

# Activation of the Sympathetic Nervous System by Galanin-Like Peptide—A Possible Link between Leptin and Metabolism

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The effects of leptin upon body weight (BW) cannot be explained by its anorectic actions alone. Part of the metabolic changes elicited by leptin includes sympathetic nervous system activation leading to increased energy expenditure. Galanin-like peptide (GALP), a recently described hypothalamic neuropeptide, is up-regulated by leptin and has anorectic effects in the mouse. We postulated that GALP mediates effects of leptin upon metabolism. To test this hypothesis, we administered GALP centrally to the leptin-deficient *ob/ob* mouse. Acutely, GALP induced a decrease in food intake and BW, both of which remained significant relative to controls for 4 d. Chronic GALP administration resulted in a sustained decrease in BW and an increase in core body temperature, de-

spite significant recovery of food intake. In a pair-fed model, chronic GALP treatment resulted in a greater decrease in BW than that seen in controls. Furthermore, GALP treatment resulted in increased body temperature and uncoupling protein 1 mRNA and protein in brown adipose tissue compared with controls. The expression of pro-opiomelanocortin (POMC) mRNA in the arcuate nucleus was decreased after chronic GALP treatment. These observations suggest that leptin's activation of the sympathetic nervous system, and ultimately thermogenesis, may be partially mediated by GALP through a melanocortin-independent mechanism. (*Endocrinology* 144: 4709–4717, 2003)

THE ADIPOCYTE-DERIVED HORMONE leptin has been well characterized as an important signaling molecule, conveying information to the central nervous system regarding energy balance (reviewed in Refs. 1–3). Leptin deficiency, as seen in the *ob/ob* mouse, is associated with obesity, hypothermia, diabetes, hyperphagia, and suppression of the reproductive axis (4–7). Replacement of leptin in the *ob/ob* model, as well as other models of leptin deficiency, is associated with the attenuation of these metabolic and behavioral abnormalities (4–9). The effects of leptin upon feeding behavior and metabolism are complex and only partially understood.

Part of leptin's effects upon body weight (BW) are mediated by its anorectic properties (reviewed in Ref. 1). In the hypothalamic arcuate nucleus, leptin modifies the expression of neuropeptides known to play a role in regulating food intake (FI). Neurons expressing POMC and cocaine- and amphetamine-regulated transcript (CART) coexpress the signaling form of the leptin receptor (Ob-R) (10, 11). Leptin up-regulates the expression of POMC and CART (12, 13), and both CART and  $\alpha$ -MSH (a cleavage product of POMC) are known to serve as satiety signals, ultimately decreasing FI (14, 15). Conversely, the orexigenic peptides neuropeptide Y

(NPY) and agouti-related protein (AgRP) are negatively regulated by leptin (16–18), and decreases in NPY and AgRP after leptin treatment are associated with decreased FI (16, 19).

In addition to leptin's role in regulating FI, it has marked effects upon metabolism (1–6). Leptin-treated *ob/ob* mice lose more weight than their pair-fed counterparts (5). Although the mechanisms involved in increased energy expenditure associated with leptin treatment are only partially understood, the increased expression of uncoupling protein 1 (UCP-1), a protein that uncouples oxidative phosphorylation from ATP synthesis (20), is known to be a factor (21–24). The increase in UCP-1 expression in brown adipose tissue (BAT) in response to leptin is dependent upon sympathetic nervous system (SNS) activation (25–27) and may be melanocortin dependent (28).

In addition to POMC/CART and NPY/AgRP neurons, GALP neurons may also play a role in the metabolic and behavioral response to leptin. GALP is a 60-amino-acid neuropeptide recently isolated from the porcine hypothalamus secondary to its ability to bind and activate galanin receptors *in vitro* (29). GALP cDNAs have subsequently been cloned from rat, mouse, macaque, and human (29–31). In the species examined to date, amino acids 9–21 are highly conserved. The expression of GALP in the central nervous system is confined to the arcuate nucleus, median eminence, and the neurohypophysis (30–35). Double-labeling studies in the rat and macaque have demonstrated that the majority ( $\geq 85\%$ ) of GALP neurons coexpress Ob-R (31, 35). Like POMC/CART and NPY/AgRP, hypothalamic GALP expression is highly

Abbreviations: aCSF, Artificial cerebrospinal fluid; AgRP, agouti-related protein; BAT, brown adipose tissue; BT, body temperature; BW, body weight; CART, cocaine- and amphetamine-regulated transcript; FI, food intake; GALP, galanin-like peptide; ICV, intracerebroventricular; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; SDS, sodium dodecyl sulfate; SNS, sympathetic nervous system; UCP-1, uncoupling protein-1.

influenced by nutritional status, with marked decreases in mRNA levels after a 48-h fast in the rat (32). Leptin treatment during the fast reverses this effect (32). Furthermore, GALP expression in the *ob/ob* mouse is essentially undetectable at baseline, but is restored to wild-type levels after central leptin treatment (30). Recently, we have demonstrated that central GALP administration has an anorectic effect in the mouse and rat (36). Additionally, acute central GALP treatment results in increases in serum LH and testosterone in the mouse (36) and LH in the rat (37). Acute GALP treatment has also been associated with increases in body temperature (BT) (38). These observations suggest that GALP neurons may serve as downstream integrators of leptin signals. To test the hypothesis that GALP mediates actions of leptin upon metabolism, we investigated the effects of acute and chronic central administration of GALP upon FI, BW, BT, and UCP-1 expression in the leptin-deficient *ob/ob* mouse.

## Materials and Methods

### Animals

Adult male *ob/ob* mice on a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME) were individually housed and maintained on a 12-h light, 12-h dark cycle (lights on at 0600 h). All animals had access to standard rodent chow and water *ad libitum* unless otherwise stated. In each experiment, animals were weight-matched before being divided into treatment groups. All procedures were approved by the Animal Care Committee of the School of Medicine of the University of Washington, in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals.

### Preparation of rat GALP

Rat GALP (1–60) peptide was prepared by chemical synthesis at 0.05- or 0.1-mmol scale on an ABI 431 or 433 peptide synthesizer (Applied Biosystems, Foster City, CA) as previously described (36). After synthesis and purification, GALP was dissolved in artificial cerebrospinal fluid (aCSF) containing 0.1% BSA for use in all experiments.

### Intracerebroventricular (ICV) injections

Freehand ICV injections were performed as described previously (39). Mice were briefly anesthetized with isoflurane (Abbott, North Chicago, IL) delivered by a vaporizer (Veterinary Anesthesia Systems, Bend, OR). After induction of adequate anesthesia, a small hole was created in the skull 1 mm lateral and 0.5 mm posterior to the bregma with a 27-gauge needle fitted with polyethylene tubing to leave 3.5 mm of the needle tip exposed. All subsequent injections were made at the same site with a 27-gauge needle similarly fitted with polyethylene tubing to leave 3.5 mm of the needle tip exposed. Mice were allowed to recover for 2 d after creation of the hole before treatment. For ICV injections, mice were anesthetized for 2–3 min with isoflurane, during which time 3  $\mu$ l solution was slowly and continuously injected into the lateral ventricle. After injections, the needle remained inserted for 30 sec to minimize backflow up the needle track. Recovery from anesthesia typically occurred within 3 min after injection. Gross examination of the brains at the time the mice were killed confirmed the absence of significant cortical damage. Tissue sections from the brains of experimental and control animals were examined in preparation for *in situ* hybridization, and no evidence of tissue damage or ventriculomegaly was noted in either group.

### Experiment 1: acute GALP treatment

Mice received a single ICV injection of aCSF ( $n = 11$ ) or 5 nmol GALP ( $n = 12$ ) between 0800 and 0930 h on d 0. FI and BW were monitored daily.

### Experiment 2: chronic GALP treatment (fed *ad libitum*)

Experimental animals were injected ICV twice daily (0700–0800 h, and 1700–1800 h) with aCSF ( $n = 8$ ) or 5 nmol GALP ( $n = 8$ ), with the

first injection occurring at 1700–1800 h on d 0. Twice daily injections were continued for 14 d (total 28 injections). FI and BW were monitored on a daily basis. Rectal temperatures were measured at a single time-point on d 14, 4–5 h after the final injection.

### Experiment 3: chronic GALP treatment (pair-fed)

Mice were injected ICV twice daily as described above ( $n = 8$  for both groups) for 14 d with the first injection on d 0 between 1700–1800 h. FI and BW were recorded on a daily basis. The GALP treatment group was started 1 d earlier than the aCSF group to allow for pair-feeding. The average FI of the GALP group for the previous 24 h was given to the aCSF animals in the evening between 1700 and 1800 h. Rectal temperatures were recorded before treatment and at the end of the experiment as described above. Animals were killed by cervical dislocation under isoflurane anesthesia. Brains were rapidly removed, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Interscapular BAT was obtained by dissection. The BAT fat pad was weighed and frozen in liquid nitrogen for subsequent RNA and protein extraction. Samples of BAT from both treatment groups were placed in Bouin's solution (Sigma, St. Louis, MO) for subsequent histology.

### $T_4$ assay

Serum total  $T_4$  concentration was determined in a single RIA (Diagnostic Products Corp, Los Angeles, CA). The limit of detection was 0.22  $\mu\text{g/dl}$ , and the intraassay coefficient of variation was 10.2%.

### UCP-1 Northern blots

Total RNA was extracted from interscapular BAT pads with Trizol (Life Technologies, Inc., Carlsbad, CA) following the manufacturer's protocol, followed by precipitation in ethanol and resuspension in ribonuclease-free  $\text{H}_2\text{O}$ . Equal amounts of RNA (5  $\mu\text{g}$ ) were fractionated by electrophoresis through 1.2% (wt/vol) agarose in 40 mM 3[*N*-morpholino]propanesulfonic acid, 10 mM sodium acetate, 1 mM EDTA, 2% (vol/vol) formaldehyde (37% solution), transferred to a nylon membrane (Roche, Indianapolis, IN), and fixed by UV illumination. Membranes were prehybridized for 1 h at 68  $^{\circ}\text{C}$  in Church & Gilbert Buffer [250 mM sodium phosphate, 1 mM EDTA, 1% (wt/vol) BSA, 7% (wt/vol) sodium dodecyl sulfate (SDS)], and probed for UCP-1 (1100-bp *Xho*I fragment from cDNA, 40), and glucose-6-phosphatase (450-bp *Pvu*II fragment from cDNA clone) as a loading control. Probes were generated by random priming (Roche, Indianapolis, IN), purified over a Sephadex G-50 column (NICK column, Amersham, Piscataway, NJ), and heat-denatured before hybridization at 68  $^{\circ}\text{C}$  overnight in Church & Gilbert buffer. After hybridization, membranes were washed twice in  $2\times$  SSC [0.3 M NaCl, 30 mM sodium citrate, 0.5% (wt/vol) SDS, 0.1% (wt/vol) sodium pyrophosphate] for 15 min at room temperature, then at 45  $^{\circ}\text{C}$ . The membranes were then washed in  $0.1\times$  SET [0.5% (wt/vol) SDS, 5 mM Tris, 2.5 mM EDTA, 0.1% sodium pyrophosphate] at 45  $^{\circ}\text{C}$ . Membranes were air-dried and exposed to x-ray film (X-Omat, Eastman Kodak, Rochester, NY) at  $-80^{\circ}\text{C}$  for 8–16 h. Two UCP-1 transcripts (1.6 and 2.0 kb) were identified after hybridization with the UCP-1-specific probe (40). The smaller transcript was used for densitometric quantitation by using NIH Image (version 1.63).

### UCP-1 immunoblotting

Interscapular BAT pads were homogenized in lysis buffer (250 mM sucrose, 20 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM EGTA, 10 mM dithiothreitol, 1% Triton X-100, and 0.5% deoxycholic acid) supplemented with protease and phosphatase inhibitors (1  $\mu\text{g/ml}$  leupeptin, 3  $\mu\text{g/ml}$  aprotinin, 40  $\mu\text{g/ml}$  soybean trypsin inhibitor, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.1  $\mu\text{M}$  microcystin-leucine arginine, 0.2  $\mu\text{M}$  NaF, 0.2  $\mu\text{M}$  orthovanadate), sonicated, and cleared by centrifugation (10,000  $\times g$ , 15 min). After the protein concentration of the soluble infranantant was determined by the Bradford method (Bio-Rad, Hercules, CA), samples were diluted into  $1\times$  sample buffer [62.5 mM Tris-HCl, 2% (wt/vol) SDS, 5% glycerol, 0.05% (wt/vol) bromophenol blue], boiled 5 min, then brought to 5% (vol/vol)  $\beta$ -mercaptoethanol. Twenty micrograms of protein per BAT pad was separated by SDS-PAGE and transferred to nitrocellulose membranes (Protran, Schleicher

& Schuell, Keene, NH). Membranes were blocked [5% (wt/vol) BSA 30 min] and probed for UCP-1 (1:1000 (vol/vol), Calbiochem, La Jolla, CA) or the protein kinase A RII $\beta$  subunit as a control (1:2000, BioMol, Plymouth Meeting, PA) at 4 C overnight. After three washes in PBS-T (10 mM sodium phosphate, 150 mM NaCl, 0.1% (vol/vol) Tween 20), membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody [1:10,000 (vol/vol) in PBS-T] containing 5% (wt/vol) nonfat dry milk. After three washes in PBS-T, bound antibodies were detected using ECL (Amersham, Piscataway, NJ) and exposure to HyperFilm ECL (Amersham). Densitometric quantitation was performed by using NIH Image (version 1.63).

### Histology

After fixation in Bouin's solution (Sigma), tissues were processed in paraffin and serially dehydrated in ethanol. Specimens were cleared in Hemo-De (d-limonene). Tissues were infiltrated in two changes of paraffin for 2.5 h total. Paraffin-embedded specimens were sectioned on a rotary microtome at 6–8  $\mu$ m and stained with hematoxylin and eosin.

### In situ hybridization

*In situ* hybridization for POMC mRNA was performed as previously described (41). Briefly, 20- $\mu$ m sections through the entire rostral-caudal extent of the arcuate nucleus were cut on a cryostat, thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA) and stored at –80 C. The plasmid vector containing a cDNA for murine POMC was kindly provided by Dr. Michael Uhler. A 925-bp *Hind*III-*Eco*RI fragment was subcloned into pSp64 (Promega, Madison, WI). After plasmid linearization with *Hind*III, an antisense POMC cRNA was transcribed from 1  $\mu$ g of template in a 20- $\mu$ l reaction containing the following: 40 U SP6 RNA polymerase (Ambion, Austin, TX); 2  $\mu$ l 10 $\times$  reaction buffer; 0.5 mM each ATP, CTP, and GTP; 0.9 mM uridine triphosphate; and 125  $\mu$ Ci <sup>35</sup>S-labeled uridine triphosphate (1250 Ci/mmol, Perkin-Elmer, Boston, MA). After 1.5 h at 37 C, template DNA was digested with ribonuclease-free deoxyribonuclease (Roche Applied Science, Indianapolis, IN). After probe purification on a NucAway Spin Column (Ambion), *in situ* hybridization was performed. Tissue was fixed, delipidated and dehydrated. The riboprobe was denatured, and dissolved in hybridization buffer with tRNA (1.9 mg/ml) and dithiothreitol (30.8 mg/ml) to a final concentration of 0.185  $\mu$ g/ml, applied to the tissue sections, covered with silanized coverslips and hybridized overnight at 55 C. Sections were then treated with ribonuclease (29  $\mu$ g/ml), washed in SSC to a stringency of 0.1  $\times$  at 60 C, and dehydrated through alcohols containing ammonium acetate. Slides were then dipped in NTB3 emulsion (Kodak, Rochester, NY) and stored at 4 C for 3 d until developed and coverslips were applied.

### Image analysis

A mouse brain atlas was used to match coronal brain sections across animals (42). Seven (aCSF) or eight (GALP) animals were analyzed per

group. Four, anatomically matched sections spanning the rostral-caudal extent of the arcuate nucleus were analyzed per animal. Slides were viewed with a Carl Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY), and silver grain clusters corresponding to POMC mRNA-expressing cells were visualized under dark-field illumination. The number of POMC-expressing cells was manually counted for each section, and summed across sections to determine total cells/animal. Clusters of silver grains judged to be POMC-expressing cells were analyzed with custom-designed software to estimate the number of silver grains/cell. The system consists of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Power Macintosh G3 computer. All slides were assigned a random three-letter code, and the investigator was blind to the slides' treatment group assignment during the analysis.

### Statistical analysis

Data are presented as the mean  $\pm$  SEM. Animal BW and FI were analyzed with single-factor ANOVA with repeated measures. Further analysis included *post hoc t* tests to evaluate significance of individual time points. Paired *t* tests were used to compare T<sub>4</sub> levels and BT within an experimental group before and after treatment. Analysis of all other data were performed by Student's *t* test. *P* < 0.05 was considered statistically significant.

## Results

### Experiment 1: acute GALP treatment

To assess the acute effects of GALP treatment upon FI and BW in the *ob/ob* mouse, we administered a single ICV injection of aCSF or 5 nmol GALP and followed FI and BW for 5 d. GALP-treated animals experienced a significant decrease in FI relative to controls over the first 24 h (1.5  $\pm$  0.2 g *vs.* 5.2  $\pm$  0.3 g, respectively, *P* < 0.0001; Fig. 1A). By 48 h, FI had markedly recovered but remained significantly lower than that of controls until the final day of the experiment. GALP-treated animals also experienced a significant decrease in BW relative to controls at 24 h (44.8  $\pm$  0.7 g *vs.* 47.8  $\pm$  0.7 g, GALP *vs.* aCSF; *P* < 0.01); however, this decrease was more sustained and BW remained significantly less than that of control animals until the final day of the follow-up period (Fig. 1B).

### Experiment 2: chronic GALP treatment (fed ad libitum)

Given that acute GALP injection resulted in significant decreases in FI and BW, we evaluated the effects of chronic

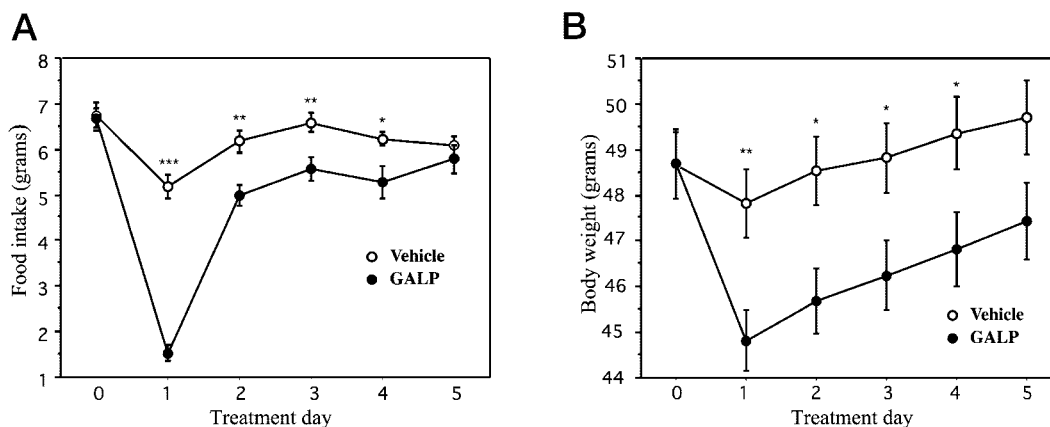


FIG. 1. The effects of acute GALP treatment in the *ob/ob* mouse. Food intake (A) and body weight (B) after a single injection of vehicle (open circles, *n* = 11) or 5 nmol GALP (closed circles, *n* = 12) on d 0. Data are presented as means  $\pm$  SEM. \*\*\*, *P* < 0.0001; \*\*, *P* < 0.01; and \*, *P* < 0.05 *vs.* aCSF.



GALP treatment in *ob/ob* mice. FI markedly decreased during the first 24 h to approximately 15% of baseline after GALP treatment (Fig. 2A). With repeated GALP dosing, FI slowly recovered and was not significantly different from controls on the last 2 d of treatment. Over the entire treatment interval, ANOVA revealed significant differences in FI between the GALP and control groups (group  $P = 0.0001$ ; interaction  $P < 0.0001$ ). Similarly, BW was also markedly decreased in the GALP-treated animals *vs.* controls (group  $P < 0.01$ ; interaction  $P < 0.0001$ ), an effect that persisted throughout the treatment interval despite the slow recovery of FI (Fig. 2B). Additionally, a significant increase in BT at the end of the treatment interval was noted in the GALP-treated animals relative to controls ( $33.2 \pm 0.4$  C *vs.*  $31.6 \pm 0.3$  C, respectively;  $P < 0.005$ , Fig. 2C).

### Experiment 3: chronic GALP treatment (pair-fed)

To differentiate the anorectic *vs.* the metabolic effects of GALP in the *ob/ob* mouse, we treated animals with central GALP injections for 14 d using a pair-feeding design (Fig. 3). Again, a significant decrease in FI was noted with chronic GALP treatment (Fig. 3A). Although FI was similar on a daily basis to that seen in the GALP-treated animals in experiment 2, FI recovery was not as marked at the end of the treatment interval. BW also significantly decreased in the GALP treatment group, and this decrease was greater than that seen in the pair-fed controls (ANOVA interaction  $P < 0.005$ , Fig. 3B). At the end of the treatment interval, GALP-treated animals had a higher BT than that of pair-fed controls ( $32.2 \pm 0.1$  C *vs.*  $30.7 \pm 0.2$  C, respectively,  $P < 0.0001$ ). Additionally, changes in BT relative to baseline were markedly greater in GALP-treated animals than in controls ( $1.7 \pm 0.3$  C *vs.*  $0.2 \pm 0.3$  C, respectively;  $P < 0.005$ , Fig. 3C). Serum  $T_4$  (Fig. 3D) and corticosterone levels (data not shown) were not significantly different between experimental groups or within groups before and after treatment.

### Effects of GALP treatment upon UCP-1 mRNA expression and protein in BAT

One mechanism whereby leptin treatment increases metabolism and BT in the leptin-deficient state is through the up-regulation of UCP-1 expression. Given that pair-fed aCSF-treated *ob/ob* mice lost less weight and had lower BT than their GALP-treated counterparts, we investigated the effects of ICV GALP treatment upon UCP-1 expression in BAT. BAT weight was not significantly different between GALP-treated and control animals ( $206 \pm 19$  *vs.*  $221 \pm 23$  mg, respectively). Northern blotting revealed a significant increase ( $\sim 2$ -fold) in UCP-1 mRNA expression in BAT of GALP-treated animals compared with pair-fed controls ( $P < 0.02$ , Fig. 4, A and B). Similarly, a 2-fold increase in UCP-1 protein in GALP-treated animals was revealed upon Western blotting of BAT protein extracts ( $P < 0.05$ , Fig. 4, C and D). BAT in *ob/ob* mice has a gross histological appearance reminiscent of white adipose tissue, containing a large number of unilocular adipocytes. Examination of BAT in GALP-treated animals revealed an increase in the appearance of multilocular brown adipocytes compared with controls (Fig. 4, E and F).

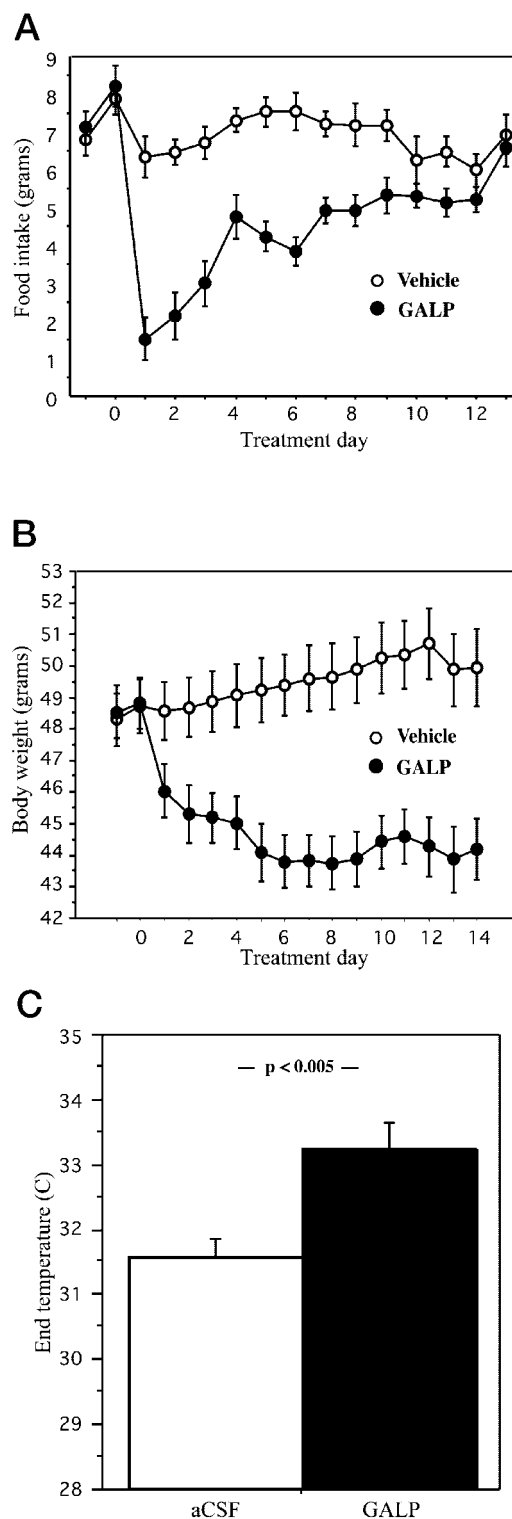


FIG. 2. The effects of chronic GALP treatment in the *ob/ob* mouse *vs.* vehicle-treated controls. Food intake (A) and body weight (B) after twice daily injection of vehicle (open circles,  $n = 8$ ) or 5 nmol GALP (closed circles,  $n = 7$ ) over 14 d. Data are presented as means  $\pm$  SEM (A) group  $P = 0.0001$ , interaction  $P < 0.0001$  by ANOVA. B, Group  $P < 0.01$ , interaction  $P < 0.0001$  by ANOVA. C, Body temperature at the end of the experimental interval ( $P < 0.005$  GALP *vs.* aCSF).

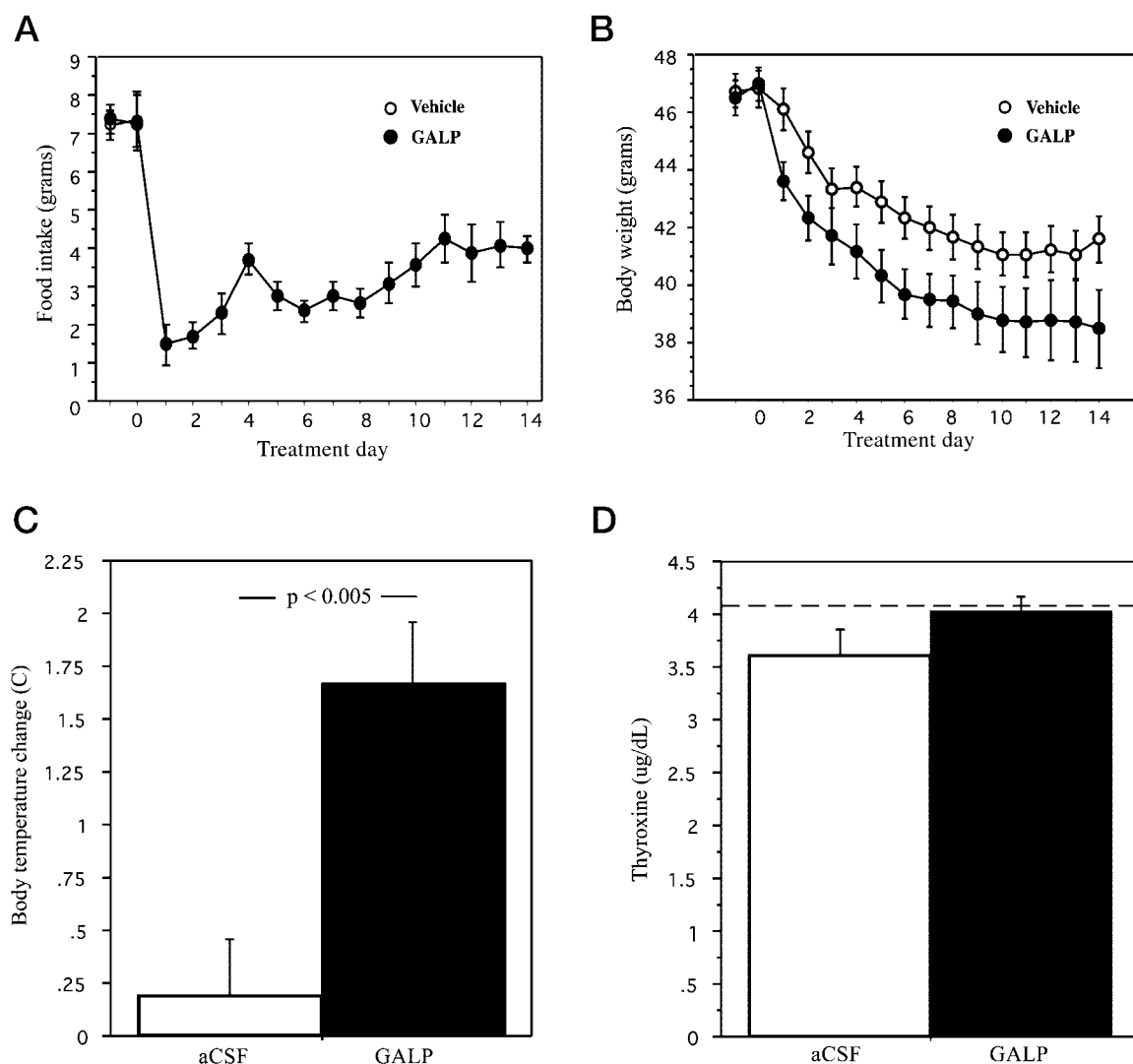


FIG. 3. The effects of chronic GALP treatment in the *ob/ob* mouse *vs.* pair-fed, vehicle-treated controls. Food intake (A) and body weight (B) after twice daily injection of vehicle (open circles,  $n = 8$ ) or 5 nmol GALP (closed circles,  $n = 8$ ) over 14 d. Treatment  $\times$  time interaction for body weight (B) was significant ( $P < 0.005$ ). C, Body temperature change from baseline ( $P < 0.005$  GALP *vs.* aCSF). D, Serum thyroxine concentration ( $\mu\text{g/dl}$ ) at the end of the treatment interval. The dashed line represents the average pretreatment thyroxine concentration ( $p = \text{NS}$  both between experimental groups and within groups before and after treatment). Data are presented as means  $\pm$  SEM.

#### Effects of chronic GALP treatment on POMC mRNA expression in the arcuate nucleus

Leptin-mediated activation of the SNS, ultimately leading to increased UCP-1 expression in BAT, may be a melanocortin-dependent event (28). To investigate the effects of chronic central GALP treatment upon hypothalamic POMC expression, *in situ* hybridization was performed on tissue sections from GALP-treated animals and pair-fed controls. GALP treatment resulted in a significant decrease in the number of identifiable cells that express POMC mRNA ( $289 \pm 16$  *vs.*  $384 \pm 25$ , GALP *vs.* control,  $P < 0.01$ , Fig. 5A); however, no statistically significant difference was seen at the grains per cell level (Fig. 5B). When stratified by anatomical regions of the arcuate nucleus, the number of cells expressing POMC mRNA remained lower in the GALP-treatment group than in the control group (data not shown).

#### Discussion

A growing body of evidence suggests that GALP neurons may be part of the circuit involved in mediating the effects of leptin upon the homeostatic regulation of BW, FI, and the reproductive axis (36–38). The expression of GALP in the arcuate nucleus is regulated by leptin (30, 32). Additionally, double-labeling studies have suggested that at least 85% of GALP neurons express the signaling form of the leptin receptor (31, 35), providing a mechanism whereby leptin concentration is directly relayed to GALP neurons. More recently, GALP has been demonstrated to effect FI and BW directly. Matsumoto *et al.* (43) demonstrated an acute orexigenic effect of central GALP treatment upon FI in the rat, a finding confirmed by ourselves and others (36, 38, 44). Despite this acute orexigenic action, both FI and BW are decreased at 24 h after central GALP treatment in the rat (36,

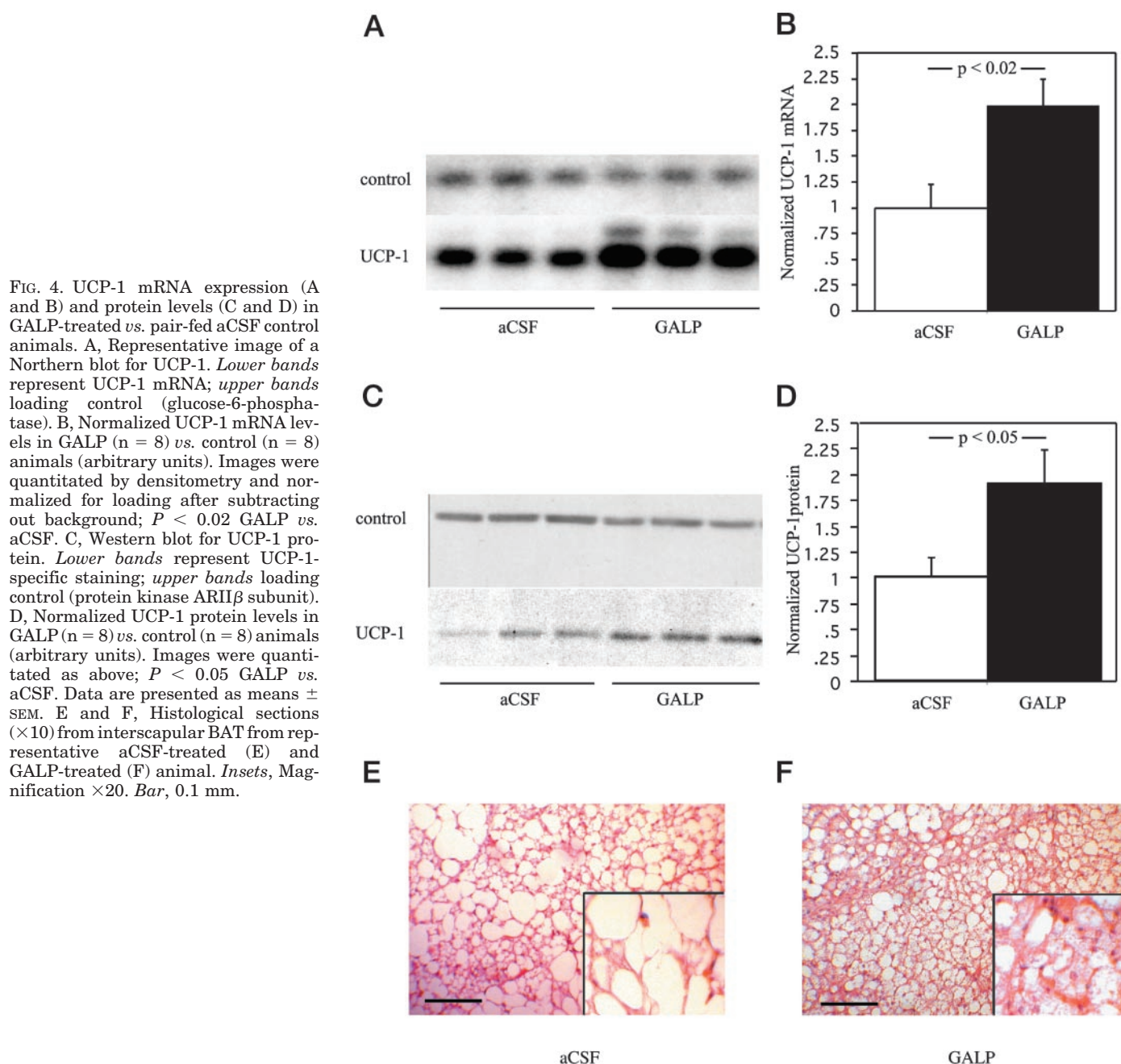


FIG. 4. UCP-1 mRNA expression (A and B) and protein levels (C and D) in GALP-treated *vs.* pair-fed aCSF control animals. A, Representative image of a Northern blot for UCP-1. Lower bands represent UCP-1 mRNA; upper bands loading control (glucose-6-phosphatase). B, Normalized UCP-1 mRNA levels in GALP ( $n = 8$ ) *vs.* control ( $n = 8$ ) animals (arbitrary units). Images were quantitated by densitometry and normalized for loading after subtracting out background;  $P < 0.02$  GALP *vs.* aCSF. C, Western blot for UCP-1 protein. Lower bands represent UCP-1-specific staining; upper bands loading control (protein kinase ARII $\beta$  subunit). D, Normalized UCP-1 protein levels in GALP ( $n = 8$ ) *vs.* control ( $n = 8$ ) animals (arbitrary units). Images were quantitated as above;  $P < 0.05$  GALP *vs.* aCSF. Data are presented as means  $\pm$  SEM. E and F, Histological sections ( $\times 10$ ) from interscapular BAT from representative aCSF-treated (E) and GALP-treated (F) animal. Insets, Magnification  $\times 20$ . Bar, 0.1 mm.

38). In the mouse, GALP appears to have only anorectic effects, with dose-dependent decreases in both FI and BW after central GALP administration (36). GALP neurons may also be important in relaying information to the hypothalamic-pituitary-gonadal axis, as acute central injections are associated with increased serum concentrations of LH in the mouse (36) and rat (37), an effect that can be blocked with a GnRH antagonist (37). If GALP does indeed play a role as an integrator of leptin signals, we reasoned that its effects upon BW may be partially mediated by changes in energy expenditure. To evaluate the effects of GALP upon FI, BW, and BT in the leptin-deficient state, we performed several experiments investigating the response to acute and chronic GALP treatment in the *ob/ob* mouse.

A single ICV injection of GALP in the *ob/ob* mouse produced a significant and sustained decrease in both FI and BW. In considering these findings, it is important to compare them to the actions of GALP in wild-type mice. Krasnow *et al.* (36) demonstrated that even with repeated GALP injections, the initial decrease seen in FI recovers (in fact, surpassing control FI) within 48 h. Similarly, BW is significantly decreased at 24 h after GALP treatment in wild-type mice but is not statistically different from vehicle-treated controls at 48 h. It is conceivable that the transitory nature of GALP's effects in wild-type mice are attributable to changes in leptin (and perhaps insulin) concentrations, which are associated with the profound decrease in BW in the first 24 h of treatment. In this model, decreases in leptin concentration would

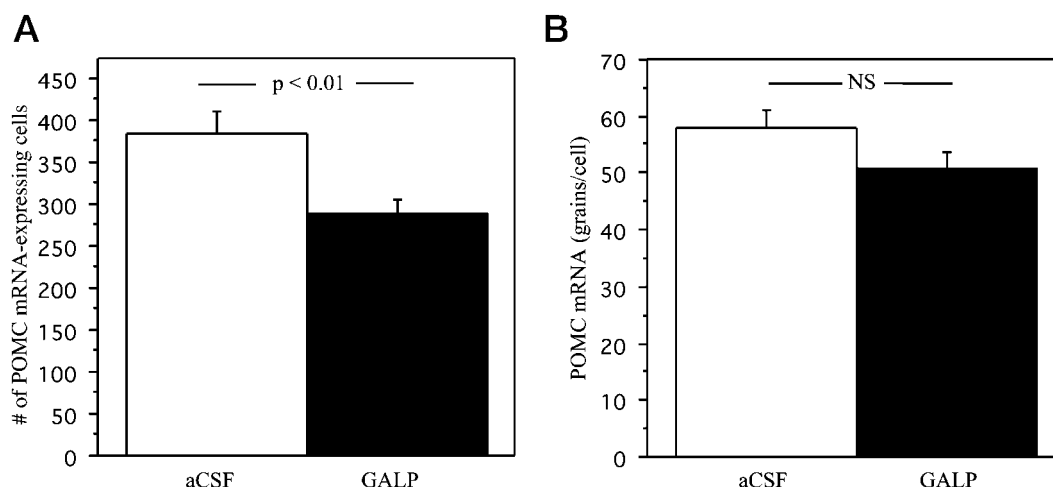


FIG. 5. Arcuate nucleus POMC expression in GALP-treated *vs.* pair-fed aCSF control animals. Total number of POMC expressing cells (A) or grains/cell (B) counted in anatomically matched sections from GALP-treated ( $n = 8$ ) or aCSF-treated ( $n = 7$ ) animals. Data are presented as the mean  $\pm$  SEM. A,  $P < 0.01$  GALP *vs.* aCSF; B,  $P = \text{NS}$ .

result in compensatory changes in the expression of neuropeptides involved in BW regulation (*i.e.* a decrease in POMC/CART and an increase in NPY/AgRP), ultimately resulting in increased FI to restore normal BW. In the leptin-deficient *ob/ob* mouse, these mechanisms are likely already maximally stimulated, and as a result, can do little to increase FI.

We also investigated the effects of chronic (14 d) GALP treatment in *ob/ob* mice. In this experiment, FI slowly recovered and was not significantly different from *ad libitum*-fed controls the last 2 d of treatment. However, BW remained significantly reduced in GALP-treated animals compared with controls throughout the treatment interval and showed no indication of recovery. At the end of the treatment interval, BT was significantly higher in GALP-treated than in control animals. Given the discrepancy between FI and BW in this experiment, we evaluated the effects of chronic GALP-treatment while controlling for FI through a pair-fed design. In this experiment, we demonstrated that central GALP treatment has effects that extend beyond its anorectic actions, with GALP-treated animals having significantly lower BW than that of pair-fed controls. Additionally, the BT of GALP-treated animals was not only higher than that of controls, but was also higher than pretreatment levels, suggesting that GALP's effects are more than simply the attenuation of BT decreases often observed with food restriction. Taken together, these observations suggest that part of GALP's effects upon BW in the *ob/ob* mouse are due to an increase in energy expenditure. Furthermore, we demonstrate that at least one component of this increase in energy expenditure is due to increased thermogenesis, an effect likely mediated by the observed increase in UCP-1 protein in BAT.

UCP-1 is a protein of the inner mitochondrial membrane that has a single known function: to increase thermogenesis by decreasing the coupling efficiency between oxidative phosphorylation and the synthesis of ATP (20, 45). It has been estimated that mitochondrial proton leak may account for 15–31% of basal metabolic rate (46, 47). As a result, changes in UCP-1 protein can lead to significant changes in overall energy expenditure and may account for the BW difference

seen in GALP-treated animals and their pair-fed controls. The regulation of UCP-1 expression occurs at the transcriptional level, with SNS activation playing an important role in this regard (48, 49). Leptin increases UCP-1 expression in BAT through activation of the SNS. Initial studies by Collins *et al.* (50) demonstrated increases in norepinephrine turnover in BAT in response to leptin. Furthermore, leptin treatment directly activates the SNS in the rat, and increases in UCP-1 expression in response to leptin can be blocked with surgical denervation and with SNS neurotoxins (25, 27, 51). These observations have been extended to the mouse, wherein dopamine  $\beta$ -hydroxylase knockout mice (which are norepinephrine and epinephrine deficient) are incapable of increasing UCP-1 expression in response to leptin (26). Taken together, these investigations have demonstrated a clear role for the SNS in mediating changes in UCP-1 expression in response to leptin treatment. Given that central (ICV) leptin treatment at much lower doses results in comparable increases in UCP-1 expression to that seen in studies using peripheral dosing, a central mechanism is likely (52).

If GALP-dependent increases in energy expenditure occur through a mechanism involving SNS activation and increased UCP-1 expression, how does the central nervous system mediate these effects? Multiple experimental observations support a role for POMC neurons in the circuits leading to increased SNS activation (28, 53–55). Therefore, we reasoned that POMC neurons may be involved in the mechanism leading to increased UCP-1 expression in BAT in response to GALP treatment. However, we found that the expression of POMC mRNA (per cell) in the arcuate nucleus of animals treated with GALP was indistinguishable from that of controls. Furthermore, the number of cells expressing POMC mRNA was actually decreased in response to GALP. These observations suggest that the effects of GALP upon UCP-1 expression are unlikely to be mediated through neuronal circuits involving the melanocortins. It is important to note that although POMC signals appear to be important in increasing SNS output and ultimately influencing UCP-1 expression, the role of POMC in mediating these changes in response to leptin is less certain. Hayes *et al.* (54) reported that



SHU9119 does not block the leptin-induced increase in SNS activation of BAT, despite the antagonist's ability to block leptin signaling to renal fat tissue. The magnitude of the change in UCP-1 expression we observed in response to GALP treatment (~2-fold increase) is less than that frequently reported in response to leptin treatment (3- to 5-fold increase); thus, it's plausible that GALP and POMC circuits function independently to increase UCP-1 expression (23, 52). A recent report has suggested that a subset (10%) of POMC neurons coexpress GALP (56). Given that a subset of POMC/CART neurons project directly to the sympathetic preganglionic column (53), perhaps these POMC neurons are those that coexpress GALP, providing a mechanism whereby GALP exerts its effects upon the SNS.

Another possible mechanism that may lead to changes in energy expenditure in GALP-treated animals would be changes in the thyroid axis. In the mouse, leptin treatment blunts the suppression of the thyroid axis observed with food restriction (8). In the *ob/ob* mouse, GALP treatment attenuated the decrease in thyroid hormone observed in pair-fed (and therefore, food restricted) control animals, although these differences did not reach statistical significance. Furthermore, the overall difference in thyroid hormone levels between the groups was small (~10% difference). Nevertheless, we cannot rule-out the possibility that changes in thyroid hormone levels are responsible for some of the BW difference between the GALP-treated and pair-fed animals. Recent observations by Seth *et al.* (44) have suggested that GALP may serve as a negative regulator of the thyroid axis. However, Matsumoto and colleagues (37) did not detect changes in TSH concentration after either ICV injections of GALP into rats or when applied to dispersed pituitary cells *in vitro*. Therefore, the role of GALP in regulating the thyroid axis, if any, remains to be elucidated.

Limitations of our work should be noted. First, although central GALP increases energy expenditure through a mechanism involving the up-regulation of UCP-1 in BAT, this should not be taken to indicate that other mechanisms are not involved. Other mechanisms by which leptin increases energy expenditure include changes in liver stearyl-coenzyme A desaturase-1 (57) and in skeletal muscle fatty-acid oxidation (58), which we did not evaluate in our study. Increased motor activity has also been noted in wild-type mice chronically treated with GALP (36) and may have contributed to the increase in energy expenditure observed in *ob/ob* mice treated with GALP. Although the effects of GALP are likely centrally mediated (*i.e.* through the activation of the SNS), the possibility exists that some of the injected GALP escapes the CNS and acts peripherally. However, the concentration of GALP reaching the periphery would likely be well below the threshold of GALP's interaction with either the GALR1 or GALR2 receptor (29). Finally, although central GALP injections are adequate to elicit the above responses, the necessity of GALP for these processes to occur in the physiological context of leptin treatment remains to be determined. A more complete assessment of these questions awaits the development of a gene-specific knockout for GALP or the development of receptor-specific antagonists.

In summary, the results of the present study demonstrate that central administration of GALP to the *ob/ob* mouse, ei-

ther acutely or chronically, is associated with significant and sustained decreases in FI and BW. Furthermore, the effects of GALP upon BW in the *ob/ob* mouse are mediated by both FI-dependent and -independent mechanisms. At least one of these FI-independent mechanisms includes an increase in thermogenesis, an effect mediated by increases in UCP-1 expression in BAT. Although there is some evidence that melanocortins are involved in the up-regulation of UCP-1 in response to leptin treatment, the effects of GALP in this regard appear to be POMC independent. Decreases in arcuate nucleus POMC expression associated with GALP treatment suggest that POMC is not mediating the effects of GALP; rather, it is responding to changes in FI and BW elicited by treatment with GALP.

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