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Supplementary Materials for

GABA neurons in the nucleus tractus solitarius express GLP-1 receptors and mediate anorectic effects of liraglutide in rats

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/12/533/eaay8071/DC1)

Data file S1 (Microsoft Excel format). Raw data.

Supplementary Materials

Materials and Methods

Adenoassociated-viral knockdown of Glp1r

For all surgical manipulations, rats were anesthetized with an intramuscular injection of a ketamine (90 mg/kg; Butler Animal Health Supply)/xylazine (2.8 mg/kg; Anased)/acepromazine (0.72 mg/kg; Butler Animal Health Supply) cocktail. All rats were given subcutaneous analgesia (Metacam; 2 mg/kg, Boehringer Ingelheim Vetmedica) immediately following surgery and for three additional postoperative days. During surgery, a bilateral infusion guide cannula (11-mm projection, 1.5-mm spacing, Plastics One) was implanted above the caudomedial NTS according to the following stereotaxic coordinates of Paxinos and Watson 2007 *(109)*: -1.0-mm posterior to the occipital ridge, ± 0.75-mm ML, -5.9-mm DV from the skull surface. A micropump-depressed (PHD 2000; Harvard Apparatus) Hamilton syringe terminating in an injector extending 2.0 mm beyond the cannula was used to bilaterally deliver 200 nl of either AAV-GLP-1R (AAV1.U6.shRGlp1r07.CB7.EGFP.SV40; serotype 1; titer=5.22e12; Penn Vector Core) or AAV-Control (a green fluoresecent protein (GFP)-expressing AAV1 downstream of the CB7 promoter; AAV1.CB7.C1.EGFP; titer= 5.22e12) viruses to body weight and food intake-matched rats. After 1 min, injectors were removed, rats were sutured closed and allowed 21 days for recovery and viral transfection prior to experimentation. Following experimentation, bilateral micropunches (1 mm^3) of the NTS were taken for qPCR analysis of *Glp1r* knockdown relative to *Gapdh*. A cryostat was used to slide mount coronal sections (30 μm) taken caudal to, at the level of, and rostral to the micropunch. Sections were used to visualize the presence of GFP-tagged virus at the rostral and caudal extents of the NTS. Animals lacking GFP expression within the NTS were excluded from analysis. Coronal sections taken at the level of the micropunch permitted visualization of GFP visualization of viral spread beyond the borders of the NTS. Animals with viral placement extending dorsally into the AP or ventrally into the DMX were excluded from analysis (n= 8), as these structures are known to densely express the GLP-1R *(8–11)*. GFP labeling extending into the adjacent nucleus gracilis was permitted, as this brain area does not express the GLP-1R *(8–11)*.

Area postrema lesion (APX)

Lesion of the area postrema was conducted via aspiration *(110)*. Rats were anesthetized and placed in a stereotaxic frame with the head flexed ventrally 90°. Skin and neck musculature was cut along midline and retracted to allow for visualization of the foramen magnum. Using a surgical microscope, dura mater was carefully dissected and cerebral spinal fluid was drained, exposing the obex and area postrema. Sham-prepared animals were sutured closed. In APX-prepared animals, the AP was aspirated using a blunted 21-gauge needle fixed to a flexible tube connected to a vacuum pump. APX animals were closed similarly and all animals were given 10 days for postoperative recovery before experimentation. Following experimentation, brains were removed and a cryostat was used to section coronal slices at 30 μm. APX animals with remaining AP tissue or damage to the adjacent NTS were excluded from analysis.

Inhibitory DREADD receptor expression in GAD1-Cre rats

Following procedures similar to those described above for the delivery of AAV-GLP-1R, a Cre-dependent inhibitory DREADD virus (pAAV-hSyn-DIO-hM4D(Gi)-mCherry; serotype 2; titer:=1.4e12; Addgene) was injected (200 nl/hemisphere) into the caudomedial NTS of GAD-Cre^+ or GAD-Cre^- littermate control rats. Animals were allowed 3 weeks for recovery and viral transfection before experimentation. Epifluorescent illumination of mCherry of cryostat-sectioned (30 μm) coronal slices was used to confirm viral transfection in Cre^+ animals and was used to exclude animals from analysis based on viral placement outside the NTS.

Food Intake and Body Weight Measurements

All injections of liraglutide were administered immediately before the onset of the dark cycle. All food intake and body weight readings were performed by calculating the cumulative change in the weight of the food hopper or the animal from the time of injection to the indicated time point. Food spillage was accounted for by subtracting the weight of crumbs, collected via papers placed beneath each hanging wire-cage, from the weight change of the food hopper.

The role of NTS GLP-1Rs in mediating the food intake and body weight-reducing effects of acutely-delivered liraglutide were examined. Chow maintained rats were prepared with either the AAV-GLP-1R (n = 8) or AAV-Control (n = 8) virus. After three weeks, rats received counterbalanced drug treatments of either vehicle (0.9% saline, 1 ml/kg) or liraglutide (50 μg/ml; 1ml/kg, gift of NovoNordisk). All injections were delivered intraperitoneally (IP) and were separated by 72 hours *(36, 111)*. We ensured that all animals had regained body weight and had restored food intake between drug treatments. Food intake measurements were made at 1, 3, 6, 24, 48 hours post-treatment and body weight measurements were made at 24 and 48 hours post-treatment.

To model the clinical useage of liraglutide as a pharmacotherapy for obesity, the role of NTS GLP-1Rs in mediating the food intake and body weight-reducing effects of chronically-delivered subcutaneous (SC) liraglutide were examined under conditions of diet-induced obesity. Two weeks prior to viral delivery, rats were switched from a chow to a high fat diet (60% kcal% fat; Research Diets). Three weeks after viral delivery, a between subjects design was used to examine the effects of once daily SC vehicle (0.9% saline; 1ml/kg) or a high dose of liraglutide (200 μg/ml; 1 ml/kg), intended to model the human drug regimen *(33, 41, 112)*, in AAV-GLP-1R (n = 10) or AAV- Control (n = 9) prepared rats. Food intake and body weight measurements during the three weeks of drug treatment were taken once daily, immediately preceding injection. Group differences throughout the testing period were analyzed by averaging the daily food intake and body weight in 1-week bins.

In order to validate the APX model, we first tested the effect of vehicle (0.9% saline; 1 ml/kg; IP) and the amylin analog salmon calcitonin (5 μg/kg; 1ml/kg; IP; Bachem) in sham-prepared (n= 9) and APX-prepared (n= 6) rats. Following a 10-day postoperative recovery period, animals received both drug treatments, separated by 72 hours in a counterbalanced design. Food intake was measured at 3 hours. Data from animals with histologically-confirmed lesions were analyzed using a two-way repeated measures ANOVA with Sidak's multiple comparison tests. To test the

necessity of the area postrema in mediating the anorectic effects of liraglutide, we examined the effects of vehicle (0.9% saline; 1ml/kg; IP) or liraglutide (50 and 200 μg/ml; 1ml/kg; IP) in sham-prepared (n = 9) and APX-prepared (n = 11) rats. Following a 10-day postoperative recovery period, all animals were given all three drug treatments in a counterbalanced design separated by 72 hours. Food intake measurements were made at 1, 3, 6, 24, 48 hours post-treatment and body weight measurements were made at 24 and 48 hours post-treatment.

To test the necessity of NTS GABAergic neurons in the food intake and body weight reducing effects of liraglutide, we examined the effects of vehicle (0.9% saline; 1ml/kg; IP) or liraglutide (200 μg/ml; 1ml/kg; IP) following chemogenetic silencing of NTS GABA neurons. Following a 3-week postoperative recovery and transfection period, GAD-Cre^{+} (n = 6) and GAD-Cre^{-} (n = 7) rats prepared with a Cre-dependent inhibitory DREADD virus delivered to the NTS received vehicle or liraglutide in combination with an IP injection of vehicle (0.5% DMSO in 0.9% saline) or CNO (1mg/ml in 0.5% DMSO, 1 ml/kg) administered 10 minutes prior. Animals were given all four drug treatments in a counterbalanced design separated by 72 hours. Food intake measurements were made at 1, 3, 6, 24, 48 hours post-treatment and body weight measurements were made at 24 and 48 hours post-treatment.

Liraglutide-induced NTS c-Fos expression

To examine whether NTS c-Fos evoked by peripherally delivered liraglutide is secondary to neural activation of the AP, we compared c-Fos responses sham and APX rats treated with vehicle (0.9% saline; 1 mg/ml; *n* =5 and 5, respectively) or liraglutide (200 μg/ml; 1 ml/kg; *n* = 4 and 7 respectively). Injections occurred at the onset of the dark cycle, at which time food was removed. Ninety minutes after injection, rats were anesthetized with ketamine (90 mg/kg)/xylazine (2.8 mg/kg)/acepromazine (0.72 mg/kg) cocktail and were transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde in PBS. Brains were extracted and stored in 4% paraformaldehyde for 24 hours before they were transferred to 20% sucrose for cryoprotection. Brains were sectioned on a cryostat in the coronal plane at 30 μm. Sections taken through the rostral-caudal extent of the NTS were collected for c-Fos processing as previously described *(113)*. Briefly, sections were blocked in 0.1 M PBS containing 3% normal donkey serum and 0.3% Triton-X at room temperature. Sections were incubated in a rabbit anti-c-Fos primary antibody (1:1000; Cell Signaling) overnight, and then, following a PBS rinse, they were incubated in a donkey anti-rabbit Alexa Fluor 488 secondary antibody (1:500, Jackson Immunoresearch) for 4 hours at 4°C. Six slide-mounted coronal sections per animal spanning the rostral-caudal extent of the NTS (-14.60 mm to – 11.96 mm from bregma, according to the stereotaxic atlas of Paxinos and Watson) were selected by an experimenter blind to treatment for quantification. Sections were imaged at 20x using a Nikon 80i fluorescence microscope with NIS-Elements AR 3.0 software. An average count of neurons expressing c-Fos within the NTS was quantified by two treatment-blind experimenters. Data are expressed as an average of the two independent counts.

Fluorescent liraglutide penetrance

To visualize the penetrance and distribution of liraglutide in the brain, Cy-3 labeled liraglutide (Cy3-Lirag; 200 μg/ml; 1ml/kg; IP gift of Novo Nordisk) was injected into a rat six hours before transcardial perfusion with 0.1 M PBS followed by 4% paraformaldehyde in PBS. Studies

examining the pharmacokinetics and brain penetrance of Cy3-Lirag and other varieties of fluorescently labeled liraglutide (Liraglutide^{VT750}, Cy-3 Liraglutide, Liraglutide^{594}, ^{3}H-Liraglutide) have demonstrated that these compounds bind the GLP-1R in a 1:1 ratio. Furthermore, there is comparable brain penetrance between these compounds and internalization of fluorescent liraglutide requires the GLP-1R *(33, 35)*. The brain was extracted and stored in 4% paraformaldehyde for 24 hours before being transferred to 20% sucrose for cryoprotection. A cryostat was used to section the brain in the coronal plane at 30 μm. Sections were coverslipped with Fluoro-Gel mounting media containing DAPI (Electron Microscopy Sciences) and visualized with a Leica SP5 X confocal microscope using the 20× and 63× oil-immersion objectives. Image *z*-stacks were collected with a step size of 1 μm. Three-dimensional rotational animations were rendered from the collected *z*-stack images using Imaris 8.1.2 (Bitplane).

Fluorescence in situ hybridization (FISH) analysis

We used fluorescent *in situ* hybridization (FISH) to confirm the presence of the *Glp1r* mRNA transcripts on NTS GABAergic neurons. FISH provides high cellular resolution and is necessary here, as the GLP-1R antibody is notorious for its lack of specificity *(114)*. Rats (n=4) were anesthetized, and brains were removed and snap frozen in hexane. Brains were coronally sectioned on a cryostat at 20 μm and mounted onto subbed slides before being placed in a vacuum chamber overnight.

Tissue was processed according to the RNAscope Fluroescent Multiplex Assay kit protocol for fresh frozen tissue (Advanced Cell Diagnostics). Probes for *Gad1* and *Glp1r* used were specific to the rat (Advanced Cell Diagnostics). Slides were coverslipped using Fluoro-Gel with DAPI (Electron Microscopy Sciences). Images were obtained on a Keyence fluorescent microscope using the protocol described above. Four sections of the NTS per rat at the level of the area postrema were randomly selected for quantification of 1) total *Glp1r*-expressing cells 2) total *Gad1*-expressing cells and 3) total co-labeled cells. Quantifications of both the percentage of NTS GABA cells that express the GLP-1R ($GABA^{+}$/$GLP\text{-}1R^{+}$) and the percentage of GLP-1R-expressing cells that are GABAergic ($GLP\text{-}1R^{+}$/ $GABA^{+}$) for the 4 sections per rat were averaged for each animal. Data are expressed as mean ± SEM of the 4 represented animals.

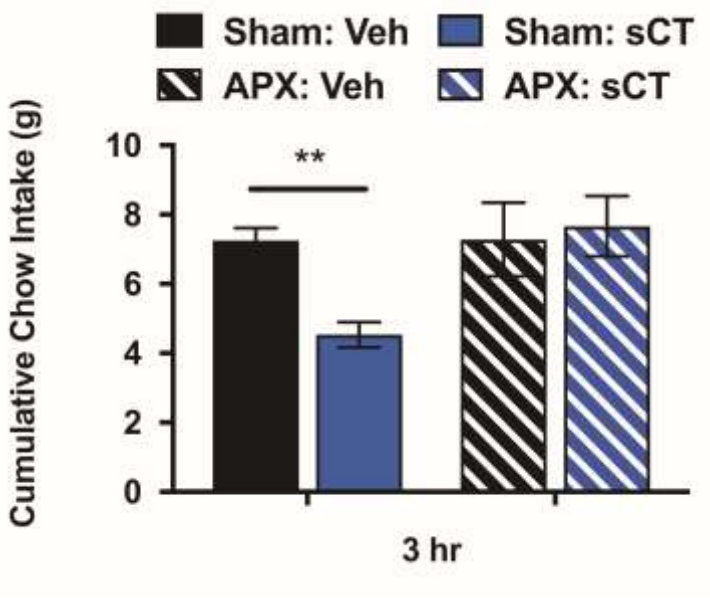

Fig. S1. AP lesioning attenuates the food intake–reducing effects of sCT. Average cumulative chow intake in sham (n=9) and APX (n=6) rats following vehicle (Veh) or salmon calcitonin (sCT; 5 μg/kg; IP) treatment. Data are expressed as means ± SEM, **p<0.01 by two-way repeated measures ANOVA with Sidak's multiple comparison tests.