

ORIGINAL ARTICLE

A hippocampus to prefrontal cortex neural pathway inhibits food motivation through glucagon-like peptide-1 signaling

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The hippocampus and the medial prefrontal cortex (mPFC) are traditionally associated with regulating memory and executive function, respectively. The contribution of these brain regions to food intake control, however, is poorly understood. The present study identifies a novel neural pathway through which monosynaptic glutamatergic ventral hippocampal field CA1 (vCA1) to mPFC connectivity inhibits food-motivated behaviors through vCA1 glucagon-like peptide-1 receptor (GLP-1R). Results demonstrate that vCA1-targeted RNA interference-mediated GLP-1R knockdown increases motivated operant responding for palatable food. Chemogenetic disconnection of monosynaptic glutamatergic vCA1 to mPFC projections using designer receptors exclusively activated by designer drugs (DREADDs)-mediated synaptic silencing ablates the food intake and body weight reduction following vCA1 GLP-1R activation. Neuropharmacological experiments further reveal that vCA1 GLP-1R activation reduces food intake and inhibits impulsive operant responding for palatable food via downstream communication to mPFC NMDA receptors. Overall these findings identify a novel neural pathway regulating higher-order cognitive aspects of feeding behavior.

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INTRODUCTION

The hippocampus and the medial prefrontal cortex (mPFC) are brain regions associated with the 'higher-order' control of memory and executive function. Recent findings reveal that these brain regions are structurally and functionally interconnected.^{1–4} For example, ventral hippocampus (vHP) inputs to the prelimbic (PL) and infralimbic (ILA) regions of the mPFC are involved in the control of contextual fear conditioning and pharmacological antidepressant responses.^{3,5,6} However, the contributions of the vHP to mPFC neural pathways in controlling other fundamental motivated behaviors are less understood. Emerging research reveals that hippocampal and mPFC neural processes independently have a critical impact on energy balance and food-motivated behaviors.^{7–14} For example, vHP neural activity has potent effects on several aspects of feeding behavior through integration of endocrine feeding signals (e.g. ghrelin and leptin) with external environmental and interoceptive stimuli.¹⁴ Similarly, the PL and ILA regions of the mPFC control feeding through dopaminergic and opioid systems^{15–17} and regulate environmental cue-driven feeding^{12,13,18} and impulsive responding for food.^{19–21} Whether a vHP to mPFC neural pathway orchestrates higher-order and cognitive aspects of feeding behavior is unknown.

Feeding behavior is regulated, in part, via various peripherally derived endocrine and centrally derived neuropeptidergic signals^{22,23} that communicate to neurons in the hypothalamus and caudal brainstem.²⁴ Glucagon-like peptide-1 (GLP-1) is a hormone that is primarily derived from the distal small intestine and from neurons in nucleus tractus solitarius of the hindbrain.²⁵ Peripheral and central activation of GLP-1 receptors (GLP-1R)

produces glucose-dependent insulin secretion and reduces food intake and body weight.^{26–30} Importantly, GLP-1 analogs were recently FDA approved for weight loss and this system is of high interest for the development of future anti-obesity pharmacotherapies.^{29,31,32} Current research into the neural substrates mediating GLP-1R-induced hypophagia has expanded from traditional lower-order hypothalamic and hindbrain 'feeding centers'^{33–36} to include structures within the mesolimbic reward system and other forebrain sites.^{37–42} For example, GLP-1Rs are expressed in the vHP,^{43,44} and recent work from our laboratory demonstrates that pharmacological activation of vHP GLP-1Rs potently reduces caloric intake and body weight.⁴⁵ The extent to which endogenous vHP GLP-1R signaling controls food-motivated behaviors, however, is unknown. Moreover, the neural circuits through which the GLP-1R system suppresses motivated responding for palatable food have not been investigated.

The present study examines whether vHP GLP-1R signaling engages downstream mPFC signaling to control higher-order aspects of feeding behavior. Specifically, we hypothesize the presence of a novel functional neural pathway whereby vHP field CA1 (vCA1) glutamatergic pyramidal neurons are activated by GLP-1R agonists, which in turn engage downstream activation of mPFC glutamate receptors to inhibit motivated and impulsive responding for palatable food. This hypothesis is investigated using vHP-targeted RNA-interference-mediated GLP-1R knockdown (KD), classic and virally mediated neural pathway tracing, fluorescent *in situ* hybridization (FISH) and pathway-specific synaptic silencing via inhibitory designer receptor exclusively activated by designer drugs (DREADDs) in combination with behavioral neuropharmacology.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Envigo, Indianapolis, IN, USA; PND 60; 320–450 g) were individually housed in a temperature controlled vivarium with *ad libitum* access (except where noted) to water and food (LabDiet 5001, LabDiet, St. Louis, MO, USA) on either 12 h:12 h light/dark cycle or 12 h:12 h reverse light/dark cycle. For all surgical procedures, rats were anesthetized via intramuscular ketamine 90 mg/kg and xylazine, 2.8 mg/kg and acepromazine, 0.72 mg/kg injections, followed by subcutaneous injections for analgesia (Ketoprofen, 5 mg/kg). All procedures were approved by the Institute of Animal Care and Use Committee at the University of Southern California.

Viral preparations and central drug injections

Central drug injections. Experiments involving central drug injections included unilateral vHP injections of exendin-4 (American Peptide, Sunnyvale, CA, USA) and mPFC injections of clozapine-n oxide (CNO, National Institutes of Health), AP-5 (Sigma-Aldrich, St. Louis, MO, USA), or CNQX (R&D Systems, Minneapolis, MN, USA). Drugs were dissolved in artificial cerebral spinal fluid (aCSF) except for CNO, which was dissolved in 33% DMSO and 66% aCSF. Drugs were administered using a microinfusion pump (Harvard Apparatus, Holliston, MA, USA) connected to a 33-Gauge microsyringe injector through the indwelling guide cannulae. Flow rate was calibrated and set to 5 μ l/min and 100 nl injection volume. Injectors were left in place for 30s to allow for complete infusion of the drug. See Supplementary Materials for further details regarding stereotaxic placement of vHP and mPFC guide cannulae and central drug administration.

AAV-mediated RNA interference for GLP-1R. For *in vivo* knockdown of GLP-1R expression, short hairpin RNA targeting GLP-1R mRNA was cloned and packaged into an adeno-associated virus (AAV1) under the control of a U6 promoter and co-expressing green fluorescent protein (GFP) downstream of a Cb7 promoter (titer = 5.22×10^{12} GC ml⁻¹), described in more detail in Schmidt *et al.*⁴⁶ and Alhadeff *et al.*⁴⁷ A scrambled shRNA, GFP-expressing AAV1 downstream of a Cb7 promoter (titer = 5.22×10^{12} GC ml⁻¹) was used as a control. AAVs or aCSF were then delivered bilaterally to the vHP [AP (anterior/posterior): -4.9 ML (medial/lateral): ± 4.8 DV (dorsal ventral): -7.8] at an injection volume of 200 nl via pressure injection with the microinfusion pump setup described above.

Following behavioral procedures (~60 days), animals were euthanized and brains were removed and flash frozen in dry-ice cooled hexane until processing as described in Alhadeff *et al.*⁴⁷ and Kanoski *et al.*⁴⁸ Tissue processing involved simultaneous histological verification and region of interest tissue micropunch collection (Supplementary Materials); collected vHP tissue was then used for quantitative PCR (qPCR) analyses for GLP-1R mRNA, described in more detail in Supplementary Materials.

AAV2-CaMKIIa-hM4di-DREADDs. CaMKIIa-promoted hM4di DREADDs (AAV2-CaMKIIa-HA-hM4Di-IRES-mCitrine) were obtained from the UNC Gene Therapy Vector Core. Animals received chronic indwelling cannulae targeting the vHP and mPFC. Following recovery, animals received 200 nl pressure injections of the AAV-CaMKIIa-hM4di DREADDs or a control virus (AAV2-CaMKIIa-GFP) to the vHP through the indwelling guide cannulae.

Immunohistochemistry

Animals were anesthetized, then transcardially perfused with ice-cold 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5). Brains were removed and immersed in 12% sucrose in PFA fixative for 20–24 h at 4 °C. The brains were then flash-frozen in dry-ice cooled isopentane before sectioning on a sliding microtome at 30 μ m. Sections are stored in antifreeze solution at -20 °C until immunohistochemistry (IHC) processing. IHC processing details are described in Supplementary Materials. Primary and secondary antibodies utilized are described in each experiment below. Photomicrographs were acquired using a Nikon 80i (Nikon DS-Q11, 1280 \times 1024 resolution, 1.45 megapixel) under epifluorescent illumination.

Fluorescent *in situ* hybridization for GLP-1R

Animals were fixation perfused and tissue was harvested and processed as described above in IHC methods. Flash-frozen brains were sectioned as above at 20 μ m and stored at -20 °C until FISH processing. Detailed FISH procedures are described in Supplementary Materials.

Anterograde neural pathway tracing

Neural pathway tracing experiments utilized two tracing strategies: anterograde tracing from the vCA1 following unilateral iontophoretic injections of PHAL (Vector Labs, Burlingame, CA, USA; 2.5% in 0.1 M sodium phosphate-buffered saline, pH 7.4) or AAV1-GFP (AAV1-hSyn-eGFP-WPRE-bGH)^{49–51} (Penn Vector Core, Philadelphia, PA, USA). Iontophoresis was performed using a precision current source (Digital Midgard Precision Current Source, Stoelting, Wood Dale, IL, USA) as described previously.⁵²

Experiment 1: the effects of vHP GLP-1R knockdown on energy balance and food-motivated behaviors

Animals ($n = 32$) received one of the following 200 nl, bilateral injections to the vHP as described above: AAV-GLP-1R shRNA (referred to as vHP GLP-1R KD group; $n = 11$), control AAV ($n = 11$), or aCSF ($n = 10$). After surgical procedures, food intake (spillage accounted for) and body weight were tracked daily for 40 days. Next, a representative subset of animals (based on representing median body weight group averages from the viral groups) (vHP GLP-1R KD $n = 6$, control AAV $n = 6$) underwent meal pattern analyses in chambers equipped with automated food intake monitors (Med Associates, Fairfax, VT, USA). aCSF controls were not included in this analyses because of equipment limitations at the time of the study, however this group of animals did not differ in GLP-1R mRNA expression or other behavioral measures from the control AAV control group (Figure 1). The automated food intake monitors record the weight of the food hopper every 10 s, allowing for ongoing analysis of meal patterns. Animals were first habituated to the chambers for 24 h with *ad libitum* access to food and water. Analyzed data are from a second 24 h period immediately following the habituation session. Meals were defined as an episode of feeding where at least 0.25 g was ingested, with meal termination defined as the beginning of a pause in ingestion of at least 10 min.⁴⁵ Data were objectively calculated with a custom Microsoft Excel macro.⁴⁵

To measure food-motivated behaviors, animals (vHP GLP-1R KD $n = 10$, control AAV $n = 11$, aCSF control $n = 9$) were trained to lever press in operant-conditioning boxes (Med Associates) for a palatable 45 mg food pellet (35% kcal fat enriched with sucrose, F05989, Bio-Serv, Flemington, NJ, USA) on a FR1 reinforcement schedule. Training occurred in 1 h sessions over a 5-day period. This procedure included one 'active' lever (right) that produced the food reinforcement, and a left 'inactive' lever that served as a dummy lever for the measurement of general lever pressing activity.

Experiment 2: investigation of vHP projections to the mPFC

Rats received either a unilateral iontophoretic injection of PHAL ($n = 4$) or AAV1-GFP ($n = 2$) targeting the vHP. Following a survival period (10 days for PHAL and 14 days for AAV1-GFP), animals were fixation-perfused and tissue was harvested and processed as described above. IHC detection of PHAL proceeded according to the described procedures using goat anti-PHAL primary antibody (1:5000, Vector Labs, Burlingame, CA, USA), followed by a donkey anti-goat secondary antibody conjugated to Cy3 (1:500, Jackson Immunoresearch). AAV1-GFP was detected based on a combination of native fluorescence of GFP and amplification of signal using a chicken anti-GFP primary antibody (1:500, Abcam) followed by a donkey anti-chicken secondary antibody conjugated to AF488 (1:500, Jackson Immunoresearch, West Grove, PA, USA). Representative images were obtained from animals with PHAL or AAV1-GFP injection sites that were confined to the vCA1.

Experiment 3: investigation of mPFC inputs from vHP GLP-1R expressing neurons

Rats ($n = 4$) received a unilateral 100 nl pressure injection of fluorogold (FG, Fluorochrome, Denver, CO, USA; 2% in 0.9% NaCl) targeting the mPFC. Following a survival period of 10 days, animals were fixation-perfused and tissue was harvested and processed as described in 'Methods' section. A representative animal with fluorogold back-labeled neurons in the vHP (field CA1) was selected for GLP-1R FISH analyses (Methods; Supplementary Materials). To observe separate GLP-1R mRNA in vHP cell bodies (as fluorogold and DAPI autofluoresce in the same channel), the protocol above was repeated in a separate untreated animal where DAPI (ACD, 320851) was placed on the slide prior to coverslipping.

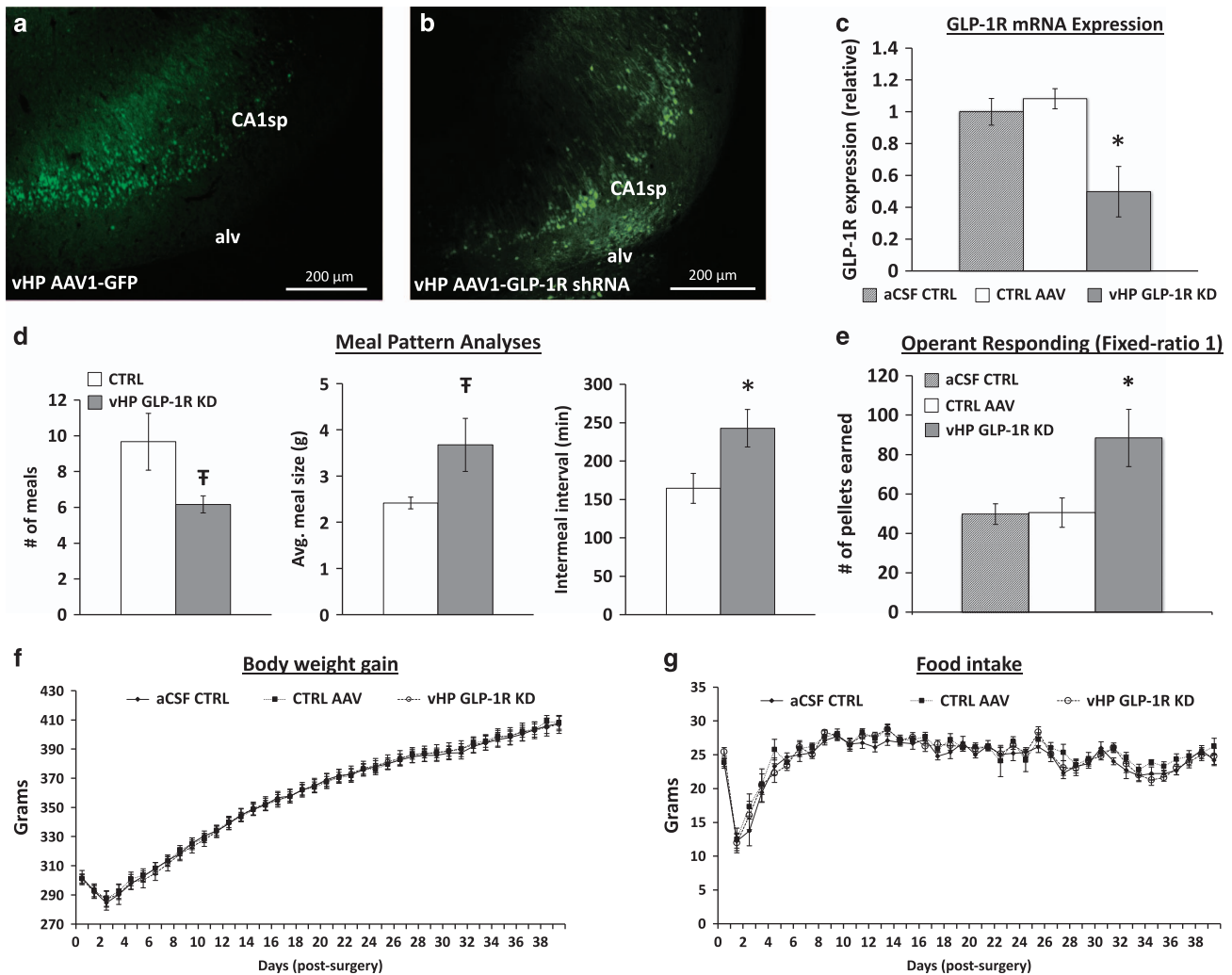


Figure 1. vHP-targeted (a) AAV-GFP and (b) AAV-GLP-1R shRNA delivery transfects vHP neurons. (c) vHP AAV-GLP-1R shRNA significantly reduces GLP-1R mRNA expression. (d) Meal pattern analyses in vHP GLP-1R KD vs CTRL AAV rats. (e) Operant responding on a FR1 reinforcement schedule in vHP GLP-1R KD vs CTRL animals. (f) Body weight gain and (g) food intake in vHP GLP-1R KD vs CTRL groups. * $P < 0.05$ vs CTRL AAV or aCSF controls; [†] $P < 0.07$. Data are mean \pm s.e.m. AAV, adeno-associated virus; alv, alveus; CA1sp, CA1 pyramidal cells; KD, knockdown; vHP, ventral hippocampus.

Experiment 4: the effects of vHP to mPFC disconnection with inhibitory DREADDs on vHP GLP-1R-mediated feeding

Rats were implanted bilaterally with vHP and mPFC-targeted cannulae ($n = 10$) and received injections of AAV2-CaMKIIa-hM4Di DREADDs to the vHP as described above. After an incubation period of 30 days, animals were given unilateral injections of CNO (3 mm) or vehicle to the mPFC, which was followed by unilateral vHP administration of exendin-4 (0.03 μ g) 1 h into the light cycle. Food intake (spillage accounted for) and body weight were measured 24 h later. This study utilized a counter-balanced within-subjects design with 2–3 intervening days between treatments. Doses for CNO and exendin-4 were based on Mahler *et al.*,⁵³ Stachniak *et al.*,⁵⁴ and Hsu *et al.*⁴⁵ As a control experiment, a separate cohort of animals ($n = 7$) received vHP AAV2-CaMKIIa-GFP and the design above was replicated.

Experiment 5: investigating the effects of mPFC glutamate receptor blockade on vHP GLP-1R-mediated food intake

Rats ($n = 11$) were implanted bilaterally with vHP and mPFC-targeted cannulae. One hour into the light cycle, a NMDA receptor antagonist, AP-5 (0.5 μ g), or vehicle was delivered unilaterally into the mPFC, which was then followed by vHP exendin-4 (0.03 μ g) or vehicle delivery. Food intake (spillage accounted for) and body weight were measured 24 h later. This study utilized a counter-balanced within-subjects design with 2–3 intervening days between treatments. This design was then replicated in a separate cohort of rats ($n = 9$) using an AMPA/Kainate receptor

antagonist, CNQX (0.3 μ g), delivered to the mPFC. Dose selection for AP-5 and CNQX are based on Mietlicki-Baase *et al.*⁴⁰

Experiment 6: investigating the molecular mechanisms underlying vHP GLP-1R to mPFC NMDA-R signaling

Animals received unilateral vHP delivery of either exendin-4 (0.03 μ g, $n = 6$) or vehicle ($n = 6$). Ninety minutes later, animals were perfused and tissue was harvested as described above. IHC detection of phosphorylated NR2B (pNR2B) was performed according to the procedures above using rabbit anti-pNR2B (1:1000, Abcam, Cambridge, UK) followed by a donkey anti-rabbit secondary antibody conjugated to biotin. The tissue was incubated in ABC reagent (1:1000, Vector) for 5 h, washed in potassium phosphate buffered saline, and then incubated in biotinylated tyramine (1:1000) for 10 min. The tissue is then incubated overnight in a Cy3-conjugated streptavidin (1:500) complex. Quantification of pNR2B signal intensity was performed using ImageJ v1.48, where the mPFC region of interest was selected and mean pixel intensity was determined for each animal. Data are represented as the mean pixel intensity relative to the vehicle group.

Experiment 7: the effects of mPFC NMDA receptor blockade on the vHP GLP-1R-regulated food impulsivity behavior

To measure food impulsivity behaviors, we adapted protocols for a task known as ‘differential reinforcement of low rates of responding’

(DRL).^{20,21,55} Briefly, rats ($n=10$) were trained to lever press in operant-conditioning boxes (Med Associates) for a palatable 45 mg food pellet (35% kcal fat enriched with sucrose, F05989, Bio-Serv) on a DRL 0 reinforcement schedule, where animals are not required to wait an allotted time between lever presses and are reinforced immediately upon a lever press. After 5 days of DRL 0, animals are then placed on DRL 5. Here, animals are required to withhold responding for 5 s before the subsequent lever press response results in food reinforcement. A lever press that occurs before the 5 s period resets the timer and no reinforcement is provided. After 5 days of DRL 5, animals underwent 5 days of DRL 10 (10 s between reinforced lever presses), and finally 10 days of DRL 20 (20 s between reinforced lever presses). During all 45 min training sessions, a brief (~1 sec) stimulus light turned on to signal reinforced lever presses. Efficiency in each DRL training schedule was calculated by the number of pellets earned/total number of lever presses.

Two days after the last day of DRL 20 training, animals were given four tests (within-subjects design, 2–3 intervening days between treatments). Prior to each test, animals were 24 h food deprived to induce high-impulsive drive.²¹ Unilateral mPFC injections of AP-5 (0.5 μ g) or vehicle and subsequent unilateral vHP injections of exendin-4 (0.03 μ g) were given 3 h before being tested in the DRL 20 task.

Data analysis. All experiments, except for GLP-1R qPCR analyses, met assumptions of normality and equality of variance and were analyzed using parametric repeated measures or one-way analysis of variance followed by Fisher least significant difference *post hoc* comparisons when significant main effects were obtained to compare individual treatments with control groups. When assumptions were not met, non-parametric Mann–Whitney tests were used. Data analyses were conducted with computer software (Statistica V7; Statsoft). The α level for significance was 0.05. All quantifiable data are displayed as mean \pm s.e.m. To ensure the minimum sample size required to be reasonably likely to detect the hypothesized effect, power analyses were conducted for each proposed experiment based on our pilot and recently published experiments using Statsoft software (Statistica 64, V7). Alpha level was set at 0.05 for power analyses; the root mean square standardized effect was estimated separately for each experiment.

RESULTS

Endogenous vHP GLP-1R signaling regulates feeding and food-motivated behaviors

To examine the relevance of endogenous vHP GLP-1R-mediated feeding and palatable food-motivated responding, animals received bilateral vCA1 administration of AAV1-GLP-1R shRNA (vHP GLP-1R KD; $n=11$), AAV1-GFP (AAV control; $n=10$) or aCSF (control; $n=10$). The latter control group is necessary in light of recent evidence that under some conditions AAVs can be neurotoxic,⁵⁶ and thus the control/scrambled AAV treatment can potentially have unintended neural or behavioral outcomes. However, in this case the aCSF control and AAV control groups did not differ significantly for any of the dependent variables (all P -values > 0.4). Following experimental procedures (~60 days post surgery), histological analyses confirmed successful transfection of both AAV-GFP and AAV-GLP-1R shRNA in vCA1 neurons (visualized with GFP, Figures 1a and b). One animal was omitted from analyses because the injection site did not include vCA1 and one KD outlier animal was removed based on excessive GLP-1R mRNA expression. Consistent with recently published work utilizing this AAV-GLP-1R shRNA knockdown approach,^{46,47} qPCR analyses of vHP AAV-GLP-1R shRNA transfected tissue showed a significant (~50%) reduction of GLP-1R mRNA compared to either CTRL AAV or aCSF controls (Figure 1c, $F(1,16)=8$, $P < 0.01$). vHP GLP-1R KD had no significant effects on long term food intake and body weight during the 5-week post-surgery period (Figures 1f and g). However, microstructural feeding analyses revealed that vHP GLP-1R KD alters meal patterning, evidenced by a trend towards greater meal size ($F(1,10)=4.5$, $P < 0.07$) with a compensatory decrease in meal frequency ($F(1,10)=4.7$, $P < 0.07$) in the vHP GLP-1R KD group ($n=6$), as well as a significant increase in inter-meal interval ($F(1,10)=6.3$, $P < 0.05$) compared to AAV controls

($n=6$) (Figure 1d). No group differences were detected for satiety ratio, calculated as inter-meal interval/meal frequency (data not shown).

To examine the endogenous relevance of vHP GLP-1R signaling on food-motivated behaviors, rats were trained to lever press for a palatable 45 mg food pellet (35% kcal fat enriched with sucrose) on a fixed ratio-1 reinforcement (FR1) schedule. Results show a significant main effect of group ($F(2,26)=4.39$, $P < 0.05$) where vHP GLP-1R KD animals ($n=10$) earned a significantly greater number of pellets across a 5-day training period compared to either CTRL AAV ($n=11$) or aCSF controls ($n=9$) ($P < 0.05$) (Figure 1e). Collectively, these data indicate that vHP GLP-1R signaling endogenously regulates meal patterning and effort-based responding for palatable food.

The vCA1 sends a monosynaptic input to the ipsilateral ILA and PL regions of the mPFC

Extending the findings of previous work,^{1,2} our results reveal an exclusively ipsilateral monosynaptic connection from the vHP to the mPFC (ILA and PL regions) using both traditional and virus-based anterograde pathway tracing methods. Animals received a unilateral vCA1 iontophoretic injection of the anterograde pathway tracer *Phaseolus vulgaris* leucoagglutinin (PHAL) or an AAV that labels axons in an anterograde fashion (AAV1-hSyn-eGFP-WPRE-bGH; henceforth referred to as 'AAV1-GFP') (representative injection sites depicted in Figures 2b and e). This AAV is used by the Allen Institute for Brain Science⁵¹ and in the literature⁵⁰ for anterograde tracing purposes. Results demonstrate extensive PHAL immunoreactive and AAV1-GFP axon terminal fields in the PL and ILA that were exclusively ipsilateral to the vCA1 injection (Figures 2a,c,d and f). Figures 2g–j display a schematic representation of the distribution AAV1-GFP axon terminal fields in the mPFC. We note that while previous reports have investigated vHP to mPFC projections,¹ here we examine a novel vCA1 subregion that is caudal and lateral to previously investigated vHP anterograde projection pathways. Moreover, this is the first report to our knowledge to directly compare PHAL with AAV1-GFP anterograde neural pathway tracing from the same injection area.

vHP GLP-1R-expressing neurons project to the mPFC

We detected GLP-1R mRNA expression in vHP (field CA1) pyramidal layer neurons (Figure 3a) via FISH, an outcome consistent with previous work utilizing a mouse model with transgenic GFP expression in GLP-1R-expressing neurons⁴⁴ and with a publication demonstrating radio-labeled *in situ* hybridization signal in this region.⁴³ In all rats that received mPFC fluorogold (FG) injections ($n=4$; representative injection site in Figure 3b), extensive FG expression in back-labeled vHP neurons was observed (representative FG-labeled cell bodies in Figure 3c). These data support the presence of a monosynaptic pathway between the vCA1 and mPFC, as also suggested in Figure 2. Moreover, vCA1 FISH analyses combined with native FG immunoreactivity from a representative mPFC FG-injected animal revealed that all FG back-labeled vCA1 cell bodies co-expressed GLP-1R mRNA (Figure 3d). Collectively, these data provide proof-of-principle evidence that vCA1 neurons express GLP-1Rs, and that mPFC-projecting vCA1 neurons are predominantly GLP-1R positive.

Reversible chemogenetic disconnection of vHP to mPFC neural circuitry abolishes vHP GLP-1R-mediated food intake reduction

Taking advantage of the ipsilateral monosynaptic connection between the vHP GLP-1R-expressing neurons and the mPFC, we utilized an hm4Di inhibitory DREADDs technique to silence synaptic communication to the mPFC from the vHP to investigate the functional relevance of this circuit to food intake. Recent

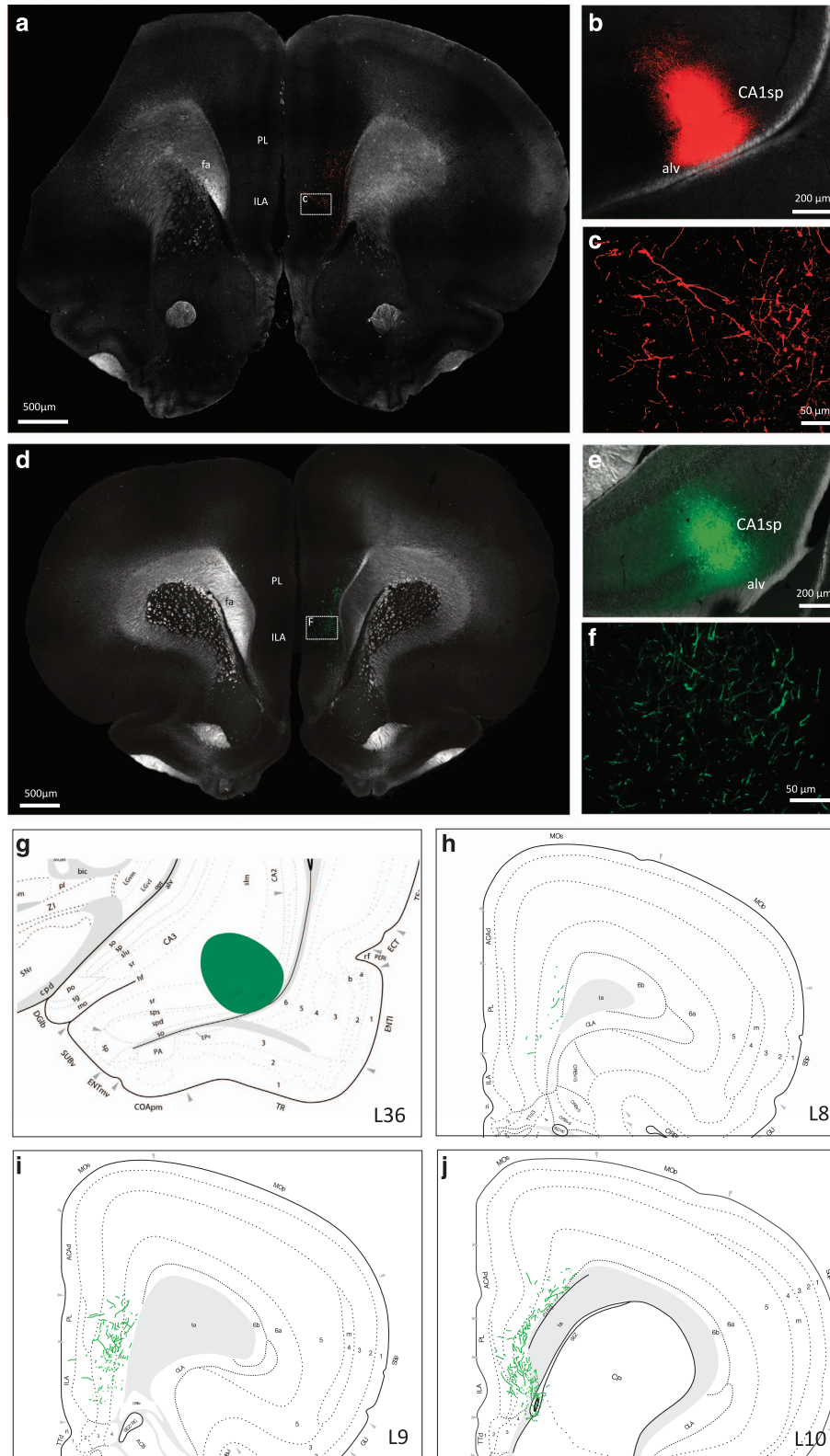


Figure 2. vCA1 neurons provide monosynaptic and unilateral input to the mPFC. Unilateral iontophoretic vCA1 delivery of either PHAL (red) or AAV1-GFP (green) (representative injection sites in **b** and **e** respectively) reveals (**a**, **c**) PHAL and (**d**, **f**) AAV1-GFP fluorescent axon terminal fields in the mPFC ipsilateral to the vCA1 injection. A schematic representation of mPFC AAV1-GFP axon terminal fields from the vHP is shown in **g–j**, where **g** displays a representative vCA1 AAV1-GFP injection site and **h–j** shows mPFC AAV1-GFP axon terminal field distribution (Swanson atlas levels 8–10). alv, alveus; CA1sp, CA1 pyramidal layer; fa, corpus callosum, anterior forceps; ILA, infralimbic area; mPFC, medial prefrontal cortex; PL, prelimbic area.

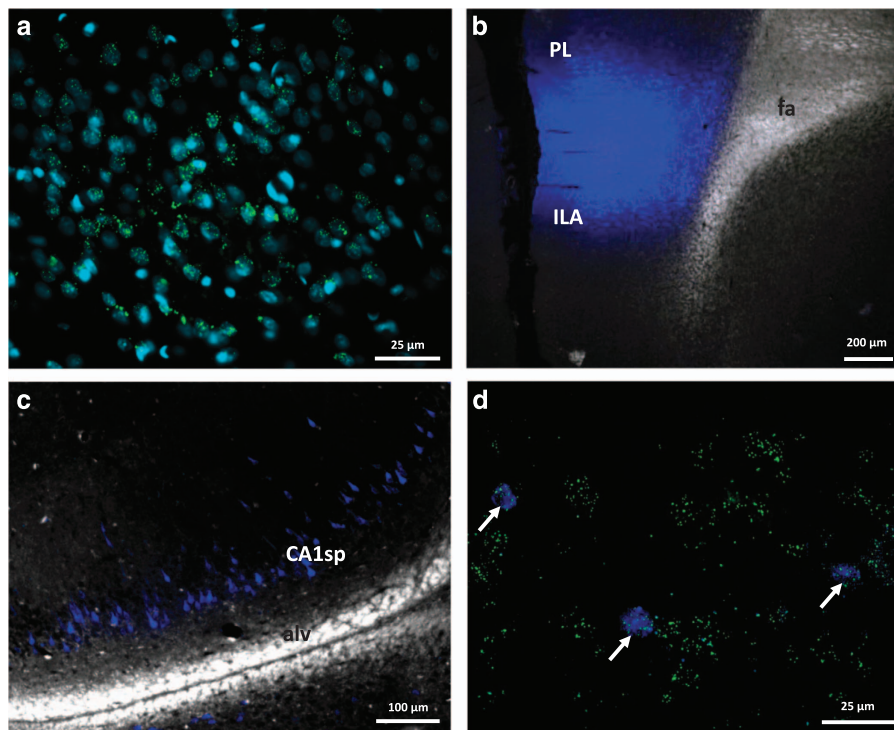


Figure 3. (a) GLP-1R mRNA expression (green) in vCA1 cell bodies (DAPI, teal). (b) Representative mPFC fluorogold injection site. (c) Distribution of backlabeled fluorogold vCA1 neurons that project to the mPFC. (d) Representative vCA1 neurons that project to the mPFC (fluorogold, blue) co-express GLP-1R mRNA (green). mPFC, medial prefrontal cortex.

findings show that AAV-hM4Di DREADDs are trafficked to axon terminals, and administration of the DREADDs ligand, CNO targeted to axon terminals silences synaptic communication to post-synaptic cells without affecting the firing rate of the presynaptic neurons.^{53,54} Utilizing the design outlined schematically in Figure 4a, animals ($n=10$) first received AAV2-CaMKIIa-hM4Di DREADDs in the vCA1, where the CamKIIa promoter allows for the selective expression of hM4Di in glutamatergic pyramidal neurons in the vCA1 (Figure 4b). Using a counterbalanced, within-subjects design, vHP hM4Di DREADDs-expressing animals received unilateral CNO (3 mM) or vehicle (VEH) targeted to the ILA and PL (representative injection site in Figure 3d following 100 nl Chicago Skyblue ink administration). Rats then received unilateral administration of the selective GLP-1R agonist, exendin-4 (EX4), or vehicle to the ipsilateral vCA1 (representative injection site in Figure 3e) and 24 h food intake and body weight were recorded. The dose of EX4 (0.03 μ g) was selected with reference to previous work⁴⁵ showing it to be without effects on feeding and body weight when delivered to the lateral ventricle. Results reveal a significant main effect of drug ($F(3,27) > 4.4$, $P < 0.01$), where the food intake and body weight reduction that is observed with vHP GLP-1R activation (Figure 4f, mPFC VEH/vHP VEH vs mPFC VEH/vHP EX4, $P < 0.05$), is eliminated when synaptic communication from the vHP to the mPFC is disconnected via mPFC CNO delivery (Figure 4f, mPFC VEH/vHP EX4 vs mPFC CNO/vHP EX4, $P < 0.05$). In contrast, animals receiving a control virus (AAV2-CaMKIIa-GFP; representative transfection in Figure 3c) in the vHP retain the food intake and body weight reduction resulting from vHP GLP-1R activation when pre-treated with CNO (Figure 4g, mPFC CNO/vHP VEH vs mPFC CNO/vHP EX4, $F(3,18) = 5.5$, $P < 0.05$; body weight mPFC CNO/vHP VEH vs mPFC CNO/vHP EX4, $F(3,18) = 4.2$, $P < 0.07$). Taken together, these data suggest that a monosynaptic neural pathway from the vHP to the mPFC is critical in regulating GLP-1R-mediated food intake.

vHP GLP-1R-mediated hypophagia requires mPFC NMDA receptor signaling

We next examined the role of different glutamate receptor subtypes in the mPFC in vHP-GLP-1R-mediated feeding. To determine the functional relevance of mPFC NMDA receptors (NMDAR) in the vHP GLP-1R-mediated hypophagic response, rats ($n=11$) first received a unilateral mPFC injection of a NMDA receptor antagonist, AP-5 (0.5 μ g) or vehicle. Immediately afterwards, animals received unilateral/ipsilateral vHP injections of EX4 (0.03 μ g). Treatments were counterbalanced using a within-subjects design. Results showed a main effect of drug ($F(3,30) = 9.8$, $P < 0.01$), where vHP EX4 administration significantly reduced food intake and body weight when animals were pretreated with mPFC vehicle (Figure 5a, $P < 0.01$). In contrast, pretreatment with mPFC AP-5 resulted in the attenuation of vHP EX4 induced food intake and body weight reduction (Figure 5a, mPFC VEH/vHP EX4 vs mPFC AP-5/vHP EX4, $P < 0.01$). Utilizing a similar experimental design ($n=9$), mPFC pretreatment with an AMPA/Kainate receptor antagonist, CNQX (0.3 μ g) had no effect on the food intake and body weight reduction induced by vHP exendin-4 administration (main effect of drug, $F(3,18) = 6.3$, Figure 5b, mPFC CNQX/vHP VEH vs mPFC CNQX/vHP EX4, $P < 0.05$). These data indicate that the full expression of vHP GLP-1R-mediated feeding requires mPFC NMDA-R signaling, but not AMPA/Kainate receptor signaling. Consistent with these findings, unilateral vHP EX4 ($n=6$; 0.03 μ g) administration significantly increased phosphorylation of NR2B, an NMDA-R subunit, in the mPFC 90 min after injection compared to vehicle treatment ($n=6$; Figure 5c, $F(1,10) = 12.6$, $P < 0.01$). Overall these data provide behavioral, neuroanatomical and molecular evidence consistent with the hypothesis that mPFC NMDA-R are a downstream target for vHP GLP-1R-mediated anorectic effects.

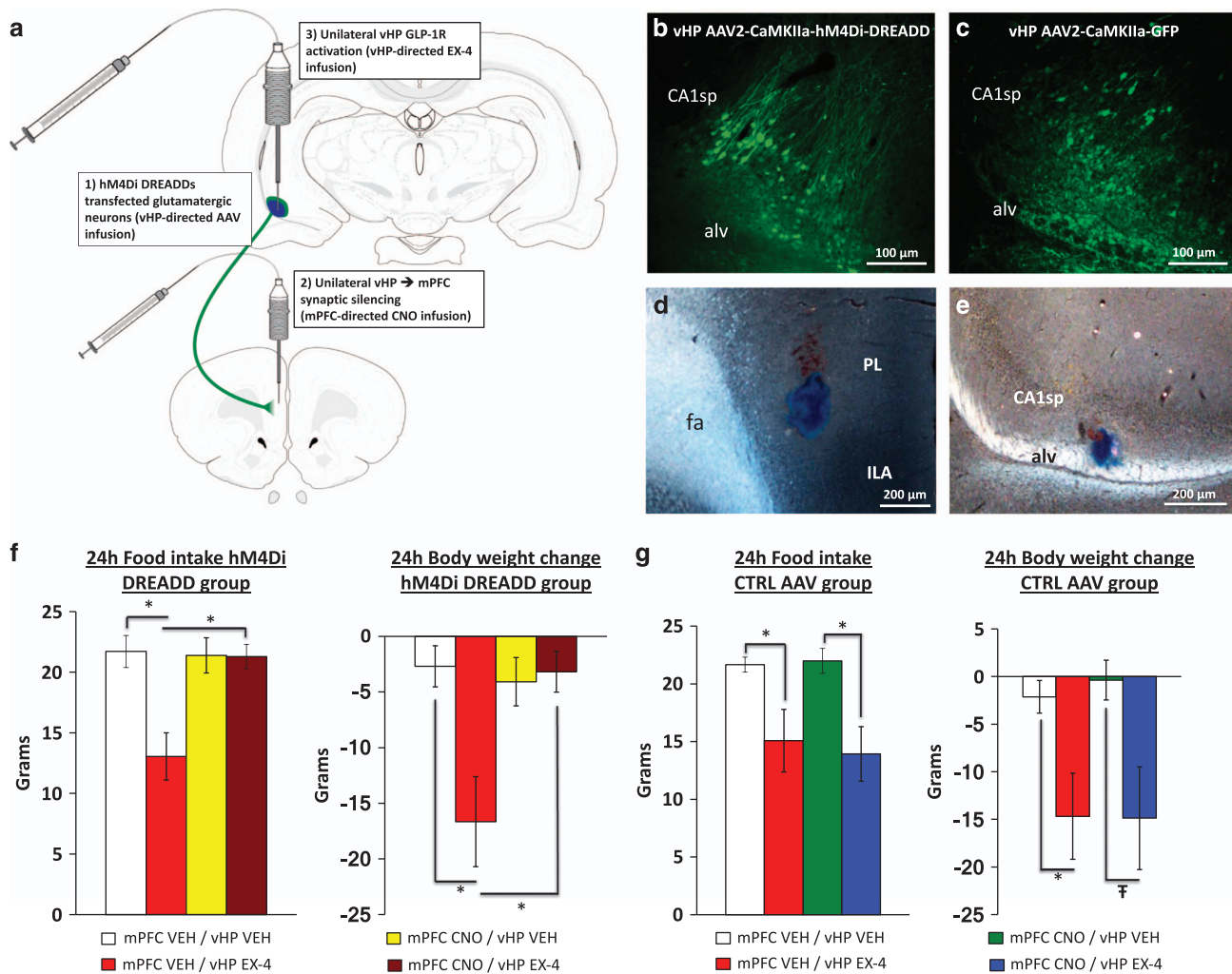


Figure 4. Reversible vHP to mPFC disconnection with inhibitory DREADDs. (a) Shows the experimental design involving DREADDs-mediated vHP-mPFC synaptic disconnection and pharmacological activation of vHP GLP-1Rs with exendin-4. (b) AAV2-CaMKIIa hM4Di DREADDs and (c) AAV2-CaMKIIa-GFP transfects vHP pyramidal neurons. (d) Representative mPFC and (e) vHP injection sites. (f) 24 h food intake and body weight following mPFC administration of CNO or vehicle (VEH) and vHP administration of exendin-4 (EX4) or VEH in vHP hM4Di DREADDs transfected animals. (g) 24 h food intake and body weight following mPFC administration of CNO or VEH and vHP administration of EX4 or VEH in vHP CTRL AAV animals. * $P < 0.05$; † $P < 0.07$. Data are mean \pm s.e.m. AAV, adeno-associated virus; DREADD, designer receptors exclusively activated by designer drugs; mPFC, medial prefrontal cortex; vHP, ventral hippocampus.

vHP GLP-1R to mPFC NMDA-R neural signaling inhibits impulsive responding for palatable food

To investigate the role of a vHP GLP-1R to mPFC NMDA-R neural pathway in food impulsivity, we utilized a behavioral procedure known as DRL as a measure of inhibitory control.^{20,21,55} Briefly, animals ($n = 10$) first learn to lever press for a palatable food reward (35% kcal fat enriched with sucrose). Following a series of training phases with escalating DRL delay criteria, rats acquired stable responding after 10 days of DRL 20 schedule, where lever presses are only reinforced if an animal waits 20 s between lever presses. Responses made before the 20 s period ends result in no food reinforcement and the timer is reset. Our data show that animals increase their efficiency (measured by pellets earned/total number of lever presses) in this task across a 10 day DRL 20 training period (Figure 6a, significant main effect of day, $F(9,108) = 11.9, P < 0.01$).

During test sessions in which impulsive food drive is upregulated via 24 h food restriction,²¹ results show that vHP EX4 administration (0.03 μ g) alone significantly increases efficiency in the DRL 20 task (main effect of drug, $F(3,30) = 6.3, P < 0.01$) (Figure 6b, mPFC VEH/vHP VEH vs mPFC AP-5/vHP EX4,

$P < 0.01$), suggesting that vHP GLP-1R activation reduces impulsive responding for food. Importantly, this effect is generated by a significant decrease in total lever presses (Figure 6b, mPFC VEH/vHP VEH vs mPFC VEH/vHP EX4, $P < 0.05$) with no differences in pellets earned, suggesting that vHP GLP-1R signaling did not entirely suppress appetitive drive, but rather increased the efficiency in earning food reinforcement. When combined with mPFC NMDA-R blockade via AP-5 pre-treatment, the increased efficiency elicited by vHP GLP-1R activation was significantly attenuated (Figure 6b, mPFC VEH/vHP EX4 vs mPFC AP-5/vHP EX4, $P < 0.01$), as demonstrated by a trend towards an increase in total lever presses for the mPFC AP-5/vHP EX4 treatment compared to mPFC VEH/vHP EX4 ($P < 0.07$) with no differences found in the number of pellets earned. Altogether these data suggest that a vHP GLP-1R to mPFC NMDA-R neural pathway regulates impulsive responding for palatable food.

DISCUSSION

The hippocampus is known for its role in regulating mnemonic processes, including those relating to declarative and visuospatial

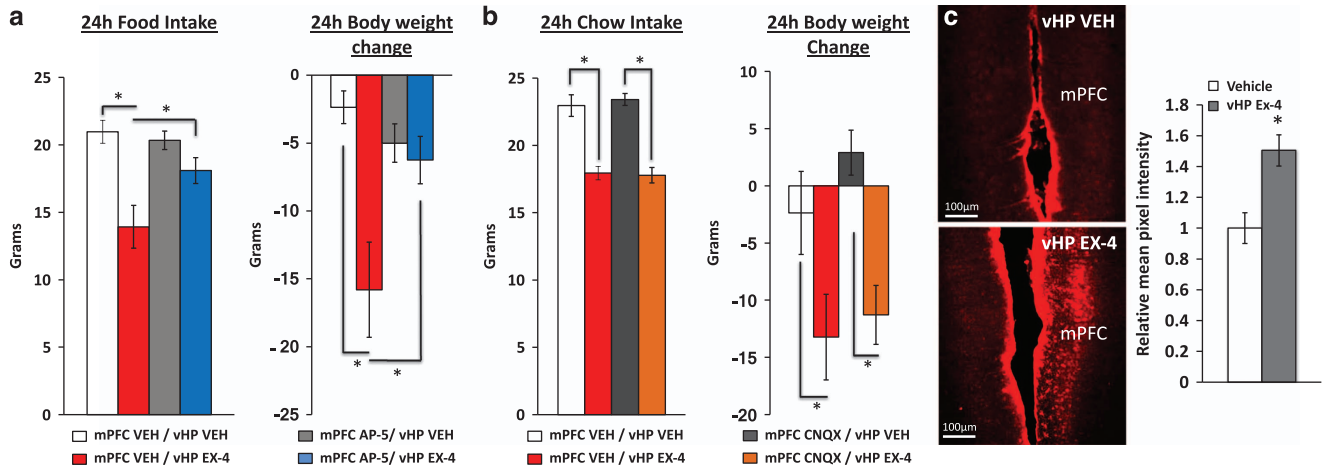


Figure 5. (a) 24-h food intake and body weight following mPFC administration of AP-5 or vehicle (VEH) and vHP administration of exendin-4 (EX-4) or VEH. (b) 24 h food intake and body weight following mPFC administration of CNQX or VEH and vHP administration of EX-4 or VEH. (c) Phosphorylated NR2B expression in the mPFC following unilateral vHP delivery of exendin-4 or vehicle. * $P < 0.05$. Data are mean \pm s.e.m. mPFC, medial prefrontal cortex; vHP, ventral hippocampus.

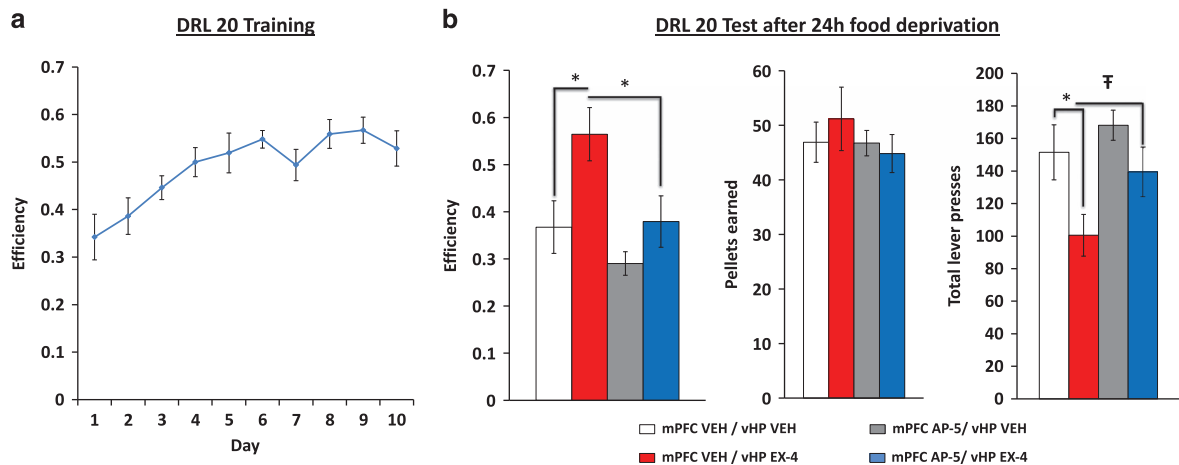


Figure 6. (a) DRL 20 efficiency across a 10 day training period. (b) DRL 20 test session after 24 h food deprivation in animals receiving mPFC AP-5 or vehicle and vHP exendin-4 or vehicle. * $P < 0.05$; $^{\dagger}P < 0.07$. Data are mean \pm s.e.m. DRL, differential reinforcement of low rates of responding; mPFC, medial prefrontal cortex; VEH, vehicle; vHP, ventral hippocampus.

memory. More recently this brain region has emerged as an important center for controlling higher-order aspects of feeding behavior.^{8,14} Hippocampal neurons respond to several types of gastrointestinal satiation signals^{57,58} and utilize endocrine signals to bi-directionally control energy balance and conditioned food-motivated behaviors.¹⁴ Using vHP-targeted RNA interference to chronically knockdown vHP gene expression of GLP-1R (a G protein-coupled receptor of clinical relevance for obesity), the present findings extend previous work by providing evidence that vHP GLP-1R signaling is endogenously relevant for both normal feeding behavior and for effort-based responding for palatable food. Relative to controls, rats with vHP GLP-1R KD demonstrated increased lever press responding (FR1 operant schedule) for a palatable high-fat, sugar-enriched food under *ad libitum* conditions. vHP GLP-1R KD also altered meal patterning by significantly increasing inter-meal interval (driven by trends towards increased meal size and compensatory decreases in meal frequency) relative to controls. This is consistent with our recent work⁴⁵ showing that pharmacological vHP GLP-1R activation decreases meal size, and with other work demonstrating that peripheral and central GLP-1R activation reduces food intake primarily through a reduction in

meal size.^{27,59–62} Despite these alterations in feeding behavior, GLP-1R KD did not influence long-term body weight or total caloric intake when animals were maintained on chow. It is unknown whether total caloric intake and/or body weight would be impacted with a greater GLP-1R KD magnitude (~50% KD in the present study), and/or with an ‘obesogenic’ dietary challenge (e.g. cafeteria diet).

GLP-1 is produced in L cells in the ileum, as well as in nucleus tractus solitarius neurons that have extensive ascending projections throughout the brain. The endogenous source of GLP-1 signaling to the vHP is unknown, but given that GLP-1-expressing nucleus tractus solitarius neurons do not project to the hippocampus,^{45,63} GLP-1 acting on vHP neurons is likely to originate from either peripheral transport of GLP-1 across the blood–brain barrier,⁶⁴ or via ‘volume transmission’ from GLP-1 released in the cerebral spinal fluid (CSF) from hindbrain GLP-1 neurons.⁴⁵ Consistent with the latter possibility, our recent work detected active levels of GLP-1 in both the vHP parenchyma and in the CSF, and GLP-1-immunoreactive terminals are abundant in the ependymal layer lining the cerebral ventricles at multiple rostral-caudal levels of the neuraxis.⁴⁵ Moreover, ~30% of GLP-1-

producing neurons are co-labeled following injection of a retrograde pathway tracer in the lateral cerebral ventricle, suggesting a subset of these neurons have axon terminals innervating the ventricular space.

To investigate the neural pathways downstream of vHP GLP-1R-mediated feeding, we utilized complementary anterograde neural pathway-tracing methods to investigate a vHP to mPFC neural connection: PHAL and AAV1-GFP pathway tracing. Building on previous work,¹ both methods revealed a robust monosynaptic connection from vCA1 to the PL and ILA that are exclusively ipsilateral (Figures 2a–f). Furthermore, combination of FISH and retrograde neuronal tracing revealed evidence that vHP neurons that project to the mPFC also express GLP-1R (Figure 3d). To examine the functional relevance of this monosynaptic vHP GLP-1R to mPFC neural connection in the control of feeding behaviors, we combined neuropharmacology with reversible chemogenetic CaMKIIa hm4di DREADDs-mediated synaptic disconnection. Results showed that vHP to mPFC disconnection eliminates the food intake reduction that accompanies vHP GLP-1R activation, suggesting that the mPFC is a critical downstream target for vHP GLP-1R-mediated feeding. Importantly these effects were not secondary to confounds arising from parenchymal CNO⁶⁵ and AAV delivery⁵⁶ because animals with vHP AAV-CaMKIIa-GFP controls were unaffected by mPFC CNO delivery.

While the current study, along with previous neuroanatomical work,^{1,2} presents strong support for a direct relationship between vHP GLP-1R signaling and the mPFC, we recognize the possibility that multisynaptic and other downstream pathways are also involved. The vCA1 sends inputs to other feeding-related regions, including the lateral hypothalamic area (LHA) and amygdala.^{1,66} Furthermore, amygdala-LHA and mPFC-LHA circuitry has been shown to regulate external cue-driven feeding.^{12,67} Interestingly, recent work from our lab has demonstrated interactions between vHP ghrelin-mediated hyperphagia and the LHA⁶⁸ and other data from Sweeney and Yang⁶⁹ has shown that activation of a vHP to lateral septum pathway reduces feeding. Additional studies will be required to determine the involvement of multisynaptic pathways and other downstream neural targets modulating vHP GLP-1R-mediated feeding effects.

vHP pyramidal neurons are primarily glutamatergic and provide an excitatory input to the mPFC,^{1,2} and hippocampal glutamatergic signaling has been shown to interact with mPFC ionotropic glutamate receptors such as NMDA and AMPA/Kainate receptors.^{2,70,71} Here we demonstrate that a glutamatergic pathway from the vHP to the mPFC is functionally relevant to feeding behaviors. First, mPFC NMDA receptor, but not AMPA/Kainate receptor blockade attenuated the food intake and body weight reduction following vHP GLP-1R activation, suggesting that downstream mPFC NMDA receptor signaling is required for the full expression of vHP GLP-1R-mediated feeding behaviors. At the molecular level, our data further suggests that vHP GLP-1R activation increases phosphorylation of the NMDA receptor subunit, NR2B, in the mPFC. mPFC NMDA receptor blockade attenuated but did not completely reverse the vHP GLP-1R-induced food intake reduction, whereas DREADDs-mediated vHP to mPFC synaptic silencing completely reversed the effects. These findings suggest that other downstream mPFC neurochemical systems are likely involved. For example, research has shown that mPFC neurons utilize metabotropic glutamate receptors (for example, mGluR 2/3 and mGluR 5) to control executive functions^{72–74} and mGluR 2/3 activation has been shown to interact with mPFC serotonin receptor signaling to control impulsive behaviors.⁷⁵ Furthermore, research has implicated dopaminergic^{11,16,17,20,55,76} and opioid systems acting in the mPFC^{15,21} in appetite control. Thus, the extent to which other neurochemical signals in the mPFC interact with vHP GLP-1R signaling requires further investigation.

The DRL task is a well-established measure of food impulsive response control in rats.^{20,55} mPFC pyramidal neuron activation has been shown to reduce impulsive feeding behaviors¹⁹ and mPFC opioid systems play a role in regulating impulsive feeding behaviors, particularly when animals are in a 'high-drive' impulsive state such as during food restriction.²¹ Our results demonstrate that vHP GLP-1R signaling is involved in controlling impulsive responding for palatable food, as vHP-targeted EX4 administration reduced impulsivity in the DRL task when animals were in a high-impulsive state induced by 24 h food deprivation. mPFC NMDA-R blockade attenuated the vHP GLP-1R induced impulsivity reduction, indicating these downstream receptors regulate both the food intake reduction and the reduced impulsive responding for palatable food triggered by vHP GLP-1R activation. Importantly we found that this effect was primarily driven by changes in lever press responses and no differences were found in pellets earned between all treatments, suggesting that vHP GLP-1R to mPFC NMDA-R signaling does not eliminate appetitive drive, suggesting a more selective effect on food impulsivity. We note that parallel analyses of impulsive responding for palatable food were not possible in the vHP GLP-1R KD animals, as these rats had increased FR1 lever-press responding at baseline (DRL 0), therefore precluding interpretation of potential group differences with longer DRL delays. These results taken together suggest that vHP GLP-1R to mPFC NMDA-R pathways are modulating higher-order aspects of feeding behavior, particularly in the realm of inhibitory control.

Both vHP and mPFC neural processing, as well as central nervous system GLP-1R signaling are capable of regulating both food and non-food motivated behaviors, particularly anxiety and stress.^{3–7,77–79} However, our results indicate that vHP GLP-1R activation does not impact anxiety-like behaviors in the Zero maze task (Supplementary Figure S1), an established rodent assay for anxiety-like behaviors. Moreover, our recent work⁴⁵ demonstrated that vHP GLP-1R activation does not induce nausea-associated behaviors (assessed via conditioned flavor avoidance). These findings suggest that vHP GLP-1R-mediated reductions in food-motivated behaviors are not secondary to alterations in visceral state or anxiety. However, comprehensive additional studies are required to carefully assess other non-food-motivated behaviors that might be regulated by vHP GLP-1R to mPFC NMDAR pathways. Moreover, provided that Experiment 1 demonstrated that vHP GLP-1R KD increases operant FR1 responding for palatable food, it is likely that activation of the novel identified vHP GLP-1R to mPFC NMDAR pathway reduces various food motivated behaviors rather than specifically reducing impulsive responding for palatable food. Other aspects of motivational feeding behaviors (for example, cue-driven feeding), as well as other measures of food impulsivity (for example, impulsive choice tested via delay discounting⁸⁰) that are potentially inhibited by this pathway will require further research.

The GLP-1 system is clinically relevant for obesity treatment based on its potent food intake and body weight reducing effects following central nervous system GLP-1R activation.²⁹ Results from the present study identify a neural pathway where vHP glutamatergic neurons are activated by GLP-1R signaling which in turn engages NMDA-R signaling in the mPFC to regulate food-motivated behaviors. Collectively these data illuminate novel neurobiological targets for the control higher-order feeding behaviors, and reveal a novel functional output of vHP-mPFC neural connectivity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)