Lesions of area postrema and subfornical organ alter exendin-4-induced brain activation without preventing the hypophagic effect of the GLP-1 receptor agonist

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Baraboi ED, Smith P, Ferguson AV, Richard D. Lesions of area postrema and subfornical organ alter exendin-4-induced brain activation without preventing the hypophagic effect of the GLP-1 receptor agonist. Am J Physiol Regul Integr Comp Physiol 298: R1098–R1110, 2010. First published January 27, 2010; doi:10.1152/ajpregu.00326.2009.—The mechanism and route whereby glucagon-like peptide 1 (GLP-1) receptor agonists, such as GLP-1 and exendin-4 (Ex-4), access the central nervous system (CNS) to exert their metabolic effects have yet to be clarified. The primary objective of the present study was to investigate the potential role of two circumventricular organs (CVOs), the area postrema (AP) and the subfornical organ (SFO), in mediating the metabolic and CNS-stimulating effects of Ex-4. We demonstrated that electrolytic ablation of the AP, SFO, or AP + SFO does not acutely prevent the anorectic effects of Ex-4. AP + SFO lesion chronically decreased food intake and body weight and also modulated the effect of Ex-4 on the neuronal activation of brain structures involved in the hypothalamic-pituitary-adrenal axis and glucose metabolism. The results of the study also showed that CVO lesions blunted Ex-4-induced expression of c-fos mRNA (a widely used neuronal activity marker) in 1) limbic structures (bed nucleus of the stria terminalis and central amygdala), 2) hypothalamus (paraventricular hypothalamic nucleus, supraoptic nucleus, and arcuate nucleus), and 3) hindbrain (lateral and lateral-external parabrachial nucleus, medial nucleus of the solitary tract, and ventrolateral medulla). In conclusion, although the present results do not support a role for the CVOs in the anorectic effect induced by a single injection of Ex-4, they suggest that the CVOs play important roles in mediating the actions of Ex-4 in the activation of CNS structures involved in homeostatic control.

food intake; energy expenditure; hypothalamic-pituitary-adrenal axis; glucose metabolism; c-fos

EXENDIN-4 (Ex-4) is a 39-amino acid polypeptide originally isolated from the venom of the Gila monster (Heloderma suspectum) (18). It binds to the same receptor as native glucagon-like peptide-1 (GLP-1), with which it shares a 53% sequence homology (22). GLP-1 is a proglucagon-derived hormone secreted from enteroendocrine L cells, which are mainly found in the distal intestine. The best understood biological effects of GLP-1 and other GLP-1 receptor (GLP-1R) agonists relate to their actions in the regulation of blood glucose levels through the stimulation of insulin biosynthesis/secretion, inhibition of glucagon release, and gastric emptying (15, 50, 64). Recently, GLP-1 was shown to inhibit food intake following central and peripheral administration in a variety of species (20, 60, 63).

In contrast to circulating GLP-1, which is rapidly degraded through the action of the enzyme dipeptidyl peptidase IV (14, 29), Ex-4 has a half-life of several hours in the circulation. Animal studies have shown antidiabetic effects of Ex-4 (67), as well as reductions in food intake and body weight following central (2, 47) or peripheral (47, 57) administration of the drug. Regardless of the route of administration, Ex-4 also induces a specific pattern of neuronal activation among brain structures involved in food intake regulation and associated behaviors (66). These structures include 1) hypothalamic centers (paraventricular hypothalamic nucleus (PVH), supraoptic nucleus (SON), and arcuate nucleus (Arc)), 2) hindbrain nuclei (parabrachial (PB) nucleus, nucleus of the solitary tract (NTS), and area postrema (AP)), and 3) limbic structures (bed nucleus of stria terminalis (BST) and central nucleus of amygdala (CeA)) (6, 66). Central injection of GLP-1 also stimulates c-fos expression in the PVH, specifically in corticotropin-releasing factor (CRF) neurons, suggesting that the GLP-1R mediates the central modulation of hypothalamic-pituitary-adrenocortical (HPA) axis activity (33, 61, 62). Finally, it has been demonstrated that Arc neurons presenting GLP-1R also coexpress glucokinase (GK) mRNA, a marker of neuronal glucose sensing (42).

The mechanism whereby peripherally administered GLP-1 or Ex-4 inhibits food intake remains largely unknown. The hypophagia induced by the peripheral injection of Ex-4 has been shown to be mediated by the GLP-1R, inasmuch as it is absent in GLP-1R-deficient mice (4). The anorectic effect of centrally injected Ex-4 is, however, insensitive to the GLP-1R antagonism (7). Furthermore, Ex-4-induced anorexia seems to be highly dependent on the peripheral GLP-1R, whereas ablation of sensory vagal pathways by systemic pretreatment with capsaicin prevents the ability of Ex-4 to suppress food intake in mice (59). More recently, the circumventricular organs (CVOs), which are brain structures located on the peripheral side of the blood-brain barrier, have also been suggested as potential targets through which peripheral GLP-1R agonists may stimulate the brain. The AP and the subfornical organ (SFO) are two CVOs with identified GLP-1 binding sites (23, 43) and GLP-1R mRNA expression (36). The AP has been shown to be a key site for the central action of peripheral GLP-1R agonists (65). On the other hand, there are no data regarding the role of the SFO in mediating the central actions of GLP-1R agonists. Consequently, the present study aimed to explore the role of the AP and SFO in mediating the metabolic and brain-stimulating effects of Ex-4. Using three different
types of animal models, created by electrolytic ablation of the AP, SFO, or AP + SFO, we investigated the roles of the CVOs in Ex-4-induced effects on food intake, energy expenditure, brain neuronal activation, and some aspects of HPA axis activity and glucose metabolism.

METHODS

Animals and Diet

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

Electrolytic Lesions

Groups of rats were subjected to electrolytic lesions, which were carried out under ketamine (60 mg/kg)-xylazine (7.5 mg/kg) anesthesia. In sham-lesioned rats, electrodes were introduced within the SFO, AP, or SFO + AP, but no current was passed.

AP lesion. The rat was placed in a stereotaxic apparatus. Its head was flexed, and the cisterna magna was opened to permit access to the fourth ventricle. The rat was then subjected to electrolytic lesion (Keithley Instruments 225 Current Source, 0.5 mA for 20 s) of the AP via a monopolar Parylene C-insulated tungsten electrode (Micro Probe) with a tip exposure of 100 µm.

SFO lesion. The rat was placed in a stereotaxic apparatus, and the head was horizontally fixed. A midline incision was made in the soft tissues above the skull, and a small hole was drilled in the cranium, so that the electrode could be advanced into the region of the SFO using its rostroventral stalk (37). Data from rats with partial damage to the SFO from its targets in the ventral forebrain. Neuronal SFO projections to the preoptic region and hypothalamus leave the structure via its rostroventral stalk (37). Data from rats with partial damage to the SFO without destruction of the rostroventral pole were not included in the analyses. SFO-sham-lesioned animals showed evidence of the electrode tracks into the hippocampal commissure but no evidence of damage to the SFO.

Postoperative Care

During postoperative days 1 and 2, all rats were carefully followed and fed ad libitum a “recovery” diet (liquid chocolate-flavored Ensure and Pablum baby cereals, containing 10.45 KJ/g, with 14.7% of energy as fat, 70.6% as carbohydrate, and 14.4% as protein) and given antibiotic-anti-inflammatory treatment [Baytril (5 mg/kg)-buprenorphine (0.5 mg/kg) sc] daily. The recovery diet was a palatable, well-balanced diet that was provided to promote resumption of normal eating and drinking after the lesions. On postoperative day 3, animals were returned to a normal laboratory chow diet (containing 12.9 KJ/g, with 4.5% of energy as fat, 77.5% as carbohydrate, and 18.0% as protein). Rats were allowed 2 wk to recover before any investigation.

Ex-4 Injections

Ex-4 was intraperitoneally injected using a 1-ml syringe connected to a 25-gauge needle. The volume of injection was 1.1 ml/kg in the feeding test and 1.25 ml/kg in experiments examining c-fos activation. Ex-4 (Sigma-Aldrich Canada, Oakville, ON, Canada) was dissolved in a solution of isotonic pyrogen-free saline and 0.1% BSA, which was also injected in vehicle control animals.

Body Weight, Food Intake, and Body Composition

Body weight and food intake were measured daily during postoperative days 1–11 and every 2nd day during the rest of the experimental period. Food spillage was carefully accounted for in the measurement of food intake. Body composition was determined by dual-energy X-ray absorptiometry (DEXA; PIXImus2, Lunar, Madison, WI) on the surgery day and 3 wk later. The PIXImus2, which was primarily designed for mice, does not allow for the assessment of the whole body composition in larger animals, such as rats. However, we chose to measure the composition of a specific area, from which it is possible to infer the whole body composition of the rat (40). During measurements, anesthetized rats were in a prone position, with the posterior legs maintained in external rotation. The hip, knee, and ankle articulations were in 90° flexion. The scanned region was defined using the following anatomic reference points: frontally, the first lumbar vertebra; caudally, the caudal limit of the iliac bone; medially, a line splitting the backbone in half; and laterally, right distal femoral metaphysis. The region of interest thus comprised the femoral region of the right hindleg and the ipsilateral half of the inferior abdomen. The machine was calibrated daily with the manufacturer’s phantom. The same region was scanned in all rats. The percentage of body fat, as well as the fat-free mass and the fat mass, was measured using this technique.

Energy Expenditure and Food Intake in Metabolic Chambers

Between postoperative weeks 4 and 6, rats were housed in rat metabolic chambers for measurement of food intake and energy expenditure. The metabolic chambers consisted of air-proof cages, which were linked to an open-circuit indirect calorimetry system (19, 54). Rats were put in metabolic cages 24 h before the refeeding test for acclimatization to the new environment before measurements. After 20 h of food deprivation, rats were intraperitoneally injected with saline or 2 µg/kg of Ex-4 and then given free access to food. The dose of Ex-4 used in this study has been shown to induce an anorectic effect without interfering with the general status and locomotor function of the rat (44). Food intake was measured at 20-min intervals for 3 h following injections, and O2 uptake (VO2) and CO2 consumption (VCO2) were also recorded every 20 min. The average of values obtained during the last 3 h before the injection was used as the basal value; during the postinjection period, hourly values were obtained from the average of three consecutive 20-min measurements. VO2 was measured with an O2 analyzer (model S-3A, Applied Electrochemistry, Sunnyvale, CA), and VCO2 was measured with a CO2 analyzer (model CD-3A, Applied Electrochemistry). The respiratory quotient was calculated as VCO2/VO2.

Brain In Situ Hybridization Histochemistry

At the end of postoperative week 6, after an overnight fast, all rats were intraperitoneally injected with Ex-4 at 1.55 µg/kg, which,
on the basis of a series of tests, was demonstrated to be the lowest dose that induces a complete pattern of neuronal activation, or saline and killed 30 min later, between 0830 and 1130. Brains were collected and prepared (see below) to determine the expression of c-fos mRNA to estimate brain activation in response to various challenges (26, 48).

Rats were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg)-xylazine (7.5 mg/kg) and, without delay, intracardially perfused with 200 ml of ice-cold isotonic saline followed by 500 ml of paraformaldehyde (4%) solution. The brains were removed and kept in paraformaldehyde (4%) for 7 days, transferred to a solution containing paraformaldehyde (4%) and sucrose (10%), and cut 12 h later using a sliding microtome (Histoslide 2000, Reichert-Jung, Heidelberg, Germany). Brain sections (30 μm) were taken from the area between the olfactory bulb and the brain stem and stored at −30°C in a cryoprotectant solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%), and glycerol (20%).

In situ hybridization histochemistry was used to localize c-fos mRNA, CRF heteronuclear RNA (hnRNA), and GK mRNA on brain sections. The protocol was adapted from the technique described by Simmons et al. (53). Briefly, brain sections (1 of every 6 brain slices) were mounted onto poly-t-l-lysine-coated slides and allowed to desiccate overnight under vacuum. The sections were then successively fixed for 20 min in paraformaldehyde (4%), digested for 30 min 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 triethanolamine, pH 8.0), and dehydrated through graded concentrations (50, 70, 95, and 100%) of alcohol. After vacuum drying for ≥2 h, 90 μl of the hybridization mixture, which contains an antisense 35S-labeled cRNA probe (107 cpm/ml), were spotted on each slide. Coverslips were mounted on the slides, which were incubated overnight at 60°C in a slide warmer. On the next day, coverslips were removed and the slides were rinsed four times with 4°C saline-sodium citrate (SSC: 0.6 M NaCl and 60 mM trisodium citrate buffer, pH 7.0) containing 1 mM DTT, digested for 30 min at 37°C with RNase A (10 mg/ml; Roche Diagnostics, Indianapolis, IN), washed in descending concentrations of SSC (2× for 10 min, 1× for 5 min, 0.5× for 5 min, and 0.1× for 30 min at 60°C) containing 1 mM DTT, and dehydrated through graded concentrations of alcohol. After 2 h of vacuum drying, the slides were exposed on X-ray film (Eastman Kodak, Rochester, NY) 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 min at 14–15°C and fixed in rapid fixer (Kodak) for 5 min. Finally, tissues were rinsed in running distilled water for 1–2 h, 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**

cRNA probes were generated from rat cDNA fragments for c-fos (Dr. I. Verma, The Salk Institute, La Jolla, CA), GK (Dr. J. Buteau, Institut Universitaire de Cardiologie et de Pneumologie de Québec), and the 530-bp genomic fragment of the CRF intron 1 for heteronuclear CRF (Dr. S. Watson, University of Michigan, Ann Arbor, MI). Radioactive riboprobes were synthesized by incubation of 250 ng of linearized plasmid in 10 mM NaCl, 10 mM DTT, 6 mM MgCl2, 40 mM Tris (pH 7.9), 0.2 mM ATP/GTP/CTP, 100 μCi of α-35S-UTP (Perkin Elmer), 40 U of RNase inhibitor (Roche Diagnostics), and 20 U of RNA polymerase (T7 or T3 for antisense and sense probes, respectively, for c-fos mRNA and GK mRNA and T7 or SP6 for antisense and sense probes, respectively, for CRF hnRNA) for 60 min 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 the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada), eluted in 150 μl of 10 mM Tris-1 mM EDTA buffer, and incorporated in a hybridization solution containing (per ml) 107 cpm of 35S probe, 52% formamide, 330 mM NaCl, 10 mM Tris (pH 8), 1 mM EDTA (pH 8), 1× Denhart’s solution, 10% dextran sulfate, 0.5 mg/ml of tRNA, 10 mM 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.

**Fig. 1.** Photomicrographs of complete lesions of the subfornical organ (SFO) and area postrema (AP) vs. respective sham operation. Bregma levels are −0.8 mm for the SFO and −13.7 mm for AP. Scale bars, 200 μm.
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 BX51 microscope (Olympus America, Melville, NY). Images were acquired with an Evolution QEi camera and analyzed with ImagePro Plus version 5.0.1.11 (Media Cybernetics, Silver Spring, MD). Saturation of the hybridization signal was avoided by adjustment of the exposure time for the image with the strongest hybridization signal sampled for each region in every series. The luminosity of the 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 to reduce to zero the number of saturated (pure white) pixels. The same exposure time was conserved throughout the analysis of the entire series.

The oval part of the BST (BSTov, 0.10–0.26 mm caudal to β), the SON (1.10–1.50 mm caudal to β), the parvo- and magnocellular subdivisions of the PVH (PVHp and PVHm, respectively, 1.80–2.00 mm caudal to β), the Arc (2.45–3.25 mm caudal to β), the lateral and lateral-external PB nucleus (PBl and PBle, 9.16–9.25 mm caudal to β), the medial part of the NTS (NTSm, at the level at which the NTS contacts the 4th ventricle, 13.28–13.60 mm caudal to β), and the ventrolateral medulla (VLM, 13.76–14.16 mm caudal to β) were outlined, and the pixel density of the hybridization signal was measured on both hemispheres of two to four 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 for each specific region was corrected by subtraction of background readings taken from areas immediately surrounding the analyzed region. The brain sections from the different groups of rats were matched for rostrocaudal levels as closely as possible.

Plasma Analyses and Tissue Weights

At the time of death, blood was collected by cardiac puncture and centrifuged (1,500 g for 15 min at 4°C), and the separated plasma was stored at −20°C for biochemical analysis. Plasma glucose concentrations were determined using an automated glucose analyzer (YSI 2300 Stat Plus, Yellow Springs Instruments, Yellow Springs, OH). Commercially available radioimmunoassay kits were used for the measurement of plasma insulin (Linco Research, St. Charles, MO) and plasma corticosterone (MP Biomedicals, Toronto, ON, Canada). Interscapular brown adipose tissue, inguinal subcutaneous white adipose tissue, epididymal subcutaneous white adipose tissue, retroperitoneal white adipose tissue, and soleus muscle were sampled and weighed.

Statistical Analysis

Values are means ± SE. Statistical differences in cumulative weight gain and food intake between sham and lesioned rats were determined within each type of lesion (AP, SFO, or AP + SFO) using repeated-measure ANOVA in a mixed-model analysis with a heterogeneous autoregressive covariance structure. Multivariate normality was verified with Mardia’s test. For all other variables, statistical differences within each type of lesion were determined by one- or two-way ANOVA. Fisher’s protected least significance difference test or Bonferroni-Dunn post hoc tests were used to identify the intergroup differences in cases of significant interactions. When necessary, data were logarithmically transformed to satisfy the variance normality criterion. Results were considered significant at P < 0.05. The correlation between the levels of expression of neuropeptide Y (NPY) mRNA in the Arc and plasma levels of corticosterone was estimated using Pearson’s correlation analysis. Repeated-measures ANOVAs were performed using SAS version 9.1.3 (SAS Institute, Cary, NC); all other statistical analyses were performed using StatView version 5.0 (SAS Institute).
RESULTS

Histological Verification of Electrolytic Lesions

In the present study, we used a total of 72 animals, which, after histological evaluation of AP and SFO, were assigned to one of six experimental groups as follows: AP lesion (n = 14), SFO lesion (n = 7), AP + SFO lesion (n = 7), AP sham lesion (n = 9), SFO sham lesion (n = 13), and AP + SFO sham lesion (n = 11). Only animals with complete lesions (see METHODS; Fig. 1) were included for further evaluation. The remaining 11 animals were excluded from analyses because of incomplete lesions or excessive lesions that damaged or destroyed adjacent structures (see METHODS).

Body Weight Gain, Food Intake, and Body Composition in Sham and Lesioned Rats

Body weight gain and food intake (normalized for body weight) during the 40 days of the study are presented in Fig. 2. On the day of surgery, there was no significant difference in body weight between groups (Fig. 2A). During postsurgery days 1 and 2, when rats were nourished with the recovery diet, food intake was significantly reduced in AP- and AP + SFO-lesioned rats compared with their sham controls (32.5–52% of controls, P < 0.05; Fig. 2B). Thereafter, daily food intake in AP-lesioned rats was restored to the sham level, while food intake in the AP + SFO-lesioned rats remained less than in controls (65.5–80% of respective sham, P < 0.05), during the next 10 days (Fig. 2B). Cumulative food intake remained lower in AP + SFO-lesioned rats than in their shams (71.7–92.3% of controls, P < 0.05; Fig. 2C). Also, AP + SFO-lesioned rats gained less body weight during days 1–32 (90.9–94.5% of controls, P < 0.05; Fig. 2A, Table 1). Reduced body weight gain of the AP + SFO-lesioned rats was associated with a smaller gain in fat mass (17.9–52.4% of controls, P < 0.05; Table 1). However, at the end of the experimental period, the AP + SFO-lesioned rats showed a trend to regain weight, and no statistically significant difference was observed in body weight (Fig. 2A) and adipose tissue mass (data not shown) in AP + SFO-lesioned compared with sham rats. No significant difference in body weight gain was observed in AP- or SFO-lesioned rats compared with their shams (71.7–92.3% of controls, P < 0.05; Fig. 2B).

Table 1. Body composition in sham-operated and lesioned rats at the end of postoperative week 3

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<tr>
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<th>Sham</th>
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<tr>
<td></td>
<td>AP</td>
<td>SFO</td>
</tr>
<tr>
<td>BW gain, g</td>
<td>35.76 ± 6.37</td>
<td>34.63 ± 3.32</td>
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<tr>
<td>FM gain, g</td>
<td>5.94 ± 1.28</td>
<td>4.70 ± 0.99</td>
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<tr>
<td>FFM gain, g</td>
<td>1.31 ± 2.10</td>
<td>3.51 ± 1.42</td>
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<tr>
<td></td>
<td>6.56 ± 2.10</td>
<td>-4.75 ± 2.49*</td>
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Values are means ± SE. AP, area postrema; SFO, subfornical organ; BW, body weight; FM, fat mass; FFM, fat-free mass. *Significant effect of lesion (P < 0.05), as assessed by 2-way ANOVA followed by post hoc tests.

Energy Expenditure and Food Intake During Refeeding in Sham and Lesioned Rats Intrapitoneally Injected With Ex-4

Food intake and VO2 during hour 1 of refeeding following food deprivation was significantly reduced in Ex-4-injected rats (28–51% of food intake of saline-treated rats and 80–95% of VO2 of saline-treated rats, P < 0.0001 for the main effect of drug in ANOVAs, including all groups; Fig. 3). During this period, food intake and VO2 (raw data and data normalized for body weight) showed no difference in AP-, SFO-, or AP + SFO-lesioned rats vs. their shams (Fig. 3), a trend that was continued for the next 2 h of refeeding (data not shown).

c-fos mRNA in Brain of Sham and Lesioned Rats After Intrapitoneal Administration of Ex-4

The hybridization signal for c-fos mRNA at 30 min after the intraperitoneal injection of Ex-4 was quantified in sham and lesioned rats in brain nuclei that are likely involved in the control of food intake and associated behaviors: BSTov, CeA, PVHv, PVHm, SON, Arc, PBI nucleus, PBle nucleus, NTSm, and VLM (Figs. 4 and 5). In saline-injected rats, no difference was observed between sham and lesioned groups for any type of surgery (data not shown). In agreement with previous data (our previous results and Refs. 5, 6, 66), c-fos mRNA expression was markedly increased after the injection of Ex-4 in all nuclei (between P < 0.0001 and P < 0.01 vs. saline; Fig. 4). In Ex-4-treated rats, the AP lesion blunted the neuronal activation in the hypothalamic Arc and in the brain stem nuclei, such as PBI and PBle, NTSm, and VLM (P < 0.05; Fig. 5, A and C, G, K, and O). In SFO-lesioned rats, Ex-4-induced neuronal activation was lower (compared with sham) in the PVHm, Arc, PBI nucleus, and VLM (P < 0.05; Fig. 5, A and D, H, L, and P). SFO ablation led to a tendency for a decrease in c-fos mRNA expression in the SON, but this effect was not statistically significant. AP + SFO lesion significantly reduced c-fos mRNA expression in almost all regions of interest: the limbic structures (BSTov and CeA), the parvo-cellular (PVHv) and magnocellular (PVHm and SON) nuclei, and the brain stem regions (NTSm and VLM; P < 0.05; Fig. 5, A and E, I, M, and R). In contrast to single lesions, AP + SFO lesion did not reduce the neuronal activation in the Arc of Ex-4-treated rats.

Intraoperative and Postoperative Energy Expenditure and Food Intake

Energy expenditure and food intake during postsurgery weeks 4–6 in the fasting state showed a lower respiratory quotient (VCO2/VO2) in SFO- and AP + SFO-lesioned rats than in sham rats: 0.707 ± 0.010 vs. 0.743 ± 0.009 (P = 0.02) for SFO-lesioned vs. sham and 0.708 ± 0.016 vs. 0.754 ± 0.011 (P = 0.01) for AP + SFO-lesioned vs. sham.
NPY and Proopiomelanocortin mRNA in the Arc of Sham and Lesioned Rats After Intraperitoneal Administration of Ex-4

Expression of NPY mRNA in the Arc was slightly increased after intraperitoneal injection of Ex-4 in sham-operated rats ($P < 0.05$ vs. saline; Fig. 6, A and C). In contrast, proopiomelanocortin (POMC) mRNA expression in the Arc was not significantly changed 30 min after Ex-4 administration (Fig. 6, D and F). However, lesions of AP and SFO (alone or in combination) did not alter the expression of NPY and POMC transcripts in the Arc of rats treated with saline (data not shown) or Ex-4 (Fig. 6, B and E). A positive significant correlation was found between NPY mRNA levels and plasma corticosterone ($r = 0.412, P = 0.018$), but not between NPY mRNA and plasma insulin and glucose.

GK mRNA in the Arc in Sham and Lesioned Rats After Intraperitoneal Administration of Ex-4

The expression of GK mRNA in the Arc was slightly increased after intraperitoneal injection of Ex-4 in sham-operated rats ($P = 0.06$ vs. saline; Fig. 7, A and C). AP + SFO lesion prevented this increase ($P < 0.05$ vs. sham-operated rats), whereas AP or SFO lesion had no effect (Fig. 7, B and D). In control, saline-injected rats, the absence of the two CVOs, AP and SFO, did not alter GK mRNA levels in the Arc (data not shown).

CRF hnRNA in the PVHp in Sham and Lesioned Rats After Intraperitoneal Administration of Ex-4

Examination of the PVHp revealed a significant increase in CRF hnRNA expression after Ex-4 injection in sham-operated rats ($P < 0.0001$ vs. saline; Fig. 8, A and C). This effect was prevented in AP + SFO-lesioned ($P < 0.05$ vs. sham-operated rats) animals (Fig. 8, B and C), but not in rats with single lesions (Fig. 8B). In saline-injected rats, AP or SFO lesion did not induce a significant modification in CRF hnRNA expression (data not shown).

Plasma Corticosterone, Insulin, and Glucose in Sham and Lesioned Rats After Intraperitoneal Administration of Ex-4

Intraperitoneal injection of Ex-4 led to a significant elevation in plasma corticosterone (2- to 5-fold, $P < 0.0001$), insulin (2- to 3.5-fold, $P < 0.001$), and glucose (1.2- to 1.3-fold, $P < 0.0001$) compared with saline (Table 2). In contrast to CRF hnRNA expression in the PVHp, plasma corticosterone levels were not influenced by the absence of one or both CVOs in saline- or Ex-4-treated rats. Similarly, plasma insulin levels were not affected by AP and SFO ablation. However, the elevation of plasma glucose was prevented by AP + SFO lesion in Ex-4-treated rats ($P < 0.05$ vs. AP + SFO sham; Table 2).

DISCUSSION

The primary objective of the present study was to investigate the role played by the AP and SFO in mediating the CNS actions of Ex-4 in the activation of central autonomic control centers and metabolic function. The results demonstrated that AP + SFO lesion did not significantly attenuate the inhibitory effects of Ex-4 on food intake. However, AP + SFO lesion altered Ex-4-induced c-fos expression in specific brain structures potentially related to the regulation of energy balance and associated behaviors. In addition, AP + SFO lesion alters the expression of genes likely involved in the central modulation of the HPA axis and glucose metabolism. This study also demonstrated that AP + SFO lesion could significantly influence long-term energy homeostasis.

The present results confirm the ability of the GLP-1R agonist Ex-4 to reduce food intake (5) and decrease $\dot{V}O_2$. Similar reductions in energy expenditure generated by administration of GLP-1R agonists in laboratory rodents and humans have been demonstrated (4, 21, 32). Studies on the effects of...
Fig. 4. c-fos mRNA expression in brain of fasted sham-operated rats 30 min after intraperitoneal injection of Ex-4. Pixel density of the hybridization signal was determined in the oval part of the bed nucleus of stria terminalis (BSTov), central nucleus of amygdala (CeA), parvocellular (PVHp) and magnocellular (PVHm) subnuclei of the paraventricular hypothalamic nucleus (PVHp and PVHm), supraoptic nucleus (SON), arcuate hypothalamic nucleus (Arc), lateral (PBl) and lateral-external (PBle) parts of the parabrachial nucleus, medial part of the nucleus of the solitary tract (NTSm), and ventrolateral medulla (VLM). Values are means ± SE. Significant effect of drug, as assessed by 1-way ANOVA: *P < 0.05, **P < 0.001, ***P < 0.0001.

GLP-1R agonists in rats (32) and humans (21) and our unpublished data (using Ex-4 in rats) suggest that the anorectic effect of Ex-4 could be the main cause for the Ex-4-induced reduction in energy expenditure. Unexpectedly, our results did not support a role for the AP or SFO in the anorectic effects of Ex-4, which suggests that these CVOs, even though they express the GLP-1R (23, 36, 43), are not essential to the inhibitory effects of GLP-1R agonists on food intake in the refeeding model that was used in the present study. The reasons for this lack of effect of CVO lesion on Ex-4-induced anorexia are not known and suggest the involvement of other mechanisms. Previous studies, showing that vagal neurons express GLP-1R mRNA (41) and that vagotomy prevents the anorectic effect of peripheral GLP-1 (1) and Ex-4 (59), suggest a role for the vagus nerve. Also, the hypothesis that Ex-4-induced hypophagia could be mediated by central receptors other than GLP-1R cannot be excluded.

Although AP and SFO lesions do not affect Ex-4-induced feeding behavior, AP and SFO lesions had significant effects on the pattern of Ex-4-induced neuronal activation shown using brain c-fos expression as a marker of neuronal activation (26, 48). AP lesion impaired Ex-4-induced neuronal activation in a number of hindbrain nuclei, including the PBl and PBle, NTSm, and VLM, which are structures connected to the AP (24, 52). In addition, AP ablation resulted in reduced c-fos mRNA expression in response to Ex-4 in the hypothalamic Arc, which maintains polysynaptic connections with the AP principally via synapses in the NTS and PBl nucleus (9, 11). SFO ablation blunted Ex-4-induced activation of the PVHm, which is known as a major site receiving SFO projections (37). In addition, c-fos expression in hypothalamic Arc and brain stem regions (such as the PBle nucleus and VLM) was reduced by SFO lesions. The SFO neurons could activate those regions via the limbic system, which receives extensive inputs from the SFO (55, 56). It is indeed well known that neurons of the limbic BST and CeA project to the hypothalamic Arc, pontine PB nucleus, and brain stem VLM (11, 12, 38). AP + SFO lesion reduced c-fos mRNA expression in limbic structures (BSTov and CeA), in the PVHp, PVHm, and SON, and in the brain stem regions (NTSm and VLM). In contrast to single AP or SFO lesions, AP + SFO ablation did not prevent Ex-4-induced neuronal activation in the Arc. This lack of effect may be explained by the activation of the Arc neurons as part of a central compensatory mechanism aimed at limiting the negative energy balance induced by AP + SFO lesion. Reasons for the suppressive effect of CVO lesions on Ex-4-induced brain c-fos expression, but not on Ex-4-induced hypophagia, are not known, although this finding suggests that CVO-mediated central effects of Ex-4 are not directly linked to the effects on feeding and metabolism associated with this behavioral paradigm. The possibility that c-fos might not be a good marker for Ex-4-induced anorexia cannot be excluded.

The attenuation of Ex-4-induced neuronal activation of the PVHp, produced by AP + SFO ablation, was accompanied by a reduction in the CRF hnRNA levels in the mediodorsal PVHp. The CRF hnRNA is the primary transcript in the CRF gene expression process and is not constitutively present in CRF-expressing brain structures (31, 46). Measurement of its expression has proved to be useful in detecting rapid changes in CRF expression in the PVH, where changes in CRF synthesis cannot easily be measured because of the high density of the CRF message (25). It is noteworthy that the inhibitory effect of AP + SFO lesions on CRF hnRNA expression in response to Ex-4 was not paralleled by a decrease in the corticosterone levels. This observation was surprising in light of the recognized importance of PVHp CRF neurons in governing the pituitary-adrenal axis (39). However, there is evidence that Ex-4 injected peripherally may also stimulate corticosteroid release through activation of GLP-1Rs located on pituitary and adrenal glands (28).

Elevation of plasma corticosterone could be partially responsible for Arc NPY mRNA expression induced by Ex-4. It is well known that Arc NPY neurons present glucocorticoid receptors, and there is evidence of a positive association between NPY and glucocorticoids (8). A recent study showed the lack of coexpression of GLP-1R and NPY mRNA in Arc neurons (49). It is therefore unlikely that the enhanced NPY expression that we found after Ex-4 injection was due to a direct neuronal activation of the Arc. On the other hand, the same study showed that 68% of GLP-1R-positive neurons coexpress POMC mRNA (49). Moreover, it has been demonstrated that POMC mRNA hypothalamic expression is increased 2 h after central administration of GLP-1 in fasted rats (51). We did not find a significant increase in Arc POMC mRNA expression after intraperitoneal injection of Ex-4, likely because our rats were killed 30 min after Ex-4, before such changes occur. Our data suggest that different mechanisms may be responsible for central regulation of Ex-4-induced
anorexia and, also, that AP and SFO are not involved in immediate effects of peripheral Ex-4 on NPY and POMC mRNA expression in the Arc.

The results of the present study show that the AP/SFO lesion reduced body weight gain and daily food intake. The reduction in weight gain in AP + SFO-lesioned rats was accompanied by a reduction in fat gain, which was significant for the first part of the study (until postoperative week 3) but tended to normalize thereafter. At the end of the experiment, AP + SFO-lesioned rats showed a tendency to regain weight.

Fig. 5. c-fos mRNA expression in brain of fasted rats 30 min after intraperitoneal injection of Ex-4. A: pixel density of the hybridization signal in lesioned rats vs. respective sham in BSTov, CeA, PVHp and PVHm, SON, Arc, PBI and PBle, NTSm, and VLM. Values are means ± SE. Significant effect of lesion, as assessed by 2-way ANOVA: *P < 0.05. B–R: dark-field photomicrographs demonstrating positive hybridization signal for c-fos mRNA in PVHp and PVHm (B–E), SON (F–I), Arc (J–M), and NTSm (N–R) in nonlesioned (B, F, J, and N), AP-lesioned (C, G, K, and O), SFO-lesioned (D, H, L, and P), and AP + SFO-lesioned (E, I, M, and R) rats. V3, 3rd ventricle. Bregma levels are −1.80 mm for the PVH, −1.30 mm for the SON, −2.60 mm for the Arc, and −13.30 mm for the NTSm. Scale bars, 200 μm.
an effect that possibly resulted from compensatory homeostatic processes aimed at counteracting the negative energy balance created by the AP + SFO lesion. The effect of single ablations of the AP and SFO on body weight and food and water intake has been reported (27, 30, 35, 58), but the impact of the absence of both CVOs on feeding and energy homeostasis had not been examined before the present investigation. Ablation of the AP and the adjacent tissue of the caudal-medial portion

Fig. 6. Neuropeptide Y (NPY) and proopiomelanocortin (POMC) mRNA expression in the Arc 30 min after intra-peritoneal injection of Ex-4 in sham-operated or lesioned rats. A and B: pixel density of the hybridization signal for NPY mRNA after Ex-4 or saline injection in fasted sham-operated rats (A) and after Ex-4 injection in fasted AP-, SFO-, or AP + SFO-lesioned vs. sham-operated rats (B). *p < 0.05 (by t-test). C: dark-field photomicrographs demonstrating positive hybridization signal for NPY mRNA in the Arc in sham-operated rats treated with Ex-4 or saline. D and E: pixel density of the hybridization signal for POMC mRNA after Ex-4 or saline injection in fasted sham-operated rats (D) and after Ex-4 injection in fasted AP-, SFO-, or AP + SFO-lesioned vs. sham-operated rats (E). F: dark-field photomicrographs demonstrating positive hybridization signal for POMC mRNA in the Arc in sham-operated rats treated with Ex-4 or saline. Bregma level, −2.85 mm. Scale bars, 200 μm.
of the NTS has been reported to cause physiological changes such as transient hypophagia and body weight loss when the animals are fed normal laboratory chow (13, 17, 27, 30). We observed similar effects on the ingestive behavior following the AP ablation even when, immediately after the surgery, the animals were fed a diet apparently more palatable than chow. Some studies have highlighted the preference in AP-ablated rats for palatable foods, but this effect was observed between 2 wk and 1 mo after surgery (16, 30, 45). Confirming previous data (10, 16, 27, 30), we also observed that, in the second part of the experimental period, energy intake was similar in AP-lesioned and control rats. In addition, body weight gain was slightly less in AP-lesioned rats than in sham animals during postlesion week 1 but was not significantly different between AP-lesioned rats and their respective shams thereafter. As mentioned above, AP lesions were restricted to the AP region, and all rats presenting damage of surrounding NTS tissue were excluded. Similar results have also been obtained by other groups using same criteria of exclusion (10). Body weight and food intake did not differ between SFO-lesioned and sham-operated animals, confirming previous data (35, 58). Ex-4-induced elevation of insulin was not affected by AP or SFO ablation. However, the elevation of plasma glucose induced by Ex-4 was prevented by AP + SFO lesion. Hyperglycemia following Ex-4 injection in fasted nondiabetic rats has been reported (3, 34). The mechanism underlying this phenomenon would seem to involve the sympathoadrenal system. Ex-4 has been shown to activate neurons in brain sites implicated in the autonomic control of the adrenal medulla (66). The prevention of this hyperglycemic effect by the
Ablation of AP + SFO suggests the presence of central mechanism involving the GLP-1R in the control of glucose metabolism. It is noteworthy that the expression of GK mRNA in the Arc neurons was increased after the intraperitoneal injection of Ex-4 in sham-operated rats and that AP + SFO lesion prevented the increase in GK mRNA, emphasizing a potential role of these two CVOs in the central regulation of glucose homeostasis.

Table 2. Plasma corticosterone, insulin, and glucose in sham-operated and lesioned rats 30 min after intraperitoneal injection of Ex-4 or saline

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>AP + SFO</th>
<th>Lesion</th>
<th>AP + SFO</th>
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<tbody>
<tr>
<td><strong>Corticosterone, nmol/l</strong></td>
<td></td>
<td></td>
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<tr>
<td>Saline</td>
<td>568.01 ± 211.97</td>
<td>318.42 ± 108.79</td>
<td>577.99 ± 188.75</td>
<td>91.47</td>
</tr>
<tr>
<td>Ex-4</td>
<td>1,347.36 ± 409.58*</td>
<td>1,520.75 ± 264.06*</td>
<td>1,654.79 ± 179.84*</td>
<td>1,878.52 ± 144.23*</td>
</tr>
<tr>
<td><strong>Insulin, pmol/l</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Saline</td>
<td>49 ± 11</td>
<td>64 ± 7</td>
<td>128 ± 33</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>Ex-4</td>
<td>167 ± 63*</td>
<td>230 ± 67*</td>
<td>218 ± 89*</td>
<td>168 ± 43*</td>
</tr>
<tr>
<td><strong>Glucose, mmol/l</strong></td>
<td></td>
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<tr>
<td>Saline</td>
<td>9.05 ± 0.41</td>
<td>8.96 ± 0.14</td>
<td>8.84 ± 0.34</td>
<td>9.04 ± 0.39</td>
</tr>
<tr>
<td>Ex-4</td>
<td>10.48 ± 1.27*</td>
<td>11.56 ± 0.49*</td>
<td>11.10 ± 0.64*</td>
<td>11.88 ± 0.54*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Ex-4, exendin-4 (1.55 μg/kg). *Significant main effect of drug \((P < 0.001)\), as assessed by 2-way ANOVA determined within each type of lesion. †Significant effect of lesion \((P < 0.001)\), as assessed by Fisher’s protected least significance difference and Bonferroni-Dunn post hoc tests.
In summary, the present study provides evidence for a role of the AP and SFO in mediating the central effects of Ex-4. Ablation of these CVOs significantly alters brain neuronal activation but not hypophagia induced by intraperitoneally injected Ex-4. However, both AP and SFO seem to be synergistically involved in central GLP-1R-mediated activation of PVH neurons governing regulation of the HPA axis and the Arc neurons involved in glucose metabolism.

Perspectives and Significance

The mechanisms underlying the anorexigenic effect of Ex-4 remain poorly understood. The present data show that the CVOs (AP and SFO) do not seem to be directly implicated in hypophagia but do play important roles in mediating the activation of a number of autonomic control centers in response to peripherally injected Ex-4. The AP and SFO also seem to be synergistically involved in the central GLP-1R-mediated regulation of the HPA axis and glucose metabolism. These findings provide new insights into the central pathways underlying the stress-related effects of the GLP-1R signaling system. It should be recognized, however, that the functional effects of these alterations in neuronal activity induced by lesions of the CVOs in response to Ex-4 have not been described by the present studies, but they do represent intriguing targets for future investigation.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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