-injection and no change in cumulative meal number (3 h: $F_{2,24}=1.5$, $P=0.24$, 4 h: $F_{2,24}=1.6$, $P=0.22$ and 5 h: $F_{2,24}=2.0$, $P=0.16$; Figure 1c–e). While the increased average meal size effect with PVT Ex9 persisted at 12 h ($F_{2,24}=7.2$, $P<0.01$) and 24 h ($F_{2,24}=7.5$, $P<0.01$; Figure 1d), it was counteracted by a reduction in cumulative meal number (12 h: $F_{2,24}=3.7$, $P<0.05$; 24 h: $F_{2,24}=6.2$, $P<0.01$; Figure 1e) resulting in no difference in cumulative chow intake between treatments (12 h: $F_{2,24}=3.2$, $P=0.06$; 24 h: $F_{2,24}=1.3$, $P=0.30$; Figure 1c).

To determine whether the intake inhibitory effects observed with PVT Ex4 delivery were secondary to a possible induction of malaise, pica (the consumption of kaolin clay), a well-established model for assessing drug-induced malaise (Takeda *et al*, 1993), was measured. Kaolin clay consumption was not different between treatments (Veh 0.1 ± 0.1 g, Ex4 0.3 ± 0.2 g; $t_{13}=1.5$, $P=0.16$), indicating that the intake inhibitory effects of PVT GLP-1R signaling are independent of malaise.

PVT GLP-1R Activation Reduced Food-Seeking and Food-Motivated Behaviors

CPP for HFD and cue-induced reinstatement of sucrose-seeking were used to assess the effect of PVT Ex4 (25 ng) delivery on food-seeking behaviors. CPP results showed that, compared to their baseline preference, Veh-treated rats increased time spent in the HFD-associated environment (baseline 301.1 ± 12.9 s, Test 501.5 ± 38.8 s). By contrast, PVT Ex4-treated rats failed to display a preference for the HFD-paired environment (baseline 372.5 ± 21.0 s, test 385.4± 78.9 s), indicating that PVT GLP-1R signaling blocked HFD-seeking behavior ($t_{15}=2.4$, $P<0.05$; Figure 2a).

Next, we showed that, in the presence of cues, Veh-treated rats reinstated sucrose-seeking behavior as evidenced by increased lever presses compared to extinction performance ($t_7=3.4$, $P<0.05$; Figure 2b). PVT Ex4 treatment blocked the expression of cue-induced reinstatement of sucrose-seeking where the number of lever presses emitted following Ex4 treatment was significantly lower than Veh-treated rats ($t_{14}=2.6$, $P<0.05$; Figure 2b). Inactive lever presses during reinstatement were not different between groups (Veh: 4.8 ± 2.7, Ex4: 1.1 ± 0.7; $t_{14}=1.3$, $P=0.22$).

To determine the effects of PVT GLP-1R signaling on palatable food-motivated behaviors, rats were tested on PR responding for sucrose. Compared to Veh-treatment, PVT Ex4 administration reduced the number of sucrose pellets earned ($F_{2,26}=5.2$, $P<0.05$) and active lever presses ($F_{2,26}=6.8$, $P<0.01$; Figure 2c and d), supporting the view that PVT GLP-1R signaling reduces food-motivated behaviors.

As PVT Ex4 treatment slightly but significantly reduced the number of inactive lever presses ($F_{2,26}=4.5$, $P<0.05$; Figure 2d), a control experiment was conducted to ensure that PVT Ex4 delivery did not negatively impact on behavior performance (Supplementary Information). Rats were trained to lever press on FR5 and FR10 schedules of reinforcement. As expected, PVT Ex4 significantly reduced the number of active lever presses ($F_{1,5}=16.4$, $P<0.01$) and sucrose pellets earned ($F_{1,5}=22.1$, $P<0.01$). Importantly, when responses under FR5 *vs* FR10 were compared, similar to Veh-treated rats, Ex4-treated rats increased lever presses during FR10 compared to FR5 ($t_5=2.7$, $P<0.05$), and obtained equivalent amounts of sucrose pellets during both schedules ($t_5=0.6$, $P=0.59$; Supplementary Figure S1A and B). The increased behavioral output under FR10 schedule of reinforcement indicates that the reduced inactive lever presses observed during PR testing in Ex4-treated rats cannot be explained by impaired lever press performance from PVT GLP-1R activation and demonstrates that feeding effects of PVT GLP-1R agonism are not secondary to behavioral suppression.

PVT Neurons Receive Direct GLP-1 Inputs from Caudal NTS PPG Neurons that are Activated by Food Intake

Ensure ingestion significantly increased the number of c-Fos-positive cells in the NTS (No Ensure: 1.1 ± 0.7 cells, Ensure: 197 ± 11.6 cells; $t_5=-10.1$, $P<0.001$). Analyses of caudal NTS sections (Bregma − 14.1 to − 14.5) revealed that 22.4% of GLP-1 cells (red) colocalize with c-Fos (green), 44.1% of FG-labeled cells (blue) colocalize with c-Fos, 32.2% of GLP-1 cells are FG-labeled, and 11.7% of GLP-1 cells are both FG-labeled and c-Fos-positive (Supplementary Table S1, Figure 3a), thus indicating that food intake activates NTS PPG neurons, PVT-projecting NTS neurons, as well as NTS PPG neurons that project monosynaptically to PVT.

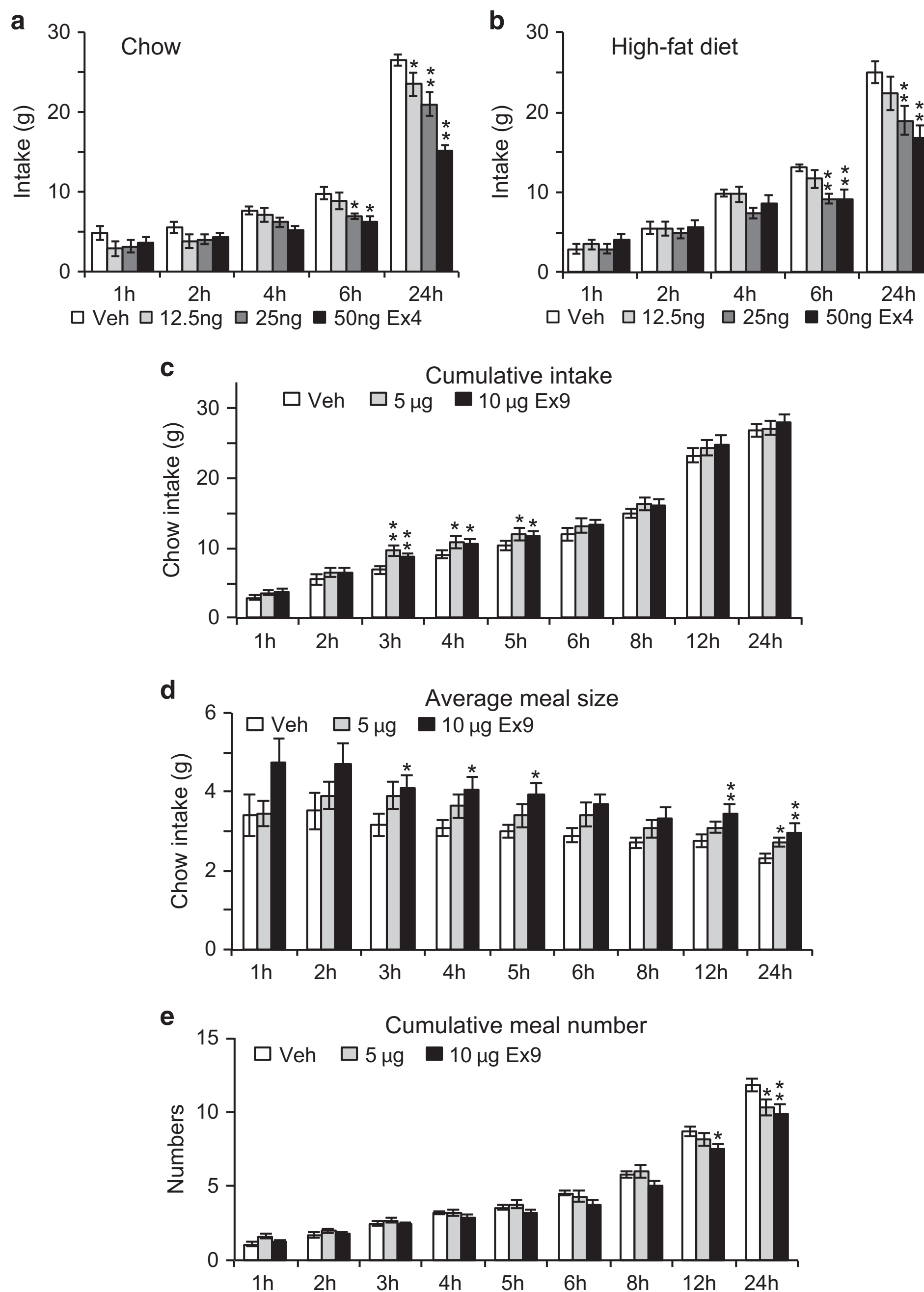

Figure 1 Effect of intra-PVT Ex4 or Ex9 delivery on food intake. (a) Chow and (b) high-fat diet (HFD) intake was reduced at 6 h (25 and 50 ng) and 24 h (12.5, 25, and 50 ng) post-injection of Ex4. PVT GLP-1R blockade increased (c) cumulative chow intake at 3–5 h via an increase in (d) average meal size, with no effect on (e) cumulative meal number. At 12 and 24 h, PVT GLP-1R blockade increased average meal size but had no effect on cumulative chow intake, likely due to a compensatory decrease in cumulative meal number. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

PVT GLP-1 Fibers Closely Appose NAc-Projecting FLEx-Labeled Cells

To identify putative anatomical interactions between GLP-1R-expressing PVT-to-NAc projecting cells and GLP-1 axons in the PVT, coronal PVT sections of rats injected with FLEx were analyzed for FLEx (green puncta), FG (blue), and GLP-1 immunoreactivity (red). Confocal analysis revealed close appositions of GLP-1 axons to NAc-projecting PVT cells that also express FLEx (white; Figure 3b). The pattern of labeling suggests possible synaptic contact between GLP-1 axons and GLP-1R-expressing PVT-to-NAc projecting neurons.

PVT GLP-1R Activation Reduced Excitability of PVT-to-NAc Projecting Neurons

Whole-cell patch clamp recordings were performed on mouse brain slices to determine the effects of Ex4 on PVT-to-NAc projecting neurons identified by retrograde transported microfluorescent beads (Supplementary Figure S2).

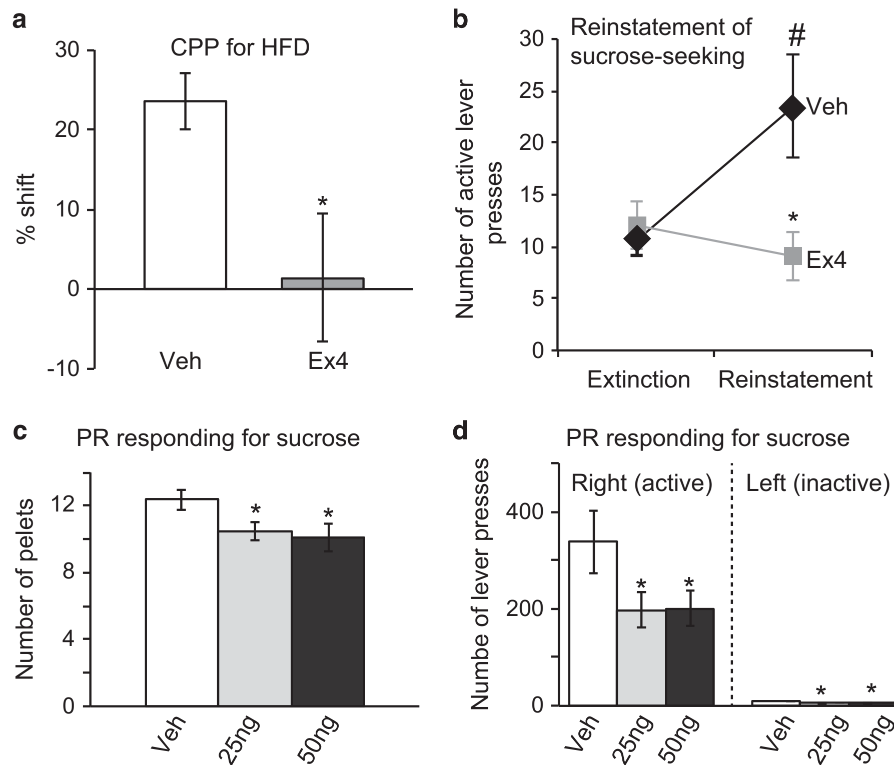

Figure 2 PVT Ex4 decreased food-seeking and food-motivated behaviors. (a) Conditioned place preference (CPP): Veh-treated rats expressed a CPP for the HFD-paired environment. PVT Ex4 (25 ng) delivery prior to testing eliminated the CPP for HFD. (b) Cue-induced reinstatement of sucrose-seeking: In the presence of cues, PVT Veh, but not Ex4 (25 ng) delivery, increased lever presses during reinstatement when compared to presses during extinction. PVT Ex4-treated rats had reduced lever presses during reinstatement compared to Veh-treated controls. (c and d) Progressive ratio (PR) responding for sucrose reward: Ex4 reduced (c) number of sucrose pellets earned and (d) lever presses. Data are presented as mean ± SEM. *$P<0.05$ Veh *vs* Ex4; #$P<0.05$ Veh extinction *vs* reinstatement.

The impact of Ex4 on spontaneous action potential (AP) generation in PVT-to-NAc core projecting neurons was first examined. Ex4 (10 nM) profoundly suppressed AP firing (Figure 4a and d) within 1 min of Ex4 application ($t_6=4.90$, $P<0.01$). These effects were reversible as spontaneous AP firing resumed after wash out. To ensure receptor specificity of Ex4 on PVT GLP-1R activation, we applied the GLP-1R antagonist, Ex9 and found that Ex9 dose-dependently blocked the inhibitory effects of Ex4 on AP firing (100 nM Ex9: $t_4=9.97$; 1 μM Ex9: $t_5=18.99$; $P<0.01$; Figure 4a and c). We then asked whether synaptic drive plays a role in the suppression of AP firing in PVT-to-NAc projecting neurons by GLP-1R activation. APs were recorded in the presence of synaptic blockers (CNQX 20 μM, APV 50 μM, to block responses mediated by glutamate receptors; PTX 50 μM, to block $GABA_A$ receptor mediated responses) to block both excitatory and inhibitory synaptic transmission. Interestingly, Ex4-induced reduction in AP firing persisted in the presence of synaptic blockers ($t_5=3.03$, $P<0.05$; Figure 4b). However, compared to the effects of Ex4 in the absence of synaptic blockers, the suppression of AP firing was smaller in magnitude and was delayed (4–5 min after Ex4 application; Figure 4b and Supplementary Figure S3). Recordings of resting membrane potential in the presence of synaptic blockers revealed hyperpolarization of PVT-to-NAc cells following Ex4 treatment (−4.27 ± 1.85 mV), suggesting a reduction in intrinsic excitability of the postsynaptic cell. This result suggests that the reduction in AP by Ex4 is mediated through both synaptic transmission-dependent and -independent mechanisms. Since the suppression of neuronal activity by GLP-1R activation was also partially mediated by synaptic inputs, the impact of Ex4 on synaptic release was assayed directly. Both spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs) were recorded in PVT-to-NAc core- and PVT-to-NAc shell- projecting neurons. Application of Ex4 decreased the frequency (core: $t_{10}=4.7$; shell: $t_{11}=5.0$; $P<0.001$) but not amplitude (core: $t_{10}=1.1$, shell: $t_{11}=0.7$; $P>0.3$) of sEPSCs in these cells (Figure 5). Moreover, in the presence of tetrodotoxin, mEPSCs frequency (core: $t_{10}=3.2$, shell: $t_5=2.8$; $P<0.05$) but not amplitude (core: $t_{10}=0.9$, shell: $t_5=0.9$; $P>0.3$) was also reduced by Ex4. These data suggest a possible presynaptic effect of GLP-1R signaling on synaptic vesicle release from nerve terminals. Spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs) in PVT-to-NAc projecting neurons were also recorded. Ex4 increased mIPSC amplitude for NAc core projecting cells only (core: $t_7=-3.8$, $P<0.01$; shell: $t_7=-1.5$, $P=0.18$) but not frequency (core: $t_7=1.4$, shell: $t_7=0.3$ $P>0.2$), suggesting that postsynaptic GLP-1R facilitate postsynaptic current sizes of inhibitory synapses (Supplementary Figure S4).

Quantification of c-Fos-immunopositive cells in the PVT of Veh or Ex4-treated rats or mice (Supplementary Materials and methods) revealed no treatment difference in PVT c-Fos

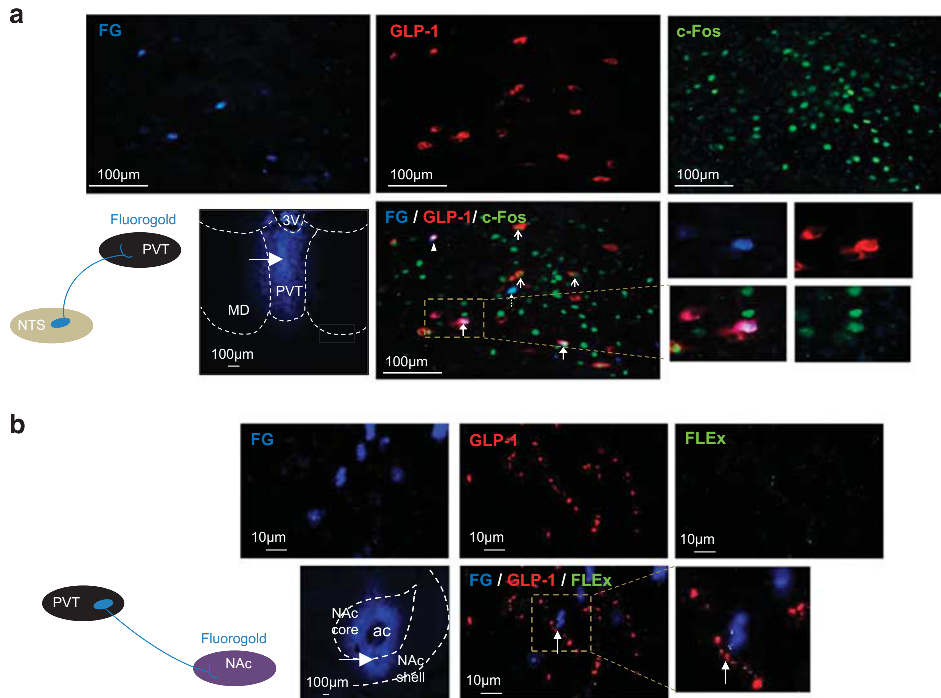

Figure 3 Photomicrographs of PVT and NTS sections. (a) Representative photomicrographs of NTS cells that are fluorogold (FG)-labeled (retrograde labeling from PVT) shown in blue, GLP-1-positive shown in red, and c-Fos-positive (cells activated by food intake) shown in green. White closed arrowhead (without shaft) indicates colocalization of NTS GLP-1-positive cells and FG-labeled cells (magenta), White open arrow shows double labeling of NTS GLP-1- and c-Fos-positive cells, white dotted arrow indicates colocalization of NTS FG-labeled and c-Fos-positive cells (teal), white closed arrow show triple labeling of NTS FG labeled, GLP-1, and c-Fos-positive cells. (b) A representative single z-stack confocal image of fluorogold (FG)-labeled NAc-projecting PVT neurons, fluorescein Ex4 (FLEx), and PVT GLP-1-positive axon terminals. White arrow shows GLP-1-positive fiber (red) in close contact with a NAc-projecting PVT cell (blue) that expresses internalized FLEx (white). FLEx (green) is a marker for GLP-1R. White horizontal arrows indicate FG injection site. All images were taken at ×2 or ×20 magnification, except for confocal images that were taken at ×40 magnification and ×2 optical zoom.

expression (rats: $t_7 = -1.5$, mice: $t_5 = 1.1$, $P > 0.1$ Supplementary Figure S5), indicating that PVT GLP-1R activation did not stimulate neuronal activity.

DISCUSSION

More than a decade ago, Ann Kelley and colleagues proposed the hypothalamic-thalamic-striatal circuity as a neural mechanism contributing to food intake control. In the proposal, the PVT is viewed as a relay center that receives inputs related to behavioral states (eg, arousal, energy status) from the hypothalamus to modulate motivational and feeding behavioral outcomes through outputs to the NAc (Kelley *et al*, 2005), an area associated with reward and motivation function. While several studies had followed up on Ann Kelley's idea to demonstrate a role for PVT neurons on food intake control (Barson *et al*, 2015; Betley *et al*, 2013; Bhatnagar and Dallman, 1999; Choi *et al*, 2012; Zhang and van den Pol, 2017), the ascending and descending pathways mediating the hypothesized food intake effects remain to be further evaluated. Here, we investigated and provided novel evidence for a role of GLP-1 inputs from NTS PPG neurons in mediating a range of feeding effects by PVT neurons. Results showed that activation of PVT GLP-1R decreased food intake, food-seeking, and food-motivated behaviors while blocking PVT GLP-1R increased food intake and meal size. We also demonstrated that PVT neurons receive monosynaptic inputs from NTS PPG neurons that are activated by food intake, thus providing physiological relevance to the feeding inhibitory effects of PVT GLP-1R signaling. In addition, electrophysiological results revealed that PVT GLP-1R signaling reduced excitability of PVT-to-NAc projecting neurons. Together, data here highlight the contribution of PVT GLP-1R signaling on food intake and reward suppression and suggest a role for PVT-to-NAc pathway in mediating the effects of PVT GLP-1R activation.

The contribution of PVT neurons in reward control was first described in studies that demonstrated intra-PVT self-stimulation reward behavior in rodents (Clavier and Gerfen, 1982; Cooper and Taylor, 1967). More recent evidence indicates a role of PVT neurons in drug-seeking behaviors (Kirouac, 2015). Given the common neural pathways mediating the effects of drugs of abuse and natural rewards, we hypothesized that PVT neurons also participate in the control of food reward. Using CPP, PR operant responding, and cue-induced reinstatement paradigms, we provided a variety of complementary behavioral evidence showing that PVT Ex4 delivery reduced the motivation to work for food reward and blocked palatable food-seeking behaviors, supporting the hypothesis that GLP-1R signaling in PVT plays a significant role in food reward-driven behaviors. Further support for PVT's role in reward function come from data showing that PVT neurons are activated in response to palatable food cues (Schiltz *et al*, 2007) and that selective activation of PVT-to-NAc neurons that express glucose transporter 2 (Glut2) increases the motivation to

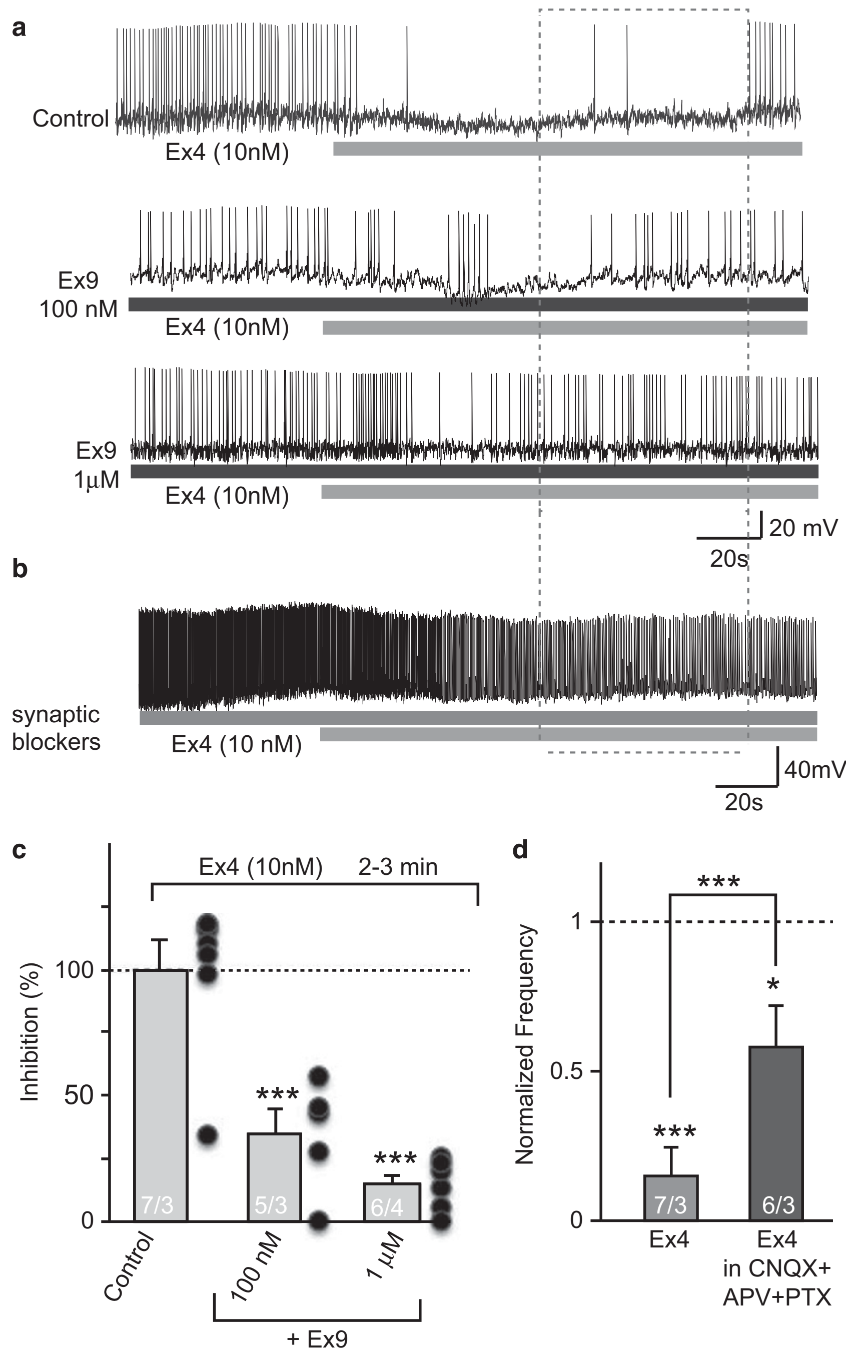

Figure 4 Ex4 suppressed spontaneous action potential (AP) firing in PVT-to-NAc core projecting neurons. (a) Representative traces of APs in response to 10 nM Ex4 application in the absence or presence of different concentrations of Ex9. AP frequency was analyzed 1 min after Ex4 application (dashed box). (b) The effects of Ex4 on AP firing in the presence of synaptic blockers. Ex4-induced suppression on AP firing was delayed in the presence of the blockers. (c) Ex9 blocked the inhibitory effects of Ex4 on the firing frequency of APs. Data are normalized to the average inhibitory effects of Ex4 on APs. (d) Normalized frequency of APs show inhibitory effects of Ex4 on APs in the absence or presence of synaptic blockers. The number of neurons recorded and the number of mice used are indicated in the bar (neurons/mice). Data are presented as mean ± SEM. *$P<0.05$, ***$P<0.001$.

work for sucrose (Labouebe *et al*, 2016). Our findings here extend the roles of PVT to include food reward control and specifically highlight a novel contribution of PVT GLP-1R signaling in mediating these food-motivated and food-seeking behaviors.

The feeding and reward inhibitory effects of PVT GLP-1R agonism reported here expand the anatomical distribution GLP-1R-expressing nuclei that contribute to food intake and reward control (Kanoski *et al*, 2016) to include the PVT. Further, we provided the first evaluation of the role of central GLP-1R signaling in blocking cue-induced reinstatement of palatable food-seeking behavior. This behavior is of clinical relevance given that brain fMRI responses to food cues predict future body weight gain (Sun *et al*, 2015) and that exposure to palatable foods and food cues increases the likelihood of reinstatement or relapse to unhealthy eating

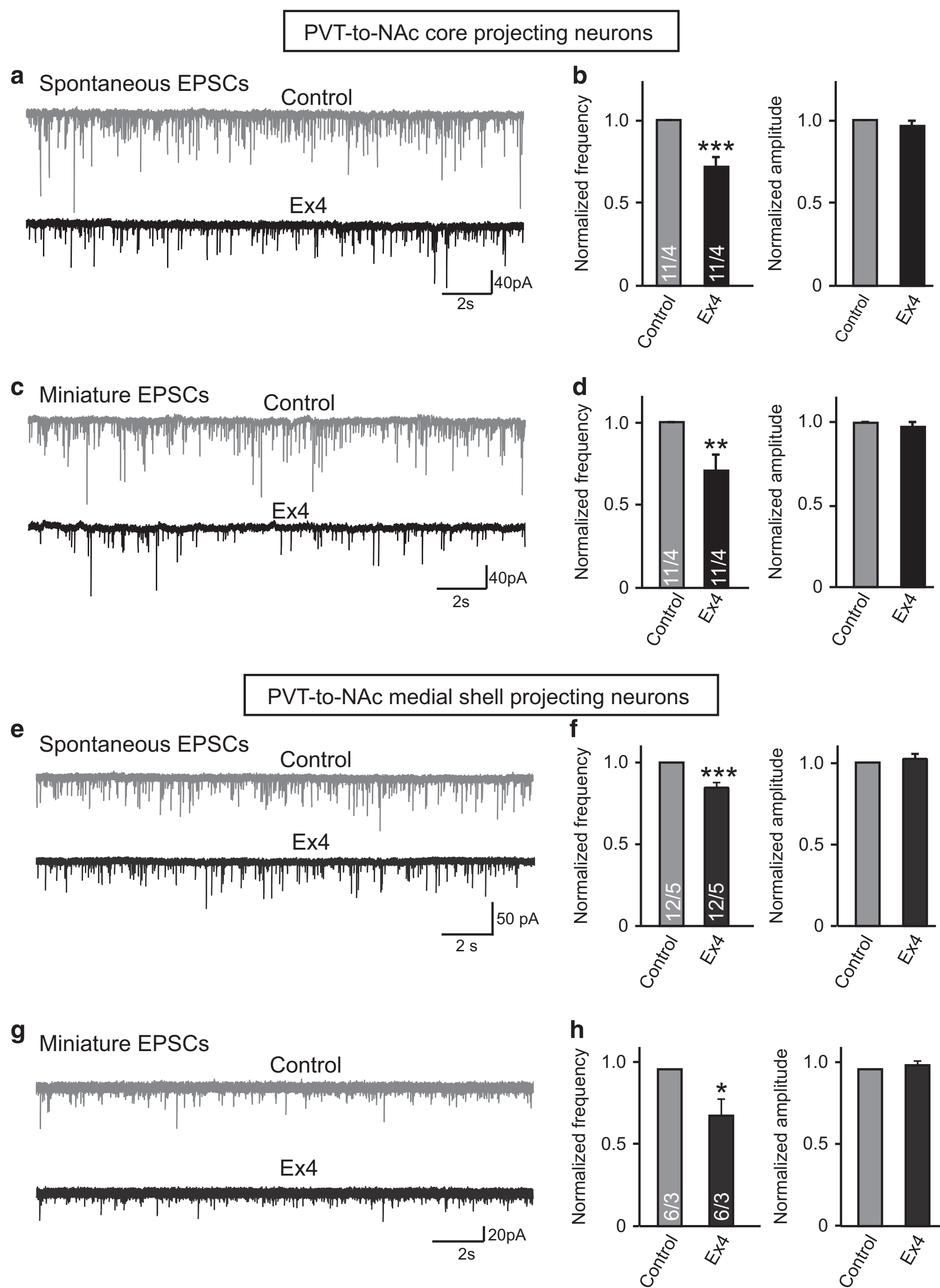

Figure 5 Ex4 suppressed both spontaneous and miniature EPSCs in PVT-to-NAc core and shell projecting neurons. (a–d) In PVT-to-NAc core projecting neurons, Ex4 application decreased the frequencies of both (a and b) sEPSCs and (c and d) mEPSCs. (e–h) In PVT-to-NAc shell projecting neurons, application of Ex4 decreased the frequencies of both (e and f) sEPSCs and (g and h) mEPSCs. The number of neurons recorded the number of mice used are indicated in the bar (neurons/mice). Data are presented as mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

habits (Stanton *et al*, 1990). Interestingly, the magnitude of fMRI responses evoked by viewing high-calorie food images (food cues) was reduced by systemic administration of long acting GLP-1 analogs (Ten Kulve *et al*, 2015; van Bloemendaal *et al*, 2015) that decreased subsequent food intake (van Bloemendaal *et al*, 2015). These data suggest a role for central GLP-1R signaling in preventing cue-induced reinstatement of food-seeking behavior, and are consistent with our findings which highlight the role of PVT GLP-1R agonism in mediating food intake and food-seeking behaviors.

We also pursued the hypothesis that PVT projections to NAc serves as a possible pathway for the effects of PVT GLP-1R signaling. PVT neurons project densely to the NAc (Vertes and Hoover, 2008) and studies show that orexin receptor or electrical stimulation of PVT neurons increases

NAc dopamine release (Choi et al, 2012; Parsons et al, 2007), a primary mediator of addiction and reward behaviors. Furthermore, PVT-to-NAc projecting neurons are activated by context-induced reinstatement of alcohol-seeking (Hamlin et al, 2009) and food-paired cues (Haight et al, 2016), and activation of Glut2-expressing PVT-to-NAc neurons increases food-motivated behaviors (Labouebe et al, 2016). These findings suggest a role for PVT-to-NAc projecting neurons in regulating reward-driven behaviors and thus might play a role in PVT GLP-1R signaling function. Here, we first identified the anatomical distribution of PVT GLP-1 fibers and putative GLP-1R-expressing PVT-to-NAc projecting cells, and then examined the impact of Ex4 on PVT-to-NAc cell excitability. We found that NTS GLP-1-positive axon terminals closely appose NAc-projecting PVT cells that express GLP-1R. Electrophysiological analyses of PVT-to-NAc projecting cells revealed that activation of PVT GLP-1R suppressed AP firing in these neurons, which suggests an inhibitory effect of Ex4 delivery to PVT cells. Ex4-induced hyperpolarization and slower attenuation of Ex4-mediated AP firing suppression in the presence of synaptic blockers suggests a cellular mechanism that involves both synaptic transmission and cell autonomous effects of GLP-1R activation. Further evaluation of these results revealed a presynaptic reduction of excitatory synaptic release and a postsynaptic increase in inhibitory efficacy by PVT GLP-1R signaling. Overall, these data show that GLP-1R signaling in PVT-to-NAc projecting neurons suppressed neuronal excitability. We propose that the inhibition comes from three sources: (1) presynaptic suppression of excitatory synaptic release; (2) postsynaptic facilitation of inhibitory synaptic strength; and (3) synaptic independent suppression of neuronal excitability.

Given that the majority of PVT-to-NAc projecting neurons are glutamatergic (Christie et al, 1987), it is possible that Ex4-induced inhibition of these efferents would reduce glutamate inputs to NAc. Glutamatergic signaling in NAc facilitates motivation and hedonic liking of palatable foods such that blocking NAc metabotropic glutamatergic signaling reduces food intake and food-liking (Richard and Berridge, 2011). On the other hand, blocking ionotropic glutamatergic signaling, for example, with AMPA receptor antagonist, increases food intake (Faure et al, 2008; Maldonado-Irizarry et al, 1995; Urstadt et al, 2013). Thus the different functional effects of NAc glutamatergic signaling depends on the type of glutamatergic receptors. Whether inhibiting PVT-to-NAc cells through PVT GLP-1R signaling impacts on metabotropic or ionotropic glutamate receptor signaling is unclear but a recent study by Labouebe et al (2016) showed that optogenetic activation of Glut2-expressing PVT-to-NAc neurons increases EPSCs on NAc cells and that this increase was inhibited by application of a AMPA receptor antagonist. While it is tempting to postulate that the motivation stimulatory effects of Glut2 PVT-to-NAc activation is mediated by activation of AMPA receptor signaling, it is important to note that AMPA receptor antagonism causes robust increases in food intake (Faure et al, 2008; Maldonado-Irizarry et al, 1995; Urstadt et al, 2013). This direction of effect is in contrast to the findings of Labouebe et al, therefore it may be more likely that NAc metabotropic glutamate receptors, which stimulation leads to increases in food intake, are mediating the effects of Glut2 PVT→ NAc activation on the motivation to procure sucrose. PVT terminals also come in close contact with NAc dopamine fibers (Pinto et al, 2003) and that blocking NAc ionotropic glutamate receptors prevents PVT stimulation-induced NAc dopamine efflux (Parsons et al, 2007). Whether the intake inhibitory effects of PVT Ex4 are mediated through reduced NAc glutamate and/or dopamine signaling are important research questions to pursue to further understand the downstream mechanisms of PVT GLP-1R signaling on food intake control. While only PVT-to-NAc neurons were examined here, future studies should also investigate the contribution of other PVT projecting targets in mediating the effects of PVT GLP-1R signaling.

Although the inhibitory effect of GLP-1R signaling on neuronal excitability of PVT-to-NAc projecting neurons contrasts with reports of excitatory effects of GLP-1R signaling in other brain regions of mice and rats (eg, hypothalamus (Acuna-Goycolea and van den Pol, 2004; Cork et al, 2015), NAc core (Mietlicki-Baase et al, 2014), ventral tegmental area (VTA) (Mietlicki-Baase et al, 2013), and parabrachial nucleus (Richard et al, 2014)), it is consistent with the c-Fos data reported here and with previous reports on GLP-1R signaling in pancreas-projecting dorsal vagal motor neurons (Wan et al, 2007), arcuate neuropeptide Y neurons (Secher et al, 2014), and NAc-projecting VTA dopamine neurons (Wang et al, 2015). Heterogeneity in the response to GLP-1R agonism is also reported within a single neural substrate: PVN GLP-1R activation increases APs in some neurons but suppresses APs in others (Acuna-Goycolea and van den Pol, 2004); in NAc core, while GLP-1R activation reduced EPSC frequency, AP was slightly increased, suggesting differential pre- and post-synaptic effects of GLP-1R activation (Mietlicki-Baase et al, 2014). Furthermore, the classical GLP-1R signaling pathway observed in pancreatic beta-cells (Goke and Conlon, 1988; Goke et al, 1989) and NTS (Hayes et al, 2011) that emphasizes the activation of adenylate cyclase via Gs protein has been expanded to include coupling of Gi/Go to GLP-1R shown in other cell systems (Galera et al, 1996; Hallbrink et al, 2001). These findings therefore suggest that the cellular mechanisms of GLP-1R signaling may be more heterogeneous than previously expected and that its differential function may be dependent upon neuronal subtype.

In the present study, we only focused on the role of GLP-1R signaling in the medial PVT (mPVT). Recent studies are beginning to show differential behavioral effects when targeting the anterior PVT (aPVT) vs the posterior PVT (pPVT). For example, orexin and substance P receptor signaling in the aPVT, but not the pPVT, increases alcohol intake (Barson et al, 2015, 2017), and activation of aPVT→ NAc pathway, but not the pPVT → NAc circuit, reduces sucrose-seeking (Do-Monte et al, 2017). Given the close proximity of mPVT to the aPVT and pPVT, it is possible that the effects observed in the present study may include the aPVT and/or pPVT. Whether GLP-1R signaling in the aPVT vs pPVT results in differential behavioral outcomes remains to be examined.

In summary, the data presented show that PVT GLP-1R signaling contributes to the motivational and appetitive aspects of feeding control. Our findings highlight the PVT as a novel site of action for GLP-1R signaling on food intake and reward and show for the first time that NTS PPG

neurons that project to the PVT are activated by food intake and that PVT GLP-1R activation reduced excitation of PVT-to-NAc projecting neurons. Future studies examining the PVT neural circuits mediating the intake inhibitory effects of PVT GLP-1R signaling are warranted to provide additional mechanistic details of the pathways involved in regulating feeding behaviors and energy homeostasis.

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