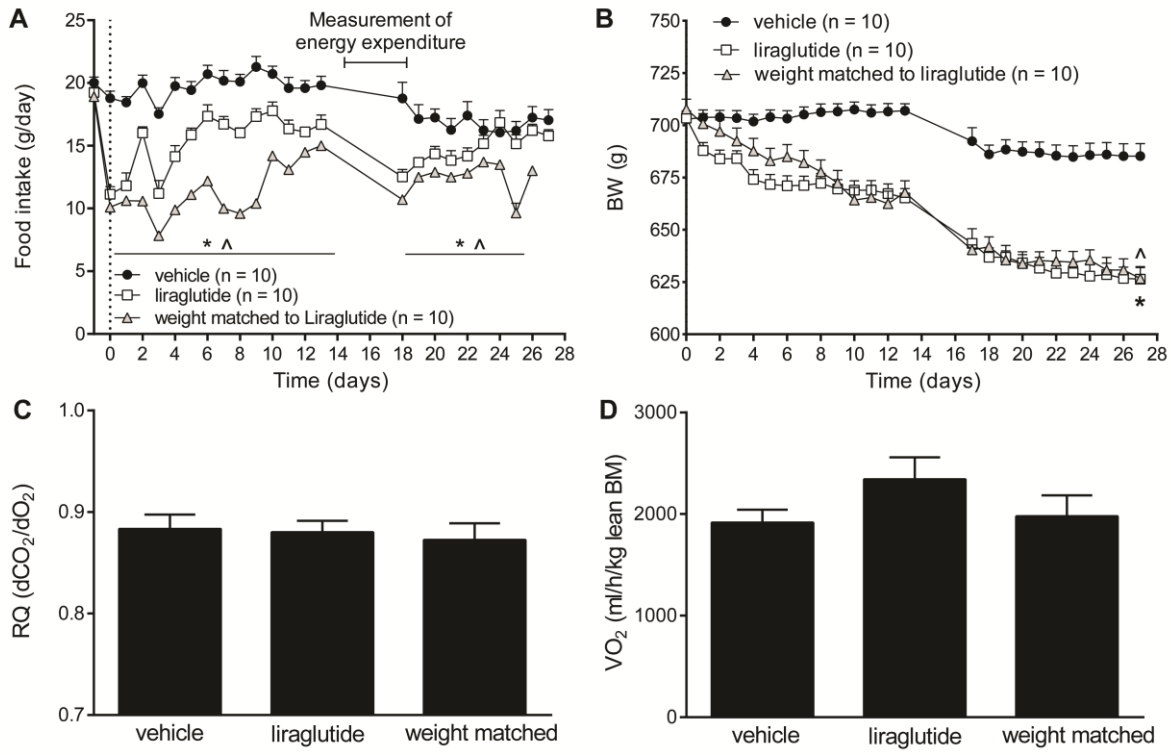
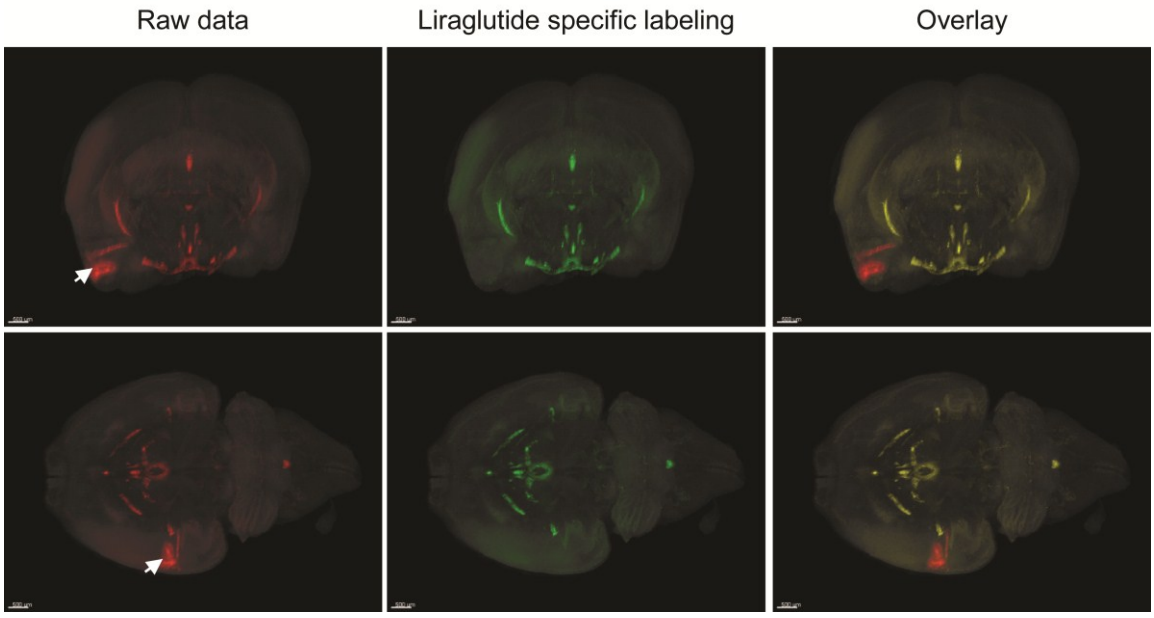
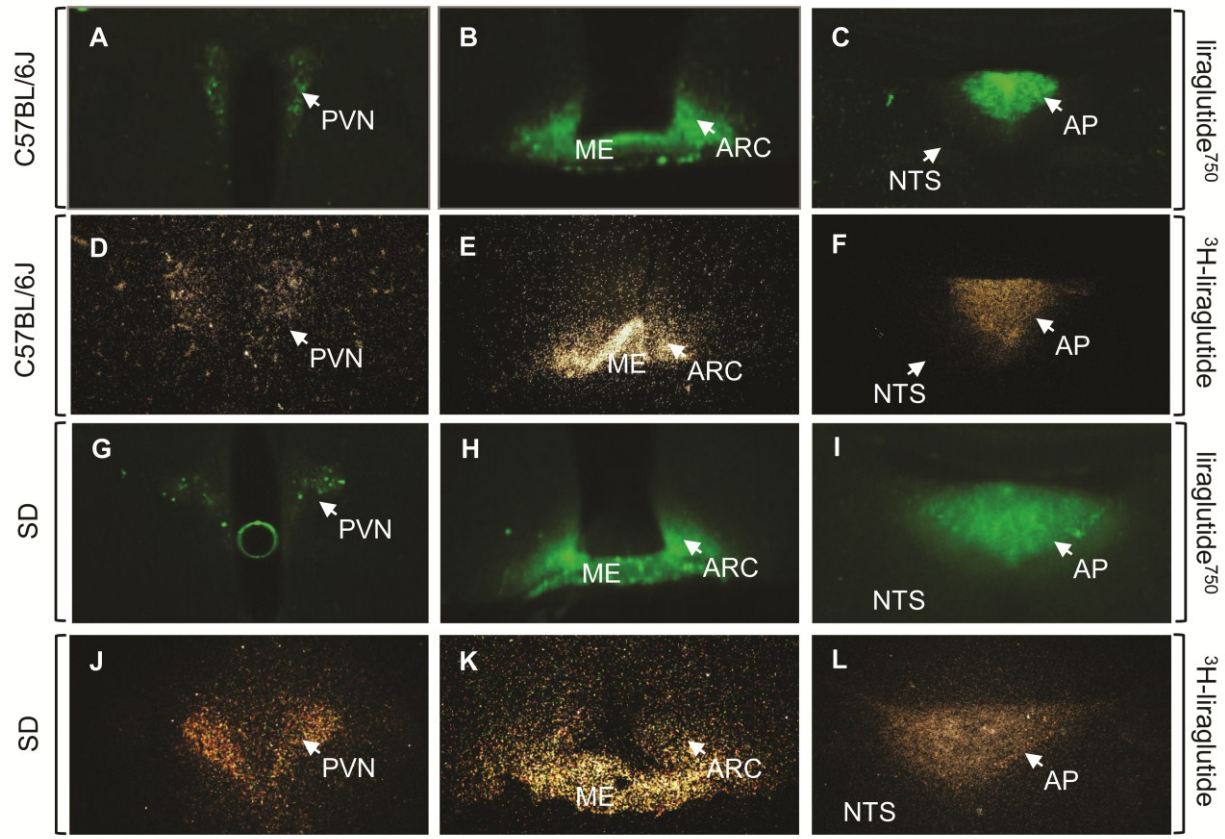
Supplementary Figures



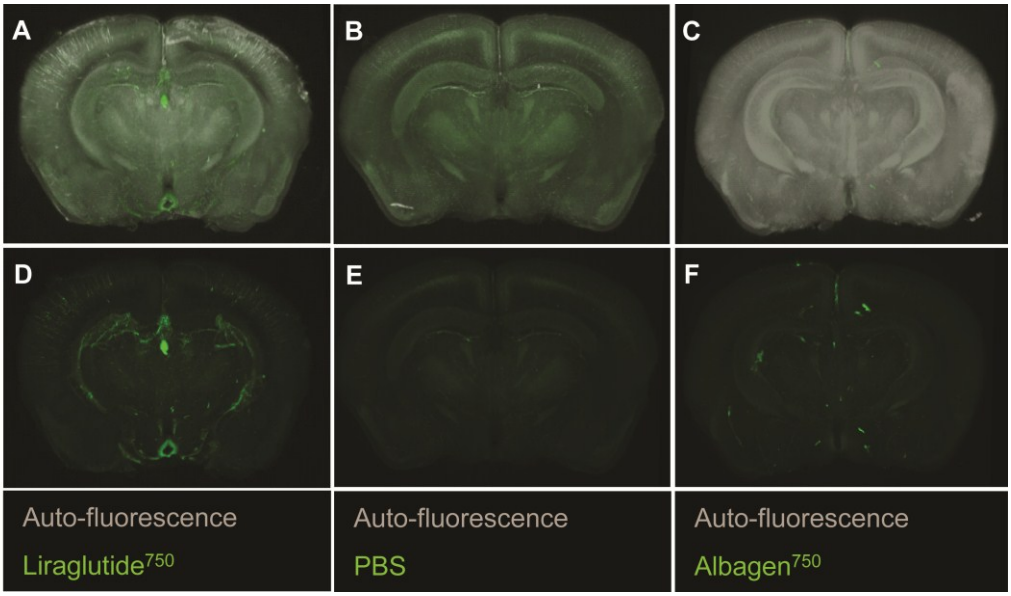
Supplementary Figure 1: Measurement of food intake, body weight and energy expenditure during administration of liraglutide to DIO rats. 28 days of liraglutide treatment initially reduced food intake compared to vehicle ($p<0.0001$; * vehicle vs. liraglutide; ^, liraglutide vs. weight matched), however, this difference leveled out during the last days of the study (A). Body weight was significantly decreased by liraglutide treatment all through the study period ($p<0.001$; * vehicle vs. liraglutide; ^, vehicle vs. weight matched) (B). Energy expenditure measurements were obtained at day 15-17 (weight matched: day 14-16). Treatment with liraglutide did not change the total mean (light + dark phase) steady state RQ (C) nor the total mean (light + dark phase) steady state VO2 (D). Data are means +/- SEM., and statistical analyses are performed using one- or two-way (repeated measures) ANOVA w/ Fishers (one-way) or Bonferroni (two-way) post-hoc test.



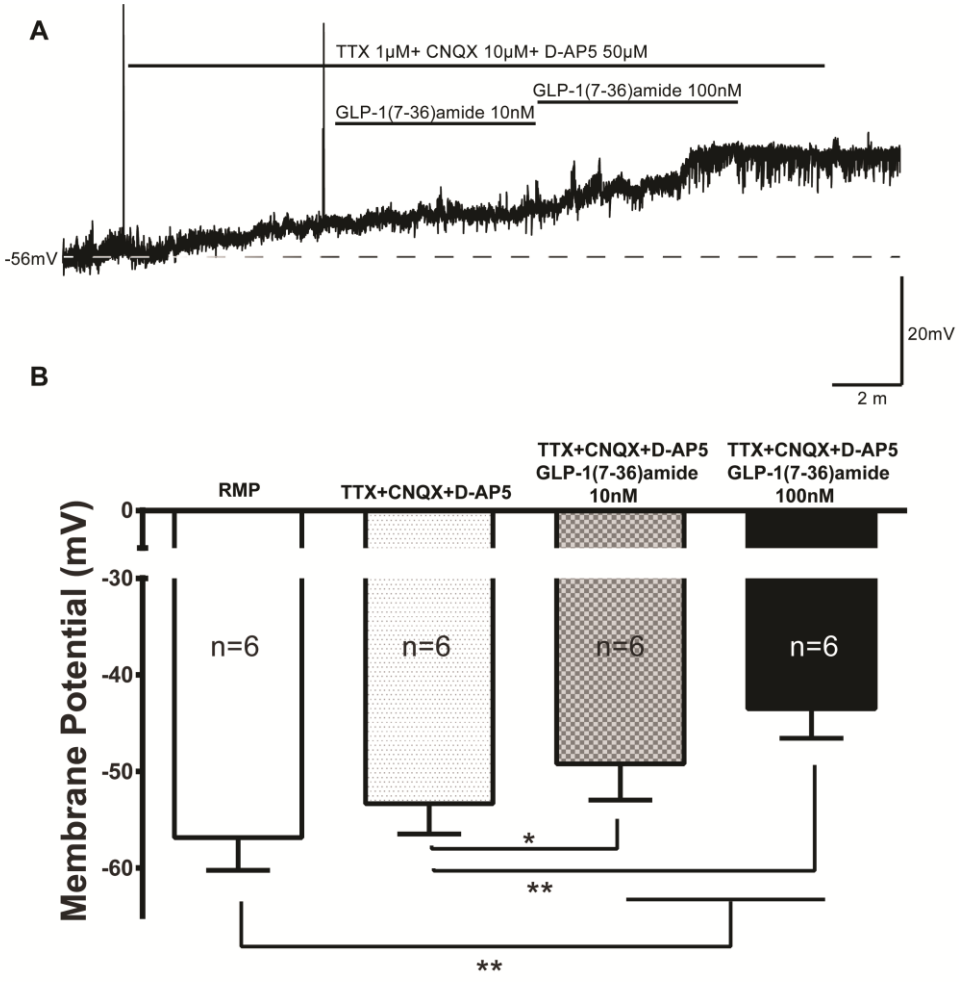
Supplementary Figure 2: Whole brain imaging of liraglutide access. A total of 12 whole brains from C57BL/6J mice dosed with liraglutide^{750} were scanned to get a representative image of the entire mouse brain. However due to the size and dense capillary network, 3D reconstruction of the whole brain was often complicated by fluorescence trapped in the vasculature (despite perfusion fixation). The brain used to illustrate the different regions of liraglutide access and distribution in Figure 3 and 4 was the only brain completely devoid of vasculature labeling but contained an unspecific signal in the right side of the brain (red channel; arrow). This signal was not observed in other samples and since it was unilateral we concluded that it was unrelated to liraglutide access. In the manuscript the whole brain images are mainly used for illustration purposes and the unspecific signal was therefore removed digitally (green channel). The overlay shows that all other signals are maintained.



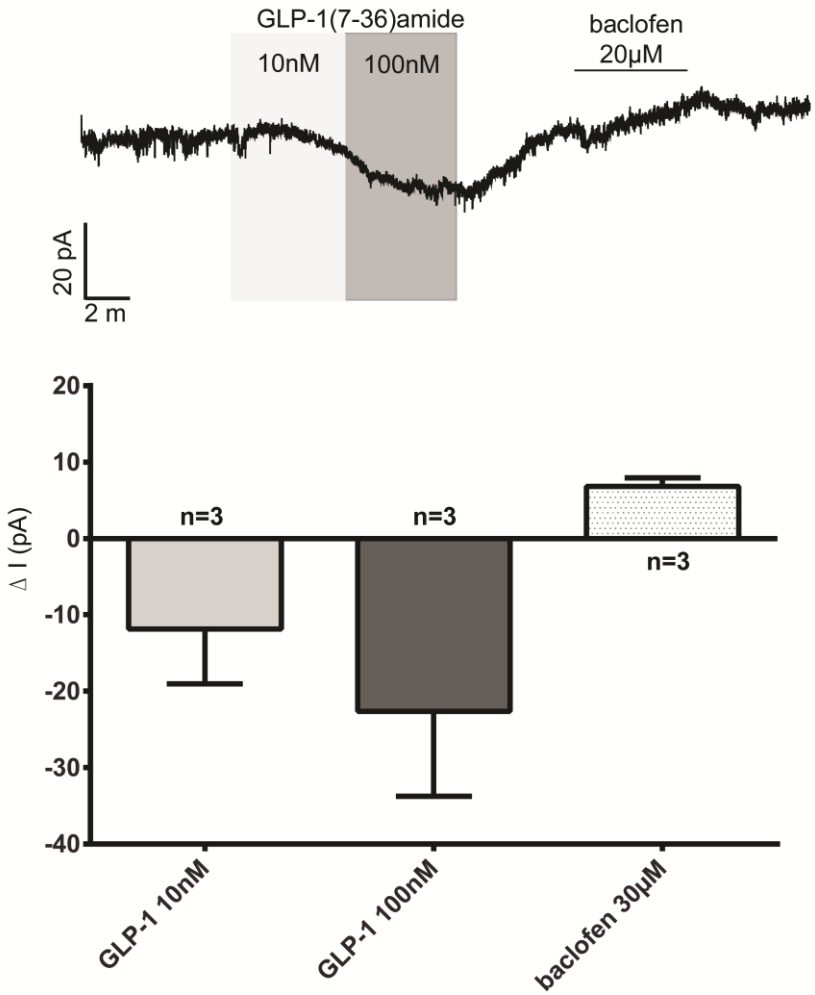
Supplementary Figure 3: Distribution of labeled liraglutide in mouse and rat brain. Liraglutide^{750} located to PVN, ARC and AP following peripheral administration in mice (A – C) and rats (G-I). The same distribution was evident with ^{3}H-liraglutide administered peripherally to mice (D-F) or rats (J-L) further suggesting, that the fluorescent signal observed in various brain regions was not due to a property by the fluorophore but rather a specific ligand binding of GLP-1R.



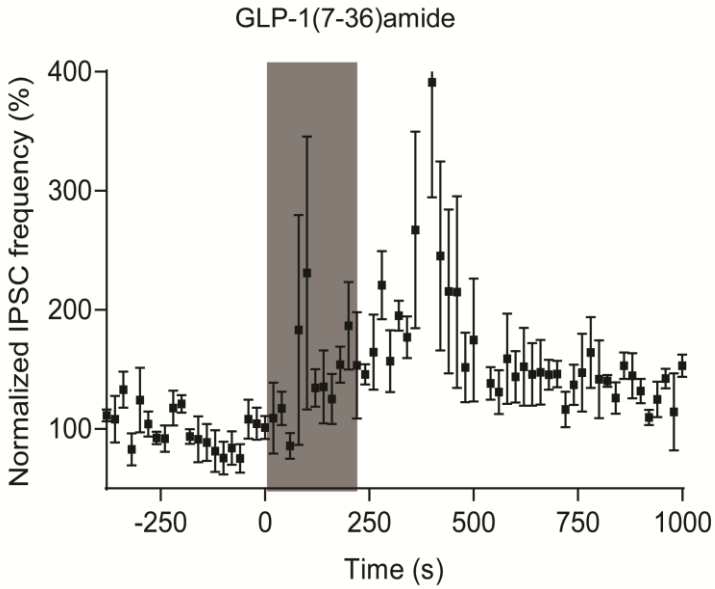
Supplementary Figure 4: Control studies for specificity of the fluorescent signal. A liraglutide^{750} signal was evident in several brain nuclei following peripheral administration (n = 5) (A and D). The signal did not correspond to auto-fluorescence of specific, anatomical structures, as no fluorescence could be observed in PBS dosed animals (n = 5) (B and E). Furthermore, no fluorescent signal could be detected with peripheral administration of albagen^{750} (n = 3) (C and F) further signifying, that the fluorescent signal observed in various brain regions was not due to a property by the fluorophore but rather a specific ligand binding of GLP-1R. Sections were scanned at 620nm and 710nm representing both auto-fluorescence from the tissue (grey; A-C) and specific signal (green; A-F).



Supplementary figure 5: GLP-1 effects on POMC neurons are postsynaptic. Representative traces of GLP-1(7-36)amide 10nM and 100nM effects on POMC neurons in the presence of 1μM Tetrodotoxin (TTX) + 10μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) + 50μM DL-2-amino-5-phosphonovaleric acid (AP5) (A). Bar graph shows the magnitude of GLP-1(7-36)amide-induced responses (B). Data are means +/- SEM., and statistical analyses are performed using one-way ANOVA with post-hoc tukey's test. *$p<0.05$, **$p<0.01$. RMP: resting membrane potential.



Supplementary figure 6: Effects of GLP-1 on POMC neurons in voltage clamp mode. Representative trace of a continuous recording of a POMC-GFP+ neuron in whole cell voltage-clamp mode in the presence of GLP-1(7-36)amide 10nM, GLP-1(7-36)amide 100nM, and baclofen 10µM (A). All traces were filter post-recording in clampfit with a lowpass filter set up at 2Hz. Bar graph shows changes in current in the presence of GLP-1 and baclofen (B).



Supplementary figure 7: GLP-1 mediated regulation of GABAergic effects on POMC neurons. Inhibitory postsynaptic current (IPSC) frequency (%) from voltage clamp recordings of POMC neurons showed an increased GABAergic IPSC frequency in the presence of GLP-1(7-36)amide.

Supplementary Methods

Selective vagal de-afferentiation

17 male SPRD rats (8 weeks of age) underwent a sub-diaphragmatic vagal de-afferentiation (SDA) and 16 animals underwent a sham operation. Rats were anesthetized with a mixture of ketamine (63 mg/kg) and xylazine (9.4 mg/kg) mixed 4:3 (0.1 ml/100 g body weight) and maintained at 36-37°C throughout surgery on a heating pad. Under microscopic observation, a hole was drilled into the base of the skull at the posterior lacerated foramen, revealing the brainstem and overlying dura. The dura was broken, permitting observation of the left vagal afferent and efferent rootlets where they attach to the brainstem. The left dorsal vagal rootlets were then severed by avulsing them with fine forceps, causing no bleeding and no damage to vagal efferent rootlets. The left rootlets were chosen because they contain not only afferents from the ventral gastric vagus and accessory celiac nerve, but also carry afferents from the hepatic vagus, which supplies the liver and proximal duodenum. Gel foam was packed into the skull hole and the wound was closed with stainless steel surgical clips. Next, a sub-diaphragmatic vagotomy of the dorsal vagal trunk was performed, disconnecting all sub-diaphragmatic vagal afferents and efferents supplying the dorsal gastric and celiac branches. The ventrum was exposed by a midline laparotomy incision and the stomach and esophagus were gently retracted. The dorsal vagal sub-diaphragmatic trunk was exposed and gently detached from the esophagus using fine forceps. A segment of the trunk oral to the celiac vagal branch was isolated and cauterized, leaving no intact nerve bundles. Finally, the muscle and skin layers of the laparotomy incision were closed with suture and clips. The sham operation was performed by exposing the left cervical vagus as it penetrates the posterior foramen of the skull, but leaving it untouched. In addition, a midline laparotomy was performed exposing the sub-diaphragmatic vagal trunks. Following the operational procedures all animals were monitored closely and provided with analgesic (one daily dosing subcutaneously (s.c.) with Carprofen (0.1 ml/100 g body weight of Rimadyl, 50 mg/ml diluted 1:9 in isotonic NaCl). Analgesic was provided for at least 4 days post-operational. For 1 week following operations, animals were provided with a liquid diet (Complan, no: 233296, Veterinær Apotek) to minimize gastro-intestinal stress following the abdominal surgery and the vagal de-afferentiation and to speed up post-operational body weight recovery. To test whether the animals from our current SDA study were completely vagotomized, the 14 vehicle treated (6 sham and 8 SDA) animals from the mentioned 14 days dosing experiment were subjected to an acute intra-peritoneal (i.p.) dose of vehicle or CCK-8 (8 µg/kg). Fifteen minutes later a pre-weighed amount of food was returned to the food hopper and food intake was measured for the following two hours at time point 15, 30, 60 and 120 minutes after food presentation. Two days later, the experiment was

repeated but with previous vehicle animals being administered with CCK-8 and CCK-8 animals with vehicle. The SDA lesion was anatomically verified in three SDA animals and three sham animals (all from the liraglutide dosing groups), by i.p. injection with fluorogold tracer, and wheat germ agglutinin (WGA) administered into the nodose ganglion where the afferent rootlets had been severed. Three days later the animals were euthanized by transcardial perfusion with 4 % paraformaldehyde. The brains were removed, sectioned into 40 µm free-floating sections and immuno-reacted to FG and WGA using DAB as a chromogene (described in Vrang N, Hansen M, Larsen PJ, and Tang-Christensen M. Characterization of brainstem preproglucagon projections to the paraventricular and dorsomedial hypothalamic nuclei. *Brain research.* 2007;1149(118-26). Sections were mounted, dried and cover slipped and examined for retrograde labeled fluorogold staining in the left DMX and absence in the right DMX using a fluorescent microscope.

In vivo and in vitro validation of liraglutide^{750}

For in vivo validation of liraglutide^{750} efficacy, DIO mice, *Glp1r*$^{-/-}$ mice or age matched controls (n=5/group, male) were fed regular chow (for *Glp1r*$^{-/-}$ and controls, no. 1324, Altromin, Brogaarden) or 60 % fat (for DIO mice, D12462, Research Diets Inc.). The mice were dosed BID s.c. with 120nmol/kg VivoTag-750-liraglutide dissolved in vehicle (PBS, pH=7.5) or PBS for four days. The liraglutide^{750} dose was titrated over the first two days. Body weight was measured at day 0 and day 4.

For in vitro validation of liraglutide^{750} efficacy, binding with ^{125}I-GLP-1 was performed on a stable transfected cell line, BHK 467-12A expressing high levels of h-GLP-1R (prepared at Novo Nordisk A/S). Cells at approximate 95 % confluence were washed once with PBS and harvested with Versene (aqueous solution of the tetrasodium salt of ethylenediaminetetraacetic acid), centrifuged 5 min at 201 x g and the supernatant removed. On ice, the cell pellet was homogenized by the Ultrathurax for 20-30 sec. in 10 ml of buffer (20 mM Na-HEPES, 10 mM EDTA, pH=7.4), centrifuged 15 min at 80.496 x g and pellet re-suspended in 10 ml of buffer (20 mM Na-HEPES, 0.1 mM EDTA, pH=7.4). The suspension was homogenized for 20-30 sec and centrifuged 15 min at 80.496 x g. Suspension in buffer, homogenization and centrifugation were repeated once and the membranes were re-suspended in buffer (20 mM Na-HEPES, 0.1 mM EDTA, pH=7.4) and stored at -80°C. The binding assay was performed in 96-well Optiplates. The membranes were diluted in assay buffer (50 mM HEPES, 5 mM EGTA, 5 mM $MgCl_2$, 0.005 % Tween 20, pH=7.4) to a final concentration of 0.1-0.2 mg/ml. Membranes, in the presence of ^{125}I-GLP-1 (final concentration 0.06nM), unlabeled ligands (liraglutide^{750} or liraglutide) in increasing concentrations

(1μM; 100nM; 10nM; 1nM; 100pM; 10; 1pM) and high/low HSA concentrations (0.0003 %, 0.6 % final, Sigma A1653) were added 0.5mg/well SPA beads (RPNQ 0001, Perkin Elmer) and incubated 2 hr. at 30°C. Following incubation, the plates were centrifuged (453 x g, 10min) and measured in a topcounter. Receptor binding was plotted using GraphPad Prism (GraphPad) and IC_{50} values were calculated.

^{3}H-liragluide administration

The specificity of the localization of liraglutide^{750} was further evaluated by injecting mice i.v. (NMRI, n=4/group) or rats (SPRD, n=8) s.c. with 150-200 μg/kg [^{3}H]-liraglutide (1.11-1.4 mCi/ml) dissolved in vehicle (PBS, pH= 7.5). The animals were euthanized at 2h (mice) or 8h (rats) following injection. The animals were anesthetized with isoflurane and euthanized by transcardial perfusion with saline. The brains were dissected and frozen in 2-methyl butane at -40°C before being sectioned 10 μm thick, around hypothalamus and AP. The sections were stored at -80°C until further processed. Before exposure, the sections were placed straight from the freezer into 2% Glutaraldehyde (in Dulbecco buffer) at 4°C for 10 min, followed by 1 min in ice cold Tris buffer (pH 8.6) and one min in ice cold H_2O and finally dehydrated in ETOH (99%) for 5 min. Dried sections were dipped in K5 emulsion (Ilford) diluted 3:1 in H_20 (containing 1.7% glycerol), dried and exposed for a total of 5 months. The slides were then developed in D19 Developer (Kodak), and counterstained in Mayers Hematoxylin.

Albagen^{750} administration

The contribution of the fluorophore on the access and distribution in the brain was further evaluated by i.v. administration of 120 nmol/kg albagen conjugated to VivoTag-S®750 NIR FLUOROCHROME LABEL (Perkin Elmer) (in PBS) to mice (n = 3). The mice were anesthetized with isoflurane 4 hours following injection and transcardially perfused with 10 ml heparinized (10U/ml) saline followed by 10 ml 10 % neutral buffered formalin (NBF). Brains were removed, immersed into 10 % NBF and cleared (THF followed by DBE) for imaging with SPIM as described in the method section.