

A Role for Galanin-Like Peptide in the Integration of Feeding, Body Weight Regulation, and Reproduction in the Mouse

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Galanin-like peptide (GALP) shares sequence homology with galanin and binds to galanin receptors *in vitro*. GALP neurons in the arcuate nucleus coexpress leptin receptors, and GALP mRNA expression is up-regulated by leptin. Based on these observations, we postulated that GALP plays a role in mediating leptin's inhibitory effects on food intake (FI) and body weight (BW), as well as its stimulatory effect on the reproductive axis. To test these hypotheses, we performed several studies in which mice received intracerebroventricular injections of either GALP or vehicle. Acute GALP treatment elicited a dose-dependent suppression of FI and BW. Long-

term treatment with GALP caused only transient reductions in FI and BW, demonstrating that the mice became refractory to continued exposure to GALP. GALP inhibited FI as early as 1 h post injection. Central injection of GALP suppressed locomotor activity and elicited the formation of a conditioned taste aversion. In male mice, serum levels of LH and testosterone were increased by GALP administration. Although we cannot rule out possible nonspecific effects of GALP on FI, the present observations are consistent with the argument that GALP is a downstream effector of leptin's actions within the central nervous system. (*Endocrinology* 144: 813–822, 2003)

THE HYPOTHALAMIC ARCUATE nucleus (Arc) contains neuronal circuitry that is involved in the regulation of energy homeostasis and reproduction. Several phenotypically discrete populations of neurons in the Arc are targets for the hormone leptin, which is synthesized and secreted by adipocytes and reduces food intake (FI) and body weight (BW) in rodents (1). In addition to its role in regulating energy homeostasis, leptin has also been shown to have a stimulatory effect on the reproductive axis (2). Leptin exerts its effects on energy balance and reproduction, in part, by modulating the activity of neuropeptide circuits within the Arc. Neurons that express proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), neuropeptide Y (NPY), and agouti-related protein (AgRP) also express leptin receptor (Ob-R) mRNA (3–6). Leptin increases POMC and CART mRNA expression (7, 8), and both α -MSH (a cleavage product of the POMC precursor gene) and CART have been demonstrated to decrease FI and BW when administered centrally (8–10). Conversely, leptin decreases mRNA levels of the orexigenic neuropeptides NPY and AgRP (11, 12). All four of these neuropeptides are thought to be downstream effectors of leptin signaling within the hypothalamus and have been implicated in the regulation of energy homeostasis and reproduction (2, 13–16). However, evidence suggests that other populations of neu-

rons in the Arc, in addition to the ones mentioned above, are also targets for leptin, including neurons that synthesize galanin-like peptide (GALP).

GALP is a 60-amino-acid neuropeptide that was recently isolated from porcine hypothalamus by virtue of its ability to bind and activate galanin receptors *in vitro* (17). GALP cDNAs have subsequently been cloned from pig, rat, mouse, macaque, and human (17–19). In all species studied to date, amino acids 9–21 of GALP are 100% homologous to the first thirteen amino acids of galanin, which are believed to confer biological activity to the galanin molecule (17–20). GALP binds to galanin receptors *in vitro*, showing a higher affinity for GalR2 ($IC_{50} = 0.24$ nM) than for GalR1 ($IC_{50} = 4.3$ nM). In contrast, galanin binds to GalR1 and GalR2 with more similar affinities ($IC_{50} = 0.1$ nM and 0.48 nM, respectively) (17). GALP has a fairly restricted distribution pattern within the central nervous system (CNS), with the expression of GALP mRNA and protein in the forebrain being confined to the Arc and median eminence, as well as the neurohypophysis (21–24).

The expression of GALP mRNA within the Arc is highly dependent upon an animal's nutritional status. In the rat, levels of GALP mRNA are reduced by a 48-h fast, an effect that is reversed by concomitant treatment with leptin (21). In the leptin-deficient *ob/ob* mouse, GALP mRNA expression is virtually nondetectable but is restored to wild-type levels by central leptin administration (18). Furthermore, the vast majority (greater than 85%) of GALP-containing cells in the Arc coexpress Ob-R mRNA (19) or protein (24). These observations suggest that GALP neurons are downstream targets of leptin within the CNS, and thus might play an intermediary role in leptin's ability to decrease FI and BW and stimulate

Abbreviations: aCSF, Artificial cerebrospinal fluid; AgRP, agouti-related protein; Arc, arcuate nucleus; BW, body weight; CART, cocaine- and amphetamine-regulated transcript; CNS, central nervous system; CTA, conditioned taste aversion; FI, food intake; GALP, galanin-like peptide; ICV, intracerebroventricular; NPY, neuropeptide Y; Ob-R, leptin receptor; POMC, proopiomelanocortin; SPR, saccharin preference ratio.

reproductive hormone secretion. To test these hypotheses, we performed a series of experiments to evaluate the effects of central GALP treatment on FI, BW, and reproductive hormone levels in the mouse.

Materials and Methods

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) employing *N*-fluorenylmethyloxycarbonyl/*t*-butyl ester protection strategy. Crude peptide and HPLC pooled fractions were analyzed by analytical reversed-phase HPLC with a Vydac (Hesperia, CA) 214TP C18 column (300Å pore size, 5 µm particle size, 0.46 × 25 cm). The conditions for this and all subsequent primary analytical reversed-phase HPLC were linear gradients of 5–50% acetonitrile in 0.1% aqueous trifluoroacetic acid over 25 min, with a flow rate of 1.0 ml/min. The effluent was monitored with a photo diode array detector from which the 220-nm absorbance profile was extracted, revealing a purity of more than 95%. HPLC fractions were analyzed by flow injection with an atmospheric pressure ionization 1 electrospray-mass spectrometer (PerSeptive Biosystems, Framingham, MA). HPLC-MS was done with an atmospheric pressure ionization 150 (PerSeptive Biosystems) in conjunction with Waters analytical HPLC system (Waters Corp., Milford, MA) by using a YMC ODS-AQC18 column, with a linear gradient of 0–60% acetonitrile in 0.1% aqueous trifluoroacetic acid over 12 min with a flow rate of 0.5 ml/min. ESI-mass spectrometry revealed a molecular weight of 6504 (observed) *vs.* 6502.7 (theoretical). Quantitative amino acid analyses were performed on a PE Applied Biosystems (Foster City, CA) 420A derivatizer with a 130A separation system. Quantitative amino acid analyses were performed in triplicate, and composition was determined by comparison of the area of each amino acid peak in the peptide to the area of a known amount of amino acid standard. The compositional amino acid ratio fit the theoretical ratio. In all experiments, GALP was dissolved in artificial cerebrospinal fluid (aCSF) containing 0.1% BSA.

Animals

Adult male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were individually housed and were maintained on a 14-h light, 10-h dark dark cycle (lights on at 0500 h), unless otherwise noted. Male Sprague Dawley rats (B&K Universal, Kent, WA) were individually housed and were 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 stated otherwise. 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 NIH Guide for Care and Use of Laboratory Animals.

Intracerebroventricular (ICV) injections

Freehand ICV injections were performed as previously described (25), with a few modifications. Briefly, mice were anesthetized with isoflurane (Abbott Laboratory, North Chicago, IL) delivered by a vaporizer (Veterinary Anesthesia Systems, Bend, OR). Upon achieving a surgical plane of anesthesia, a small hole was bored in the skull 1 mm lateral and 0.5 mm posterior to bregma with a Hamilton syringe attached to a 27-gauge needle fitted with polyethylene tubing, leaving 3.5 mm of the needle tip exposed. Once the initial hole was made, all subsequent injections were made at the same site. Mice were allowed to recover for at least 2 d before treatment. For ICV injections, mice were anesthetized with isoflurane for a total of 2–3 min, during which time 3 µl solution were slowly and continuously injected into the lateral ventricle. The needle remained inserted for approximately 30 sec after injection to minimize backflow up the needle track. Mice typically recovered from the anesthesia within 3 min after the injection.

Experiment 1: dose-response

Mice received two ICV injections (1800 h and 0800 h the following day) of either aCSF (*n* = 7) or 1 nmol (*n* = 6), 5 nmol (*n* = 8), or 10 nmol

GALP (*n* = 5). FI, BW, and rectal temperature were measured 24 h after the first injection.

Experiment 2: long-term GALP treatment

Mice received two ICV injections/d (0800 h and 1800 h) for 4.5 d, with the first injection occurring at 1800 h (d 0). Mice were treated with either aCSF (*n* = 8) or 5 nmol GALP (*n* = 8). FI and BW were monitored daily.

Experiment 3: locomotor activity

Mice were group-housed (four per cage) and were maintained on a 12-h light, 12-h dark cycle (lights on at 0700 h). Either aCSF (*n* = 7) or 5 nmol GALP (*n* = 8) was injected ICV twice daily (0800 h and 1800 h) for 4.5 d, with the first injection occurring at 1800 h. Locomotor activity was measured by placing the mice in individual transparent Plexiglas cages (40 × 20 × 20 cm) surrounded by aluminum frames equipped with infrared beams (San Diego Instruments, San Diego, CA). Activity levels were monitored for 14 h overnight (starting at 1830 h) after the first and ninth injections. Activity levels are expressed as the number of ambulations (two consecutive beam breaks) recorded in each 30-min interval, as well as the total number of ambulations over the entire 14-h observation period.

Experiment 4: conditioned taste aversion

Mice were placed on a water restriction schedule, during which time they received two water bottles per cage from 0830–0900 h and from 1400–1900 h each day for 7 d. Food and water intake were measured daily. On d 8, the mice received a single bottle of water containing 0.15% saccharin (Sigma, St. Louis, MO) at 0830 h. Bottle position in the cage was randomized. The saccharin water was removed 30 min later, and then the mice received an ICV injection of either aCSF (*n* = 9) or 5 nmol GALP (*n* = 8). A third group of mice received an ip injection (2% BW) of 0.15 M LiCl (J. T. Baker, Inc., Phillipsburg, NJ) dissolved in saline (*n* = 4). The mice were allowed to recover for 48 h, during which time they were still water restricted. Food and water intake returned to preinjection levels in all treatment groups within 48 h. After 48 h, the mice received two bottles (one containing water and the other containing 0.15% saccharin water, with bottle position randomized) at 0830 h. Fluid intake was measured from each bottle after 2 h. Data are expressed as saccharin preference ratios (SPR = volume of saccharin water consumed/total fluid volume consumed during the 2-h period).

Experiment 5: time-course

Mice received a single ICV injection of either aCSF (*n* = 7) or 5 nmol GALP (*n* = 7) at 1800 h. FI was measured 2, 4, 6, 8, 14, and 24 h post injection, and BW was measured 14 and 24 h post injection.

Experiment 6: morning GALP injection in mice

Mice received a single ICV injection of either aCSF (*n* = 8) or 5 nmol GALP (*n* = 8) at 0900 h. FI was measured after 1 and 2 h, and both FI and BW were measured 24 h post injection.

Experiment 7: morning GALP injection in rats

Rats (320–350 g) were anesthetized with an injection (0.1 ml/100 g BW, im) of a ketamine (100 mg/ml)/xylazine (20 mg/ml)/acepromazine maleate (10 mg/ml) cocktail. The animals were placed in a stereotaxic instrument with the incisor bar positioned 3 mm below the horizontal plane that intersected the center of the ear bars. A permanent stainless steel cannula was implanted into the third ventricle at 0 mm from midline, –2.2 mm caudal to bregma, and –7.5 mm ventral to dura (26). The animals were allowed to recover for 8 d before treatment, during which time they were handled daily. On d 9, the rats received a single injection of either aCSF or 5 nmol GALP at 0900 h. Injections were delivered in a volume of 3 µl over the course of 2 min. FI was measured 1, 2, and 24 h post injection; BW was measured 24 h post injection. The experiment was repeated 4 d later in a cross-over design, such that each animal received both treatments (*n* = 10 per treatment). Correct cannula placement was determined by injecting 0.5 nmol NPY

2 d after the first round of injections. Only rats that increased baseline FI by at least 4 g during the first hour after NPY treatment were included in the study.

Experiment 8: reproductive hormones

Mice were handled daily for 2 wk before treatment. Mice received a single ICV injection of either aCSF ($n = 9$) or 5 nmol GALP ($n = 8$) between 0800 h and 0830 h. The mice were lightly anesthetized with isoflurane 30 min later and were then brought to a separate room. Blood was obtained by a combination of orbital bleed and by collecting trunk blood after cervical dislocation and rapid decapitation. Sera were stored at -20°C for hormone measurements.

RIAs

Serum LH and FSH concentrations were measured with reagents obtained from NIH. For LH, the antiserum used was anti-rLH-S-11 and the standard was rLH-RP3. The assay sensitivity was 0.2 ng/ml and the intraassay coefficient of variation was 7%. For FSH, the antiserum used was anti-rFSH-S-11 and the standard was rFSH-RP2. The assay sensitivity was 1.0 ng/ml and the intraassay coefficient of variation was 6%. Testosterone was measured with a double antibody kit (ICN Biomedicals, Inc., Costa Mesa, CA). The assay sensitivity was 0.02 ng/ml, and the intraassay coefficient of variation was 3%. All hormones were measured in single assays.

Statistical analysis

All data are expressed as a mean \pm SEM for each group. Differences between groups were assessed by one-way or two-way ANOVA. When the ANOVA indicated significant differences, Fisher's *post hoc* test was used to identify differences between individual treatment groups. Two group comparisons were performed using Student's *t* test. Differences were considered significant when $P < 0.05$.

Results

Experiment 1: dose-response

To determine whether acute GALP treatment alters FI and/or BW, we gave mice two ICV injections (14 h apart) of vehicle or 1, 5, or 10 nmol GALP. Central GALP treatment elicited a dose-dependent suppression of both FI (Fig. 1A) and BW (Fig. 1B) when measured 24 h after the first injection, with significant decreases in FI and BW occurring with injection of 5 or 10 nmol GALP. At 24 h after the first injection, rectal temperature was depressed in the mice that received 10 nmol GALP ($P < 0.005$ vs. vehicle-treated mice) but was unaffected by either 1 or 5 nmol GALP (data not shown). In addition to reducing FI and BW, treatment with the higher doses of GALP had pronounced effects on motor behavior. The mice treated with 5 or 10 nmol GALP consistently remained immobile for a longer period of time than the vehicle-treated mice (*i.e.* they did not appear to recover from the anesthesia as quickly as the vehicle-treated animals). Within the first hour post injection, tremors were often observed in the mice that received 10 nmol GALP. There was no evidence of tremors associated with either the 1- or 5-nmol doses.

Experiment 2: long-term GALP treatment

To assess the effects of long-term GALP treatment on FI and BW, mice were given two daily ICV injections of either vehicle or 5 nmol GALP for 4.5 d. Long-term GALP treatment had transient effects on both FI (Fig. 2A) and BW (Fig. 2B). At 24 h after the first injection (d 1), FI and BW were significantly reduced in the GALP-treated mice ($P < 0.005$ and

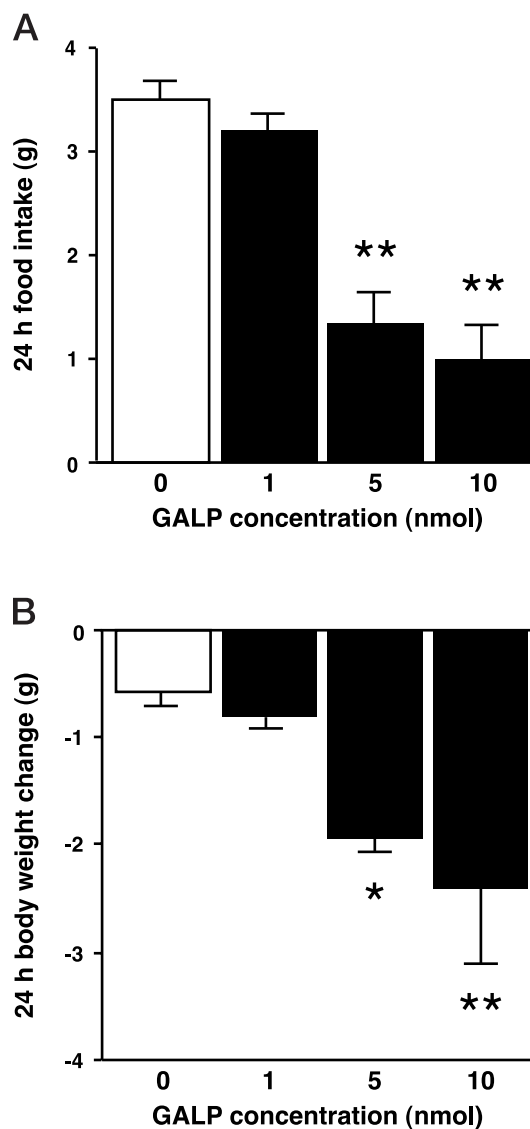


FIG. 1. A, Food intake; and B, body weight change 24 h after the first of two ICV injections of vehicle or GALP (1, 5, or 10 nmol). *, $P < 0.005$; **, $P < 0.0005$ vs. vehicle-treated mice.

$P < 0.05$ vs. vehicle-treated mice, respectively). However, at 48 h after the first injection (d 2), FI and BW no longer differed between the vehicle- and GALP-treated groups. FI recovered to baseline levels in the GALP-treated mice by d 2, whereas BW took longer to return to pretreatment levels. FI was significantly elevated in the GALP-treated mice on d 4 ($P < 0.05$ compared with vehicle-treated mice).

Experiment 3: locomotor activity

Before drawing any conclusions about whether ICV GALP administration induces satiety in mice, we considered the possibility that the reduction in FI observed following central GALP treatment might be secondary to other behavioral effects of ICV GALP administration. Because we had observed altered motor behavior following ICV injection of 5 or 10 nmol GALP, it seemed possible that the acute suppression of FI following GALP treatment might be a consequence of

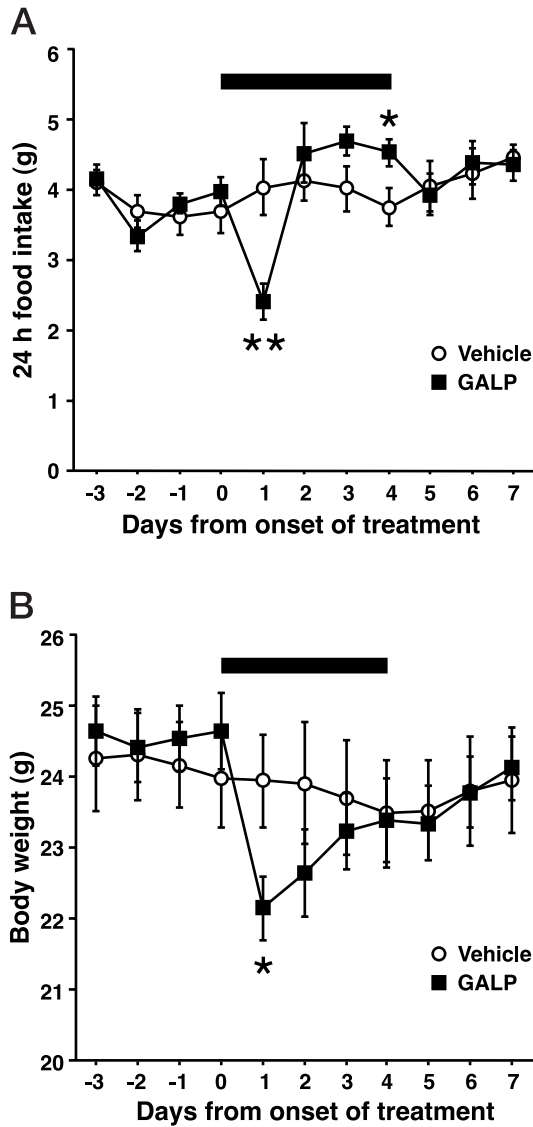


FIG. 2. A, Twenty-four-hour food intake; and B, body weight over the course of long-term treatment with vehicle (○) or 5 nmol GALP (■). Mice received two ICV injections/d for 4.5 d. Solid black bars indicate duration of treatment. *, $P < 0.05$; **, $P < 0.005$ vs. vehicle-treated mice.

impaired motor function, thus rendering the mice physically incapable of eating as much as the vehicle-treated animals. To further investigate this possibility, we performed an experiment to assess whether locomotor activity is altered following both acute and long-term GALP treatment. Mice were given two daily ICV injections of vehicle or 5 nmol GALP for 4.5 d, as in experiment 2. For 14 h following the first injection (Fig. 3A), locomotor activity was significantly suppressed by 5 nmol GALP ($P < 0.005$ vs. vehicle-treated mice). Following the first injection, there was very little evidence of movement in the GALP-treated mice until the final few hours of the observation period, coinciding with the timing of the lights coming on (Fig. 3B). Surprisingly, locomotor activity was significantly elevated in the GALP-treated mice during the 14-h period following the ninth injection ($P < 0.005$ vs. vehicle-treated mice). After the ninth injection, the GALP-

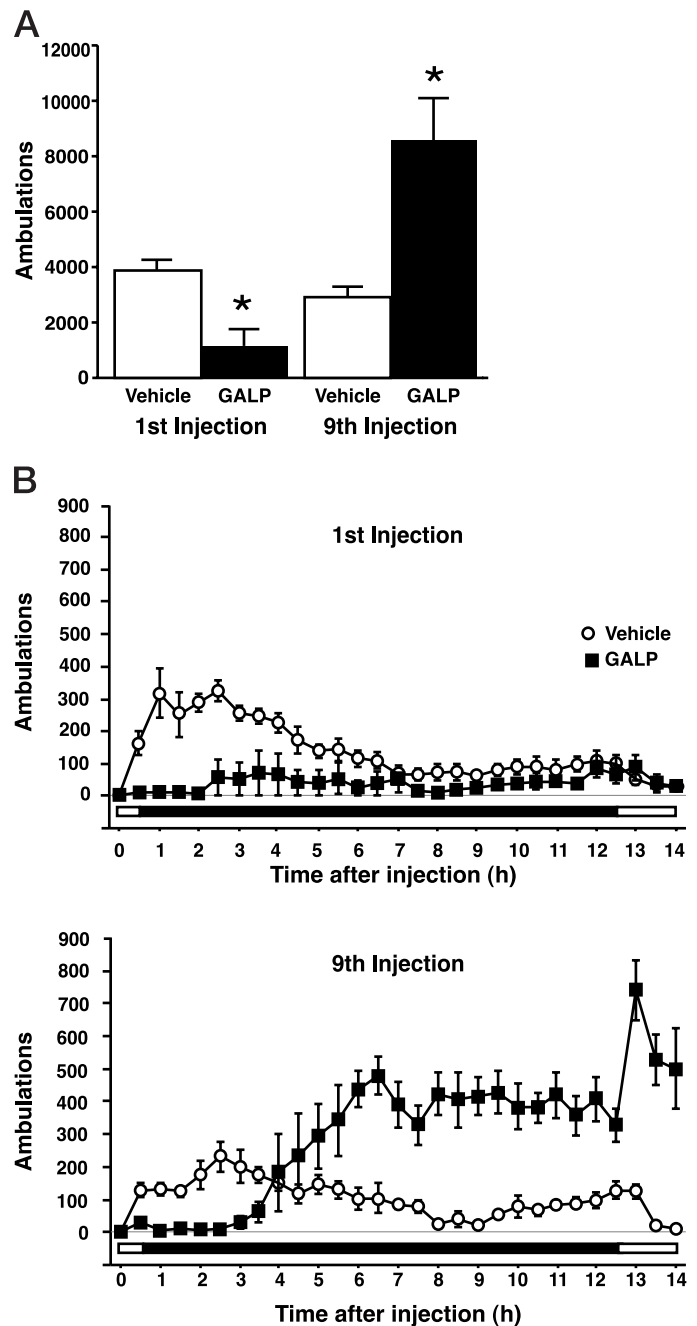


FIG. 3. A, Locomotor activity (presented as total number of ambulations over 14 h) following ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars). Mice received two ICV injections/d for 4.5 d, and activity levels were measured for 14 h following the first and ninth injections. *, $P < 0.005$ vs. vehicle-treated mice. B, Fourteen-hour activity profiles of mice treated with vehicle (○) or 5 nmol GALP (■) after the first and ninth injections. Light/dark bars at the bottom of the graphs indicate the timing of lights on and lights off.

treated mice were immobile for approximately 4 h but then became increasingly hyperactive for the remainder of the observation period.

Experiment 4: conditioned taste aversion

Another conceivable mechanism underlying the reduction of FI following ICV GALP treatment is that central admin-

istration of 5 nmol GALP is aversive to mice. Conditioned taste aversion (CTA) paradigms are commonly employed to assess whether substances might have aversive properties in rodents (27). Therefore, we performed an experiment to determine whether ICV GALP treatment would elicit the formation of a CTA in mice (Fig. 4). Using a two-bottle paradigm, we observed that ICV treatment with vehicle did not appear to induce a CTA, as evidenced by a high saccharin preference ratio (SPR) in the vehicle-treated group (SPR = 0.83 ± 0.03). Another group of mice received an ip injection of LiCl, an emetic drug that is well known to elicit the formation of a CTA in rodents. As expected, the LiCl-treated mice had a low SPR (SPR = 0.12 ± 0.06 ; $P < 0.0001$ vs. vehicle-treated mice), indicating that LiCl treatment induced the formation of a CTA. Mice that received an ICV injection of 5 nmol GALP also formed a strong CTA (SPR = 0.02 ± 0.01 ; $P < 0.0001$ vs. vehicle-treated mice).

Experiment 5: time course

To assess the time-course of GALP's effects on FI and BW, we measured FI and BW at various intervals up to 24 h following a single ICV injection of vehicle or 5 nmol GALP (administered 1 h before lights off). GALP's inhibitory effect on FI was rapid, since FI was significantly suppressed in the GALP-treated mice as early as 2 h post injection (Fig. 5). FI remained significantly reduced at all subsequent time points ($P < 0.001$ vs. vehicle-treated mice at all post-injection time points). BW was significantly decreased in the GALP-treated mice 14 h post injection ($P < 0.05$ vs. vehicle-treated mice) but no longer differed from the vehicle-treated group by the 24-h time point (data not shown).

Experiment 6: morning GALP injection in mice

Galanin has been shown to acutely stimulate FI in the rat (28). Furthermore, Matsumoto *et al.* (29) recently reported that in the rat, FI is acutely stimulated 90 min following injection of GALP (0.3 or 1 nmol) into the third ventricle. Although we observed that a single injection of 5 nmol GALP inhibits 2 h FI in mice when administered in the

evening (experiment 5), we decided to test GALP's effect on acute FI when administered during the daytime. We reasoned that a stimulatory effect of GALP on feeding might be revealed in the morning, when mice are normally satiated, instead of in the evening when there is already a near-maximal physiological drive to eat. When mice were given a single ICV injection of 5 nmol GALP at 0900 h, FI was significantly suppressed during the first hour post injection ($P < 0.05$ vs. vehicle-treated mice). FI during the second hour did not differ from that of the vehicle-treated group (Fig. 6A). FI (Fig. 6B) and BW (Fig. 6C) in the GALP-treated group were significantly reduced 24 h post injection ($P < 0.05$ vs. vehicle-treated mice). Lower doses of GALP (0.2 and 1 nmol) also tended to reduce FI during the first hour post injection compared with vehicle treatment, although the differences did not reach statistical significance (data not shown). Although no formal measures of locomotor activity were made in this experiment, treatment with 1 nmol GALP appeared to have minimal effects on motor behavior and the 0.2 nmol dose appeared to be sub-threshold for any detectable motor effects. Although the morning FI measurements in all treatment groups were only slightly above the level of detection, there was no evidence of stimulation of FI at these lower doses of GALP, even in the absence of pronounced effects on motor activity.

Acute FI was also measured in mice following a morning ICV injection of 1 nmol galanin, a dose that has been previously demonstrated to acutely stimulate FI in the rat (28). Compared with vehicle treatment, galanin had no discernible effect on FI 1, 2, or 24 h post injection and was without effect on 24 h BW (data not shown). Furthermore, galanin treatment did not appear to have any obvious effects on motor behavior.

Experiment 7: morning GALP injection in rats

To confirm the earlier report by Matsumoto *et al.* (29) of GALP's acute stimulatory effect on FI in the rat, rats were given a single third ventricle injection of vehicle or 5 nmol

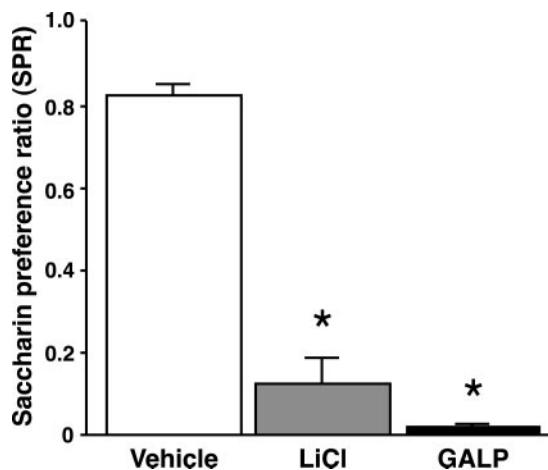


FIG. 4. Two-hour saccharin preference ratios in mice treated with vehicle (ICV), LiCl (0.15 M, 2% BW; ip) or GALP (5 nmol; ICV) 48 h earlier. *, $P < 0.0001$ vs. vehicle-treated mice.

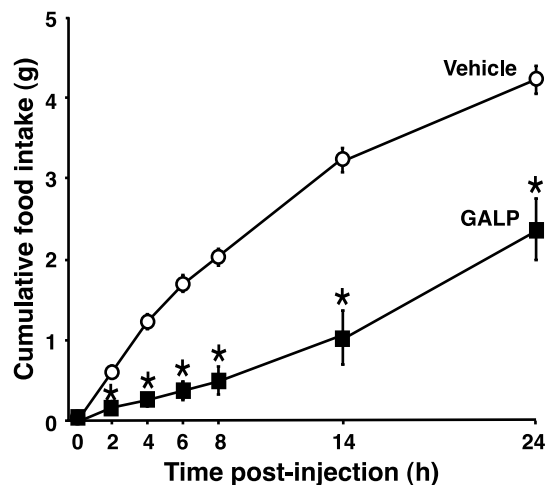


FIG. 5. Cumulative food intake 0, 2, 4, 6, 8, 14, and 24 h following a single ICV injection of vehicle (○) or 5 nmol GALP (■). *, $P < 0.001$ vs. vehicle-treated mice.

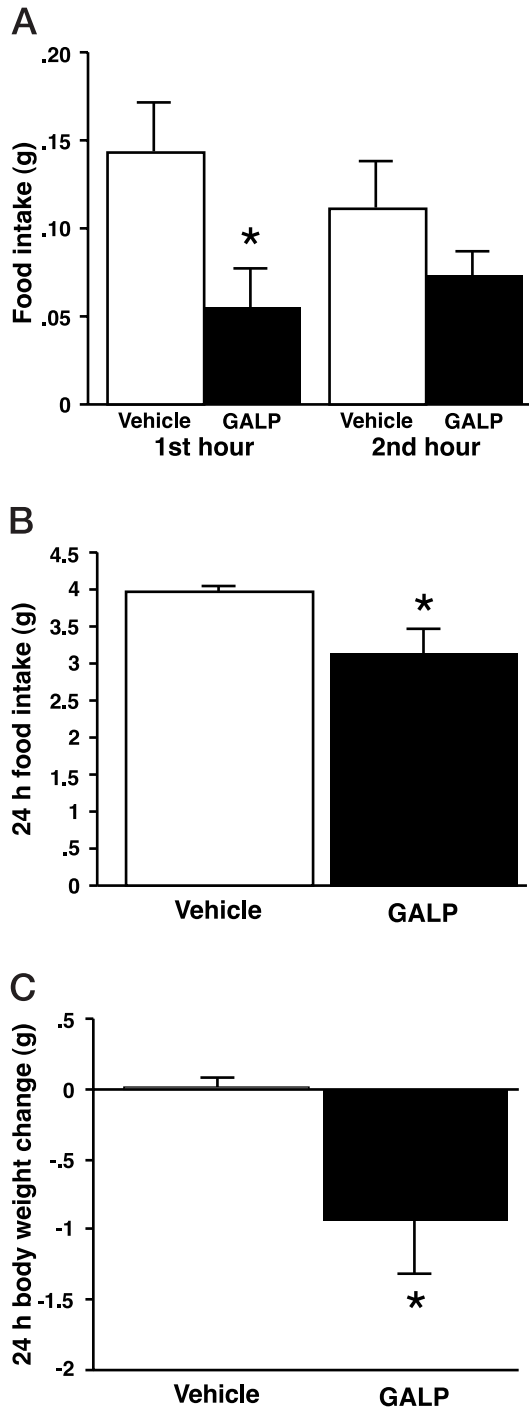


FIG. 6. A, One- and 2-h food intake; B, 24-h food intake; and C, 24-h BW change in mice following a single morning ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars). *, $P < 0.05$ vs. vehicle-treated mice.

GALP at 0900 h. FI was significantly elevated in the GALP-treated group during the first hour post injection ($P < 0.05$ vs. vehicle-treated rats) but did not differ between the two groups during the second hour (Fig. 7A). Despite the initial increase in FI with GALP treatment, FI (Fig. 7B) and BW (Fig. 7C) were significantly reduced in the GALP-treated rats 24 h post injection ($P < 0.005$ and $P < 0.05$ vs. vehicle-treated rats,

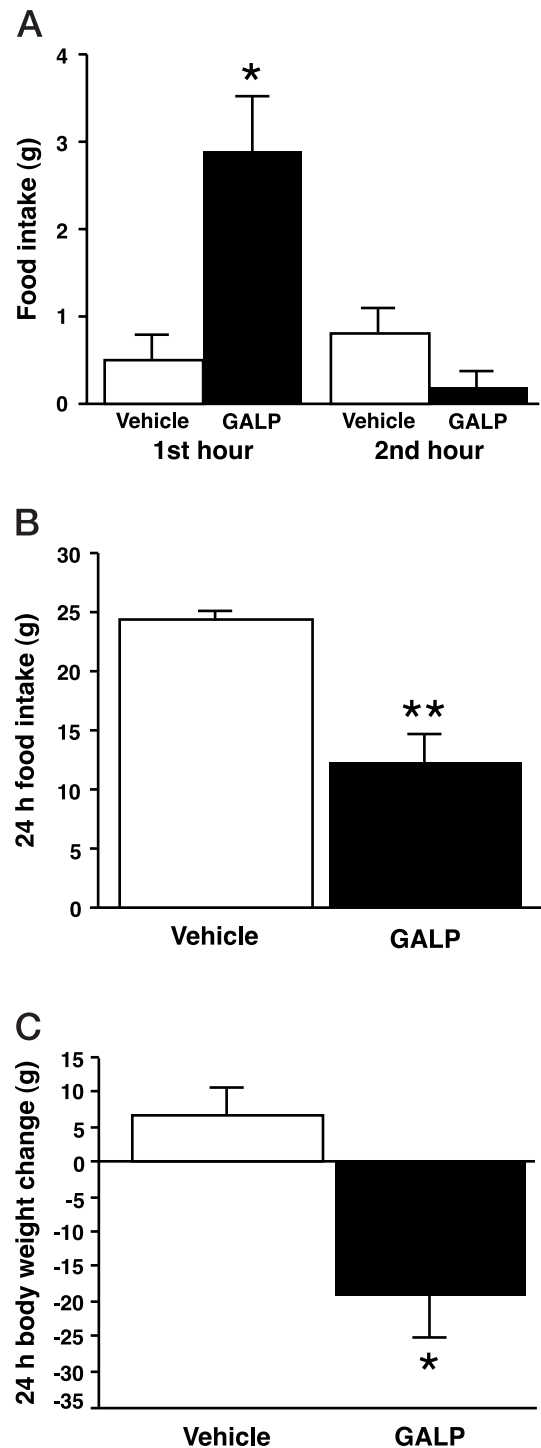


FIG. 7. A, One- and 2-h food intake; B, 24-h food intake; and C, 24-h BW change in rats receiving a single morning ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars). *, $P < 0.05$; **, $P < 0.005$ vs. vehicle-treated rats.

respectively). Motor behavior did not appear to be altered in the rats by administration of 5 nmol GALP.

Experiment 8: reproductive hormones

To determine whether ICV GALP treatment has an acute stimulatory effect on the hypothalamic-pituitary-gonadal

axis in the mouse as has been previously reported in the rat (30), we measured serum gonadotropin and testosterone levels in male mice after GALP treatment (Fig. 8). 30 min following an ICV injection of either vehicle or 5 nmol GALP, serum levels of LH and testosterone were significantly higher in the GALP-treated mice compared with the vehicle-treated group ($P < 0.0005$). Serum levels of FSH were modestly elevated in the GALP-treated animals, but the difference did not reach statistical significance ($P = 0.06$ vs. vehicle-treated mice).

Discussion

These experiments demonstrate an inhibitory effect of central GALP treatment on FI and BW in the mouse. GALP's suppressive effect on FI is rapid (evident within the first hour post injection) and transient in nature. The recovery of both FI and BW during long-term GALP administration occurred despite continuing treatment, indicating that the mice became refractory to repeated GALP injections. It is unclear why this occurred; however, it is conceivable that the observed recoveries of FI and BW are attributable to the activation of compensatory neural feeding pathways, driving the animals to eat in an attempt to restore BW back to normal. The observation that FI was slightly enhanced on d 3 and 4 of long-term GALP treatment lends further support to the notion that activation of orexigenic circuits (*e.g.* NPY or AgRP) or inhibition of anorectic pathways (*e.g.* POMC or CART) might be responsible for the rapid recovery of FI. Alternatively, it is plausible that the mice became desensitized to long-term GALP treatment due to receptor down-regulation in response to chronic exposure to ligand, much like the down-regulation of hypothalamic Ob-R that occurs with chronic leptin treatment (31).

Matsumoto *et al.* (29) recently demonstrated that in the rat, central GALP administration stimulates FI during the first 90 min post injection, suggesting that GALP is orexigenic. This observation seems to be counterintuitive, considering reports that the expression of GALP mRNA is up-regulated by leptin, a hormone that acts centrally to reduce FI. The expression of orexigenic neuropeptides, such as NPY and AgRP, is generally inhibited by leptin and induced by fasting and leptin deficiency (13, 14). In contrast, the expression of GALP mRNA is reduced in cases of fasting and leptin de-

iciency, in a manner similar to that of the anorectic peptides α -MSH and CART (14). In an attempt to reconcile our observations in the mouse with previous reports that GALP stimulates FI in the rat, we investigated the effects of GALP on short-term FI in the mouse, in the event that we might have been overlooking an acute stimulatory effect of GALP on FI. However, when mice were injected with GALP in the morning, we observed a reduction in FI during the first hour following the injection. Although mice eat very little food during the daytime, it is possible to observe a statistically significant increase in acute FI in the morning. In our hands, we have observed an approximately 2-fold increase in FI in mice 1 h following an ICV injection of NPY in the morning (unpublished observations). Furthermore, the use of isoflurane anesthesia before and during the ICV injection of NPY did not block the increase in FI in that study; thus, we would conclude that the use of isoflurane was unlikely to have masked a possible stimulatory effect of GALP on FI in the present study.

The observation that GALP had no discernible stimulatory effect on FI in the mouse, in contrast to a previously published report of GALP's effects on FI in the rat, initially raised the concern that the GALP we were using might display differences in biological activity and/or potency compared with the GALP preparation used by Matsumoto *et al.* (29). Therefore, we tested the bioactivity of our GALP within a different physiological context. Central GALP administration has been shown to acutely stimulate LH secretion in the male rat (30). We reasoned that if the GALP we were using was bioactive, we would likely observe an increase in LH secretion in mice following GALP administration, as is the case in the rat. Indeed, we found that ICV treatment with our GALP preparation acutely increased circulating levels of both LH and testosterone in male mice, thus corroborating results in the rat and confirming the efficacy of our GALP preparation.

To further examine whether mice and rats exhibit differential feeding responses to acute GALP treatment, we administered GALP to rats. We confirmed the occurrence of a transient stimulatory effect of GALP on FI in the rat, yet also observed a significant decrease in FI and BW 24 h later, as we have demonstrated in the mouse. These findings corroborate a recent report by Lawrence *et al.* (32) demonstrating that FI

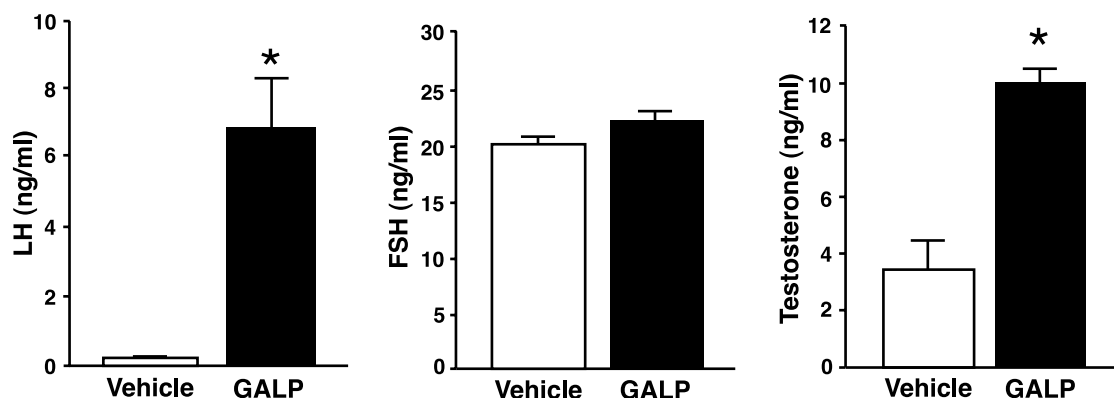


FIG. 8. Serum LH, FSH, and testosterone concentrations 30 min following a single ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars). *, $P < 0.0005$ vs. vehicle-treated mice.

and BW are reduced 24 h following a single ICV injection of GALP in the rat. One possible explanation for the disparate effects of GALP on FI between the 1- and 24-h intervals in the rat may be that GALP activates different neural pathways over the short and long term. It is conceivable that GALP's rapid stimulatory effect on FI reflects an activation of galanin receptors, perhaps mimicking the orexigenic effect of central galanin administration in the rat. This supposition is supported by the observation that GALP's acute stimulatory effect on FI in the rat is similar in its time-course to that of galanin (29). However, over a more extended time interval GALP might activate a different population of receptors (either another galanin receptor subtype or perhaps a yet-to-be-identified GALP-specific receptor), resulting in the activation of neural circuits that ultimately decrease FI and BW. If this were the case, the different response of the rat and the mouse to an acute GALP challenge might be attributable to the inaccessibility of exogenously administered GALP to orexigenic galaninergic pathways in the mouse, or perhaps to the absence of orexigenic galaninergic pathways in the mouse (which do exist in the rat) (33). The fact that there was no apparent stimulatory effect of galanin on acute FI in the mouse supports these arguments.

Centrally administered GALP elicits temporary immobilization in the rat (29). In the present study, spontaneous locomotor activity was reduced in mice after both acute and long-term GALP treatment. Compared with a single GALP injection, locomotor activity was suppressed for a shorter duration after long-term GALP treatment and was subsequently followed by pronounced hyperactivity. We did not measure activity levels at any intermediate time points during the course of long-term GALP treatment, so we do not know precisely when during the 3-d window this shift in motor response occurred. The physiological significance of this response is not immediately apparent. One possibility is that endogenous GALP is involved in the generation of circadian rhythms and/or sleep/wake cycling, as was recently shown to be the case with the orexins, a family of hypothalamic neuropeptides that is also implicated in regulating feeding behavior (34). On the other hand, it is possible that GALP's effects on motor behavior occur as a consequence of widespread diffusion of GALP following ICV injection. Galanin receptors are widely distributed throughout the CNS, including regions involved in motor function (35), and thus it is conceivable that the ICV injection of GALP leads to activation of receptors that ordinarily do not receive input from GALP neurons.

Given the acute suppressive effect of GALP treatment on locomotor activity, it is plausible that the reduced FI observed following ICV GALP treatment is a consequence of impaired motor function. Similar concerns have been raised about the specificity of CART's inhibitory effect on FI. Central administration of CART(55–102) reduces FI in rats but also causes movement-associated tremors and aberrant body postures (8, 36). Additional experiments have provided support for the argument that CART has specific effects on feeding behavior that are independent of its effects on motor function. Central administration of CART antiserum significantly increases night-time feeding in rats (8), and ICV administration of CART(42–89) induces Fos expression in re-

gions of the brain that are involved in the regulation of FI, including several hypothalamic nuclei (37). When similar experiments are performed with GALP, they should help to elucidate whether the effects of exogenous GALP on FI are specific to modulation of central feeding pathways. Although we did not formally measure locomotor activity with doses of GALP lower than 5 nmol in this study, doses of both 0.2 and 1 nmol GALP had minimal effects on gross motor function, yet we still observed a trend for reduced FI at these lower doses. A transient suppression of locomotor activity occurred after both the first and ninth GALP injections during long-term treatment with GALP, but FI was suppressed only during d 1 of the 4.5-d treatment period. This suggests that changes in FI cannot be entirely explained by reduced levels of motor activity immediately after the GALP injection.

The central administration of GALP elicited the formation of a strong CTA in mice. The induction of a CTA has been used for several decades as a behavioral assay to ascertain whether a particular substance might be toxic or cause visceral illness in rodents. One interpretation of experiment 4 is that central administration of 5 nmol GALP produces a pharmacological toxicity, and this explains its inhibitory effects on feeding and BW. Notwithstanding, there has been an ongoing debate about the interpretation of CTA studies. Several investigators have proposed that the formation of a CTA may not reflect either toxicity or illness, nor does it prove that the administered substance is truly aversive to the subject (27, 38, 39). Many drugs that elicit the formation of a CTA in an experimental setting do not appear to cause nausea or visceral illness and are even self-administered by animals, such as morphine and cocaine (39). It is also worth noting that not all agents that elicit a CTA reduce FI. The central administration of NPY results in the formation of a CTA in the rat, despite its simultaneous ability to markedly stimulate FI (40). Given the uncertainty of interpreting the results of CTA experiments, it is conceivable that the induction of a CTA by central GALP treatment does not reflect simple toxicity. Perhaps the central administration of GALP modulates the activity of neural pathways that are normally activated under aversive circumstances, thus leading to the formation of a CTA. Alternatively, if ICV GALP treatment does indeed have aversive properties, it might be argued that such an effect may still be physiologically relevant, in the sense that aversion may simply lie at one extreme of the range of appetitive behavior.

In addition to confirming the biopotency of our GALP preparation, the observation that central GALP administration stimulates the secretion of reproductive hormones suggests that GALP is involved in the neuroendocrine control of reproduction. GALP-immunoreactive fibers are found in close apposition to a subset of GnRH neurons in the medial preoptic area and the bed nucleus of the stria terminalis, and ICV GALP administration induces Fos expression in GnRH neurons (24, 30). GALP's stimulatory effect on LH secretion in the rat is blocked by pretreatment with a GnRH receptor antagonist, suggesting that exogenous GALP treatment stimulates LH secretion via a GnRH-dependent mechanism (30). These observations implicate a role for GALP in modulating the hypothalamic-pituitary-gonadal axis in the rat. The present demonstration that central GALP treatment in-

creases serum LH and testosterone concentrations in the mouse suggests that GALP has a modulatory effect on the reproductive axis of this species as well as the rat.

We have demonstrated here that central administration of GALP decreases FI and BW in the mouse and the rat. Together with previous demonstrations that the expression of GALP mRNA is up-regulated by leptin, the present observations are consistent with GALP playing a physiological role in the regulation of energy homeostasis. However, it is conceivable that the observed reduction in FI following central GALP treatment is not due to a specific action of GALP on neural circuits controlling feeding but is instead secondary to impaired motor behavior and/or aversive properties of ICV GALP treatment. Additional experiments will be necessary to further resolve questions about the specificity of GALP's effects on feeding behavior. The effects of GALP on FI appear to be species dependent because we (and others) have shown that GALP acutely stimulates FI in the rat but not in the mouse. We have also demonstrated a stimulatory effect of GALP on reproductive hormone secretion in the mouse. Together, these observations support the argument that GALP is a downstream effector of leptin's actions within the CNS. Furthermore, the putative involvement of GALP in the regulation of both feeding and reproduction implicates GALP as a potential molecular link between these two physiological processes.

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References

- Friedman JM, Halaas JL 1998 Leptin and the regulation of body weight in mammals. *Nature* 395:763–770
- Cunningham MJ, Clifton DK, Steiner RA 1999 Leptin's actions on the reproductive axis: perspectives and mechanisms. *Biol Reprod* 60:216–222
- Cheung CC, Clifton DK, Steiner RA 1997 Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* 138:4489–4492
- Elias CF, Lee CE, Kelly JF, Ahima RS, Kuhar M, Saper CB, Elmquist JK 2001 Characterization of CART neurons in the rat and human hypothalamus. *J Comp Neurol* 432:1–19
- Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Morgan PJ, Trayhurn P 1996 Coexpression of leptin receptor and preproneuropeptide Y

- mRNA in arcuate nucleus of mouse hypothalamus. *J Neuroendocrinol* 8:733–735
- Wilson BD, Bagnol D, Kaelin CB, Ollmann MM, Gantz I, Watson SJ, Barsh GS 1999 Physiological and anatomical circuitry between Agouti-related protein and leptin signaling. *Endocrinology* 140:2387–2397
- Thornton JE, Cheung CC, Clifton DK, Steiner RA 1997 Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. *Endocrinology* 138:5063–5066
- Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S 1998 Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 393:72–76
- McMinn JE, Wilkinson CW, Havel PJ, Woods SC, Schwartz MW 2000 Effect of intracerebroventricular α -MSH on food intake, adiposity, c-Fos induction, and neuropeptide expression. *Am J Physiol Regul Integr Comp Physiol* 279:R695–R703
- Rohner-Jeanrenaud F, Craft LS, Bridwell J, Suter TM, Tinsley FC, Smiley DL, Burkhardt DR, Statnick MA, Heiman ML, Ravussin E, Caro JF 2002 Chronic central infusion of cocaine- and amphetamine-regulated transcript (CART 55–102): effects on body weight homeostasis in lean and high-fat-fed obese rats. *Int J Obes Relat Metab Disord* 26:143–149
- Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriaciunas A, Mackellar W, Rostek Jr PR, Schonher B, Smith D, Tinsley FC, Zhang X, Heiman M 1995 The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 377:530–532
- Mizuno TM, Mobbs CV 1999 Hypothalamic agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. *Endocrinology* 140:814–817
- Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG 2000 Central nervous system control of food intake. *Nature* 404:661–671
- Elmquist JK, Elias CF, Saper CB 1999 From lesions to leptin: hypothalamic control of food intake and body weight. *Neuron* 22:221–232
- Lebrethon MC, Vandersmissen E, Gerard A, Parent AS, Junien JL, Bourguignon JP 2000 *In vitro* stimulation of the prepubertal rat gonadotropin-releasing hormone pulse generator by leptin and neuropeptide Y through distinct mechanisms. *Endocrinology* 141:1464–1469
- Stanley SA, Small CJ, Kim MS, Heath MM, Seal LJ, Russell SH, Gbatei MA, Bloom SR 1999 Agouti related peptide (Agrp) stimulates the hypothalamic pituitary gonadal axis *in vivo*, *in vitro* in male rats. *Endocrinology* 140:5459–5462
- Ohtaki T, Kumano S, Ishibashi Y, Ogi K, Matsui H, Harada M, Kitada C, Kurokawa T, Onda H, Fujino M 1999 Isolation and cDNA cloning of a novel galanin-like peptide (GALP) from porcine hypothalamus. *J Biol Chem* 274:37041–37045
- Jureus A, Cunningham MJ, Li D, Johnson LL, Krasnow SM, Teklemichael DN, Clifton DK, Steiner RA 2001 Distribution and regulation of galanin-like peptide (GALP) in the hypothalamus of the mouse. *Endocrinology* 142:5140–5144
- Cunningham MJ, Scarlett JM, Steiner RA 2002 Cloning and distribution of galanin-like peptide mRNA in the hypothalamus and pituitary of the macaque. *Endocrinology* 143:755–763
- Bedecs K, Berthold M, Bartfai T 1995 Galanin—10 years with a neuroendocrine peptide. *Int J Biochem Cell Biol* 27:337–349
- Jureus A, Cunningham MJ, McClain ME, Clifton DK, Steiner RA 2000 Galanin-like peptide (GALP) is a target for regulation by leptin in the hypothalamus of the rat. *Endocrinology* 141:2703–2706
- Larm JA, Gundlach AL 2000 Galanin-like peptide (GALP) mRNA expression is restricted to arcuate nucleus of hypothalamus in adult male rat brain. *Neuroendocrinology* 72:67–71
- Kerr NC, Holmes FE, Wynick D 2000 Galanin-like peptide (GALP) is expressed in rat hypothalamus and pituitary, but not in DRG. *Neuroreport* 11:3909–3913
- Takatsu Y, Matsumoto H, Ohtaki T, Kumano S, Kitada C, Onda H, Nishimura O, Fujino M 2001 Distribution of galanin-like peptide in the rat brain. *Endocrinology* 142:1626–1634
- Hohmann JG, Teal TH, Clifton DK, Davis J, Hruby VJ, Han G, Steiner RA 2000 Differential role of melanocortins in mediating leptin's central effects on feeding and reproduction. *Am J Physiol Regul Integr Comp Physiol* 278:R50–R59
- Paxinos G, Watson C 1986 The rat brain in stereotaxic coordinates. 2nd ed. San Diego: Academic Press, Inc.
- Bernstein IL 1999 Taste aversion learning: a contemporary perspective. *Nutrition* 15:229–234
- Crawley JN 1999 The role of galanin in feeding behavior. *Neuropeptides* 33:369–375
- Matsumoto Y, Watanabe T, Adachi Y, Itoh T, Ohtaki T, Onda H, Kurokawa T, Nishimura O, Fujino M 2002 Galanin-like peptide stimulates food intake in the rat. *Neurosci Lett* 322:67–69
- Matsumoto H, Noguchi J, Takatsu Y, Horikoshi Y, Kumano S, Ohtaki T, Kitada C, Itoh T, Onda H, Nishimura O, Fujino M 2001 Stimulation effect of galanin-like peptide (GALP) on luteinizing hormone-releasing hormone-

- mediated luteinizing hormone (LH) secretion in male rats. *Endocrinology* 142:3693–3696
31. **Martin RL, Perez E, He YJ, Dawson Jr R, Millard WJ** 2000 Leptin resistance is associated with hypothalamic leptin receptor mRNA and protein down-regulation. *Metabolism* 49:1479–1484
 32. **Lawrence CB, Baudoin FM, Luckman SM** 2002 Centrally administered galanin-like peptide modifies food intake in the rat: a comparison with galanin. *J Neuroendocrinol* 14:853–860
 33. **Kyrkouli SE, Stanley BG, Seirafi RD, Leibowitz SF** 1990 Stimulation of feeding by galanin: anatomical localization and behavioral specificity of this peptide's effects in the brain. *Peptides* 11:995–1001
 34. **Willie JT, Chemelli RM, Sinton CM, Yanagisawa M** 2001 To eat or to sleep? Orexin in the regulation of feeding and wakefulness. *Annu Rev Neurosci* 24:429–458
 35. **O'Donnell D, Ahmad S, Wahlestedt C, Walker P** 1999 Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: distinct distribution from GALR1. *J Comp Neurol* 409:469–481
 36. **Aja S, Sahandy S, Ladenheim EE, Schwartz GJ, Moran TH** 2001 Intracerebroventricular CART peptide reduces food intake and alters motor behavior at a hindbrain site. *Am J Physiol Regul Integr Comp Physiol* 281:R1862–R1867
 37. **Vrang N, Tang-Christensen M, Larsen PJ, Kristensen P** 1999 Recombinant CART peptide induces c-Fos expression in central areas involved in control of feeding behaviour. *Brain Res* 818:499–509
 38. **Grant VL** 1987 Do conditioned taste aversions result from activation of emetic mechanisms? *Psychopharmacology* 93:405–415
 39. **Hunt T, Amit Z** 1987 Conditioned taste aversion induced by self-administered drugs: paradox revisited. *Neurosci Biobehav Rev* 11:107–130
 40. **Woods SC, Figlewicz DP, Madden L, Porte Jr D, Sipols AJ, Seeley RJ** 1998 NPY and food intake: discrepancies in the model. *Regul Pept* 75–76:403–408