BRAIN ACTIVATION FOLLOWING PERIPHERAL ADMINISTRATION OF
THE GLP-1 RECEPTOR AGONIST EXENDIN-4

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Running head: Brain effects of Exendin-4

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ABSTRACT

The aim of our study was to investigate the anorectic and brain stimulatory effects of various doses of Exendin-4 (Ex-4) and to investigate the role of the vagus nerve in Ex-4-induced brain activation. A dose-related increase in c-fos mRNA expression was observed following Ex-4 administration (0.155 - 15.5 μg/kg). Doses of Ex-4 that caused anorexia without aversive effects (0.155; 0.775 μg/kg) induced c-fos expression in the hypothalamic arcuate and paraventricular (PVH - parvocellular) nuclei as well as in the limbic and brainstem structures. Doses of Ex-4 that caused aversion (1.55; 15.5 μg/kg) stimulated the same regions (in a more intense way) and additionally activated the magnocellular hypothalamic structures (supraoptic nucleus and PVH magnocellular). The brain c-fos pattern induced by Ex-4 showed both similarities and differences with that induced by refeeding. Subdiaphragmatic vagotomy significantly blunted the stimulation of c-fos mRNA expression induced by Ex-4 in the nodose ganglion, the medial part of nucleus of the solitary tract and the parvocellular division of the PVH. Pre-treatment with the Ex-9-39 (330 μg/kg ip) impaired the neuronal activation evoked by Ex-4 in all brain regions and in the nodose ganglion. The effects of Ex-4 on the hypothalamic-pituitary-adrenal axis activity were not altered by vagotomy. The results of this study demonstrate and relate the anorectic and brain stimulatory effects of aversive and non-aversive doses Ex-4 and indicate that the activation of specific central regions induced by the peripheral administration of Ex-4 is, at least in part, dependent on the integrity of the vagus nerve.

Keywords: Subdiaphragmatic vagotomy, refeeding, c-fos, hypothalamic pituitary axis.
INTRODUCTION

The glucagon-like peptide-1 receptor (GLP-1R) agonist Exendin-4 (Ex-4) is a 39-amino acid polypeptide initially isolated from the venom of the Gila monster (*Heloderma suspectum*) (11). Both Ex-4 and native GLP-1 are highly homologous (13). Unlike GLP-1, Ex-4 displays increased resistance to the proteolytic activity of dipeptidyl peptidase IV (DPP IV) and has a half-life of several hours in circulation (11). Similar to GLP-1, Ex-4 has anti-diabetic properties that have been described in both animals and humans (33, 57). In humans, Ex-4 (Exenatide) treatment has also been associated with a progressive dose-dependent weight loss (9). Consistently, treatment with Ex-4 in animal studies has been associated with significant reductions in food intake and body weight gain following either central (3, 37) or peripheral (26, 37, 46) administrations.

The mechanisms whereby peripherally administered Ex-4 exerts hypophagia remain incompletely elucidated. The inhibitory effect of Ex-4 on gastric motility and emptying has been suggested as a potential factor reducing food intake (17). In addition, peripheral injection of Ex-4 has been shown to induce neuronal activation of brainstem and hypothalamic structures potentially involved in conveying satiety signals (3, 54, 55). Ex-4 and other GLP-1R agonists also seem to influence neuronal activity in other brain regions, such as the forebrain limbic system (4, 38). In humans, Ex-4 has been shown to induce dose-related nausea (9) potentially through activation of limbic regions and associated brainstem structures such as area postrema (AP) and parabrachial nucleus (PB), two nuclei described to be involved in the development of conditioned taste aversion (CTA) (56).
There is no consensus on how signals generated by peripherally administered GLP-1R agonists such as Ex-4 reach the central nervous system. It has previously been suggested that the effect could be mediated through the activation of two specific circumventricular organs (CVOs), the AP and the subfornical organ (SFO) (10, 23). Those two CVOs are known for their blood-brain barrier permeability and their high level of GLP-1R expression and we recently reported that electrolytic ablation of AP and SFO alters Ex-4-induced c-fos expression in brain areas involved in the regulation of energy balance and associated behaviors (5). Paralelly, contribution of the vagus nerve in GLP-1R agonist action has also been addressed by various groups (1, 20, 46, 53). Subdiaphragmatic vagotomy and transection of the brainstem-hypothalamic pathway abolishes the anorectic effects of intraperitonealey (ip) injected GLP-1 in refed rats (1) and ablation of sensory neural pathways by systemic pretreatment with capsaicin prevents the ability of Ex-4 to suppress food intake in mice (46). More recently, it has been demonstrated that the satiating effect of ip administrated GLP-1 requires vagal afferent signaling in rats (39). It has also been been reported that the GLP-1 increase in c-fos expression in the hypothalamic arcuate nucleus (ARC) is prevented by bilateral truncal vagotomy (1) but the effect of the vagotomy on Ex-4-induced c-fos expression in the brain has yet to be described.

The present study was designed to examine the anorectic and brain stimulatory effects of aversive and non aversive doses of Ex-4 and to assess the contribution of the vagus nerve in those effects. Brain and nodose neuronal activation were assessed by evaluating c-fos mRNA expression, which has been extensively and reliably used for the characterization of brain activation in response to various challenges (32, 40). The pattern
of Ex-4-induced brain c-fos expression was also compared to that of refeeding. Plasma levels of glucose, insulin, and corticosterone together with corticotropin-releasing factor (CRF) expression in the brain were also assessed as all those variables have previously been shown to be influenced by Ex-4 (21, 27, 57).

METHODS

Animals and diet

Male Wistar rats (250-300 g body weight) were purchased from Charles River Canada (St-Constant, QC, Canada). All rats were cared for and handled according to the Canadian Guide for the Care and Use of Laboratory Animals and our institutional animal care committee approved the present protocol. The animals were housed individually in wire-bottom cages suspended above absorbent paper and, unless otherwise specified, fed ad libitum with a stock diet (rodent laboratory chow 5075, Purina, Strathroy, ON, Canada). They were exposed to a 12h-12h dark-light cycle and kept under an ambient temperature of 23 ± 1° C. The rats were accustomed to these conditions during a period of seven days.

Experimental design

Experiment Series 1: Dose-related effects of Ex-4 on brain neuronal activation, food intake, CTA, hypothalamic-pituitary-adrenal (HPA) axis activity and glucose metabolism.
Fasted (24 hours) rats were ip injected with Ex-4 (0.155; 0.775; 1.55 and 15.5 μg/kg) or saline (n=3-11/group) and then returned to their cages for 30 min prior to being sacrificed. Other groups of fasted rats (n=5-7) were injected with either saline or Exendin 9-39 (Ex-9-39, 330 μg/kg) and offered ad libitum refeeding for 30 (only for saline) or 60 min (for both saline and Ex-9-39) and then sacrificed. Brains were collected to determine the c-fos mRNA and CRF hnRNA expression and plasma samples were collected for corticosterone, insulin and glucose measurements. Additional fasted rats (24 hours) rats (n=5-7/group) were injected with Ex-4 (0.155; 0.775; 1.55 and 15.5 μg/kg) and refed ad libitum for 60 min to measure food and water intake at 30 and 60 min time intervals during refeeding. Finally, extra groups of rats (n=5-6/group) were used to evaluate the dose-response effect of Ex-4 (0; 0.155; 0.775; 1.55 and 15.5 μg/kg) on CTA.

**Experiment Series 2: Effects of vagotomy and Ex-9-39 on Ex-4-induced changes in neuronal activation, HPA axis activity and glucose metabolism.**

After a 24h fast, vagotomized and sham-operated rats (230-350g) (n=5-7/group) were injected with saline and 5 minutes later with Ex-4 (1.55 μg/kg) or saline to assess the effects of vagotomy on Ex-4-induced c-fos mRNA expression in the brain and nodose ganglion. It has previously been reported that 1.55 μg/kg of Ex-4 induces an anorexigenic effect without interfering with the general status and locomotor functions in rats (35). An additional group of sham-operated rats received the GLP-1R antagonist, Ex-9-39 (330 μg/kg) followed by Ex-4 (1.55 μg/kg) to evaluate the effects of the GLP-1R blockade on Ex-4-induced c-fos mRNA expression. This dose of Ex-9-39 *per se* does not stimulate c-
*fos* expression in the brain (51). In order to produce an adequate antagonism of GLP-1R, Ex-9-39 was administrated 5 min prior to the injection of Ex-4 (21, 24, 34). Thirty minutes after the second injection, rats were anaesthetized and sacrificed, and blood, brains, and nodose ganglia were collected for the measurements of *c-fos* expression.

**Assessment of CTA**

A one-bottle CTA test was performed in order to provide higher sensitivity in evaluating differential aversion between groups (7). The design was adapted from the one described by Grigson et al. (16). All the behavioral testing was conducted in the rat’s homecage. Animals were acclimated using a deprivation schedule, which allowed unlimited access to deionized water for 30 min only each morning (10:30 to 11:30). During this period, a drinking tube (graduated 30 ml glass feeding tube, Dyets Inc., Bethlehem, PA, USA) was affixed to an empty food container and positioned in the rat’s cage. On day 7, water intake had stabilized and the conditioning trial was performed. Rats were then allowed free access to a 0.15% saccharose solution for 30 min and injected with a 0.15 M solution of LiCl (2% body weight) or Ex-4 (0; 0.155; 0.775; 1.55 and 15.5 μg/kg) immediately after the bottle was removed. Between day 7 and 10, access to water was still limited to 30 min each morning. On day 10, rats were again offered the saccharose solution and fluid intake was evaluated after 30 min.

**Vagotomy**

Rats were fasted overnight before surgery. Subdiaphragmatic vagotomy and pyloroplasty were carried out in 15 rats, under isoflurane anaesthesia. The stomach and
lower esophagus were exposed after an upper midline laparotomy. The stomach was gently retracted down beneath the diaphragm and, under microscope, anterior and posterior vagal trunks were visualized and isolated from the esophagus about 2 cm above the stomach. Two sutures were tied around each vagal trunk with sterile 4.0 silk (Ethicon Inc., Markham, ON, Canada), directly above the hepatic branch and beneath the diaphragm, and at least 1 cm of nerve tissues was dissected between the sutures. In addition, all neural and connective tissues surrounding the esophagus were removed. A pyloroplasty was performed in order to prevent gastric stasis (31). The pyloric sphincter was severed by an incision parallel to its axis and reconstructed with coated vicryl 8.0 taper-point sutures (Ethicon Inc.) perpendicular to the pylorus axis. The stomach was repositioned to its original location and the muscular abdominal wall and skin were successively closed with a sterile 4.0 silk suture. In sham-operated rats (n=15), a similar abdominal incision was made, stomach was retracted, vagal trunks were identified and manipulated and pyloroplasty was performed. Four out of 15 vagotomized rats died shortly after the procedure due to post-surgery complications. The remaining 11 vagotomized rats survived and maintained good health. All operated rats were allowed 1 week to recover prior to any experimental procedure. During that period, all animals were handled each day in order to familiarize them to the injection procedure.

Body weight and food intake was assessed daily. On postoperative days 1-3, an analgesic treatment (Ketoprofen 5 mg/kg) was subcutaneously administered daily. Rats had *ad libitum* access to water and a liquid diet (Clinical Resource Standard, Novartis Nutrition, Whitby, ON, Canada) for 24h after surgery. From postoperative day 2, animals were given a standard rat laboratory chow. Rats recovered for 1 week prior to the
initiation of the Ex-4 treatment. During the first 4 postoperative days, vagotomized rats gained less body weight than sham-operated animals but afterwards returned to their preoperative daily body weight gain. At the end of the recovery period, no difference in body weight gains was detected between vagotomized and sham-operated animals. The day before surgery, daily body weight gain was 8.35 ± 2.89 vs. 8.21 ± 2.31 in vagotomized vs. sham rats whereas one week after surgery, it was 7.81 ± 1.78 vs. 8.03 ± 1.92 (Supplementary figure 1).

Immediately after sacrifice, exhaustive visual inspection of the vagus nerve and surrounding tissues was performed in order to verify whether the vagotomy was adequate and complete. The ends of the vagal trunks were identified, and the integrity of the peri-oesophageal nervous tissue, the appearance of the oesophagus and stomach, the presence of food in the stomach and oesophageal reflux were recorded. The stomach was weighed and its size measured. Criteria for complete vagal transection were the lack of continuity in the nervous tissue along with the presence of gastric distension and oesophageal reflux (25). The vagotomy was successfully achieved in 11 rats. Vagotomized rats displayed a significantly greater stomach volume compared to sham-operated rats [length of the greater gastric curvature (cm) was 9.90 ± 1.25 vs. 6.10 ± 0.05, \( P=0.002 \), and gastric diameter (cm) was 7.45 ± 0.74 vs. 4.96 ± 0.09, \( P<0.001 \), respectively] (Supplementary figure 2). A stomach-to-body weight ratio was also calculated to further confirm the successful achievement of the vagotomy. It has been previously shown that a stomach-to-body weight ratio greater than 0.02 in fasted rats is a good indicator of a complete vagotomy (30). The stomach-to-body weight ratio in the vagotomized rats was 0.049 ±
0.016 vs. 0.009 ± 0.0003 in sham animals ($P=0.008$) (Supplementary figure 2). All sham-operated rats (n=15) had intact vagal innervation and normal stomach size.

**Drug administration**

Ex-4 and Ex 9-39 (at doses indicated above) were ip injected using a 1-ml syringe with a 25-gauge needle. The volume of injection was adjusted according to the weight of each animal (1.1 mL/kg). Ex-4 was purchased from Sigma-Aldrich Canada, Oakville, ON, Canada and Ex 9-39 (AC 2706) was kindly provided by Amylin Pharmaceuticals, San Diego, CA, USA. Both peptides were dissolved in a solution of isotonic pyrogen-free saline and 0.1% BSA. Treatments were achieved during the early light phase (between 08:30 and 10:30 h) after 24h of food deprivation.

**Tissue preparation for in situ hybridization histochemistry**

Rats were anaesthetized with ketamine (60mg/kg) / xylazine (7.5mg/kg) ip. They were then intracardially perfused with 200 ml of ice-cold isotonic saline followed by 500 ml of paraformaldehyde (4%) solution. The brains were then removed and kept in paraformaldehyde (4%) for 7 days. They were transferred to a solution containing paraformaldehyde (4%) and sucrose (10%) before being cut 12 hours later using a sliding microtome (Histoslide 2000, Reichert-Jung, Heidelberger, Germany). Brain sections (30 μm) were taken from the olfactory bulb to the brainstem and stored at -30°C in a cryoprotectant solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%) and glycerol (20%). Immediately after the brain removal, cervical vagi were identified and nodose ganglia were collected and fixed in paraformaldehyde. Then,
ganglia were transferred in a cryoprotectant solution and stored at -30°C prior to being sliced. Fifteen-µm-thick longitudinal sections of the nodose ganglion were obtained using the microtome and mounted on slides immediately before in situ hybridization histochemistry.

**In situ hybridization histochemistry**

*In situ* hybridization histochemistry was used to identify c-fos mRNA and CRF hnRNA in tissue sections taken from brain and nodose ganglia. The protocol for *in situ* hybridization was largely adapted from the technique described by Simmons et al. (44). Briefly, brain sections (one out of every six brain sections) or nodose ganglion sections were mounted onto poly-L-lysine-coated slides and allowed to desiccate overnight under vacuum. The sections were then successively fixed for 20 minutes in paraformaldehyde (4%), digested for 30 minutes at 37°C with proteinase K (10 µg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M trietholamine, pH 8.0), and dehydrated through graded concentrations (50, 70, 95, and 100%) of alcohol. After vacuum drying for at least 2 hours, 90 µl of the hybridization mixture, containing an antisense ³⁵S-labeled cRNA probe (10⁷ cpm/ml), was spotted on each slide. Coverslips were mounted on the slides and incubated overnight at 60°C in a slide warmer. On the next day, coverslips were removed and the slides were rinsed four times with 4x saline-sodium citrate (SSC: 0.6 M NaCl, 60 mM trisodium citrate buffer, pH 7.0) containing 1mM 1,4-dithiothreitol (DTT), digested for 30 minutes at 37°C with RNase A (10mg/ml; Roche Diagnostics, Indianapolis, IN, USA), washed in descending concentrations of SSC (2x for 10 minutes, 1x for 5 minutes, 0.5x for 5 minutes, 0.1x for
30 minutes at 60°C) containing 1mM DTT, and dehydrated through graded concentrations of alcohol. After 2 hours of vacuum drying, the slides were exposed on an X-Ray film (Eastman Kodak, Rochester, NY, USA) for 24 h. Once removed from the autoradiography cassettes, the slides were defatted in toluene and dipped in NTB2 nuclear emulsion (Kodak). After the slides were exposed for 7 days, they were developed in D19 developer (Kodak) for 3.5 minutes at 14-15°C and fixed in rapid fixer (Kodak) for 5 minutes. Finally, tissues were rinsed in running distilled water for 1-2 hours, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, and cleared in toluene, and coverslips were applied with dibutylphthalate-xylol mounting medium.

**Antisense 35S-labeled riboprobes**

The c-fos cRNA probe was generated from the EcoRI fragment of rat c-fos cDNA (Dr. I. Verma, The Salk Institute, La Jolla, CA, USA) subcloned into a pBluescript SK-1 plasmid (Stratagene, La Jolla, CA, USA), and linearized with Sma I and Xho I (Pharmacia Biotech Inc., Oakville, ON, Canada) for antisense and sense probes, respectively. The heteronuclear (hn) CRF cRNA probe was generated from 530 bp fragment of the CRF intron 1 (Dr. S. Watson, University of Michigan, Ann Arbor, MI) subcloned into pGem-3 plasmid (Stratagene, La Jolla, CA, USA), and linearized with Hind III and EcoRI (Pharmacia Biotech Inc.) for antisense and sense probes, respectively. Radioactive riboprobes were synthesized by the incubation of 250 ng of linearized plasmid in 10 mM NaCl, 10 mM DTT, 6 mM MgCl, 40 mM Tris (pH 7.9), 0.2 mM ATP/GTP/CTP, 100 μCi α-35S-UTP (Perkin Elmer, Montreal, QC, Canada), 40 U
RNase inhibitor (Roche Diagnostics), and 20 U of RNA polymerase (T7 or T3 for antisense and sense probes, respectively, for e-fos mRNA and T7 or SP6 for antisense and sense probes, respectively, for CRF hnRNA) for 60 minutes at 37°C. The DNA templates were treated with 100 μl of DNase solution containing 0.1 U/ml DNase (Roche Diagnostics), 0.25 mg/ml of tRNA and 50 mM Tris/10 mM MgCl₂. The riboprobes were purified using RNeasy Mini Kit (Qiagen, Missisauga, ON, Canada), eluted in 150 μl of 10 mM Tris-1 mM EDTA buffer, and incorporated in a hybridation solution containing (per ml) 10⁷ cpm of ³⁵S probe, 52% formamide, 330 mM NaCl, 10 mM Tris (pH 8), 1 mM EDTA (pH 8), 1X Denhart’s solution, 10% dextran sulphate, 0.5 mg/ml of tRNA, 10mM DTT, and diethyl pyrocarbonate water. This solution was mixed and heated at 65°C and then spotted on slides. The specificity of the probe was confirmed by the absence of positive signal in sections hybridized with sense probe.

Quantitative analysis of the hybridization signals

The hybridization signals revealed on NTB2-dipped nuclear emulsion slides were examined under dark-field microscopy using an Olympus BX60 microscope (Olympus America, Melville, NY, USA). Images were acquired with a camera (RT Slider, model 2.3.0, Diagnostic Instruments) and analyzed with ImagePro-Plus software version 4.5.1.23 (MediaCybernetics, Silver Spring, MD, USA). Saturation of the hybridization signal was avoided by adjusting the exposure time for the image with the strongest hybridization signal sampled for each region in every series. The luminosity of system was set to the maximum, and the saturation warning option was used to visualize saturated regions in the image preview. Thereafter, according to the pixel distribution
histogram, the exposure time was adjusted in order to reduce to zero the number of saturated (pure white) pixels. The same exposure time was used throughout the analysis of the entire series.

The oval part of the bed nucleus of stria terminalis [BSTov, 0.10-0.26 mm caudal to bregma (β)], the supraoptic nucleus (SON, 1.10-1.40 mm caudal to β), the magnocellular paraventricular hypothalamic nucleus (PVHm, 1.80-2.00 mm caudal to β) the parvocellular paraventricular hypothalamic nucleus (PVHp) including dorsal (d), medio-ventral (mv) and medio-dorsal (md) subdivisions (1.80-2.00 mm caudal to β), the central nucleus of amygdala (CeA, 2.00-2.45 mm caudal to β), the arcuate nucleus (ARC, 2.54-3.25 mm caudal to β), the lateral, lateral-external and medial parabrachial nuclei (PBl, PBle and PBm respectively, 9.16-9.25 mm caudal to β), the locus coeruleus (LC, 9.8-10.04 mm caudal to β), the medial, central, and lateral subnuclei of the NTS (NTSm, NTSce, and NTSl, respectively) – at the level at which the NTS touches the fourth ventricle (13.28-13.60 mm caudal to β) and caudal part of the NTSm (caudal NTSm) – caudal to the obex (13.68-14.36 mm caudal to β), and the area postrema (AP, 13.76-14.16 mm caudal to β) were outlined and the pixel density of the hybridization signal was measured on both hemispheres of 2-4 brain sections for each animal assigned to a given treatment. When no hybridization signal was visible under dark-field illumination, the brain structures of interest were outlined under bright-field illumination and then subjected to densitometric analysis under dark-field illumination. The pixel density of each specific region was corrected by subtracting background readings taken from areas immediately surrounding the region of interest. The brain sections from the different groups of rats were matched for rostrocaudal levels as closely as possible.
Cells expressing c-fos mRNA were counted on all longitudinal slices of the two nodose ganglia in each animal. The maximal number of c-fos-expressing cells found over all nodose slices belonging to each animal was considered for statistical analysis.

**Plasma Determinations**

At the time of sacrifice, blood was collected by cardiac puncture and centrifuged (1,500 g for 15 minutes at 4 °C) and plasma was stored at -20 °C for further biochemical analyses. Plasma glucose concentrations were determined using an automated glucose analyzer (YSI 2300 Stat Plus, Yellow Springs Instruments, Yellow Springs, OH, USA). Commercially available radioimmunoassay kits were used for the measurements of plasma insulin (Linco Research, St. Charles, MO, USA) and plasma corticosterone (MP Biomedicals, Toronto, ON, Canada).

**Statistical analysis**

The values presented in tables and graphs are means ± standard errors of the means (SEM). Statistical differences between groups were determined by unpaired Student’s t-test or one-way analysis of variance (ANOVA). When necessary, data were logarithmically transformed to satisfy the variance normality criterion. The Fisher’s PLSD post hoc test was also used to identify significant differences among means. Results were considered as being significant at \( P < 0.05 \). Statistical analyses were performed using StatView version 5.0 software (SAS Institute, Cary, NC, USA).
RESULTS

Dose-related effects of Ex-4 administration on brain neuronal activation, food intake, and CTA.

Figure 1 illustrates the pattern of c-fos mRNA expression in the brain following the ip administration of different doses of Ex-4 and after ingestion of a meal. Twenty-four hours of food deprivation did not per se stimulate c-fos expression in the brain. The lowest dose of Ex-4 used (0.155 μg/kg) induced neuronal activity in the limbic structures (BSTov and CeA), in the SFO, and in the PBle (see Figure 1 for the distribution pattern and Table 1 for the quantification of c-fos mRNA expression). At 30 min, Ex-4 (0.155 μg/kg) did not significantly reduce food intake (Figure 2A). However, the anorectic effect of the 0.155 μg/kg dose of Ex-4 became significant at 60 min (Figure 2C). In addition to stimulating the BSTov, CeA and PB, the intermediary dose of Ex-4 (0.775 μg/kg) stimulated c-fos mRNA expression in the NTSm (at V4- and caudal levels), AP, ARC, PVHm and PVHp (mv and md subdivisions) (see Table 1 and Figure 1). The 0.775 μg/kg dose of Ex-4 (and larger doses) produced significant anorectic effects during 0-30, 30-60 and 0-60 min periods of refeeding (see Figure 2A, 2B, and 2C, respectively). Ultimately, the highest Ex-4 doses (1.55 μg/kg and 15.5 μg/kg) induced extra c-fos expression in the hypothalamic magnocellular structures (PVHm and SON) and locus coeruleus (LC) (Table 1 and Figure 1). In contrast to the effects of the lower doses of Ex-4 (0.155 μg/kg and 0.775 μg/kg), administration of either 1.55 or 15.5 μg/kg of Ex-4 (Figure 2) also caused CTA (Figure 3). The anorectic effects of the various doses of Ex-4 were also significant when values were corrected for body weight (Supplementary figure 3).
The present data indicate that virtually most nuclei activated by refeeding were also stimulated by Ex-4 at doses of 1.55 and 15.5 μg/kg (Figure 1). These regions included the NTS, AP, PB, LC, CeA, BST, PVT, SON, PVHm, and PVHp. Within most of these nuclei however, Ex-4 and feeding induced different patterns of neuronal activation (Figure 4). During early refeeding, the amount of ingested food and the brain neuronal activation patterns were similar at 30 and 60 min. Supplementary table 1 shows quantification of the c-fos mRNA expression in BSTov, CeA, PVHp, PVHm, SON, ARC, LC, PBle, NTSm, and AP, food intake and body weight in rats refed for either 30 or 60 min. Compared to refeeding, Ex-4 strongly increased c-fos mRNA expression in the limbic structures (BSTov, CeA) and in PBle and AP, which are important sources of neuronal input for the forebrain limbic system. In the hypothalamus, Ex-4 significantly increased c-fos mRNA expression in the PVHp and ARC. In contrast, refeeding strongly stimulated the activation of the magnocellular hypothalamic nuclei, NTS and PBm (Figure 4). The non-aversive dose of Ex-4 (0.775 μg/kg) specifically induced c-fos mRNA expression in the limbic structures (BSTov, CeA), PBle and AP, and in hypothalamic regions such as the PVHp (md and mv subdivisions) and ARC. In addition, the Ex-4 aversive doses (1.55 and 15.5 μg/kg) significantly increased neuronal activation in the magnocellular structures (PVHm and SON) and LC while amplifying c-fos mRNA expression in the PVHp and AP (Table 1 and Figure 4).

Ex-9-39 significantly prevented refeeding-induced c-fos mRNA expression in the PVHm, PVHp-mv and ARC. Additionally, a general trend for a decrease in c-fos mRNA expression following Ex-9-39 was observed in almost all brain nuclei. The latter effects fell however short of statistical significance (Supplementary figure 4).
We also observed that Ex-4 dose-dependently stimulated the HPA axis (Figure 5). The expression of CRF hnRNA, the primary transcript in CRF expression, was significantly induced by Ex-4, starting with the dose of 0.775 μg/kg. Levels of plasma corticosterone dose-dependently increased following Ex-4 (0.155 - 15.5 μg/kg). It is noteworthy that doses of 1.55 and 15.5 μg/kg of Ex-4 evoked similar levels of plasma corticosterone and CRF hnRNA expression in PVHp (Figure 5). In contrast to Ex-4, refeeding did not change CRF hnRNA expression and plasma corticosterone levels (Figure 5).

Acute treatment with Ex-4 in fasted rats induced a dose-dependent rise in plasma glucose levels. The effect on plasma glucose started at the lowest dose (0.155 μg/kg). The secretion of insulin was on the other hand stimulated only by the highest doses of Ex-4 (1.55 and 15.5 μg/kg) (Table 2). As expected, plasma glucose and insulin concentrations were higher at the end of the 60 min refeeding period than following food deprivation (Table 2).

**Effects of vagotomy and Ex-9-39 on Ex-4-induced changes in neuronal activation, HPA axis activity and glucose metabolism.**

In line with the results of experiment series 1, Ex-4 (1.55 μg/kg) significantly induced c-fos mRNA expression in the PVH (PVHm, PVHp-d, PVHp-mv and PVHp-md), ARC, SON, PB (PBl and PBle subnuclei), NTSm (V4- and caudal parts) and AP (P<0.05, Figure 6). Subdiaphragmatic vagotomy reduced the neuronal activation induced by Ex-4 in the NTSm (P<0.05) and in the PVHp-md (P<0.05). Vagotomy led to a marginal non-significant decrease in Ex-4-induced c-fos mRNA expression in the ARC.
(P=0.07). In the PVHp-d and PVHp-mv, vagotomy-induced alterations in c-fos expression fell also short of statistical significance (Figure 6). In contrast, pre-treatment with Ex-9-39 (330 μg/kg) prevented the activation induced by Ex-4 injection in the hypothalamic (PVHp, PVHm, SON, and ARC) and brainstem (NTSm and PBl) nuclei (P ranged between 0.001 and 0.01). Ex-9-39 only slightly reduced c-fos expression evoked by Ex-4 in the AP and PBle (Figure 6). It is well known that PBle receives massive projections from AP neurons (19). Increases in Ex-4-induced c-fos mRNA expression (1.55 μg/kg) in BST (P<0.05) and CeA (P<0.05) were not affected by subdiaphragmatic vagotomy (data not shown). Pre-treatment with Ex-9-39 tended to decrease c-fos expression in BST (P=0.07) but did not influence neuronal activation in CeA (data not shown).

Subdiaphragmatic vagotomy did not prevent elevations in CRF hnRNA expression and plasma corticosterone induced by Ex-4 (1.55 μg/kg) (Figure 7). In contrast, pre-treatment with Ex-9-39 significantly altered the induction of CRF hnRNA expression in the PVHp (P<0.05). However, the GLP-1R antagonist did not significantly influence the rise in plasma corticosterone evoked by Ex-4 (Figure 7). The Ex-4-induced increased levels in plasma insulin and glucose were prevented by pre-treatment with Ex-9-39 but not by subdiaphragmatic vagotomy (Table 3).

We also examined the c-fos mRNA expression in the nodose ganglion in different feeding conditions (ad libitum feeding, 24 hours of food deprivation and following a 1 hour refeeding period after 24 hours of fasting), as well as in vagotomized and sham-operated rats ip injected with saline, Ex-4 or Ex-9-39 (Figure 8). Twenty-four hours of fasting did not stimulate c-fos mRNA expression in the nodose ganglion more relative to
Aversive and non-aversive doses of Ex-4 elicited an anorectic effect and stimulated neuronal activity in several brain regions. At the lowest dose (0.155 μg/kg), Ex-4 caused an anorectic effect and stimulated neuronal activity in the SFO, BST, CeA, and PB. At the intermediate dose of 0.775 μg/kg, Ex-4 induced a potent anorexigenic response coupled to a stimulation of brain structures such as the NTS, AP, PVH, and ARC. At the highest doses (1.55 μg/kg and 15.5 μg/kg), which were concomitantly anorectic and aversive, Ex-4 activated the hypothalamic magnocellular structures and LC in addition to further activating the regions stimulated by the smallest doses. The present results also demonstrate that the neuronal activation induced by Ex-4 (1.55 μg/kg) in the nodose ganglion and two brain nuclei, namely the medial NTS and PVHp, was partially prevented by the subdiaphragmatic vagotomy. Finally, it was also observed that some of the Ex-4 effects, such as those on the HPA axis activity (as
assessed by CRF hnRNA expression in the PVHp and plasma corticosterone) and glucose homeostasis were not influenced by the subdiaphragmatic vagotomy.

The present data support the view that the activation by Ex-4 of the limbic regions (CeA and BST), certain hypothalamic nuclei (PVHp and ARC) and vagus-associated brain structures (NTS, AP, PBle) occurs concomitantly with the non-aversive anorectic effect of the GLP-1R agonist. In that respect, Baumgartner et al. (8) observed that the induction of c-fos in the NTS, AP, and CeA was related to the eating-inhibitory activity of GLP-1, when it was infused into the hepatic-portal vein. Central administration of GLP-1 has also been found to stimulate catabolic neurons in the ARC (43). It is noteworthy that aversive doses of Ex-4 appear to cause a pattern of c-fos induction fairly similar to that seen following non-aversive doses, which let suppose that the strength of the stimulation (rather than its mere presence) in certain regions might dictate the aversive response. Stimulation of the AP, PB, and CeA has been seen to be implicated in the CTA acquisition (12, 36, 45). It is noteworthy that the highest doses (1.55 and 15.5 μg/kg) nonetheless specifically induced c-fos in magnocellular hypothalamic structures hosting neurons that express vasopressin and oxytocin, which are two peptides whose neurohypophyseal secretion has been reported to be stimulated by LiCl in a dose-dependent manner (52). Furthermore, the acquisition of LiCl-induced CTA appears to be mediated (at least in part) by aversion-related pathways involving oxytocin-expressing neurons. Also, high doses of vasopressin have been shown to amplify the effects of LiCl on CTA (18).
Distinct patterns of neuronal activation were observed in response to refeeding and treatment with Ex-4. In contrast to refeeding, administration of an anorectic, non-aversive dose of Ex-4 induced c-fos expression in the limbic structures (BST and CeA), PVHp (md and mv subdivisions), ARC, PBle and AP. The high, aversion-inducing dose of Ex-4 stimulated further PVH, LC and AP. Contrarily, refeeding more intensely stimulated neuronal activation in the NTSce, NTSI and PBm. In the PVHp, Ex-4 (but not refeeding) strongly activated the medio-ventral and dorsal subregions (known for their influence in the regulation of the autonomic control) and the medio-dorsal subregion (hosting corticotrophic neurons). In agreement with our previous study (50), we only observed modest activation of the PVHp in contrast to the strong stimulation of the magnocellular regions present during refeeding. Magnocellular hypothalamic neurons were stimulated by both refeeding and Ex-4 (especially, taste aversion-inducing doses). In addition, Ex-9-39 prevented feeding-induced activation of magnocellular neurons, suggesting that endogenous GLP-1 could be partially responsible for the postprandial stimulation of the magnocellular system, especially in its oxytocinergic component. For the needs of the present study, neurochemical characterizations of activated magnocellular neurons were not achieved. However evidence suggests that feeding and GLP-1R agonism stimulate particular activation patterns in vasopressin and oxytocin neurons (24, 48). We have previously described that vasopressin neurons were activated upon refeeding and that oxytocin neurons were not (48). In contrast, it has been demonstrated that the central administration of GLP-1 induces c-fos expression in approximately 38% of the oxytocinergic and in very small proportion of vasopressinergic (-10%) magnocellular neurons (24).
The present study also emphasizes that the peripheral administration of Ex-4 might also significantly increase expression of CRF hnRNA in the PVH and plasma corticosterone levels. CRF hnRNA is not constitutively produced in the brain, but its expression can be rapidly triggered in response to effective stimuli (5, 22, 49). It is noteworthy that the threshold dose necessary to stimulate CRF hnRNA expression (0.775 μg/kg) was higher than the dose required inducing a significant increase in plasma corticosterone (0.155 μg/kg). Low doses of Ex-4 injected peripherally seem to stimulate directly the adrenocortical cells and to induce the release of corticosterone by the adrenal glands (29). The activation of corticotrophic cells of PVHp appears to require the ip administration of higher doses of Ex-4. The activation of PVH following treatment with high doses of Ex-4 is consistent with some of the effects of GLP-1R agonists on the HPA axis (27, 28, 42, 47) and cardiovascular system (6, 15, 55, 58). It has previously been reported that central GLP-1 increases the sympathetic outflow, which translates into an increase in blood pressure and heart rate, and into the induction of Fos-IR in the adrenal medulla (6, 55).

We observed that acute treatment with Ex-4 in fasted rats induced a dose-dependent increase in plasma glucose levels. A similar hyperglycemic effect was observed following an acute Ex-4 injection in fasted non-diabetic rats. One mechanism for this could be a Ex-4-induced sympathoadrenal activation (2, 28). Consistent with this hypothesis, Ex-4 was shown to activate neurons in brain sites involved in autonomic and adrenal medulla controls (55).

Ex-4 and feeding strongly and comparably activated neurons of both the nodose ganglion and NTSm, which host afferent nerves from abdominal vagus. In contrast, the
NTSce, which receives projections from the oesophagus and the NTSl, which is innervated by laryngeal and pharyngeal afferent nerves (41) were activated by feeding, but not by Ex-4 administration. These data possibly suggest that all vagal afferent nerves are activated in response to feeding but that only abdominal vagal afferents are triggered by the GLP-1R agonism. Doses of Ex-4 that mediated neuronal activation in the NTS also induced c-fos expression in the nodose ganglion and AP. Our data show that complete subdiaphragmatic vagotomy significantly decreased Ex-4-induced c-fos expression in the nodose ganglion and NTSm as well as in the PVHp-md that receives projections from the NTSm (48). In other regions such as the PVHm, SON, ARC, and PB the stimulation of c-fos expression observed in response to Ex-4 was not influenced by vagotomy while being reduced by the pretreatment with the GLP-1R antagonist Ex-9-39. These results suggest that subdiaphragmatic vagal afferents play an important role in transduction of the GLP-1 signal from the digestive tract to the NTSm and PVH. As demonstrated in the first experiment series, the activation of these regions is not specifically related to aversives doses of Ex-4 as non-aversive doses of Ex-4 significantly induced c-fos mRNA expression in these regions. Therefore, it seems that the vagus nerve is not particularly involved in the mediation of the aversive effects of Ex-4.

Other brain regions such as the PB, limbic regions, PVHm and SON might be activated by Ex-4 directly or via the CVOs. There is evidence that two CVOs, namely the organum vasculosum of lamina terminalis and SFO, strongly project to the magnocellular structures of the hypothalamus (14). At the same dose of Ex-4 administrated in the present study, we have recently reported that the concomitant ablation of the AP and SFO blunts the stimulation of c-fos mRNA expression in specific brain structures including the
BST, CeA, PVH, SON, PB, and NTS (5). The present data suggest that the Ex-4-induced activation of AP was not affected by the subdiaphragmatic vagotomy despite the strong connection between the AP, NTS and the vagal pathway. Therefore, AP neurons seem to be directly stimulated by circulating Ex-4 rather than via the vagus nerve per se. It has been shown that the AP is characterized for its high density of GLP-1 binding sites (14). However, the neuronal activation of the NTSm seems to be dependent on both the vagal and CVO-mediated pathways involved in GLP-1 signaling. Ex-4-induced c-fos expression in the BST and CeA was not altered by vagotomy (present study) but by AP + SFO ablations (5), suggesting that the limbic response to Ex-4 could be, at least partially, mediated by receptors localized in these CVOs. Meanwhile, the relatively weak effect of Ex-9-39 pretreatment on the Ex-4-induced neuronal activation in limbic structures suggests that central receptors other than GLP-1R could mediate some of limbic-related Ex-4 central effects.

The elevation of CRF hnRNA expression following administration of an aversive dose of Ex-4 was not altered by vagotomy, but it was prevented by the pretreatment with the GLP-1R antagonist, Ex-9-39. Ex-4-induced plasma corticosterone levels were neither affected by subdiaphragmatic vagotony nor by Ex-9-39 pretreatment. These results suggest that the effect of Ex-4 on the HPA axis has a central component and the induction of the CRF expression is probably mediated by the central activation of GLP-1R.

In conclusion, the results of this study demonstrate and relate the anorectic and brain stimulatory effects of aversive and non-aversive doses Ex-4. They also demonstrate that the activation of specific central regions induced by the peripheral administration of Ex-4 is, at least in part, dependent on the integrity of the vagus nerve.
PERSPECTIVES AND SIGNIFICANCE

The present study was conducted in the context of a series of studies whose ultimate goal was to determine the mechanisms and neuronal circuits whereby GLP-1R agonism influences energy balance regulation. GLP-1, the main endogenous GLP-1R agonist, is secreted by the gastrointestinal L-cells. Its incretin or anorectic action, even though short-lasting because of the enzyme DPP IV, can be exerted at different levels, which complexifies the identification of GLP-1R-mediated metabolic pathways. Its anorectic action, which ultimately involves the brain, appears mediated by the vagus nerve afferents, the CVOs and the direct brain passage of the peptide. By testing the anorectic effects of the long-lasting GLP-1R agonist Ex-4 at various doses while assessing CTA and brain neuronal activation, we were able to further relate the anorectic and brain stimulatory effects of aversive and non-aversive doses of Ex-4. We also were able to demonstrate that Ex-4 produces neuronal activations that can be distinct from those brought about by the ingestion of food per se in a refeeding paradigm. Finally, the results of the present study, together with those of a recently published investigation (5), provide evidence for a role of the vagus and the CVOs in the effect of the GLP-1R agonism on brain neuronal activation.
We thank Julie Plamondon and Marie-Claude Roy for assistance with *in situ* hybridization, Josée Lalonde for plasma determination, Marie-Noëlle Cyr and Sébastien Poulin for their help in animal care along with Serge Simard for assistance with statistical analyses.
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FIGURE LEGENDS

**Figure 1.** Representative rostro-caudal distribution of c-fos mRNA expression following ip injection of saline or different doses of Exendin-4 (Ex-4) and after refeeding (RF) after 24h of food deprivation. The pictures represent the x-ray film autoradiographs of coronal brain sections hybridized with an antisense riboprobe complementary to rat c-fos mRNA. Fasted rats were injected with saline, Ex-4 (0.155; 0.775; 1.55; 15.5 μg/kg) or re-fed for 60 min. BST - bed nucleus of the stria terminalis, CeA - central amygdala, PVHp – paraventricular hypothalamic nucleus, parvocellular part, PVHm - paraventricular hypothalamic nucleus, magnocellular part, SON – supraoptic nucleus, ARC- arcuate nucleus, PB - parabrachial nucleus, LC - locus coeruleus, NTSm – medial part of nucleus of the solitary tract, AP - area postrema. Bregma levels (caudal to bregma) are: -0.26 mm for BSTov, -1.1 mm for SFO, -1.8 mm for PVH and SON, -3.14 mm for ARC and CeA, -9.16 mm for PB, -10.04 mm for LC, -13.3 mm for NTS, -13.8 mm for AP, -14.08 mm for caudal NTSm.

**Figure 2.** Food intake during 0-30 min (A), 30-60 min (B) and 0-60 min (C) refeeding periods following ip injection of different doses of Exendin-4 (Ex-4: 0.155; 0.775; 1.55; 15.5 μg/kg) or saline. Values are means ± SEM. Significant effect between groups differently labeled, \( P < 0.05 \).

**Figure 3.** Intakes of the saccharose solution 0.15% during the conditioning and test trials in rats injected with different doses of Exendin-4 (Ex-4: 0.155; 0.775; 1.55; 15.5 μg/kg),
saline or LiCl. Values are means ± SEM. Significant effect between groups differently labeled, $P < 0.05$.

**Figure 4.** C-fos mRNA expression in brain of rats fasted for 24h and injected ip with saline (FD saline) or Exendin-4 (FD Ex-4 0.775 or 15.5 μg/kg), or refed for 60 min and injected with saline (RF saline). (A’-A”) Mean pixel density of the hybridization signal for c-fos mRNA in the oval part of the bed nucleus of the stria terminalis (BSTov), central amygdala (CeA), dorsal (d), medio-dorsal (md) and medio-ventral (mv) subdivisions of the parvo cellular part of the paraventricular hypothalamic nucleus (PVHp), in the magnocellular part of the paraventricular hypothalamic nucleus (PVHm), supraoptic nucleus (SON), arcuate nucleus (ARC), locus coeruleus (LC), in the lateral part (PBl), lateral-external part (PBle), and medial part (PBm) of the parabrachial nucleus, in the medial subnucleus (NTSm), central subnucleus (NTSce), and lateral subnucleus (NTSl) of the nucleus of the solitary tract, and in the area postrema (AP) in fasted rats injected with saline or Ex-4 (0.775 or 15.5 μg/kg), or after 60 min of refeeding. Values are means ± SEM. Significant effect between groups differently labeled, $P < 0.05$ (B-M) Darkfield photomicrographs demonstrating positive hybridization signal for c-fos mRNA in the PVHp and PVHm (B and C), SON (D and E), CeA, (F and G), PBI, PBle and PBm (H and I), NTSm, NTSce, NTSI, caudal part of the NTSm, and AP (J-M) after Ex-4 injection (B, D, F, H, J, and L) or 1h of refeeding (C, E, G, I, K, and M). Scale bars, 100 μm. Bregma levels (caudal to bregma) are: -1.8 mm for PVH and SON, -3.14 mm for CeA, -9.16 mm for PB, -13.3 mm for NTS, -13.8 mm for AP.
**Figure 5.** Quantification of heteronuclear corticotropin releasing factor (CRFhn) RNA in the paraventricular hypothalamic nucleus and plasma corticosterone following ip injection of different doses of Exendin-4 (Ex-4: 0; 0.155; 0.775; 1.55; 15.5 μg/kg) and 60 min of refeeding. Values are means ± SEM. Bars not sharing a common superscript are significantly different from each other, *P* < 0.05.

**Figure 6.** C-fos mRNA expression in brain of vagotomized and sham-operated rats ip injected with saline saline or Exendin-4 following saline or Exendin 9-39. (A) Mean pixel density of the hybridization signal for c-fos mRNA in the medio-dorsal (md), dorsal (d) and medio-ventral (mv) subdivisions of the parvocellular part of the paraventricular hypothalamic nucleus (PVHp), in the area postrema (AP), lateral (PBl) and lateroexternal (PBle) parts of the parabrachial nucleus, medial part of the nucleus of the solitary tract (NTSm) – at V4 and caudal levels, in the magnocellular part of the paraventricular hypothalamic nucleus (PVHm), the supraoptic nucleus (SON) and in the arcuate nucleus (ARC). SS - sham rats injected twice with saline, VS - vagotomized rats injected twice with saline, SE4 – sham-operated rats injected with Exendin-4 following saline, VE4 - vagotomized rats injected with Exendin-4 following saline, SE9E4 - sham rats injected with Exendin-4 following Exendin 9-39. Values are means ± SEM. Significant effects of SE9E4 vs. SE4 (*P*<0.05; **P<0.001) and VE4 vs. SE4 (‡P<0.05). (B-M) Darkfield photomicrographs depicting positive hybridization signal for c-fos mRNA in the PVHp and PVHm (*B, C, and D*), SON (*E, F, and G*), V4- level NTSm (*H, I, and J*), and caudal NTSm and AP (*K, L, and M*) in sham-operated rats injected with Exendin-4 (*B, E, H, and
in vagotomized rats injected with Exendin-4 ($C, F, I$, and $L$), and in sham rats injected with Exendin 9-39 and Exendin-4 ($D, G, J$, and $M$). V3 – third ventricle, V4 – fourth ventricle, och – optic chiasm. Scale bars, 100 µm. Bregma levels (caudal to bregma) are -1.8 mm for PVH and SON, -13.3 mm for NTS, -13.8 mm for AP.

**Figure 7.** Mean pixel density of hybridization signal for CRF hnRNA in paraventricular nucleus of hypothalamus and plasma corticosterone in vagotomized and sham-operated rats. Rats were ip injected with saline or Exendin-4 following saline or Exendin 9-39. SS – sham-operated rats injected twice with saline, VS - vagotomized rats injected twice with saline, SE4 – sham-operated rats injected with Exendin-4 following saline, VE4 - vagotomized rats injected with Exendin-4 following saline, SE9E4 – sham-operated rats injected with Exendin-4 following Exendin 9-39. Values represent means ± SEM. Bars not sharing a common superscript are significantly different from each other ($P<0.05$ for CRF hnRNA and $P<0.005$ for corticosterone).

**Figure 8.** Quantification of c-fos mRNA expressing cells ($A$) and darkfield photomicrographs ($B-E$) in nodose ganglia of vagotomized ($V$) and sham-operated ($S$) rats. Rats were fed *ad libitum* (AL), food deprived for 24h (FD) or re-fed for 1h after 24h of fast (RF). Rats were ip injected with saline, Exendin-4 (Ex-4) (1.55 µg/kg) following saline or Exendin 9-39 (Ex-9) (330 µg/kg). ($A$) Fos-positive cell number is represented by the solid bars in sham rats (S), black right-rising hatched bars in vagotomized rats (V) and white left-rising hatched bars in sham-operated rats injected with Exendin 9-39 (Ex-9). Values are means ± SEM. Bars not sharing a common superscript are significantly
different from each other, \( P < 0.001 \). (B-E) Darkfield photomicrographs demonstrating positive hybridization signal for \( c-fos \) mRNA in nodose ganglia of sham-operated rats injected with saline (B and B1), sham rats injected with Ex-4 (1.55 \( \mu \text{g/kg} \)) (C and C1), vagotomized rats injected with Ex-4 (1.55 \( \mu \text{g/kg} \)) (D and D1), sham-operated rats injected with Ex-9 (330 \( \mu \text{g/kg} \)) and Ex-4 (1.55 \( \mu \text{g/kg} \)) (E and E1). B1, C1, D, and E1 panels are the higher magnification of the areas depicted by rectangles on the B, C, D, and E panels. Scale bars, 100 \( \mu \text{m} \).
**SUPPLEMENTARY FIGURE LEGENDS**

**Supplementary figure 1.** Daily body weight gain in sham-operated and vagotomized rats. Data represents means ± SEM.

**Supplementary figure 2.** Complete vagotomy criteria: length of the greater gastric curvature (A), gastric diameter (B), and stomach-to-body weight ratio (C) in sham-operated and vagotomized rats. Data represents individual values.

**Supplementary figure 3.** Food and water intake at 30 (A and C, respectively) and 60 (B and D, respectively) min following ip injection of different doses of Exendin-4 (Ex-4: 0.155; 0.775; 1.55; 15.5 μg/kg) or saline. Values are means ± SEM. Significant effect between groups differently labeled, \( P < 0.05 \).

**Supplementary figure 4.** Mean pixel density of hybridization signal for c-fos mRNA expression in brain of rats refed 60 min and injected with saline or Ex-9-39 (330 μg/kg). CeA - central amygdala, PVHp – paraventricular hypothalamic nucleus, parvocellular part with medio-dorsal (md), dorsal (d) and medio-ventral (mv) subdivisions, PVHm - paraventricular hypothalamic nucleus, magnocellular part, SON – supraoptic nucleus, ARC- arcuate nucleus, PB - parabrachial nucleus with lateral (PBl) and lateroexternal (PBle) subdivisions, NTS – nucleus of the solitary tract with medial (NTSm) and central (NTSce) subdivisions, AP - area postrema. Values are means ± SEM. * - significant effect between groups, \( P < 0.05 \).
Table 1. Quantification of c-fos mRNA expression in brains of rats following ip injection of saline or different doses of Exendin-4 (Ex-4): 0.155, 0.775, 1.55, and 15.5 μg/kg.

Data are mean pixel density of the hybridization signal in the oval part of bed nucleus of the stria terminalis (BSTov), central amygdala (CeA), magnocellular part of the paraventricular hypothalamic nucleus (PVHm), dorsal (d), medio-dorsal (md) and medio-ventral (mv) subdivisions of the parvocellular part of the paraventricular hypothalamic nucleus (PVHp), supraoptic nucleus (SON), arcuate nucleus (ARC), locus coeruleus (LC), lateral-external part of parabrachial nucleus (PBle), medial part of nucleus of the solitary tract (NTSm), area postrema (AP), subfornical organ (SFO). Values are means ± SEM. Significant effect between groups differently labeled as assessed by one-way ANOVA, \( P < 0.05 \).

<table>
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<tr>
<th></th>
<th>Saline</th>
<th>Ex-4 0.155μg/kg</th>
<th>Ex-4 0.775μg/kg</th>
<th>Ex-4 1.55μg/kg</th>
<th>Ex-4 15.5μg/kg</th>
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<td>BSTov</td>
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<td>18.69 ± 3.83</td>
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<td>CeA</td>
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<td>24.98 ± 5.13</td>
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<td>PVHm</td>
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<td>25.80 ± 1.26</td>
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<td>PVHp (d)</td>
<td>5.72 ± 2.10</td>
<td>8.30 ± 0.99</td>
<td>9.70 ± 0.96</td>
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<td>PVHp (md)</td>
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<td>PVHp (mv)</td>
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<td>20.08 ± 6.04</td>
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<td>SON</td>
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<td>5.35 ± 0.81</td>
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<td>ARC</td>
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<td>16.44 ± 2.04</td>
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Table 2. Plasma insulin and glucose following the ip injection of different Exendin-4 (Ex-4) doses and after 1h of refeeding. Data are means ± SEM. Significant effect between groups differently labeled as assessed by one-way ANOVA, \( P < 0.05 \).

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<th>Ex-4 15.5μg/kg</th>
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<td>b,c</td>
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<td><strong>Glucose</strong> (mmol/l)</td>
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</table>

\( P^{(0.06)} \), c,d
Table 3. Plasma insulin and glucose in vagotomized (V) and sham-operated (S) rats. Blood was collected 30 minutes after an ip injection of either: saline, Exendin-4 (Ex-4) (1.55 µg/kg) or Ex-4 preceded by Exendin 9-39 (Ex-9) (330 µg/kg). Data are means ± SEM. Significant effect between groups differently labeled as assessed by Ficher’s PLSD test following one-way ANOVA, $P < 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Saline $S$</th>
<th>Saline $V$</th>
<th>Ex-4 $S$</th>
<th>Ex-4 $V$</th>
<th>Ex-9 + Ex-4 $S$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin (nmol/l)</strong></td>
<td>0.046 ± 0.004 a</td>
<td>0.048 ± 0.008 a</td>
<td>0.190 ±0.027 b</td>
<td>0.218 ± 0.021 b</td>
<td>0.112 ± 0.023 c</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>7.50 ± 0.38 a</td>
<td>7.13 ± 0.63 a</td>
<td>10.43 ± 0.98 b</td>
<td>9.92 ± 0.63 b</td>
<td>8.02 ± 0.47 a</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.
Figure 3.

Graph showing 30-min sucrose intake (ml) for different LiCl doses (μg/kg) during Conditioning trial and Test trial.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
**Supplementary table 1.** Quantification of c-fos mRNA expression in brains of rats and food intake at 30 and 60 min of refeeding.

Data are mean pixel density ± SEM of the hybridization signal in the oval part of bed nucleus of the stria terminalis (BSTov), central amygdala (CeA), magnocellular part of the paraventricular hypothalamic nucleus (PVHm), dorsal (d), medio-dorsal (md) and medio-ventral (mv) subdivisions of the parvocellular part of the paraventricular hypothalamic nucleus (PVHp), supraoptic nucleus (SON), arcuate nucleus (ARC), locus coeruleus (LC), lateral-external part of parabrachial nucleus (PBle), medial part of nucleus of the solitary tract (NTSm), area postrema (AP), subfornical organ (SFO). FI – food intake, BW – body weight. Non-directional Student’s t-test.

<table>
<thead>
<tr>
<th></th>
<th>Refeeding 30min n=5</th>
<th>Refeeding 1h n=4</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSTov</td>
<td>15.31 ± 3.56</td>
<td>13.80 ± 0.42</td>
<td>0.13</td>
</tr>
<tr>
<td>CeA</td>
<td>15.04 ± 1.80</td>
<td>15.61 ± 1.73</td>
<td>0.82</td>
</tr>
<tr>
<td>PVHm</td>
<td>18.25 ± 5.44</td>
<td>22.70 ± 3.64</td>
<td>0.58</td>
</tr>
<tr>
<td>PVHp (d)</td>
<td>3.07 ± 1.37</td>
<td>2.59 ± 0.31</td>
<td>0.77</td>
</tr>
<tr>
<td>PVHp (md)</td>
<td>7.51 ± 1.56</td>
<td>6.85 ± 1.04</td>
<td>0.75</td>
</tr>
<tr>
<td>PVHp (mv)</td>
<td>4.53 ± 2.02</td>
<td>3.82 ± 1.06</td>
<td>0.78</td>
</tr>
<tr>
<td>SON</td>
<td>35.05 ± 5.29</td>
<td>45.88 ± 5.01</td>
<td>0.19</td>
</tr>
<tr>
<td>LC</td>
<td>7.44 ± 2.26</td>
<td>10.46 ± 3.36</td>
<td>0.46</td>
</tr>
<tr>
<td>PBle</td>
<td>13.31 ± 2.32</td>
<td>18.18 ± 3.82</td>
<td>0.29</td>
</tr>
<tr>
<td>NTSm</td>
<td>18.19 ± 2.76</td>
<td>14.08 ± 3.16</td>
<td>0.96</td>
</tr>
<tr>
<td>AP</td>
<td>6.46 ± 1.45</td>
<td>8.63 ± 2.37</td>
<td>0.44</td>
</tr>
<tr>
<td>ARC</td>
<td>9.76 ± 1.72</td>
<td>8.91 ± 1.57</td>
<td>0.73</td>
</tr>
<tr>
<td>FI (g)</td>
<td>6.91 ± 0.45</td>
<td>7.47 ± 1.07</td>
<td>0.61</td>
</tr>
<tr>
<td>BW (g)</td>
<td>315.94 ± 2.51</td>
<td>314.95 ± 4.70</td>
<td>0.84</td>
</tr>
</tbody>
</table>
Supplementary Figure 1.
Supplementary Figure 2.
Supplementary Figure 3.
Supplementary Figure 4.