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GLP-1 and estrogen conjugate acts in the supramammillary nucleus to reduce food-reward and body weight

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ABSTRACT

The obesity epidemic continues unabated and currently available pharmacological treatments are not sufficiently effective. Combining gut/brain peptide, GLP-1, with estrogen into a conjugate may represent a novel, safe and potent, strategy to treat diabesity. Here we demonstrate that the central administration of GLP-1-estrogen conjugate reduced food reward, food intake, and body weight in rats. In order to determine the brain location of the interaction of GLP-1 with estrogen, we avail of single-photon emission computed tomography imaging of regional cerebral blood flow and pinpoint a brain site unexplored for its role in feeding and reward, the supramammillary nucleus (SUM) as a potential target of the conjugated GLP-1-estrogen. We confirm that conjugated GLP-1 and estrogen directly target the SUM with site-specific microinjections. Additional microinjections of GLP-1-estrogen into classic energy balance controlling nuclei, the lateral hypothalamus (LH) and the nucleus of the solitary tract (NTS) revealed that the metabolic benefits resulting from GLP-1-estrogen injections are mediated through the LH and to some extent by the NTS. In contrast, no additional benefit of the conjugate was noted on food reward when the compound was microinjected into the LH or the NTS, identifying the SUM as the only neural substrate identified here to underlie the reward reducing benefits of GLP-1 and estrogen conjugate. Collectively we discover a surprising neural substrate underlying food intake and reward effects of GLP-1 and estrogen and uncover a new brain area capable of regulating energy balance and reward.

1. Introduction

The obesity epidemic continues unabated and is associated with adverse health consequences - diabetes, cardiovascular disease, disability, and increased cancer risk. The currently available pharmacological treatments are less efficient than expected. Therefore, there is an urgent need for novel therapies that reduce body weight more potently than currently available pharmaceutics without producing undesirable side effects. One potential way to achieve a more potent weight loss may be by combining multiple anti-obesity drug targets in one molecule. Polypharmaceutical approaches, for example targeting the incretin system with an unimolecular glucagon-like peptide 1 (GLP-1)-estrogen co-agonist,

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offer promising solutions to treat diabesity (Finan et al., 2012).

GLP-1, a gut/brain peptide primarily produced in the intestinal tract in response to ingested nutrients and by the neurons of the nucleus of the solitary tract (NTS) of the brainstem (Holst, 2007), gained interest due to its glucoregulatory (Drucker, 2006) and appetite suppressing effects (Barrera et al., 2011). The highly selective GLP-1 receptor (GLP-1R) agonist, Exendin-4 (Ex4), is approved as antidiabetic agent in the treatment of type 2 diabetes (Byetta®) and anti-obesity treatment (Saxenda®). The steroid hormone estradiol, mainly produced by the ovaries, has also been implicated as a potential anti-obesity agent (Mauvais-Jarvis et al., 2013). However, the reproductive endocrine toxicity and oncogenicity limit its clinical application.

The conjugation of GLP-1 to estrogen allows for specific delivery of estrogen to GLP-1R expressing tissues without producing the undesirable side effects. In addition, this selective targeting of estrogen receptors to GLP-1R expressing cells is much more effective in reducing obesity and improving dyslipidemia and hyperglycemia than activation of either GLP-1 or estrogen receptors (ER) alone (Finan et al., 2012). The site of action of the weight and food intake reducing impact remains unknown. Here we hypothesize that the anti-obesity action of the conjugated GLP-1-estrogen is mediated by its direct action on CNS GLP-1 and estrogen receptors, which are co-expressed in several brain areas regulating homeostatic feeding behavior and metabolism (Shughrue et al., 1997; Merchenthaler et al., 1999). Hedonically driven feeding may be one of the key components driving overeating (Berthoud, 2002, 2011; Alsio et al., 2012). Furthermore the potential impact of the GLP-1-estrogen conjugate on food-reward behavior has not yet been explored.

In the present study we determine whether peripheral or central co-activation of GLP-1 and estrogen receptors impacts on food-reward behavior. Using single-photon emission computed tomography (SPECT) imaging of regional cerebral blood flow (Kolodziej et al., 2014) and site-specific microinjections we identify the supramammillary nucleus (SUM), a brain area largely unexplored for its role in feeding or reward, as a potential target for the synergistic activity of the conjugate to regulate food reward. The latter finding not only reveals a surprising neural substrate underlying food intake and reward effects of GLP-1 and estrogen but also uncovers a new brain area capable of regulating energy balance and reward.

2. Material and methods

2.1. Animals

Adult Sprague-Dawley rats (200–250 g, Charles River, Germany) were housed in a 12 h light/dark cycle (lights on at 6 am) with regular chow and water available *ad libitum* in their home cages. All experiments were performed in male rats as the initial study by Finan et al. (2012) already demonstrated that the GLP-1-estrogen conjugate exerts the metabolic benefits independently of the gender. All animal procedures with rats were carried out with ethical permission and in accordance with the University of Gothenburg Institutional Animal Care and Use Committee guidelines. For functional neuroimaging adult eight week old male C57Bl/6 mice (Charles River, Germany) were used and studies were carried out in accordance with the German animal welfare laws and approved by the animal ethics committee of Sachsen-Anhalt.

2.2. Brain surgery

Brain cannulation was performed as previously described (Skibicka et al., 2011). Briefly, rats were implanted with a guide cannula under ketamine/xylazine anesthesia at the following coordinates: lateral ventricular guide cannula (26 gauge, Plastics One Roanoke, VA, USA; coordinates: ±1.6 mm from midline, 0.9 mm posterior to bregma, and 2.5 mm ventral from the surface of the skull, with injector aimed 4.5 ventral to skull); ventral tegmental area (VTA) guide cannula (±0.75 mm from midline, 5.7 mm posterior to bregma, and 6.5 mm ventral from the surface of the skull, with injector aimed 8.5 ventral to skull; (Skibicka et al., 2011); SUM guide cannula (on the midline, 4.7 mm posterior to bregma, and 7.1 mm ventral from the surface of the skull, with injector aimed 9.1 ventral to skull); lateral hypothalamus (LH) guide cannula (±1.5 mm from midline, 2.8 mm posterior to bregma, and 6.8 mm ventral from the surface of the skull, with injector aimed 8.8 ventral to skull); NTS guide cannula (±0.75 mm from midline, on occipital suture, and 6.9 mm ventral from the surface of the skull, with injector aimed 8.9 ventral to skull, modified from Richard et al. (2015); were attached to the skull with dental acrylic and jeweler's screws. Following a surgical recovery period (7 days) placement of the lateral ventricle cannula was verified by angiotensin II (2 μl; 10 ng/μl) administration. Rats, which consumed >5 ml/water within 30 min following injection of angiotensin II were included in the studies. Correct placement of the VTA, LH and SUM cannula was confirmed *post mortem* by injection of India ink at the same microinjection volume as used for the experiment. NTS injection sites were evaluated by injection of 24 μg of 5-thio-*d*-glucose in 0.3 μl of artificial cerebrospinal fluid (aCSF, Tocris, Bristol, UK), which induce a sympathoadrenal-mediated glycemic response if the injections correctly reach the NTS (Ritter et al., 1981). Only rats with correct placement were included in the study.

2.3. Drugs

GLP-1 control compound and GLP-1-estrogen stable conjugate were provided by R. DiMarchi (Indiana University, Bloomington, IN, USA). The conjugate is likely to target nuclear ER, both ERα and ERβ may have a contribution since we previously demonstrated that both ER knockout mice have a blunted effect of the conjugate (Finan et al., 2012). The conjugate initiates the classic estrogen responsive element (ERE)-mediated transcriptional events, though a contribution from plasma membrane receptors cannot be excluded (especially considering the relatively rapid onset of the conjugate impact on reward). In the first study we confirmed that estrogen attachment does not influence the inherent activity of the peptide as the binding affinity and the biochemical signaling potency of the conjugate is similar to the parent peptides (Finan et al., 2012). This allows the ligand-activated endocytosis of GLP-1R at target cells and finally drives the intracellular transport of the conjugated estrogen to reach the intracellular receptors (Finan et al., 2012). We previously reported that for all metabolic parameters measured the stable conjugate offered an advantage compared to the GLP-1 compound alone, or compared to a labile conjugate, with a 7.5% weight loss in mice given the labile conjugate and 23% weight loss in mice that received the stable conjugate (Finan et al., 2012). Food intake followed the same pattern. Since the labile conjugate did not have any additional benefits over GLP-1 alone it was proposed that the efficacy of the conjugate likely relies on concentrating the estrogen into energy balance relevant tissues, since the estrogen is guided by GLP-1 and released intracellularly only in tissues expressing GLP-1 receptors. Importantly, the conjugate was not only designed to maximize the metabolic benefits above those of the single agonists but also to avoid the side effects often seen with GLP-1 activation and to bypass the reproductive endocrine toxicity and oncogenicity of estrogen (Finan et al., 2012). β-Estradiol was purchased from Sigma (E4389; St Louis, MO, US), dissolved in saline (vehicle for subcutaneous injections) or aCSF (vehicle for central injections).

2.4. Operant conditioning procedure

To test the impact of GLP-1-estrogen on food-motivated behavior the sucrose-driven progressive ratio (PR) operant conditioning test was used. Food-induced operant conditioning training and testing were performed in rat conditioning chambers (30.5 × 24.1 × 21.0 cm; Med-Associates, Georgia, VT, USA) containing a metal grid floor, two retractable levers with light bulbs above, and a food pellet dispenser that can deliver 45 mg of sucrose pellets (Test Diet, Richmond, IN, USA). The training included four stages: first three sessions on fixed ratio (FR) starting with FR1 (single press on the active lever = delivery of one sucrose pellet) followed by FR3 (3 presses/pellet) and finally FR5 (5 presses/pellet), where a minimum of 50 presses per session was required to graduate to the next FR level. The FR5 was followed by the PR schedule where the cost of a reward was progressively increased (Skibicka et al., 2011). PR sessions were continued until a stable baseline (15% for three consecutive sessions) was reached. During the FR and the first three PR sessions all rats were mildly food restricted resulting in a gradual loss of about 10% of their initial body weight over a period of one week.

For the drug testing the following conditions were used: (1) For subcutaneous (sc.) drug application (1 ml per kg body weight): vehicle, 2 μg GLP-1-estrogen, 1.87 μg GLP-1, and 0.13 μg estrogen per kg body weight. (2) For intracerebroventricular (ICV) drug application (1 μl): vehicle, 0.125 μg GLP-1-estrogen, 0.117 μg GLP-1, and 0.008 μg estrogen. (3) For VTA (0.5 μl), and SUM (0.3 μl) drug application: vehicle, 0.075 μg GLP-1-estrogen, 0.07 μg GLP-1, and 0.005 μg estrogen. Satiated rats received the injections early in the light phase 90 min (sc. injections) or 30 min (central injections) before the 60 min sucrose reinforced PR operant conditioning.

2.5. SPECT-imaging of regional cerebral blood flow (rCBF)

SPECT-imaging of rCBF was performed similarly as described in detail in Kolodziej et al. (2014). In brief, awake unrestrained C57Bl/6J mice were intravenously injected via jugular vein catheters with the blood flow tracer 99m-technetium hexamethylpropyleneamine oxime (99mTc-HMPAO) after treatment with GLP-1-estrogen, GLP-1, or PBS as control. After 99mTc-HMPAO-injection animals were anesthetized and scanned using a small-animal SPECT/CT scanner. 99mTc-HMPAO is a lipophilic compound that, after flow-dependent wash-in, is rapidly converted to a hydrophilic compound that remains trapped in the brain and shows no redistribution. The 99mTc-brain distribution as determined in anesthetized animals in the scanner thus reflects the spatial pattern of the average blood flow during the injection period in the awake state. Jugular vein catheters (ALZET, Cupertino, USA; 44.5 mm PU, OD: 0.84 mm, ID: 0.36 mm, connected to a 50 mm ALZET connection, OD: 1.02 mm OD, ID: 0.61 mm, total catheter length 9.5 cm) were implanted into the right external jugular vein. Following a two days recovery period, mice were intraperitoneally injected either with 400 μg per kg body weight (1 ml per kg body weight) GLP-1 (n = 8), GLP-1-estrogen (n = 7), or PBS (n = 7). After substance application the jugular vein catheter was connected via a saline-filled Teflon tube (Tefzel-Tube, CS-Chromatographie Service GmbH, D-52379 Langerwehe, Germany, OD: 1/16 inch ID: 0.5 mm) of 60 length to a saline filled syringe. 30 min after substance application a syringe filled with the 99mTc-HMPAO-injection solution was connected to the Teflon tube and the tracer injection started. 99mTc-HMPAO was freshly prepared from frozen aliquots of kit preparations for use in humans (Ceretec™, GE-Healthcare, Buchler, Braunschweig, Germany) (Kolodziej et al., 2014). Compared to the previous study (Kolodziej et al., 2014) the HMPAO-concentration in the aliquots was doubled so that the volumes of the aliquots could be reduced (125 μl as compared to 250 μl). Injections were made using a syringe pump (Harvard Instruments, Holliston, MA, USA). The tracer was injected over 18 ± 2 min. In principle, the tracer can be injected in much shorter time periods (Kolodziej et al., 2014). We here hypothesized that averaging blood flow over longer periods of time might be a more suitable approach for visualizing longer-lasting drug effects. The animals were injected with 99mTc-HMPAO-solutions in volumes of 340 ± 83 μl, the differences in volume being due to variations in 99mTc-contents in the eluate from the 99mTc-generators. The flow rate of the tracer-infusion pump was adjusted according to the differing volumes (16 μl–25 μl/min) of the tracer-solutions. After tracer injection, animals were anesthetized with 2% isoflurane and transferred to the SPECT/CT-scanner (four-head NanoSPECT/CT™ Mediso/Hungary). The amounts of 99mTc remaining in the syringe, and teflon tube were determined using a radionuclide calibrator (Aktivimeter Isomed, 2010; Nuklear-Medizin-Technik Dresden GmbH, Germany) and subtracted from the initial activity of the tracer solution to calculate the injected dose. The animals were injected on average with 60 ± 8 MBq of 99mTc.

For SPECT/CT imaging mice were scanned under gas anesthesia (1.0–1.5% isoflurane in 2:1 O2:N2O volume ratio). CT and SPECT images were co-registered. CT scans were made at 45 kVp, 177 μA, with 180 projections, 500 msec per projections, 96 μm isotropic spatial resolution, and reconstructed with the manufacturer's software (InVivoScope 1.43) at isotropic voxel-sizes of 100 μm. SPECT scans were made using ten-pinhole mouse brain apertures with 1.0 mm pinhole diameters providing an isotropic spatial resolution of about 0.7 mm FWHM (Kolodziej et al., 2014). 24 projections were acquired during a total scan time of 2 h. Axial FOV was 20.9 mm. Energy windows were set to the default values of the NanoSPECT/CT (140 keV ± 5%). SPECT images were reconstructed using the iterative algorithm of the manufacturer's software (HiSPECT™, SCIVIS, Goettingen) at isotropic voxel output sizes of 338 μm.

For data analysis brain 99mTc-distributions were compared in mice injected with PBS; GLP-1, or GLP-1-estrogen. SPECT/CT images were manually aligned to a high-resolution MR mouse brain data set (Ma et al., 2005) based on skull-landmarks of the CTs using the MPI-Tool™ software (version 6.36, ATV, Advanced Tomo Vision, D-50169 Kerpen, Germany). SPECT brain data were cut out of the SPECT-data in the Osirix™ DICOM-viewer (64-bit version 5.7.1) using a whole-brain volume-of-interest (VOI) made from the template provided by Ma and colleagues (Ma et al., 2005). Brain SPECT data were global mean normalized using the MPI-Tool™ software. In the voxelwise analysis unpaired t-tests were made to compare brain tracer distributions in PBS versus GLP-1 and GLP-1-estrogen using the MagnAn-software (version 2.4, BioCom GbR, D-90180 Uttenreuth, Germany). Following common procedures in small-animal radionuclide imaging (Endepols et al., 2010; Wyckhuys et al., 2010; Thanos et al., 2013) uncorrected p-values were used. As a major result at the level of $p < 0.01$ we found when testing GLP-1-estrogen against PBS a deactivation in a mid-rostrocaudal part of the cingulate cortex and a prominent activation centered on the SUM. Illustrations of the results were made in Osirix™. Images in Osirix™ were exported as TIFF files and arranged using the Photoshop™ software (version CS4).

In order to calculate the differences in mean tracer uptake in the volume covered by the $p < 0.01$ voxels in the SUM-region in the probability maps, significant voxels from GLP-1-estrogen versus PBS injection extending from Bregma −2.1 to −2.8 according to

Franklin and Paxinos (George Paxinos, 2012) were grouped to one VOI using the Plugin function "Growing region" in Osirix™. Compared to the PBS-group mean tracer uptake in this VOI increased by 12% in the GLP-1-estrogen group and by 6% in the GLP-1 group.

2.6. Chow intake and body weight

Immediately after operant testing rats were moved to their home cages and 1 h and 24 h chow intake was measured. Body weight was determined immediately before the injection and 24 h postinjection.

2.7. Pica response

To investigate whether GLP-1 injection induces malaise, the intake of kaolin (Research Diets, Lane New Brunswick, NJ, USA) (Mitchell et al., 1976), a non-nutritive substance, was measured in parallel to chow intake measurements. All rats were exposed to kaolin before the experimental injections.

2.8. Locomotor activity

For activity measurements rats were injected centrally (ICV) with vehicle, GLP-1-estrogen, GLP-1 or estrogen, placed into the activity chamber 30 min later and spontaneous horizontal activity was recorded for 60 min. Rats had no access to food during the test.

2.9. Statistical analysis

For statistical analysis all parameters were initially tested with Levene's statistics for homogeneity of variances. At equal variances data were analyzed by repeated measures analysis of variance (ANOVA) followed by *post hoc* Bonferroni or LSD test. If the variances were not homogeneous parameters were analyzed using the Generalized Linear Model. All statistical analyses were conducted using the SPSS software. A p-value <0.05 was considered significant and values are expressed as means $\pm$ SEM, unless otherwise stated.

3. Results and discussion

3.1. Subcutaneous co-activation of GLP-1 and estrogen receptors reduces body weight and food reward

Given the initial data of Finan et al. (2012) demonstrating the anorexigenic properties of the GLP-1-estrogen conjugate in mice we first tested whether peripheral injection of GLP-1-estrogen in rats can similarly reduce food intake and body weight. In rats, a single peripheral, s.c., injection of GLP-1-estrogen (2 μg/kg) significantly decreased body weight and 24 h food intake (Fig. 1A,B), whereas 1 h chow intake was unaltered in all groups (data not shown). The equimolar doses of GLP-1 or estrogen alone were subthreshold for the anorexic effect, indicating a synergistic effect of GLP-1-estrogen combination. The reduced feeding response in GLP-1-estrogen treated animals was not accompanied by the induction of a malaise response, as the ingestion of kaolin (a pica response) was similar in all compared groups 24 h postinjection (Fig. 1B). We have previously shown that in mice the weight loss after the conjugate administration is primarily due to fat loss (Finan et al., 2012). While water intake was not measured in the current study, recent literature suggests it is possible that GLP-1 alone reduces water intake (McKay et al., 2011; McKay and Daniels, 2013) in addition to reducing food intake, it is less likely, however, that the conjugate further reduced water intake since to date the water intake reducing effects of estrogen were primarily seen in females (Santollo and Daniels, 2015), and the current study is performed in males. Furthermore the conjugate is equally effective at reducing body weight in males and females (Finan et al., 2012).

GLP-1 effects on body weight regulation and feeding are largely mediated by the CNS. Recent literature demonstrates that peripheral or central application of GLP-1 or GLP-1 analogs into the VTA, NAc, NTS, or parabrachial nucleus alters food-reward behavior (Dickson et al., 2012; Alhadeff et al., 2014; Richard et al., 2015). NTS GLP-1-producing neurons project directly to the mesolimbic VTA and NAc (Rinaman, 2010; Dossat et al., 2011; Alhadeff et al., 2012; Dickson et al., 2012). So far the impact of estrogen on food-motivated behavior is largely unknown. To determine whether the GLP-1-estrogen conjugate can change the motivational value of palatable food, a correlate of food-reward behavior (Hodos, 1961), we examined the number of sucrose rewards earned under a progressive ratio (PR) reinforcement schedule after peripheral, s.c., administration of the GLP-1, estrogen or the conjugate of the two. Conjugate-injected rats reduced food-motivated behavior to a greater extent than rats treated with vehicle, estrogen, or GLP-1 alone as the number of active-lever presses (Fig. 1C) and sucrose pellets earned (Fig. 1D) were significantly decreased. Taken together these data demonstrate a new role for conjugating GLP-1 with estrogen in the regulation of food reward. The potentiated reward-suppression of these two substances applied together is in line with recent findings demonstrating that estrogen is a critical regulator of the impact of a GLP-1 analogue, exendin-4, on food reward in both males and females (Richard et al., 2016).

3.2. Central co-activation of GLP-1 and estrogen receptor is sufficient to decrease food intake and food reward

Central activation of GLP-1R reduces food intake, whereas central GLP-1R blockade induces hyperphagia (Tang-Christensen et al., 1996; Turton et al., 1996; Schick et al., 2003). Several studies documented that estradiol (E2) suppresses food intake via central ERα and ERβ (Heine et al., 2000; Geary et al., 2001; Asarian and Geary, 2002; Liang et al., 2002; Gao et al., 2007). Here we demonstrate that central, ICV, application of GLP-1-estrogen was sufficient to reproduce the effect of peripheral injection on body weight and chow intake (Fig. 2A,C), whereas 1 h food intake was unaffected (Fig. 2B). Conjugate injection was more effective at reducing body weight and food intake than estrogen alone and tended to be more potent in comparison to GLP-1, though this difference did not reach significance. The significantly reduced 24 h chow intake after central injection of GLP-1-estrogen or GLP-1 was not accompanied by a malaise response (Fig. 2C). We then examined whether central co-activation of GLP-1 and estrogen receptors affects food-motivated behavior. Acute central GLP-1-estrogen treatment significantly reduced operant PR response for sucrose, whereas the equimolar doses of GLP-1 or estrogen failed to reduce food reward when applied individually (Fig. 2D,E). Moreover, in a separate experiment, GLP-1 or GLP-1-estrogen did not affect general locomotor activity at doses that significantly reduced chow intake and food-motivated behavior (Fig. 2F). These data support a role of the CNS in mediating GLP-1-estrogen effects on food intake and reward. We additionally show for the first time that the beneficial metabolic effects of GLP-1 and estrogen stimulation are not necessarily accompanied by general locomotor impairment or malaise, side-effects often seen with GLP-1 activation in rodents or humans, at the doses used in the study (Turton et al., 1996; Seeley et al., 2000; Kinzig et al., 2002; Madsbad et al., 2011; Dickson et al., 2012). It is of course possible that the results obtained would differ if higher doses of the conjugate or GLP-1 were used than those applied in the current study.

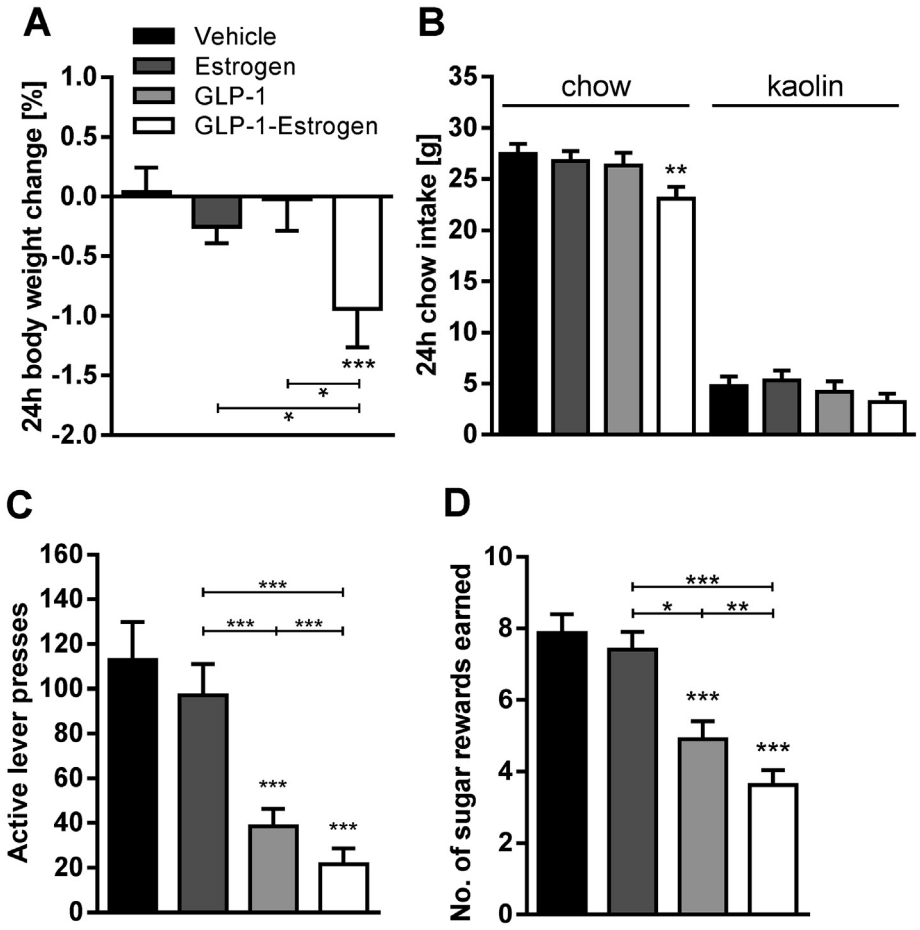

Fig. 1. Combining GLP-1 and estrogen in a subcutaneous injection was effective at reducing body weight and food reward. Conjugation of GLP-1 to estrogen led to a synergistic effect on weight loss (A) and anorexia (B), without inducing malaise as measured by the PICA response (B). The changes in weight and chow intake were associated with a striking suppression of sucrose-driven food reward behavior as indicated by reduced active lever presses (C) and rewards earned (D). Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 by comparing vehicle to compound injections unless otherwise stated. Differences between groups were calculated with repeated measures ANOVA followed by post hoc Bonferroni test (food intake 24h: $F_{(3,45)}$=5.6, p<0.005; sucrose pellets: $F_{(3,45)}$=24.4, p<0.0001) or generalized linear model (body weight change: $X^2_{(3)}$=10.4, p<0.05; active lever presses: $X^2_{(3)}$=45.1, p<0.0001). n=16.

3.3. The supramammillary nucleus (SUM) is a direct target site for GLP-1-estrogen actions on body weight, food intake, and food-motivated behavior

To find the CNS-sites underlying GLP-1-estrogen driven food reward changes we used a recently developed protocol for SPECT-imaging of regional cerebral blood flow (Kolodziej et al., 2014) as a screening tool. The voxelwise analysis revealed significant differences in rCBF after intraperitoneal injection of GLP-1-estrogen, and GLP-1 in contrast to vehicle-treated animals at $p < 0.001$ (Fig. 3 and Supplementary Fig. 1). The 99mTc brain uptake was significantly increased after GLP-1-estrogen injection in an area extending from the SUM to the posterior hypothalamus and substantia nigra and was significantly reduced in the cingulate area as compared to vehicle injections (Fig. 3A and Supplementary Fig. 1A). Few significant voxels were also detected in the left primary somatosensory cortex and left dorsal thalamus indicating reduced rCBF after GLP-1-estrogen injection in these regions (Supplementary Fig. 1B). After injection of GLP-1 the 99mTc brain uptake was found to be significantly reduced in the left ectorhinal/entorhinal area and in few voxels within the primary somatosensory cortex (Supplementary Fig. 1C,D). Isolated voxels indicating increased rCBF after injection of GLP-1 were found in the right amygdala, cingulum/anterior cingulate area and left superior colliculus (Supplementary Fig. 1C,D). If the significance level is increased to $p < 0.01$, a significant increase in rCBF was also found in the SUM and posterior hypothalamus after GLP-1 injection alone (Fig. 3B). VOI analysis, representing the mean differences within the SUM/posterior hypothalamus after injection of GLP-1-estrogen, GLP-1, or vehicle obtained from added global mean normalized data of single animals, revealed an increase in 99mTc uptake in this region from vehicle injection (1.10 μ) to GLP-1 injection (1.17 μ) and to GE injection (1.25 μ) of about 6% in each case (Fig. 3C,D). Thus, SPECT-imaging revealed the SUM as the main GLP-1-estrogen target area. The SUM is rather unexplored in the fields of energy balance regulation or reward. Nevertheless, a few studies have suggested that the SUM may participate in reward and feeding control. The medial part of SUM contains dopamine neurons and receives dense projections from the LH, including LH orexin neurons (Peyron et al., 1998; Swanson, 1982). Administration of $GABA_A$ receptor antagonists into the SUM potently induces intracranial drug self-administration. This nucleus also mediates reward triggered by administration of nicotine or the glutamate receptor agonist AMPA (Ikemoto et al., 2004, 2006). ERβ, GPR30, and GLP-1R have been detected in the SUM (Shughrue et al., 1997; Merchenthaler et al., 1999; Hazell et al., 2009) suggesting a potential for a direct effect of the GLP-1-estrogen in this area. GLP-1-estrogen injections into the SUM performed next further supported this idea as the sucrose-

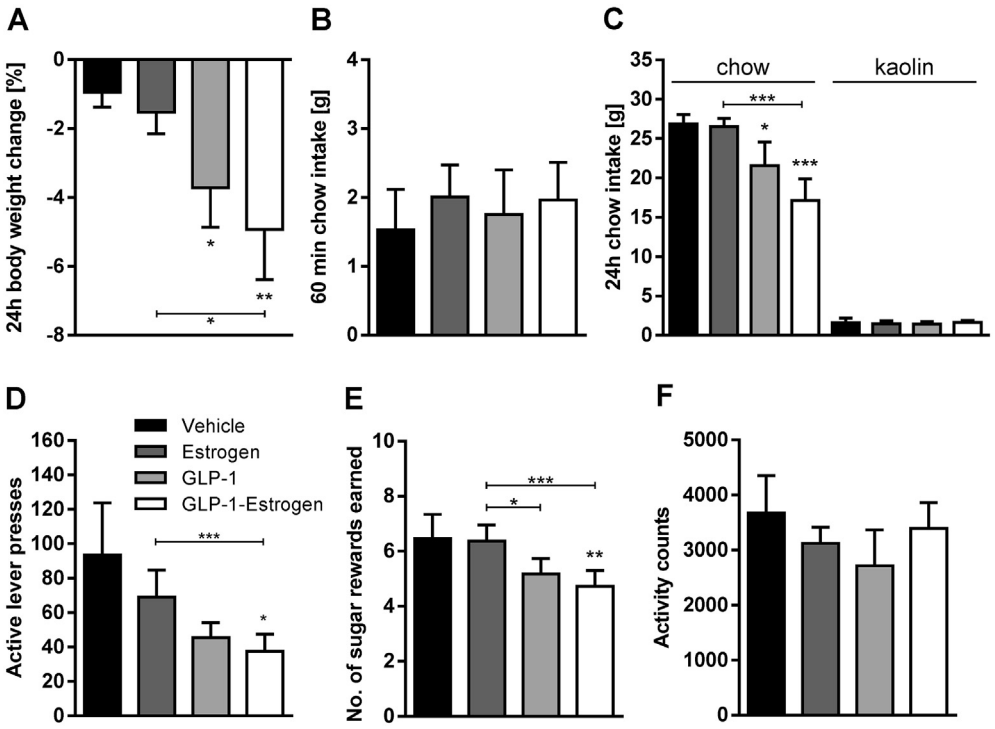

Fig. 2. Central injection of GLP-1-estrogen was sufficient to reduce body weight and food reward. Body weight was significantly reduced after central (intra lateral ventricle) injection of GLP-1 or GLP-1-estrogen conjugate (A). The impact of the treatment on food intake emerged at 24h after injection (B-C). No changes in malaise were noted (C). The sucrose-driven food reward behavior was suppressed by the GLP-1-estrogen conjugate as measured by reduced number of active lever presses (D) and sucrose pellets (E). Importantly the reduced reward behavior was not associated with non-specific reduction in general motor activity (F). Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 by comparing vehicle to compound injections unless otherwise stated. Differences between groups were calculated with generalized linear model (body weight change: $X^2_{(3)}$=15.4, p<0.005; food intake 24h: $X^2_{(3)}$=25.5, p<0.00005; active lever presses: $X^2_{(3)}$=19.4, p<0.0005; sucrose pellets: $X^2_{(3)}$=26.8, p<0.0001). n=11–12.

driven food reward and chow intake after GLP-1-estrogen administration were reduced to a much greater extent than by estrogen or GLP-1 alone (Fig. 4A–D). It is impossible to entirely exclude the possibility that some injection fluid also reached the VTA, the neighboring structure that co-express GLP-1 and estrogen receptors (Shughrue et al., 1997; Merchenthaler et al., 1999). However, current results demonstrate that the conjugate is ineffective at the level of the VTA (Fig. 4F,G). The administration of GLP-1 alone into the VTA resulted in a significant suppression of food reward, which is consistent with previous findings (Dickson et al., 2012). Thus we show that, surprisingly, traditional reward-controlling areas do not contribute to the reward-suppressing effect of the conjugated GLP-1 with estrogen but instead discover a novel candidate area, the SUM, to underlie the reward-reducing impact of GLP-1-estrogen. Since both, GLP-1 and estrogen receptors, are present in the SUM and direct intra-SUM microinjections of GLP-1-estrogen potently suppressed food reward it is reasonable to conclude that the molecule hybrid targets the reward system through a direct action in the SUM. Accordingly we demonstrate that brain circuitry beyond the classical mesolimbic dopaminergic system drives food-motivated behavior and place the SUM on the food reward regulation map certainly warranting future studies on the role of this nucleus in the regulation of food reward.

3.4. *GLP-1-estrogen enhances the metabolic benefits when injected into the LH and the NTS*

Since both, GLP-1R and ER, are also co-expressed in more classical energy balance regulating areas (Shughrue et al., 1997; Merchenthaler et al., 1999) we determined whether these sites comprise the CNS target sites of GLP-1-estrogen actions on energy homeostasis. The compounds were microinjected into the LH and the NTS; brain areas associated with feeding behavior control (Schwartz, 2006; Simpson et al., 2009). The LH was a highly sensitive target site for the body weight reduction of the conjugate (Fig. 5A). Although the NTS GLP-1-estrogen injections failed to reach significance there was an obvious trend ($p = 0.16$) to body weight reduction. In addition targeting of the LH and NTS with the GLP-1-estrogen compound induced a robust reduction in 24 h feeding response (Fig. 5B). Moreover, in both cases conjugate injection was more efficacious at lowering body weight and food intake than either GLP-1 or estrogen alone. Thus, by site-specific injections we demonstrate that the LH and to some extent the NTS are involved in GLP-1-estrogen synergy on food intake and body weight regulation. These results are in line with previous literature suggesting these two brain nuclei are key neural substrates for energy balance effects of GLP-1. Direct administration of GLP-1 into the LH significantly reduces food intake, whereas injection of Ex9 increases food intake (McMahon and Wellman, 1998; Schick et al., 2003) indicating a physiological role of GLP-1R in this area in feeding behavior control.

In addition, neuronal processing restricted to the NTS was also sufficient to mediate the intake-suppressive effects of the conjugate. The NTS is the first central area processing the gastrointestinal signals to the brain. Vagal-afferent signals synapse in specific subnuclei within the NTS to regulate food intake (Schwartz, 2000). Direct NTS injections of Ex9 or knockdown of preproglucagon, the precursor of GLP-1, in the NTS causes hyperphagia and weight gain (Hayes et al., 2009; Barrera et al., 2011). Activation of GLP-1R in the NTS, by direct injection of GLP-1 or Ex4, reduces both food intake and engages NTS noradrenergic neurons (Richard et al., 2015). Also estrogen can act via ERα on NTS neurons to inhibit feeding (Asarian

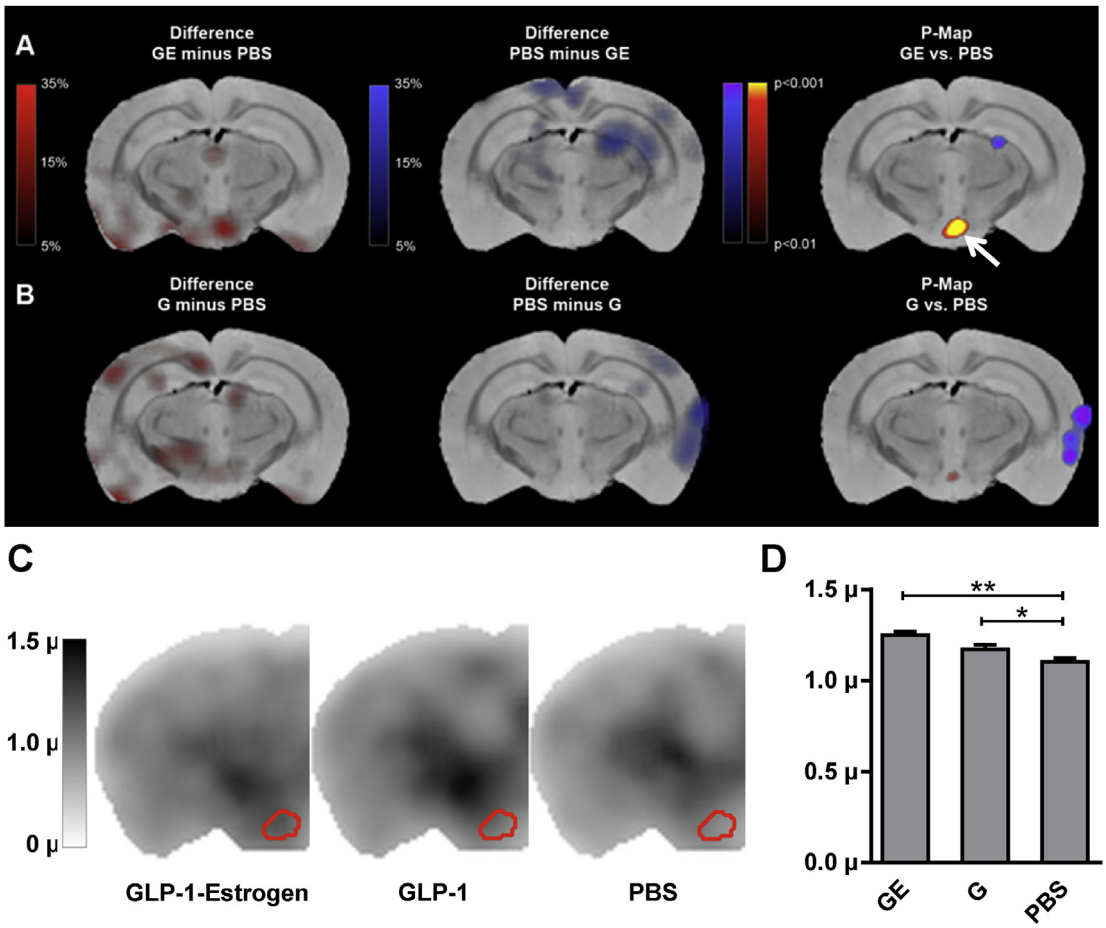

Fig. 3. SPECT-imaging revealed the supramammillary nucleus (SUM), a nucleus largely unexplored in the field of feeding and reward, as the main GLP-1- estrogen target area. Differences in mean tracer uptake in the SUM region (arrow) after injection of GLP-1-estrogen (row A) and GLP-1 (row B) versus vehicle (PBS) and corresponding probability maps (A, B, right column) clearly indicate that blood flow in the SUM was increased specifically after the administration of GLP-1 conjugated to estrogen. Differences in mean tracer uptake were calculated from normalized group data. Increases in mean tracer uptake were displayed in red, decreases in blue. Mean tracer uptake within the SUM/posterior hypothalamus in GLP-1-estrogen, GLP-1, or PBS injected mice (C, D). Hemisections of added global mean normalized data are shown in C. The ROI is highlighted in red including the SUM and posterior hypothalamus and provides a significant increase in tracer uptake after injection of GLP-1-estrogen (**p<0.001) and GLP-1 (*p<0.01) in comparison to PBS injection (D). G: GLP-1; GE: GLP-1-estrogen.

and Geary, 2007; Thammacharoen et al., 2008). Current data indicating a potent food-intake reduction after intra-NTS conjugate infusion are in line with these previous studies showing a role for both GLP-1 and estrogen in NTS in food intake regulation. That both hindbrain and hypothalamic nuclei have a key contribution to the anorexic properties of GLP-1-estrogen is perhaps not surprising considering that the neural control of energy balance is distributed across the neuraxis and many food intake regulating signals exert a similar effect from several CNS sites (Grill, 2006; Skibicka and Grill, 2009; Kanoski et al., 2016). We also note that the SUM, LH, and NTS may not be the only neural substrates targeted by the conjugate, and further studies examining more metabolic parameters (for example blood glucose changes) and different time points may reveal additional neural targets. One such possible target is the arcuate nucleus, suggested to be an important mediator of food intake effects of GLP-1R activation by some studies (Beiroa et al., 2014; Secher et al., 2014), or an important mediator of blood glucose changes induced by GLP-1 by other studies (Sandoval et al., 2008).

Recent literature demonstrates that peripheral or central application of GLP-1 or GLP-1 analogs into the VTA, NAc, NTS, or parabrachial nucleus alters food-reward behavior (Dickson et al., 2012; Alhadeff et al., 2014; Richard et al., 2015). NTS GLP-1-producing neurons project directly to the mesolimbic VTA (Rinaman, 2010; Dossat et al., 2011; Alhadeff et al., 2012; Dickson et al., 2012). So far the impact of estrogen on food-motivated behavior is largely unknown. Nevertheless, a wide range of behaviors and neurobiological mechanisms are modulated by estrogen, including alterations in a conditioned place preference test and the neuroprotection of dopamine cells (Küppers et al., 2000; Walf et al., 2007). In the current study microinjections of GLP-1-estrogen into the traditional reward-associated area, the VTA, failed to show a synergistic effect of the conjugate in food-motivated behavior changes. Thus we speculated that, in addition to SUM, the LH may be another good candidate neural substrate for the GLP-1-estrogen impact on food reward since the LH also regulates reward behavior and serves as an interface between the hypothalamus and the mesolimbic system (Geisler and Zahm, 2005; Teitelbaum and Epstein, 1962; Thompson and Swanson, 2010). While microinjections into the LH revealed a significantly suppressed food reward by GLP-1-estrogen, GLP-1 alone reduced reward to the same level (Fig. 5C) indicating that estrogen and GLP-1 do not synergise to reduce food reward in the LH. Similar results were obtained with the intra-NTS microinjections, indiating that consistent with previous studies (Alhadeff and Grill, 2014; Richard et al., 2015) NTS is an important brain nucleus for GLP-1-induced

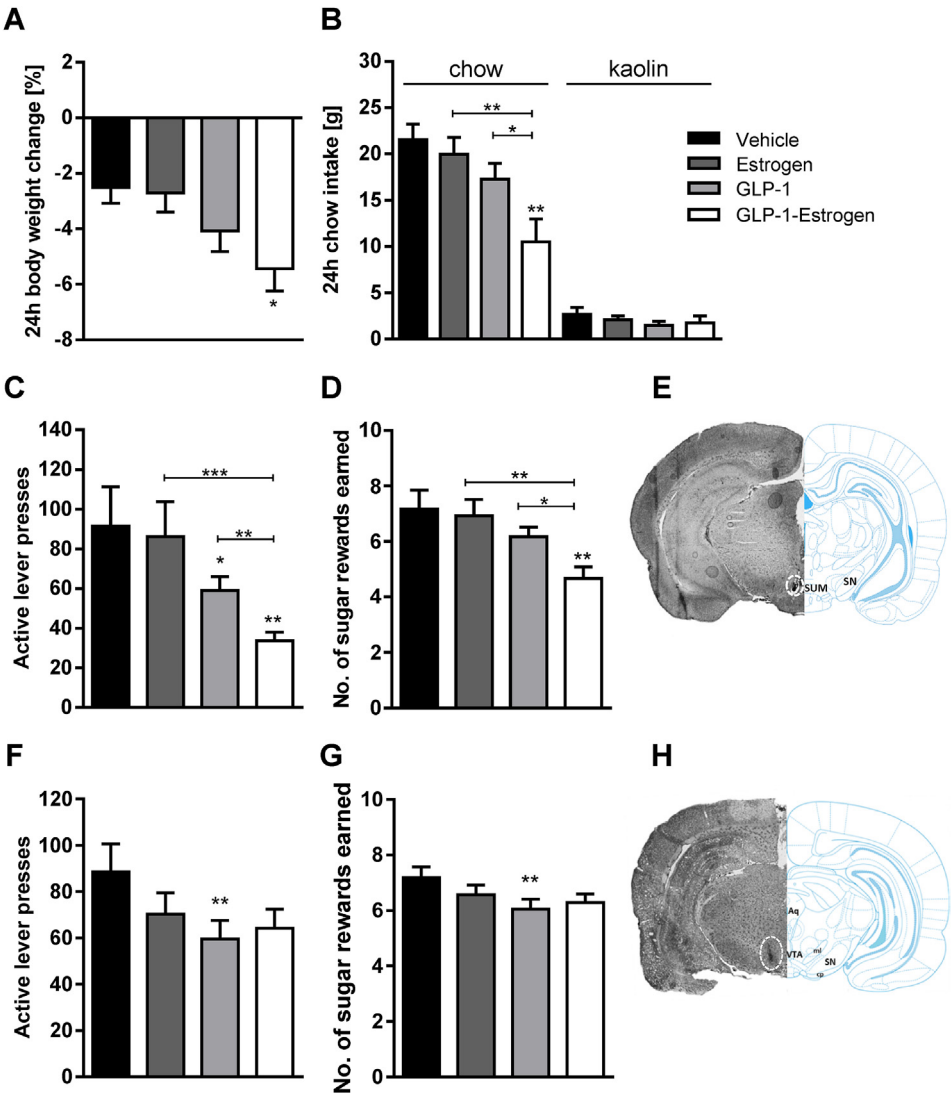

Fig. 4. The SUM is a direct target site for the impact of the GLP-1-estrogen conjugate on food-motivated behavior. Intra-SUM microinfusion of GLP-1 conjugated to estrogen, but not free GLP-1 or estrogen, reduced body weight (A) and food intake (B) but did not induce malaise measured by kaolin intake (B). Food reward was also potently reduced by intra-SUM microinfusion of both GLP-1 alone and GLP-1 conjugated to estrogen (C-D). Notably the conjugate resulted in a synergistic effect on both parameters of food-reward behavior (active lever presses (C) and number of sucrose rewards earned (D)). Representative tissue section demonstrating SUM injection site (left side) and corresponding rat brain atlas section (right side) (E). In contrast, the same dose of GLP-1-estrogen conjugate injected into the VTA, the well-established target of GLP-1R action of food reward, did not affect food-motivated behavior whereas injection of GLP-1 alone reduces the number of sucrose rewards. Active lever presses (F) and number sucrose rewards (G) earned in an operant lever-pressing paradigm after compound injection into the VTA (n = 21). VTA injection site and corresponding rat brain atlas section (H). Data represent mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by comparing vehicle to compound injections unless otherwise indicated. Differences between groups were calculated with repeated measures ANOVA followed by *post hoc* Bonferroni (SUM, food intake 24h: $F_{(3,33)}=10.0$, $p<0.0001$; VTA, active lever presses: $F_{(3,60)}=6.2$, $p<0.001$; VTA, sucrose pellets: $F_{(3,60)}=5.6$, $p<0.01$) or LSD test (SUM, body weight change: $F_{(3,33)}=4.9$, $p<0.001$; SUM, sucrose pellets: $F_{(3,33)}=7.3$, $p<0.001$) or generalized linear model (SUM, active lever presses: $X^2_{(3)}=17.3$, $p<0.001$). SN: substantia nigra; SUM: supramammillary nucleus; Aq: aquaduct; cp: cerebral peduncle; ml: medial lemniscus; VTA: ventral tegmental area.

reward supression, but does not play a role in the interaction of GLP-1 and estrogen to reduce food reward. These results highlight the differential responsivity of different neural substrates to the conjugate, and show that only select sites mediate the synergistic effects of the conjugate. Thus it is possible that our effect in ventricle-injected animals gets diluted since it provides access to many GLP-1 expressing sites, many of which may not participate in the added benefit of combining GLP-1 with estrogen, in contrast to the injection performed at other sites (eg SUM).

In summary, microinjections into key energy balance controlling nuclei revealed that the metabolic benefits resulting from GLP-1-estrogen injections are mediated through the LH and to some extent by the NTS. In contrast, no additional benefit of the conjugate was noted on food reward when the compound was microinjected into the LH or the NTS, leaving the SUM as the only neural substrate identified here to underlie the reward reducing benefits of GLP-1 and estrogen conjugate.

3.5. Conclusions

In conclusion, the data presented here provide clear evidence that the CNS is a crucial target for GLP-1-estrogen mediated actions on energy homeostasis and outline a previously unidentified role of this compound on food reward. These new insights into the mechanisms of the combined GLP-1 and estrogen action highlight the therapeutic potential of a new class of polypharmaceutical agents for the treatment of the metabolic syndrome. To localize the site of GLP-1-estrogen synergy on food reward we used a novel

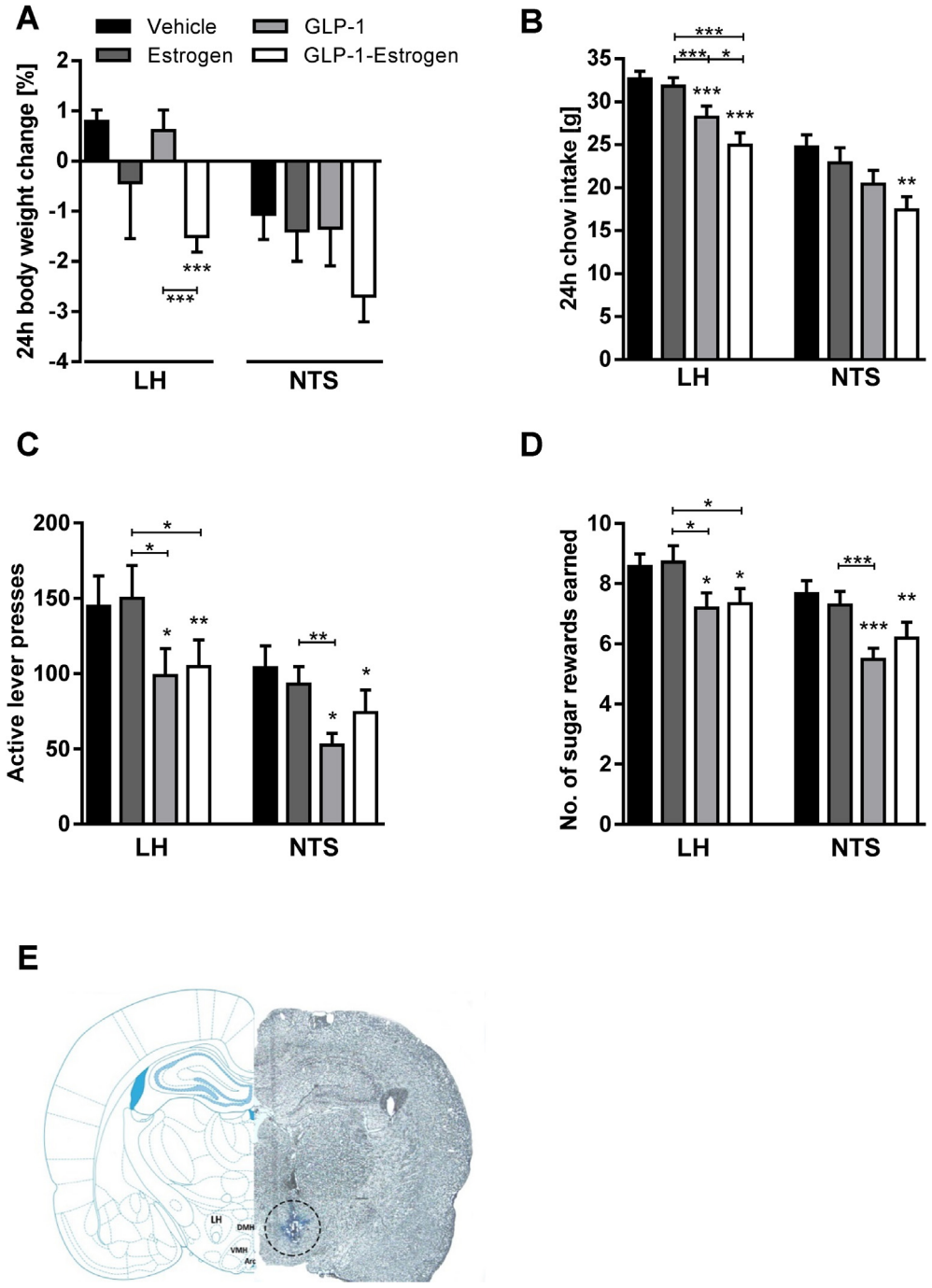

Fig. 5. GLP-1-estrogen enhances the metabolic benefits when injected into the LH and the NTS. Microinjections of GLP-1-estrogen reduces (A) 24h body weight change and (B) 24h chow intake after compound injection into the LH and NTS (n=21). No beneficial effects were noted on food reward when GLP-1-estrogen was microinjected into the LH and NTS as the number of active lever presses (C) and sucrose pellets (D) earned in an operant leverpressing paradigm was reduced to the same level as GLP-1 alone. LH injection site and corresponding rat brain atlas section (E). Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 by comparing vehicle to compound injections unless otherwise stated. Differences between groups were calculated with repeated measures (ANOVA) followed by *post hoc* Bonferroni test (NTS, Food intake 24h: $F_{(3,60)}$=4.6, p<0.01; LH, active lever presses: $F_{(3,60)}$=6.4, p<0.001; LH, sucrose pellets: $F_{(3,60)}$=13.5, p<0.001; NTS, active lever presses: $F_{(3,60)}$=6.5, p<0.001; NTS, sucrose pellets: $F_{(3,60)}$=10.0, p<0.0001) and Generalized Linear Model (LH, Body weight change: $X^2_{(3)}$=72.8, p<0.0001; LH, Food intake 24h: $X^2_{(3)}$=98.5, p<0.0001). LH: lateral hypothalamus; DMH: dorsomedial hypothalamus; VMH: ventromedial hypothalamus; Arc: arcuate nucleus.

approach of functional neuroimaging. An important and unexpected finding of the present study is that coactivation of GLP-1 and estrogen receptors results in a unique activation of the SUM, a brain area clearly capable of regulating energy balance and reward that has not been previously indicated for this role. These findings may be clinically relevant, since peripheral injections, the application route used in diabetic and obese patients prescribed GLP-1-based drugs, activated the SUM.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.07.039.

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