Galanin-Like Peptide: A Molecular Link Between Energy Homeostasis and Reproduction

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Abstract

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Galanin-like peptide (GALP) shares partial sequence identity with galanin and exhibits agonistic activity at galanin receptors (GALR1 and GALR2) *in vitro*. GALP neurons almost exclusively reside within the arcuate nucleus, a region of the hypothalamus that plays a pivotal role in the control of body weight and reproduction. GALP gene expression is highly induced by leptin, which implicates GALP neurons as potential downstream effectors of leptin's actions within the brain. To determine whether GALP mimics leptin's suppressive effect on body weight and/or its stimulatory effect on gonadotropin secretion, I administered intracerebroventricular (ICV) GALP injections to wild-type and mutant mice. I observed that ICV GALP elicits rapid but transient reductions in feeding and body weight in wild-type mice. However, acute ICV GALP treatment also reduces locomotor activity and elicits the formation of a conditioned taste aversion, which raises the possibility that GALP's anorectic effect does not reflect a homeostatic regulation of energy balance. I also observed that ICV GALP

treatment robustly stimulates luteinizing hormone (LH) and testosterone secretion in wild-type mice. In contrast to GALP's transitory effect on body weight in wild-type mice, leptin-deficient *ob/ob* mice exhibit a sustained reduction in body weight during chronic GALP treatment as a consequence of reduced feeding and increased thermogenesis. Although male *ob/ob* mice release LH in response to acute GALP treatment, long-term GALP administration does not significantly improve reproductive function in these infertile animals. Finally, I investigated whether GALP's actions in the brain are mediated by galanin receptor signaling. I found that GALR1 knockout and GALR2 knockout mice exhibit normal responses to ICV GALP treatment with respect to feeding, body weight, and LH secretion. Furthermore, a truncated GALP fragment containing the galanin-homologous sequence of the GALP molecule does not mimic the effects of full-length GALP on any of these parameters in wild-type mice. Collectively, these observations implicate GALP neurons as likely components of the neural circuitry that regulates and integrates energy homeostasis and reproduction. These findings do not support the hypothesis that GALP signals solely through galanin receptors *in vivo*, and instead hint at the existence of a yet-to-be-identified GALP-specific receptor.

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GLOSSARY

α-MSH .....alpha-Melanocyte-stimulating hormone

aCSF .....Artificial cerebrospinal fluid

AgRP .....Agouti-related protein

ANOVA..... Analysis of variance

Arc.....Arcuate nucleus

BAT .....Brown adipose tissue

BNST..... Bed nucleus of the stria terminalis

BSA .....Bovine serum albumin

cAMP .....Cyclic 3',5'-adenosine monophosphate

CART .....Cocaine- and amphetamine-regulated transcript

cDNA .....Complementary deoxyribonucleic acid

CNS .....Central nervous system

CTA .....Conditioned taste aversion

CV .....Coefficient of variation

DEPC..... Diethyl pyrocarbonate

DMN..... Dorsomedial nucleus

EB..... Estradiol benzoate

EDTA .....Ethylenediaminetetraacetic acid

FSH .....Follicle-stimulating hormone

GALP ..... Galanin-like peptide

GALR1 ..... Galanin receptor subtype 1

GALR1 KO..... Galanin receptor subtype 1 knockout

GALR2 ..... Galanin receptor subtype 2

GALR2 KO..... Galanin receptor subtype 2 knockout

GALR3 ..... Galanin receptor subtype 3

GLP-1 ..... Glucagon-like peptide-1

GnRH ..... Gonadotropin-releasing hormone

h..... hour

HPG ..... Hypothalamic-pituitary-gonadal

ICV ..... Intracerebroventricular

im ..... intramuscular

ip..... intraperitoneal

LH ..... Luteinizing hormone

MAPK ..... Mitogen-activated protein kinase

min..... minute

MPOA ..... Medial preoptic area

mRNA ..... Messenger ribonucleic acid

NPY ..... Neuropeptide Y

Ob-R ..... Leptin receptor

Ob-Rb ..... Long form of the leptin receptor

OVX ..... Ovariectomized

PeN ..... Periventricular nucleus

POA ..... Preoptic area

POMC ..... Proopiomelanocortin

PVN ..... Paraventricular nucleus

RIA ..... Radioimmunoassay

RNA ..... Ribonucleic acid

SEM ..... Standard error of the mean

SNS ..... Sympathetic nervous system

SPR ..... Saccharin preference ratio

SSC ..... Sodium saline citrate

TEA ..... Triethanolamine

tRNA ..... Transfer ribonucleic acid

UCP-1 ..... Uncoupling protein-1

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DEDICATION

To my father.

Chapter I:

Introduction

A. The Central Regulation of Energy Homeostasis and Reproduction

Organisms maintain energy homeostasis through the coordinated regulation of energy intake, utilization, and storage. To this end, mammals possess physiological control systems that defend them against dramatic fluctuations in energy availability. The central nervous system (CNS) is a critical component of these regulatory feedback circuits. In particular, the hypothalamus plays an important role in regulating fuel availability by adjusting food intake to meet the body's energetic requirements, and conversely, by modulating energy expenditure to compensate for alterations in food intake. Provided that these central regulatory mechanisms are intact and food is readily available, mammals match energy intake and energy expenditure over the long-term with remarkable precision, thereby maintaining energy balance as well as a relatively constant quantity of energy reserves.

The hypothalamus also plays a pivotal role in both the initiation and maintenance of reproductive activity, for it comprises the upstream element of the hypothalamic-pituitary-gonadal (HPG) axis. Neurons that express gonadotropin-releasing hormone (GnRH) are scattered throughout the hypothalamus and preoptic area (POA) [233]. Hypophysiotropic GnRH neurons project to the median eminence, where GnRH is secreted into the pituitary portal circulation in a pulsatile manner [174]. GnRH binds to GnRH receptors on gonadotropes in the adenohypophysis, eliciting the release of the

gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the circulation. The gonadotropins influence gonadal activity by promoting spermatogenesis, ovulation, and the synthesis of sex steroids.

B. Energetics and Reproduction

Reproduction is an energetically costly endeavor. The metabolic requirements for reproduction differ not only between mammalian species, but also between the sexes. Relatively speaking, spermatogenesis requires little energy. Except for the rare instances in which males provide considerable parental care to their offspring, a male's energetic investment in reproduction usually ends after insemination. Gametogenesis has very different implications for female mammals. Although ovulation *per se* does not require much energy, once a female ovulates, she incurs the risk of becoming pregnant. If this occurs, she will then be faced with the enormous energetic demands of gestation, lactation, and nurturing her offspring until they achieve independence. Although their energetic requirements differ, mammals of both sexes must achieve and maintain a sufficient nutritional plane to successfully meet the metabolic demands of reproduction. Consequently, an individual's reproductive capacity is gated by its prevailing metabolic status.

The shared goal of all reproductive strategies is to maximize reproductive success, which mammals achieve in a variety of ways. One common strategy is to only reproduce when environmental conditions are conducive for bearing and nurturing offspring (and conversely, to preclude reproductive efforts when environmental

conditions are unfavorable). In an extensive overview of mammalian reproductive biology, Frank Bronson wrote, "Of the many environmental factors that can influence a mammal's reproduction, food availability must be accorded the most important role" [33]. If food availability is diminished to the point at which an animal simply cannot consume enough calories to meet all of its body's competing energetic demands, energy is preferentially diverted to those physiological processes that are essential for immediate survival, including cellular maintenance, thermoregulation, and the locomotor activity required to forage for food and flee from predators [33]. This re-partitioning of metabolic fuels often occurs at the expense of those processes that are not obligatory for survival, including reproduction. Sacrificing reproductive efforts in favor of more pressing energy-consuming processes is an adaptive response, for it would be disadvantageous for an animal to jeopardize its own survival (and thus its reproductive potential) by expending large amounts of precious energy on producing and providing for offspring that are themselves unlikely to survive if food is in short supply.

If a mammal is unable to consume and assimilate enough calories to meet its body's energetic requirements, a state of negative energy balance ensues (i.e., energy expenditure exceeds energy intake). If severe and/or prolonged in duration, negative energy balance can have deleterious effects upon the HPG axis. Chronic undernutrition delays the onset of puberty in many species, including rats [31, 126], mice [101], and sheep [84]. In adult mammals, food deprivation or chronic food restriction commonly results in reduced serum gonadotropin and sex steroid concentrations, disrupted spermatogenesis, anovulation, loss of estrous cyclicity, and impaired sexual behaviors

[1, 20, 30, 110, 182]. Humans are also susceptible to reproductive impairment in response to inadequate nutrition. Acute fasting alters the secretory levels and patterns of LH and/or gonadal steroids in men [38, 131, 205] and women [193]. Chronically undernourished women with anorexia nervosa often experience primary or secondary amenorrhea and display prepubertal gonadotropin secretory profiles as adults [25]. Regardless of the reproductive stage at which caloric restriction is imposed in humans and experimental animals, the adverse effects of undernutrition on sexual maturation and adult reproductive function are usually rapidly reversed when caloric intake increases, sometimes even within minutes to hours of refeeding [32, 37, 39, 85, 110, 234].

Mammals also experience negative energy balance when their energetic requirements are substantially augmented in the absence of a proportionate increase in caloric intake. Reproductive disturbances are often evident under these circumstances. Laboratory animals that expend large amounts of energy on physical activity or maintaining body temperature in a cold environment commonly experience impaired reproductive function, but only if they are not permitted to compensate for their increased energetic demands by increasing their food intake [70, 156, 157, 198, 201, 279]. Reproductive function is generally preserved or restored in these animals if they are allowed to consume more food while they are subjected to increased energetic demands [70, 157, 201, 280]. Highly trained female athletes (particularly those individuals who participate in sports that emphasize leanness for performance and/or aesthetic purposes, such as gymnasts, ballet dancers, and distance runners) experience amenorrhea and other forms of menstrual dysfunction with greater frequency than

sedentary women [90, 270]. If training is temporarily interrupted due to injury or vacation, menses often rapidly commences or resumes in these athletes [66, 269].

Although severe metabolic challenges can disrupt reproductive function at all three levels of the HPG axis, the brain has often been implicated as the primary locus of impaired reproductive function in response to negative energy balance. Under many circumstances in which energy expenditure is increased and/or caloric intake is inadequate, LH pulse frequency is significantly reduced [36-38, 61, 115, 158, 254], but LH release in response to exogenous GnRH treatment is normal or even enhanced [37, 39, 85, 206, 260, 277]. Together, these observations implicate a reduced hypothalamic drive to the pituitary as the primary cause of reproductive impairment. Observations that exogenous GnRH treatment can reverse the adverse effects of food restriction or increased physical activity on reproductive function are consistent with the argument that endogenous GnRH secretion is diminished under these circumstances [3, 16, 31, 69, 129, 158, 160, 189]. This was recently confirmed to be the case in undernourished sheep, as evidenced by a reduction in GnRH pulse frequency and amplitude in food-restricted lambs [113]. Collectively, these observations suggest that impaired reproductive function in response to diminished energy availability is often attributable to a primary central defect, either at or above the level of the GnRH neuron.

To effectively synchronize reproductive efforts with states of energy sufficiency (and conversely, to prevent reproductive activity when energy availability is inadequate), the neural circuits governing GnRH secretion must be provided with information about peripheral energy availability. For this to occur, the brain must be receptive to a

chemical or neural signal(s) from the periphery that reflects the animal's metabolic status. According to the "adipostat" hypothesis, the brain receives information about peripheral energy availability by way of a chemical signal that carries information about the quantity of an animal's adipose reserves; the brain in turn modulates food intake and energy expenditure to maintain a relatively constant mass of body fat [125, 164]. Conceivably, the same chemical signal that conveys metabolic information to the neural circuits controlling feeding and metabolism could also impinge upon the neuroendocrine reproductive axis. The most direct means by which the brain could be informed about the adequacy of an animal's fat reserves would be via a signal originating within adipose tissue itself. For more than 40 years, scientists had speculated about the existence of a fat-derived chemical signal that communicates nutritional information to the CNS [125], but their suppositions suffered for lack of any plausible candidate molecule to fulfill such a function until the discovery of leptin in 1994.

C. Leptin

Leptin is the protein product of the *ob* gene [287]. Leptin is primarily produced by adipocytes, where its synthesis and secretion are correlated with adipose mass and body mass index [59, 155, 287]. In rodents and humans, circulating leptin concentrations are reduced when body fat stores are diminished by fasting or chronic food restriction, but are restored in undernourished individuals by refeeding and subsequent weight recovery [155, 273]. Plasma leptin levels are also reduced in extremely lean humans, including women with anorexia nervosa and highly trained endurance athletes [94, 144,

255, 274]. Conversely, plasma leptin concentrations are typically elevated in obese individuals due to their excess adiposity [59, 155]. Because adipose tissue is the body's primary site for energy storage, circulating leptin concentrations are usually an accurate reflection of the size of a mammal's energy reserves.

Leptin's actions are mediated by leptin receptors (Ob-R), of which there are at least six isoforms [147, 251, 263]. Ob-Rb is believed to be the primary signaling isoform, because it is the only one that possesses a long intracellular tail containing tyrosine residues that are requisite for activating the full complement of leptin's intracellular signaling pathways, which include Janus kinase/ signal transducer and activator of transcription and phosphatidylinositol-3 kinase signaling pathways [4, 19, 185]. Ob-Rb mRNA and protein are expressed in regions of the brain that control body weight and/or reproductive function, including several hypothalamic nuclei [73, 80, 98, 173, 224]. Although leptin can cross the blood-brain barrier via a saturable transport system [5], the presence of Ob-R immunoreactivity in several of the circumventricular organs reveals an alternate route by which leptin can gain access to the CNS [167].

Leptin influences several determinants of energy balance, including feeding, thermogenesis, glucose and lipid metabolism, and physical activity [89, 259]. Exogenous leptin treatment reduces body weight and adiposity by suppressing food consumption and enhancing energy expenditure [40, 99, 197, 272]. The fact that intracerebroventricular (ICV) injections of leptin also modulate feeding and energy expenditure— typically at lower doses than are required when leptin is administered peripherally— implicates a central locus for leptin's weight-reducing actions [40, 242,

258]. Collectively, these observations suggest that leptin serves as the afferent limb of a negative feedback circuit whose function is to defend organisms against dramatic fluctuations in the quantity of their energy reserves. When adipose mass increases, the attendant increase in circulating leptin concentrations signals the brain to reduce food intake and increase energy expenditure, which has the net effect of reducing body fat mass back toward normal. Conversely, if body fat begins to be depleted and leptin levels fall, food consumption increases and energy expenditure is reduced as part of a coordinated effort to replenish fuel reserves.

In addition to its effects on energy intake and energy expenditure, leptin also plays an important role in modulating reproductive function. Ob-R mRNA and protein are expressed at all three levels of the HPG axis [41, 98, 208, 240, 286]. Leptin stimulates the secretion of GnRH and the gonadotropins *in vitro* [191, 285] and *in vivo* [262, 271, 285]. Pretreatment with GnRH antiserum blocks leptin's stimulatory effect on LH secretion in prepubertal female rats, which suggests that leptin stimulates LH release via a GnRH-dependent mechanism [67]. Neither Ob-R mRNA nor Ob-R immunoreactivity have been detected on GnRH neurons, which underscores the important role of leptin-sensitive interneurons in relaying leptin's stimulatory signal to GnRH neurons [81, 98].

Although the effects of exogenous leptin treatment on HPG function are often subtle in well-fed animals, there have been many accounts of leptin reversing the adverse effects of acute or chronic undernutrition on reproductive function. Peripheral or central leptin injections prevent or attenuate the fasting-induced decline in LH secretion

in rats [123, 187], mice [1], sheep [188], macaques [81], and humans [43]. In female mice and hamsters, leptin treatment reverses the inhibitory effects of fasting on estrous cyclicity, ovulation, and sexual receptivity [1, 220]. Central or peripheral leptin administration also prevents a delay in the timing of puberty in moderately food-restricted female rats and mice [48, 50, 95]. Observations that the central infusion of leptin antiserum suppresses pulsatile LH secretion, disrupts estrous cyclicity, and abolishes the estrogen- and progesterone-induced LH surge in female rats implicate a role for endogenous leptin signaling in the neuroendocrine control of reproductive function in non-energetically challenged animals [42, 132].

For several decades prior to the discovery of leptin in 1994, much was already known about the metabolic and reproductive consequences of leptin deficiency, thanks to a strain of obese mice known as *ob/ob* mice [116]. *Ob/ob* mice possess a nonsense mutation in the leptin gene, which results in the production of a truncated, non-functional protein [287]. *Ob/ob* mice are obese, hyperphagic, hypometabolic, and profoundly diabetic [29, 54, 116]. Acute or chronic leptin treatment (peripheral or ICV) reduces body weight in *ob/ob* mice by suppressing food intake and enhancing thermogenesis, metabolism, and locomotor activity [40, 99, 197, 272]. Other genetic models of impaired leptin signaling include *db/db* mice and *fa/fa* rats, both of which have markedly elevated plasma leptin concentrations but are resistant to leptin's actions due to inactivating mutations in the Ob-R gene [46, 51, 147, 199]. *db/db* mice and *fa/fa* rats exhibit metabolic perturbations that are similar to those of *ob/ob* mice [28, 54, 111, 288]. The loss of central leptin signaling appears to be particularly relevant to the

development of obesity in these rodents, because transgenic mice bearing a neuron-specific disruption of Ob-R expression exhibit increased adiposity in inverse correlation with the amount of residual Ob-R expression in the hypothalamus [53]. Conversely, *db/db* mice that are transgenically modified to express functional Ob-R only in neurons are less obese than non-transgenic *db/db* mice [134].

In addition to their pronounced metabolic abnormalities, both sexes of *ob/ob* mice exhibit hypogonadotropic hypogonadism and are infertile [11, 244]. Leptin replacement for two weeks improves reproductive function in male and female *ob/ob* mice, as reflected by increases in serum gonadotropin concentrations, reproductive organ weights, and histological markers of gametogenesis and steroidogenesis [6]. More prolonged leptin treatment regimens (thirty or sixty days) are sufficient to restore fertility in both male and female *ob/ob* mice [45, 184]. *Ob/ob* mice that are pair-fed to leptin-treated *ob/ob* mice do not show signs of improved reproductive capacity, which indicates that leptin's restorative effect on reproductive function in *ob/ob* mice is not attributable to weight loss or the reduction in food intake in response to leptin treatment [6, 45, 184]. Leptin-resistant *db/db* mice and *fa/fa* rats also show signs of pronounced reproductive impairment [111, 119, 212, 288]. A critical role for central leptin signaling in the control of reproduction can be inferred from the observation that fertility is restored in male (but not female) *db/db* mice by the neuron-specific transgenic restoration of Ob-R expression [134].

Over the past decade, a handful of morbidly obese humans with leptin or Ob-R gene mutations have been identified [52, 181, 243]. In addition to their pronounced

hunger and adiposity, the individuals who were of reproductive age exhibited hypogonadotropic hypogonadism and had not spontaneously advanced through puberty [52, 243]. In the leptin-deficient patients, daily injections of leptin for 12-18 months resulted in rapid and sustained reductions in body weight [78, 150]. In two separate case studies, Licinio et al. and Farooqi et al. documented the outcomes of chronic leptin treatment on HPG function in leptin-deficient individuals. Eighteen months of leptin treatment reversed the hypogonadism and initiated the onset of puberty in a 27-year old man, as well as induced regular ovulatory cycles in a 40-year old woman [150]. Although one of the leptin-deficient female patients was of prepubertal age when leptin replacement commenced, she exhibited nocturnal patterns of gonadotropin secretion consistent with an early stage of puberty after one year of daily leptin injections [78].

Many of leptin's effects on body weight and reproductive function have been attributed to central leptin signaling [74]. Identifying the downstream targets of leptin's actions within the brain has been a major research focus of those working in the fields of body weight regulation and reproduction. Specifically, many investigators have examined the involvement of various populations of neurons in the hypothalamic arcuate nucleus (Arc) in transmitting leptin's signal to the effector circuits controlling feeding, metabolism, and pulsatile gonadotropin secretion. Because the ventral portion of the Arc functionally resides outside the blood-brain barrier due to its proximity to the adjacent median eminence, neurons in the Arc are anatomically poised to detect chemical signals from the periphery [58, 167]. Ob-Rb mRNA and protein are highly concentrated in the Arc and have been localized to many populations of neuropeptidergic neurons within

this nucleus, including those that express neuropeptide Y (NPY)/ agouti-related protein (AgRP) or proopiomelanocortin (POMC)/ cocaine- and amphetamine-regulated transcript (CART) [47, 72, 98, 172, 282].

NPY and AgRP are orexigenic/anabolic neuropeptides, and thus increase food consumption, reduce energy expenditure, and increase body weight when administered centrally [183]. α-MSH (a cleavage product of the POMC precursor gene) and CART are anorectic/catabolic peptides which reduce feeding, increase energy expenditure, and suppress body weight [213]. Of the four neuropeptides, only NPY has been accorded an important role in the regulation of GnRH and gonadotropin secretion [122]. NPY/AgRP and POMC/CART neurons are transcriptionally regulated by leptin, with leptin increasing the expression of POMC and CART mRNAs, and reducing the expression of NPY and AgRP transcripts [138, 180, 223, 242, 256]. The opposite trends in neuropeptide expression levels are observed in animals with reduced leptin concentrations (e.g., fasted rodents) [138, 180, 221, 225] and in leptin-deficient *ob/ob* mice [138, 232, 256, 278]. NPY and POMC-derived peptides have been demonstrated to play important roles in mediating the effects of leptin or leptin deficiency on energy balance and/or reproductive function [75, 76, 106, 202, 214, 226].

Of all the neuropeptidergic neurons within the Arc, NPY/AgRP and POMC/CART neurons have thus far received the most attention from those who study body weight regulation and/or the neuroendocrine control of reproduction. However, NPY/AgRP and POMC/CART neurons are not the only cell populations in the Arc that are regulated by leptin or that have been implicated in mediating leptin's effects on

energy homeostasis and reproduction. Of particular interest to our laboratory has been one of the lesser-studied neuropeptides in the Arc, galanin-like peptide (GALP). Before beginning to build an argument in favor of a role for GALP neurons in mediating leptin's actions within the CNS, it is first important and relevant to briefly describe the physiology and pharmacology of yet another neuropeptide that is expressed in the Arc, galanin.

D. Galanin and Galanin Receptors

Galanin was isolated from the porcine small intestine by Viktor Mutt's laboratory at the Karolinska Institute during a chemical screen for C-terminally amidated peptides [252]. Since its discovery over twenty years ago, galanin mRNAs have been cloned from over a dozen species [13]. In almost all species in which it has been studied to date, galanin is comprised of 29 amino acids and contains an amidated C-terminus [13]. Human and macaque galanin are 30 amino acids in length and contain an extra serine residue at the C-terminus that is non-amidated [18, 62, 77, 219]. Since its discovery over two decades ago, a broad range of physiological and pathophysiological functions have been attributed to galanin, including ones related to gut secretion and contractility, cognitive function, nociception, nerve regeneration, growth, feeding, sexual behavior, and neuroendocrine function [8, 27].

Galanin is unusual among neuropeptides in that its biological activity is conferred by its N-terminus, rather than by the C-terminal portion of the molecule as is the case with most other neuropeptides [7]. The main pharmacophores of galanin that are

important for its biological activity are Gly^1, Trp^2, Asn^5, and Tyr^9 [140]. Galanin(1-16) is a full agonist at galanin receptors *in vitro* and is capable of mimicking many of galanin's effects when injected peripherally or centrally [8]. Shorter truncated N-terminal galanin fragments [e.g., galanin(1-15), galanin(1-14), galanin(1-13), and galanin(1-12)] also bind to galanin receptors with submicromolar affinity and recapitulate some of galanin's actions *in vitro* and *in vivo* [8, 140]. Although their half-lives and receptor binding affinities are significantly reduced compared to full-length galanin, galanin(1-9) and galanin(1-10) retain some biological activity in certain *in vitro* and *in vivo* systems [8]. The fact that the first fifteen amino acids of galanin are almost 100% conserved between all species examined to date further underscores the functional significance of the N-terminal amino acid residues for galanin's biological activity [8].

Galanin is expressed in the brain, spinal cord, pituitary, gastrointestinal tract, adrenal medulla, and sympathetic nerves [12, 71, 124, 235, 261]. Within the CNS, galanin mRNA and peptide are most densely concentrated within the hypothalamus and brainstem, but are also expressed in the olfactory bulb, neocortex, hippocampus, amygdala, thalamus, cerebellum, sensory dorsal root ganglion neurons, and spinal interneurons [24, 49, 168, 204, 235]. Galanin is coexpressed with many classical and peptide neurotransmitters in the brain and is believed to play a neuromodulatory role in regulating their presynaptic and postsynaptic actions [7, 8].

In general, the distribution pattern of galanin-immunoreactive nerve terminals coincides well with [^{125}I] galanin binding sites in the rat brain [170, 236]. To date, three galanin receptor subtypes have been cloned and characterized in the rat, mouse, and

human: GALR1 [35, 97, 195, 266], GALR2 [23, 79, 109, 194, 238, 264] and GALR3 [239, 265]. All three receptor subtypes are expressed within the CNS, albeit with differing abundance and only partially overlapping distribution patterns. GALR1 mRNA is widely expressed throughout the brain, including the hypothalamus, thalamus, amygdala, hippocampus, and brainstem [35, 96, 107, 176, 195]. The distribution of GALR1 immunoreactivity in the rat brain has also been mapped, and for the most part corresponds with the expression pattern of GALR1 mRNA [34, 143]. GALR2 mRNA is expressed in most major regions of the rat CNS, but its distribution is more restricted than that of GALR1 [79, 177, 190]. GALR3 mRNA is expressed in discrete regions of the brain, primarily within the hypothalamus, preoptic area, and brainstem [171, 239]. GALR3 mRNA is expressed at much lower levels in the rat CNS than either GALR1 or GALR2 transcripts [171].

In addition to their differing distribution patterns, the three cloned galanin receptor subtypes possess distinct pharmacological profiles and are coupled to different intracellular signaling pathways [27]. Although all three receptor subtypes bind galanin and several N-terminal galanin fragments with high affinity, the receptor subtypes vary in the rank order of their affinities for full-length galanin, truncated galanin fragments, and other galanin analogues [27]. Galanin receptors are G-protein coupled receptors that share approximately 40% sequence homology [27]. GALR1 is coupled to $G_{i/o}$, and thus inhibits adenylyl cyclase activity and reduces intracellular cyclic 3',5'-adenosine monophosphate (cAMP) concentrations when bound by ligand [97, 195, 267]. GALR1 receptor activation has also been reported to enhance mitogen-activated protein kinase

(MAPK) activity and to hyperpolarize cells by opening inwardly rectifying K^+ channels [239, 267]. GALR2 is primarily coupled to $G_{q/11}$-type G proteins. GALR2 activation results in pertussis toxin-insensitive inositol phosphate hydrolysis, Ca^{+2} mobilization, and Ca^{+2}-dependent Cl^- channel activation [238]. In some *in vitro* assays, GALR2 activation reduces intracellular cAMP accumulation and stimulates MAPK activity in a pertussis toxin-sensitive manner, which is consistent with a coupling to $G_{i/o}$-type G proteins [267]. More similar to GALR1 than to GALR2 with respect to its intracellular signaling repertoire, GALR3 is coupled to $G_{i/o}$ and signals by inhibiting adenylyl cyclase and opening inwardly rectifying K^+ channels [133, 239]. Because of the differential signaling capabilities of the three galanin receptor subtypes, it is plausible that galanin's inhibitory effects on neuronal function result from its interactions with GALR1 and/or GALR3, while its stimulatory effects on neuronal activity arise from GALR2 activation.

Like many other neuropeptides that are expressed within the Arc, galanin has been implicated in the central regulation of feeding behavior and energy metabolism. Galanin rapidly but transiently stimulates food consumption in satiated animals when it is injected into the cerebral ventricles or into discrete regions of the brain [60]. The orexigenic effect of galanin is most potent when it is administered directly into the hypothalamic paraventricular nucleus (PVN), but galanin also increases food intake following injection into the Arc, dorsomedial nucleus (DMN), ventromedial nucleus, amygdala, and the area postrema/nucleus of the solitary tract [60]. Repeated galanin injections increase daytime feeding in rats, but long-term galanin treatment does not affect 24 h food intake or body weight gain over the duration of the treatment period

[237]. Centrally-administered galanin decreases the firing rate of sympathetic nerves innervating brown adipose tissue (BAT) in the rat, which implicates a role for galanin in the central control of thermogenesis [186].

Galanin influences reproductive function at both the hormonal and behavioral levels. Galanin is expressed by a subset of GnRH neurons and is co-secreted with GnRH into the pituitary portal circulation in a pulsatile manner [153, 175]. A small population of GnRH neurons in the rostral POA expresses GALR1 mRNA [178], and GALR2 mRNA is expressed by a subset of gonadotropes in the anterior pituitary [68]. Thus, galanin has the potential to modulate LH secretion through both hypothalamic and pituitary mechanisms. Indeed, galanin stimulates GnRH secretion from Arc-median eminence fragments harvested from intact male or steroid-primed ovariectomized (OVX) female rats [152, 210]. *In vivo*, galanin potentiates GnRH-induced LH secretion in intact male and estrogen-treated OVX female rats, but inhibits GnRH-induced LH release in the absence of gonadal steroids [218, 241]. Although galanin stimulates basal LH secretion from dispersed anterior pituitary cells [153], galanin does not alter LH secretion when it is injected into the cerebral ventricles of male rats [162, 169]. In contrast, ICV galanin administration does elicit LH secretion in estrogen- and progesterone-treated OVX female rats [209]. Furthermore, pre-treatment with galanin antiserum or a galanin receptor antagonist markedly blunts the proestrous LH surge in cycling female rats and the ovarian steroid-induced LH surge in OVX female rats, which implicates an important role for central galanin signaling in the neural events that trigger ovulation [154, 210].

Galanin's effects on sexual behavior are somewhat controversial and appear to be dependent upon the site of injection, as galanin inhibits sexual behavior in male rats when it is injected into the lateral cerebral ventricle, but facilitates copulatory behavior in both male and female rats when it is injected directly into the medial preoptic nucleus [14, 21, 22, 200]. ICV injection of the galanin receptor antagonist galantide facilitates sexual behavior in male rats, but ICV injection of the galanin receptor antagonist M40 prolongs the duration of copulation in sexually sluggish male mice [14, 15].

In contrast to the pronounced effects of caloric restriction on the expression levels of other neuropeptides in the Arc that influence feeding and/or reproductive function, galanin mRNA expression in the hypothalamus is unaltered by food deprivation or chronic food restriction [26, 166, 222]. Evidence in the literature in support of regulation of galanin mRNA in the Arc by leptin has also been relatively scant. ICV leptin treatment for 3 days reduces galanin mRNA levels in the mediobasal hypothalamus of *ad libitum* fed rats, an effect that is not reproduced in pair-fed animals [211]. Compared to wild-type mice, *ob/ob* mice have reduced expression levels of galanin mRNA in the hypothalamic periventricular nucleus (PeN). However, exogenous leptin treatment restores the expression of galanin mRNA in the PeN of *ob/ob* mice to wild-type levels [49]. In contrast, galanin mRNA levels in the Arc and DMN are unaffected by leptin deficiency or leptin replacement [49]. Although a small number of galanin neurons in the rat Arc are immunoreactive for Ob-R [98], Ob-Rb mRNA is not detectable in galanin neurons in the mouse hypothalamus [49]. Collectively, these observations suggest that galanin neurons in the hypothalamus (especially in the Arc) are

unlikely to play a major role in mediating leptin's effects on energy balance or neuroendocrine reproductive function.

Until recently, galanin was believed to be unrelated to any known family of neuropeptides. However, several lines of evidence had hinted at the existence of one or more unidentified galanin-like peptides. For example, galanin-like immunoreactivity exhibits molecular heterogeneity within the rat brain and ileum, in that galanin antibodies have been shown to cross-react with unknown peptides possessing chromatographic profiles that are distinct from that of galanin [207]. Furthermore, Wang et al. demonstrated the existence of galanin-like immunoreactivity in rat pancreatic islets that could be detected with an antibody against porcine galanin but not with an antibody against rat galanin [268]. Finally, many chimeric peptides that are full agonists at each of the three cloned galanin receptors *in vitro* function as antagonists *in vivo* [7, 82, 238, 239]. One possible explanation for this discrepancy is that the chimeric peptides interact with an unidentified receptor *in vivo*, one whose endogenous ligand is structurally similar to galanin. Despite these tantalizing hints about the existence of one or more galanin-like peptide(s), it was not until late 1999 that the identity of one such molecule was finally revealed.

E. GALP

In 1999, a group of scientists at Takeda Chemical Industries isolated a novel galanin-like peptide from porcine hypothalamic extracts [192]. The mature peptide was 60 amino acids in length, was derived from a 120 amino acid precursor protein, and

possessed a non-amidated C-terminus. GALP was given its name based upon its considerable degree of sequence identity with galanin; specifically, amino acids 9-21 of GALP are identical to galanin(1-13). GALP cDNAs have subsequently been cloned from the pig, rat, mouse, macaque, and human [62, 121, 192]. GALP is highly conserved at both the nucleotide and amino acid levels across all species in which it has been examined. Notably, the amino acid sequence of the galanin-homologous region of the GALP molecule is 100% conserved across species. Despite their shared sequence identity, GALP and galanin are encoded by separate genes that are located on different chromosomes in humans and mice [63, 192].

Given that GALP shares sequence identity with the biologically active region of the galanin molecule, one might predict that GALP would also be capable of interacting with galanin receptors. Indeed, GALP was initially discovered based upon its ability to bind and exhibit agonistic activity at galanin receptors *in vitro* [192]. Competitive binding studies in GALR1- and GALR2-transfected cells revealed that GALP is capable of binding to both receptor subtypes. Whereas galanin binds with higher affinity to GALR1 (IC_{50}=0.097 nM) than to GALR2 (IC_{50}=0.48 nM), GALP binds with higher affinity to GALR2 (IC_{50}=0.24 nM) than to GALR1 (IC_{50}=4.3 nM). Measuring [^{35}S]GTPγS binding to membrane preparations of galanin-receptor-transfected cells demonstrated that galanin exhibits 180-fold greater potency than GALP at GALR1 (EC_{50}=0.16 nM *vs.* EC_{50}=30 nM, respectively). In contrast, galanin and GALP display similar agonistic activities at GALR2 (EC_{50}=5.2 nM *vs.* EC_{50}=2.4 nM, respectively). Although galanin interacts with GALR1 and GALR2 relatively non-discriminately, it

appears that GALP preferentially binds and activates GALR2 *in vitro*. Unfortunately, GALP's ability (or lack thereof) to bind and activate GALR3 *in vitro* was not tested in this initial report [192].

Soon after its discovery, several groups independently reported on the neuroanatomical distribution of GALP mRNA in the forebrain. Compared to galanin, GALP has a much more restricted distribution pattern in the CNS, with GALP mRNA-expressing cell bodies being confined to the Arc, median eminence, and infundibular stalk [62, 120, 121, 128, 142]. GALP mRNA is also expressed in pituicytes in the neurohypophysis [91, 230]. Although there are some subtle species differences in the distribution of GALP mRNA within the Arc between the rat, mouse, and macaque, GALP mRNA-expressing cells are located in the periventricular region and are primarily concentrated in the caudal aspect of the nucleus in all three species [62, 120, 121, 128, 142]. Using a monoclonal antibody directed against the N-terminus of the GALP molecule, Takatsu et al. confirmed the localization of GALP-containing cell bodies in the Arc and median eminence of the rat at the protein level [246]. The same group also mapped the distribution of GALP-immunoreactive fibers in the forebrain. Dense GALP fiber projections were observed in the parvicellular PVN, anterodorsal preoptic nucleus, bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), and the lateral septal nucleus. A small number of GALP-immunoreactive fibers were also seen in the PeN [246].

Despite numerous attempts to demonstrate colocalization of GALP with other neuropeptides or enzymes that are expressed in the Arc, most of these efforts have

yielded negative results. GALP neurons do not express appreciable levels of galanin, NPY, AgRP or somatostatin [64, 246, 247]. Furthermore, the distribution of GALP mRNA in the Arc does not appear to overlap the distribution patterns of growth hormone releasing hormone or tyrosine hydroxylase [142]. Although a small percentage (approximately 7%) of GALP neurons in the rat Arc are immunoreactive for α-MSH [247], it is questionable whether this finding is truly indicative of colocalization of the two neuropeptides in a select group of neurons, or whether the observed degree of coexpression was within the limits of background staining. Thus, with the possible exception of a small number of GALP neurons that might express α-MSH, GALP neurons in the Arc appear to be phenotypically distinct from other known neuronal populations in this nucleus.

The fact that many populations of neurons in the Arc (e.g., NPY/AgRP and POMC/CART neurons) are sensitive to metabolic manipulations and are regulated by leptin led members of our laboratory to investigate whether GALP neurons in the Arc might also be downstream targets for leptin. GALP mRNA levels are reduced in hypoleptinemic or leptin-resistant states. Food deprivation for 48 h induces a modest but significant reduction in the expression levels of GALP mRNA in male rats [87] and macaques [64]. The expression of GALP mRNA is dramatically increased in fasted rats that are given peripheral leptin injections during the fast [120, 139]. Rats that are rendered diabetic by streptozotocin treatment are also hypoleptinemic and have reduced expression levels of GALP mRNA in the Arc [87, 231]. In these animals, GALP mRNA is restored to non-diabetic levels by exogenous leptin treatment [87]. GALP mRNA

levels are markedly reduced in genetic models of leptin deficiency (*ob/ob* mice) or leptin resistance (*db/db* mice and *fa/fa* rats) [121, 139, 215, 231]. In *ob/ob* mice, daily ICV leptin injections restore GALP mRNA expression to wild-type levels, indicating that leptin regulates GALP gene expression via a central mechanism(s) [121]. Moreover, GALP neurons appear to be direct downstream targets for leptin, because the vast majority (>85%) of GALP neurons in the Arc express Ob-Rb mRNA [62] or Ob-R immunoreactivity [246].

GALP neurons in the Arc are responsive to metabolic challenges and to alterations in plasma or CNS leptin concentrations, which implicates GALP as a potential downstream effector of leptin's central actions on feeding and metabolism. A growing body of neuroanatomical evidence supports the argument that GALP neurons are components of the neural circuitry controlling feeding and metabolism. In addition to expressing Ob-R, a proportion of GALP neurons also express mRNAs for the NPY Y1 receptor (42%), the serotonin 5-HT_{2C} receptor (24%), and the orexin receptor-1 (10%), all of which are believed to play important roles in the hypothalamic control of body weight regulation [64, 248]. GALP neurons send fiber projections to the parvicellular PVN, a region of the hypothalamus that receives and integrates feeding-related signals from hypothalamic and extra-hypothalamic sites [246]. Finally, ICV injection of GALP in the rat induces the expression of the immediate early gene product Fos (a marker for neuronal activation [44]) in neurons that are located in feeding-related regions of the brain, including the lateral hypothalamus, DMN, and the nucleus of the solitary tract [146]. Collectively, these observations demonstrate that GALP neurons are poised not

only to receive and process humoral and neural feeding-related signals, but also to transmit these signals to regions of the brain that control feeding behavior.

In addition to the possibility that GALP neurons convey leptin's signal to the neural circuits that control feeding and energy expenditure, there is also evidence supporting the notion that GALP neurons relay leptin's stimulatory signal to the neural networks that govern gonadotropin secretion. GALP fibers project to the MPOA, where they appose GnRH-containing perikarya and dendrites. GALP fibers have also been observed in close contact with GnRH fibers in the MPOA and BNST [246]. ICV GALP administration induces Fos expression in approximately 36% of GnRH neurons in the rat MPOA, which suggests that these GnRH neurons are either directly or indirectly transcriptionally activated by GALP [162]. A possible functional role for GALP in the regulation of gonadotropin secretion was first proposed by Matsumoto et al., who demonstrated that plasma LH levels were significantly elevated in male rats between 10 and 60 min after GALP was injected into the third cerebral ventricle [162]. Moreover, GALP's stimulatory effect on LH secretion was abolished in rats that were pre-treated with a GnRH receptor antagonist, which demonstrates that GALP stimulates LH secretion via a GnRH-dependent mechanism [162].

F. Statement of the Problem

At the time that I commenced my dissertation research, very little was known about GALP's functional significance within the brain. Despite this major gap in our knowledge, our laboratory and others had already begun to build a strong case in favor

of the argument that GALP neurons in the hypothalamus are downstream targets for leptin. The expression of GALP mRNA in the Arc is reduced in hypoleptinemic states and is markedly increased by exogenous leptin treatment, which suggests that GALP neurons are transcriptionally regulated as a function of prevailing leptin concentrations [87, 121, 139]. Furthermore, GALP neurons appear to be direct targets for leptin, because the majority (>85%) of GALP neurons express Ob-R [62, 246]. In fact, the degree of Ob-R expression by GALP neurons exceeds that of many other populations of leptin-sensitive neurons within the Arc, including NPY/AgRP (50%) and POMC/CART (50-80%) neurons [10, 47]. Because leptin plays a pivotal role in the neuroendocrine control of energy balance and reproduction, I questioned whether GALP neurons participate in relaying leptin's signal to the neural circuits controlling body weight and gonadotropin secretion. Due to a lack of the appropriate genetic or pharmacological tools to address this question directly (e.g., GALP knockout mice, GALP receptor antagonists, etc.), I had to settle for an indirect approach to begin to explore this possibility. I hypothesized that if GALP neurons are downstream effectors of leptin's actions within the CNS, then central injections of GALP should mimic leptin's effects on feeding, body weight, and the secretion of reproductive hormones. Thus, the initial aim of my dissertation research was to investigate the effects of centrally-administered GALP on food intake, body weight, and serum gonadotropin and testosterone concentrations in male wild-type mice.

Upon observing that exogenously-administered GALP mimics many of leptin's effects on feeding, body weight, and gonadotropin secretion in wild-type mice, I next

sought to determine whether GALP exerts similar actions in *ob/ob* mice. Leptin typically elicits more prolonged reductions in food intake and body weight in leptin-deficient *ob/ob* mice than in leptin-replete wild-type mice [40, 99, 103, 148, 197]. I reasoned that if leptin's suppressive effects on feeding and body weight are mediated by an increase in central GALP activity, then ICV GALP treatment might also elicit differential responses in wild-type and *ob/ob* mice with respect to feeding and body weight. Therefore, I devoted the next part of my dissertation research to comparing the effects of acute GALP treatment on feeding and body weight in male wild-type and *ob/ob* mice.

Chronic leptin treatment not only elicits marked reductions in food intake and body weight in *ob/ob* mice, but also significantly improves reproductive function in these infertile animals [6, 45, 184]. I hypothesized that if exogenously-administered leptin reduces body weight and activates the HPG axis in *ob/ob* mice via GALP-dependent signaling pathways, then repeated central injections of GALP would also induce sustained weight loss and improve reproductive function in *ob/ob* mice. To test this hypothesis, I assessed whether long-term central GALP treatment would recapitulate the effects of chronic leptin treatment on feeding, body weight, and various indices of reproductive function in male *ob/ob* mice.

GALP was discovered by virtue of its capacity to bind and exhibit agonistic activity at GALR1 and GALR2 *in vitro* [192]. GALP's ability to interact with galanin receptors *in vitro* is not entirely unexpected, given that GALP shares sequence identity with the biologically active region of the galanin molecule [192]. Although these observations implicate GALP as another endogenous ligand for galanin receptors, a

growing body of evidence suggests that GALP might not signal solely through galanin receptors *in vivo*. Notably, centrally-administered galanin and GALP have many disparate effects on feeding, body weight, and LH secretion in the rat [145, 162]. Furthermore, galanin and GALP induce different patterns of neuronal activation in the rat forebrain following their ICV injection [86, 146]. These latter observations suggest that galanin and GALP utilize different signaling pathways *in vivo*, and are consistent with the existence of an unidentified GALP-specific receptor that is distinct from the cloned galanin receptor subtypes. Because it was both conceptually and technically more feasible to attempt to rule out the contribution of GALR1 and GALR2 to central GALP signaling than to evaluate the possible participation of an unknown "GALP receptor", I devoted the final part of my dissertation research to examining whether GALP's effects on feeding, body weight, and gonadotropin secretion in the mouse are mediated by galanin receptor signaling.

Chapter II:

Materials and Methods

A. Animals and Accommodations

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. Unless otherwise noted, all mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and all rats were purchased from Animal Technologies, Ltd. (previously B&K Universal; Kent, WA). I chose to use male animals in most of the experiments to avoid the potential confounding effects of the female estrous cycle on responsiveness to central GALP treatment. The animals were housed in individual cages (except in Experiments 6 and 10) in light- and temperature-controlled rooms. With the exception of Experiment 7, food and water were always available to the animals *ad libitum*. For each experiment, the animals were weight-matched prior to being divided into treatment groups.

Mice

Experiments 1-4, 7, and 8: Adult male C57BL/6 mice were maintained on a 14-h light, 10-h dark schedule with lights on at 0500 h.

Experiments 5 and 6: Adult male C57BL/6 mice were maintained on a 12-h light, 12-h dark schedule with lights on at 0700 h.

Experiments 9 and 16-18: Adult male C57BL/6 mice were maintained on a 12-h light, 12-h dark schedule with lights on at 0600 h.

Experiments 11 and 12: Adult male wild-type and/or *ob/ob* mice (both on a C57BL/6 background) were maintained on a 14-h light, 10-h dark schedule with lights on at 0500 h.

Experiment 13: Adult male *ob/ob* mice on a C57BL/6 background were maintained on a 12-h light, 12-h dark schedule with lights on at 0600 h.

Experiment 14: Adult male GALR1 knockout mice and their wild-type littermates (on a C57BL/6 background) were provided by Dr. John Shine at the Garvan Institute for Medical Research and were received from the Jackson Laboratory. The mice were maintained on a 12-h light, 12-h dark schedule with lights on at 0600 h.

Experiment 15: Adult male GALR2 knockout mice and their wild-type littermates (on a 129S1/SvImJ background) were provided by Nura, Inc. (Seattle, WA). The mice were maintained on a 12-h light, 12-h dark schedule with lights on at 0600 h.

Rats

Experiment 4: Adult male Sprague-Dawley rats were maintained on a 12-h light, 12-h dark schedule with lights on at 0600 h.

Experiment 10: Adult female Sprague-Dawley rats were maintained on a 14-h light, 10-h dark schedule with lights on at 0700 h.

B. GALP, GALP Fragments, and GALR2 Agonist

The rat GALP that I used in Experiments 1-9, 11-14, 17, and 18 was chemically synthesized by Amgen, Inc. (Boulder, CO). The rat GALP that I used in Experiments 15 and 16 was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA). Fragments of the full-length rat GALP peptide [GALP(1-21), GALP(22-60), GALP(1-56), and GALP(3-60)] were chemically synthesized by Amgen, Inc. The GALR2 agonist AR-M1896 was custom synthesized by Phoenix Pharmaceuticals, Inc. All peptides were >95% pure. GALP and the various GALP fragments were usually dissolved in artificial cerebrospinal fluid (aCSF) containing 0.1% bovine serum albumin (BSA). In Experiment 16, GALP and AR-M1896 were dissolved in aCSF containing 0.1% BSA and 25% dimethyl sulfoxide (DMSO).

C. Freehand ICV Injections in Mice

Mice were anesthetized with isoflurane (Abbott Laboratory, North Chicago, IL) delivered by a vaporizer (Veterinary Anesthesia Systems, Bend, OR). A small hole was bored in the skull 1 mm lateral and 0.5 mm posterior to bregma with a 27 gauge needle attached to a Hamilton syringe. The needle was fitted with polyethylene tubing, leaving 3.5 mm of the needle tip exposed. Once the initial hole was bored in the skull, all subsequent injections were made at the same site. Mice were allowed to recover for at least 2 days prior to treatment. For each ICV injection, mice were anesthetized with isoflurane for approximately 3 min, during which time 3 μl of solution were slowly and continuously injected into the lateral ventricle. The needle was held in place for

approximately 1 min post-injection to allow time for the solution to absorb and to minimize backflow through the needle track. Mice typically recovered from the anesthesia within 5 min after the injection.

D. ICV Injections in Rats

Rats (320-350 g) were anesthetized with an injection (0.1 ml/100 g body weight, im) of a ketamine (100 mg/ml)/ xylazine (20 mg/ml)/ acepromazine maleate (10 mg/ml) cocktail. The animals were placed in a stereotaxic frame with the incisor bar positioned 3 mm below the horizontal plane that intersected the center of the ear bars. A permanent 26 gauge stainless steel cannula (Plastics One, Roanoke, VA) was implanted into the third cerebral ventricle at 0 mm from the midline, -2.2 mm caudal to bregma, and –7.5 mm ventral to dura. The cannula was secured to the skull with dental acrylic and screws. A 33 gauge stainless steel stylus (Plastics One) was inserted to occlude the guide cannula when it was not in use. The animals were allowed to recover for 8 days prior to treatment. Injections were delivered in a volume of 3 μl over the course of 2 min. Before euthanizing the animals, correct cannula placement was verified by injecting the rats with 0.5 nmol NPY. Only rats that increased their baseline food intake by at least 4 g during the first hour after the NPY injection were included in the data analysis.

E. Blood Collection

To minimize the occurrence of stress-induced alterations in circulating hormone concentrations, mice were handled daily for at least one week prior to blood sampling.

Blood was obtained from the mice by orbital bleed under isoflurane anesthesia. When repeated blood sampling was necessary, the mice were allowed to recover for at least 2 weeks in between consecutive orbital bleeds. For terminal experiments, blood was collected from both the orbital sinus and from the trunk after the animals were decapitated. In Experiment 10, trunk blood was obtained from the rats following decapitation. Blood was collected in serum microtainer tubes (VWR Scientific, West Chester, PA) stored on ice and was spun at 14,000 rpm for 2 min. Sera were stored at –20 C until hormones were measured.

F. Radioimmunoassays

Serum LH and FSH concentrations were measured with reagents obtained from the 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 intra-assay and inter-assay coefficients of variation (CV) were 10% and 13%, respectively. For FSH, the antiserum used was anti-rFSH-S-11 and the standard was rFSH-RP2. The assay sensitivity was 1.0 ng/ml, the intra-assay CV was 4%, and the inter-assay CV was 16%. Testosterone was measured with a double antibody kit (ICN Biomedicals, Inc., Costa Mesa, CA). The assay sensitivity was 0.02 ng/ml, the intra-assay CV was 3%, and the inter-assay CV was 6%. Within each experiment, all hormones were measured in single assays.

G. Measurement of Locomotor Activity

Spontaneous locomotor activity was measured by placing mice in individual transparent Plexiglas cages (40 cm x 20 cm x 20 cm) surrounded by aluminum frames equipped with infrared beams (San Diego Instruments, San Diego, CA). Activity levels were monitored for 14 h overnight, beginning at 1830 h. Activity levels are reported 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 14 h observation period.

H. Conditioned Taste Aversion Testing

Mice were placed on a water restriction schedule, during which time they each received two water bottles from 0830-0900 h and from 1400-1900 h each day for 7 days. Food and water intake were measured daily. On the eighth day, the mice received a single bottle of water containing 0.15% saccharin (Sigma, St. Louis, MO) at 0830 h. The position of the bottle in the cage was randomized. The saccharin water bottle was removed 30 min later, at which time the mice were given an ICV injection of aCSF or 5 nmol GALP. A third group of mice received an ip injection (2% body weight) of 0.15 M LiCl dissolved in saline. The mice were allowed to recover for 48 h, during which time they remained on the water-restriction schedule. Food and water intake returned to pre-injection levels in all treatment groups by 48 h post-injection. At this time, 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 from each bottle was

measured after 2 h. Data are expressed as saccharin preference ratios (SPR = volume of saccharin water consumed/ total fluid volume consumed).

I. Tissue Preparation

The brains that were harvested in Experiment 10 were sectioned on a cryostat into 20 μm coronal sections through the entire rostral-caudal extent of the Arc. The sections were distributed among eight sets (with 140 μm between consecutive sections in each set) and were thaw-mounted onto SuperFrost Plus slides (VWR Scientific). The slides were stored at –80 C until they were processed for *in situ* hybridization. At this time, one set of brain tissue was removed from the freezer and the slides were placed into baked metal racks on dry ice. The slides were then rapidly thawed with a hair dryer until they no longer had any detectable drops of moisture. The brain sections were then fixed in 4% paraformaldehyde (pH 7.4) for 5 min. The sections were rinsed twice in 0.1 M phosphate buffer (0.02 M NaH_2PO_4, 0.08 M Na_2HPO_4, pH 7.4) for 5 min each, briefly dipped in diethyl pyrocarbonate (DEPC)-treated water followed by 0.1 M triethanolamine (TEA, pH 8.0), and then acetylated in 0.1 M TEA containing 0.25% acetic anhydride for 10 min on a stir plate. The sections were then rinsed in 2x sodium saline citrate (SSC; 1x SSC = 150 mM NaCl, 15 mM sodium citrate) for 3 min, dehydrated in a series of increasing ethanol concentrations (70, 95, and 100%) for 3 min each, and delipidated in chloroform for 5 min. The sections were subsequently rehydrated by submerging them in 100% and 95% ethanol for 3 min each. The slides

were allowed to air dry, and were then stored at room temperature until they were hybridized with radiolabeled probe.

J. Probe Preparation

A 120 bp rat GALP probe (corresponding to bases 218-337 of the rat GALP precursor mRNA) which had been cloned into a pAMP1 plasmid was linearized with EcoR1, purified by phenol-chloroform extraction, and reconstituted in TE at a concentration of 1 μg/μl. For the *in vitro* transcription of antisense GALP riboprobe, the following ingredients were added to each reaction tube in a total volume of 20 μl: 250 μCi ^{33}P-UTP (Perkin-Elmer Life Sciences, Boston, MA); 2 μg linearized DNA; 2 μl 10x transcription buffer (Roche Applied Science, Indianapolis, IN); 0.5 mM each ATP, CTP, and GTP; 1 μl RNase inhibitor (Roche Applied Science); 1 μl SP6 RNA polymerase (Roche Applied Science); 12 μl DEPC-treated water. The reactions were incubated at 37 C for 1.5 h, and were spiked with an additional 1 μl SP6 polymerase midway through the incubation period. The DNA template was then degraded by adding 2 μl DNase (Roche Applied Science), and the reaction was terminated 30 min later by adding 1 μl transfer ribonucleic acid (tRNA) and 4 μl of 0.5 M EDTA (pH 8). The radiolabeled probe was separated from unincorporated nucleotides with NucAway Spin Columns (Ambion, Austin, TX). A small volume of the riboprobe was run on a polyacrylamide gel to confirm its integrity.

K. *In Situ* Hybridization

Radiolabeled antisense GALP riboprobe (118 µl) was combined with 4.88 ml tRNA (1.9 mg/ml), denatured by boiling for 3 min, and placed on ice for 5 min. The probe + tRNA mixture was added to 20 ml hybridization buffer [62.5% deionized formamide (v/v), 12.5% dextran sulfate (w/v), 0.375 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.02% BSA (w/v), 0.02% Ficoll (w/v), 0.02% polyvinylpyrrolidone (w/v)], such that the final probe concentration was 0.3 pmol/ml. 100 µl of probe solution was applied to each slide. The slides were covered with silanized coverslips, placed in moist chambers, and incubated overnight at 55 C.

The following morning, the coverslips were removed from the slides. The slides were returned to metal racks and were washed twice in 2x SSC for 15 min each. The slides were next treated with RNase A (30 µg/ml) in RNase buffer [10 mM Tris (pH 8.0), 1 mM EDTA, 500 mM NaCl] for 30 min at 37 C, followed by 30 min in RNase buffer alone at 37 C. The slides were then subjected to an increasingly stringent series of washes, including a 30 min wash in 1x SSC at room temperature, a 30 min wash in 0.1x SSC at 60 C, and a 15 min wash in 0.1x SSC at room temperature. The sections were dehydrated by placing them for 3 min each in 50% ethanol with 300 mM ammonium acetate, 85% ethanol with 300 mM ammonium acetate, and 100% ethanol. The slides were allowed to air dry before being dipped in NTB-3 emulsion (Kodak, Rochester, NY) that had been pre-warmed to 44 C. After the emulsion dried, the slides were placed in aluminum foil-wrapped light-proof containers and were stored at 4 C. After two days of exposure, the slides were developed (4 min in D-19 Kodak developer at 15 C, 1 min in

water at 15 C, 5 min in Kodak fixer at 15 C) and then rinsed in running water for 30 min. The sections were then counterstained with cresyl violet, dehydrated (2 min each in 70%, 95%, and 100% ethanol), and rinsed twice for 5 min each in HemoDe (Fisher, Pittsburgh, PA). Coverslips were mounted on the slides with Permount (Alban Scientific, St. Louis, MO).

L. Image Analysis

To ensure that I was blinded to the treatment group assignments of the slides when I analyzed the *in situ* hybridization assay, the slides were assigned a computer-generated three letter code and were read in random order. The sections were viewed with a Carl Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) under darkfield illumination, and were analyzed with an automated image processing system that consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Power Macintosh G3 computer (Apple, Cupertino, CA). Images were captured with a Cohu 4910 camera (Cohu, Inc., San Diego, CA). I consulted a rat brain atlas to anatomically match brain sections across animals [196]. From each animal, three brain sections containing the Arc (corresponding to Figures 28, 31, and 33 of [196]) were analyzed. The number of GALP mRNA-positive cells in each section was counted manually, while the number of silver grains per cluster was determined using a custom-designed grain-counting program. The number of GALP mRNA-containing cells was summed across each of the three sections to generate a single value for each animal. The specificity of the hybridization signal had been previously validated by hybridizing brain

sections with either an excess of unlabeled antisense probe or with a radiolabeled sense GALP probe, both of which abolished all specific signal [120].

Chapter III:

Experimental Design and Results

A. GALP and Body Weight Regulation

GALP neurons reside within the Arc, a region of the hypothalamus that plays a critical role in the central regulation of energy homeostasis [62, 120, 121, 128, 142]. GALP gene expression is reduced in hypoleptinemic rats and leptin-deficient mice, but is dramatically upregulated in these animals by exogenous leptin treatment [87, 120, 121, 139]. Leptin's stimulatory effect on GALP gene expression is most likely attributable to a direct action on GALP neurons, given that >85% of GALP neurons in the Arc express Ob-R [62, 246]. Together, these observations implicate GALP neurons as plausible candidates for mediating leptin's central weight-reducing actions. To determine whether GALP is capable of recapitulating leptin's suppressive effects on feeding and body weight, I performed a series of experiments in which I examined the effects of ICV GALP administration on food intake and body weight in male wild-type mice. I first performed a dose-response study, in which I compared the effects of four doses of GALP on food intake and body weight (Experiment 1). Upon finding that feeding and body weight were reduced by acute GALP treatment, I next investigated whether GALP's suppressive effects on feeding and body weight are sustained during the course of long-term GALP treatment (Experiment 2). In Experiment 3, I examined the time course of GALP's inhibition of 24 h food intake by measuring food consumption at various time points over a 24 h interval following a central GALP

injection. To determine whether GALP's effects on feeding and body weight are species-dependent, I compared the effects of ICV GALP administration on food intake and body weight in mice and rats (Experiment 4). Finally, in an attempt to elucidate whether GALP's anorectic effect in mice might be secondary to impaired motor function and/or illness, I measured locomotor activity (Experiments 5 and 6) and assessed whether mice form a conditioned taste aversion (Experiment 7) following ICV GALP treatment.

Experiment 1. GALP Dose-Response: Feeding and Body Weight

In this experiment, I tested the effects of central administration of four doses of GALP on 24 h food intake and body weight change in male mice. I hypothesized that if GALP mediates leptin's suppressive effects on feeding and body weight, then mice that receive central GALP injections would consume less food and lose more weight than their vehicle-treated counterparts.

Methods: Adult male mice received two ICV injections (1800 h and 0800 h the following morning) of the vehicle (aCSF; n=7) or 1 nmol (n=6), 2.5 nmol (n= 6), 5 nmol (n=8) or 10 nmol GALP (n=5). Food intake and body weight were measured 24 h after the first injection. Differences in food intake and body weight were assessed by one-way ANOVA followed by Fisher's post hoc test to identify differences between individual treatment groups. Differences were considered significant when $P < 0.05$. All data are presented as means ± SEM.

Results: Central GALP treatment elicited a dose-dependent suppression of both food intake (Figure 1A) and body weight (Figure 1B) when measured 24 h after the first

injection. Compared to vehicle treatment, food intake was significantly suppressed by the 5 and 10 nmol GALP doses ($P < 0.0005$), while significant reductions in body weight occurred following injection of 2.5 nmol ($P < 0.05$), 5 nmol ($P < 0.005$) or 10 nmol GALP ($P < 0.005$). In addition to reducing feeding and body weight, treatment with the higher doses of GALP had pronounced effects on motor behavior. The mice that were treated with 5 or 10 nmol GALP typically remained immobile for a longer period of time than the vehicle-treated mice (i.e., they did not 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 the lower GALP doses.

Experiment 2. Effects of Long-Term GALP Treatment on Feeding and Body Weight

In the previous experiment, I observed that treatment with the two highest doses of GALP elicited acute reductions in feeding and body weight in mice. In this experiment, I assessed whether GALP's suppressive effects on feeding and body weight are sustained during the course of long-term GALP treatment.

Methods: Adult male mice received two ICV injections per day (0800 h and 1800 h) for 4.5 days, with the first injection occurring at 1800 h (Day 0). Mice were injected with either the vehicle (aCSF; n=8) or 5 nmol GALP (n=8). Food intake and body weight were measured daily for one week following the commencement of treatment. Differences in food intake and body weight were assessed by two-way repeated measures ANOVA (treatment x time). When the ANOVA indicated a significant effect

of treatment, time, and/or a significant interaction between treatment and time, unpaired t-tests were used to identify differences between the treatment groups at individual time points. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Long-term GALP treatment had transient effects on both food intake (Figure 2A) and body weight (Figure 2B). At 24 h after the first injection (Day 1), food intake and body weight were significantly reduced in the GALP-treated mice ($P < 0.005$ and $P < 0.05$ *vs.* vehicle-treated mice, respectively). However, by 48 h after the first injection (Day 2), food intake and body weight no longer differed between the vehicle- and GALP-treated groups. Food intake in the GALP-treated mice was significantly greater than that of the vehicle-treated mice on Day 4 ($P < 0.05$). The recovery of body weight in the GALP-treated animals was more gradual than the recovery of food intake.

Experiment 3. Time Course of GALP's Inhibitory Effect on Feeding

In Experiments 1 and 2, I observed that centrally-administered GALP reduces food intake and body weight in mice over a 24 h period. To examine the feeding patterns of GALP-treated mice on an abbreviated time scale, I assessed the time course of GALP's inhibition of feeding by measuring food intake in mice at regular intervals over a 24 h period following an ICV GALP injection.

Methods: Adult male mice were given a single ICV injection of the vehicle (aCSF; n=7) or 5 nmol GALP (n=7) at 1800 h. Food intake was measured at 0, 2, 4, 6, 8, 14, and 24 h post-injection, and body weight was measured at 24 h post-injection.

Differences in food intake were assessed by two-way repeated measures ANOVA (treatment x time). When the ANOVA revealed a significant effect of treatment, time, and/or a significant interaction between the two factors, unpaired t-tests were performed to identify differences between the treatment groups at individual time points. A difference in body weight was assessed by an unpaired t-test. Differences were considered statistically significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Compared to the vehicle-treated mice, food intake was significantly reduced in the GALP-treated mice as early as 2 h post-injection and remained significantly suppressed at all subsequent time points ($P < 0.001$ at all post-injection time points; Figure 3). Compared to the vehicle-treated mice, the GALP-treated animals experienced a greater degree of weight loss at 24 h post-injection (-0.09 ± 0.19 g vs. −1.75 ± 0.42 g, respectively; $P < 0.005$).

Experiment 4. Comparison of GALP's Effects on Feeding and Body Weight in Mice and Rats

At the time that I was completing Experiments 1-3, two papers appeared in the literature in which the authors reported that ICV injection of GALP *stimulates* food intake in rats during the first 1 or 2 h post-injection [145, 163]. These findings initially appeared to be at odds with the results of Experiment 3, which had revealed an anorectic effect of GALP in mice as early as 2 h post-injection. Upon closer examination of the papers by Matsumoto et al. and Lawrence et al., it became evident that both groups had treated their rats with GALP during the light phase of the light/dark cycle, a time at

which the animals would have been relatively satiated. However, in Experiment 3, I injected my mice with GALP in the evening just prior to lights off, at which time the mice were presumably already experiencing a near-maximal physiological drive to eat. In the event that my decision to administer GALP in the evening in Experiment 3 had obscured my ability to detect an acute orexigenic effect of GALP in mice, I compared the effects of a morning GALP injection on short-term (1 and 2 h) and longer-term (24 h) food intake in mice and rats. I hypothesized that if mice and rats exhibit similar feeding responses to central GALP treatment, then an ICV injection of GALP in the morning would stimulate short-term food intake, but would ultimately suppress feeding and body weight in both species after 24 h.

Methods: Adult male mice received a single ICV injection of the vehicle (aCSF; n=8) or 5 nmol GALP (n=8) at 0900 h. Food intake was measured after 1, 2, and 24 h, and body weight was measured at 24 h post-injection. Differences in food intake during the first and second hours after treatment were analyzed by a repeated measures two-way ANOVA (treatment x time), which was followed by unpaired t-tests to identify differences between the treatment groups at each time point. Differences in food intake and body weight change after 24 h were assessed by unpaired t-tests. Differences were considered statistically significant when $P < 0.05$. Data are expressed as means ± SEM.

Adult male rats (310-360 g) received a single ICV injection of the vehicle (aCSF) or 5 nmol GALP at 0900 h. Food intake was measured at 1, 2, and 24 h post-injection, and body weight was measured at 24 h post-injection. The experiment was repeated 4 days later in a cross-over design, such that each rat received both treatments in a

randomized order (n=10 per treatment). Differences in food intake and body weight change at each time point were assessed by paired t-tests. Differences were considered statistically significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: When the mice were given a single ICV injection of GALP at 0900 h, food intake was significantly suppressed during the first hour post-injection ($P < 0.05$ *vs.* vehicle-treated mice; Figure 4A). Food intake during the second hour did not differ between the two treatment groups. Food intake (Figure 4B) and body weight (Figure 4C) in the GALP-treated mice were significantly reduced at 24 h post-injection ($P < 0.05$ *vs.* vehicle-treated mice).

Food intake was significantly greater in the GALP-treated rats than in the vehicle-treated rats during the first hour post-injection ($P < 0.05$; Figure 5A). Although food intake was reduced in the GALP-treated rats at the 2 h time point, the difference fell just short of reaching statistical significance ($P = 0.051$). Despite their initial increase in food intake after ICV GALP administration, food intake (Figure 5B) and body weight (Figure 5C) were significantly reduced in the GALP-treated rats at 24 h post-injection ($P < 0.005$ and $P < 0.05$ *vs.* vehicle-treated rats, respectively).

Experiment 5. GALP Dose-Response: Locomotor Activity

Before drawing any conclusions about whether ICV GALP administration induces satiety in mice, I considered the possibility that GALP's anorectic effect might not reflect a homeostatic response, but might instead occur secondary to other visceral or behavioral consequences of ICV GALP treatment. While conducting the previous

studies, I had observed that the mice that were injected with high doses of GALP (5 or 10 nmol) consistently remained immobile for longer periods of time after the injection than the vehicle-treated mice. Therefore, it seemed plausible that GALP's anorectic effect might be attributable to impaired motor function, which could theoretically render the GALP-treated mice physically incapable of getting to their food and eating as much as the vehicle-treated mice. To assess whether reduced motor activity might underlie GALP's inhibitory effect on feeding behavior, I measured spontaneous locomotor activity in mice over the 14 h period following a single ICV injection of one of two doses of GALP. Based upon my qualitative observations of impaired motor behavior in the GALP-treated mice in the previous experiments (with motor dysfunction being more pronounced with the 10 nmol dose than with the 5 nmol dose), I hypothesized that ICV GALP administration would dose-dependently suppress spontaneous locomotor activity.

Methods: Adult male mice received a single ICV injection of the vehicle (aCSF; n=6), 2.5 nmol GALP (n=6) or 5 nmol GALP (n=7) at 1800 h. Spontaneous locomotor activity (as measured by the total number of ambulations) was measured for 14 h overnight, beginning at 1830 h. Food intake and body weight were also measured at 14 h post-injection. Differences between treatment groups were analyzed by one-way ANOVA. When the ANOVA indicated a significant difference, Fisher's post hoc test was used to identify differences between individual treatment groups. Differences were considered significant when $P < 0.05$. All data are presented as means ± SEM.

Results: Food intake was significantly reduced in the mice that received 5 nmol GALP (Vehicle: 4.25 ± 0.08 g, 5 nmol GALP: 1.58 ± 0.41 g; $P < 0.0001$). Whereas the

mice that were injected with the vehicle or 2.5 nmol GALP gained weight over the 14 h period (+0.32 ± 0.16 g and +0.10 ± 0.30 g, respectively), the mice that were injected with 5 nmol GALP lost weight (-1.8 ± 0.44 g; $P < 0.0005$ *vs.* vehicle-treated mice). ICV GALP treatment suppressed activity levels in a dose-dependent manner (Figure 6). Compared to the vehicle-treated mice, the animals that received 2.5 nmol ($P < 0.05$) or 5 nmol GALP ($P < 0.0005$) displayed significantly fewer ambulations over the 14 h recording period.

Experiment 6. Effects of Long-Term GALP Treatment on Locomotor Activity

The previous experiment demonstrated that ICV GALP dose-dependently reduces spontaneous locomotor activity in mice during the first 14 h post-injection. Although not directly tested in Experiment 5, the observation of an acute depression of locomotor activity following ICV GALP treatment is consistent with the argument than a reduction in motor activity either partially or wholly underlies GALP's inhibitory effect on food intake. Although food intake was significantly reduced at 24 h after treatment commenced in the GALP-treated mice in Experiment 2, feeding completely recovered to pre-treatment levels within 48 h after the first GALP injection (and was even slightly but significantly greater than that of the vehicle-treated animals on the final day of treatment). It is conceivable that the GALP-treated mice in Experiment 2 ate less food over the first 24 h of long-term GALP treatment as a consequence of impaired motor function; however, these animals were clearly capable of feeding normally within 48 h after long-term GALP treatment commenced, which suggests that the mice were

not severely impeded by motor dysfunction at the 48 h and subsequent time points. In the present experiment, I administered nine ICV injections of GALP to mice over the course of 4.5 days and measured spontaneous locomotor activity for 14 h following the first and ninth injections. Based on the outcomes of Experiments 2 and 5, I hypothesized that locomotor activity would be reduced in the GALP-treated mice following the first GALP injection, but would be restored to control levels following the ninth GALP injection.

Methods: Mice were group-housed for this experiment (four per cage), except for when the animals were individually placed in the activity chambers and activity levels were recorded. Adult male mice received twice daily ICV injections (0800 h and 1800 h) of the vehicle (aCSF; n=7) or 5 nmol GALP (n=8) for 4.5 days, with the first injection occurring at 1800 h. Spontaneous locomotor activity was measured for 14 h overnight (starting at 1830 h) after the first and ninth injections. Food intake was also measured during these two 14 h time periods. Differences in food intake and locomotor activity were determined by repeated measures two-way ANOVA (treatment x time). When the ANOVA indicated a significant effect of treatment, time, and/or a significant interaction between the two factors, differences between the two treatment groups at individual time points were assessed by unpaired t-tests. Differences were considered significant when $P < 0.05$. Data are presented as means ± SEM.

Results: During the 14 h following the first injection, locomotor activity was significantly suppressed in the GALP-treated mice ($P < 0.005$ *vs.* vehicle-treated mice; Figure 7). Following the first injection, there was very little evidence of movement in the

GALP-treated animals until the final few hours of the observation period, coinciding with the timing of the lights coming on (Figure 8A). Unexpectedly, locomotor activity was markedly 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-treated mice were immobile for approximately 3 h, but then became increasingly hyperactive over the remainder of the observation period (Figure 8B). The GALP-treated animals ate significantly less food that the vehicle-treated mice after both the first (Vehicle: 4.22 ± 0.24 g, GALP: 1.52 ± 0.35 g; $P < 0.0001$) and ninth (Vehicle: 4.25 ± 0.23 g, GALP: 3.24 ± 0.28 g; $P < 0.05$) injections.

Experiment 7. Conditioned Taste Aversion

Another conceivable explanation for the observed reduction in food intake following acute central GALP treatment is that injecting 5 nmol GALP into the cerebral ventricles is toxic and/or causes visceral illness in mice. Conditioned taste aversion (CTA) studies are commonly employed to assess whether drugs or other exogenously-administered substances might have aversive properties in rodents [17, 275]. I hypothesized that if centrally-administered GALP causes visceral illness or is in some other way aversive to mice, then ICV GALP treatment would elicit the formation of a CTA.

Methods: Adult male mice were exposed to a novel flavor (0.15% saccharin water) for 30 min prior to receiving an ICV injection of the vehicle (aCSF; n=9) or 5 nmol GALP (n=8) at 0900 h. A third group of mice received an ip injection of LiCl (0.15

M, 2% body weight, n=4), a prototypical emetic agent that induces the formation of a CTA in mice [117]. Forty-eight hours later (by which time food and water intake in all three treatment groups had returned to pre-injection levels), the animals were given two bottles (one containing water and the other containing 0.15% saccharin water) at 0830 h. Fluid intake from each bottle was measured after 2 h. Data are expressed as saccharin preference ratios (SPR = volume of saccharin water consumed/ total fluid volume consumed). Differences in SPR were assessed by one-way ANOVA, which was followed by Fisher's post hoc test to identify differences between individual treatment groups. Differences were considered statistically significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: ICV injection of the vehicle did not appear to induce a CTA, as evidenced by the high SPR in the vehicle-treated mice (SPR=0.83 ± 0.03; Figure 9). As expected, the LiCl-treated mice had a low SPR (SPR=0.12 ± 0.06; $P < 0.0001$ *vs.* vehicle-treated mice), which demonstrates 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).

B. GALP and Gonadotropin Secretion

Leptin's stimulatory effect on GnRH secretion is likely an indirect one, because neither Ob-R mRNA nor protein has been detected on GnRH neurons [81, 98]. The lack of neuroanatomical evidence in support of a direct action of leptin on GnRH neurons suggests that leptin-sensitive interneurons are responsible for relaying leptin's

stimulatory signal to GnRH neurons. Several lines of evidence implicate GALP neurons as likely candidates for fulfilling this role. First, GALP neurons in the Arc are leptin-sensitive, because they express Ob-R and are transcriptionally regulated by central leptin treatment [62, 121, 246]. Second, GALP fibers are found in close contact with GnRH perikarya and fibers in the MPOA [246]. Third, ICV injection of GALP induces Fos expression in approximately 1/3 of GnRH neurons in the MPOA [162]. Fourth, central GALP administration stimulates LH secretion in male rats, an effect that is blocked by pre-treatment with a GnRH receptor antagonist [162]. Collectively, these observations strongly implicate GALP neurons in the central regulation of gonadotropin secretion, as well as hint at a possible role for GALP neurons in mediating leptin's stimulatory effects on the HPG axis. If these suppositions are correct, then I would predict that central GALP administration would mimic the stimulatory effects of exogenous leptin treatment on gonadotropin secretion.

To determine whether centrally-administered GALP stimulates gonadotropin secretion in mice as it does in rats, I first evaluated the effects of acute ICV GALP treatment on serum gonadotropin and testosterone concentrations in male mice (Experiment 8). Upon finding that serum LH concentrations are elevated in GALP-treated mice, I next tested GALP's efficacy at eliciting LH release by performing a dose-response study with three different doses of GALP (Experiment 9). Because I observed a positive effect of centrally-administered GALP on LH secretion in Experiments 8 and 9, I next evaluated whether changes in endogenous GALP gene expression might mediate the inhibitory and/or stimulatory effects of gonadal steroids on LH secretion in the

female. In Experiment 10, I assessed whether the expression of GALP mRNA in the Arc is subject to negative feedback regulation by estrogen, as well as whether GALP gene expression is increased coincident with the estrogen/progesterone-induced LH surge.

Experiment 8. Effects of GALP on Serum Reproductive Hormone Concentrations

In male rats, ICV injection of GALP induces Fos expression in a subset of GnRH neurons in the MPOA and stimulates LH secretion in a GnRH-dependent manner [162]. In this experiment, I hypothesized that centrally-administered GALP would increase serum gonadotropin and testosterone levels in male mice.

Methods: Adult male mice received a single ICV injection of the vehicle (aCSF; n=9) or 5 nmol GALP (n=8) between 0800 h and 0830 h. Thirty minutes later, blood was obtained by a combination of orbital bleed and by collecting trunk blood after cervical dislocation and rapid decapitation. Serum concentrations of LH, FSH, and testosterone were measured by radioimmunoassay (RIA). Differences in hormone concentrations between treatment groups were assessed by unpaired t-tests, and were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Serum levels of LH (Figure 10A) and testosterone (Figure 10C) were significantly elevated in the GALP-treated mice compared to the vehicle-treated mice ($P < 0.0005$). Serum levels of FSH were modestly elevated in the GALP-treated animals, but the difference did not reach statistical significance ($P = 0.07$ *vs.* vehicle-treated mice; Figure 10B).

Experiment 9. GALP Dose-Response: Serum LH Concentrations

Having observed that an ICV injection of 5 nmol GALP potently increased serum LH concentrations in male mice, I next evaluated GALP's efficacy at stimulating LH secretion by comparing the effects of three GALP doses on serum LH levels in male mice.

Methods: Adult male mice received a single ICV injection of the vehicle (aCSF; n=8), 0.5 nmol GALP (n=9), 1 nmol GALP (n=9) or 5 nmol GALP (n=9) between 0900 h and 1130 h. Blood was obtained by orbital bleed at 30 min following the injection, and serum LH concentrations were measured by RIA. Differences in serum LH levels were determined by one-way ANOVA. Because the ANOVA indicated a significant effect of treatment, differences between individual treatment groups were assessed by Fisher's post hoc test. Differences were considered significant when $P < 0.05$. Data are presented as means ± SEM.

Results: Compared to the vehicle-treated mice, serum LH levels were significantly elevated in the animals that received 5 nmol GALP ($P < 0.0001$; Figure 11). In contrast, the 0.5 and 1 nmol GALP doses were without effect on serum LH concentrations.

Experiment 10. Analysis of the Regulation of GALP mRNA by Ovarian Steroids

Centrally-administered GALP potently stimulates LH secretion in male mice (Experiments 8 and 9), rats [162], and monkeys [64]. Although injecting a pharmacological dose of GALP into the cerebral ventricles increases CNS GALP

concentrations and subsequently elicits the release of LH in several species, it is currently unknown whether GALP modulates LH secretion under physiological circumstances. In this experiment, I evaluated whether changes in endogenous GALP expression might mediate the negative and/or positive feedback effects of ovarian steroids on gonadotropin secretion in female rats. Specifically, I hypothesized that if a reduction in endogenous GALP tone mediates the inhibitory effect of estrogen on GnRH/LH secretion (such as occurs during most of the estrous cycle in intact female rats), then estrogen treatment should reduce GALP mRNA levels in OVX female rats. I also hypothesized that if an increase in endogenous GALP tone mediates the inductive effects of estrogen and progesterone on GnRH/LH secretion (such as occurs during the preovulatory LH surge), then GALP mRNA expression would be elevated in ovarian steroid-treated OVX rats at the time of the LH surge. In the latter scenario, estrogen and progesterone could induce GALP gene expression by acting directly on GALP neurons and/or by modulating the activity of afferent inputs to GALP neurons. If estrogen and progesterone treatment increases GALP gene expression in OVX rats, and if this occurs due to a steroid-dependent activation of excitatory neural inputs to GALP neurons, then a pharmacological blockade of afferent input to GALP neurons should abolish the inductive effects of estrogen and progesterone on GALP gene expression. Thus, I hypothesized that if GALP mRNA levels are elevated during the steroid-induced LH surge due to the steroid-dependent transsynaptic activation of GALP neurons, then GALP mRNA expression would not be elevated in estrogen- and progesterone-treated OVX rats that are treated with the general anesthetic pentobarbital, a non-specific CNS

inhibitor which has previously been shown to block the steroid-induced LH surge in OVX rats [159].

Methods: Adult female Sprague-Dawley rats were group-housed (three per cage) in this experiment. The rats were OVX under ketamine (100 mg/ml)/xylazine (20 mg/ml) anesthesia (5.0:1.6 ratio, respectively), and were then allowed to recover for 26 days. The rats were then split into four treatment groups. One group received a subcutaneous injection of peanut oil, while the other three groups received a subcutaneous injection of 30 μg β-estradiol 3-benzoate (EB; Sigma-Aldrich, St. Louis, MO) at 1030 h on Day 0. Between 1000 h and 1200 h on Day 1 (24-26 h post-injection), all the oil-treated rats (Oil; n=7) and one group of the EB-treated rats (EB; n=8) were killed by rapid decapitation following CO_2 inhalation. On the following day (Day 2) at 1200 h, all the remaining EB-treated rats were given a subcutaneous injection of 5 mg progesterone (Schein Pharmaceuticals, Florham Park, NJ). At 1400 h, half the rats were given an ip injection of saline (Surge; n=7), while the other half were given an ip injection of sodium pentobarbital (40 mg/kg; Abbott Laboratories, North Chicago, IL; Block; n=6). The animals in the Surge and Block groups were killed between 1800 h and 1930 h on Day 2 in the same manner as the rats on the previous day. Trunk blood was obtained from all rats at the time of sacrifice, and serum LH levels were measured by RIA. Brains were also collected when the animals were killed, and GALP mRNA levels were measured by *in situ* hybridization. Differences in GALP mRNA expression (number of GALP mRNA-expressing cells and grains/cell) and hormone concentrations were assessed by one-way ANOVA. When the ANOVA revealed a significant treatment

effect, differences between individual treatment groups were assessed by Fisher's post hoc test. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Compared to the Oil group, serum LH concentrations were significantly reduced in the EB group ($P < 0.05$; Figure 12). Although serum LH concentrations were significantly elevated in the rats that were killed during the LH surge (Surge; $P < 0.0001$ *vs.* the Oil-treated rats), the rats in which the LH surge was blocked by pentobarbital (Block) had serum LH concentrations that did not differ from those of the rats in the EB group. Neither the number of GALP mRNA-expressing cells (Figure 13A) nor the number of grains/cell (which reflects the cellular content of GALP mRNA; Figure 13B) differed between the four treatment groups.

C. Acute and Chronic GALP Treatment in *ob/ob* Mice

The first two sets of experiments revealed that centrally-administered GALP has pronounced effects upon feeding, body weight, and gonadotropin secretion in wild-type mice, many of which are reminiscent of leptin's effects on these parameters. Although leptin reduces food consumption and body weight in wild-type mice, leptin's anorectic and weight-reducing actions are usually more pronounced in magnitude and prolonged in duration in leptin-deficient *ob/ob* mice [40, 99, 103, 148, 197]. To assess whether like leptin, GALP also exerts differential effects in wild-type and *ob/ob* mice, I compared the food intake and body weight responses of wild-type and *ob/ob* mice to acute ICV GALP treatment (Experiment 11). Because the *ob/ob* mice in Experiment 11 exhibited

prolonged reductions in feeding and body weight following acute GALP treatment, I also assessed whether these effects of GALP in *ob/ob* mice would be sustained during the course of long-term GALP treatment (Experiment 13).

Both sexes of *ob/ob* mice exhibit hypogonadotropic hypogonadism and are infertile [11, 116, 244]. However, chronic leptin treatment significantly improves reproductive function in male and female *ob/ob* mice, and can even correct their sterility when administered for an extended period of time [6, 45, 184]. I reasoned that if leptin's stimulatory effects on HPG function in *ob/ob* mice are mediated by enhanced central GALP signaling, then long-term GALP treatment should recapitulate leptin's activational effects on the reproductive axis in these animals. Before testing this hypothesis, I first assessed whether male *ob/ob* mice respond to acute GALP treatment by releasing LH (Experiment 12). After observing that *ob/ob* mice exhibit a robust release of LH and FSH in response to acute GALP treatment, I next investigated whether long-term ICV GALP administration improves reproductive function in male *ob/ob* mice (Experiment 13).

Experiment 11. Comparison of GALP's Effects on Feeding and Body Weight in Wild-type and* ob/ob *Mice

Leptin-deficient *ob/ob* mice exhibit more prolonged reductions in food intake and body weight in response to exogenous leptin treatment than do wild-type mice, and are generally more sensitive than lean animals to leptin's anorectic and weight-reducing actions [40, 99, 103, 148, 197]. In this experiment, I compared the effects of an acute

ICV GALP injection on feeding and body weight in wild-type and *ob/ob* mice. I hypothesized that if leptin's inhibitory effects on feeding and body weight are mediated by central GALP-dependent pathways, then *ob/ob* mice would display longer-lasting responses to exogenous GALP treatment than wild-type mice with respect to feeding and body weight.

Methods: Adult male wild-type and *ob/ob* mice received a single ICV injection of the vehicle (aCSF; n=8 for wild-type and n=11 for *ob/ob*) or 5 nmol GALP (n=9 for wild-type and n=12 for *ob/ob*) between 0800 h and 1000 h on Day 0. Food intake and body weight were monitored for 5 days post-injection. Within each genotype, differences in food intake and body weight were assessed by two-way ANOVA (treatment x time). When the ANOVA revealed a significant effect of treatment, time, and/or a significant interaction between the two factors, unpaired t-tests were performed to identify differences between treatment groups at individual time points. Differences were considered significant when $P < 0.05$. Data are presented as means ± SEM.

Results: Marked differences were observed between the two genotypes with respect to the time course of recovery of food intake and body weight following central GALP administration. The GALP-treated wild-type mice ate significantly less food than the vehicle-treated wild-type mice on Day 1 ($P < 0.0005$), but ate significantly more food than their vehicle-treated counterparts on the following four days ($P < 0.005$ on Days 2, 3, and 4, $P < 0.01$ on Day 5; Figure 14A). Food intake was markedly reduced in the GALP-treated *ob/ob* mice on Day 1 ($P < 0.0005$ *vs.* vehicle-treated *ob/ob* mice; Figure 14C). Although food intake in the GALP-treated *ob/ob* mice began to show signs

of recovery on Day 2, food intake remained significantly lower in this group than in the vehicle-treated *ob/ob* mice on Days 2 ($P < 0.005$), 3, and 4 ($P < 0.05$). Body weight was significantly reduced in the GALP-treated wild-type mice on Day 1 ($P < 0.05$ *vs.* vehicle-treated wild-type mice), but no longer differed from that of the vehicle-treated wild-type mice on the following four days (Figure 14B). In contrast, body weight was significantly reduced in the *ob/ob* mice for four days after ICV injection of GALP ($P < 0.05$ *vs.* vehicle-treated *ob/ob* mice; Figure 14D).

Experiment 12. Effects of Acute GALP Treatment on Serum Gonadotropin Levels in* ob/ob *Mice

Male *ob/ob* mice have inappropriately reduced serum gonadotropin concentrations in the presence of low testosterone levels, which is indicative of hypogonadotropic hypogonadism [244]. However, serum gonadotropin concentrations (primarily FSH) in male *ob/ob* mice are elevated after two weeks of twice daily leptin injections [6]. Furthermore, the observation that circulating FSH levels in male *ob/ob* mice are increased after ten days of ICV leptin treatment indicates that leptin can stimulate gonadotropin release via a central mechanism(s) [106]. I predicted that if leptin's stimulatory effect on gonadotropin secretion in *ob/ob* mice is mediated by GALP (which potently elicits LH secretion in wild-type mice), then long-term GALP treatment should also improve gonadotropin secretion (and perhaps other indices of reproductive function) in male *ob/ob* mice. Before attempting to address the question of whether the HPG axis of male *ob/ob* mice responds to long-term GALP treatment, I first

examined whether male *ob/ob* mice release LH and FSH in response to acute ICV GALP treatment.

Methods: Adult male *ob/ob* mice received a single ICV injection of the vehicle (aCSF; n=11) or 5 nmol GALP (n=12) between 0800 h and 1000 h. Blood was obtained by orbital bleed at 30 min following the injection. Serum LH and FSH concentrations were measured by RIA. Differences in hormone concentrations between treatment groups were assessed by unpaired t-tests, and were considered significant when $P < 0.05$. Data are presented as means ± SEM.

Results: Compared to the vehicle-treated mice, serum levels of LH (Figure 15A) and FSH (Figure 15B) were significantly elevated in the GALP-treated animals ($P < 0.0001$).

Experiment 13. Effects of Long-Term GALP Treatment on Feeding, Body Weight, and Reproductive Parameters in* ob/ob *Mice

The previous two experiments revealed that *ob/ob* mice respond to acute central GALP treatment by reducing their food intake and body weight, and by releasing gonadotropins into the circulation. In this experiment, I hypothesized that long-term ICV GALP administration would mimic the effects of chronic leptin treatment in male *ob/ob* mice, thereby eliciting sustained reductions in food intake and body weight, increasing thermogenesis, and improving various indices of reproductive function.

Methods: Adult male *ob/ob* mice received twice daily ICV injections of the vehicle (aCSF; n=8) or 5 nmol GALP (n=7) at 0700 h and 1700 h, with the first injection

occurring at 1700 h on Day 0. The animals received 28 injections over the course of 14 days. Food intake and body weight were measured daily. Rectal temperature was measured at 6-8 h after the final injection on Day 14. The animals were then anesthetized with isoflurane, bled by orbital bleed, and killed by cervical dislocation. The testes and seminal vesicles were removed and weighed. Serum LH, FSH, and testosterone concentrations were measured by RIA. Differences in food intake and body weight were determined by two-way repeated measures ANOVA (treatment x time). When the ANOVA indicated a significant effect of treatment, time, and/or a significant interaction between the two factors, unpaired t-tests were used to identify differences between treatment groups at individual time points. Differences in temperature, organ weights, and hormone concentrations were determined by unpaired t-tests. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Food intake was significantly reduced in the GALP-treated mice within 24 h after GALP treatment commenced (Figure 16A). Food intake in the GALP-treated mice gradually recovered toward pre-treatment levels, and was no longer significantly different from that of the vehicle-treated mice by Day 10. Body weight was significantly reduced in the GALP-treated mice on Day 2 (Figure 16B). Despite their gradual recovery of food intake, weight loss was sustained in the GALP-treated animals for the remainder of the treatment period. At the end of the experiment, the GALP-treated animals had lost approximately 10% of their initial body weight. Body temperature was significantly elevated in the GALP-treated animals at the end of the 14 day treatment period ($P < 0.005$ *vs.* vehicle-treated mice; Figure 17).

In contrast to the pronounced effects of long-term ICV GALP treatment on feeding and body weight, there were no obvious signs of improved reproductive function in the GALP-treated *ob/ob* mice, as evidenced by reproductive organ weights, FSH levels, and testosterone levels that did not differ from those of the vehicle-treated *ob/ob* mice (Figure 18A-D). Serum LH concentrations were below the assay's limit of detection in most of the animals, which indicates that GALP treatment did not markedly increase LH secretion.

D. GALP and Galanin Receptors

GALP shares sequence identity with the portion of the galanin molecule that confers its biological activity at galanin receptors, and GALP binds and exhibits agonistic activity at GALR1 and GALR2 *in vitro* [192]. Despite the similarities between galanin and GALP with respect to structure and pharmacology, the two neuropeptides exert only partially overlapping actions within the CNS. Although both neuropeptides stimulate acute feeding in the rat, only GALP has an inhibitory effect on 24 h food intake and body weight [145]. Furthermore, GALP but not galanin stimulates LH secretion in male rats [162]. In addition to their disparate effects on energy homeostasis and gonadotropin secretion, centrally-administered galanin and GALP induce different patterns of neuronal activation in the rat CNS [86, 146]. These differences between galanin and GALP suggest that the two neuropeptides might signal through different receptors *in vivo*. To assess the role of galanin receptors (specifically the two galanin receptor subtypes that GALP has been demonstrated to interact with *in vitro*, GALR1

and GALR2) in mediating GALP's central actions, I evaluated whether GALP modulates food intake, body weight, and LH secretion in male mice in which the GALR1 or GALR2 genes had been disrupted (Experiments 14 and 15). Because GALP displays a higher affinity for GALR2 than for GALR1 *in vitro*, I also examined whether the central administration of a GALR2 agonist would mimic GALP's effects on feeding, body weight, and LH secretion in wild-type mice (Experiment 16). Finally, I injected wild-type mice with truncated GALP fragments in an effort to determine whether GALP's biological activity is conferred by the galanin-homologous region of the GALP peptide (Experiments 17 and 18).

Experiment 14. Effects of GALP in GALR1 Knockout Mice

Although GALP has been demonstrated to bind and exhibit agonistic activity at GALR1 *in vitro*, it is unknown whether GALP signals through GALR1 *in vivo*. In this experiment, I hypothesized that if GALP's effects on feeding, body weight, and LH secretion in wild-type mice are mediated solely by its interactions with GALR1, then mice lacking functional GALR1 receptors (GALR1 knockout mice; GALR1 KO) would not be responsive to exogenous GALP treatment with respect to these parameters.

Serum LH Concentrations

Methods: Adult male wild-type (n=6) and GALR1 KO mice (n=7) received a single ICV injection of the vehicle (aCSF) or 5 nmol GALP between 0900 h and 1100 h. Blood was obtained by orbital bleed at 30 min following the injection. After two weeks,

the experiment was repeated in a cross-over design, such that each mouse received both treatments in a randomized order. Serum LH concentrations were measured by RIA. Differences in serum LH concentrations were assessed by two-way repeated measures ANOVA (genotype x treatment). When the ANOVA revealed a significant effect of genotype, treatment, and/or a significant interaction between the two factors, paired t-tests were used to assess differences between treatments within each genotype. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: ICV GALP treatment elicited significant increases in serum LH concentrations in both wild-type and GALR1 KO mice compared to when the mice were treated with the vehicle alone ($P < 0.0005$; Figure 19).

Feeding and Body Weight

Methods: One week following the second round of the LH experiment, the same wild-type (n=5) and GALR1 KO mice (n=7) received two ICV injections of the vehicle (aCSF) or 5 nmol GALP at 1700 h and 0800 h on the following morning. Food intake and body weight were measured at 24 h after the first injection. The experiment was repeated in a cross-over design one week later. Differences in food intake and body weight were determined by two-way repeated measures ANOVA (genotype x treatment). When the ANOVA revealed a significant effect of genotype, treatment, and/or a significant interaction between the two factors, differences between the

treatments within each genotype were assessed by paired t-tests. Differences were considered significant when $P < 0.05$. Data are presented as means ± SEM.

Results: The wild-type and GALR1 KO mice responded similarly to central GALP injections with respect to feeding and body weight change. Compared to vehicle treatment, ICV GALP injection significantly reduced 24 h food intake in wild-type ($P < 0.005$) and GALR1 KO mice ($P < 0.0005$; Figure 20A). The GALP-treated wild-type ($P < 0.005$) and GALR1 KO mice ($P < 0.0005$) also lost more body weight compared to when the mice were treated with the vehicle alone (Figure 20B).

Experiment 15. Effects of GALP in GALR2 Knockout Mice

Although GALP binds and exhibits agonistic activity at both GALR1 and GALR2 *in vitro*, GALP has a higher affinity for GALR2. Therefore, I hypothesized that if GALP's effects on feeding, body weight, and LH secretion are mediated solely by GALR2 signaling, then these effects of GALP would not be observed in mice lacking functional GALR2 receptors (GALR2 knockout mice; GALR2 KO).

Serum LH Concentrations

Methods: Adult male wild-type (n=5) and GALR2 KO mice (n=6) received an ICV injection of the vehicle (aCSF) or 5 nmol GALP between 0730 h and 0930 h. Blood was obtained by orbital bleed at 30 min following the injection. The experiment was repeated two weeks later in a cross-over design, such that each mouse received both treatments in a randomized order. Serum LH levels were measured by RIA. Differences

in serum LH concentrations were assessed by two-way repeated measures ANOVA (genotype x treatment). When the ANOVA revealed a significant effect of genotype, treatment, and/or a significant interaction between the two factors, paired t-tests were used to assess differences between treatments within each genotype. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Serum LH levels were significantly elevated in the GALP-treated wild-type and GALR2 KO mice compared to when the mice were injected with the vehicle alone ($P < 0.0001$; Figure 21).

Feeding and Body Weight

Methods: Three weeks after the second round of the LH experiment, the same wild-type (n=4) and GALR2 KO mice (n=6) received two ICV injections of the vehicle (aCSF) or 5 nmol GALP at 1700 h and 0800 h the following morning. Food intake and body weight were measured at 24 h after the first injection. The experiment was repeated in a cross-over design one week later. Differences in food intake and body weight were assessed by two-way repeated measures ANOVA (genotype x treatment). When the ANOVA revealed a significant effect of genotype, treatment, and/or a significant interaction between the two factors, paired t-tests were employed to assess differences between treatments within each genotype. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Compared to vehicle treatment, 24 h food intake was significantly reduced by ICV GALP treatment in both the wild-type ($P < 0.05$) and GALR2 KO mice

($P < 0.005$; Figure 22A). Body weight was also reduced in the GALP-treated wild-type and GALR2 KO mice ($P < 0.05$ and $P < 0.005$ *vs.* vehicle treatment, respectively; Figure 22B).

Experiment 16. Effects of a GALR2 Agonist on Feeding, Body Weight, and LH Concentrations

The previous experiment revealed that GALR2 KO mice retain normal responsiveness to centrally-administered GALP with respect to feeding, body weight, and LH secretion. One interpretation of this experimental outcome is that GALP's effects on energy balance and gonadotropin secretion occur independently of GALR2 signaling. However, given the possibility of functionally redundant signaling systems and/or developmental compensation in mice that are genetically manipulated to lack a specific gene throughout prenatal and postnatal life, it is still conceivable that GALR2 is involved in mediating GALP's actions within the brain. As an alternative approach to assess the role of GALR2 in mediating GALP's effects on feeding, body weight, and LH secretion in the mouse, I treated wild-type mice with central injections of the GALR2 agonist AR-M1896 [Galanin(2-11)-NH_2] [151]. I hypothesized that if GALP's effects on feeding, body weight, and LH secretion are mediated by GALR2 signaling, then AR-M1896 would mimic these effects of GALP.

Feeding and Body Weight

Methods: Adult male wild-type mice received two ICV injections of the vehicle (25% DMSO in aCSF; n=7), 5 nmol GALP (n=5) or 5 nmol AR-M1896 (n=7) at 1630 h and 0730 h the following morning. Food intake and body weight were measured at 24 h after the first injection. Differences in food intake and body weight were assessed by one-way ANOVA. When the ANOVA indicated a significant treatment effect, Fisher's post hoc test was used to identify differences between the individual treatment groups. Differences were considered significant when $P < 0.05$. Data are presented as means ± SEM.

Results: Twenty-four hour food intake and body weight were significantly reduced in the GALP-treated mice compared to the vehicle-treated animals ($P < 0.0001$; Figure 23A and 23B). Feeding and body weight were not altered by AR-M1896 treatment.

Serum LH Concentrations

Methods: Eleven days after the feeding/body weight experiment, the same wild-type mice received an ICV injection of the vehicle (25% DMSO in aCSF; n=7), 5 nmol GALP (n=6) or 5 nmol AR-M1896 (n=7) between 0800 h and 1000 h. Blood was obtained by orbital bleed at 30 min following the injection. Serum LH concentrations were measured by RIA. Differences in serum LH concentrations were assessed by one-way ANOVA, which was followed by Fisher's post hoc test to identify differences

between individual treatment groups. Differences were considered significant when $P < 0.05$. Data are presented as means ± SEM.

Results: Serum LH concentrations were significantly elevated in the GALP-treated mice ($P < 0.0005$ *vs.* vehicle-treated mice), but did not differ between the mice in the vehicle and AR-M1896 groups (Figure 24).

Experiment 17. Effects of Short GALP Fragments on Feeding, Body Weight, and Serum LH

The N-terminal region of the galanin molecule confers its biological activity at galanin receptors [13]. Several truncated N-terminal galanin fragments [e.g., galanin(1-13), galanin(1-15), and galanin(1-16)] bind to hypothalamic galanin receptors with submicromolar affinity [140] and mimic many of galanin's actions *in vivo* [8]. GALP(9-21) is 100% homologous to the first thirteen amino acids of galanin, and the full-length GALP peptide also binds to galanin receptors with submicromolar affinity *in vitro* [192]. If GALP signals through galanin receptors *in vivo*, then it would seem likely that the galanin-homologous region of the GALP molecule is responsible for conferring its biological activity at galanin receptors. Alternatively, it is possible that GALP's biological activity resides within the C-terminal portion of the peptide, as is the case with most other neuropeptides [7]. The fact that amino acids 38-54 of GALP are highly conserved across species implicates a potentially important biological role for this region of the GALP molecule [63, 192]. In this experiment, I investigated the effects of two truncated GALP fragments [GALP(1-21) and GALP(22-60)] on feeding, body weight,

and LH secretion in wild-type mice. I hypothesized that if the galanin-homologous region of the GALP molecule confers its biological activity (presumably at galanin receptors), then a fragment containing this thirteen amino acid sequence [GALP(1-21)] would mimic the effects of full-length GALP. However, if GALP's biological activity is not conferred by its shared sequence with galanin and is instead imparted by the C-terminal portion of the GALP molecule, then I predicted that GALP(22-60) would mimic the effects of full-length GALP.

Feeding and Body Weight

Methods: Adult male wild-type mice received two ICV injections of the vehicle (aCSF; n=7), 5 nmol GALP (n=6), 5 nmol GALP(1-21) (n=6) or 5 nmol GALP(22-60) (n=6) at 1700 h and 0800 h the following morning. Food intake and body weight were measured at 14 and 24 h after the first injection. Differences in food intake and body weight were determined by one-way ANOVA. When the ANOVA indicated a significant treatment effect, differences between individual treatment groups were assessed by Fisher's post hoc test. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Although the GALP-treated mice ate less food over the 24 h period than the vehicle-treated mice, the difference only reached statistical significance at the 14 h time point ($P < 0.005$; Figure 25A and 25B). However, body weight was significantly reduced in the GALP-treated mice at 24 h after the first injection ($P < 0.0001$ *vs.* vehicle-treated mice; Figure 25C). Food intake and body weight in the mice injected

with GALP(1-21) or GALP(22-60) did not differ from those of the vehicle-treated animals.

Serum LH Concentrations

Methods: Adult male wild-type mice received a single ICV injection of the vehicle (aCSF; n=7), 5 nmol GALP (n=7), 5 nmol GALP(1-21) (n=7) or 5 nmol GALP(22-60) (n=7) between 0900 h and 1130 h. Blood samples were obtained by orbital bleed at 30 min following the injection. Serum LH concentrations were measured by RIA. Differences in serum LH levels were determined by one-way ANOVA, which was followed by Fisher's post hoc test to identify differences between individual treatment groups. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Serum LH levels were significantly elevated in the GALP-treated mice at 30 min following the injection ($P < 0.0001$ *vs.* vehicle-treated mice; Figure 26). In contrast, ICV injections of GALP(1-21) or GALP(22-60) were without effect on serum LH concentrations.

Experiment 18. Effects of Long GALP Fragments on Feeding, Body Weight, and Serum LH

In the previous experiment, neither GALP(1-21) nor GALP(22-60) had any discernable effect on food intake, body weight, or LH release in wild-type mice. This observation could indicate that a larger component of the GALP peptide is necessary for

activation of its cognate receptor(s). In this experiment, I tested the effects of two longer GALP fragments [GALP(1-56) and GALP(3-60)] on feeding, body weight, and LH secretion in wild-type mice. Given that these fragments are only three or four amino acid residues shorter in length than full-length GALP, I hypothesized that both GALP fragments would mimic the effects of full-length GALP on the above-mentioned parameters.

Feeding and Body Weight

Methods: Adult male wild-type mice received two ICV injections of the vehicle (aCSF; n=5), 5 nmol GALP (n=6), 5 nmol GALP(1-56) (n=6) or 5 nmol GALP(3-60) (n=5) at 1700 h and 0800 h the following morning. Food intake and body weight were measured at 14 and 24 h after the first injection. Differences in food intake and body weight were determined by one-way ANOVA. When the ANOVA indicated a significant treatment effect, differences between individual treatment groups were assessed by Fisher's post hoc test. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Food intake was significantly reduced in the mice that were treated with GALP, GALP(1-56) or GALP(3-60) after both 14 and 24 h ($P < 0.0005$ *vs.* vehicle-treated mice; Figure 27A and 27B). Likewise, body weight was significantly reduced in the mice that were injected with GALP ($P < 0.005$), GALP(1-56) ($P < 0.0005$) or GALP(3-60) ($P < 0.005$) compared to the vehicle-treated animals (Figure 27C).

Serum LH Concentrations

Methods: One week following the feeding/body weight experiment, the treatment groups were shuffled and the same wild-type mice were given an ICV injection of the vehicle (aCSF; n=7), 5 nmol GALP (n=6), 5 nmol GALP(1-56) (n=6) or 5 nmol GALP(3-60) (n=6) between 0800 h and 1100 h. Blood was obtained by orbital bleed at 30 min following the injection, and serum LH concentrations were measured by RIA. Differences in serum LH concentrations were assessed by one-way ANOVA, which was followed by Fisher's post hoc test to identify differences between individual treatment groups. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SEM.

Results: Mice injected with GALP ($P < 0.05$) or GALP(3-60) ($P < 0.0005$) had significantly elevated serum LH levels compared to the vehicle-treated animals (Figure 28). Serum LH levels were also elevated in the mice that were treated with GALP(1-56); however, the difference did not reach statistical significance ($P = 0.18$).

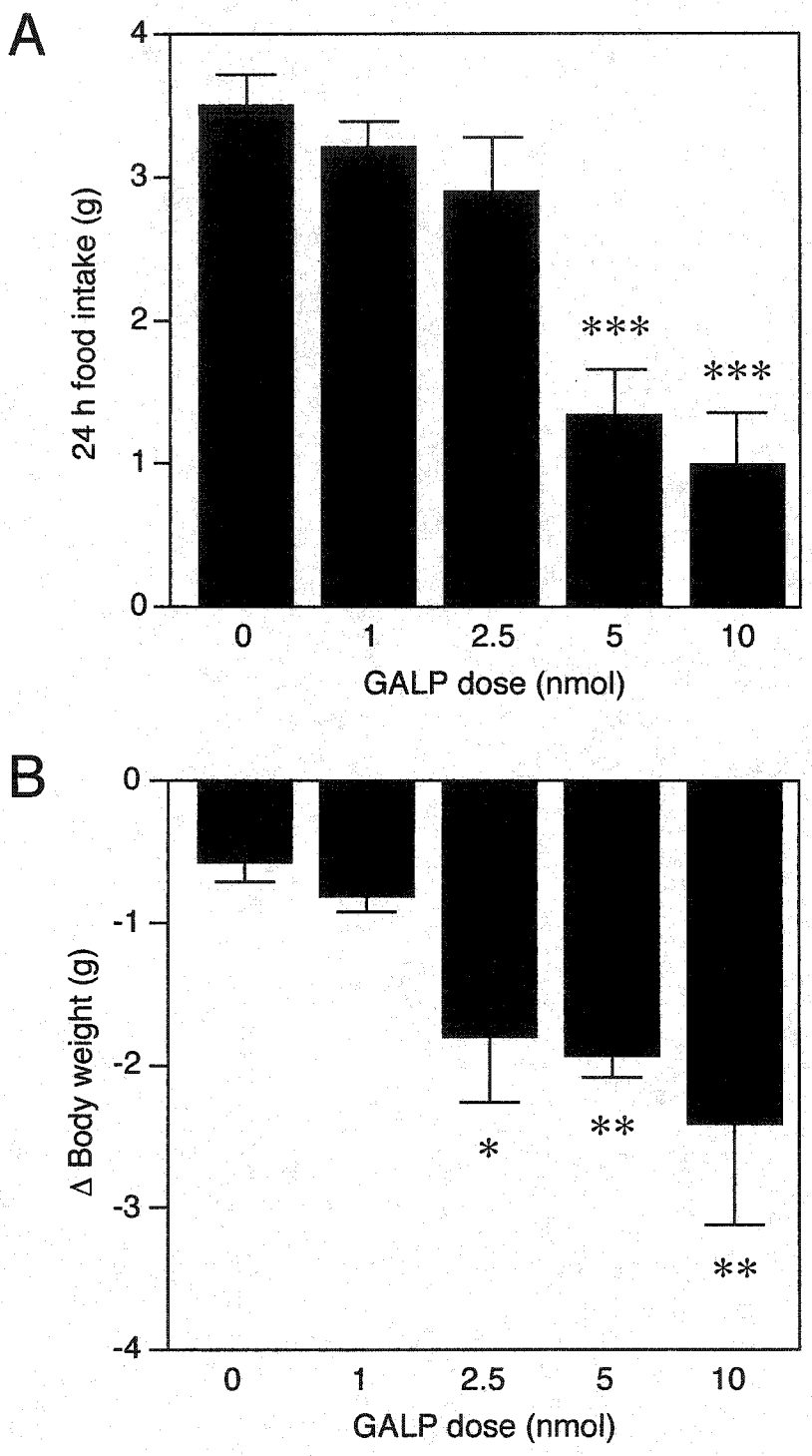
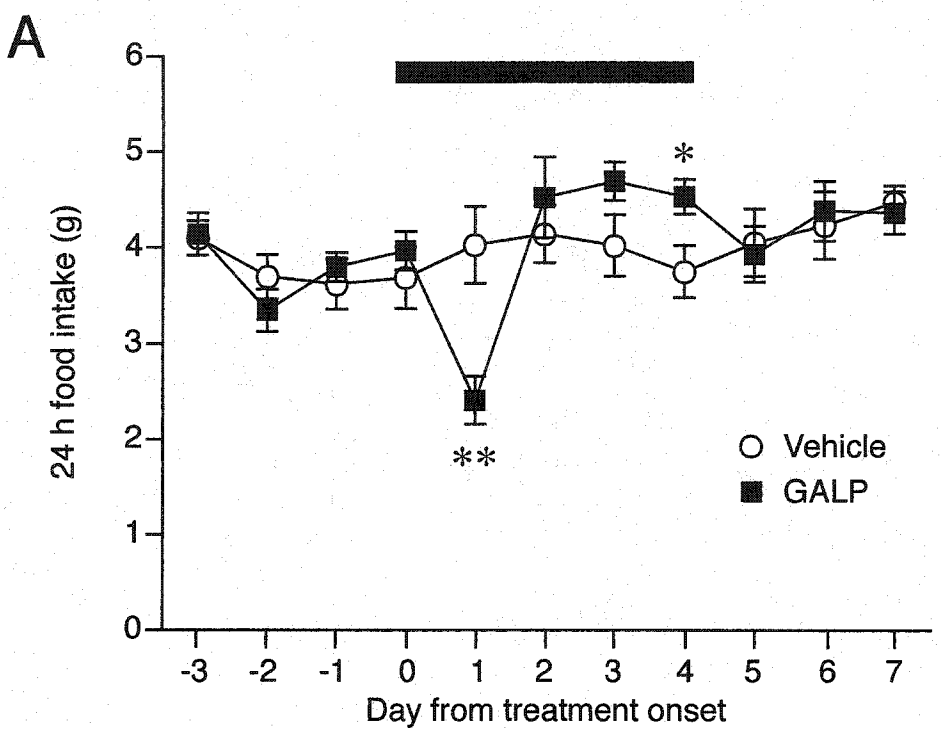
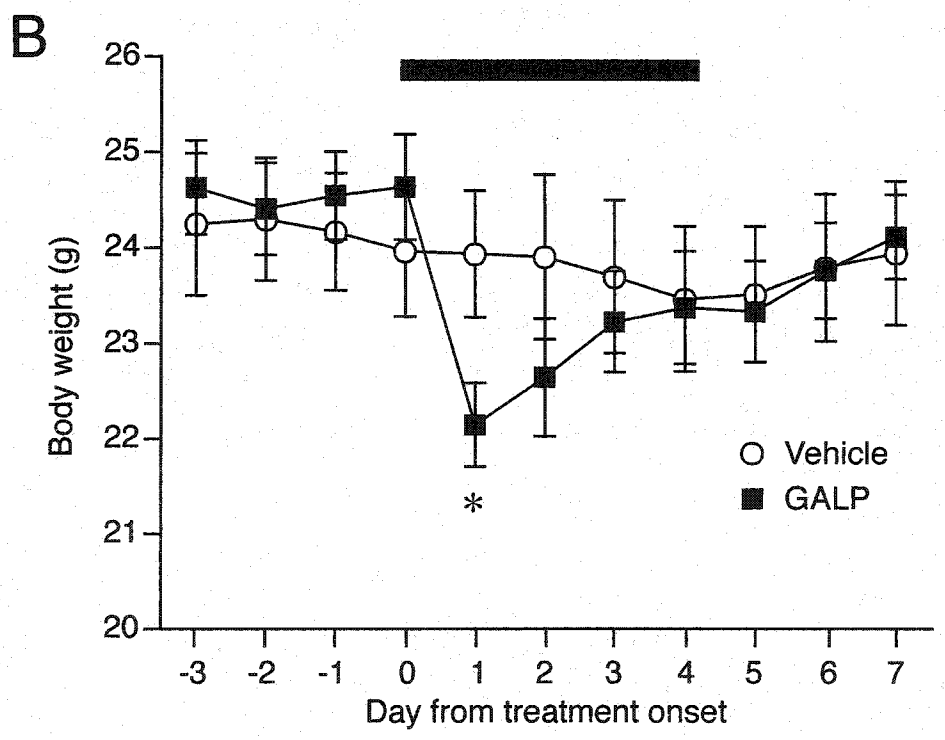


Figure 1. Dose-Dependent Effects of GALP on Feeding and Body Weight.
A. Food intake and B. body weight change in male mice at 24 h after the first of two ICV injections of vehicle or GALP (1, 2.5, 5 or 10 nmol). Data are presented as means ± SEM. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$ *vs.* vehicle-treated mice.

Figure 2. Effects of Long-Term GALP Treatment on Feeding and Body Weight. A. Daily food intake and B. body weight during the course of long-term treatment with vehicle (○) or 5 nmol GALP (■). Male mice received two ICV injections per day for 4.5 days. The solid black bars at the top of the graphs indicate the duration of treatment. Data are presented as means ± SEM. $*P < 0.05$; $**P < 0.005$ *vs*. vehicle-treated mice.


A
24 h food intake (g)
0
1
2
3
4
5
6
-3
-2
-1
0
1
2
3
4
5
6
7
Day from treatment onset
*
**
Vehicle
GALP


B
Body weight (g)
20
21
22
23
24
25
26
-3
-2
-1
0
1
2
3
4
5
6
7
Day from treatment onset
*
Vehicle
GALP

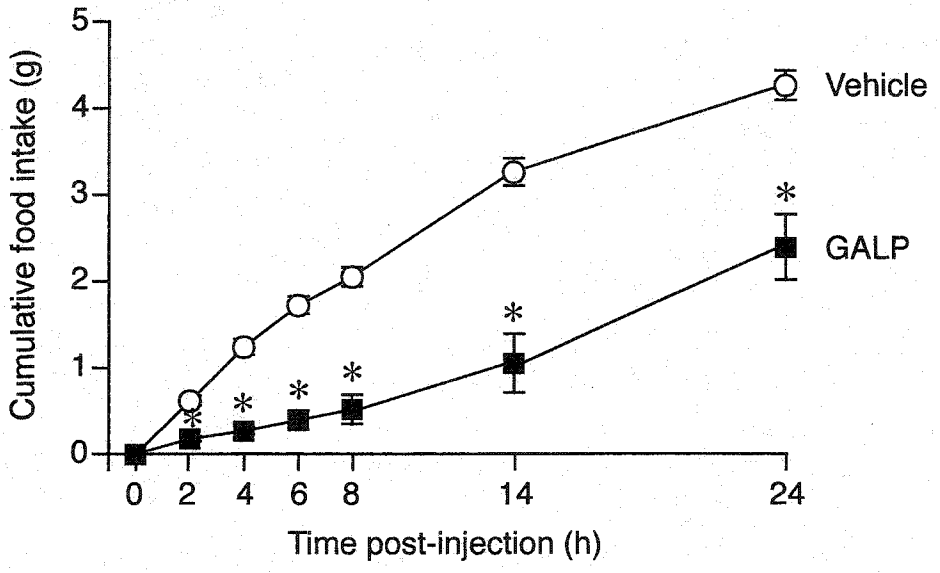


Figure 3. Time-Course of GALP's Inhibition of Feeding. Cumulative food intake in male mice at 0, 2, 4, 6, 8, 14, and 24 h following a single ICV injection of vehicle (○) or 5 nmol GALP (■). Data are presented as means ± SEM. *$P < 0.001$ *vs.* vehicle-treated mice.

Figure 4. Effects of ICV GALP on Feeding and Body Weight in Mice. A. 1 and 2 h food intake, B. 24 h food intake, and C. 24 h body weight change in male mice receiving a single morning ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars). Data are presented as means ± SEM. $^{*}P < 0.05$ *vs*. vehicle-treated mice.

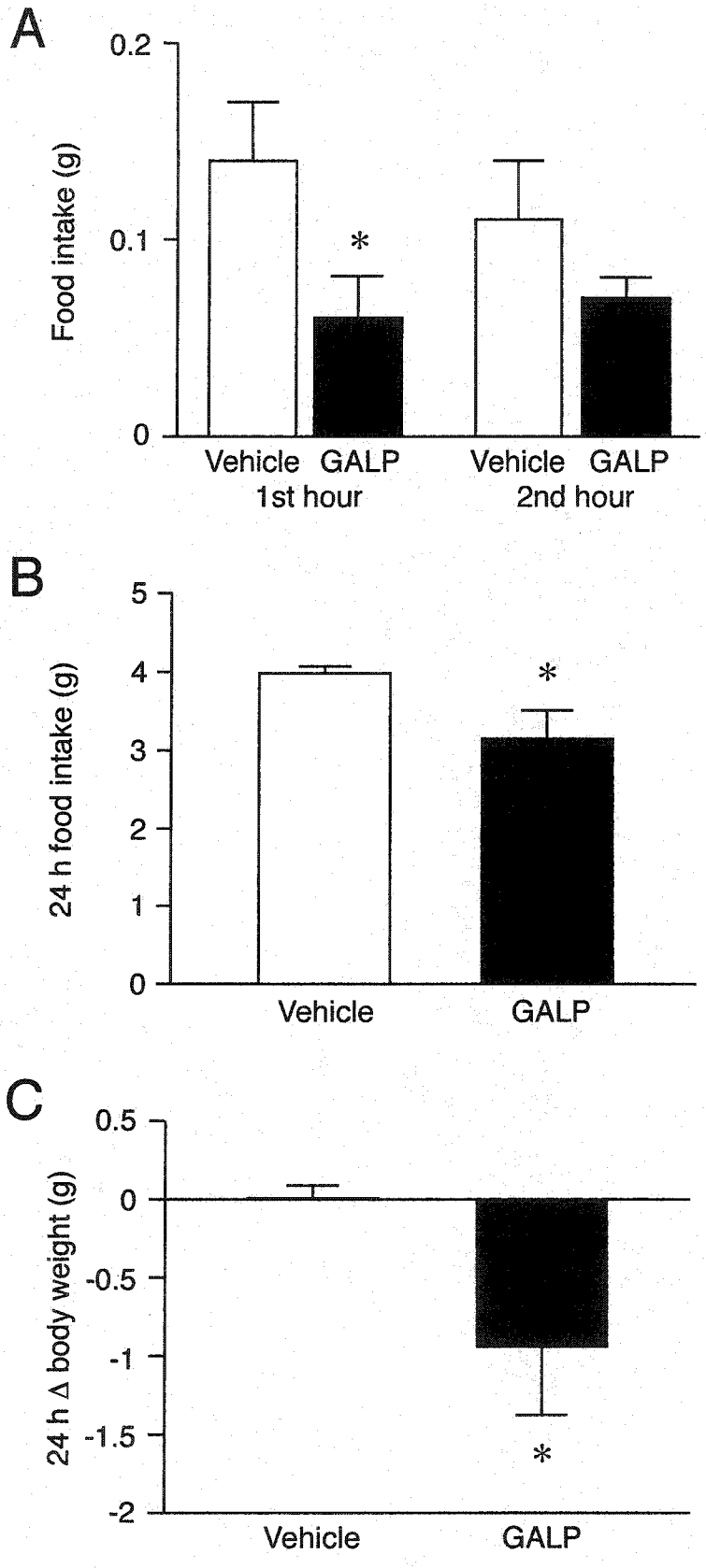
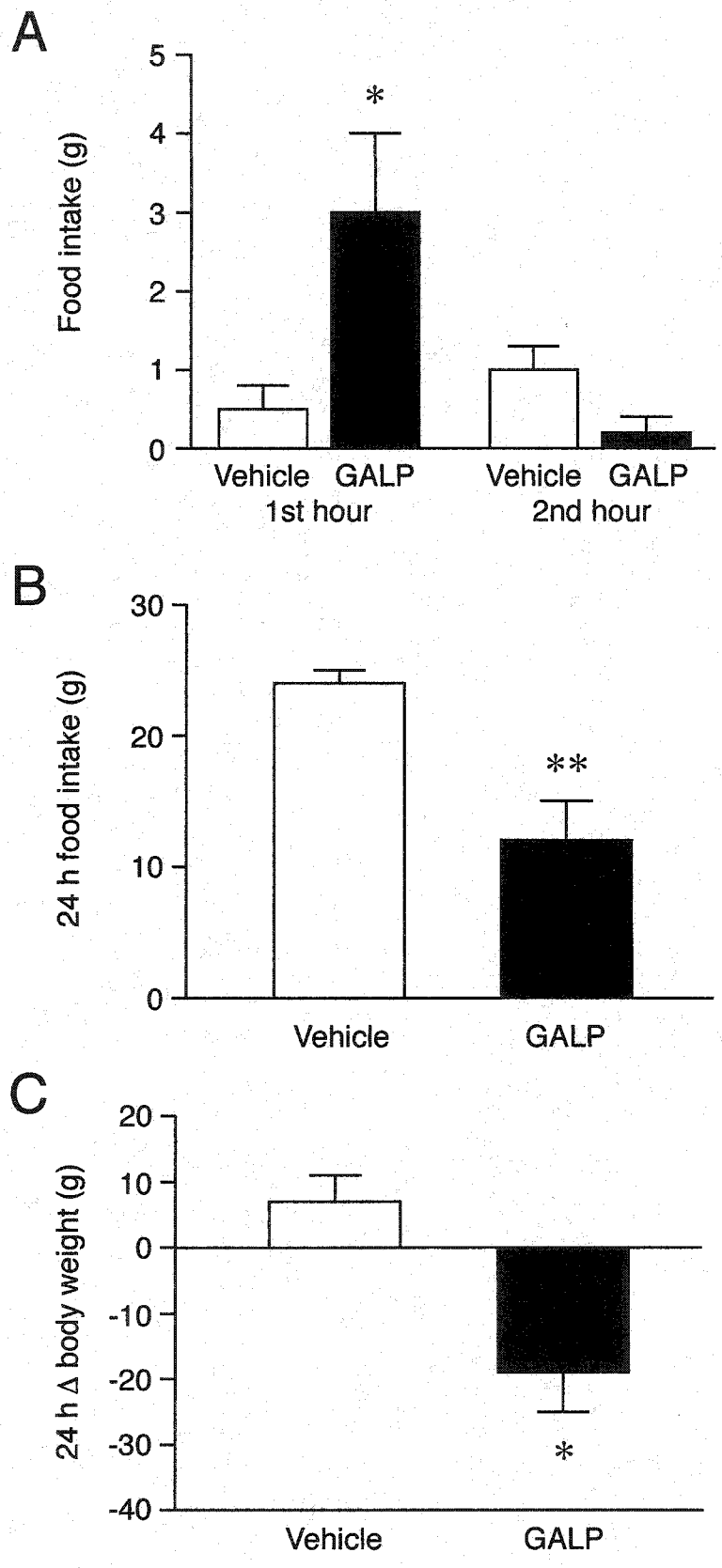

A
0.2
0.1
0
Food intake (g)
*
Vehicle
GALP
1st hour
Vehicle
GALP
2nd hour
B
5
4
3
2
1
0
24 h food intake (g)
*
Vehicle
GALP
C
0.5
0
-0.5
-1
-1.5
-2
24 h Δ body weight (g)
*
Vehicle
GALP

Figure 5. Effects of ICV GALP on Feeding and Body Weight in Rats. A. 1 and 2 h food intake, B. 24 h food intake, and C. 24 h body weight change in male rats receiving a single morning ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars). Data are presented as means ± SEM. $*P < 0.05$; $**P < 0.005$ *vs*. vehicle-treated rats.


A
Food intake (g)
5
4
3
2
1
0
*
Vehicle
GALP
1st hour
Vehicle
GALP
2nd hour
B
24 h food intake (g)
30
20
10
0
**
Vehicle
GALP
C
24 h Δ body weight (g)
20
10
0
-10
-20
-30
-40
*
Vehicle
GALP

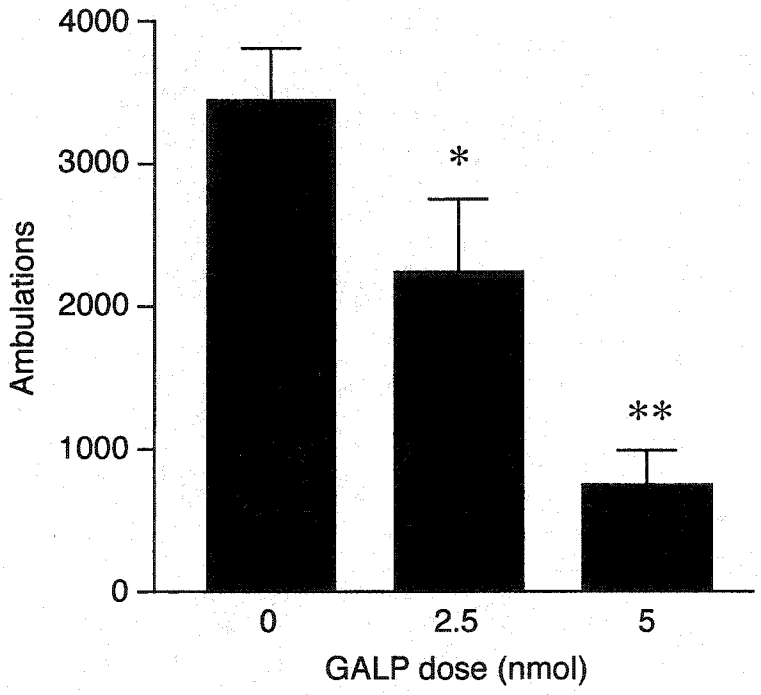


Figure 6. Dose-Dependent Effects of GALP on Locomotor Activity. Locomotor activity (presented as the total number of ambulations over 14 h) in male mice following a single ICV injection of 0, 2.5 or 5 nmol GALP. Data are presented as means ± SEM. $*P < 0.05$; $**P < 0.0005$ *vs*. vehicle-treated mice.

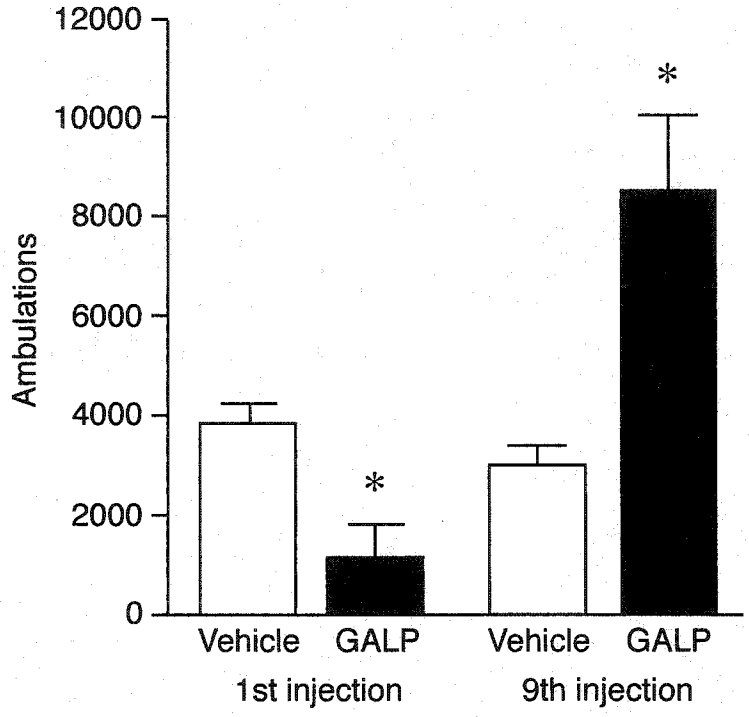
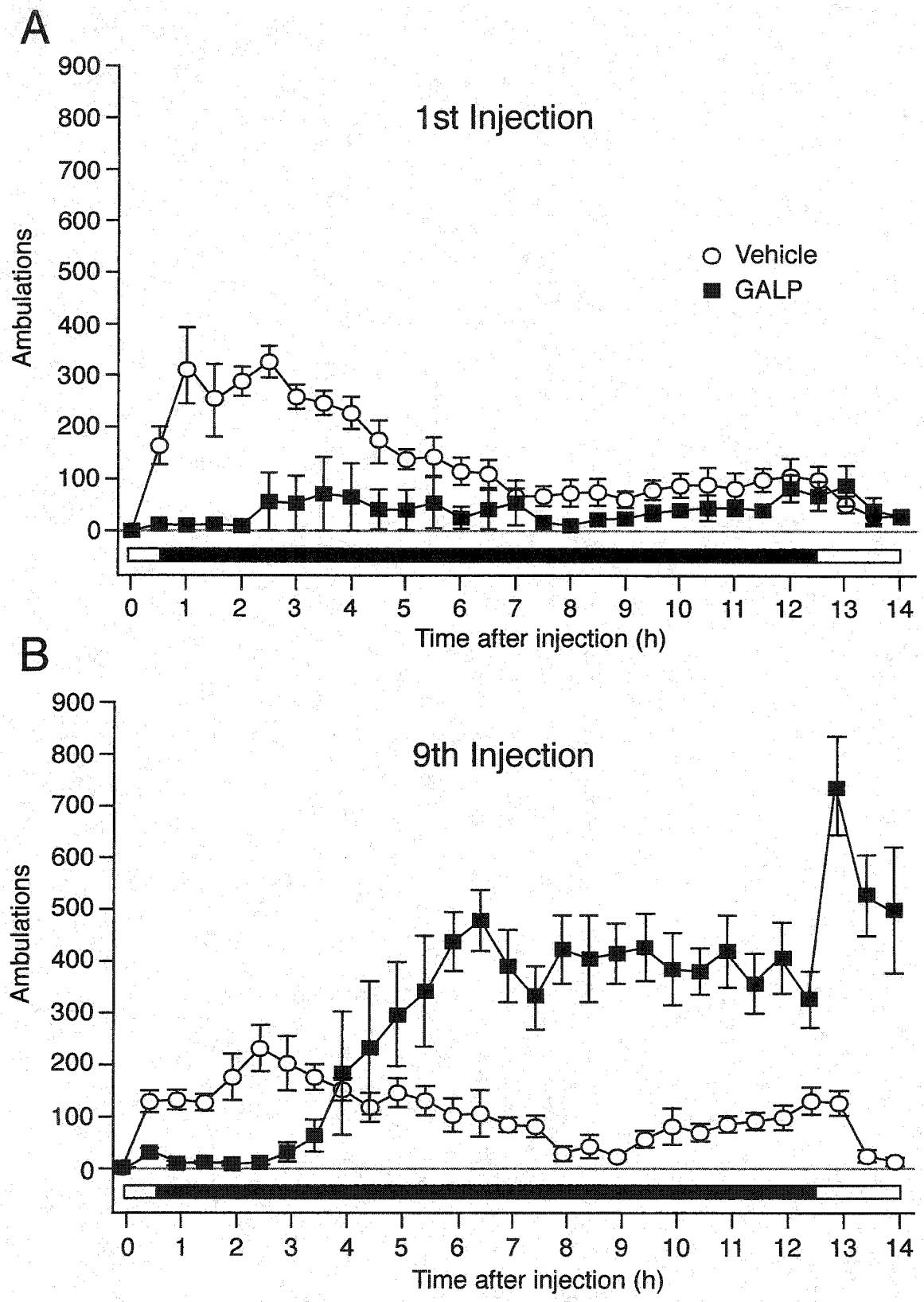


Figure 7. Differential Effects of Acute and Long-Term GALP Treatment on Locomotor Activity. Locomotor activity (presented as the total number of ambulations over 14 h) during long-term treatment with vehicle (open bars) or 5 nmol GALP (closed bars). Male mice received two ICV injections per day for 4.5 days, and activity levels were measured for 14 h following the first and ninth injections. Data are presented as means ± SEM. *P< 0.005 *vs*. vehicle-treated mice.

Figure 8. Time Course of GALP's Acute and Long-Term Effects on Locomotor Activity. Locomotor activity profiles in male mice that received nine ICV injections of vehicle (○) or 5 nmol GALP (■) over the course of 4.5 days. Locomotor activity was measured after the first and ninth injections and is plotted in 30 minute bins. The white/black bars at the bottom of each graph indicate the timing of lights on (white) and lights off (black). Data are presented as means ± SEM.


A
1st Injection
Vehicle
GALP
Ambulations
900
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time after injection (h)
B
9th Injection
Ambulations
900
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time after injection (h)

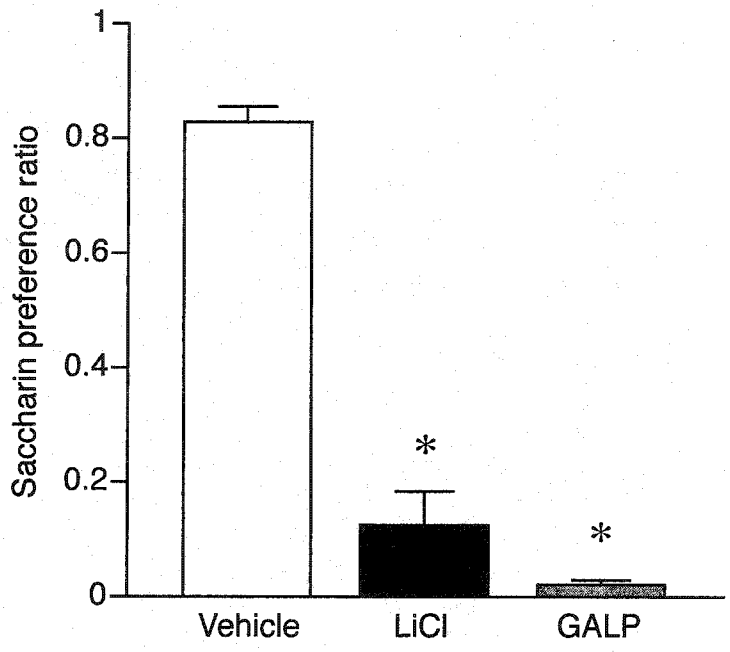
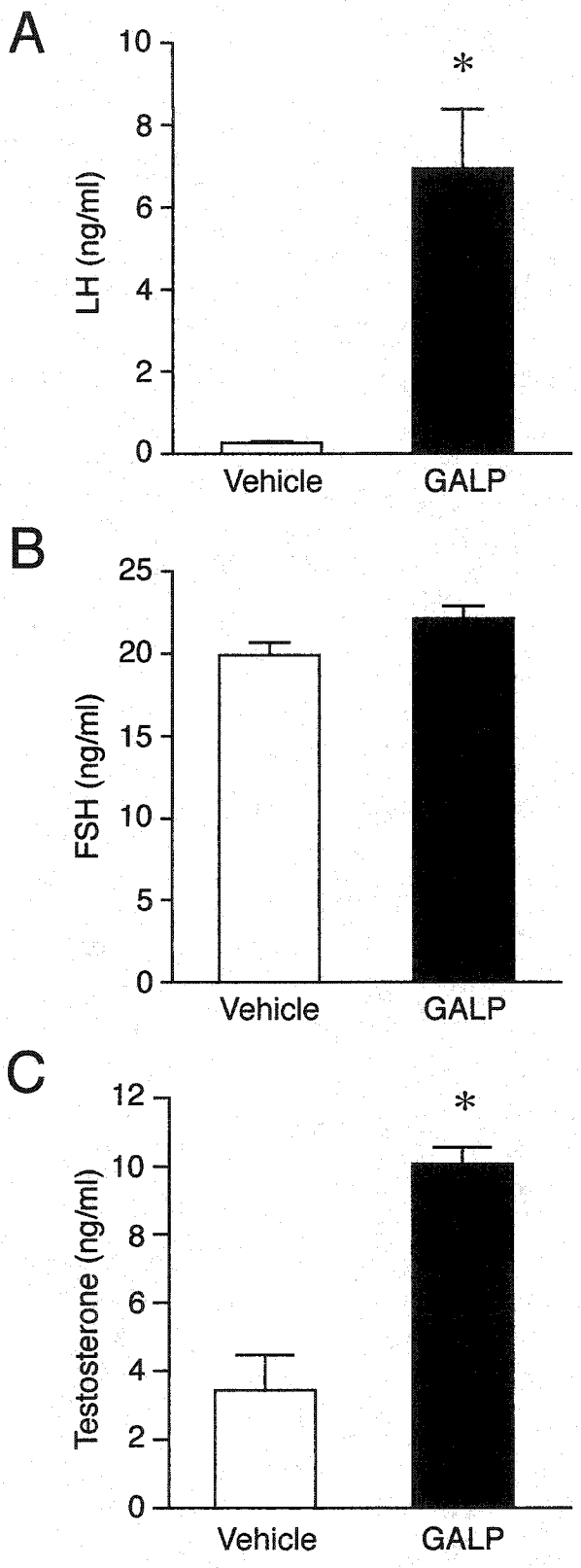


Figure 9. Conditioned Taste Aversion Learning in GALP-Treated Mice. 2 h saccharin preference ratios in male mice that were treated with vehicle (ICV), LiCl (0.15 M, 2% body weight; ip) or GALP (5 nmol; ICV) 48 h earlier. Data are presented as means ± SEM. *$P < 0.0001$ *vs.* vehicle-treated mice.

Figure 10. Effects of GALP on Serum Gonadotropin and Testosterone Concentrations in Male Wild-Type Mice. Serum concentrations of A. LH, B. FSH, and C. testosterone in male mice at 30 min following a single ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars). Data are presented as means ± SEM. *$P < 0.0005$ *vs*. vehicle-treated mice.


A
LH (ng/ml)
10
8
6
4
2
0
*
Vehicle
GALP
B
FSH (ng/ml)
25
20
15
10
5
0
Vehicle
GALP
C
Testosterone (ng/ml)
12
10
8
6
4
2
0
*
Vehicle
GALP

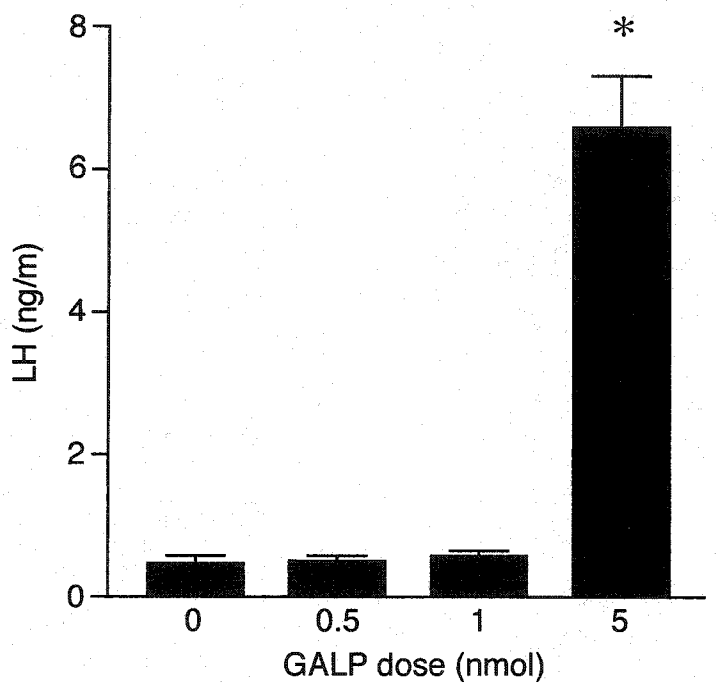


Figure 11. Dose-Dependent Effect of GALP on Serum LH Concentrations. Serum LH concentrations in male mice at 30 min following a single ICV injection of 0, 0.5, 1 or 5 nmol GALP. Data are presented as means ± SEM. *$P < 0.0001$ *vs*. vehicle-treated mice.

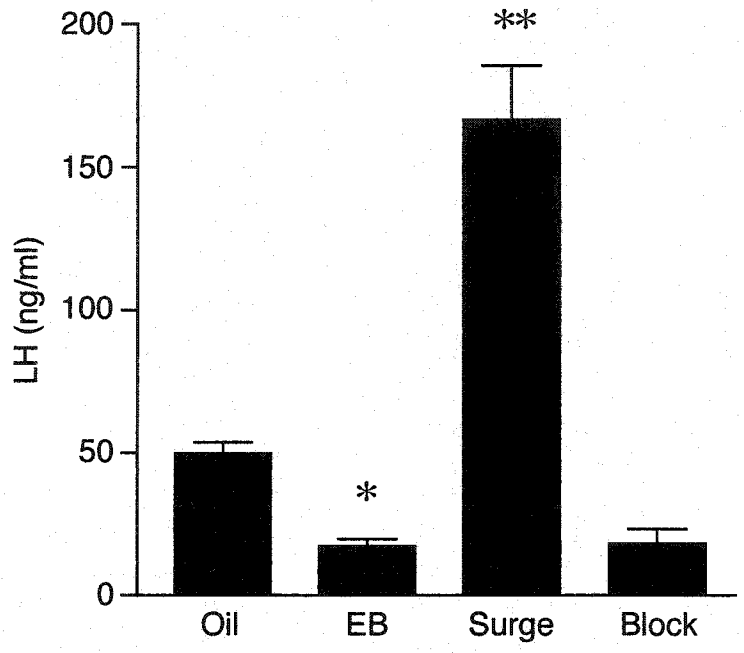


Figure 12. Serum LH Concentrations in Ovarian Steroid-Treated Female Rats. Serum LH concentrations in OVX female rats that were treated with peanut oil (Oil), estradiol benzoate (EB), estradiol benzoate, progesterone, and saline (Surge) or estradiol benzoate, progesterone, and pentobarbital (Block). The Oil and EB groups were killed at 24-26 h after the injections, while the Surge and Block groups were killed in the evening during the anticipated time of the LH surge. Data are presented as means ± SEM. $*P < 0.05$; $**P < 0.0001$ *vs.* Oil group.

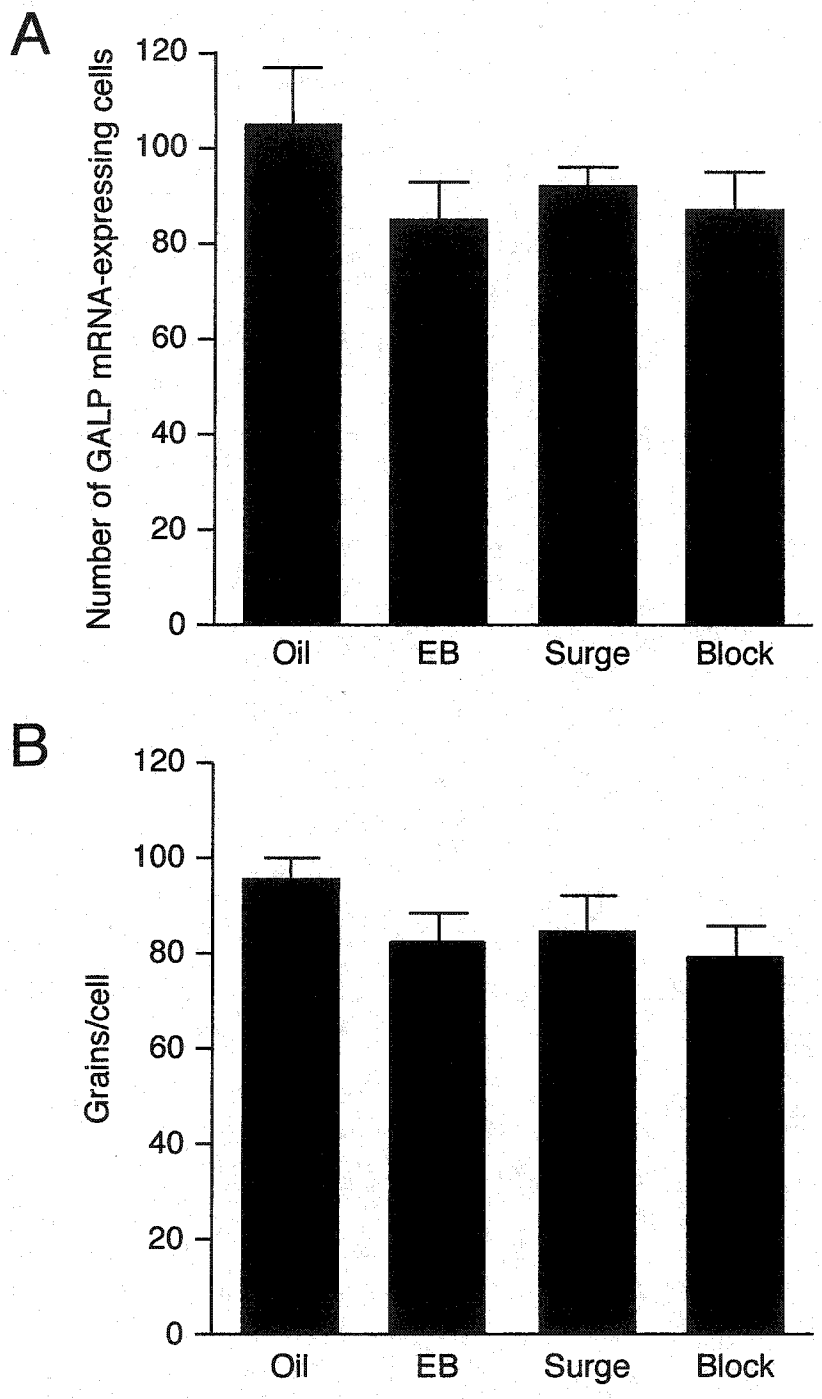


Figure 13. GALP mRNA Expression in Ovarian Steroid-Treated Female Rats. A. Number of GALP mRNA-expressing cells and B. cellular GALP mRNA content in OVX female rats that were treated with peanut oil (Oil), estradiol benzoate (EB), estradiol benzoate, progesterone, and saline (Surge) or estradiol benzoate, progesterone, and pentobarbital (Block). Data are presented as means ± SEM.

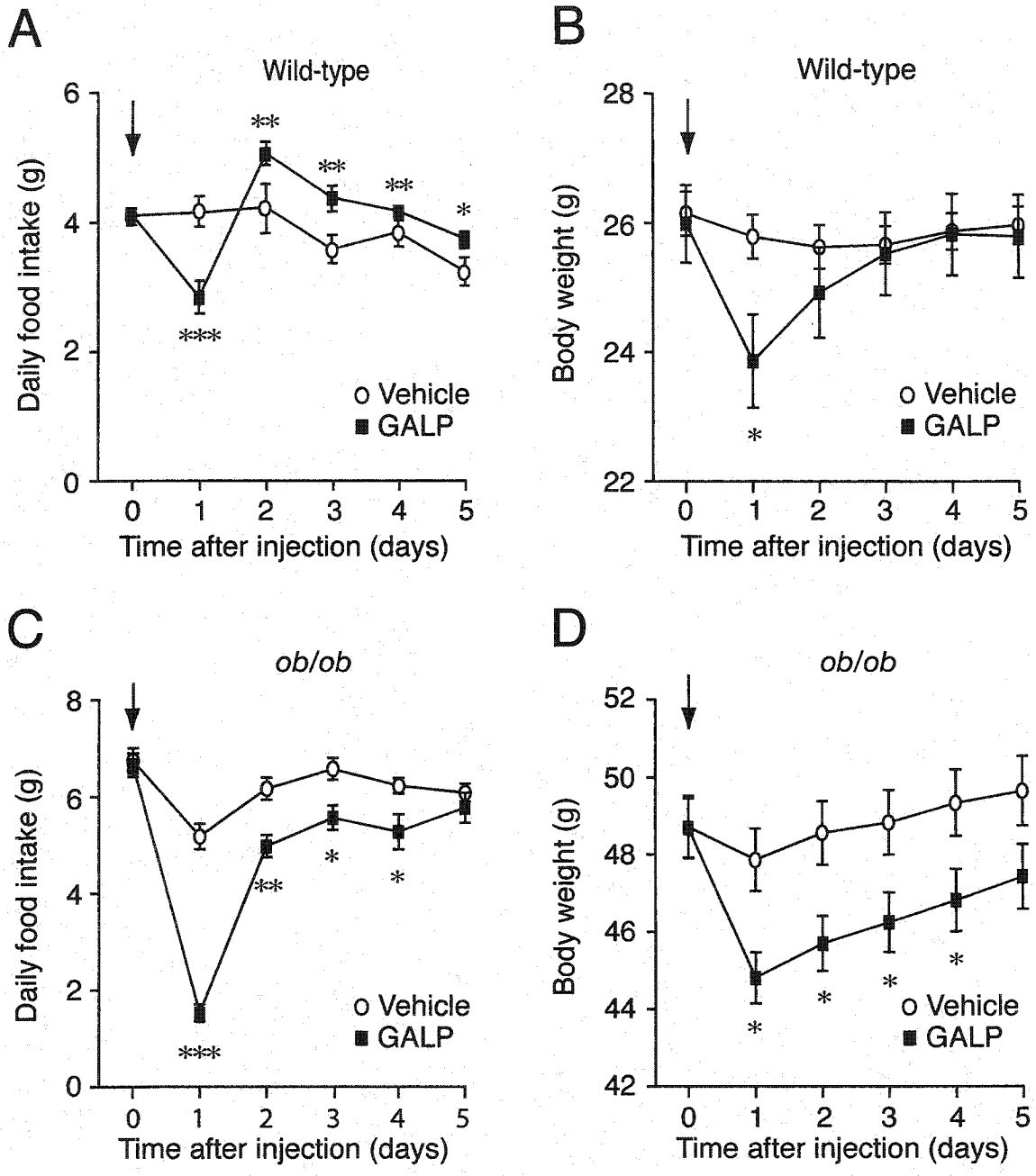


Figure 14. Effects of GALP on Feeding and Body Weight in Wild-Type and *ob/ob* Mice. A. Daily food intake and B. body weight in male wild-type mice that received a single ICV injection of vehicle (o) or 5 nmol GALP (■) on Day 0. C. Daily food intake and D. body weight in male *ob/ob* mice that received a single ICV injection of vehicle (o) or 5 nmol GALP (■) on Day 0. The arrow in each graph indicates when the injections were given. Data are presented as means ± SEM. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$ *vs*. vehicle-treated mice.

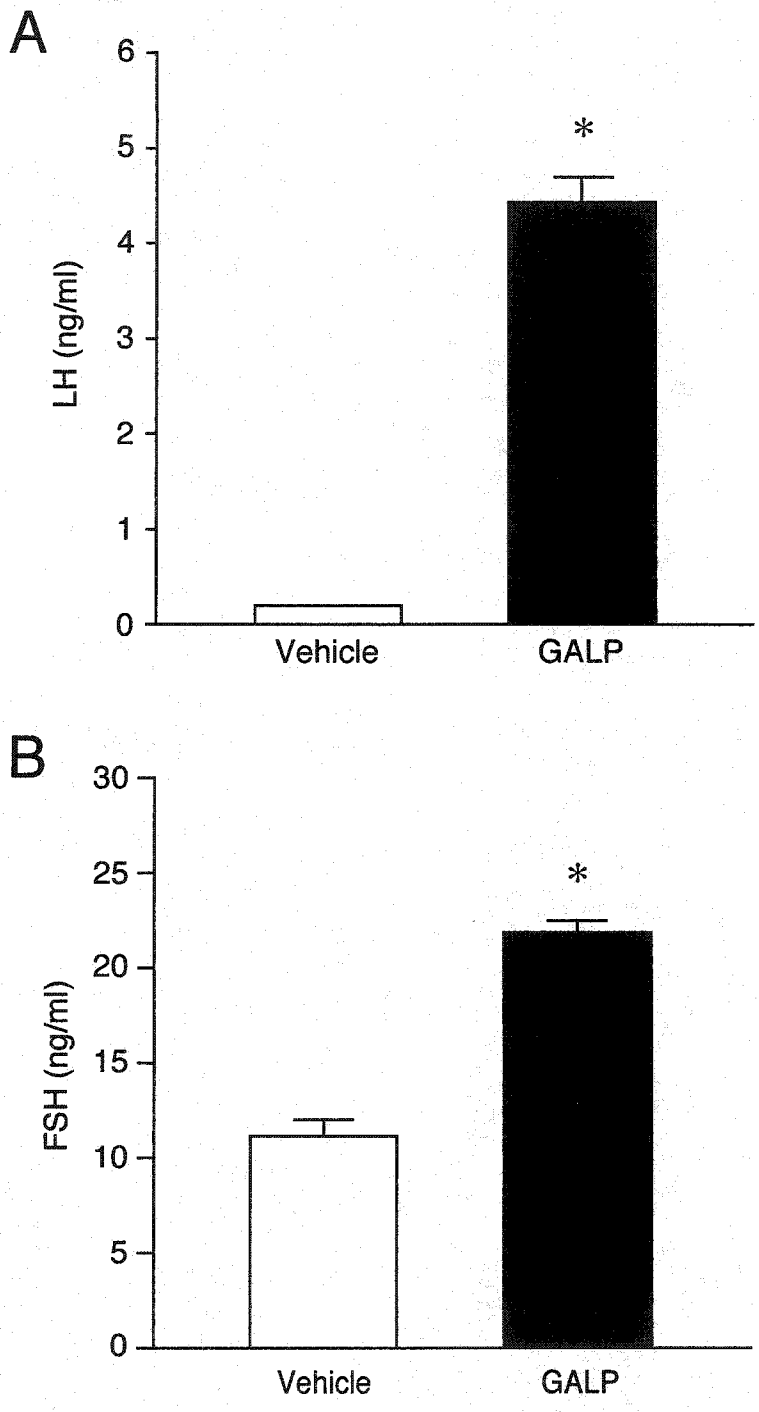


Figure 15. Effects of GALP on Serum Gonadotropin Concentrations in Male *ob/ob* Mice. Serum concentrations of A. LH and B. FSH in male *ob/ob* mice at 30 min following a single ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars). Data are presented as means ± SEM. $^*P < 0.0001$ *vs.* vehicle-treated mice.

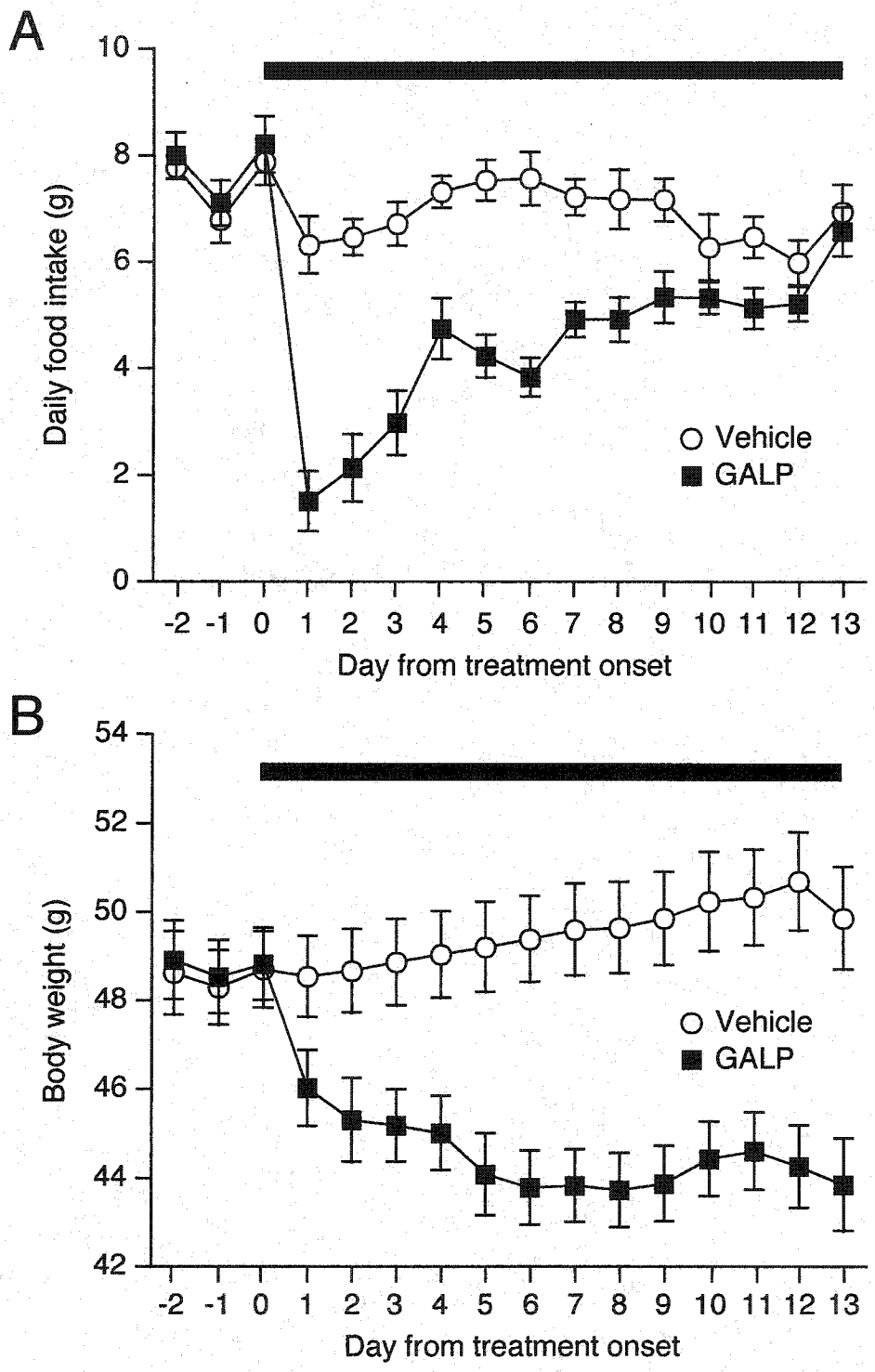


Figure 16. Effects of Long-Term GALP Treatment on Feeding and Body Weight in *ob/ob* Mice. A. Daily food intake and B. body weight in male *ob/ob* mice during long-term treatment with vehicle (○) or 5 nmol GALP (■). Mice received two ICV injections per day for 14 days, beginning on Day 0. The solid black bars at the top of the graphs indicate the duration of treatment. Data are presented as means ± SEM.

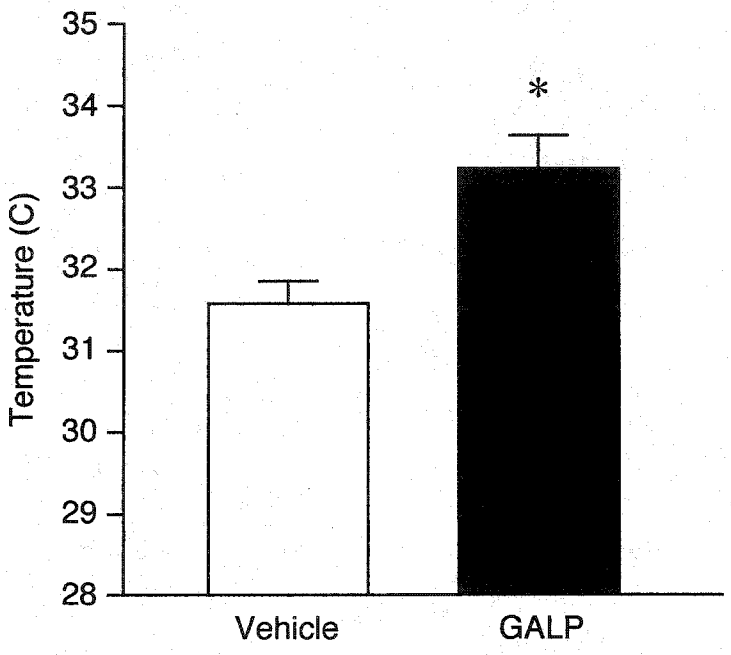


Figure 17. Effect of Long-Term GALP Treatment on Body Temperature in *ob/ob* Mice. Rectal temperature in male *ob/ob* mice following long-term treatment with vehicle (open bar) or 5 nmol GALP (closed bar). The mice received two ICV injections per day for 14 days, and temperature was measured 6-8 h after the final injection. Data are presented as means ± SEM. *$P < 0.005$ *vs.* vehicle-treated mice.

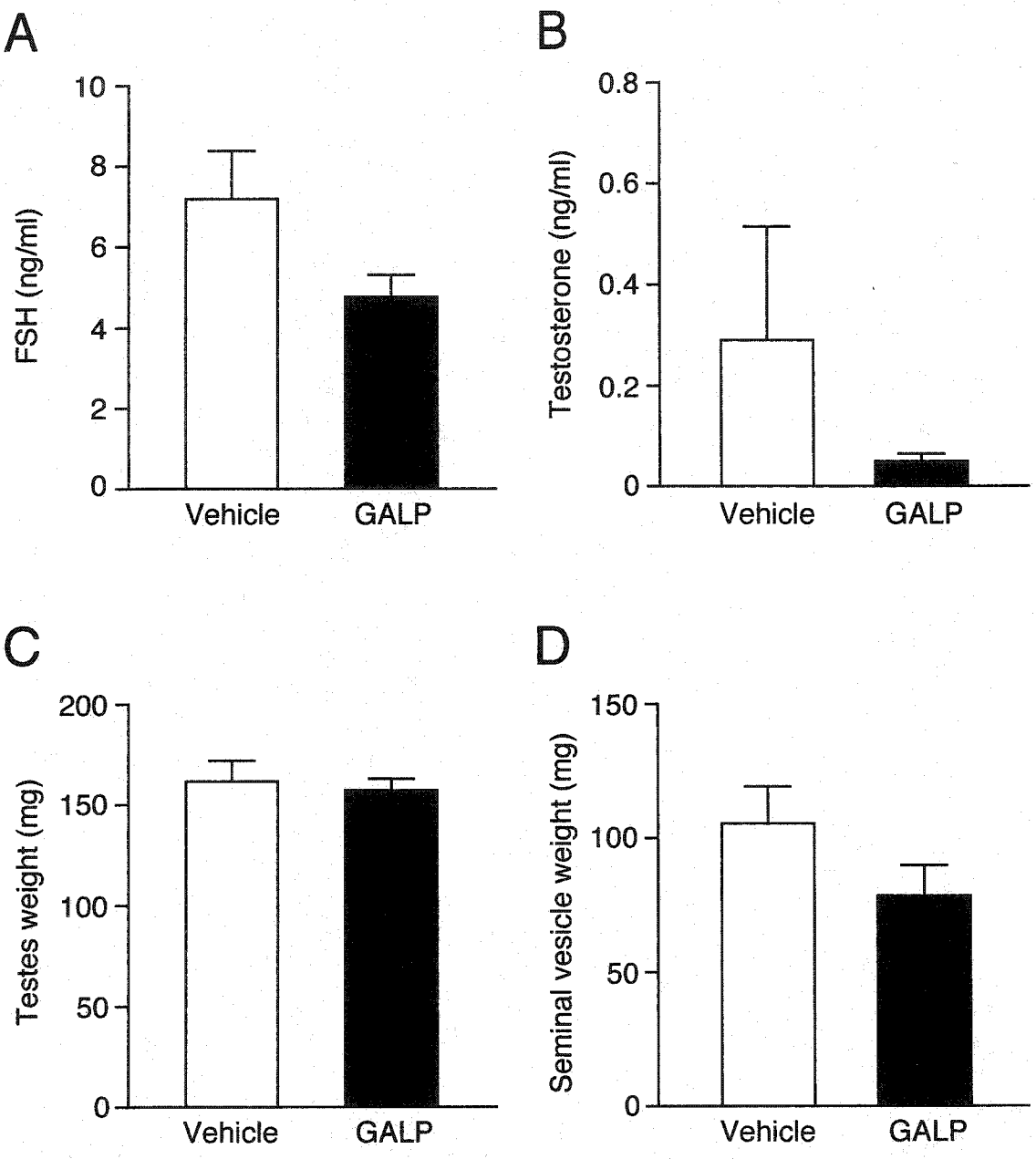


Figure 18. Effects of Long-Term GALP Treatment on Hormone Concentrations and Reproductive Organ Weights in *ob/ob* Mice. A. Serum FSH levels, B. serum testosterone levels, C. testes weight, and D. seminal vesicle weight in male *ob/ob* mice following long-term treatment with vehicle (open bars) or 5 nmol GALP (closed bars). The mice received two ICV injections per day for 14 days, and blood and organs were harvested at 6-8 h after the final injection. Data are presented as means ± SEM.

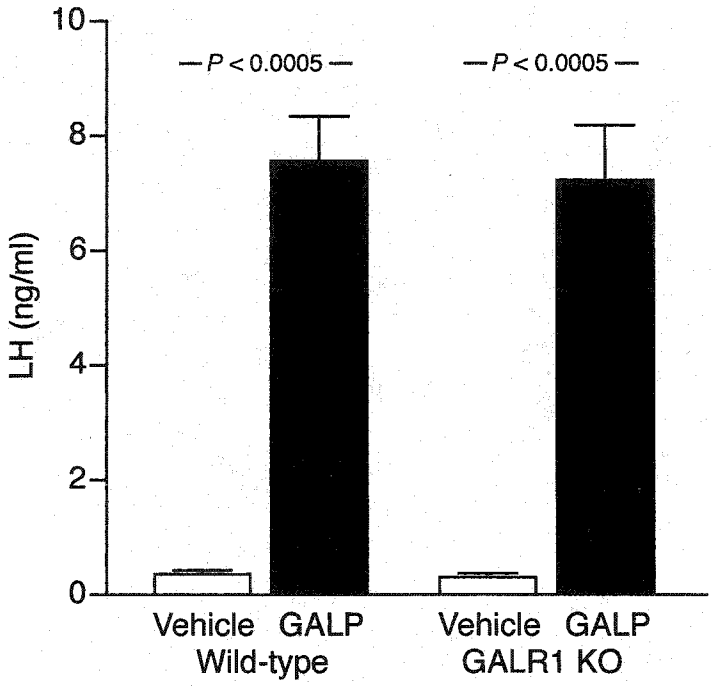


Figure 19. Serum LH Concentrations in GALP-Treated Wild-Type and GALR1 KO Mice. Serum LH concentrations at 30 min following a single ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars) in male wild-type and GALR1 KO mice. Data are presented as means ± SEM.

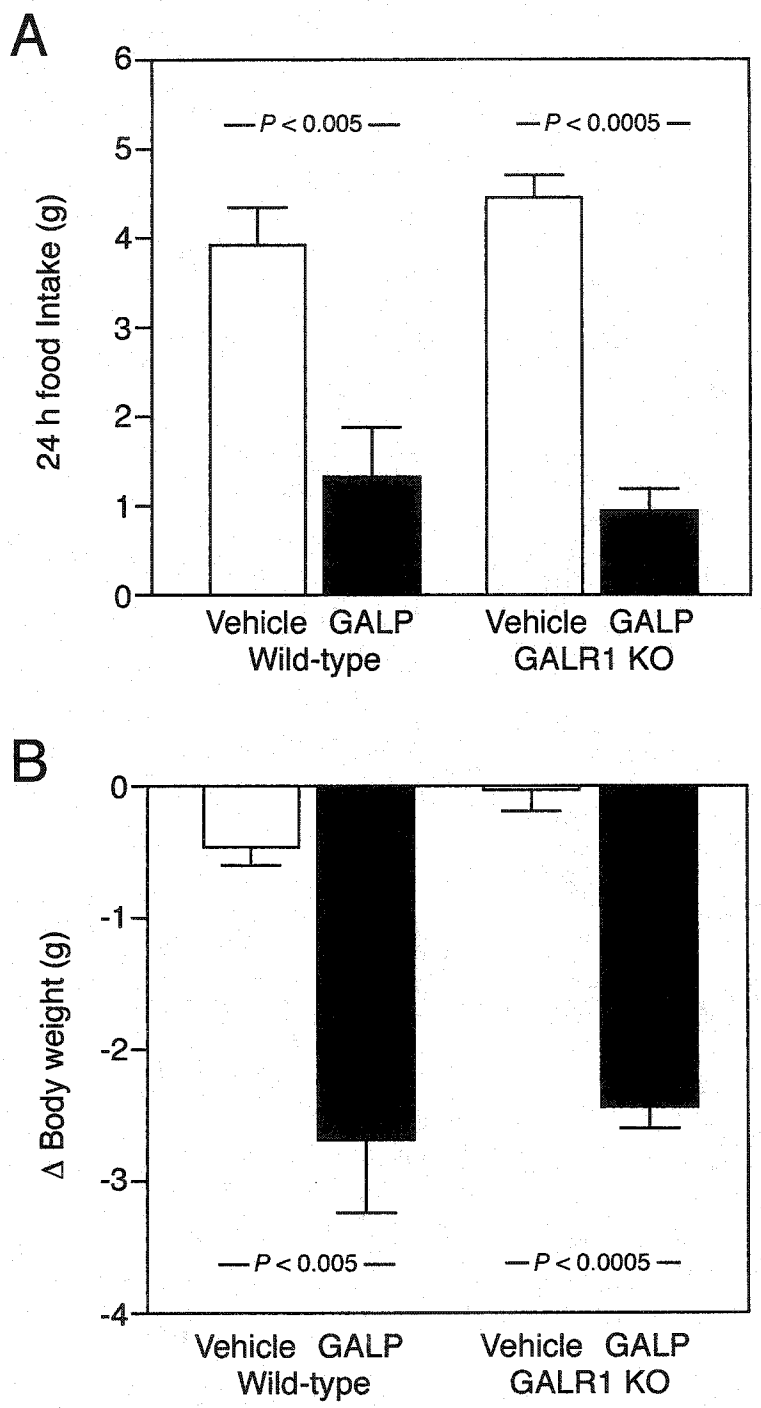


Figure 20. Feeding and Body Weight in GALP-Treated Wild-Type and GALR1 KO Mice. A. Food intake and B. body weight change over 24 h following the first of two ICV injections of vehicle (open bars) or 5 nmol GALP (closed bars) in male wild-type and GALR1 KO mice. Data are presented as means ± SEM.

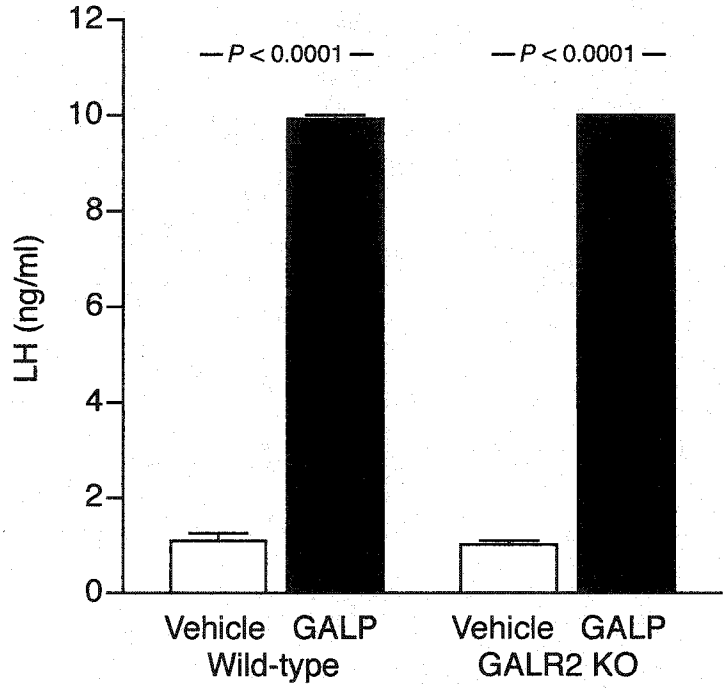


Figure 21. Serum LH Concentrations in GALP-Treated Wild-Type and GALR2 KO Mice. Serum LH concentrations at 30 min following a single ICV injection of vehicle (open bars) or 5 nmol GALP (closed bars) in male wild-type and GALR2 KO mice. Data are presented as means ± SEM.

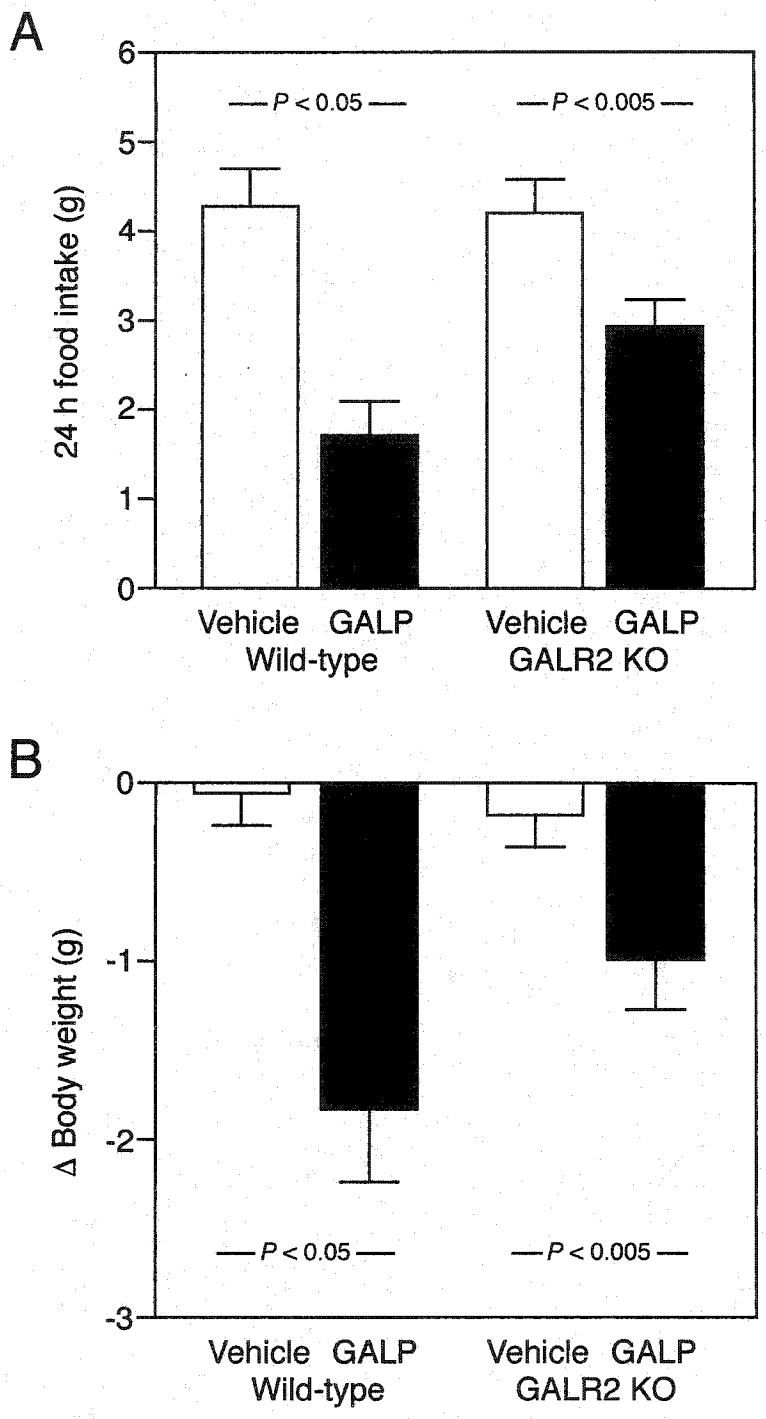


Figure 22. Feeding and Body Weight in GALP-Treated Wild-Type and GALR2 KO Mice. A. Food intake and B. body weight change over 24 h following the first of two ICV injections of vehicle (open bars) or 5 nmol GALP (closed bars) in male wild-type and GALR2 KO mice. Data are presented as means ± SEM.

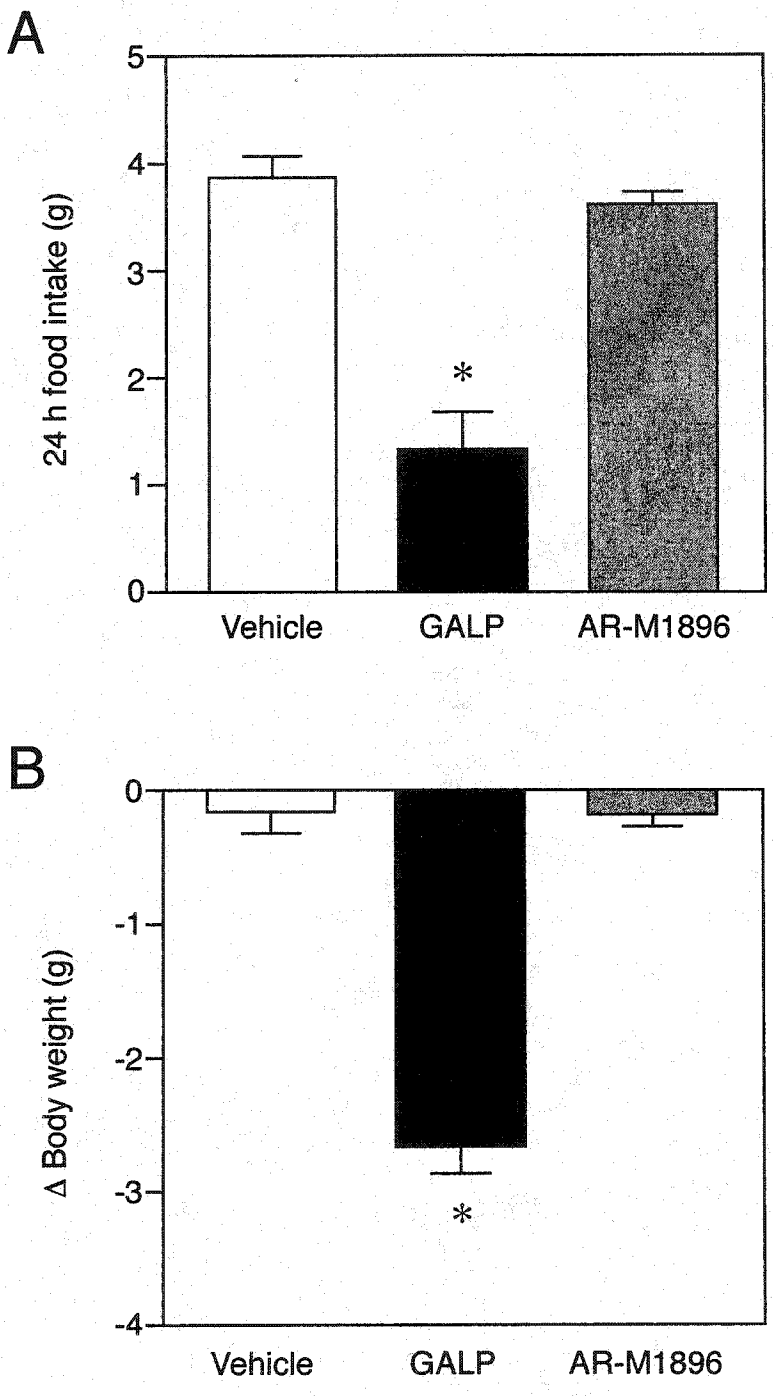


Figure 23. Effects of a GALR2 Agonist on Feeding and Body Weight. A. Food intake and B. body weight change at 24 h following the first of two ICV injections of vehicle, 5 nmol GALP or 5 nmol AR-M1896 in male wild-type mice. Data are presented as means ± SEM. *$P < 0.0001$ *vs.* vehicle-treated mice.

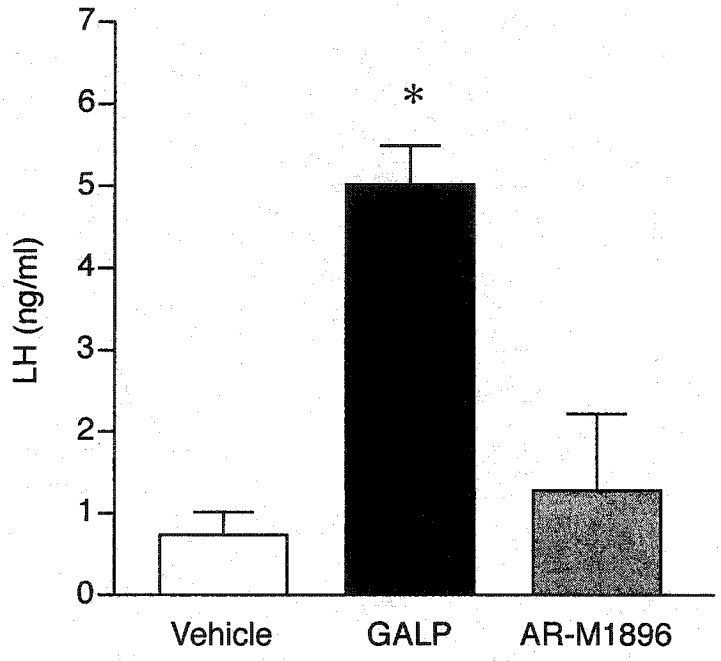
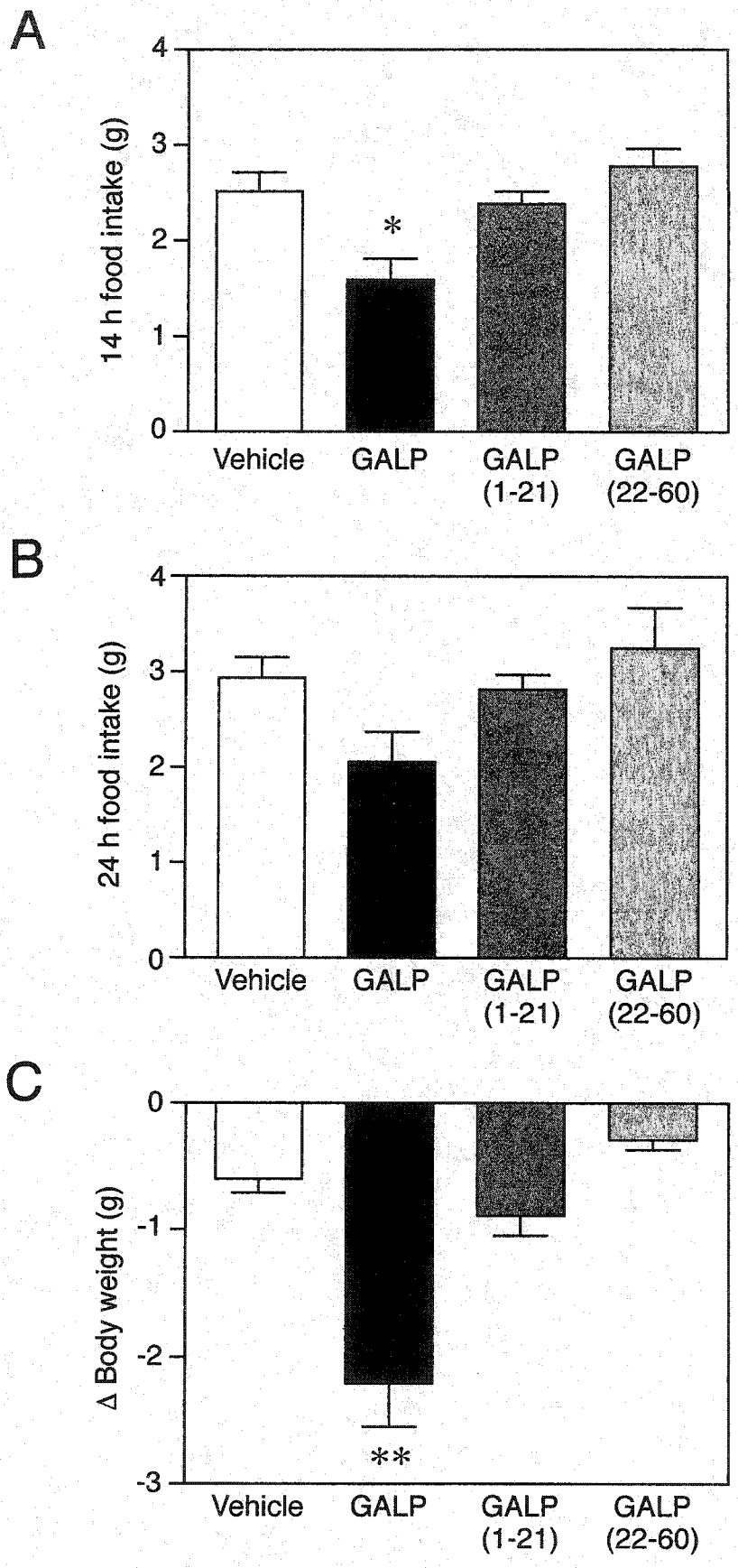


Figure 24. Effect of a GALR2 Agonist on Serum LH Concentrations. Serum LH concentrations at 30 min following a single ICV injection of vehicle, 5 nmol GALP or 5 nmol AR-M1896 in male wild-type mice. Data are presented as means ± SEM. *$P < 0.0005$ *vs*. vehicle-treated mice.

Figure 25. Effects of GALP(1-21) and GALP(22-60) on Feeding and Body Weight. A. 14 h food intake, B. 24 h food intake, and C. 24 h body weight change in male wild-type mice following two ICV injections of vehicle, 5 nmol GALP, 5 nmol GALP(1-21) or 5 nmol GALP(22-60). Data are presented as means ± SEM. $*P < 0.005$; $**P < 0.0001$ *vs*. vehicle-treated mice.


A
14 h food intake (g)
4
3
2
1
0
*
Vehicle
GALP
GALP (1-21)
GALP (22-60)
B
24 h food intake (g)
4
3
2
1
0
Vehicle
GALP
GALP (1-21)
GALP (22-60)
C
Δ Body weight (g)
0
-1
-2
-3
**
Vehicle
GALP
GALP (1-21)
GALP (22-60)

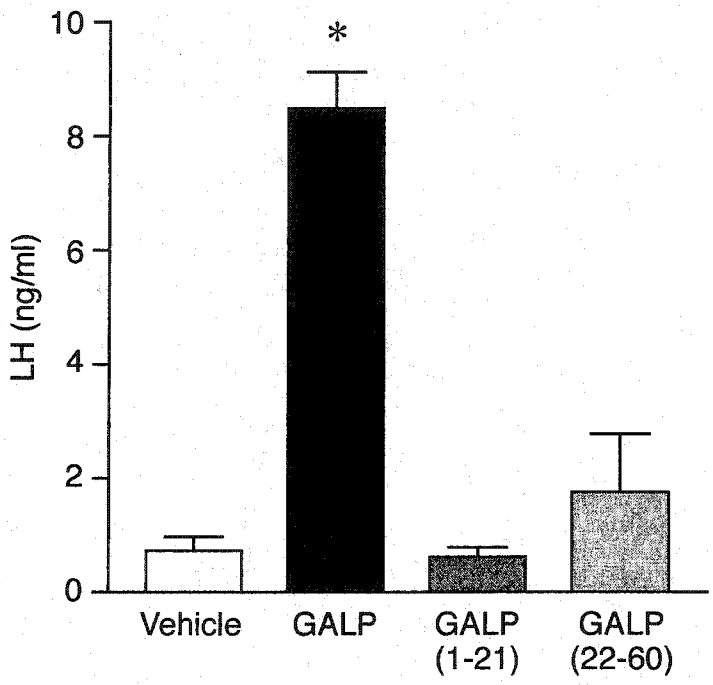
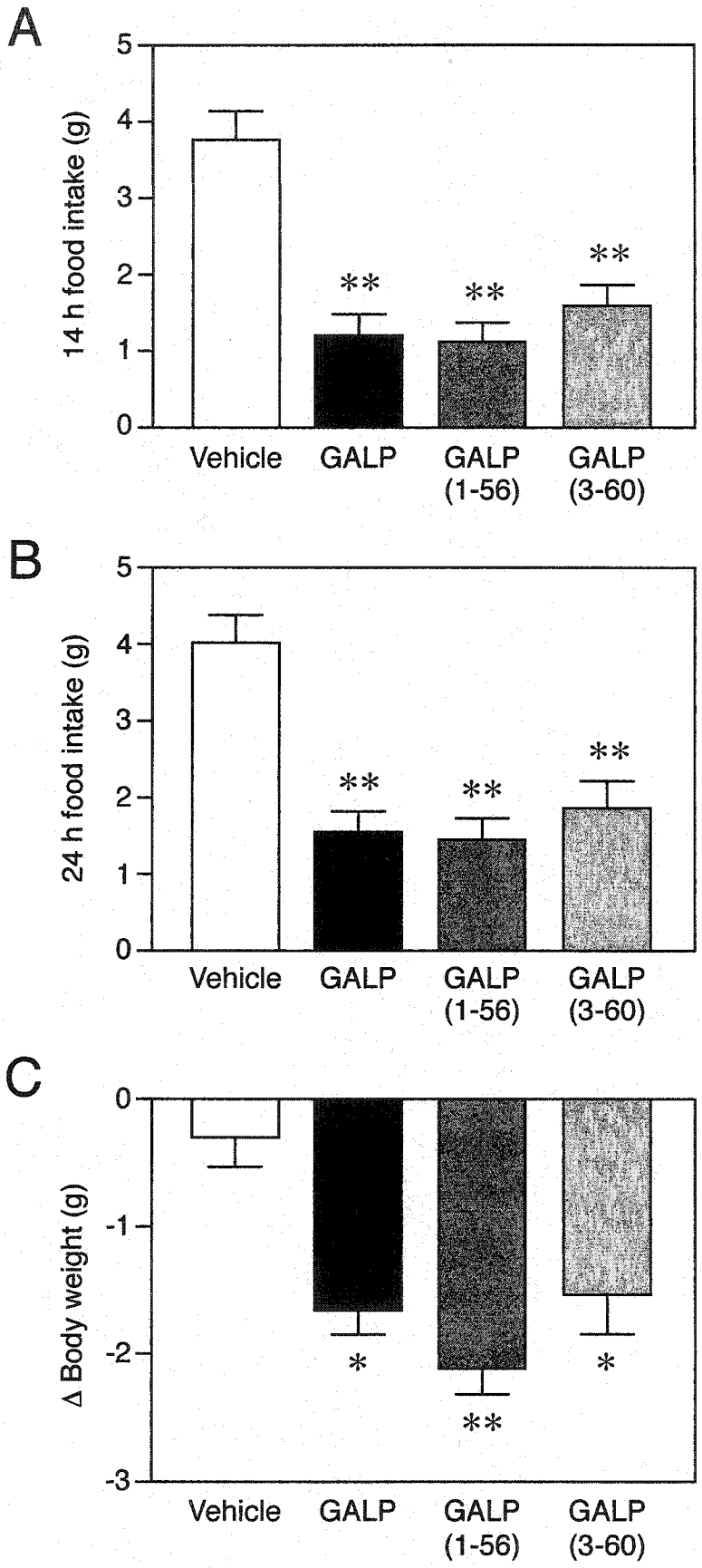


Figure 26. Effects of GALP(1-21) and GALP(22-60) on Serum LH Concentrations. Serum LH concentrations in male wild-type mice at 30 min following a single ICV injection of vehicle, 5 nmol GALP, 5 nmol GALP(1-21) or 5 nmol GALP(22-60). Data are presented as means ± SEM. $^{*}P < 0.0001$ *vs.* vehicle-treated mice.

Figure 27. Effects of GALP(1-56) and GALP(3-60) on Feeding and Body Weight. A. 14 h food intake, B. 24 h food intake, and C. 24 h body weight change in male wild-type mice following two ICV injections of vehicle, 5 nmol GALP, 5 nmol GALP(1-56) or 5 nmol GALP(3-60). Data are presented as means ± SEM. $*P < 0.005$; $**P < 0.0005$ *vs*. vehicle-treated mice.


A
14 h food intake (g)
5
4
3
2
1
0
**
**
**
Vehicle
GALP
GALP (1-56)
GALP (3-60)
B
24 h food intake (g)
5
4
3
2
1
0
**
**
**
Vehicle
GALP
GALP (1-56)
GALP (3-60)
C
Δ Body weight (g)
0
-1
-2
-3
*
**
*
Vehicle
GALP
GALP (1-56)
GALP (3-60)

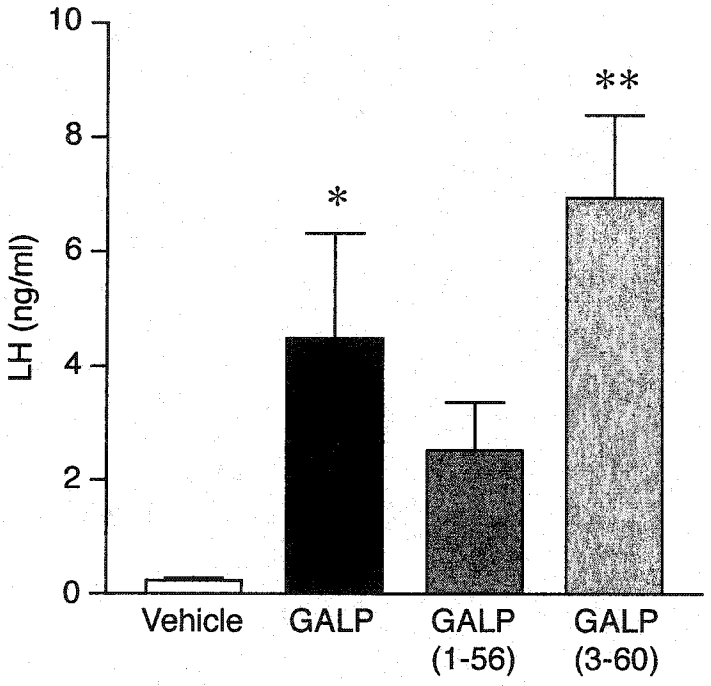


Figure 28. Effects of GALP(1-56) and GALP(3-60) on Serum LH Concentrations. Serum LH concentrations in male wild-type mice at 30 min following a single ICV injection of vehicle, 5 nmol GALP, 5 nmol GALP(1-56) or 5 nmol GALP(3-60). Data are presented as means ± SEM. $*P < 0.05$; $**P < 0.0005$ *vs.* vehicle-treated mice.

Chapter IV:

Discussion

A. GALP and Energy Homeostasis

Prior to the discovery that exogenously-administered GALP modulates feeding and energy expenditure in rodents, several key observations supported a role for GALP in the central regulation of energy balance. First, GALP neurons are almost exclusively located in the Arc, a region of the hypothalamus that plays a critical role in body weight regulation [62, 120, 121, 128, 142]. Second, the expression of GALP mRNA in the Arc is reduced in response to various metabolic challenges (e.g., food deprivation, experimentally-induced diabetes, etc.), but is upregulated by metabolic signals that reflect the adequacy of an animal's energy reserves, including leptin [120, 121, 139] and insulin [87]. Third, the majority of GALP neurons express Ob-R, which likely indicates a role for these neurons in carrying out some of leptin's physiological actions [62, 246]. Fourth, GALP neurons express receptor mRNAs for several classical neurotransmitters and neuropeptides that regulate feeding behavior, including NPY, orexin A, and serotonin [64, 248]. Fifth, GALP neurons project to regions of the forebrain where feeding-related signals are integrated (e.g., PVN), and centrally-administered GALP induces Fos expression in several feeding-related nuclei in the hypothalamus and brainstem [86, 146, 246]. Collectively, these observations implicate GALP neurons in the Arc as components of the hypothalamic circuitry regulating feeding behavior and body weight. Because GALP gene expression is positively regulated by leptin and

insulin, both of which suppress food intake and body weight when they are injected directly into the brain [40, 100, 104, 114, 250, 283], I hypothesized that centrally-administered GALP would also reduce food consumption and body weight. Indeed, Experiment 1 revealed a dose-dependent inhibitory effect of ICV GALP treatment on 24 h food intake in mice [136]. Additionally, the mice that were treated with the two highest doses of GALP (5 and 10 nmol) lost significantly more body weight than the vehicle-treated animals over the 24 h period following the first injection.

Although feeding and body weight are suppressed by acute GALP treatment, GALP's anorectic and weight-reducing effects are not sustained during the course of long-term GALP administration. In the GALP-treated mice in Experiment 2, food intake and body weight recovered to pre-treatment levels while the animals continued to receive twice daily GALP injections, indicating that the mice became refractory to repeated exposure to GALP. Although it is not immediately apparent why this occurred, it is conceivable that the GALP-treated mice exhibited an activation of compensatory neural feeding pathways in an effort to restore their body weight back to normal. The modest hyperphagia that was observed in the GALP-treated mice during the final two days of treatment lends support to the notion that the activation of orexigenic circuits (e.g., NPY or AgRP) and/or inhibition of anorectic pathways (e.g., α-MSH or CART) might have been responsible for the rapid recovery of food intake in these animals. Although I did not measure serum leptin concentrations in this experiment, leptin levels were presumably reduced in the GALP-treated mice after the first 24 h of treatment due to their pronounced weight loss, which could have triggered a compensatory feeding

response on the subsequent treatment days. Alternatively, it is plausible that the mice became refractory to long-term GALP treatment due to a downregulation of GALP receptors in response to chronic exposure to GALP, much like the downregulation of hypothalamic Ob-R that occurs with chronic leptin treatment [161]. Desensitization of post-receptor signaling mechanisms might also have contributed to the rapid recovery of food intake and body weight in the GALP-treated mice in this experiment.

Soon after I first observed an inhibitory effect of GALP on feeding and body weight in mice, a few reports appeared in the literature claiming that centrally-administered GALP *stimulates* feeding in rats. Specifically, two groups independently demonstrated that GALP-treated rats consume significantly more food than vehicle-treated rats during the first 1 or 2 h after GALP is injected into the cerebral ventricles [145, 163]. Seth and colleagues have more recently reported that GALP stimulates acute food intake in rats when it is injected directly into the PVN [228]. These reports describing an orexigenic effect of GALP in the rat seemed to be counterintuitive, given that GALP gene expression is highly induced by leptin and insulin, two hormones that act within the brain to reduce food intake [40, 100, 104, 114, 250, 283]. Expression levels of orexigenic neuropeptides (e.g., NPY and AgRP) are typically reduced by leptin and insulin [180, 203, 242, 276]. In contrast, GALP gene expression is regulated by leptin and insulin in a manner similar to that of the anorectic peptides α-MSH and CART [130, 138, 149, 256]. Despite this apparent contradiction, I decided to measure food intake in mice at various intervals over the course of 24 h following an ICV GALP injection, in the event that I had overlooked an acute orexigenic effect of GALP in mice

by only measuring food intake at the 24 h time point in the previous experiments. However, when I injected mice with GALP in the evening (just prior to lights off), I observed that GALP inhibited feeding within the first 2 h post-injection (Experiment 3).

In two of the three studies reporting an orexigenic effect of GALP in the rat, GALP was administered during the early part of the light phase, a time at which rats are typically satiated [145, 228]. However, I injected my mice with GALP in the evening in Experiment 3, a time at which they were presumably already experiencing a near maximal physiological drive to eat. To increase my chances of observing an acute orexigenic effect of GALP in mice, I next examined whether GALP stimulates feeding in mice when it is administered in the morning (Experiment 4). However, when mice were injected with GALP in the morning, I still observed an inhibition of food intake during the first hour post-injection, as well as reductions in food intake and body weight after 24 h. Thus, even when GALP is administered at a time of day when a stimulatory effect on food intake is most likely to be manifest, it appears that mice (unlike rats) do not exhibit an acute orexigenic response to ICV GALP treatment. Although mice eat very little food during the daytime, it is possible to detect a statistically significant increase in acute food intake in mice in the morning. For example, I have detected an approximately two-fold increase in food intake in mice within 1 h following an ICV injection of NPY in the morning [135]. Furthermore, the use of isoflurane anesthesia prior to and during the ICV injection of NPY did not prevent the mice from consuming more food; thus, I believe that the use of isoflurane was unlikely to have masked an orexigenic effect of GALP in the mice in Experiment 4.

In an attempt to replicate the published reports of an acute orexigenic effect of GALP in the rat (as well as to confirm the biological activity of my GALP preparation, which I was beginning to question!), I also injected rats with GALP in the morning. In Experiment 4, the GALP-treated rats ate significantly more food than the vehicle-treated rats within the first hour after the injection, thus corroborating the findings of Matsumoto et al. and Lawrence et al. [145, 163]. However, food intake and body weight were significantly reduced in the GALP-treated rats at 24 h post-injection, an observation that has also been reported by Lawrence and colleagues [145]. Thus, whereas both rats and mice exhibit a reduction in 24 h food intake in response to ICV GALP treatment, only rats exhibit an acute orexigenic response to centrally-administered GALP.

One possible explanation for GALP's disparate effects on food intake in the rat between the 1 and 24 h intervals is that GALP activates different neural pathways over the short- and long-term. It is conceivable that GALP's rapid stimulation of food intake reflects an activation of galanin receptors, perhaps mimicking the orexigenic effect of central galanin administration in the rat [60]. This supposition is supported by the observation that GALP's acute stimulatory effect on feeding in the rat is similar in its time-course to that of galanin [145, 163]. In the rat, mRNAs for all three of the cloned galanin receptor subtypes are expressed in the PVN [171, 190], and both galanin and GALP stimulate acute food intake when injected directly into this hypothalamic nucleus [228]. Thus, the PVN could be a potential neural substrate for the shared acute orexigenic effects of galanin and GALP in the rat. However, over a more extended time

interval, GALP might activate a different population of receptors (either another galanin receptor subtype or perhaps an unidentified GALP-specific receptor), resulting in the activation of neural circuits that ultimately decrease food intake and body weight. If this were the case, the different responses 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 an absence of orexigenic galaninergic pathways in the mouse (which do exist in the rat). Although there is at least one report in the literature in which the ICV injection of a high dose of galanin modestly stimulates food intake in mice [108], I have been unable to detect an orexigenic effect of ICV galanin treatment in mice (unpublished observations), which is consistent with the argument that orexigenic galaninergic pathways are either inaccessible to peptides that are injected into the ventricular system or are altogether lacking in mice.

In the course of conducting the first four experiments, I noticed that the GALP-treated mice remained immobile for a considerably longer amount of time than the vehicle-treated mice following the first ICV GALP injection (i.e., the GALP-treated mice did not appear to recover from the injection/anesthesia as quickly as their vehicle-treated counterparts). Hence, I was not particularly surprised to find that centrally-administered GALP has a dose-dependent inhibitory effect on spontaneous locomotor activity within the first 14 h post-injection (Experiment 5). However, the observation that GALP has disparate effects on locomotor activity when it is administered acutely or repeatedly was unexpected. In Experiment 6, spontaneous locomotor activity was

reduced in the GALP-treated mice immediately following both the first and ninth injections. Whereas locomotor activity remained suppressed for most of the 14 h interval following the first GALP injection, a period of pronounced hyperactivity followed the transient suppression of activity after the ninth GALP injection. I did not measure activity levels at any intermediate time-points during the course of long-term GALP treatment, so I do not know precisely when during the three day window this shift in motor response occurred.

The physiological significance (if any) of the GALP-induced hypoactivity and hyperactivity observed in Experiments 5 and 6 is not immediately apparent. One possibility is that endogenous GALP is involved in the generation of circadian activity 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 involved in regulating feeding behavior [281]. Alternatively, it is possible that GALP influences motivation or anxiety. In support of the latter hypothesis, Matsumoto and colleagues reported that rats that are treated with 1 nmol GALP exhibit "alert immobility" for approximately 15 min post-injection, which the authors attributed to an anxiogenic effect of GALP [163]. On the other hand, it is possible (and perhaps likely) that the effects of ICV GALP treatment on motor behavior do not reflect a physiological role for GALP in the regulation of motor activity, but are instead pharmacological responses to the widespread diffusion of GALP throughout the ventricular system. Galanin receptors are located in motor-related areas of the brain [190], and thus it is conceivable that the central injection of GALP

elicits a widespread activation of receptors that ordinarily do not receive input from GALP neurons.

Given the acute suppressive effect of centrally-administered GALP on locomotor activity in mice, it is possible that the reduction in feeding observed during the first 24 h following ICV GALP treatment does not reflect satiety, but is instead a secondary consequence of impaired motor function. Similar concerns have been raised about the specificity of CART's inhibitory effect on food intake. Central administration of CART(55-102) reduces feeding in rats but also causes movement-associated tremors and aberrant body postures [2, 138]. However, the observation that centrally-administered CART antiserum significantly increases nighttime food consumption in rats suggests that endogenous CART normally exerts a tonic restraint upon feeding [138]. As genetic and pharmacological tools that suppress endogenous GALP signaling (e.g., GALP neutralizing antibodies, GALP receptor antagonists, GALP knockout mice, etc.) become available, they will hopefully aid in determining whether GALP's anorectic effect is specific to the modulation of central feeding pathways.

The central administration of GALP elicited the formation of a strong CTA in mice (Experiment 7). CTA learning is commonly used as a behavioral assay to ascertain whether a particular substance might be toxic and/or cause visceral illness in rodents [17, 275]. One interpretation of Experiment 7 is that centrally-administered GALP makes mice sick, and that the inhibitory effects of ICV GALP treatment on feeding and body weight do not reflect a homeostatic regulation of energy balance. Notwithstanding this possibility, there has been an ongoing debate about the interpretation of CTA studies

over the past several years. Several investigators have proposed that the formation of a CTA does not necessarily reflect toxicity or illness, nor does it prove that the administered substance is truly aversive to the subject [17, 93, 112]. Many drugs that elicit the formation of a CTA in an experimental setting do not appear to be particularly aversive, and are sometimes even self-administered by animals, such as morphine and amphetamine [112]. It is also worth noting that not all agents that elicit a CTA reduce food intake. For example, the central administration of NPY results in the formation of a CTA in the rat, despite its simultaneous ability to markedly increase food consumption [284]. Given the uncertainty of interpreting the results of CTA experiments, the induction of a CTA by central GALP treatment might not reflect simple toxicity. It is possible that centrally-administered GALP interacts with the neural pathways that mediate taste aversion learning, thus leading to the formation of a CTA without actually possessing aversive properties. Alternatively, it could be argued that if ICV GALP is truly aversive to mice, such an effect might still implicate a biologically important role for endogenous GALP in the suppression of feeding, in the sense that aversion may simply lie at one extreme of the range of appetitive behaviors. For example, GALP signaling in the CNS might play a specific role in inhibiting food intake under pathophysiological circumstances, such as in the case of patients with cancer and other wasting diseases.

Several of the effects of ICV GALP treatment closely resemble those of another neuropeptide, glucagon-like peptide-1 (GLP-1). ICV injection of GLP-1 reduces acute food intake and induces the formation of a CTA in rats [249, 253, 257]. Seeley and

colleagues have recently obtained evidence suggesting that GLP-1 signaling in the CNS mediates LiCl-induced anorexia, thus implicating a role for GLP-1 in the regulation of food intake under non-homeostatic conditions [227]. In addition to this putative function for GLP-1 signaling in the CNS, GLP-1 also appears to be involved in the homeostatic control of feeding behavior. Notably, injection of GLP-1 directly into the PVN reduces food intake without inducing the formation of a CTA [165], and feeding is increased in rats that are treated with a GLP-1 receptor antagonist [257]. Furthermore, central GLP-1 signaling plays an essential role in mediating leptin's anorectic effect in rats, because leptin does not reduce food intake in animals that are treated with a GLP-1 receptor antagonist [92], Hence, neuropeptides can be involved in both the homeostatic and non-homeostatic control of ingestive behavior— the two functions need not be mutually exclusive. We will hopefully be able to distinguish between the potential homeostatic and/or non-homeostatic roles for GALP in regulating feeding behavior when the appropriate experimental tools become available.

Collectively, these first seven experiments demonstrated that centrally-administered GALP reduces food intake and body weight in mice and rats. GALP's effects on feeding behavior appear to be species-dependent, because rats (but not mice) exhibit an acute orexigenic response to ICV GALP treatment. GALP's inhibitory effects on feeding and body weight in mice are transient in nature. These observations are consistent with the notion that GALP plays a physiological role in the regulation of energy homeostasis. However, it is conceivable that the observed reduction in food intake following central GALP treatment is not due to a specific action of GALP on

neural feeding circuits, but is instead a consequence of impaired motor function and/or aversive properties of centrally-administered GALP. Additional experiments will be required to further resolve questions about the specificity of GALP's effects on feeding behavior. Together with the finding that GALP gene expression is highly induced by leptin, many of these observations are consistent with the argument that GALP neurons are downstream effectors of leptin's actions on feeding and body weight within the CNS. However, this hypothesis must be directly tested using the appropriate experimental tools (e.g., GALP knockout mice, GALP receptor antagonists, etc.) before one can draw any firm conclusions on this matter.

B. GALP and Reproduction

In addition to the putative role of endogenous GALP in regulating feeding and body weight, there is also compelling evidence supporting the notion that central GALP signaling participates in the regulation of GnRH and gonadotropin secretion. GALP neurons project to regions of the basal forebrain where GnRH neurons reside, and GALP-immunoreactive fibers have been observed in close contact with GnRH cell bodies and fibers in these areas [246]. ICV injection of GALP induces Fos expression in a sub-population of GnRH neurons in the MPOA, which suggests that these GnRH neurons are transcriptionally activated by GALP [162]. Central GALP treatment also elicits a robust elevation in circulating LH concentrations in male rats and rhesus macaques [64, 162].

In Experiments 8 and 9, acute ICV GALP treatment potently increased serum LH levels in male mice, thus extending the LH-releasing action of GALP to another mammalian species. Only the highest dose of GALP (5 nmol) elicited a significant increase in serum LH concentrations; the lower two doses were without any obvious effect on LH release. A similar sensitivity of LH release to ICV GALP treatment has been documented in male rats [162]. As would be expected of a neuropeptide that enhances LH release, GALP also induced a significant elevation in plasma testosterone levels at 30 min post-injection. Although serum FSH levels were modestly elevated in the GALP-treated mice in Experiment 8, the difference fell just short of reaching statistical significance. Matsumoto and colleagues were also unable to detect an effect of centrally-administered GALP on plasma FSH levels in male rats [162], and thus it appears that GALP has divergent effects on LH and FSH release in both species.

Although I injected GALP directly into the brains of the mice in Experiments 8 and 9, it is possible that some of the GALP leaked out of the brain and into the hypophyseal or general circulations. Hence, it is difficult to establish with certainty whether GALP elicits LH secretion in mice through direct actions on the brain and/or pituitary. GALP's ability to stimulate LH secretion is completely abolished in rats and monkeys that are pre-treated with a GnRH antagonist, which implicates a hypothalamic site of action [64, 162]. *In vitro*, GALP stimulates GnRH release from hypothalamic explants and GnRH-expressing GT1-7 cells [229]. However, GALP does not stimulate basal or GnRH-induced LH secretion from dispersed anterior pituitary cells, which argues against a direct effect of GALP at the level of the pituitary [162]. Collectively,

these observations are consistent with the argument that GALP stimulates LH secretion via a GnRH-dependent pathway(s).

Although injecting GALP into the cerebral ventricles of male mice and rats unequivocally stimulates LH release, this is clearly a pharmacological manipulation of central GALP signaling. In Experiment 10, I sought evidence supporting a role for endogenous GALP in modulating LH secretion within a physiological context, namely in the events preceding and culminating in the preovulatory LH surge. In the female, gonadal steroids can exert both negative and positive feedback effects upon GnRH/LH secretion. During most of the estrous cycle, estrogen suppresses the pulsatile secretion of LH; however, during the afternoon of proestrus, the continuously rising levels of estrogen (and progesterone) ultimately trigger an LH surge. I hypothesized that if alterations in endogenous GALP tone underlie or contribute to the negative and/or positive feedback regulation of GnRH/LH secretion by ovarian steroids, then GALP synthesis would also be subject to hormonal regulation. However, this hypothesis was not supported by the data in Experiment 10, which revealed that neither the number of GALP mRNA-expressing cells in the Arc nor the cellular content of GALP mRNA was altered by any of the hormonal treatments. This is consistent with recent demonstrations of a lack of effect of either the removal or replacement of gonadal steroids on GALP mRNA levels in castrated male rats and in ovarian steroid-treated spayed female rhesus macaques [64, 65]. Hence, it appears that alterations in endogenous GALP tone do not play a major role in effecting the negative or positive feedback actions of gonadal steroids on GnRH/LH secretion, at least at the transcriptional level. These observations

do not preclude an important role for GALP in mediating the effects of gonadal steroids on GnRH/LH secretion, for it remains possible that gonadal steroids influence GALP neuronal activity via post-transcriptional mechanisms.

Although not directly tested in these experiments, observations that centrally-administered GALP potently stimulates LH secretion in male mice, rats, and macaques are consistent with the hypothesis that GALP neurons in the Arc mediate leptin's stimulatory effects on GnRH and gonadotropin secretion [67, 271, 285]. GnRH neurons do not appear to express Ob-R, which implicates an important role for leptin-sensitive interneurons in relaying leptin's stimulatory signal to GnRH neurons [81, 98]. GALP neurons are ideally suited to serve this function. GALP neurons are direct downstream targets for leptin, which not only increases GALP mRNA expression in rats and mice [120, 121, 139], but also stimulates GALP secretion from hypothalamic explants *in vitro* [229]. Perhaps the most compelling evidence supporting a role for GALP in mediating leptin's effects on the neuroendocrine reproductive axis is the observation that incubating hypothalamic explants with GALP antiserum completely abolishes leptin's stimulatory effect on GnRH release [229]. Whether central GALP signaling is obligatory for leptin-induced GnRH secretion *in vivo* remains to be determined.

C. Acute and Long-Term GALP Treatment in *ob/ob* Mice

Body Weight Regulation

Ob/ob mice are obese, hyperphagic, and hypometabolic as a consequence of a congenital leptin deficiency [54, 116]. Exogenous leptin treatment significantly reduces body weight and adiposity in *ob/ob* mice by decreasing food intake and increasing energy expenditure [40, 99, 197, 272]. Although wild-type mice also eat less food and lose weight in response to exogenous leptin treatment, they are typically less sensitive to leptin than *ob/ob* mice [103]. Whereas wild-type mice exhibit transient reductions in food intake and body weight in response to chronic treatment with relatively high doses of leptin, lower doses of leptin elicit prolonged reductions in feeding and body weight in *ob/ob* mice [40, 99, 103, 148, 197].

In Experiment 11, I hypothesized that if leptin's anorectic and weight-reducing actions are mediated by enhanced GALP signaling in the brain, then *ob/ob* mice would exhibit more prolonged reductions in feeding and body weight than wild-type mice in response to a single ICV GALP injection. Although feeding and body weight in the GALP-treated wild-type mice were reduced at 24 h post-injection, the animals exhibited pronounced hyperphagia on the following four days. Food intake and body weight in the GALP-treated *ob/ob* mice were also reduced at the 24 h time point and showed signs of recovery within 48 h after the injection; however, both feeding and body weight remained suppressed in these animals for an additional three days. There are several possible explanations for why the *ob/ob* mice exhibited a longer-lasting inhibition of

feeding (and body weight) in response to acute ICV GALP treatment. In the GALP-treated wild-type mice, circulating leptin levels presumably decreased as the animals lost weight, which could have been responsible for eliciting the compensatory orexigenic response observed in these animals. Because the orexigenic circuits of *ob/ob* mice are likely to already be maximally or near-maximally stimulated due to their complete lack of leptin, *ob/ob* mice might not be as capable of sensing a reduction in body weight and/or as efficient at enacting a compensatory orexigenic response as wild-type mice. Alternatively, it is conceivable that *ob/ob* mice display more prolonged feeding and body weight responses to acute GALP treatment due to an upregulation of GALP receptor expression levels in the brain in response to their low endogenous GALP expression. This has been shown to be the case for Ob-Rb mRNA levels in the Arc, which are approximately two-fold higher in *ob/ob* mice than in wild-type mice [9]. Regardless of the mechanism(s) by which GALP elicits prolonged reductions in feeding and body weight in *ob/ob* mice, it is tempting to speculate that *ob/ob* mice are more sensitive than wild-type mice to the feeding and weight-reducing effects of exogenous GALP treatment. However, this hypothesis can be neither confirmed nor refuted until dose-response curves are generated and compared between the two genotypes.

In Experiment 13, long-term GALP treatment elicited a more prolonged reduction in food intake in *ob/ob* mice than was observed in the wild-type mice in Experiment 2. Although feeding was significantly reduced in the GALP-treated *ob/ob* mice for most of the two week treatment period, their food intake no longer differed from that of the vehicle-treated mice on the final two days of treatment. Also unlike the

GALP-treated wild-type mice in Experiment 2, body weight in the GALP-treated *ob/ob* mice was suppressed for the duration of the treatment period. The observation that the GALP-treated mice did not show any signs of recovering their body weight, despite the fact that their food intake was gradually restored to control levels over the course of the two week interval, suggests that GALP reduces body weight both by decreasing food intake and increasing energy expenditure. A role for GALP in enhancing energy expenditure is also supported by the observation that GALP-treated *ob/ob* mice lose more body weight than pair-fed *ob/ob* mice [102].

The observation that body temperature was significantly elevated in the GALP-treated *ob/ob* mice at the end of the two week treatment period indicates that thermogenesis was enhanced in these animals. Mitochondrial uncoupling proteins allow rodents to dissipate large quantities of energy in the form of heat by dissipating the proton gradient across the inner mitochondrial membrane, thereby uncoupling fuel oxidation from ATP synthesis [83]. Uncoupling protein-1 (UCP-1) is expressed in BAT and is regulated at the transcriptional level via activation of the sympathetic nervous system (SNS) [105]. UCP-1 mRNA and protein levels are reduced in *ob/ob* mice compared to wild-type mice [56]. However, Hansen et al. reported an induction of UCP-1 mRNA and protein expression in *ob/ob* mice following two weeks of central GALP treatment [102]. Leptin treatment (peripheral or ICV) also increases UCP-1 mRNA and protein levels in rodents (including *ob/ob* mice) [56, 57, 216], an effect which is dependent upon an intact SNS [55, 179, 217]. It is likely that centrally-administered GALP also increases UCP-1 expression and thermogenesis in *ob/ob* mice by enhancing

sympathetic output to BAT; however, this hypothesis must be directly tested (e.g., by administering GALP to *ob/ob* mice in which the sympathetic supply to BAT has been chemically or surgically disrupted) before this can be stated conclusively.

Reproduction

Male *ob/ob* mice remain suspended in a prepubertal state throughout adulthood, possessing diminutive reproductive organs, reduced circulating concentrations of gonadotropins and testosterone, and an enhanced sensitivity to the negative feedback actions of testosterone on gonadotropin secretion [244]. The observation that male *ob/ob* mice release LH in response to an acute GnRH challenge suggests that reproductive failure in these animals is at least partially attributable to impaired GnRH secretion; however, the possibility that *ob/ob* mice also have a pituitary defect has not been ruled out [245]. Chronic leptin treatment for two weeks significantly improves reproductive function in male *ob/ob* mice, as evidenced by increased serum FSH concentrations, enlarged testes and seminal vesicles, and improved histological signs of spermatogenesis and testosterone production compared to vehicle-treated animals [6]. Even longer leptin treatment regimens can restore fertility in these animals [184]. The stimulatory effects of leptin on endocrine and gonadal function are not recapitulated in *ob/ob* mice that are pair-fed to leptin-treated animals, which indicates that leptin's stimulatory effects on the HPG axis are not secondary to leptin-induced weight loss [6, 184]. Although leptin was administered peripherally in these experiments and therefore could have acted directly on the pituitary, testes, and/or accessory organs, the results of several other experiments

suggest that leptin's stimulatory effects on the HPG axis are at least partially due to a direct action of leptin on the brain [42, 67, 106, 132, 285].

The central mechanisms by which leptin improves reproductive function in *ob/ob* mice are poorly understood. I hypothesized that if leptin stimulates the HPG axis of *ob/ob* mice through a GALP-dependent pathway(s), then exogenously-administered GALP would mimic leptin's positive effects on various indices of reproductive function in these animals. Before treating *ob/ob* mice with repeated injections of GALP in an effort to improve their reproductive capacity, I first examined whether *ob/ob* mice would be responsive to acute central GALP treatment (Experiment 12). Serum LH concentrations in male *ob/ob* mice were significantly elevated in response to a single ICV GALP injection, albeit to a slightly lesser magnitude than in the GALP-treated wild-type mice in Experiment 8. Unlike the wild-type mice in Experiment 8, GALP treatment also significantly increased serum FSH levels in *ob/ob* mice. Thus, despite their profound disturbances of GnRH and gonadotropin secretion, male *ob/ob* mice are capable of releasing LH and FSH in response to a single GALP injection.

Despite GALP's acute stimulatory effect on gonadotropin secretion in male *ob/ob* mice, I did not observe any obvious signs of improved reproductive function in male *ob/ob* mice that were chronically treated with GALP (Experiment 13). In fact, there was a trend toward a decrease in three of the four measured indices of reproductive function in the GALP-treated animals. This is in direct contrast to the results of Barash et al., who demonstrated that two weeks of twice daily leptin injections improved several measures of reproductive function in male *ob/ob* mice [6]. One difference between the

two studies is that in the experiment by Barash et al., the control mice were pair-fed to the leptin-treated mice. However, in Experiment 13, the vehicle-treated mice were allowed to feed *ad libitum*. In the former study, although the leptin-treated *ob/ob* mice had higher gonadotropin concentrations than the pair-fed, vehicle-treated *ob/ob* mice, leptin treatment did not increase gonadotropin concentrations above pre-treatment levels. Thus, Barash and colleagues concluded that leptin treatment prevented the decline in reproductive function that resulted from food restriction in the pair-fed animals, but did not have any further stimulatory effect on the HPG axis [6]. Because I did not control for food intake in Experiment 13, it is possible that the hypophagia and consequent loss of body weight in the GALP-treated mice resulted in more pronounced reproductive dysfunction in these animals than in the vehicle-treated mice. The fact that leptin treatment, but not a similar regimen of GALP injections, improves reproductive function in *ob/ob* mice suggests that leptin's activational effects on the HPG axis of *ob/ob* mice might occur independently of central GALP signaling [6, 45, 184]. On the other hand, I cannot exclude the possibility that GALP does mediate leptin's stimulatory effects on reproductive function in *ob/ob* mice, but that twice daily GALP injections were insufficient to elicit sustained elevations in GnRH/gonadotropin secretion. If this were the case, then chronically infusing GALP into the brain (either at a constant release rate or in a pulsatile manner) might prove to be more effective at stimulating the HPG axis of *ob/ob* mice.

D. GALP and Galanin Receptors

In the five years since GALP's discovery, we have come to learn a good deal about the effects of exogenous central GALP treatment on feeding, body weight, and gonadotropin secretion in multiple species. However, the neural pathways and signaling mechanisms by which GALP exerts these effects in the brain have so far remained elusive. GALP shares sequence homology with the region of the galanin molecule that confers galanin's biological activity at galanin receptors [13, 192]. The observation that GALP binds and activates GALR1 and GALR2 (with higher affinity for GALR2 than for GALR1) in transfected cell lines demonstrates that *in vitro*, GALP has the ability to interact with at least two of the galanin receptor subtypes [192]. However, it is unknown whether GALP interacts with galanin receptors *in vivo*. To begin to address this conundrum, I assessed whether GALP's actions in the brain are mediated by GALR1 and/or GALR2 signaling. I used two mouse models of impaired galanin receptor signaling to address this question, namely mice in which the GALR1 or GALR2 genes had been disrupted. If GALP's effects on food intake, body weight, and gonadotropin secretion in the mouse occur solely through GALP's interaction with either GALR1 or GALR2, then mice lacking the relevant receptor subtype would not be expected to respond to exogenous GALP treatment. This was clearly not the case in Experiments 14 and 15, because both the GALR1 and GALR2 KO mice exhibited feeding, body weight, and LH responses to GALP that were indistinguishable from those of wild-type mice [137].

Although the outcomes of Experiments 14 and 15 demonstrated that neither GALR1 nor GALR2 are essential for GALP's effects on feeding, body weight, and LH secretion in the mouse, I cannot definitively rule out an important role for either receptor subtype in central GALP signaling. For example, it is conceivable that GALP is capable of signaling through both GALR1 and GALR2 *in vivo*. If this were the case, then the functional implications of losing one receptor subtype would be obscured by a redundancy of function; addressing this possibility would require the generation of animals with deletions of both GALR1 and GALR2. It is also plausible that GALP signals through only one of the two galanin receptor subtypes (GALR1 or GALR2), and that if the critical receptor is lost during development (by mutation), the other "compensates" for the lost receptor by upregulating its own expression and functional relevance to GALP signaling. This compensation does not appear to occur in the GALR1 KO mice, at least at the transcriptional level, because the expression of GALR2 mRNA in the brain is comparable to that of wild-type mice [118]. The possibility of compensation by GALR1 has yet to be determined in the GALR2 KO mice. Although it is conceivable that compensation occurs through post-transcriptional mechanisms in the knockout animals, the fact that GALR1 and GALR2 utilize primarily non-overlapping intracellular signaling pathways (which is unusual for receptor subtypes within the same family) makes it unlikely that the responsiveness of GALR1 and GALR2 KO mice to GALP is attributable to enhanced signaling by the alternate galanin receptor subtype [238, 267]. Notwithstanding the possibilities of functional redundancy and/or

compensation, the present observations are consistent with the notion that GALP is capable of utilizing receptors other than GALR1 or GALR2 *in vivo*.

Because GALP displays considerably higher affinity for GALR2 than for GALR1 *in vitro*, I used another strategy to re-evaluate the functional significance of GALR2 to central GALP signaling. AR-M1896 is a selective GALR2 agonist that binds to GALR2 with a roughly similar affinity as GALP and has been shown to mimic the effects of galanin on nociception and dorsal root ganglion neuron excitability [127, 151]. I reasoned that if GALP's effects on feeding, body weight, and LH secretion in mice result from activation of GALR2, then GALP and AR-M1896 would have similar effects on these measures (Experiment 16). However, AR-M1896 did not mimic GALP's effects on food intake, body weight or gonadotropin secretion, which provides further evidence against a role for GALR2 in mediating GALP's actions within the CNS.

While not directly tested in these experiments, it is conceivable that GALR3 mediates GALP's actions within the brain. Although GALP's ability to interact with GALR3 was not tested in the original paper reporting GALP's binding affinities for GALR1 and GALR2, Lang et al. have recently demonstrated that GALP binds with higher affinity to GALR3 (IC_{50}=10 nM) than it does to GALR1 (IC_{50}=77 nM) or GALR2 (IC_{50}=28 nM) in transfected human neuroblastoma cell lines [141]. In the rat brain, the expression of GALR3 mRNA is restricted to discrete regions, including the preoptic area and several hypothalamic nuclei [171]. Notably, the expression pattern of GALR3 mRNA coincides well with the distribution pattern of GALP-immunoreactive fibers [246]. Thus, I cannot rule out the possibility that GALP signals through GALR3 *in vivo*.

It is also plausible that the biological effects of GALP are mediated by an unidentified galanin receptor subtype or a member of the galanin receptor family; however, this cannot be directly tested until such putative receptors are discovered.

The results of several studies comparing the effects of centrally-administered galanin and GALP suggest that the two neuropeptides work through different receptor systems. Although both galanin and GALP have an acute stimulatory effect on food intake in the rat, only GALP has a suppressive effect on feeding and body weight after 24 h [145]. GALP increases energy expenditure in *ob/ob* mice (presumably via activation of the SNS), whereas galanin suppresses SNS activity in rats [102, 186]. Matsumoto and colleagues demonstrated that GALP, but not an equimolar dose of galanin, acutely stimulates LH secretion through a GnRH-dependent mechanism in male rats [162]. Furthermore, GALP, but not galanin, stimulates GnRH secretion from GT1-7 cells, which do not appear to express any of the known galanin receptor subtypes [229]. Moreover, treatment with galantide, a nonspecific galanin receptor antagonist that binds to all three cloned galanin receptor subtypes [27], does not block GALP's ability to stimulate GnRH release from GT1-7 cells, and only partially attenuates GALP's stimulatory effect on GnRH secretion from hypothalamic explants [229]. Whereas Fraley et al. have documented a pronounced stimulatory effect of central GALP treatment on sexual behavior in male rats, this group and others have demonstrated that ICV galanin administration suppresses copulatory behavior in male rats [14, 88, 200]. In addition to the differential biological effects of centrally-administered GALP and galanin, the two peptides induce different patterns of Fos expression in the rat forebrain

following their central injection, which indicates that the two peptides can activate different neuronal populations [86, 146]. Together with the observed normal responsiveness to GALP in the GALR1 and GALR2 KO mice, these observations collectively point toward galanin and GALP signaling through different receptor systems.

Although the results from my experiments with the GALR1 and GALR2 KO mice argue against the notion that GALR1 or GALR2 mediate the effects of central GALP administration in the mouse, there are also several lines of evidence that are consistent with the hypothesis that GALP is capable of signaling through galanin receptors *in vivo*. In addition to the structural and pharmacological similarities between galanin and GALP, the two neuropeptides share some common biological effects following central injection in the rat, which could indicate a shared receptor mechanism(s). For example, both galanin and GALP have an acute stimulatory effect on food intake in rats when injected into either the cerebral ventricles or the PVN [145, 163, 228]. Seth and colleagues reported that injection of either galanin or GALP into the PVN also inhibits the secretion of thyroid-stimulating hormone [228]. The same group has also recently demonstrated that galanin and GALP both stimulate GnRH secretion from hypothalamic explants [229], which could be attributable to a common interaction with the small population of GnRH neurons in the rostral POA that express GALR1 mRNA [178]. Galanin receptors are anatomically poised to mediate the above-mentioned common effects of galanin and GALP, given that galanin receptor mRNAs are expressed in areas of the brain that are involved in the control of feeding and/or neuroendocrine

function, including most hypothalamic nuclei and the preoptic area [171, 190]. Together, these observations raise the possibility that the shared biological actions of galanin and GALP are mediated by galanin receptor signaling and are consistent with the argument that GALP is capable of interacting with galanin receptors *in vivo*.

In Experiments 17 and 18, I took a pharmacological approach to address the question of whether GALP signals through galanin receptors *in vivo*. In these experiments, I attempted to distinguish between whether GALP's effects on feeding and gonadotropin secretion in mice require its shared sequence with galanin [GALP(9-21)] or whether the biologically active moiety of the GALP molecule is distinct from its galanin-homologous sequence. To accomplish this task, I first examined the effects of centrally-injecting mice with short fragments of the full-length GALP peptide (Experiment 17). I postulated that if GALP's effects on feeding, body weight, and LH secretion in the mouse are mediated by galanin receptors, then a fragment of the GALP peptide [GALP(1-21)] containing the same sequence as the biologically active region of the galanin molecule might mimic the effects of full-length GALP. However, GALP(1-21) had no detectable effect on feeding, body weight or LH secretion. Likewise, GALP(22-60) was without effect on any of the measured parameters, which suggests that the C-terminal portion of the GALP molecule is not sufficient for its biological activity. The lack of effect of either GALP(1-21) or GALP(22-60) might indicate that the biologically active region of the GALP peptide spans the two fragments, or could instead simply reflect the fact that a larger component of the GALP peptide is necessary for activation of its physiologically-relevant cognate receptor(s). Both of these

interpretations are consistent with the observation that injection of longer fragments of the GALP peptide, GALP(1-56) and GALP(3-60), recapitulated the effects of full-length GALP on feeding and body weight, and GALP(3-60) mimicked GALP's stimulatory effect on LH secretion in the mouse (Experiment 18). Alternatively, it is possible that the biologically active region of the GALP molecule is contained within one of the two shorter fragments but that the fragment did not assume the proper tertiary structure necessary for receptor recognition and/or activation. Experiments utilizing progressively shorter fragments of the full-length GALP molecule will help to further delineate the biologically active region of the GALP peptide, and will hopefully provide insight into the biological significance (if any) of the galanin-homologous sequence of the molecule.

This final set of experiments revealed that neither GALR1 nor GALR2 are essential for GALP to exert its effects on feeding, body weight or LH secretion in the mouse. Furthermore, the galanin-homologous region of the GALP molecule is not sufficient to recapitulate the effects of the full-length GALP peptide on any of these parameters. These observations argue against the hypothesis that GALP signals solely through galanin receptors and are consistent with the existence of a yet-to-be-identified GALP-specific receptor. Although it is possible that the shared biological actions of GALP and galanin are attributable to a common activation of galanin receptors, it is likely that the unique biological effects of GALP are mediated by a GALP-specific receptor(s), one that is distinct from the known galanin receptor subtypes.

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