Extended Research Design and Methods

Study 1. Samples from the parietal cortex were taken from the postcentral parietal gyrus while the hypothalamus and medulla oblongata were removed en-bloc. Tissues were subsequently fixed in buffered formalin for 8-10 days and afterwards sectioned in 2 mm slices and embedded in paraffin blocks. Hematoxylin-Eosin stain was performed in slides obtained from each block in order to select the paraffin blocks to locate GLP-1 receptors immunohistochemically. Paraffin blocks originated 2 mm above the Obex (+/- 1mm) were selected from medulla oblongata since they included regions of area postrema, nucleus solitarius and the dorsal motor nucleus of the vagus. Paraffin blocks from the vicinity of the Infundibulum (+2, 0 and -2 mm) were selected from the hypothalamus, including the arcuate nucleus, paraventricular nucleus and the complex of ventromedial nucleus. All adequately fixed paraffin blocks from parietal cortex were chosen.

Formalin fixed parafin-embedded tissue sections (4 mm thick) were deparafinized in xylene and rehydrated in a graded series of alcohol solutions. A monoclonal antibody specific for GLP-1 receptor (GLP-1R) was used for immunostaining (1:300 dilution, overnight incubation at 4°C, pH 7.4). Heat induced antigen retrieval was carried out using preheated target retrieval solution (Dako, Glostrup, Denmark). Thermoscientific LP Detection System kit was used for HRP polymer and DAB chromogen. Based on the intensity of immunostaining, GLP-1R expression level was further graded as absent, weak, moderate, strong, or very strong. Pancreatic tissue samples showing expression of GLP-1R exclusively at Islets of Langerhans served as positive controls for antibody reactivity. The slides were reviewed and scored by an experienced pathologist.

Study 2. Participants were excluded from the study if their DM was not well controlled (i.e. HbA1c>8.9% or FPG>250 mg/dL), they were taking any medications for DM aside from metformin, women were breastfeeding, pregnant or planning to become pregnant, or they had moderate or severe renal disease, had a history of congestive heart failure, had an allergy to the ingredients of liraglutide, had inflammatory conditions such as rheumatoid arthritis, had a history of gastroparesis, pancreatitis or gallstones, were taking any hormonal medications aside from contraceptives, had a family history of thyroid cancer, were vegetarians or had any specific dietary restrictions (since images would include non-vegetarian/various items), had any diagnosed psychiatric conditions, any history of weight loss or brain surgery, or were unsafe for MRI by having metal implants, claustrophobia, or body dimensions that could not fit into the scanner.

No prior studies had examined the mechanisms underlying GLP-1 induced weight loss in humans in an in vivo human model and fMRI technology and thus no data were available from prior calculations. We had planned to have evaluable, full data sets on 20 subjects, after participant attrition. A paired comparison between baseline and end of the study, was expected to have 99% power to detect a difference in brain area activation of 0.6 (SD=0.53, two sided), while an unmatched comparison would provide 80% power to detect a difference in brain area activation of 0.6 (SD=0.53, two sided) with 5% chances of committing type I error. This study was expected to be able to form the basis of power calculations for future studies.

Visual analog scales (VAS) consisted of a 10 cm line on which participants marked their answers to specific questions: “How hungry do you feel right now?”; “How much do you think you could eat right now?”; “How pleasant would it be eat right now?”; and “How nauseous do you feel right now?” where the closer to the beginning of the line they marked, the less they felt

that attribute (for instance for the first question, this would indicate that the participant was not at all hungry and give a rating of 0), and the far end of the line indicated higher feelings of that attribute (e.g. extremely hungry or a rating of 10). The lines were measured using a ruler from the start of the line to where the participant had marked it to the nearest tenth of a cm.

Biochemical measurements and analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS), v.19 and first summarized with descriptive statistics. For example, continuous variables are presented as mean ± standard error of the mean (SE). Data for categorical variables are presented as numbers and/or percentages. Kolmogorov-Smirnov test and frequency histograms were used to check the normality of distribution of the continuous variables. We obtained the skew statistic and standard error of skew (using the descriptives command in SPSS) to identify variables that are non-normal. The difference in values between visit 4 and visit 1 and visit 8 and visit 5 as well as the percent change between each period [(value in visit 4 – value in visit 1)/value in visit 1*100 vs. (value in visit 8 – value in visit 5)/value in visit 5 *100] were also compared and assessed as dependent variables. In the text, F-statistics are reported from the SPSS output of the mixed model and degrees of freedom are reported in parentheses, showing the SPSS reported degrees of freedom values calculated using the Satterthwaite approximation. T-statistics are reported from a paired t-tests and degrees of freedom are shown in parentheses.

fMRI protocol and analysis

We acquired task-related blood-oxygenation level-dependent (BOLD) fMRI data using an echoplanar imaging (EPI) sequence [repetition time (TR)=3000 ms; time to echo (TE)=25 ms; flip angle=90°; field of view (FOV)=230x230 mm; slice thickness=2 mm; gap=0.8 mm; matrix size=128x128]. To minimize the impact of distortions, we used the following measures: a)

performing oblique slice acquisition (approximately at 40 degrees), b) using a sensitivity encoded single-shot EPI sequence (SENSE acceleration factor R = 1.2), and c) using thin slices. The first three volumes were discarded to allow for equilibration effects. We also acquired high-resolution T1-weighted magnetization prepared rapid gradient echo (MPRAGE) structural scans (TE=3.2 ms; TR=6.92 ms; flip angle=8°; FOV=256 mm; resolution=256x256; slice thickness=1.2 mm; no gap; voxel size: 1x1x1.2 mm).

BOLD data was preprocessed using the SPM8 (Statistical Parametric Mapping; The Wellcome Trust Centre of Neuroimaging; London, UK). Briefly, fMR images of each individual subject were flipped, realigned (motion-corrected), normalized to an EPI template with affine registration followed by nonlinear transformation, and smoothed with a Gaussian kernel of 6mm. A general linear model (GLM) was constructed for each individual subject, using the onsets of the food or non-food image blocks with realignment parameters in 6 dimensions. The data were high-pass filtered to remove low-frequency signal drifts. A statistical analytical design was constructed for each individual subject, using the general linear model (GLM), including each block and realignment parameters in all 6 dimensions as regressors. The data were high-pass filtered (1/128 Hz cutoff) to remove low-frequency signal drifts. The GLM estimated the component of variance that could be explained by each of the regressors.

To confirm that the high fat or high calorie images were more desirable than the lower calorie or low fat images, independent panelists, who were not patients or researchers in the study and who did not know the protocol or study hypotheses, were invited from the community. These individuals rated the images for desirability on a 1-5 Likert scale, where 5 was the most desirable. Panelists rated the highly desirable images as more desirable (rating 3.74 ± 1.5) as compared to the less desirable images (2.17 ± 1.4; $t(284)= 11.18$; $p<.001$). The cross-over design

of the study allows each patient to serve as his or her own control, which would eliminate baseline differences in food preference from the final analysis (paired t-test analyses were used for the fMRI data). Also, given the sheer breadth and depth of images presented (180 highly desirable food images and 180 less desirable food images in blocks of 5 images each at each scanning session) slightly different preferences for specific types of food would be inconsequential.

Since the parietal cortex was significant at $p<.001$ from the whole brain analysis, effect sizes (z scores) from the parietal cortex (the region with peak activation from the main effect; size: 297 mm^3) was extracted using marsbar (http://marsbar.sourceforge.net/) and this was correlated with VAS data using a Pearson correlation, controlling for treatment. We also ran whole brain regression analyses with the VAS data for the contrast of highly desirable > less desirable food images during the fasting state while participants were on liraglutide. Activations significant at $p<.001$ and with an extent of 30 voxels or more are reported.

In a secondary analysis, we also performed small volume corrections (SVC) on fMRI data during the contrast of highly desirable as compared to less desirable images in the fasting state for the areas of interest as previously described in order to be able to compare our results to those which were previously found [1]. Briefly, spherical regions of interest were created in marsbar with radii 5-mm (for amygdala) or 10-mm (for insula, putamen, and OFC) for SVC. As the hypothalamus is also a key area of interest, we also performed SVC for the hypothalamus using a sphere of 10-mm radius as defined previously [2].

References

[1] van Bloemendaal L, RG IJ, Ten Kulve JS, et al. (2014) GLP-1 receptor activation modulates appetite- and reward-related brain areas in humans. Diabetes 63: 4186-4196
[2] Farr OM, Fiorenza C, Papageorgiou P, et al. (2014) Leptin therapy alters appetite and neural responses to food stimuli in brain areas of leptin-sensitive subjects without altering brain structure. The Journal of clinical endocrinology and metabolism 99: E2529-2538