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Characterization of Galanin in the Murine Brain

By

John George Hohmann

**A dissertation submitted in partial fulfillment
of the requirements for the degree of**

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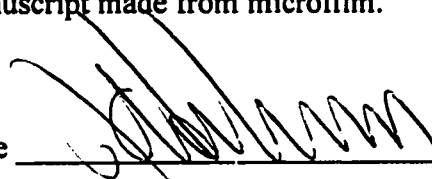
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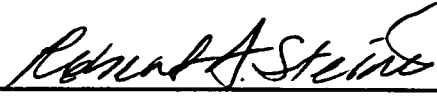
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Abstract

Characterization of Galanin in the Murine Brain

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The neuropeptide galanin has been implicated in a many central nervous system functions. These range from fundamental processes, such the ability to successfully breed and regulate metabolism, to higher order functions such as the need to learn and remember tactics for finding food, avoiding predators, and choosing mates. To learn more about the role of galanin in the brain, I examined the effects of permanently altered levels of galanin in genetically altered mice. My hypothesis was that enduring changes in galanin levels in mutant animals would produce phenotypes that in turn could provide clues about the roles galanin has in the brain. First, I examined the distribution of galanin gene expression in the brain of the mouse. I found that galanin is widely expressed throughout the brain, in a pattern unique to mice. Second, I sought to determine the role of galanin by using transgenic mice (GALTG), in whom the galanin gene is overexpressed. I found that while GALTG mice had normal neuroendocrine phenotypes, they had significant alterations in levels of galanin receptor mRNA in the hypothalamus, and altered neuropeptide Y (NPY) mRNA in the nucleus accumbens (Acb). I infer from

this that compensatory mechanisms act in GALTG mice to keep galanin levels within the physiological range in the hypothalamus, and may serve to counteract the overabundance of galanin in the Acb. Third, I studied the effects of targeted ablation of the galanin gene in knockout mice (GKO). I found that GKO mice had some neuroendocrine abnormalities, but had no changes in galanin receptors or NPY mRNAs in the hypothalamus. This suggests that developmental compensation by galanin receptors or other neuropeptides may not be a generalized strategy in the complete absence of galanin. Fourth, I tried to determine whether NPY and galanin have overlapping roles in the brain by producing and performing an initial characterization of mice with both the galanin and NPY genes ablated (DKO). I discovered that the loss of both galanin and NPY led to phenotypes more severe than seen with single knockouts of these molecules, suggesting that functional overlaps exist between these two widely expressed neuropeptides.

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To my wonderful and very patient sons

1
Chapter 1

Introduction

I. Neuropeptides

The astonishing complexity of the mammalian central nervous system would seem to demand an armada of communication mechanisms to organize and deliver coherent messages. Yet, despite the fact that there are an estimated 10^{11} neurons in the human brain, only a handful of classical neurotransmitters are thought to be primarily responsible for neuron-to-neuron signaling. These include the small molecule transmitter acetylcholine (ACh), 5 biogenic amines, and a few amino acid transmitters.

Understanding how these few molecules can effectively orchestrate the cognitive functions of the brain and maintain control of the physiological processes of the body has engaged neurobiologists for the greater part of the last 100 years. Granted, the presence of multiple receptor sub-types and post-synaptic cellular signaling components may account for much of the diversity of neuronal responses expected in a complex system. However, the existence of other neuromodulators that can expand the range of neuronal signaling frequencies and amplitudes is a reasonable postulate.

In 1953, Du Vigneaud et al. determined the amino acid sequence for oxytocin, the first chemically identified member of a new classification of molecules, the neuro-active peptides, or neuropeptides (Du Vigneaud, et al., 1953). Since this initial discovery more than 40 years ago dozens of neuropeptides have been discovered, and in recent years, many of these molecules have been recognized as important mediators of neuronal communication systems.

What is a neuropeptide? Hokfelt et al suggests that these molecules are defined by having “a physiologic involvement.....in CNS functions” (Hokfelt, et al., 2000). A more explicit definition is offered by Civelli et al, who state that “neuropeptides are defined as peptides that modulate CNS neurotransmission through activation of specific receptors” (Civelli, et al., 1999). A common characteristic of these small proteins is the initial synthesis of a larger precursor protein, which is enzymatically cleaved to form one or more biologically active peptides. Initial synthesis usually takes place in the endoplasmic reticulum. This is followed by transport to the Golgi apparatus where the precursor peptide is either packaged into vesicles for entry into the regulated secretory pathway, or transported centrifugally to the nerve terminal (reviewed in (Kandel, et al., 2000)). Here they are often processed by endopeptidases into mature peptides, which are often less than 40 amino acids in length. In contrast to small molecule transmitters, which are released at active zones in response to high Ca^{2+} concentrations, peptide transmitters are likely to be released extra-synaptically, responding to a general increase in axoplasmic Ca^{2+} levels (Verhage, et al., 1991).

After release into the synaptic cleft or extracellular space, the vast majority of neuropeptides exert their effects by activating G-protein-coupled receptors (GPCR). This may occur either pre- or post-synaptically. A further contrast between neuropeptide and classical neurotransmitter signaling are the mechanisms that exist for attenuating the transmitter's actions. While small molecule transmitters are inactivated either by specific enzymes in the synaptic cleft (such as acetylcholinesterase) or through cellular uptake mechanisms, no such specific enzymes or transporters are known for peptides. Most peptides are either degraded by relatively non-specific peptidases, or are removed by

diffusion. This gives neuropeptide transmitters the capacity to exert a longer-lasting effect after neuronal release, rather than the ‘sharper’ signals mediated by classical transmitters (reviewed in (Hokfelt, et al., 2000)). Also, while small molecule transmitters can be synthesized locally in the nerve terminal to quickly “recharge the batteries”, neuropeptides are produced in the cell body and transported to their site of action—a temporally and spatially much longer process.

What are the major roles of neuropeptides in the brain? It has been suggested that a primary function of these molecules is to act as modulators of classical neurotransmitter activity. This has been inferred partly by the extensive co-localization of one or more neuropeptides in neurons that contain classical transmitters, a phenomenon that seems now to be the rule rather than the exception (reviewed in ((Hokfelt, et al., 1987; Kandel, et al., 2000; Lundberg, 1996)). Numerous studies have shown that peptides can exert stimulatory or inhibitory actions on the release and/or post-synaptic actions of Ach and amine transmitters (for examples see (Hokfelt, et al., 1998; Kupfermann, 1991).

Tomas Hokfelt has championed the concept that there are 3 main types of neuropeptide activity in the brain (Hokfelt, et al., 2000). In the first case are peptides that are normally expressed abundantly in the brain, indicating that they are active and available for use at all times. The second type are peptides that are normally produced at extremely low levels, but are upregulated during times of extraordinary need, such as extreme stress or after neuronal injury. In the third case are neuropeptides that are expressed preferentially during development, where they may have important ontogenic roles that diminish in adulthood. In all cases these molecules may act independently, as neuromodulators of classical transmitter activity, or in cooperation with other peptides.

In some circumstances, neuropeptides may act as the primary transmitter in a neuron, and may even have their actions modulated by small molecule transmitters (for example (Meister and Hokfelt, 1988). The best examples of these are neuropeptides that control neuroendocrine function. Anterior pituitary hormone release is tightly regulated by the hypothalamic neuropeptides, including corticotropin-releasing factor (CRF), thyrotropin-releasing hormone (TRH), growth-hormone-releasing hormone (GHRH), somatostatin (SS), and gonadotropin-releasing hormone (GnRH). All of these peptides release their contents at the median eminence into the portal circulation, and are thought to be the primary transmitter molecules in their respective neurons. Also, the hypothalamic magnocellular neurons that project to the neurohypophysis contain multiple neuropeptides, but apparently no classical neurotransmitters (Brownstein and Mezey, 1986). It may be that hormone release into the bloodstream requires different temporal modalities than neuronal signaling in the brain, needing control over periods of minutes or hours rather than milliseconds; if this were the case, hormonal regulation may be more suited to slower, peptidergic mechanisms.

Another physiological process that seems to be substantially regulated by peptides is the system that controls feeding and body weight (reviewed in(Elmqvist, et al., 1999; Elmqvist, et al., 1998b; Schwartz, et al., 1999)). While several of these molecules are synthesized outside the brain as hormones and are transported into the brain to exert their effects (reviewed in (Baskin, et al., 1999b)), an ever increasing cast of brain-derived neuropeptides that regulate ingestive behaviors and metabolism are being identified. Interestingly, many of the hypothalamic areas that are involved in other neuroendocrine roles are the sites of synthesis and action of these molecules. These include the arcuate

nucleus (Arc), the paraventricular nucleus (PVN), the ventromedial nucleus (VMN), the dorsomedial nucleus (DMN) and the lateral hypothalamic area (LH). Lesions of these neuropeptide-producing regions lead to well described metabolic abnormalities (Bellinger, et al., 1979; Choi and Dallman, 1999), and overexpression or ablation of hypothalamic neuropeptides and their receptors in mice can cause ingestive and metabolic deficits (Erickson, et al., 1996a; Graham, et al., 1997; Huszar, et al., 1997; Marsh, et al., 1998; Shimada, et al., 1998). While it is very likely that important roles exist for classical neurotransmitters such as norepinephrine (NE), dopamine (DA) and serotonin (5-HT) in the brain's control of food intake and metabolism, current evidence suggests that hypothalamic neuropeptides also have a significant role in these processes.

II. Introduction to Galanin

During the early 1980's, the lab of Victor Mutt at the Karolinska Institute in Stockholm discovered many new biologically active peptides using a strategy of identifying molecules with C-terminal amidation, using thin-layer chromatography. Several peptides were isolated from extracts of the porcine intestine and brain with this procedure, including peptide HI, peptide PYY, and most notably neuropeptide tyrosine (NPY) ((Tatemoto, et al., 1982; Tatemoto and Mutt, 1981)). During the purification of PYY, extracts from porcine intestine were found to contain a molecule with a C-terminal alanine amide. This new peptide was purified and sequenced, and subsequently named galanin after the first and last of its 29 amino acids, glycine and alanine (Tatemoto, et al., 1983). No significant sequence homology or structural similarity was seen when

compared to any other known peptide, placing galanin as the first member in a new family of peptides. Initial studies confirmed the biological activity of this peptide by demonstrating that galanin causes contraction of smooth muscle in the rat, and induces a mild but long-lasting hyperglycemia in dogs (Tatemoto, et al., 1983).

Subsequently, the rat galanin gene was cloned in 1987 by two independent groups (Kaplan, et al., 1988b; Vrontakis, et al., 1987). The human gene was identified in 1991 (Evans and Shine, 1991), the mouse gene in 1995 (Lundkvist, et al., 1995) and to date the galanin gene has been cloned from more than a dozen different species. There is a high degree of sequence identity among species in the coding region of the galanin gene, and this evolutionary conservation suggests a significant biological role for this peptide. In fact, the first 15 amino acids of galanin are identical in all species in which it has been examined, with the exception of tuna fish, which has 2 residue changes. In all known species, galanin is 29 amino acids long, except in humans, where it is not amidated at its C-terminal and has 30 amino acids (Evans and Shine, 1991).

Using mouse galanin as a reference, the murine preprogalanin gene has 6 exons, of which exons 3-5 are translated into a 123 amino acid precursor peptide (Kofler, et al., 1996). After cleavage of the signal peptide portion, the prepeptide consists of galanin and the less well-conserved 58 amino acid galanin message-associated peptide, which is also cleaved in a tissue specific manner.

Galanin has a widespread distribution in the body, with highest levels seen in the gastrointestinal, peripheral and central nervous systems (Rokaeus, et al., 1984). Galanin is also present in many mammalian organ systems, notably in the pancreas, adrenal, pituitary, urinary tract, genital tract and respiratory tract (reviewed in (Rokaeus, 1987)).

The distribution of galanin in the central nervous system has been best studied in the rat, where it is widely distributed in the brain. Galanin appears before birth, being expressed as early as E15 in rodents (Gabriel, et al., 1989; Ryan, et al., 1997). In the brain of the adult rat, galanin is abundantly produced in multiple nuclei of the hypothalamus, including the supraoptic (SON), preoptic (POA), dorsomedial (DMN) and arcuate (Arc) nuclei. The other major areas of expression are in the dorsal raphe nucleus and locus coeruleus (LC) (Melander, et al., 1986a; Skofitsch and Jacobowitz, 1985; Skofitsch and Jacobowitz, 1986). Moderate numbers of galanin-containing cells are located in many other areas of the rat brain, including the basal forebrain, amygdala, thalamus, hippocampus and nucleus of the solitary tract.

In the primate brain, the distribution of galanin is somewhat different from that in the rat, and also varies considerably among primate species. In the human brain, the distribution of galanin-positive cell bodies is somewhat limited, with largest concentrations seen in the hypothalamus, basal nucleus of Meynert and LC (Gentleman, et al., 1989; Kordower, et al., 1992b). Curiously, while the pattern of galaninergic profiles is quite similar between the human and baboon, striking differences exist between the human and Cebus monkey. For example, while in other species the LC contains hundreds of galanin cells, in the monkey it contains only galanin fibers. This is also the case in the hypothalamic SON, where in the human there are abundant perikarya whereas none exist in the monkey. Whether these differences between species represents actual differences in functions of galanin or are simply reflections of expression in areas where galanin has no real role, is not yet known.

Little is known about galanin in the central nervous system of the mouse, despite

the growing importance of murine models for studying basic neurobiological mechanisms and neurological diseases. In one study of the development of the mouse preoptic area, it was reported that galanin-positive cells can be visualized as early as E18, and that in the adult, expression of galanin in the POA is sexually dimorphic (Brown, et al., 1999). In the only other anatomical report of galanin in the mouse brain, galanin immunoreactivity has been found in the basal forebrain and hypothalamus, and the distribution of galanin in these areas is sexually dimorphic (Rajendren, et al., 2000). One goal in this thesis was to elucidate where in the mouse brain the galanin gene is expressed, and to contrast this expression with that found in other species.

III. Regulation of Galanin

One of the most striking findings regarding galanin is that expression of this peptide is very labile in the brain, particularly after almost any kind of neuronal insult. The first hint of this was in an early anatomy study, where it was shown that galanin is induced in the sacral spinal cord following transection or treatment with capsaicin, suggesting a galanin response to neuronal injury (Ch'ng, et al., 1985). This finding soon spurred other studies in the rodent brain, where it has been convincingly demonstrated that galanin is upregulated after many different types of lesions. Cortes et al. found that galanin mRNA and peptide are higher in the dorsal raphe after removal of the cortex, and that electrocoagulation lesions of the ventral hippocampus lead to higher levels of galanin in the medial septum and diagonal band (Cortes, et al., 1990a; Cortes, et al., 1990b). This suggests that the galanin gene is induced as a response to damage in the terminal fields of its neurons, since the medial septum galanin cells project preferentially to the ventral hippocampus (Senut, et al., 1989). A similar effect is also seen after chemical lesions in

the medial septum/diagonal band, with the neurotoxin ibotenic acid (de Lacalle, et al., 1997). Interestingly, this effect can be mimicked by tetrodotoxin-induced blockade of neuronal activity in this same pathway (Agoston, et al., 1994), suggesting that perturbations of electrical activity are sufficient to alter galanin gene expression.

Several studies have also demonstrated that injections of the axon-degrading neurotoxin colchicine upregulate the galanin gene in many areas of the brain, providing further evidence for a robust galanin response to neuronal injury (Cortes, et al., 1990a; Cortes, et al., 1990b; Pu, et al., 1999). Unfortunately, these observations about colchicine's effects also cloud the interpretation of many early anatomical studies, where this neurotoxin was used to "improve" the visualization of galanin immunoreactivity in cell bodies (Melander, et al., 1986a; Skofitsch and Jacobowitz, 1985).

Galanin and its mRNA are also highly responsive to changes in hormone levels in the brain, with the most dramatic changes seen with estrogen. In ovariectomized rats treated with estradiol, galanin concentrations are induced in several areas of the brain and pituitary (Rugarn, et al., 1999a). Curiously, this effect is noted in many brain regions not commonly associated with classical neuroendocrine function, such as the hippocampus and occipital cortex. This raises the possibility that estrogen and galanin may interact functionally in non-neuroendocrine roles, such as in the learning and memory circuitry. Galanin is also induced by estrogen in the LC, where this same treatment has no effect on tyrosine hydroxylase gene expression, implying that galanin and norepinephrine synthesis may be regulated independently (Tseng, et al., 1997). It should be noted though that there are many avenues of regulation aside from transcriptional control, so galanin and tyrosine hydroxylase may well be regulated in concert at some other level. A direct effect of

estrogen (acting through its receptor) on the galanin gene is suggested by the presence of a functional estrogen response element in the galanin promoter (Howard, et al., 1997b). Other hormones that increase galanin concentrations in the brain include progesterone (Rugarn, et al., 1999b), corticosterone (Akabayashi, et al., 1994b), and thyroid hormone (Ceccatelli, et al., 1992). This induction is not universal, as the pancreatic hormone insulin reduces galanin gene expression (Tang, et al., 1997). There is also evidence that galanin regulates the expression of its own mRNA. Peripheral infusions of the peptide reduce levels of galanin mRNA in both the hypothalamus and the pituitary, suggesting the presence of a long-loop autofeedback mechanism (Giustina, et al., 2000).

The highly inducible nature of galanin indicates a potential role for this neuropeptide during periods of neuronal stress or injury, and the regulation of galanin by many hormones suggests this molecule may be intimately involved in neuroendocrine functions.

IV. Coexpression and Regulation of Other Neurotransmitters by Galanin

One notable feature of galanin is its extensive presence as a cotransmitter in many parts of the central nervous system, with both classical and peptide neurotransmitters. These include ACh, NE, dopamine (DA), 5-HT, vasopressin, NPY, GnRH and GHRH. The best-studied anatomical and regulatory interaction is between galanin and ACh. Early studies found that galanin coexists in 50-70% of cholinergic neurons that project to the hippocampus in the rat (Melander, et al., 1985). However, these early studies

employed the galanin gene-inducing neurotoxin colchicine, clouding the interpretation of these results. More recent studies in rats not treated with colchicine show a more modest level of coexpression, in the range of 15-25%, with a fairly discrete distribution of double-labeled cells within the basal forebrain (Miller, et al., 1998). The extent of coexpression of galanin with ACH is very species dependent. In the monkey, for example, galanin colocalizes with most cholinergic basal forebrain neurons (Kordower and Mufson, 1990; Melander and Staines, 1986). On the other hand, virtually no coexpression of galanin with ACh is seen in the basal forebrain of the human, and galanin seems to be generally less abundant in this region than in other species (Kordower and Mufson, 1990; Walker, et al., 1991).

Although the relative abundance of galanin within ACH neurons may be variable among species, its inhibitory effects on cholinergic release are unequivocal. This was initially described in 1987 by Fisone et al., who found that injections of galanin into the cerebral ventricles (ICV) inhibits the evoked release of ACH in the ventral hippocampus (Fisone, et al., 1987). It was also observed that galanin inhibits the potassium-stimulated release of ACH in ventral hippocampal slices, but has no effect in the dorsal hippocampus. This finding was confirmed by Ogren et al. who observed that galanin, when microinjected directly into the ventral hippocampus, reduces both basal and stimulated ACH release in the rat (Ogren, et al., 1996). However, the opposite effect though is seen in the striatum, where galanin consistently stimulates ACH release, an effect that can be blocked with the galanin antagonist M15 (Ogren and Pramanik, 1991; Pramanik and Ogren, 1992), suggesting the presence of different receptor subtypes. Most

of these effects have been ascribed to a presynaptic action, as galanin has not been convincingly shown to have significant effects on ACh postsynaptically. Galanin does block the slow cholinergic EPSP in the ventral hippocampus induced by ACh release from hippocampal slices (Dutar, et al., 1989), and appears to do so via a pertussis toxin sensitive G_i mediated mechanism (Wang, et al., 1999).

The other well-studied phenomenon of galanin's coexistence with a classical neurotransmitter is with NE. In the rat LC, several studies have shown that galanin is expressed in the majority of noradrenergic neurons (Holets, et al., 1988; Melander, et al., 1986c; Moore and Gustafson, 1989). Galanin also exists in other brainstem noradrenergic cell groups to varying extents (Melander, et al., 1986c). In the LC of the human, there are fewer NE neurons that contain galanin.(Chan-Palay, et al., 1990). It is likely that a majority of the galaninergic innervation of the cerebral cortex and hippocampus originates in the LC. Disruption of the ascending fiber bundles from this region leads to a significant depletion of galanin fibers in the forebrain, particularly in the hippocampal region (Melander, et al., 1986d; Xu, et al., 1998). Lending further credence to this view is the finding that injections of the retrograde tracer Fast Blue into the cerebral cortex and hippocampus accumulate in galanin-containing cells in the LC (Holets, et al., 1988).

Similar to its actions on the cholinergic system of the basal forebrain, galanin is generally thought to be inhibitory to the activity of NE neurons. LC cells fire spontaneously at rates from 0.2-5 Hz, and this spontaneous firing can be inhibited in pontine slices by bath application of galanin (Sevcik, et al., 1993). This is accompanied by a hyperpolarization of LC neurons and a decrease in input resistance, suggesting that

galanin facilitates the opening of potassium channels. These actions of galanin can be reversed by application of the galanin antagonist MIS, presumably by blocking galanin receptors (Bartfai, et al., 1991). In slices of the medulla oblongata and hypothalamus of the rat, galanin inhibits the stimulated release of NE (Tsuda, et al., 1990; Tsuda, et al., 1989). Galanin's inhibitory effects on the actions of NE are apparently widespread in the brain. Even in the cerebral cortex, where galanin fibers only are present in modest numbers, this peptide inhibits the NE-induced accumulation of cyclic AMP in a slice preparation (Nishibori, et al., 1988). The dose of galanin used in this latter study had no effect by itself in altering cyclic AMP, but did inhibit the effects of NE in a dose-dependent manner.

Although the interactions of galanin with DA have not been extensively studied, considerable coexpression has been demonstrated in the arcuate nucleus (Melandar, et al., 1986c). Interestingly, galanin either stimulates or inhibits DA activity depending on the site of action. When injected into the hypothalamus, galanin stimulates DA release in the striatum, while at the same time lowering ACH release (Jansson, et al., 1989; Rada, et al., 1998). This effect seems to be specific to injections into the PVN, as direct application of galanin onto striatal slices actually inhibits stimulated DA release, while having no effect on basal release (Tsuda, et al., 1998). In the Arc, galanin inhibits the actions of DA, and this inhibition has been linked to a stimulation of prolactin release from the anterior pituitary (Gopalan, et al., 1993; Jansson, et al., 1989; Melandar, et al., 1987; Nordstrom, et al., 1987). The rapid effect of this stimulation after infusion of galanin (within 20 minutes) suggests that galanin may act through its receptors directly on dopaminergic neurons at the palisade zone of the median eminence. Here, it could conceivably block

DA release into the portal circulation, relieving the tonic inhibition of DA on prolactin release.

Galanin also is expressed in 5-HT neurons in the dorsal raphe of the rat brain (Melander, et al., 1986c), but apparently not in the human brain (Kordower, et al., 1992a). Although it has been demonstrated that galanin reduces 5-HT release from nerve terminals, this may be due more to the dense plexus of galanin fibers impinging on 5-HT neurons than from galanin produced in these cells. Galanin has not yet been found in the axons of serotonergic cells (reviewed in (Pieribone, et al., 1998)).

Galanin coexists with NPY in the LC (Holets, et al., 1988), with vasopressin in the basal forebrain and amygdala (Miller, et al., 1993), and with neurotensin and CRF in the PVN (Ceccatelli, et al., 1989). Little is known about the functional significance of coexistence in these neuropeptide systems. Galanin also is present in the majority of GnRH neurons in the rat preoptic area (Coen, et al., 1990; Marks, et al., 1992; Merchenthaler, et al., 1990), and in many GHRH cells in the arcuate nucleus (Niimi, et al., 1990), which is further discussed in the following section.

To summarize, the extensive coexpression phenomenon of galanin with both classical and peptide transmitters suggests that this molecule may play—in an as yet undefined manner—a role in regulating neuronal communication mechanisms.

V. Galanin and Reproduction

Among the most extensively studied of galanin's roles concerns its control of gonadotropin release (reviewed in (Hohmann, et al., 1998)). The first evidence of this came in 1987, when Sahu et al. discovered that galanin injected in the cerebral ventricles induces the release of luteinizing hormone (LH) in ovariectomized rats (Sahu, et al.,

1987). Galanin may do this by stimulating the release of GnRH from the median eminence, as has been shown in fragments of medial basal hypothalamus *in vitro* (Lopez and Negro-Vilar, 1990b). Neutralizing the effects of galanin with either antiserum or the galanin antagonist galantide blunts basal LH release and reduces the magnitude of the LH surge (Lopez, et al., 1993; Sahu, et al., 1994).

Although galanin may act directly in the brain to stimulate the release of gonadotropins, it also appears to act directly in the pituitary. Galanin levels are high in the portal circulation (Lopez, et al., 1991), perhaps reflecting its release from neurosecretory cells in the median eminence. Galanin mRNA is expressed in the pituitary, and is highly induced by estrogen (Gabriel, et al., 1990; Kaplan, et al., 1988a). Interestingly, the question of whether galanin always acts in the pituitary to stimulate LH release remains controversial. Early studies showed that galanin increases LH content in dispersed pituitary cells and potentiates the stimulatory effect of GnRH on LH release (Lopez, et al., 1991). However, a more recent study suggests that galanin may in certain circumstances be a paracrine inhibitor of gonadotrophs, as dispersed pituitary cells harvested at proestrus release less LH after galanin treatment in a dose-dependent fashion (Todd, et al., 1998).

Although the site and mode of action of galanin in the regulation of gonadotropin release is still uncertain, its co-expression in GnRH neurons has been well described. Consistent with a role in the activation of the reproductive axis, it has been shown that galanin mRNA increases markedly during the peripubertal period in rats. In adult rats, galanin mRNA in GnRH cells is 3 times higher in females than in males (Rossmannith, et al., 1994). Also, prepubertally castrated animals do not show the characteristic rise of

galanin mRNA across puberty, suggesting that this event is dependent on the prepubertal rise in titers of sex steroids (Finn, et al., 1996). Further evidence that galanin expressed in GnRH neurons is involved in reproductive processes comes from observations that galanin mRNA in these cells varies considerably over the estrous cycle in the rat (Marks, et al., 1993a), with a dramatic rise in gene expression seen during and after the LH surge at proestrus. The curious finding that galanin mRNA in GnRH cells remains high for at least 24 hours after the surge hints that this peptide may also play a role in dampening the pulsatility of GnRH release. This may help to restore the low basal levels of LH release seen during diestrus.

Correlative evidence for galanin as a regulator of GnRH neuronal activation comes from a study examining the time course of the activation marker *c-fos* mRNA in these cells (Finn, et al., 1998). Here it was seen that at 1200h on proestrus, *c-fos* message and galanin mRNA are both low in GnRH cells, as are serum LH levels. However, at 1500h on proestrus, *c-fos* message rises dramatically, and this is temporally well correlated with the LH surge. Galanin gene expression rises soon thereafter, while GnRH message does not change, suggesting that *c-fos* may be preferentially activating the cotransmitter galanin in these cells.

Galanin is robustly expressed in GnRH neurons in the rat and mouse (Rajendren and Gibson, 1999). However, its presence in these cells in the primate is less certain. In the pig-tailed macaque, galanin mRNA has not been detected in the populations of GnRH cells thought to be involved in the LH surge, although peripheral infusions of galanin do stimulate LH release in this species (Finn, et al., 2000). Finn et al. found a separate population of GnRH neurons in the substantia innominata that do express galanin, but the

functional roles of these newly discovered cells are as yet unknown.

The above mentioned studies suggest a prominent role for galanin in the control of the reproductive axis. This led me to hypothesize that long-term alterations in levels of galanin in the brain would cause phenotypic changes that could shed light on the relative importance of this molecule as a neuropeptide in reproduction.

VI. Galanin and Growth

The tightly regulated control of growth hormone (GH) synthesis and release is primarily orchestrated by 2 neuropeptides expressed in the hypothalamus, GHRH and SS. In the complex circuit where GHRH stimulates GH release and SS inhibits it, galanin may play an important modulating role. Numerous studies in humans have demonstrated that infusions of galanin reliably stimulate GH secretion (Arvat, et al., 1995; Cuerda, et al., 1998; De Marinis, et al., 2000; Todd, et al., 2000). Galanin may act by promoting GHRH release, as it potently stimulates GHRH content in the medium of median eminence fragments (Nishiki, et al., 1997). Alternatively, galanin may inhibit SS release and thereby disinhibit GH secretion (Chan, et al., 1996). Galanin stimulates GH levels at doses that do not alter the secretion of other hormones known to be influenced by galanin, such as LH, follicle-stimulating hormone (FSH), thyrotropin, and prolactin (Arvat, et al., 1995). This argues for a physiological role for galanin in stimulating the somatotropic axis, whereas the effects of this peptide on other pituitary hormones may require pharmacological doses.

Analysis of the pattern of galanin gene expression in GHRH neurons reveals a developmental and sexually dimorphic regulation of coexpression (Delemarre-van de

Waal, et al., 1994). During development in the rat, galanin message in GHRH cells increases at each age examined from neonates to adult rats. In the pre-pubertal rat, there is no difference between males and females in levels of galanin expression in GHRH neurons. However, in adults there is significantly more galanin mRNA in GHRH neurons of males than in females. This sexually dimorphic expression pattern of galanin in GHRH neurons may underlie part of the sexually differentiated pattern of pulsatile GH secretion that develops with the onset of puberty. While the coexpression of galanin with GHRH has not been examined in humans, extensive colocalization has been observed in the hypothalamus of and in nerve fiber varicosities of the median eminence of the monkey, confirming an anatomically relevant interaction in primates (Meister, et al., 1990).

These observations led me to hypothesize that either the chronic overabundance or complete lack of galanin would cause observable changes in growth patterns, and that these changes may be sexually dimorphic.

VII. Galanin and Feeding

Neuropeptides are intimately involved in the central control of body weight, and regulate both food intake and metabolism in mammals. The array of peptides that are involved in these processes continues to increase, and includes orexigenic peptides such as NPY, agouti-related peptide, melanin-concentrating hormone, beta-endorphin, and the orexins, and appetite-suppressing peptides such as leptin, CRF, alpha melanocyte-stimulating hormone and bombesin (reviewed in (Smith, 2000)). Galanin joined this list in 1986, when Kyrkouli et al. provided evidence that injections of galanin into the medial basal hypothalamus in rats acutely increased feeding (Kyrkouli, et al., 1986). Further anatomical specificity for the actions of galanin in appetite control was gleaned with the

demonstration that galanin injected into the PVN stimulates feeding, whereas microinjections into other hypothalamic areas are without effect (Kyrkouli, et al., 1990). Galanin also stimulates appetite when injected into the amygdala (Kyrkouli, et al., 1990) and nucleus of the solitary tract (Koegler, et al., 1999), and its orexigenic actions can be blocked by galanin antagonists (Crawley, et al., 1993). Thus far, significant effects of galanin on feeding have only been demonstrated in rats. This being the case, one objective of my research was to determine whether galanin is orexigenic in mice.

The mechanisms that underlie regulation of body weight by galanin remain obscure, but may involve the metabolic hormone leptin. This so-called "satiety" hormone, produced mainly in adipose tissue, acts centrally on the appetite-regulatory circuitry to reduce food intake and increase metabolic activity (Campfield, et al., 1995; Dunbar, et al., 1997; Halaas, et al., 1997; Halaas, et al., 1995; Hohmann, et al., 2000; Pellemounter, et al., 1995; Zhang, et al., 1994). Leptin signals in the brain through the long form of its receptor, which is expressed in many of the same neuroendocrine centers of the brain as galanin, such as the Arc, DMN, PVN and PeN hypothalamic nuclei (Elmquist, et al., 1998a). Leptin is thought to act directly on neuropeptide-containing cells in the hypothalamus, as it has been shown that leptin receptors are expressed on most proopiomelanocortin (POMC) neurons in the arcuate nucleus (Cheung, et al., 1997), and are also present in many NPY-expressing cells in the Arc (Baskin, et al., 1999a; Mercer, et al., 1996a). Convincing evidence has not yet been adduced for robust coexpression of the leptin receptor in galanin-containing cells, although it has been reported that some galanin-positive cells in the Arc contain leptin receptor immunoreactivity (Hakansson, et al., 1998). On the other hand, Cheung et al found no evidence for leptin receptor mRNA

in galanin-expressing cells in any hypothalamic region examined (Cheung, et al., 2001).

The question of whether galanin levels in the brain are significantly changed in obese rodents who have altered leptin signaling is also equivocal, raising doubts about the importance of galanin and leptin in modulating each other's actions. Leptin-deficient *ob/ob* mice, which harbor a mutation in the leptin gene, are grossly obese, diabetic and infertile (Ingalls, et al., 1950). Brain levels of several appetite-regulating peptides including NPY, POMC and neurotensin are different between obese and lean animals (Thornton, et al., 1997; Wilding, et al., 1993), but total hypothalamic galanin content is apparently unchanged in the face of chronic leptin deficiency (Williams, et al., 1991a; Williams, et al., 1991b). Nevertheless, in one specific area of the hypothalamus, the PeN, galanin gene expression is higher in *ob/ob* mice than in lean controls (Cheung, et al., 2001). Leptin administration in *ob/ob* mice reduces galanin mRNA in the PeN to that of lean controls, while having no effect in either the Arc or DMN, suggesting that the PeN may be an important area of interaction between these 2 peptide hormones. The situation regarding galanin is similar in the *fa/fa* Zucker rat, which has deficient leptin signaling, and is also very obese. Hypothalamic concentrations of galanin are not changed in food-restricted or freely fed *fa/fa* animals compared to lean rats, while those of neuromedin and NPY are increased (Williams, et al., 1991b). A more finely grained analysis of galanin by radioimmunoassay (RIA) does reveal a significant increase in 2 hypothalamic areas, the PVN and SON, and a reduction in the median eminence of obese Zucker rats when compared to lean rats (Beck, et al., 1993).

Central injections of NPY, MCH or galanin induce feeding in satiated rats, overcoming the signals that normally inhibit further food intake. The effects of these

orexigens can be prevented by prior treatment with leptin administered ICV, placing galanin into the category of neuropeptides that could conceivably be affected by leptin (Sahu, 1998b). Furthermore, chronic infusions of leptin into the brain down-regulates expression of galanin in concert with reductions in NPY and MCH mRNA (Sahu, 1998b), suggesting an interplay among these various molecules. George Bray has suggested that the seminal link between all these peptide hormones is their effect on sympathetic activity (Bray, 2000). A strong reciprocal relationship exists between suppression of feeding and suppression of the sympathetic nervous system.

Neuropeptides such as NPY, beta-endorphin, and MCH all reduce sympathetic activity while stimulating feeding (reviewed in (Bray, 2000)), perhaps ultimately by reducing the actions of NE. This is also the case for galanin, which has been shown to reduce sympathetic nerve actions on interscapular brown adipose tissue (Nagase, et al., 1996). On the other hand, anorectic peptides such as leptin, CRF, and alpha-MSH all increase sympathetic outflow, providing further support for this hypothesis (Bray, 2000).

The above observations suggest that an anatomical and physiological interaction exists between galanin and leptin, but the importance of this interaction is unclear. To further understanding of the relevance of the galanin/leptin link, I undertook a series of experiments assessing the differences in response to leptin in various models of altered galanin signaling.

An unsolved controversy surrounding galanin and food intake is the hypothesis that galanin is involved in the selection of macronutrient choice. The work of Leibowitz and colleagues has shown that in a small area of the anterior PVN, a high fat diet leads to an increase in galanin mRNA, which is associated with hyperglycemia and obesity

(Leibowitz, et al., 1998). When antisense oligonucleotides against galanin mRNA are injected into the anterior PVN, fat ingestion decreases and body weight drops (Akabayashi, et al., 1994a). The physiological importance of this finding has been questioned by other investigators, who have shown that acute galanin injections into either the PVN or the amygdala increase feeding by rats given a choice of either a high-fat or low-fat diet, with no preference given to either (Smith, et al., 1996). In another study, galanin given into either the solitary tract or the third ventricle had no effect on macronutrient selection, while stimulating overall food intake (Koegler, et al., 1999). Another observations related to fat ingestion and galanin is that maintenance on a high-fat diet lowers galanin gene expression in the PVN of obese Zucker rats (Mercer, et al., 1996b). Also, diabetic Brattleboro rats, who show a high preference for a lipid diet, have increased galanin mRNA in the SON and PVN (Odorizzi, et al., 1999), although the sub-region in the PVN where galanin is overexpressed is not the same as reported by the Leibowitz group (Leibowitz, et al., 1998).

In summary, there is no clear consensus about whether galanin is important for body weight regulation or fat metabolism. One goal of my research was to examine whether genetically induced alterations in galanin would lead to significant changes in body mass and food intake pattern, in the hope of shedding light on this controversy.

VIII. Galanin and NPY

Numerous lines of evidence suggest a functional overlap among various neuropeptide systems in the control of important physiological processes, including reproduction and feeding. Two systems that are plausible candidates for this putative “role-sharing” are those of galanin and NPY. Both neuropeptides can stimulate the

reproductive axis, although NPY may under some circumstances be inhibitory as well (McDonald, et al., 1985; Raposinho, et al., 1999; Reznikov and McCann, 1993).

Interestingly, administration of the galanin receptor antagonist galantide blocks the NPY-induced LH surge in steroid-primed ovariectomized rats (Horvath, et al., 1996), suggesting that some of NPY's stimulatory effects may be mediated downstream by galanin. Both galanin and NPY induce feeding when introduced into the cerebral ventricles or hypothalamus, although the effects of NPY are considerably more robust (reviewed in (Bray and York, 1998; Schwartz, et al., 1993)).

Like galanin, NPY is abundantly produced in the neuroendocrine hypothalamus, notably in the Arc (Gehlert, et al., 1987). NPYergic neurons project strongly to the PVN from the Arc (Broberger, et al., 1999), and galaninergic neurons send prominent axonal projections to the PVN, from the LC and the DMN (Levin, et al., 1987). One intriguing piece of evidence for direct contact between galanin and NPY comes from the demonstration that in the Arc and the POA, correlated light and electron microscope double-immunolabeling reveals direct contacts between NPY fibers and galanin-containing perikarya (Horvath, et al., 1996). While co-localization studies have not yet been performed in the Arc, in the brainstem NPY is present in the majority of galanin-containing neurons of the LC (Xu, et al., 1998).

The functional and anatomical overlap between galanin and NPY suggests that these two neuropeptide systems may complement at least some of each other's roles in the hypothalamus. A major component of my research was to test the hypothesis that galanin and NPY are functionally related. I postulated that the importance of this relationship could best be examined in circumstances where one or both of these

neuropeptides are permanently altered.

IX. Galanin and Cognition

Galanin is apparently unique as a neuropeptide that has a role as an inhibitory modulator of learning and memory processes. While many neuropeptides, including vasopressin, NPY, CRH, TRH, and Substance P have been shown to be cognitive enhancers, only galanin has demonstrated consistent inhibition of cognitive performance (reviewed in (Bennett, et al., 1997)). One of the first clues that galanin may have a role in cognition came from anatomical studies showing a dramatic upregulation of galanin in the degenerating basal forebrain of people with Alzheimer's disease (AD) (reviewed in (Mufson, et al., 1998)). A major pathological consequence of this disorder is the severe loss of cholinergic neurons on the basal forebrain, which may contribute to the dementia exhibited in AD.

Accompanying this anatomically specific loss of cholinergic cells is the dramatic hyperinnervation by galanin-containing fibers in the same regions of the basal forebrain. (Chan-Palay, 1988; Chan-Palay, 1990; Mufson, et al., 1993). Confocal microscopic analysis of the basal forebrain of AD patients reveals a hypertrophied and dense plexus of galaninergic fibers surrounding and in close apposition to the remaining cholinergic cells (Bowser, et al., 1997). As there are relatively few galanin-producing cells in the human basal forebrain (Chan-Palay, 1988), it is presumed that the neuronal source of this hyperinnervation is extrinsic to this area. Curiously, in the hippocampal terminal fields of the basal forebrain, galanin binding is significantly increased in AD while galanin binding is spared in the degenerating basal forebrain (Ikeda, et al., 1991).

These anatomical findings, considered together with the putative role of ACh in

learning and memory (reviewed in (Mufson, et al., 1998)), and the demonstrated inhibition of cholinergic tone by galanin, suggests that an excess of galanin might cause behavioral deficits in learning and memory-related tasks. In the first demonstration of this, Mastropalo et al. found that in rats with basal forebrain lesions, ICV or ventral hippocampal injections of ACH reverses deficits in a delayed alternation T-maze task, and that galanin injections blocks improvements seen with ACH (Mastropalo, et al., 1988). A subsequent study revealed that administration of the galanin antagonist M40, when coupled with a muscarinic agonist, improves performance in basal forebrain-lesioned rats in the delayed non-matching to position task (DNMPT) (McDonald, et al., 1998). Another galanin antagonist, galantide, improves performance on a social memory task in rats (Arletti, et al., 1997), and galanin reduces the step-down latency in a passive avoidance task (Ukai, et al., 1995). Further evidence of a negative role for galanin in the learning and memory circuitry comes from studies where galanin has amnestic action on active avoidance, one-trial reward learning, and the Morris watermaze (Malin, et al., 1992; Schott, et al., 2000; Shandra, et al., 1994)

Anatomical analysis of the specific sites of galanin-induced impairments has shown that the ventral hippocampus is the region where galanin injections are most effective (Orgen, et al., 1996; Robinson and Crawley, 1994; Schott, et al., 1998). Retrograde tracing studies have shown that galanin is an important component of the septo-hippocampal pathway, providing an anatomical link between the basal forebrain and the ventral hippocampus. Furthermore, microinfusion of galanin directly into the medial septal region also have been shown to impair working memory, as assessed by the T-maze task (Givens, et al., 1992). Also, in a study of aged-rats, specific galanin binding

is increased in an age-associated manner in several areas of the brain, and this increase correlates well with behavioral impairments on the Morris watermaze (Krzywkowski, et al., 1994). An intriguing recent finding is that galanin synergizes with the toxic 1-42 form of the beta-amyloid protein to reduce ACH release, which perhaps leads to further cognitive decline in the already degenerating AD brain (Wang, et al., 1999).

Taken together, the evidence strongly suggests that galanin has a substantial role in modulating cognitive functions. This might occur either through galanin acting directly on basal forebrain or hippocampal neurons that mediate memory acquisition, storage and retrieval, or by galanin modifying the actions of other neurotransmitters involved in these processes.

X. Galanin Receptors

As is the case with most neuropeptide transmitters, galanin signals through G-protein-coupled receptors (GPCR). There are now three cloned galanin receptor subtypes, which are all expressed to varying degrees in the brain (reviewed in (Branchek, et al., 2000)). Early binding studies with [¹²⁵I]-labeled galanin provided the first evidence of a wide distribution of galanin receptors in the rodent and primate brains (Kohler, et al., 1989; Melander, et al., 1988; Skofitsch, et al., 1986). The highest density of binding is seen in the olfactory bulb, the septum, the bed nucleus of the stria terminalis, the medial thalamus, various nuclei of the hypothalamus, the piriform and entorhinal cortices, the substantia nigra, the locus coeruleus, and the dorsal vagal complex. In general, fairly good agreement in anatomical localization of galanin binding is observed among species.

The use of galanin fragments and peptide galanin analogues strongly suggests the existence of multiple receptor subtypes in the brain, based upon differences in binding

and affinity. For example, the highly conserved 1-15 galanin fragment binds with high affinity in regions of the hippocampus where full-length galanin binds only weakly (Hedlund, et al., 1992). Valkna et al have shown dramatically differing IC50 values for the galanin-induced inhibition of adenylyl cyclase activity between ventral and dorsal hippocampus (Valkna, et al., 1995). In addition, the galanin 1-15 fragment hyperpolarizes dorsal hippocampal CA3 pyramidal neurons under conditions where full-length galanin and other galanin fragments are ineffective (Xu, et al., 1999). In the human brain, differences have been seen in binding affinity and labeling between various chimeric galanin peptides and galanin in the hypothalamus and basal forebrain, again suggesting the presence of different subtypes in these 2 regions (Deecher, et al., 1995).

Additional support for the existence of more than one galanin receptor in the brain comes from studies of galanin and galanin fragment-analogue interactions with various signal transduction pathways. Galanin has been shown to work through $G_{i/o}$ proteins to reduce cAMP levels, close voltage-sensitive Ca^{2+} channels, and open ATP-sensitive K^+ channels (reviewed in (Branchek, et al., 1998; Kask, et al., 1997)). These pathways are thought to underlie galanin's hyperpolarizing effects at the nerve terminal and inhibition of exocytosis. However, in many cases galanin stimulates transmitter release, as has been seen with GnRH and GHRH release at the median eminence, and with ACH release in the striatum. Galanin may promote exocytosis by mobilizing Ca^{2+} through activation of phospholipase C and/or phospholipase A (Hardwick and Parsons, 1992; Mulvaney and Parsons, 1995). Additional evidence for galanin linking to multiple signaling mechanisms comes from the demonstration that it can both increase and inhibit inositol phospholipid turnover (Malm, et al., 1997) and can increase cAMP levels in gastric cells (Gu, et al.,

1994).

The first galanin receptor (GAL-R1) was cloned from a human Bowes melanoma cell line (Habert-Ortoli, et al., 1994), followed soon by the cloning of the rat (Parker, et al., 1995), and the mouse version in 1997 (Jacoby, et al., 1997). Human, mouse and rat GAL-R1 are 92-93% identical at the amino acid level, and show the characteristic 7 transmembrane structure of GPCRs. GAL-R1 couples consistently to $G_{i/o}$ -type G proteins. It reduces cAMP levels in a pertussis-toxin sensitive manner, opens inwardly-rectifying G-protein-linked K^+ channels, and stimulates MAP kinase activity (Wang, et al., 1998). Like galanin, GAL R1 is widely expressed in the rat brain. *In situ* hybridization studies examining GAL-R1 mRNA have found high levels of hybridization in the hypothalamus, thalamus, hippocampus, amygdala, and brain stem (Burgevin, et al., 1995; Gustafson, et al., 1996; Parker, et al., 1995).

As galanin has been extensively studied as a neuroendocrine-regulating peptide, several studies have carefully examined the distribution of GAL R-1 in the hypothalamus of the rat. In a semi-quantitative study of the distribution of GAL-R1-labeled cells, Mitchell et al. found the highest expression within the medial POA, in all regions of the PVN, the SON, and in the ventromedial nucleus (VMN) (Mitchell, et al., 1997). The greatest cell count is seen in the POA, with the greatest intensity of labeling seen in the POA and PVN. GAL R-1 is also expressed robustly in the Arc, the LH, and in the DMN. Two further areas of high expression outside the hypothalamus are the lateral septum (LS) and vertical limb of the diagonal band (VDB). An interesting observation made by these authors was that the distribution and intensity of galanin and its receptor GAL-R1 is remarkably similar.

To date, no published reports exist regarding the distribution of GAL-R1 in the mouse. A goal of my research was to examine the expression patterns of GAL-R1 mRNA, to lay the anatomical framework for studies on the regulation of this receptor subtype.

The expression of GAL-R1 is altered by many different experimental manipulations—reminiscent of how galanin itself is regulated (although not always in the same direction). For example, while colchicine treatment up-regulates expression of the galanin gene, it lowers GAL-R1 in the magnocellular neurons of the hypothalamus (Landry and Hokfelt, 1998). Hypophysectomy also increases galanin in these cells, while markedly decreasing GAL-R1. In contrast, salt-loading increases the expression of both galanin and its receptor, while lactation reduces levels of both in magnocellular neurons. In another study, GAL-R1 was observed to be much higher in magnocellular neurons of the SON and PVN of the diabetic Brattleboro rat (Landry, et al., 1999). Again, in the magnocellular hypothalamus increases in GAL-R1 are seen following inhibition of glucose metabolism either by 2-deoxy-d-glucose or inhibition of fatty acid metabolism by sodium mercapto-acetate (Gorbatyuk and Hokfelt, 1998). As the magnocellular neurons in the SON and PVN coexpress galanin and GAL-R1 (Landry and Hokfelt, 1998), it would appear that galanin may regulate its own actions, at least in part, through this receptor subtype. One aim of my research was to determine if genetic alterations in galanin gene expression would lead to changes in mRNA levels of GAL-R1. I hypothesized that if GAL-R1 is an inhibitory auto-receptor for galanin, then message levels of this receptor would be higher when galanin levels are high in the brain, and would be lower when galanin levels are low or absent.

GAL-R1 also coexists with galanin in a high proportion of DMN and POA cells in the hypothalamus (Landry, et al., 1998), in the bed nucleus of the stria terminalis and medial amygdala (Miller, et al., 1997), and in the neuroendocrine cells of the human ocular epithelium (Ortego and Coca-Prados, 1998). A possible role for GAL-R1 in reproductive processes has been postulated reflecting, in part, the observation that this receptor subtype is upregulated in the POA during proestrus and estrus (Faure-Virelizier, et al., 1998b), also times when galanin gene expression in this region is high (Finn, et al., 1998). It is not known whether the cells that have higher GAL-R1 gene expression during these periods are the same ones that have increased galanin mRNA. It has been shown that a subset of GnRH neurons in the POA contain GAL-R1 (Mitchell, et al., 1999b) and many of these neurons also contain galanin (Marks, et al., 1992). In addition, a population of cells in the rostral Arc that preferentially project to the POA and are regulated by testosterone express both POMC and GAL-R1 (Bouret, et al., 2000).

The other major receptor subtype that is expressed in the brain is GAL-R2. This receptor was cloned almost simultaneously from rat hypothalamus by several different pharmaceutical company groups (Fathi, et al., 1997a; Howard, et al., 1997a; Wang, et al., 1997a). This was followed soon by cloning of the human and mouse GAL-R2 homologues (Borowsky, et al., 1998; Fathi, et al., 1998a; Pang, et al., 1998). GAL-R2 is slightly longer than GAL-R1, and shares only 40% amino acid identity with GAL-R1. In contrast to GAL-R1, which clearly signals through a $G_{i/o}$ -mediated mechanism, GAL-R2 apparently couples to several signaling pathways. The best described pathways involve phospholipase C- and G_q -mediated transduction. GAL-R2 stimulates inositol phosphate hydrolysis in a pertussis toxin resistant manner, promotes intracellular Ca^{2+} mobilization,

and induces the opening of Ca^{2+} -dependent Cl^- channels (Fathi, et al., 1998a; Smith, et al., 1997; Wang, et al., 1998). In some cells lines, GAL-R2 seems to couple to Gi/o , as activation of the receptor leads to a reduction in forskolin-stimulated cAMP accumulation (Smith, et al., 1997; Wang, et al., 1998). To further confuse the issue, it has been reported that stable transfection of human GAL-R2 leads to both an accumulation of inositol phosphate and Ca^{2+} mobilization from internal stores, combined with a pertussis toxin-sensitive reduction in cAMP (Fathi, et al., 1998b; Fathi, et al., 1998c).

The GAL-R2 receptor subtype has a much wider distribution in the body than GAL-R1, yet has a somewhat more limited and different distribution in the brain of the rat, the species where all the major anatomical studies have been performed to date. The expression of GAL-R2 is highest in discrete areas of the brain—the hypothalamus, the dentate gyrus, and the cerebellar cortex (Fathi, et al., 1997a; Kolakowski, et al., 1998a; O'Donnell, et al., 1999). This is in sharp contrast to GAL-R1, which is expressed in neither the cerebellum nor dentate gyrus. In the latter area, dense labeling is seen over the granule cells, with no signal in Ammon's horn. Moderate levels of GAL-R2 expression have been reported in the piriform, cingulate and entorhinal cortices, in various basal forebrain nuclei, and in the dopaminergic area of the substantia nigra (O'Donnell, et al., 1999).

As is the case with GAL-R1, the distribution of GAL-R2 has been best described in the hypothalamus, where it is expressed to some degree in virtually all hypothalamic nuclei. In a careful study, Mitchell et al found that several areas which express high levels of GAL-R2 do not express GAL-R1 at all, including the suprachiasmatic nucleus, the retrochiasmatic nucleus, and all divisions of the mammillary bodies (Mitchell, et al.,

1999b). In contrast, magnocellular areas of the PVN and SON are virtually devoid of GAL-R2 labeling, suggesting no significant role for this subtype in mediating delivery of neurohormones to the posterior pituitary. Other hypothalamic regions show high expression levels for both GAL-R1 and GAL-R2, including the preoptic area and dorsomedial nuclei, suggesting some overlapping roles for these 2 subtypes.

GAL-R2 has not been shown to be expressed in cells that contain galanin, but as is the case with GAL-R1, a subset of cells in the rostral arcuate nucleus that produce POMC also synthesize the GAL-R2 receptor (Bouret, et al., 2000). Using a novel co-labeling strategy of digoxigenin and fluorescence immunohistochemistry, Nichol et al have shown a distinct pattern of co-localization of GAL-R2 with somatostatin and tyrosine hydroxylase in the Arc nucleus and zona incerta (Nichol, et al., 1999).

There are no reports of GAL-R2 regulation as yet in the forebrain of the rat, but this subtype is dramatically upregulated after damage to motor neurons or primary sensory neurons (Burazin and Gundlach, 1998; Sten Shi, et al., 1997). Nerve crush injury in the facial nucleus leads to a 4-fold increase in GAL-R2 concomitant with a 10-fold increase in galanin gene expression, suggesting that GAL-R2 may mediate some of the neuro-regenerative effects of galanin. Also, inflammation causes a significant increase in levels GAL-R2 mRNA, along with the characteristic rise in galanin seen as a result of this insult. Oddly, axotomy of dorsal root ganglia reduces GAL-R2 message while increasing galanin mRNA, further demonstrating the complexity and tissue specificity of action of this receptor (Sten Shi, et al., 1997). The above studies demonstrate that, as is the case with GAL-R1 gene expression, GAL-R2 message levels differ significantly in experimental conditions that also lead to changes in mRNA levels of galanin. I

hypothesized that, like the GAL-R1 receptor subtype, regulation of GAL-R2 message would be one way for the brain to counteract long-lasting exposure to changes in galanin. To test this hypothesis, I examined GAL-R2 mRNA in the brains of mice with genetically altered levels of galanin.

Little is known about the central nervous system actions of the third cloned galanin receptor (GAL-R3). This subtype has been cloned in the rat (Smith, et al., 1998b; Wang, et al., 1997b), and human (Smith, et al., 1998b) but has not yet been reported in the mouse. The rat and human clones share 90% amino acid identity, and GAL-R3 has amino acid similarities of 36% with GAL-R1 and 55% to GAL-R2. The limited description of the functional coupling of GAL-R3 to signal transduction pathways resembles that of GAL-R1, and is consistent with $G_{i/o}$ -coupled mechanisms (reviewed in (Branchek, et al., 2000)). GAL-R3, when transfected into *Xenopus* oocytes, promotes the opening of inwardly rectifying K^+ channels in a pertussis toxin sensitive manner (Smith, et al., 1998a). In a melanophore dispersion assay, GAL-R3 activation led to a dose-dependent aggregation of pigment as has been seen previously when adenylyl cyclase is inhibited (Kolakowski, et al., 1998a).

The anatomical localization of the GAL-R3 transcript is still somewhat controversial. Using solution hybridization/Rnase protection assays Smith et al found high levels of GAL-R3 mRNA in the hypothalamus and pituitary, with no expression observed in the hippocampus (Smith, et al., 1998a). In contrast, another study utilizing RT-PCR methods reports that both the hippocampus and hypothalamus exhibit a strong GAL-R3 mRNA signal, Whereas nothing was seen in the pituitary (Waters and Krause, 2000). In a limited *in situ* hybridization study, GAL-R3 is reported to have a distribution

similar to that of GAL-R2, with abundant message in the hypothalamus, dentate gyrus, and substantia nigra (Kolakowski, et al., 1998a). Within the hypothalamus, GAL-R3 expression is highest in the VMN, Arc, PVN and SON, with some labeling noted in the mamillary bodies. Whether GAL-R3 colocalizes with galanin or any classical or neuropeptide transmitters is as yet unknown.

XI. The Mouse as a Model System

The appropriate choice of experimental model is a critical aspect of scientific discovery. Certainly, if methods exist that provide answers to important questions in biology which do not require the use of animals, these techniques should be used, for both ethical and practical reasons. Methodologies such as cell culture and computer modeling have been powerful tools in helping to understand the fundamental workings of the brain. However, when attempting to elucidate physiologically important processes such as the control of neuroendocrine systems, the use of living animals is often the only way to ask the relevant questions.

The vast majority of studies exploring the behavioral, anatomical and neurochemical aspects of mammalian neurobiology have employed rats as the preferred model system. Rats are inexpensive, easy to maintain and handle, and are available in well-defined in-bred strains that offer the homogeneous sets of animals useful for controlling experimental variables. Rats have been used extensively for many different disease models, and are a well understood species physiologically and anatomically, giving the researcher a rich database to draw from. Importantly, rats are apparently well suited to life in a small space, and live long and healthy lives in captivity. A major disadvantage of the rat model is the current lack of techniques to effectively perform

targeted genetic manipulations in this species. While several transgenic rats have been produced (reviewed in (Murphy, et al., 2000; Voigt, et al., 2000), the lack of developed stem cell technology in rats make them as yet unsuited for gene ablation studies.

The other major mammalian model used for the study of physiology is the primate. The major advantage of the primate model is its similarity to ourselves, which often allows for closer experimental comparisons to the human condition than rodent models can provide. Primates are long-lived, intelligent in a way that makes cognitive studies easier to interpret than results from rodents, and suffer from many of the same diseases that humans do. However, for many studies in basic scientific discovery, primates do not fit the ethical mandate of using the most simplistic model possible to provide answers to the important questions being investigated. Primates are also extremely expensive to house and care for, have very long gestation times and low birth numbers, and like rats lack a defined gene-targeting methodology.

In recent years, the mouse has been gaining precedence as the preferred model system for many types of studies. Mice share with rats practical advantages such as being inexpensive, easy to house and handle, and are readily available in well-characterized strains. The availability of multiple different lines of well-characterized inbred mice dates as far back as 1910, when Clarence Little established the Dilute Brown Agouti (DBA) mouse (reviewed in (Monastersky and Gulezian, 1997)). During the next 60 years these and many other lines of mice were used for medically relevant studies in areas such as immunology research, but for behavioral and neuroendocrine studies the rat remained the preferred model. However, in the last 30 years this has been changing—due largely to the advent of transgenic and knockout technology. The ability to insert extra copies of a

defined gene or inactivate a gene discretely with knockout strategies has revolutionized the way rodents are used in biomedical research (Capecchi, 1989; Palmiter and Brinster, 1986; Picciotto and Wickman, 1998; Tsien, et al., 1996). There are disadvantages to relying on the mouse model, such as difficulty of surgeries due to small size and the relative lack of a behavioral and pharmacological database to work with. Nevertheless, the overwhelming advantage of being able to make important comparisons by altering the genome makes the mouse the model of choice for studies revolving around the interface between gene regulation and physiology. For this reason, I chose to primarily focus on the mouse as a research model for studying the role of galanin in the brain.

XII. Statement of the Problem

Because neuropeptides have a widespread distribution in the brain, and may have subtle, modulatory roles in the nervous system, revealing the precise function of these molecules in the brain has provided researchers with a difficult set of challenges. Research on galanin is illustrative of these difficulties. Galanin has been implicated in a host of central nervous system functions. These range from fundamental processes, such as the ability to successfully breed and regulate metabolism, to higher order functions such as the need to learn and remember tactics for finding food, avoiding predators, and choosing mates. Most of the myriad reports on galanin have employed either pharmacological or biochemical approaches to elucidate the regulation of the galanin gene and the workings of galanin in the brain. While these approaches have been informative, studies in cell culture systems and injections of galanin or its antagonists into the brain can only reveal part of the story. Neurons in culture lack the important afferents and efferent projections that are essential components of a complex neuronal

circuit. In addition, the peptide antagonists developed thus far are poorly selective for the 3 cloned galanin receptors, and to further confound the issue many of these antagonists show partial agonistic effects. As a result, the functions of galanin suggested thus far are often conflicting and unconvincing.

The approach I have taken is to examine the effects of permanently altered levels of galanin in genetically altered mice. My essential hypothesis was that enduring changes in galanin gene expression of mutant animals would produce phenotypes that in turn could provide clues to the specific roles galanin has in the brain. First, to provide an anatomical substrate for further studies, I examined the distribution of galanin gene expression in the brain of the mouse. Second, I sought to determine the role of galanin by using transgenic mice, in whom the galanin gene is overexpressed in noradrenergic neurons. Third, I studied the effects of targeted ablation of the galanin gene in knockout mice. Fourth, I tried to determine whether NPY and galanin have overlapping roles in the brain by producing and performing an initial characterization of mice with both the galanin and NPY genes ablated.

Chapter 2

Materials and Methods

I. Animals and Accommodations

All animals used in these studies were from the mouse colony at the University of Washington. All animals were housed in the Department of Comparative Medicine animal care facilities. All animal procedures were approved by the University of Washington Animal Care Committee, in accordance with the National Institute of Health Guide to Care and Use of Laboratory Animals. All reasonable steps were taken to minimize animal suffering, and only the minimum numbers of animals necessary were used for each study. Animals were grouped housed except during actual experimental procedures, when single housing was used as required. GALTG mice were produced at the University of Washington as described in (Steiner, et al., 2001). GKO mice were produced at Bristol University, Bristol, England as described in (Wynick, et al., 1998). DKO mice were produced by crossing GKO mice and NPYKO mice (both maintained in the mouse colony at the University of Washington) together. The resulting compound heterozygotes were mated, and offspring genotyped by southern blot to identify DKO, GKO and WT animals. DKO male and female mice from different sets of original founders were bred together to develop the DKO colony, and GKO single mutants were developed using the same strategy. WT animals resulting from the mating of compound heterozygotes were also bred together for use as age-matched experimental controls.

II. Assessment of General Health and Physiology

All mutant strains of mice were examined for general health and several physiological parameters were assessed. Animals were weighed by putting them in a plastic beaker placed on a table-top balance (200 x 0.01g; Ohaus Corp. Florham Park, NJ). Mutant mice and their appropriate WT control mice were always weighed at the same time of day to minimize variation in body weight. Food intake was measured at 24 h periods for daily food intake, or at 2 h intervals for assessment of circadian food intake patterns. Water intake was measured at 24 h intervals, using graduated water bottles or by weight. Body length was measured in anesthetized mice by placing them on a small ruler with mm increments, and measuring from the tip of the nose to the anus. Core body temperatures were obtained using a digital thermometer (Fisher Scientific, Pittsburgh, PA). Mice were gently restrained by hand, and a rectal probe inserted was approximately 15 mm into the anus, until temperatures stabilized.

III. Blood Collection and Glucose Measurement

Blood was collected by two different methods. Orbital eye bleeds were used to collect small amounts of blood for glucose and subsequent hormone measurements. Mice were anesthetized with an isoflurane inhalant. Using a 100 μ l heparinized glass capillary tube, rotating pressure was applied to the medial canthus of the eye until rupture of the capillary bed of the orbital sinus. Blood was collected into a serum separator tube (Microtainer Brand, Becton Dickinson, Franklin Lakes, NJ) and allowed to clot briefly on ice. The blood was spun at 14,000 g for 3 minutes, and the separated serum pipetted into microcentrifuge tubes and stored at -20° until needed. Larger volumes of blood were collected at sacrifice, by cardiac puncture. Animals were deeply anesthetized using an

isoflurane inhalant. An superficial incision was made in the chest, and the skin pulled back to reveal the xyphoid process. A 22g x 1 1/2" needle was inserted at an approximately 30° angle under the xyphoid process and into the heart. Blood was drawn into a 1 ml or 5 ml disposable syringe, transferred to a serum separator tube, and processed as previously described. Glucose measurements were made by using a hand-held glucometer (Glucometer Elite XL; Bayer, Elkhart, IN). Animals were gently anesthetized using isoflurane anesthesia, and blood collected by orbital eye bleed, as described above. A small spot of blood was placed in the test strip, which was inserted into the glucometer, and readings recorded as serum/plasma glucose in mg/dl.

IV. Radioimmunoassays

Leptin: Serum leptin concentrations for GALTG mice were measured in the Laboratory of Joseph Beavo at the University of Washington. Samples were measured in duplicate volumes of 50 μ l each, using a commercial RIA kit (Linco St. Louis, MO). The sensitivity of the assay was 0.1 ng/ml. Serum leptin concentrations for GKO and DKO mice were measured in the Core Laboratory of the Center for Nutritional Research Unit at the University of Washington, using a solid phase enzyme immunoassay kit (Research Diagnostics, Flanders NJ). Samples were measured in duplicate volumes of 50 μ l each.

Insulin: Serum insulin concentrations were measured in the Immunoassay Core Laboratory of the Diabetes Research Center at the University of Washington. Samples were measure in duplicate volumes of 100 μ l each, and the sensitivity of the assay was 2.5 μ U/ml.

Glucagon: Serum insulin concentrations were measured in the Immunoassay Core Laboratory of the Diabetes Research Center at the University of Washington. Samples

were measured in duplicate volumes of 100 μ l each, and the sensitivity of the assay was 20 pg/ml.

Corticosterone: Serum corticosterone concentrations were measured in the laboratory of Jon Levine, Department of Neurobiology and Physiology, Northwestern University, Evanston, IL. Samples were measured in duplicate volumes of 25 μ l each.

Prolactin: Serum prolactin concentrations were measured in the laboratory of Jon Levine, Department of Neurobiology and Physiology, Northwestern University, Evanston, IL. Samples were measured in duplicate volumes of 60 μ l each.

Insulin-like growth factor-1: Insulin-like growth factor concentrations were measured in the laboratory of Gloria Tannenbaum, Department of Pediatrics, McGill University, Montreal, Quebec. Samples were measured in duplicate volumes of 25 μ l each.

T4: Serum T4 concentrations were measured in the laboratory of Jon Levine, Department of Neurobiology and Physiology, Northwestern University, Evanston, IL or by Phoenix Laboratories, Everett, WA. Samples were measured in duplicate volumes of 25 μ l each.

Luteinizing hormone: Serum luteinizing hormone concentrations were measured in the Endocrine Laboratory at Colorado State University, Fort Collins, Colorado, using reagents from the National Institute of Health. The standards used were rLH-CSU-S1. Samples were measured in duplicate volumes of 50 μ l each. The sensitivity of the assay was 0.05ng/ml.

Follicle-stimulating hormone: Serum follicle-stimulating hormone concentrations were measured in the laboratory of Jon Levine, Department of Neurobiology and

Physiology, Northwestern University, Evanston, IL. Samples were measured in duplicate volumes of 50 μ l each.

Testosterone: Serum testosterone concentrations were measured in the laboratory of Dr. William Bremner of the Puget Sound Veterans Administration under the auspices of the NICHD Population Research Center, using a Delphia fluoroimmunoassay kit (EG & G Wallac, Turku, Finland). Samples were measured in duplicate volumes of 25 μ l each, and the sensitivity of the assay was 5pg/100 μ l of serum.

Galanin: Tissue galanin concentrations were measured by Dr. Mohammad Ghatei, Department of Investigative Science, Imperial College of Medicine, London, England. Tissues were dissected fresh, and quick frozen in micro-centrifuge tubes on dry ice until assay. Tissues were extracted by boiling in 0.5M acetic acid. The antibody was raised in New Zealand White rabbits against synthetic rat galanin conjugated to bovine serum albumin (BSA) by glutaraldehyde and used at a final dilution of 1:80,000. This antibody cross-reacted 35 and 15% with porcine and human galanin respectively, and did not show any cross-reactivity with any other known neuropeptides. I-125 galanin label was prepared by the iodogen method and the product was purified by HPLC. The galanin label was used at 45 Bq/tube. The assay was performed in a total volume of 800 μ l of 0.06M phosphate-EDTA buffer (pH 7.4) containing 0.3% BSA and incubated at 4 ° C for 3 days. Bound and free fractions were separated by the addition of 6 mg of dextran-coated charcoal and counted. The detection limit of the assay was 1.2 fmol between adjacent tubes and the inter- and intra-assay variation was established to be less than 10%.

V. Administration of Hormones

Leptin: All leptin used in these studies was supplied by Zymogenetics, Corp. Seattle, WA. Recombinant full-length human leptin protein was produced in *Saccharomyces cerevisiae*. Leptin was purified to near homogeneity (>95%) by analytical HPLC, and quantified by mass spectroscopy. The control solution was 50mM sodium borate (pH 8.0). Leptin was administered once or twice daily just before lights out. Leptin was injected into the peritoneal cavity of gently restrained, unanesthetized animals, using a 27g x 1/2" needle. Animals were then immediately returned to their home cages.

Freehand intracerebroventricular (ICV) injections of galanin and NPY: Galanin (American Peptide Company, Sunnyvale, CA) was dissolved in artificial cerebrospinal fluid (aCSF) at a final concentration of 4 µg/20 µl. NPY (Sigma, St. Louis, MO). was dissolved in aCSF at a final concentration of 3 µg/5 µl. Both peptides were administered in a volume of 5 µl. Mice were anesthetized with an isoflurane inhalant, and placed in a sternal recumbent position. A 27g x 1/2" needle fitted with a 0.8 cm plastic sheath (leaving 0.4 cm needle exposed) was attached to the Luer-Lok hub of a 25 µl Hamilton syringe. Slight pressure was applied to the ears in a downward direction to level and stabilize the head during injection. The injections were given into the lateral ventricle with the needle inserted perpendicularly to the head, at 1.0 mm posterior to bregma and 0.5 mm lateral to the midline. After a steady continuous injection, the needle was kept in place for several more seconds, to prevent backflow up the needle track. An initial hole was made 1 day prior to the beginning of the experiment, and the hormones were administered through this hole.

Administration of galanin via Alzet mini-pump: Galanin (American Peptide Company, Sunnyvale, CA) was dissolved in aCSF at a final concentration of 1.5 $\mu\text{g}/\mu\text{l}$, and loaded into a 100 μl Alzet mini-pump (Model 1003D Alzet, Mountain View, CA). Animals were anesthetized with isoflurane, and kept unconscious with an inhalant nose tube for the duration of the surgery. The head was shaved, and incision made from just behind the eyes to the nape of the neck. The skull was cleaned with alcohol, and a small hole was made 1.0 mm posterior to bregma and 0.5 mm lateral to the midline. The Alzet brain infusion cannula was placed into the lateral ventricles at a depth of 3 mm, and glued to the skull with superglue. A subcutaneous hole was made between the scapulas, and the Alzet pump was inserted under the skin and attached to the cannula with a 1 cm long plastic tube. The scalp was closed, and sealed with Vet-bond. Mice were removed from anesthesia, and allowed to recover on a heating pad before being returned to their home cage. At the end of the treatment period, the animals were sacrificed, and the pumps were removed. Cannulas were tested for patency with blue dye, and any remaining contents of the pump were evacuated and measured with a Hamilton syringe.

VI. Catecholamine Measurements

Catecholamines were measured by HPLC in the laboratory of Alvin Matsumoto of the Veterans Administration Puget Sound Health Care System, Seattle, WA. Mice were sacrificed under isoflurane anesthesia, and brains were immediately removed and dissected, then frozen on dry ice and kept at -80°C . To extract catecholamines, tissues were homogenized in the following volumes of 0.1M perchloric acid with 0.01% cysteine: brainstem, 400 μl ; hippocampus, 200 μl , hypothalamus, 200 μl , forebrain 600

μl . 3,4-dihydroxybenzylamine HBr was used as an internal standard at 10,000 $\mu\text{g}/10 \mu\text{l}$. A 400 μl aliquot was then sonicated. Sonicates were centrifuged at 18,700g for 10 min. A 100 μl aliquot of supernatant was added to 20 mg of acid-washed alumina and 400 μl of 0.5 M Tris buffer with 2% EDTA and rotated overnight. The alumina was washed twice with 1 ml of distilled water and then catecholamines were extracted with 100 μl of 0.1M perchloric acid buffer with 0.01% cysteine. The HPLC was run at a flow rate of 1 ml/min, and an injection volume of 20 μl was used. The working potential of the electrochemical detector was +0.8 V, and the full-scale sensitivity was 5 nA for all tissues. Quantification of catecholamines was done by comparing the peak heights of unknowns to those of known quantities of catecholamine standards and DHBr, using an HP 3393A integrator. The lower limit of detection ranged from 10 to 100 μg . Samples that were below the limit of detection were assigned a value equal to the limit of detection. All catecholamine levels were normalized per mg of protein. Protein was determined, using the Bio-Rad (Hercules, CA) protein microassay.

VII. Tissue Preparation

In all experiments, mouse brains were kept frozen at -80°C until processing. Brains were then placed into the chamber of a Leica CM 1900 cryostat (Bartels and Stout, Bellevue, WA) to equilibrate to -20°C . Brains were mounted onto a frozen chuck with Tissue-Tek OCT (Miles Scientific, Naperville, IL) and coronal sections were cut at a thickness of 20 μm . Individual sections were mounted onto Superfrost Plus slides (VWR, West Chester, PA), with 4-8 sections on a single slide. Sections were collected on four sets of slides, with each slide representing a one-in-four series of sections from the rostral

to caudal extent of the brain region of interest. Slides were placed in airtight plastic slide boxes, and stored at -80°C until *in situ* hybridization.

VIII. Probe Preparation

³⁵S-labeled mouse galanin probe (Experiments 1 and 5). The plasmid vector pGemT-Easy containing a 493 bp cDNA corresponding to the entire coding region of preprogalanin was kindly provided by Dr. James Hyde of University of Kentucky. The plasmid was linearized with SacII and transcribed with SP6 to generate a cRNA antisense probe complementary to mouse galanin mRNA. A sense probe was generated by linearizing the plasmid with PstI, and transcribed with T7. The isotopically-labeled mouse galanin antisense riboprobe was generated in an *in vitro* transcription reaction with the following reagents: 25 μl (Exp 1) or 15 μl (Exp 5) of 12.5 mCi/ml ³⁵S-labeled UTP, which represents 25% of UTP, and 2 μl (Exp 1) or 0.9 μl (Exp 5) cold thio-labeled UTP in a final concentration of 50 mM α -thio-UTP; 2 μl mixture of 50 μM each of ATP, CTP, and GTP; 2 μl RNase block; 3 μl SP6; 2 μg linearized mouse galanin plasmid vector DNA; 2 μl 10X transcription buffer (Boehringer Mannheim, Indianapolis, IN); and 10 μl DEPC-treated water. The transcription reaction was incubated at 37°C for 1.5 h. The reaction mixture was then mixed with 1 μl DNase I for 15 min before the reaction was terminated with the addition of 4 μl EDTA and 1 μl tRNA. The probe mixture was brought to a final volume of 50 μl with DEPC-treated water, purified with a Quickspin G-50 Sephadex spin column (Boehringer Mannheim, Indianapolis, IN) and run on a polyacrylamide gel to check for probe integrity. This riboprobe was used at a concentration of 0.25 $\mu\text{g}/\text{ml}\cdot\text{kb}$.

³⁵S-labeled rat galanin probe (Experiments 2 and 4): The plasmid vector Bluescript 13 (-) containing a 680 bp cDNA to rat galanin was kindly provided by Dr. Maria Vrontakis, University of Manitoba, Winnipeg, Canada. The cDNA consists of 372 bp of coding sequence, flanked by 124 bp of 5' non-coding sequence and 184 bp of 3' non-coding sequence. The plasmid was linearized with HindIII for antisense and SmaI for sense probes. The riboprobe was generated as described above, using 23 μ l (Exp 2) or 24 μ l (Exp 4) 12.5 mCi/ml ³⁵S-labeled UTP, 2 μ l cold UTP, and 1 μ g of linearized galanin template, using T7 polymerase for antisense transcription. The riboprobe was used at a concentration of 0.25 μ g/ml \cdot kb.

³⁵S-labeled mouse POMC probe (Experiment 3): The plasmid vector pMKSU16 containing a 920 bp mouse POMC cDNA was kindly provided by Dr. Michael Uhler. This fragment was subcloned into pSP64 plasmid, and consists of 98 bp of 5' non-coding sequence, the entire 705 bp coding sequence, and 105 bp of 3' non-coding sequence. The plasmid was linearized with HindIII for antisense, and transcribed with SP6 polymerase. The riboprobe was generated as described above, using 11 μ l of 12.5 mCi/ml ³⁵S-labeled UTP, 3 μ l of cold UTP, and 1 μ g of linearized POMC template. The riboprobe was used at a concentration of 0.20 μ g/ml \cdot kb.

³⁵S-labeled rat NPY probe (Experiment 17 and 25): The plasmid vector pBLNPY-1, containing a 511 bp rat NPY cDNA was kindly provided by Steven Sabol, and was subcloned into the plasmid pBSKS. The plasmid was linearized with PVUII for antisense, and with SmaI for sense probes. Antisense transcription was performed with T3 polymerase, and with T7 for sense transcription. The antisense riboprobe was generated as described above, using 27 μ l (Exp 25) or 25 μ l (Exp 17) 12.5 mCi/ml ³⁵S-labeled UTP,

2 μ l cold UTP, and 0.9 μ g (Exp 17) or 2 μ g (Exp 25) linearized NPY template. The riboprobe was used at a concentration of 0.25 μ g/ml \cdot kb.

³³P-labeled rat galanin receptor 1 (GAL-R1) probe (Experiment 13): A 390 bp GAL-R1 cDNA was cloned by Ellen Grafstein-Dunn, and inserted into the plasmid pBSKS. The plasmid was linearized with EcoRI for antisense and with XbaI for sense probes. Antisense transcription was performed with T7 polymerase, and with T3 for sense transcription. The riboprobe was generated as above, using 0.25 mCi of 10 mCi/ml ³³P-labeled UTP and 1 μ g of linearized template per 20 μ l reaction. The riboprobe was used at a concentration of 0.37 pmol/ml.

³³P-labeled rat galanin receptor 2 (GAL-R2) probe (Experiment 14): A pCDNA plasmid containing the open reading frame with contiguous 5' and 3' non-coding regions for GAL-R2 was kindly provided by Andrew Howard, and a 452 bp fragment was subcloned into plasmid PDP18 CU-. The plasmid was linearized with HindIII for antisense and EcoRI for sense probes. Antisense transcription was performed with T3 polymerase, and with T7 for sense transcription. The antisense riboprobe was generated as above, using 0.25 mCi of 10 mCi/ml ³³P-labeled UTP and 1 μ g of linearized template per 20 μ l reaction. The riboprobe was used at a concentration of 1 pmol/ml.

³³P-labeled mouse galanin receptor 1 (GAL-R1) probe (Experiments 15, 16 and 24): A 377 bp segment containing the coding region of GAL-R1 cDNA was cloned by Anders Jureus, and inserted into the plasmid pAMP. The plasmid was linearized with EcoRI for antisense and with HindIII for sense probes. Antisense transcription was performed with SP6 polymerase, and with T7 for sense transcription. The antisense riboprobe was generated as above, using 0.25 mCi of 10 mCi/ml ³³P-labeled UTP and

2.2 µg of linearized template per 20 µl reaction. The riboprobes were used at a concentration of 0.32 pmol/ml.

IX. *In situ* Hybridization Histochemistry

Tissue fixation and delipidation: All slides were processed through fixation and delipidation steps prior to *in situ* hybridization. Slides were removed from the -80° C freezer, and racked into baked metal slide holders on dry ice. Slides were then dried with a warm air hair dryer, until no droplets of moisture could be seen. Slides were then placed into a fixative solution of 4% paraformaldehyde (pH 7.4) for 5 minutes, then washed with stirring in 0.1M phosphate buffer (0.02M NaH₂PO₄, 0.08M NaHPO₄, pH 7.4) twice for 5 minutes each. Slides were then dipped in Nanopure water, followed by 0.1M TEA (pH 8.0), then placed into stirring 0.1M TEA with 0.25% acetic anhydride for 10 minutes. This was followed by 3 minutes equilibration in 2x standard sodium citrate (SSC), and 3 minutes each of ascending alcohol dehydrations (70%, 95%, 100%). Slides were delipidated in chloroform for 5 minutes, then rehydrated for 3 minutes each in 100% and 95% alcohol, before being allowed to air dry. Slides were wrapped in foil, and stored at room temperature until hybridization.

Prehybridization: For the galanin receptor assays (Experiments 13, 14, 15, 16, 24) slides were prehybridized to maximize the signal-to-noise ratio. A solution of 75% hybridization buffer (50% deionized formamide; 10% dextran sulfate; 0.3M NaCl; 10mM Tris, pH 8.0; 1 mM EDTA; 0.02% BSA; 0.02% Ficoll; 0.02% polyvinylpyrrolidone) and 25% tRNA (10 mg/ml) was applied in a volume of 100 µl to each slide. Silanized coverslips were placed over the tissue sections, and the slides incubated at 60° C for two hours. The coverslips were removed, and the slides rinsed for 3 minutes in 2x SSC,

before dehydration for 3 minutes each in ascending alcohol steps (70%, 95%, 100%).

Slides were air-dried and covered in foil until hybridization.

Hybridization : Slides were placed in moistened Tupperware containers for hybridization. Probes were applied in a total volume of 40-75 μ l per slide, depending on the amount of probe available, with the same amount applied for all slides in a given assay. The volume of hybridization solution consisted of 80% hybridization buffer (50% deionized formamide; 10% dextran sulfate; 0.3M NaCl; 10mM Tris, pH 8.0; 1 mM EDTA; 0.02% BSA; 0.02% Ficoll; 0.02% polyvinylpyrrolidone) with the remaining 20% consisting of probe (at the appropriate concentration detailed in the previous section) and tRNA (10 mg/ml). The probe/tRNA mixture was denatured by boiling for 3 minutes , then placed in a slushy ice bath for a further 5 minutes, before being added to the hybridization buffer. For 35 S-labeled probes, 200 mM DTT was added to the hybridization buffer to reduce disulfide bond formation between riboprobes. After applying the probe, slides were covered with silanized coverslips, then hybridized overnight at the appropriate temperature, as detailed in the Results section for each experiment.

RNAse treatment and stringency washes: After hybridization, coverslips were removed from the slides, and slides were rinsed with stirring twice at 15 minutes each in 2x SSC. Single-stranded and unbound probe was removed by a 30 minute incubation at 37° C with RNAse A (30 μ g/ml in 10 mM Tris, pH 8.0; 1 mM EDTA; 50 mM NaCl). Following this slides were rinsed at 37° C for 30 minutes in RNase buffer (10 mM Tris, pH 8.0; 1 mM EDTA; 50 mM NaCl). After a 30 minute wash in 1x SSC, slides were subjected to a final stringency wash of 0.1x SSC twice at 30 minutes each, at the

appropriate temperature as detailed in the Results section for each experiment. Slides were equilibrated for 3 minutes in 0.1x SSC at room temperature, before being dehydrated in an ascending series of alcohols (70% with 300 mM ammonium acetate, 85% with 300 mM ammonium acetate, 100%). Slides were allowed to air dry, then stored at room temperature.

Autoradiography: Slides were dipped for autoradiographic exposure in either NTB-2 (Kodak, Rochester NY) for ^{35}S -labeled riboprobes or NTB-3 for ^{33}P -labeled riboprobes. NTB-2 was diluted 1:1 in 600 mM ammonium acetate, and NTB-3 was used undiluted. Emulsions were heated to 45° C in a water bath, and poured into a dipping chamber in the absence of white light. Slides were dipped back-to-back by hand, then allowed to air dry for two hours in the complete darkness, before being placed in light-proof containers, wrapped in foil, and stored at 4° C until development. Test slides were developed each few days (4 minutes D-19 Kodak developer at 15° C, 1 minute Nanopure water at 15° C, 5 minutes Kodak fixer at 15° C), until the specific signal-to-noise ratio was determined to be optimal. The slides were then developed (as above) and rinsed for 20 minutes in running deionized water. Slides were counterstained with cresyl violet to visualize cell bodies, dehydrated for 2 minutes each in alcohol (70%, 95%, 100%) and cleared for 15 minutes in three changes of Hemo-De (Fisher, Pittsburgh, PA). Coverslips were mounted onto slides with Permaslip (Alban Scientific, St. Louis, MO) to preserve tissue for image analysis and archiving.

X. Image analysis

Slides were anatomically matched using a mouse atlas (Franklin and Paxinos, 1997) so that an equivalent number of slides from each region of interest could be

analyzed for each experimental group. mRNA-positive cells were identified under dark-field using 40x magnification, as clusters of silver grains. Cresyl violet staining was used to orient specific nuclei within the brain under bright-field at 2.5x and 10x magnification. Analysis was performed with a Zeiss Axioscope (Carl Zeiss, New York, NY), using a 40x epi-illumination objective, and side-illumination reflected dark field. Sections were read by a blinded observer, using an automated image analysis system, developed by Don Clifton, University of Washington. This system consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN), attached to a Power Macintosh G3 computer. Images were captured with a CCD camera (COHU, San Diego, CA), and both the number of identifiable mRNA-positive cells and the number of grains per cell were calculated by the image analysis software.

For some areas, it was not possible to count individual cells, either because clusters were too tightly packed together, or because the signal was identifiable but diffusely distributed in a particular region. In these cases, sections were analyzed using the MCID image analysis system (Imaging Research, St. Catherines, Ontario). Sections were visualized with a Nikon Optiphot II microscope (Meridian Instruments, Kent, WA) with a 10x objective under dark-field illumination. Video images were captured with a CCD camera (Dage-MTI, Michigan City, IN) and were projected to a monitor. The mRNA-expressing region was then outlined freehand, and the average gray levels of the area of interest were measured. A similar sized region devoid of specific signal was then outlined, and the average gray levels of this region calculated, to achieve a signal-to-background ratio (SBR) for each area.

Chapter 3

Experimental Design and Results

I Galanin Gene Expression in the Mouse Brain

As a foundation for further gene regulation studies, I performed the following experiments to determine the anatomical localization and relative abundance of galanin in the mouse brain. I also examined the gene expression patterns of galanin and POMC in response to the absence of NPY. In Experiment 1, I determined the distribution of galanin in the adult male mouse brain, from the olfactory bulb to the caudal medulla. In Experiment 2, I examined and compared levels of galanin mRNA in the hypothalamus of wild-type (WT) and NPY knockout mice (NPYKO), hypothesizing that the galanin gene would be upregulated when NPY is absent. In Experiment 3, I analyzed POMC gene expression in both WT and NPYKO mice. I hypothesized that the POMC gene, whose products are also involved in neuroendocrine processes, would also be differentially regulated in NPYKO mice.

A. Experiment 1

I hypothesized that galanin mRNA would be widely distributed in the brain of the mouse, as it is in the rat and primate.

Materials and Methods

Experimental Design: Adult male CBL6/J x 129SV mixed strain mice (n=3) bred

in the animal colony at the University of Washington were sacrificed by CO₂ asphyxiation, and brains were collected and quick-frozen to -80 ° C. Brains were sectioned at 20 µm thickness according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the olfactory bulb (plate 1) to the caudal portion of the medulla (plate 93).

Riboprobe Preparation: A 493 bp mouse galanin cDNA was used to generate sense and antisense ³⁵S-labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *In situ* hybridization was used and is described in Chapter 2. ³⁵S-labeled probes were used at a concentration of .25µg/kb·ml. Sections were hybridized overnight at 60° C, and washed 2 times at 65° C. Slides were coated in NTB-2 emulsion and exposed for 22 days before development.

Image Analysis: The entire mouse brain, from the olfactory bulb to the caudal medulla was inspected by reflected-light, dark-field microscopy for the presence of silver grain clusters (indicating the presence of galanin mRNA-containing cells) in defined brain regions. Each brain region with galanin-expressing cells was subjectively scored for both the number of cells and relative levels of galanin mRNA expression. Approximately 150 sections at 20 µm intervals were examined for each animal.

Results

Galanin mRNA-containing cells were identified in anatomically distinct nuclei throughout the central nervous system of the mouse (**Fig.1-6 and Table 1**). In the olfactory bulb, moderate numbers of labeled cells were found throughout the glomerular layer, but few cells were seen elsewhere. The prosencephalon contained numerous

galaninergic cells, from the diagonal band of Broca to the supramammillary nucleus.

Labeling was generally moderate in the telencephalon, with the highest numbers of cells found in the bed nucleus of the stria terminalis, the horizontal limb of the diagonal band of Broca, and the subfornical organ. The dentate gyrus had moderate labeling, primarily in the caudal polymorphic layer. Several divisions of the amygdala had only modest cell counts; however, these few cells were intensely labeled. Virtually no labeling was seen in the cortex, nucleus accumbens, or striatum.

In the diencephalon, the thalamus contained relatively few galanin cells, but a continuous ring of weakly labeled cells was seen extending from the central lateral to the central medial thalamic nuclei. The hypothalamus contained the largest number and densest labeling of any forebrain region. Most divisions of the preoptic (POA) area were intensely labeled, particularly in the medial and ventromedial divisions. The area surrounding the rostral third ventricle had many highly expressing cells—most notably in the anteroventral PeN and throughout the rostral PeN. The supraoptic, perifornical, paraventricular (PVN), and Arc nuclei all contained moderate numbers of cells, while the lateral hypothalamic area had scattered cells throughout its rostral-caudal extent. The most densely labeled nucleus of the hypothalamus was the DMN, with intensely labeled, tightly packed cells found in all divisions—most notably in the rostral portions.

The mesencephalon contained the fewest number of galanin-producing cells in the brain, with scattered cells noted in the Edinger-Westphal and oculomotor nuclei, and only occasional labeled cells observed elsewhere. In the rhombencephalon, most noradrenergic cell groups contained galanin mRNA, including A7, A5, A1 and A2. The locus coeruleus (LC) contained large numbers of tightly clustered, intensely labeled cell

bodies, throughout its rostro-caudal extent. Many brightly-labeled cells were also found in the vagal complex, most obviously in the solitary tract nucleus (NTS) and the dorsal motor nucleus of vagus (DMV). Distinct, small groups of cells were noted in the inferior salivatory, paragigantocellular, and lateral reticular nuclei. No specific labeling was detected in the cerebellum.

B. Experiment 2

I hypothesized that because galanin and NPY have several overlapping functions and partially overlapping distributions, one molecule might partly compensate for the absence of the other in the neuroendocrine hypothalamus. NPYKO mice have normal food intake, body weight, reproductive capacity and endocrine profiles (Erickson 1996, hollolpeter 1998, erickson 1997). If galanin helps to normalize these physiological functions, then the galanin gene might be upregulated in regions of NPYKO mice where galanin and NPY both have roles.

Materials and methods

Experimental Design: Adult female NPYKO mice from the lab of Richard Palmiter (n=5) and their WT female littermates (n=6) were sacrificed by CO₂ asphyxiation, and brains were collected and quick-frozen to -80 ° C. Brains were sectioned at 20 μm according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the decussation of the anterior commissure (plate 29) to the end of the Arc (plate 54).

Riboprobe Preparation: A 680 bp rat galanin cDNA was used to generate sense and antisense ³⁵S-labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *In situ* hybridization was used and is described in Chapter 2. ³⁵S-labeled probes were used at a concentration of 0.25µg/kb·ml. Sections were hybridized overnight at 60° C, and washed in 0.1x SSC at 60° C. Slides were coated in diluted NTB-2 emulsion and exposed for 5 days before development.

Image Analysis: Galanin gene expression in the Arc and amygdala was analyzed by reflected-light dark field microscopy for the presence of silver grain clusters in defined brain regions, as described in Chapter 2. Total numbers of galanin-expressing cells and mean silver grains per cell were calculated using Image Analysis software. Due to the overlapping clustering of cells in the DMN, this region was analyzed on the MCID grain counting system, as described in Chapter 2. Total grain counts were measured for the DMN. For all regions, 3 anatomically matched slides from each animal that contained the Arc, DMN and central nucleus of the amygdala were analyzed, and all identifiable cells were counted in each slide.

Statistical Analysis: Each region was analyzed independently, and Student's unpaired t-test was used to compare galanin mRNA levels between WT and NPYKO animals. Results of statistical tests were considered significant at p<0.05. Results are expressed as mean values ± SEM.

Results

Galanin mRNA was upregulated in the Arc and DMN of NPYKO, but was

unchanged in the amygdala. In the Arc, the mean number of cells that expressed measurable amounts of galanin message was significantly increased in NPYKO as compared to WT (NPYKO = 138 ± 16 vs WT = 86 ± 8 ; $p < 0.02$) (Fig. 7). There was no difference in the mean grains/cell between genotypes (NPYKO = 44 ± 2 vs WT = 40 ± 3 ; NS) (Fig. 7). This result suggests that although there was no increase in the amount of galanin being produced by each cell in the Arc, new cells were being recruited which could now be identified as galanin-expressing neurons.

In the DMN, there was a significant increase in the proportional area of the DMN that contained silver grains in NPYKO as compared to WT (NPYKO = 0.028 ± 0.007 vs WT = 0.012 ± 0.002 ; $p < 0.05$) (Fig. 8). While the total grain count was more than double in the NPYKO, the high standard error between animals resulted in no significant difference between genotypes (NPYKO = 17581 ± 4560 vs WT = 8157 ± 798 ; $p = 0.05$) (Fig. 8). These results concur with those in the Arc, as the increase in silver grain area suggests that more galanin-expressing cells are detectable in the NPYKO than in WT.

In the amygdala, scattered cells throughout the central nucleus expressed galanin mRNA. However, no differences were detected between genotypes in this region in either cell count (NPYKO = 55 ± 6 vs WT = 56 ± 8 ; NS) (Fig. 9) or in grains/cell (NPYKO = 34 ± 3 vs WT = 36 ± 2 , NS) (Fig. 9). This result suggests that in this region, the loss of NPY does not lead to an alteration in galanin gene expression.

C. Experiment 3

As the findings of Experiment 2 showed that the galanin gene was up-regulated in the Arc of NPYKO mice, I hypothesized that other genes that are preferentially expressed

in this region may also be regulated differently. A primary candidate for regulation is POMC, which like NPY and galanin is abundantly produced in the Arc. As NPY and at least one product of the POMC gene, α -MSH, have been shown to have opposing roles on several metabolic processes (reviewed in Schwartz 2000), I hypothesized that the chronic lack of NPY might also lead to a reduction in POMC gene expression.

Materials and Methods

Experimental Design: Adult female NPYKO mice from the lab of Richard Palmiter (n=5) and their WT female littermates (n=6) were sacrificed by CO₂ asphyxiation, and brains were collected and quick-frozen to -80 ° C. Brains were sectioned at 20 μ m according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the decussation of the anterior commissure (plate 29) to the end of the Arc (plate 54).

Riboprobe Preparation: A 920 bp mouse POMC cDNA was used to generate sense and antisense ³⁵S-labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *In situ* hybridization was used and is described in Chapter 2. ³⁵S-labeled probes were used at a concentration of 0.20 μ g/kb·ml. Sections were hybridized overnight at 58° C, and washed 2 times in 0.1x SSC at 60° C. Slides were coated in diluted NTB-2 emulsion and exposed for 8 days before development.

Image Analysis: POMC gene expression in the Arc was analyzed by reflected-light dark field microscopy for the presence of silver grain clusters in defined brain regions, as described in Chapter 2. Total numbers of POMC-expressing cells and

mean silver grains per cell were calculated using Image Analysis software. The Arc nucleus was divided into 3 regions, and each region was analyzed separately. The regions were as follows: rostral, atlas plates 41-44; mid, atlas plates 45-48; posterior, atlas plates 49-53. One anatomically matched slide from each animal was analyzed for each region of the Arc, and all identifiable POMC-expressing cells were assessed on each slide.

Statistical Analysis: Each region was analyzed independently, and Student's unpaired t-test was used to compare POMC mRNA levels between WT and NPYKO animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

POMC-expressing cells were found throughout all regions of the Arc, with the greatest number of POMC-positive cells found in the rostral Arc. Moderate numbers of cells were observed in the mid-Arc, and only scattered cells were found in the posterior Arc. No differences were detected in any region for number of POMC-containing cells between genotypes ([Rostral: NPYKO = 83 ± 29 vs WT = 68 ± 19] [Mid: NPYKO = 54 ± 15 vs WT = 42 ± 5] [Posterior: NPYKO = 18 ± 12 vs WT = 14 ± 6]; All NS). No differences were detected in mean grains per cell between genotypes in any area examined ([Rostral: NPYKO = 19 ± 2 vs WT = 17 ± 1] [Mid: NPYKO = 21 ± 2 vs WT = 18 ± 1] [Posterior: NPYKO = 19 ± 4 vs WT = 17 ± 2] All NS). When the data for all 3 areas were collapsed together, no differences were seen in cell count (NPYKO = 150 ± 30 vs WT = 121 ± 20 ; NS) or in grains/cell (NPYKO = 20 ± 2 vs WT = 18 ± 1 ; NS) (**Fig. 10**).

II. Galanin Transgenic Mice

To investigate the role of the major population of galaninergic neurons in the brainstem, I performed several studies using galanin transgenic mice (GALTG). These animals carry a transgene in which the mouse galanin gene has been placed under the control of the dopamine beta-hydroxylase promotor, which leads to expression of galanin in all noradrenergic cells. Details of the construct and the creation of these mice are described in Chapter 2. Using the colony of mice maintained here at the University of Washington, I first examined by *In situ* hybridization the extent of galanin overexpression in the locus coeruleus of GALTG mice, in Experiment 4. Next, in Experiment 5, I examined the patterns of expression of galanin throughout the brain of GALTG mice. In Experiment 6, galanin content in GALTG mice, as measured by radioimmunoassay. In Experiment 7, I assessed general physiological parameters such as body weight, temperature, and reproductive capacity. In Experiment 8, the effects of galanin overexpression on blood hormone profiles were determined. I hypothesized that overexpression of galanin would result in changes in an animal's adaptation to metabolic challenges. To test this hypothesis, GALTG and WT mice were fasted for 48 hours in Experiment 9. To further test this, I treated GALTG mice with leptin chronically in Experiment 10, and measured the metabolic effects of this treatment. I also hypothesized that the overexpression of galanin in noradrenergic neurons would alter basal levels of norepinephrine. To test this, catecholamine levels were measured in several brain regions in Experiment 11. In Experiment 12, I determined the effects of lesioning the terminal

fields of noradrenergic/galaninergic neurons in GALTG mice. As overexpression of galanin might lead to alterations in levels of galanin receptors, I examined the gene expression patterns of these receptors in Experiments 13-16. Finally, in Experiment 17, levels of NPY mRNA were measured in various brain regions of GALTG mice in to test the hypothesis that chronic overexpression of galanin would to alterations in levels of this potentially compensatory neuropeptide.

A. Experiment 4

To confirm that the galanin transgene was being transcribed in the brainstem, I performed *In situ* hybridization and analyzed the area of greatest galanin gene expression in the brainstem, the LC. I hypothesized that galanin mRNA would be significantly higher in the LC of GALTG mice than in WT mice.

Materials and Methods

Experimental Design: Adult male GALTG mice (n=7) and their WT male littermates (n=7) were sacrificed by CO₂ asphyxiation, and brains were collected and quick-frozen to -80 ° C. Brains were sectioned at 20 μm according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the appearance of the 4th ventricle (plate 74) to the end of the LC (plate 79).

Riboprobe Preparation: A 680 bp rat galanin cDNA was used to generate sense and antisense ³⁵S-labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *In situ* hybridization was used and is described in Chapter 2. ³⁵S-labeled probes were used at a concentration of

0.25 μ g/kb·ml. Sections were hybridized overnight at 55° C, and washed 2 times in 0.1x SSC at 60° C. Slides were exposed to hyperfilm for 3 days, before being coated with NTB-2 emulsion, diluted 1:1 with ammonium acetate. Slides were exposed for 7 days before development.

Image Analysis: Sections were analyzed on the MCID Image Analysis system, as described in Chapter 2. A minimum of 2 anatomically matched slides were chosen from each animal, and the mean relative optical density (ROD) was determined for the LC in each slide analyzed.

Statistical Analysis: Student's unpaired t-test was used to assess galanin mRNA levels between WT and GALTG animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

GALTG mice had nearly a five-fold increase in relative galanin mRNA levels as compared to WT mice (GALTG = 69 ± 13 vs WT = 15 ± 3 ; $p < 0.005$) (Fig. 11). These results confirmed that the transgene is expressed abundantly in noradrenergic neurons in the LC.

B. Experiment 5

To characterize the extent of galanin transgene expression in GALTG mice, I examined galanin mRNA levels in the entire mouse brain, and compared them to WT. I hypothesized that some expression of the transgene would be evident in the forebrain of

GALTG mice, as previous studies using the hDBH promoter have revealed some ectopic expression of reporter genes in areas of the brain that do not normally express DBH (Hoyle 1994, Mercer 1991).

Materials and Methods

Experimental Design: Adult female GALTG mice (n=5) and their WT male littermates (n=5) were sacrificed by CO₂ asphyxiation, and brains were collected and quick-frozen to -80 ° C. Brains were sectioned at 20 µm according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the beginning of the piriform cortex (plate 10) to the end of the medulla (plate 93).

Riboprobe Preparation: A 493 bp mouse galanin cDNA was used to generate sense and antisense ³⁵S-labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *in situ* hybridization was used and is described in Chapter 2. ³⁵S-labeled probes were used at a concentration of 0.25 µg/kb·ml. Sections were hybridized overnight at 60° C, and washed 2 times in 0.1x SSC at 65° C. Slides were dipped in diluted NTB-2 emulsion, and were exposed for 23 days before development.

Image Analysis: All sections from each animal were examined by reflected-light dark field microscopy for the presence of silver grain clusters in defined brain regions, as described in Chapter 2. The relative abundance of galanin-containing cells and the relative intensity of grain density were assessed qualitatively, and scored as weak, moderate, high, or very high. No Statistical Analysis was performed for this study.

Results

I found several major areas of galanin overexpression in GALTG mice, many of which are not known to normally express DBH (Table 2). With the exception of the noradrenergic cell groups in the brainstem, virtually none of the regions that were seen to overexpress the transgene have previously been found to synthesize appreciable amounts of galanin. Starting in the rostral-most areas of the brain, the piriform cortex expressed very high levels of galanin (Fig. 12). This expression pattern extended throughout the brain until the disappearance of the piriform cortex at the level of the mammillary bodies. Brightly labeled, tightly clustered cells were observed throughout layer II, and spread into layer III of the piriform cortex. In the rostral forebrain, a continuous ring of cell was seen extending from the piriform cortex ventrally into the olfactory tubercle and tenia tecta. Scattered cells were observed in the olfactory nucleus. Above the rhinal fissure, a weak band of labeled cells was seen in the lateral orbital and ventral orbital cortices. Another band, again in cortical layer II, often extended dorsally on both sides of the median fissure.

In some GALTG mice, scattered moderately labeled cells could be seen in the caudate putamen and nucleus accumbens. Also, in some GALTG animals, a continuum of cells was seen ringing the entire cortical layer II/III from the level of the rostral basal forebrain to the anterior hypothalamus. The basal forebrain seemed to have normal levels of galanin expression in all regions where galanin has been identified. The same was true for the hypothalamus, where no apparent dramatic changes in galanin mRNA levels compared to WT were identified.

Proceeding caudally, as the piriform cortex extended into the cortex-amygdala

transition area and anterior cortical amygdaloid nucleus, a robust band of highly expressing cells was observed. The amygdala itself appeared to have levels of galanin mRNA indistinguishable from WT. At this level, a distinct moderately labeled band of cells was seen in retrosplenial granular cortex, again in layer II.

The hippocampus and its associated regions also exhibited unusual galanin expression patterns in GALTG mice. Although scattered cells were seen in the polymorphic layer of the dentate gyrus of both GALTG and WT mice, occasional moderately-expressing cells were observed in all hippocampal layers uniquely in the GALTG, particularly in the ventral oriens layer and the dorsal CA3 region.

One of the regions observed with the highest levels of galanin mRNA in GALTG mice was the entorhinal cortex. Beginning at the caudal-most part of the piriform cortex, a very highly labeled band of cells extended throughout the entorhinal cortex, particularly in caudal portions. Here, both the lateral and medial entorhinal cortex were very robustly labeled (Fig. 13). At the level of the caudal hippocampus, the subiculum also was moderately labeled. Extending caudally, the subiculum and parasubiculum were often seen to have densely packed cells whose expression levels ranged from weak to very high.

In the brainstem, many cell groups expressed galanin more robustly in the GALTG than in WT mice. A ring of cells was observed surrounding the caudal cerebral aqueduct, and this continued into the rostral 4th ventricle. Rostrally, the central nucleus of the inferior colliculus exhibited many weakly labeled cells, occasionally extending into the external cortex. No other labeled cells were observed dorsal to the 4th ventricle, in either GALTG or WT mice.

All noradrenergic cells groups were labeled with robustly expressing cells.

These groups included the Kolliker-Fuse nucleus (A7), the LC (Fig. 14), the dorsal and ventral subcoerulear nuclei, and all of A5. Interestingly, only moderate expression was observed in the area marked in the atlas as A2 (Franklin 1997). Also, although a few cells in the C1-C3 adrenergic regions displayed scattered labeling, it was hard to ascribe galanin-positive cells specifically to these areas.

Other regions of the brainstem that exhibited robust expression patterns in GALTG mice included the rostral parabrachial nucleus (all subdivisions) and just dorsal to this, in the cuneiform nucleus. Heavily labeled cells were observed extending from the cuneiform nucleus ventrally down through the medial parabrachial all the way to the ventral subcoeruleus (Fig. 15). Further caudal in the medulla, a portion of the anteroventral cochlear nucleus also had moderately labeled cells in its lateral portions. All other brainstem cell groups that express galanin in WT mice appeared to have normal levels of galanin mRNA in GALTG mice.

C. Experiment 6

To confirm that the overexpression of galanin mRNA in GALTG was being translated into higher amounts of galanin, I sent dissected tissue to the lab of Dr. Steven Bloom for measurement of galanin by radioimmunoassay. In the first round, several organs were assayed to test if galanin was being overexpressed in both the brain and periphery. In the second round, several brain regions were microdissected, and analyzed for galanin content. I hypothesized that all regions which in the mouse normally either

produce norepinephrine, or receive significant noradrenergic projections, would have higher galanin levels in GALTG mice as compared to WT. Also, due to the ectopic expression of the galanin transgene in several brain regions, I hypothesized that galanin concentrations would be higher in the forebrain of GALTG mice.

Materials and Methods

Experimental Design: For galanin content in whole organs, adult female GALTG (n=3) and their female WT controls (n=3) were sacrificed by CO₂ asphyxiation, and organs were immediately dissected and weighed fresh. Organs were quick-frozen to -80° C, and sent on dry ice for RIA measurements, which are described in Chapter 2. For galanin content in regions of the brain, adult male GALTG mice (n=6) and their age-matched male WT controls (n=6) were sacrificed under isoflurane anesthesia, and brains were dissected in sucrose buffer and quick-frozen to -80 ° C. Brains were sent on dry ice for RIA measurements, which are described in Chapter 2.

Results

Galanin concentrations were calculated as pmol/gram of wet tissue weight (Table 3). I found that galanin content in the forebrain was nearly 2-fold higher in the GALTG than in WT mice (GALTG = 7.33 ± 0.13 vs WT = 3.80 ± 0.32 ; $p < 0.001$). These results confirmed that overexpression of the galanin gene leads to an increase in measurable galanin peptide. Adrenal galanin concentrations were also significantly higher in the GALTG mice (GALTG = 206 ± 17.6 vs WT = 103 ± 15.6 ; $p < 0.05$). No significant differences were seen in any other organ examined, although pituitary concentrations

were numerically much higher in the GALTG mice (**Table 3**).

In the second round of measurements, galanin content in the total forebrain excluding the hypothalamus and hippocampus was 83% higher in the GALTG compared to WT (GALTG = 49.6 ± 6.1 fmol/mg vs WT = 27.6 ± 3.1 fmol/mg; $p < 0.01$) (**Fig. 16**). Galanin concentrations in the hippocampus were markedly higher in the GALTG brain (GALTG = 125 ± 17 fmol/mg vs WT = 29.6 ± 4.7 fmol/mg; $p < 0.001$) (**Fig. 16**), as was galanin content in the brainstem (GALTG = 123 ± 5 fmol/mg vs WT = 82.8 ± 2.5 fmol/mg; $p < 0.0001$). In contrast, no significant differences in galanin concentrations between genotypes in the hypothalamus (GALTG = 581 ± 33 fmol/mg vs WT = 560 ± 37 fmol/mg; NS) (**Fig. 16**).

These results show that the overexpression of galanin in GALTG mice translates into higher than normal peptide levels in several areas of the animal. The highest levels of galanin were observed in the hypothalamus, pituitary and adrenals, underscoring the importance of these areas as centers of galaninergic activity. However, hypothalamic galanin content was equally high in both WT and GALTG mice, suggesting that compensatory mechanisms may act in this region to keep levels of this neuropeptide near the physiological norm.

D. Experiment 7

Galanin has been implicated in the control of many neuroendocrine functions, including growth, food intake, metabolic homeostasis and reproduction. To investigate whether the overexpression of galanin in noradrenergic (and some forebrain) neurons leads to deficits in any of these functions, I assessed basic physiological profiles in

GALTG mice. I hypothesized that if galanin were to have a critical neuroendocrine role in areas where it is overexpressed in GALTG mice, this could be revealed by measuring indices such as body weight and reproductive success.

Materials and Methods

Experimental Design: Several different cohorts of GALTG and WT mice (n=5-10 per cohort) were examined at various ages for body weight, daily food intake, rectal temperature, and fertility, as described in Chapter 2. Animals were sacrificed by CO₂ asphyxiation or under isoflurane anesthesia, and organ systems were dissected, examined and weighed.

Statistical Analysis: Student's unpaired t-test was used to compare the measured variables between WT and GALTG animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as means \pm SEM.

Results

Growth patterns in GALTG mice appeared normal compared to WT in both sexes throughout development, and adult body weights were not significantly different between genotypes—although GALTG mice tended to be slightly smaller than WT (Fig. 17). No differences were seen at any age in general health, rectal temperatures, or daily food intake (Table 4). All organ systems seemed grossly normal, and no unusual growths or tumors were seen in the GALTG mice. Organs were removed and weighed, and I found that GALTG mice had much smaller pituitaries than did WT (GALTG = 1.1 ± 0.1 mg vs WT = 1.8 ± 0.2 mg; $p < 0.001$) (Fig. 18). There were no differences in the weights of other

organs between genotypes, including the adrenals (GALTG = 9 ± 1 mg vs WT = 8 ± 1 mg; NS) (Fig. 18).

GALTG mice were reproductively competent. Before GALTG mice were backcrossed into the C57BL6 background, it appeared that smaller litter sizes resulted when male GALTGs were bred with WT females. This difference disappeared when the animals were backcrossed for several generations, and C57BL6 transgenic mice had normal litters. No deficits were noted in the ability of male GALTG mice to fertilize females, or in the capacity of female GALTG mice to carry pups to term. GALTG mothers nursed and cared for their young adequately, and no differences in pup survival were seen between genotypes. Reproductive organs in male and female GALTG appeared morphologically normal, and no differences were observed in either organ size or weight (Table 5).

E. Experiment 8

The results of Experiment 6 demonstrated that galanin levels in the GALTG are very high in the brainstem, forebrain, pituitary and adrenals. I hypothesized that the chronically high levels of galanin in GALTG mice would lead to changes in neuroendocrine-related hormones, as central and peripheral infusions of galanin have been shown to alter levels of several of these hormones, including insulin, corticosterone, LH and GH. To test this postulate, serum blood levels of several circulating hormones were measured by radioimmunoassay.

Materials and Methods

Experimental Design: Baseline blood samples were collected from male and female GALTG and WT mice, either by orbital eye bleed under isoflurane anesthesia, or at sacrifice under isoflurane anesthesia. Details of blood collection procedures are described in Chapter 2. Serum hormone levels were measured by radioimmunoassay, and serum glucose levels were measured with a hand-held glucometer, as described in Chapter 2.

Statistical Analysis: Student's unpaired t-tests were used to compare hormone levels between WT and GALTG animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Analysis of gonadotropin levels revealed no serious deficits in either gender of GALTG mice (**Table 6**). Serum values of LH were not different between genotypes of either sex. Levels of Follicle-stimulating hormone (FSH) were moderately lower in GALTG males than in WT males, but were still well within the physiological range for this strain of mice. FSH values were not different between female GALTG and female WT mice. Testosterone was also measured, and no differences were noted between genotypes (**Table 6**).

Since galanin has been shown to alter the release of pancreatic hormones, glucagon and insulin were measured in male mice of both genotypes. No differences were seen in either metabolic hormone between genotypes (**Table 6**). Fasting glucose levels were obtained, and were not different between GALTG and WT. Corticosterone levels were also not different between genotypes. These results show that despite having nearly

double the amount of galanin in the brain, neuroendocrine profiles are by and large completely normal in GALTG mice, and virtually indistinguishable from those of WT mice.

F. Experiment 9

Many studies have shown that injections of galanin into the brain of the rat leads to moderate increases in food intake, but the role of galanin in an animal's adaptive response to starvation has not been directly tested. To determine whether the overexpression of galanin in GALTG mice alters either body weight or food intake in response to starvation, I fasted mice for 48 hours, and measured body weight loss during fasting, and feeding and body weight responses during the recovery period.

Materials and Methods

Experimental Design: Young adult male GALTG (n=9) and their age-matched male WT controls (n=9) were housed in individual cages for 11 days prior to fasting. When body weights did not change significantly for 4 consecutive days, food was removed for 48 hours at 0930 am. Body weight was measured at 24 and 48 hours, and food was given back at 0930 on the second day. Food intake and body weights were monitored for another 2 weeks post-fast.

Statistical Analysis: Analysis of variance (ANOVA) with repeated measures was used to compare body weights and food intake between WT and GALTG animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Mean body weights were not different between genotypes during the pre-fast period (Fig. 19). When food was removed, both groups of mice lost approximately the same amount of weight at both 24 hours (GALTG = $-17.0 \pm 0.6\%$ vs WT = $-18.4 \pm 0.9\%$; NS) and 48 hours (GALTG = $-30.1 \pm 1.7\%$ vs WT = $-28.2 \pm 0.6\%$; NS) (Fig. 19). Upon refeeding, all animals regained weight and recovered to baseline within 2 days, and there were apparently no differences in the rates of recovery between GALTG and WT at any time during the 2 week post-fast period.

Mean food intake was not different between genotypes during the pre-fast period. After refeeding, both genotypes ate significantly more than baseline levels for 10 days post-fast (Fig. 19). GALTG mice ate significantly more during the first 5 hours after refeeding (GALTG = 5.29 ± 0.25 g vs WT = 4.62 ± 0.18 g; $p < 0.05$), but no differences were seen between genotypes in food intake at any other time during the post-fast period (Fig. 19). These results show that overexpression of galanin in GALTG mice does not lead to significant changes in the response to starvation in these animals.

G. Experiment 10

The results of Experiment 9 show that GALTG mice have a normal response to fasting, but do not reveal whether an animal's response to the satiety hormone leptin is dependent on differing levels of galanin in the brain. I hypothesized that as an orexigenic peptide, galanin might oppose the central nervous system effects of leptin, and thus GALTG mice would be resistant to the reductions in food intake and body weight caused

by leptin treatment. To test this, I administered leptin chronically to GALTG and WT mice, and measured several parameters known to be responsive to leptin treatment.

Materials and Methods

Experimental Design: Adult female GALTG mice (n=12) and their female age-matched WT controls (n=12) were housed individually and monitored until body weights and food intake stabilized. Mice of each genotype were assigned to either leptin-treatment or saline-treatment groups, so that there were no significant differences in initial body weight between groups. Mice were treated ip with either leptin (100 µg/mouse in 500 µl saline with 50 mM boric acid) or saline (500 µl with 50 mM boric acid) for 32 days. Injections were given one hour before lights out. Body weights and food intake were monitored daily. At the end of the study, mice were sacrificed under isoflurane anesthesia. Blood was collected, fat pads and adrenals were removed and weighed, and brains collected and quick-frozen at – 80° C.

Statistical Analysis: Body weight and food intake patterns over the course of the study were analyzed by two-way ANOVA (treatment and genotype) with repeated measures. Blood levels and tissue weights were analyzed by two-way factorial ANOVA. When significant differences in interactions between genotype and treatment were found, individual groups were analyzed by Fisher's PLSD test. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Leptin treatment led to a significant loss in body weight ($p < 0.001$) in both

GALTG and WT mice (GALTG = $-5.3 \pm 0.5\%$ vs WT = $-6.5 \pm 1.7\%$; NS). No difference was seen between genotypes in weight response to leptin or saline, and there was no significant interaction between genotype and treatment (Fig. 20). Leptin treatment also caused a modest but highly significant ($p < 0.001$) reduction in mean daily food intake in both genotypes (GALTG leptin = 3.18 g vs saline 3.40 g, WT leptin = 3.01 g vs saline 3.61 g). As was observed with body weight, there were no differences in food intake between genotypes, and while the WT leptin-treated group ate less than the other groups, the interaction between genotype and treatment did not quite reach statistical significance ($p = 0.07$) (Fig. 20).

Adrenal weights were significantly lower in the leptin-treated groups of both genotypes, but there was no difference in adrenal weight between GALTG and WT in response to either saline or leptin treatment (Table 7). When fat pads were weighed, I found that leptin treatment led to a significant reduction in adipose pad mass (Table 7). White fat pad weight was reduced equally between GALTG and WT mice, but brown fat mass was reduced only in the GALTG leptin-treated group ($p < 0.001$) (Table 7). No significant differences in either genotype or treatment were detected in serum leptin or insulin levels (Table 7). These results indicate that overexpression of galanin in GALTG mice does not lead to demonstrable changes in the response of these animals to chronic leptin administration.

H. Experiment 11

In this experiment, I tested the hypothesis that chronically high levels of galanin

in noradrenergic neurons of GALTG mice would lead to lower catecholamine levels in several brain regions, based on previous studies demonstrating inhibitory effects of galanin on noradrenergic activity .

Materials and Methods

Experimental Design: Young adult male GALTG (n=9) and WT age-matched controls (n=9) were sacrificed under isoflurane anesthesia, and brains rapidly removed. Dissections were performed in 0.32M sucrose buffer, and individual hypothalamus, hippocampus and brainstem sections were immediately placed in cryostat tubes and quick-frozen to -80° C. Total dissection time from sacrifice to freezer was approximately 10 minutes. Brain regions were analyzed for NE, DA and dihydroxyphenylacetic acid (DOPAC) content by HPLC in the lab of Dr. Alvin Matsumoto, as described in Chapter 2.

Statistical Analysis: Student's unpaired t-test was used to compare differences between WT and GALTG animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

NE levels were very high in the hypothalamus, somewhat lower in the brainstem, and lowest in the hippocampus (**Table 8**). No differences were detected between genotypes in NE content in any brain region measured. DA levels were also high in the hypothalamus, and were very low in the brainstem and hippocampus (**Table 8**). No differences were found in between genotypes in DA content in any area examined.

Concentrations of the dopamine metabolite DOPAC were quite low in all brain regions, and no differences were seen between genotypes (Table 8). These results show that GALTG mice have normal levels of the catecholamines NE and DA compared to WT mice in each brain region examined.

I. Experiment 12

In this experiment, I examined the effects of lesioning terminal fields of NE projections in GALTG and WT mice by administering the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA). To preferentially lesion NE terminals without significantly altering DA levels, I administered 6-OHDA into the lateral ventricles. The purposes of this experiment were 2-fold. First, I sought to determine whether lesioning NE terminal fields would affect the physiology of GALTG mice differently than in WT mice. Second, I hypothesized that these lesions would lead to differential galanin receptor changes between GALTG and WT mice, and these results are described in Experiment 16.

Materials and Methods

Experimental Design: Young adult male GALTG and age-matched WT mice were individually housed and monitored for 1 week until body weights and food intake stabilized. Mice were separated into 4 weight-matched groups, and treatments were administered into the lateral ventricles via freehand ICV injections, as described in Chapter 2. The groups were as follows: WT aCSF (n=5); WT 6-OHDA (n=5); GALTG

aCSF (n=8); GALTG 6-OHDA (n=8). The dose of 6-OHDA was 50 μg in 5 μl aCSF with 0.04% ascorbic acid, and control animals were dosed with 5 μl aCSF with 0.04% ascorbic acid. Rectal temperatures were obtained each hour for the first 3 hours after treatment, and then again on day 5. Body weights and food intakes were monitored daily throughout the course of the experiment. At experiment's end, mice were sacrificed under isoflurane anesthesia, blood was collected via cardiac puncture, and brains dissected and quick-frozen to -80°C .

A separate group of WT mice were given either 6-OHDA (n=5) or aCSF (n=5), and sacrificed after 14 days for catecholamine content measurements, as described in Experiment 11 and Chapter 2.

Statistical Analysis: Bodyweights, temperatures and food intakes were compared using 2-way ANOVA (genotype and treatment) with repeated measures. When significant differences were seen in main effects, individual time-points were analyzed by Fisher's PLSD test. Serum blood levels were compared by 2-way factorial ANOVA. Catecholamine levels were compared by Student's unpaired t-test for each region. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

The effectiveness of the 6-OHDA lesion was confirmed by measuring rectal temperatures after treatments. Core temperature dropped significantly in 6-OHDA-treated mice of both genotypes ($p < 0.0001$), and rebounded back to baseline by 3 hours after treatment (Fig. 21). Body temperatures remained unchanged from baseline at 24 hours

and 5 days. There was no significant difference in rectal temperatures between genotypes at any time point, confirming the normal thermogenic responses of GALTG mice in both basal and repeated measurement conditions.

6-OHDA treatment did not cause a significant change in food intake at any time-point during the study, although there was a transient drop at 24 hours in both genotypes (Table 9). Body weight was moderately lower at 24 hours after 6-OHDA injection (Fig. 21), but did not differ from aCSF treated animals at any other time. Control animals treated with aCSF lost weight initially, but recovered back to baseline by the end of the study. Neither corticosterone nor reproductive hormone levels were affected by 6-OHDA treatment (Table 9).

In the group of animals examined for catecholamine concentrations, highly significant reductions resulting from 6-OHDA treatment were seen in NE levels (Fig. 22). NE levels in the hypothalamus were reduced by 47% (aCSF = 34.1 ± 1.1 ng/ml vs 6-OHDA = 18.1 ± 3.9 ; $p < 0.01$). Brainstem NE concentrations were reduced by 50% (aCSF = 18.4 ± 0.3 ng/ml vs 6-OHDA = 9.2 ± 0.9 ; $p < 0.0001$). The greatest reduction in NE was observed in the hippocampus, where the lesion led to an 86% loss (aCSF = 12.1 ± 0.7 ng/ml vs 6-OHDA = 1.7 ± 0.9 ; $P < 0.0001$). DA levels were not reduced as a result of 6-OHDA administration (Fig. 22), confirming the specificity of this treatment paradigm for lesioning NE terminal fields.

J. Experiment 13

The previous experiments all showed that metabolic, reproductive and ingestive parameters of GALTG mice are remarkably normal, considering the effects that acute

injections of galanin have on these physiological systems. One plausible consequence of galanin overexpression would be compensatory regulation of galanin receptors as a mechanism for counteracting chronically high peptide levels. I hypothesized that the normal neuroendocrine phenotype of GALTG mice would, at least in part, be due to changes in galanin receptor gene expression. I tested this in Experiment 13 by comparing mRNA levels of GAL-R1 in the hypothalamus of GALTG and WT mice.

Materials and Methods

Experimental Design: Adult female GALTG mice (n=5) and their WT female littermates (n=6) were sacrificed by under isoflurane anesthesia, and brains were collected and quick frozen to -80°C . Brains were sectioned at $20\ \mu\text{m}$ according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997), from the beginning of the piriform cortex (plate 10) to the end of the medulla (plate 93).

Riboprobe Preparation: A 390 bp rat GAL-R1 cDNA was used to generate sense and antisense ^{33}P -labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *in situ* hybridization was used and is described in Chapter 2. ^{33}P -labeled probes were used at a concentration of $0.37\ \mu\text{mol/ml}$. Slides were pre-hybridized for 2 hours at 55°C in hybridization buffer. Sections were hybridized overnight at 61°C , and washed 2 times in $0.1\times\ \text{SSC}$ at 60°C . Slides were coated in undiluted NTB-3 emulsion and exposed for 20 days before development.

Image Analysis: Cellular levels of GAL-R1 mRNA were analyzed under dark-

field microscopy on the MCID Image Analysis system, as described in Chapter 2.

Selected hypothalamic areas were measured from anatomically matched sections from GALTG and WT mice, and outlined with a freehand selection tool. Optical densities from each region were recorded, and background level densities were recorded from size-matched areas devoid of specific signal on each slide. Relative optical densities were calculated as a ratio of density in the outlined nuclei/density of outlined background (SBR), with a ratio of 1 representing no specific signal measured above background levels.

Statistical Analysis: Student's unpaired t-test was used to compare GAL-R1 mRNA levels between WT and GALTG animals in each area analyzed. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Several neuroendocrine-related areas of the hypothalamus were found to express GAL-R1 in WT and GALTG mice. These included the PeN, PVN, Arc, DMN, VMN, posterior hypothalamus (PH) and mammillary nuclei (MM). Signal to background ratios were numerically higher in GALTG mice than in WT mice in every region examined. Nuclei where GAL-R1 gene expression levels were significantly higher included the PeN (GALTG = 1.24 ± 0.05 vs WT = 1.08 ± 0.03 ; $p < 0.05$) the DMN (GALTG = 1.53 ± 0.07 vs WT = 1.17 ± 0.03 ; $p < 0.01$) and the VMN (GALTG = 1.34 ± 0.07 vs WT = 1.16 ± 0.03 ; $p < 0.05$) (Fig. 23). These results show that, at least in the hypothalamus, GALTG mice respond to the permanent overexpression of galanin in the brain by upregulating a

major subtype of galanin receptor.

K. Experiment 14

The other major galanin receptor subtype in the brain is GAL-R2. Although not as abundantly expressed as GAL-R1, this subtype has been shown to be present in moderate levels in the rat hypothalamus. I hypothesized that as GAL-R1 is induced in neuroendocrine nuclei of GALTG mice, GAL-R2 mRNA levels would also be altered in response to galanin overexpression. Because GAL-R1 and GAL-R2 are coupled to different signal transduction pathways, I reasoned that patterns of altered GAL-R2 gene expression might become manifest in different areas and directions than was seen with GAL-R1.

Materials and Methods

Experimental Design: Adult female GALTG mice (n=5) and their WT male littermates (n=5) were sacrificed under isoflurane anesthesia, and brains were collected and quick frozen to -80°C . Brains were sectioned at $20\ \mu\text{m}$ according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the beginning of the piriform cortex (plate 10) to the end of the medulla (plate 93).

Riboprobe Preparation: A 452 bp rat GAL-R2 cDNA was used to generate sense and antisense ^{33}P -labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *in situ* hybridization was used and is described in Chapter 2. ^{33}P -labeled probes were used at a concentration of 1pmol/ml . Sections were hybridized overnight at 55°C , and washed 2 times in $0.1\times\text{SSC}$

at 60° C. Slides were coated in undiluted NTB-3 emulsion and exposed for 28 days before development.

Image Analysis: GAL-R2 gene expression levels were analyzed under dark-field microscopy on the MCID Image Analysis system, as described in Chapter 2. Selected hypothalamic areas were measured from anatomically matched sections from GALTG and WT mice, and outlined with a freehand selection tool. Optical densities from each region were recorded, and background level densities were recorded from size-matched areas devoid of specific signal on each slide. Relative optical densities were calculated as a ratio of density in the outlined nuclei/density of outlined background (SBR), with a ratio of 1 representing no specific signal measured above background levels.

Statistical Analysis: Student's unpaired t-test was used to compare levels of GAL-R2 mRNA between WT and GALTG animals in each area analyzed. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

GAL-R2 mRNA was identified in several nuclei of the mouse hypothalamus, including the anteroventral periventricular nucleus (AVPe), PVN, suprachiasmatic nucleus (SCN), Arc, DMN, VMN and MM. Faint expression was observed in several other hypothalamic areas, but analysis was not possible in these nuclei due to the inability to cleanly outline the expressing regions. Levels of GAL-R2 were generally lower in GALTG mice than in WT (**Table 10**), but signal-to-background ratio differences were only significant in the PVN and Arc (**Fig. 24**). GAL-R2 mRNA was also observed in the LC, but mRNA levels were unchanged in this area between GALTG and WT mice

(Table 10). These results suggest that in contrast to the general upregulation of GAL-R1 in the hypothalamus of GALTG mice, levels of mRNA for another major subtype of galanin receptor, GAL-R2, are either unchanged or down-regulated in these animals.

L. Experiment 15

The previous 2 studies demonstrated that the expression of galanin receptor subtypes is different between GALTG and WT—at least in the female. Galanin gene expression is sexually dimorphic in several areas of the hypothalamus. Thus, I was curious to learn whether the regulation of galanin receptor mRNA observed in female GALTG might also be evident in the male. To test this, I compared levels of GAL-R1 mRNA in male GALTG and WT mice using a mouse riboprobe. To obtain a more complete localization of GAL-R1 message in the mouse, I also examined the distribution of GAL-R1 mRNA in the entire forebrain of the mouse.

Materials and Methods

Experimental Design: Young adult male GALTG (n=6) and age-matched WT (n=6) controls were sacrificed under isoflurane anesthesia, and brains were collected and quick-frozen to -80°C . Brains were sectioned at $20\ \mu\text{m}$ according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the nucleus accumbens (plate 19) to the end of the hippocampus (plate 65).

Riboprobe Preparation: A 377 bp mouse GAL-R1 cDNA was used to generate sense and antisense ^{33}P -labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *in situ* hybridization was used

and is described in Chapter 2. ³³P-labeled probes were used at a concentration of 0.32pmol/ml. Slides were pre-hybridized for 2 hours at 58° C in hybridization buffer. Sections were hybridized overnight at 59° C, and washed 2 times in 0.1x SSC at 60° C. Slides were coated in undiluted NTB-3 emulsion and exposed for 7 days before development.

Image Analysis: GAL-R1 mRNA distribution patterns were assessed qualitatively for cell count and intensity of expression, from the rostral Acb/lateral septal area to the end of the hippocampus. GAL-R1 gene expression levels in the hypothalamus were analyzed quantitatively using dark-field microscopy on the NIH Image computerized analysis system, as described in Chapter 2. Selected hypothalamic areas were measured from anatomically matched sections from GALTG and WT mice, and outlined with a freehand selection tool. Optical densities from each region were recorded, and background level densities were recorded from size-matched areas devoid of specific signal on each slide. Relative optical densities were calculated as a ratio of density in the outlined nuclei/density of outlined background (SBR), with a ratio of 1 representing no specific signal measured above background levels.

Statistical Analysis: Student's unpaired t-test was used to compare levels of GAL-R1 mRNA between WT and GALTG animals in each area analyzed. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

GAL-R1 mRNA was widely distributed in the forebrain of the male mouse (Table 11). Starting in the rostral-most sections, the striatum was labeled throughout its

rostral-caudal extent with scattered GAL-R1 expressing cells. All divisions of the lateral septum were heavily labeled, and this region was one of highest in the forebrain in total numbers of cells. A few scattered expressing cells were seen in the Acb, in both the core and shell. The ventral palladium had moderate numbers of expressing cells, as did the olfactory tubercle. Both the vertical and horizontal limbs of the diagonal band had moderate cell counts, but the medial septum was virtually devoid of specific signal. Distinct but weakly labeled cells were seen in the dorsal peduncular nucleus. Most divisions of the bed nucleus of the stria terminalis had moderate to high numbers of GAL-R1 expressing cells. The basal nucleus of Meynert and substantia innominata also had many, scattered GAL-R1 expressing cells.

In the hypothalamus, most major nuclear groups exhibited at least some GAL-R1 mRNA labeling. The most intensely labeled areas were the DMN, the lateroanterior hypothalamic nucleus (LA), the Pen, the VMN, and the MPA. Other hypothalamic regions expressing moderately included the AVPe, PVN, supraoptic, LH and posterior hypothalamic area.

The thalamus was the densest labeled region of any examined. In the nuclei of the thalamus that expressed GAL-R1 message, the labeled cells were usually so tightly packed that it appeared as though a continuum of cells covered the entire nucleus. Regions that were intensely labeled were the paraventricular nuclei (PVA and PV), the central medial nucleus (CM), the paracentral nucleus, and the mediodorsal nucleus.

The amygdala and associated nuclei were labeled with GAL-R1 mRNA in several areas. The most intensely expressing area was a continuous band of cells extending from the medial amygdaloid nucleus (dorsal and ventral) to the basomedial amygdala. The

caudal hippocampus exhibited only low to moderate levels of GAL-R1 expression.

Identifiable expressing areas included the pyramidal cell regions of CA1 and CA3, and in the ventral-most part of the dentate gyrus. A group of expressing cells was reliably seen in the amygdalohippocampal area (AHiPM).

In the caudal regions of the forebrain, a number of areas expressed low to moderate numbers of cells. The areas of highest expression were the various subdivisions of the mammillary nuclei, the medial and lateral habenula, the superior colliculus, and in a region of the periaqueductal gray (PAG) just dorsal to the aqueduct of Sylvius. Interestingly, many low-expressing cells were seen throughout the extent of the retrosplenial granular cortex, and the parts of the entorhinal cortex were densely labeled with GAL-R1 message. These two areas were the only cortical regions in the entire forebrain seen to express appreciable amounts of GAL-R1 mRNA.

Several regions of the hypothalamus were examined for differences in levels of GAL-R1 mRNA between WT and GALTG mice. GAL-R1 message was significantly higher in several areas of GALTG mice. These areas included the PeN (+20%), the DMN (+45%) and the VMN (+54%) (Fig. 25 and Table 12). Other hypothalamic areas, including the PVN, the SO, the LA and the AH had GAL-R1 that were not significantly different between genotypes (Table 12).

These results show that GAL-R1, like galanin itself, is widely distributed and abundantly expressed in the brain of the male mouse. These results also demonstrate that the same areas where I previously had found GAL-R1 to be upregulated in female GALTG mice also had increased receptor mRNA in male GALTG mice. This finding confirms these areas as potentially important regions for regulatory compensation in

response to galanin overexpression.

M. Experiment 16

The changes exhibited in galanin receptor gene expression in the hypothalamus may be the result of “rewiring” in response to high levels of galanin during development. Another plausible explanation for altered galanin receptor levels is that GALTG mice are continually responding to high levels of galanin by up- or down- regulating receptor subtypes on a “moment-to-moment” basis. To test this, I compared GAL-R1 mRNA between 6-OHDA lesioned and aCSF-treated GALTG mice. I hypothesized that lesioning noradrenergic cells and their projections to the hypothalamus would reduce galanin concentrations in the hypothalamus of the GALTG, which would lead to a reduction in GAL-R1 gene expression.

Materials and Methods

Experimental Design: Brains from mice used in the 6-OHDA studies of Experiment 12 were collected and frozen at -80° C. Brains were sectioned at 20 μ m according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the nucleus accumbens (plate 19) to the end of the hippocampus (plate 65).

Riboprobe Preparation: A 377 bp mouse GAL-R1 cDNA was used to generate sense and antisense 33 P-labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *in situ* hybridization was used and is described in Chapter 2. 33 P-labeled probes were used at a concentration of 0.30pmol/ml. Slides were pre-hybridized for 1.5 hours at 55° C in hybridization buffer.

Sections were hybridized overnight at 55° C, and washed 2 times in 0.1x SSC at 63° C. Slides were coated in undiluted NTB-3 emulsion and exposed for 10 days before development.

Image Analysis: GAL-R1 gene expression levels were analyzed under reflected-light dark-field microscopy on the NIH Image computerized analysis system, as described in Chapter 2. Selected hypothalamic areas were measured from anatomically matched sections,, and outlined with a freehand selection tool. Optical densities from each region were recorded, and background level densities were recorded from size-matched areas devoid of specific signal on each slide. Relative optical densities were calculated as a ratio of density in the outlined nuclei/density of outlined background (SBR), with a ratio of 1 representing no specific signal measured above background levels.

Statistical Analysis: Student's unpaired t-test was used to compare levels of GAL-R1 mRNA between 6-OHDA-lesioned and aCSF-treated animals in each area analyzed. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

I examined GAL-R1 mRNA in hypothalamic areas that in Experiments 13 and 15 exhibited higher message levels in GALTG compared to WT. I also compared expression levels in the PVN, where no altered regulation between genotypes was observed in previous experiments. No significant differences could be detected in any region examined between 6-OHDA- and aCSF-treated mice (**Fig. 26 and Table 16**).

These results indicate that lesioning the ascending noradrenergic fibers in GALTG mice does not lead to altered levels of GAL-R1 mRNA. As these fibers probably also contain galanin, lesioning this extrahypothalamic source of galanin is not sufficient to reduce receptor message back to WT levels.

N. Experiment 17

Galanin and NPY have many potential overlapping roles, and are both abundant in the neuroendocrine hypothalamus. One conceivable reason for the relatively normal hypothalamic-associated phenotypes of GALTG mice might be compensatory down-regulation of NPY in these animals. To test this hypothesis, I compared levels of NPY mRNA between GALTG and WT mice in 3 areas where both NPY and galanin are thought to regulate metabolic, ingestive and reproductive behaviors.

Materials and Methods

Experimental Design: Adult female GALTG mice (n=5) and their WT male littermates (n=5) were sacrificed under isoflurane anesthesia, and brains were collected and quick-frozen to -80°C . Brains were sectioned at $20\ \mu\text{m}$ according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the beginning of the piriform cortex (plate 10) to the end of the medulla (plate 93).

Riboprobe Preparation: A 511 bp rat NPY cDNA was used to generate sense and antisense ^{35}S -labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *in situ* hybridization was used and is described in Chapter 2. ^{35}S -labeled probes were used at a concentration of

0.25 μ g/kb·ml. Sections were hybridized overnight at 62° C, and washed 2 times in 0.1x SSC at 66° C. Slides were coated in NTB-2 emulsion diluted 1:1 with ammonium acetate, and exposed for 6 days before development.

Image Analysis: NPY gene expression in the nucleus accumbens (Acb) and LC was analyzed by reflected-light dark field microscopy for the presence of silver grain clusters in defined brain regions, as described in Chapter 2. Total numbers of galanin mRNA-expressing cells and mean silver grains per cell were calculated with Image Analysis software for the Acb, and total cell count was recorded for the LC. Due to the overlapping clustering of cells in the Arc, this region was analyzed on the MCID grain counting system, as described in Chapter 2. SBR and grain areas were measured for the Arc. For all regions, at least 3 anatomically matched slides from each animal for each area were analyzed, and all identifiable cells were counted in each slide.

Statistical Analysis: Each region was analyzed independently, and Student's unpaired one-tailed t-test was used to compare levels of NPY mRNA between WT and GALTG animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Robust expression of NPY message was observed in many areas of the mouse brain, including the Acb, most cortical regions, the hippocampus, and the Arc. Many other brain regions expressed NPY mRNA at lower levels, including the inferior colliculus and LC in the brainstem. In the hypothalamus, the only region with substantial expression of the NPY gene was in the Arc, where very bright, overlapping silver grain

clusters were seen in the ventromedial portion of this nucleus. No NPY message was observed in the DMN of either genotype. In the Acb, NPY mRNA levels were significantly lower in GALTG compared to WT, both in numbers of identifiable mRNA expressing cells (GALTG = 72 ± 8 vs WT = 130 ± 26 ; $p < 0.05$) and in grains per cell (GALTG = 18 ± 3 vs WT = 29 ± 4 ; $p < 0.05$) (Fig. 27). In the Arc, both genotypes exhibited abundant expression of NPY, and no differences were detected in either signal-to-background ratio or total grain area (Table 14). In the LC, only scattered cells were detected, and there were no differences noted between genotypes (Table 14). These results indicate that NPY message levels are regulated in the forebrain of GALTG mice in a region-specific manner, and that the Acb may be a region where galanin and NPY have overlapping and conceivable compensatory roles.

III. Galanin Knockout Mice

The results of Part 2 demonstrate that overexpression of galanin in GALTG mice does not lead to serious alterations in the ability of these animals to regulate neuroendocrine and metabolic systems. However, there are changes in the expression of certain genes that may in part compensate for permanently high levels of galanin. Another way to study the role of galanin in the brain would be to analyze the consequences of its absence. To do this, mice lacking a functional galanin gene (GALKO) were obtained from the lab of Dr. David Wynick, and a colony of these mice was established and maintained in the 6th floor animal facility at the University of Washington.

As one of my primary interests has been to contrast the effects of the complete lack of galanin with that of overexpression of galanin, I performed many of the same experiments in GALKO mice as were described in Part 2. I began my characterization of GALKO mice with an assessment of general health, body weight, food intake, and reproductive capacity, in Experiment 18. In Experiment 19, I tested the hypothesis that the lack of galanin would lead to significant deficits of serum values of neuroendocrine hormones, and also sought to determine whether any differences found between genotypes would be sexually dimorphic. In Experiment 20, I postulated that GALKO mice would exhibit significant differences compared to WT mice in basal levels of catecholamines. In Experiment 21, I tested the hypothesis that the lack of galanin in GALKO mice would lead to deficits in the capability of these mice to respond normally to chronic injections of leptin. To extend this hypothesis, I examined the effects of a 48 hour fast, both with and without accompanying leptin treatment, in Experiment 22. In Experiment 23, I hypothesized that the capacity of GALKO mice to respond normally to central injections of galanin and NPY would be compromised. Since GALTG mice have altered levels in the hypothalamus of GAL-R1 mRNA, I hypothesized in Experiment 24 that the complete lack of galanin in GALKO mice would also result in changes in message levels of GAL-R1 when compared to WT mice. Finally, in Experiment 25, I sought evidence for compensatory changes in levels of NPY mRNA by comparing message levels of this neuropeptide in both GALKO and WT mice.

A. Experiment 18

GALKO mice have previously been reported to be phenotypically deficient in

several pituitary-related functions (Wynick 1998), but in this initial description of the mice nothing was reported about basic physiological parameters such as body and organ weights, food consumption, or reproductive capacity. To assess these physiological parameters, and to set the foundation for further studies in GALKO mice, I took baseline measurements from adult male and female mutant and WT mice.

Materials and Methods

Experimental Design: Several different cohorts of mixed strain (C57BL6 x 129) GALKO and age-matched WT mice (n=6-12 per cohort) were examined at various ages for body weight, daily food intake, rectal temperature, and fertility, as described in Chapter 2. Animals were sacrificed by CO₂ asphyxiation or under isoflurane anesthesia, and organ systems were dissected, examined and weighed.

Statistical Analysis: Student's unpaired t-tests were used to assess differences between WT and GALKO animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Growth patterns in GALKO mice appeared normal in both genders throughout development, and adult body weights were not significantly different between genotypes. (Fig. 28). No genotype differences were seen at any age in general health, rectal temperatures, body length, body mass index (BMI) or daily food intake (Table 15). All organ systems seemed grossly normal, and no unusual growths or tumors were seen in the GALKO mice. When organs were removed and examined, no gross morphological

differences were noted, and adrenal weights were not different between genotypes (Table 15).

GALKO mice appeared to be reproductively competent. Mutant mice were able to conceive and give birth to normal litters, and no dramatic differences were seen in pup survival rate. Female GALKO mice were able to lactate and nurse their young normally, as evidenced by the appearance of milk in the infant stomach, and by the normal growth rate of pups. Reproductive organs appeared morphologically normal in GALKO mice, and were of normal weight, except for seminal vesicles, which were heavier in the knockouts (Table 16). These results indicate that, despite the complete loss of a major neuropeptide in the brain and periphery, GALKO mice are virtually indistinguishable from WT animals in terms of body weight regulation and reproductive competency.

B. Experiment 19

I collected blood from male and female GALKO and WT mice for analysis of serum hormone levels. I hypothesized that the chronic loss of galanin would lead to significant alterations in the mutant animal's ability to regulate reproductive and metabolic hormones within a narrow physiological range. I also hypothesized that such alterations might be sexually dimorphic, given the demonstrated differences in galanin levels in the brain between males and females.

Materials and Methods

Experimental Design: Baseline bloods were collected from male and female mixed-strain GALKO and WT mice, either by orbital eye bleed under isoflurane

anesthesia, or at sacrifice under isoflurane anesthesia. Details of blood collection procedures are described in Chapter 2. Serum hormone levels were measured by radioimmunoassay, and serum glucose levels were measured with a hand-held glucometer, as described in Chapter 2.

Statistical Analysis: Student's unpaired t-tests were used to compare hormone levels between WT and GALKO animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Several metabolic hormone levels were significantly different in GALKO mice (**Table 17 and Fig. 29**). Fasting glucose levels were 43% higher in female GALKO than in female WT, but were not different between male GALKO and male WT. Insulin levels were markedly higher in male GALKO than in male WT (+854%), while not being different between female GALKO and female WT. Levels of glucagon were also higher in GALKO males (+40%) than in WT males, but again there were no differences in females between genotypes. No differences were found between genotypes of either gender in T4, leptin or corticosterone values (**Table 17**).

Reproductive hormone levels were also normal in both sexes of GALKO mice, with the exception of FSH levels, which were 30% higher in GALKO males than in WT males (**Table 17**). LH levels were also 48% higher in GALKO males, but this difference did not reach statistical significance. These results indicate that in contrast to the normal appearance and reproductive capacity of GALKO mice, the lack of galanin in these animals causes significant deficits in baseline hormone levels, and that several of these

measured variables were sexually dimorphic.

C. Experiment 20

In this experiment, I measured and compared DA and NE content in several brain regions of GALKO and WT mice. I hypothesized that the chronic loss of galanin in GALKO mice would lead to altered catecholamine levels in these animals compared to WT mice.

Materials and Methods

Experimental Design: Young adult male 129 strain GALKO (n=6) and WT age-matched controls (n=5) were sacrificed under isoflurane anesthesia, and brains rapidly removed and frozen at -80° C. Brains were gently thawed on ice, and dissections were performed in 0.32M sucrose buffer. The hypothalamus was dissected from the rest of the forebrain, and individual hypothalamic, forebrain and brainstem sections were immediately placed in cryostat tubes and quick-frozen to -80° C. Total dissection time was approximately 10 minutes. Brain regions were analyzed for NE and DA content by HPLC in the lab of Dr. Alvin Matsumoto, as described in Chapter 2.

Statistical Analysis: Student's unpaired t-tests were used to compare differences between WT and GALKO animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

NE content was very high in the hypothalamus, somewhat lower in the brainstem, and lowest in the rest of the forebrain (**Table 18**). No significant differences were seen between genotypes in NE levels in any brain region examined. DA content was much higher in the total forebrain (-hypothalamus) than in the hypothalamus alone, and was very low in the brainstem (**Table 18**). As with NE, no differences were seen in DA content between genotypes in any brain region. These results suggest that the loss of galanin has no demonstrable effects on levels of either NE or DA in the brain of the mouse.

D. Experiment 21

In this experiment, I assessed the effects of chronic leptin injections in GALKO and WT mice. As galanin is thought to be an orexigenic neuropeptide, it may oppose the actions of leptin in regulating ingestive behaviors and metabolic profiles. I hypothesized that without galanin, GALKO mice would be more sensitive to the weight loss and feeding inhibitory effects of leptin.

Materials and Methods

Experimental Design: Adult male and female mixed strain GALKO mice (n=6 each) and their age-matched WT male and female controls (n=6 each) were housed individually and monitored until body weights and food intake stabilized. Mice of each genotype were assigned to either leptin-treatment or saline-treatment groups, with each group containing 3 females and 3 males. Mice were treated via ip injections with either

leptin (100 $\mu\text{g}/\text{mouse}$ in 500 μl saline with 50 mM boric acid) or saline (500 μl with 50 mM boric acid) for 21 days. Injections were given one hour before lights out. Body weights and food intake were monitored daily. At the end of the study, mice were sacrificed under isoflurane anesthesia, blood was collected, fat pads were removed and weighed fresh, and brains were collected and quick-frozen at -80°C .

Statistical Analysis: Body weight and food intake patterns over the course of the study were analyzed by two-way ANOVA (treatment and genotype) with repeated measures. Fat pads weights were analyzed by two-way factorial ANOVA. When significant differences were found, treatment groups within each genotype were analyzed with Student's unpaired t-tests. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Leptin treatment caused a significant loss in body weight in both GALKO and WT mice (GALKO leptin = $-7.50 \pm 0.97\%$ vs GALKO saline = $-0.12 \pm 0.75\%$; WT leptin = $-3.30 \pm 0.53\%$ vs WT saline = $1.05 \pm 2.08\%$; $p < 0.0001$) (Fig. 30). There was a significant genotype effect on final body weight change ($p < 0.05$), but there was no significant interaction between genotype and treatment. Leptin administration did not lead to a reduction in mean daily grams food intake, and there was no effect of genotype on feeding (GALKO leptin = 3.31 ± 0.16 vs GALKO saline = 3.75 ± 0.18 ; WT leptin = 3.82 ± 0.29 vs WT saline = 3.85 ± 0.28 ; NS) (Fig. 30). When adipose tissue was dissected and weighed, I found a significant effect of treatment on white adipose tissue (Table 19). GALKO leptin-treated mice had lower white adipose tissue weights than did

the other treatment groups (as a percentage of total body weight), but the difference between genotypes did not reach significance, and there was no significant interaction between genotype and treatment. Also, no differences were recorded between genotypes or treatments in brown adipose tissue, when measured as a percentage of total body weight (Table 19).

These results suggest that while GALKO mice may be moderately more sensitive to the chronic effects of leptin than are their WT counterparts, the differences are modest. For the most part, GALKO mice are able to regulate their body weight and food intake normally in response to a long-term leptin challenge.

E. Experiment 22

Experiment 21 demonstrated that GALKO mice are only mildly perturbed in their ability to respond to leptin treatment. However, the abnormal baseline metabolic hormone levels in these animals suggest that they might respond aberrantly to a metabolic challenge. To test this, I deprived GALKO and WT mice of food for 48 hours, either with or without leptin treatment. I hypothesized that if galanin were important during periods of physiological duress, then the absence of galanin in GALKO mice would lead to a compromised adaptive response to starvation.

Materials and Methods

Experimental Design: Age-matched adult male mixed strain GALKO and WT mice (n=12 each) were housed individually, and allowed to acclimatize for 3 weeks. Body weights and food intakes were monitored daily throughout the experiment. At

1100h on day 1, mice were moved to new cages, and food was removed for 48 hours. Mice were provided with water at all times. At 1100h on day 3, blood was collected and mice were refed. At 1200h on day 7, food was once again removed for 48 hours. At 2000h on day 7 and day 8, mice were treated with 50 μ g leptin in 400 μ l saline. At 1200h on day 9, blood was collected, and 6 animals of each genotype were refed. The other 6 animals of each genotype were sacrificed, organs collected and weighed fresh, and brains collected and quick-frozen at -80° C. At 1200h on day 15, the remaining mice were sacrificed, blood was collected, organs were collected and weighed, and brains collected and quick-frozen to -80° C. Serum hormone levels were measured by radioimmunoassay, and serum glucose levels were measured with a hand-held glucometer, as described in Chapter 2.

Statistical Analysis: Food intake and body weights were analyzed by 2-way (genotype and treatment) ANOVA with repeated measures. Serum hormone and glucose levels in fasted and fasted with leptin groups were analyzed by 2-way (genotype and treatment) factorial ANOVA. When significant differences were found, treatment groups within each genotype were analyzed by Student's unpaired t-tests. Testosterone levels were analyzed non-parametrically with the Mann-Whitney U-test, due to the unequal variance between genotypes and treatment groups. Organ weights in the ad lib-fed and fasted with leptin-treated groups were analyzed by 2-way (genotype and treatment) factorial ANOVA. When significant differences were found, treatment groups within each genotype were analyzed by Student's unpaired t-tests. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Baseline body weights and food intakes were not significantly different between genotypes. Fasting for 48 hours caused significant reductions of body weight in both GALKO and WT mice, with each genotype losing an equivalent amount of weight (Fig. 31). Upon refeeding, both genotypes recovered their original weights within 1 day, and maintained this weight gain throughout the recovery period. Both genotypes ate more food than baseline levels for 3 days following refeeding, with intake returning to normal by day 7. During the second 48 hour fast with leptin, again no differences were noted between genotypes in body weight loss or recovery after refeeding. All mice again regained their original body weights within 24 hours. Refeeding patterns were not affected by leptin treatments during starvation in either genotype.

A significant interaction was found between treatment and genotype in testis weight ($p < 0.05$). GALKO fasted with leptin mice had significantly lower testicular weights than did ad-lib fed GALKO mice, while no such differences were seen in the WT groups ([GALKO leptin fasted = 211 ± 4 mg vs GALKO ad-lib = 231 ± 9 mg; $p < 0.05$] [WT leptin fasted = 228 ± 7 mg vs WT ad-lib = 214 ± 6 mg; NS]) (Fig. 32). I found a significant treatment ($p < 0.0001$) and genotype effect ($p < 0.05$) in seminal vesicle weight, and a significant interaction between genotype and treatment ($p < 0.0001$). Fasting with leptin treatment led to significantly decreases in both genotypes, but the magnitude of the decrease was greater in GALKO mice ([GALKO leptin fasted = 199 ± 5 mg vs GALKO ad-lib = 388 ± 16 mg; $p < 0.0001$] [WT leptin fasted = 238 ± 8 mg vs WT ad-lib = 295 ± 11 mg; $p < 0.01$]) (Fig. 32). No differences were found between genotypes or treatments in adrenal weight (GALKO fasted leptin = 8.0 ± 1.0 mg; GALKO ad-lib = 6.0

± 1.0 mg; WT fasted leptin = 6.0 ± 1.0 mg; WT ad-lib = 8.0 ± 1.0 mg; NS).

Many changes were seen in hormone levels due to treatment, and several of these were different between genotypes (Table 20). In WT mice, testosterone levels were significantly elevated in fasted with leptin-treated animals compared to fasted alone animals ($p < 0.05$), while no differences were found between treatment groups in GALKO mice (Fig. 33). I found a significant effect of treatment on FSH levels ($p < 0.01$), as fasting with leptin-treated groups had higher values than fasting alone groups (Fig. 33). While there was no significant effect of genotype on FSH levels, analysis within genotypes revealed a significant difference between the GALKO treatments ($p < 0.01$), while there were no differences between the WT treatment groups. LH levels were not different between genotype or treatments. T4 levels were dramatically higher in the fasting with leptin-treated groups than with fasting alone ($p < 0.0001$). However, no genotype difference was detected, and there was no interaction between genotype and treatment (Fig. 34). In contrast, corticosterone levels were dramatically lower in the fasting with leptin compared to fasting alone groups ($p < 0.0001$) (Fig. 34). There was a significant interaction between genotype and treatment ($p < 0.05$), and within genotype analysis revealed a greater decrease between the GALKO treatment groups than was observed between the WT treatment groups. There was no significant effect of either genotype or treatment on glucose levels, but a significant interaction was detected ($p < 0.05$). Within genotypes, GALKO fasted with leptin animals had higher glucose levels than the fasted alone mice ($p < 0.05$), while no differences were seen between WT groups (Table 20).

The results of this experiment indicate that GALKO mice respond normally to a starvation challenge in regard to body weight and food intake subsequent to a starvation

challenge, and this response was not different between GALKO and WT mice after leptin injections. These results also indicate that reproductive organ weights are differentially altered in GALKO mice after fasting. While mutant mice have a normal hormonal response to a 48 h fast, these animals respond quite differently than do WT mice when leptin is administered during the fasting period.

F. Experiment 23

The purposes of this experiment were two-fold. The first objective was to determine whether central infusions of galanin increase food intake and body weight in mice, as all prior published studies examining the orexigenic properties of galanin have been performed in rats. I hypothesized that galanin, as it does in rats, would cause a modest increase in food intake and body weight. The second objective was to ascertain whether GALKO, when compared to WT, would respond normally to infusions of both galanin and NPY. I hypothesized that GALKO mice would be more sensitive to the food-inducing actions of these peptides.

Materials and Methods

Experimental Design: Young adult male 129 strain GALKO mice (n=14) and age-matched WT male controls (n=12) were housed individually and allowed to acclimatize for 1 week. Body weights and food intake was monitored during this period. Mice were separated into weight-matched groups, and given either NPY (3 μ g in 5 μ l aCSF) or 5 μ l aCSF between 1300 and 1500h. The dose of NPY was chosen based on pilot studies

where 3 μg caused significant increases in feeding and body weight. The method of administration for all NPY treatments was by freehand ICV injections, as described in Chapter 2. Body weights and food intakes were monitored at 2 and 24 hours after treatments. After an 11 day recovery period, all mice were cannulated, and an Alzet brain infusion kit connected to a 7 day osmotic mini-pump was placed in each animal, as described in Chapter 2. Pumps were filled with either aCSF (n=6 each genotype) or galanin (0.75 $\mu\text{g}/\text{hour}$ in aCSF) (n=6 WT, n=8 GALKO). This dose of galanin was chosen based on pilot studies where an acute ICV dose of 20 μg galanin caused a significant feeding response. Mice were monitored daily for 6 days, when they were sacrificed under isoflurane anesthesia, blood collected, and brains removed and quick frozen at -80°C . Brainstems were dissected, and sent out for catecholamine measurement, as described in Chapter 2. All pumps were evacuated and checked for residual fluid, and dye was infused through the brain infusion kits to check for cannula patency. Three mice were removed from the study after $>50\ \mu\text{l}$ residual galanin was found in their pumps.

Statistical Analysis: Food intake and body weights were analyzed by 2-way (genotype and treatment) ANOVA with repeated measures. When significant differences were found, treatment groups within each genotype were analyzed by Student's unpaired t-tests. Serum hormone levels were analyzed by 2-way (genotype and treatment) factorial ANOVA. When significant differences were detected, treatment groups within each genotype were analyzed by Student's unpaired t-test. Results of statistical tests were considered significant at $p<0.05$. Results are expressed as mean values \pm SEM.

Results

NPY injections led to a highly significant increase in the cumulative food intake at 2h compared to aCSF-treated animals in both genotypes (GALKO = +212%, WT = +256%; $p < 0.0001$) (Fig. 35). There was no significant effect of genotype on 2 h intake, and no interaction between genotypes was detected. There was no discernible effect of NPY on food intake at 24 h in either genotype (Table 21). Body weights were not significantly altered from pre-injection weights in either genotype at 2 or 24 hours (Table 21). These results indicate that GALKO mice respond normally to the orexigenic effects of an acute dose of NPY.

Galanin infusions for 1 week led to a modest, but significant, increase in food intake when compared to aCSF-treated animals in both genotypes (GALKO = +17%, WT = +18%; $p < 0.001$) (Table 22). No differences were noted between WT and GALKO mice in mean food intake. Treatment with galanin also resulted in a significant increase in percent body weight change when compared to aCSF-treated mice ($p < 0.01$) (Fig. 35). As with food intake, no significant differences were observed in body weight response to galanin or aCSF infusions between GALKO and WT mice.

Galanin infusions had no effect on levels of either NE or DA in the brainstem (Table 22). I found a significant genotype effect in T levels, as galanin treatment led to an increase in T in WT mice compared to controls ($p < 0.05$), but did not raise T levels in GKO mice (Table 22). No differences were observed in LH, FSH, corticosterone or leptin levels between genotypes or treatment groups (Table 22). These results show that galanin has a modest orexigenic effect in mice, and that GALKO mice have a nearly normal response to galanin infusions.

G. Experiment 24

In Experiment 15, I demonstrated that GALTG mice show an induction in GAL-R1 mRNA in response to high levels of galanin in the hypothalamus, when compared to WT mice. Since GALKO mice produce no galanin, a different pattern of hypothalamic receptor regulation might be evident in these mutants. I hypothesized that the chronic loss of galanin would lead to a down regulation of GAL-R1 mRNA in the hypothalamus of GALKO mice.

Materials and Methods

Experimental Design: Tissue for this study was obtained from Dr. David Wynick's mouse colony at the University of Bristol. All mice were young adult 129 strain (n=6 each genotype). Whole brains were received frozen, and stored at -80° C until sectioning. Brains were sectioned at 20 μ m according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the nucleus accumbens (plate 19) to the end of the hippocampus (plate 65).

Riboprobe Preparation: A 377 bp mouse GAL-R1 cDNA was used to generate sense and antisense 33 P-labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *in situ* hybridization was used and is described in Chapter 2. 33 P-labeled probes were used at a concentration of 0.32pmol/ml. Slides were pre-hybridized for 2 hours at 58° C in hybridization buffer. Sections were hybridized overnight at 59° C, and washed 2 times in 0.1x SSC at 60° C. Slides were coated in undiluted NTB-3 emulsion and exposed for 7 days before

development.

Image Analysis: GAL-R1 distribution patterns were assessed qualitatively for cell count and intensity of expression, from the rostral Acb/lateral septal area to the caudal extent of the hippocampus. GAL-R1 gene expression levels in the hypothalamus were analyzed quantitatively under dark-field microscopy on the NIH Image computerized analysis system, as described in Chapter 2. Selected hypothalamic areas were measured from anatomically matched sections of GALKO and WT mice, and outlined with a freehand selection tool. Optical densities from each region were recorded, and background level densities were recorded from size-matched areas devoid of specific signal on each slide. Relative optical densities were calculated as a ratio of density in the outlined nuclei/density of outlined background (SBR), with a ratio of 1 representing no specific signal measured above background levels.

Statistical Analysis: Student's unpaired t-test was used to compare GAL-R1 mRNA levels between WT and GALKO animals in each area analyzed. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM

Results

I found that GAL-R1 was abundantly expressed in the brain of GKO mice, as it is in WT animals. By visual inspection, no dramatic changes in GAL-R1 were observed in any area of the GKO brain. In the hypothalamus, quantitative analysis revealed no significant differences in any region examined (Table 23 and Fig. 36). These results contrast sharply with those observed in GALTG mice, and suggest that the complete loss of galanin—at least in the hypothalamus—does not lead to demonstrable changes at the

mRNA level in GAL-R1.

H. Experiment 25

The results of Experiment 17 showed that while no significant alterations in NPY gene expression were observed in the Arc or LC of GALTG mice compared to WT mice, mRNA for this neuropeptide was reduced in the Acb of transgenic mice. To assess whether the lack of galanin would have a different effect on NPY mRNA levels in these regions, I examined NPY gene expression in the Arc, LC and Acb of GALKO and WT mice. I hypothesized that GALKO mice might compensate for the lack of galanin by up-regulating NPY gene expression in these regions.

Materials and Methods

Experimental Design: Tissue for this study was obtained from Dr. David Wynick's mouse colony at the University of Bristol. All mice were young adult 129 strain (n=6 each genotype). Whole brains were received frozen, and stored at -80° C until sectioning. Brains were sectioned at 20 µm according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) from the nucleus accumbens (plate 19) to the end of the hippocampus (plate 65), and through the locus coeruleus (plate 75-79).

Riboprobe Preparation: A 511 bp rat NPY cDNA was used to generate sense and antisense ³⁵S-labeled riboprobes. Details are found in Chapter 2.

In situ Hybridization: The standard procedure for *in situ* hybridization was used and is described in Chapter 2. ³⁵S -labeled probes were used at a concentration of .

0.25 μ g/kb-ml. Sections were hybridized overnight at 65° C, and washed 2 times in 0.1x SSC at 65° C. Slides were coated in NTB-2 emulsion diluted 1:1 with ammonium acetate, and exposed for 6 days before development.

Image Analysis: Levels of NPY mRNA were analyzed by reflected-light dark field microscopy for the presence of silver grain clusters in defined brain regions, as described in Chapter 2. Total numbers of NPY mRNA-expressing cells and mean silver grains per cell were calculated with image analysis software for the Acb and LC. Due to the overlapping clustering of cells in the Arc, this region was analyzed on the MCID grain counting system, as described in Chapter 2. SBR and grain areas were measured for the Arc. For all regions, at least 3 anatomically-matched slides from each animal for each area were analyzed, and all identifiable cells were counted in each slide.

Statistical Analysis: Each region was analyzed independently, and Student's unpaired t-tests were used to compare NPY mRNA levels between WT and GALKO animals. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Robust expression of NPY message was observed in many areas of both the WT and GALKO mouse brain, including the Acb, most cortical regions, the hippocampus, and the Arc. Many other brain regions expressed NPY mRNA at lower levels, including the inferior colliculus and LC in the brainstem. In the hypothalamus, the only region abundantly expressing NPY was the Arc, where very bright, overlapping silver grain clusters were seen in throughout the ventromedial portion of this nucleus. Scattered NPY

mRNA-expressing cells were noted in the DMN and posterior hypothalamus in both genotypes. In the Acb, there were fewer cells that containing NPY message in GALKO mice, and fewer grains per cell, but neither of these differences quite reached statistical significance (Fig. 37). Robust expression of NPY mRNA was seen in both genotypes in the Arc, but no differences were detected between GALKO and WT mice in either SBR or area of mRNA-expressing cells (Table 24). In the LC, moderately-labeled cells were seen in both genotypes, but again no differences were noted between GALKO and WT mice in either number of cells or grains per cell (Table 24). These results suggest that levels of NPY mRNA remain relatively normal in the brain following targeted deletion of the galanin gene.

IV. Galanin/NPY knockout mice

Both GALKO and NPYKO mice are quite normal in terms of reproductive capacity and body weight regulation, even though mild disturbances are evident in hormone profiles and neurochemistry. One conceivable reason for this relative lack of demonstrable phenotypes may be that galanin and NPY have overlapping and redundant roles, and thus one molecule would “take over” some functions of the other, missing neuropeptide. In order to test this hypothesis, I produced double knockout mice that lacked both the galanin and NPY genes (DKO), and established a colony of these mice and their WT controls. As a complete characterization of these animals was beyond the scope of this thesis, I performed several targeted experiments designed to reveal phenotypic changes. The first goal, in Experiment 26, was to test the hypothesis that DKO mice would have readily observable changes in their basic physiological behaviors.

Next, I examined serum hormone levels in Experiment 27, to test the hypothesis that the lack of both galanin and NPY leads to deficits in metabolic and reproductive hormones. In Experiment 28, I examined open-field behaviors in DKO mice, hypothesizing that changes in basal activity levels might be partly responsible for the body weight differences between DKO and WT mice. As both galanin and NPY have been associated with fat ingestion and fat metabolism, I weaned a group of DKO mice onto a high-fat diet in Experiment 29, hypothesizing that DKO mice would be less able to respond to this metabolic challenge. Finally, in Experiments 30 and 31, I treated DKO and WT mice with leptin chronically, with the hypothesis being that mice lacking both galanin and NPY would be more sensitive to the weight-loss effects of leptin.

A. Experiment 26

In this experiment I examined growth rate, food intake, and reproductive indices in DKO mice. I hypothesized that the lack of two potentially important metabolic neuropeptides, NPY and galanin, would lead to animals that have significant deficits in body weight and ingestive behaviors. I also hypothesized that if galanin and NPY were critical molecules in the central reproductive circuitry, their combined absence would result in mice with fertility deficits.

Materials and Methods

Experimental Design: GALKO mice were bred together with NPYKO mice to produce mixed strain (C57BL6 x 129) double mutants, as described in Chapter 2. Several cohorts of DKO mice were tracked during development, with body weights and general

health assessed biweekly from post-natal day 42 until adulthood. At 8 weeks of age, male DKO (n=24) and WT mice (n=23) were housed individually, rectal temperatures measured, and food and water intakes monitored for 4 days. Young adult male and female mice of both genotypes (n=8-12 per group) were weighed, daily and circadian food intake patterns monitored, body length measured, and reproductive capacity assessed, as described in Chapter 2. Mice were sacrificed under isoflurane anesthesia, brains and blood collected, and reproductive organs examined and weighed as fresh tissue.

Statistical Analysis: Body weights during development for each gender were compared by one-way (genotype) ANOVA with repeated measures. All other indices were analyzed for each gender by Student's un-paired t-tests. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Both male and female DKO mice were significantly heavier than their WT controls during development (Fig. 38). Body weights in males were moderately higher in DKO mice at 40, 70 and 100 days of age ([40 day: DKO = 22.0 ± 0.5 g vs WT = 19.4 ± 0.6 g; $p < 0.01$] [70 day: DKO = 28.8 ± 0.4 g vs WT = 26.6 ± 0.5 g; $p < 0.01$] [100 day: DKO = 34.6 ± 0.6 g vs WT = 31.4 ± 0.8 g; $p < 0.01$]). By 200 days of age, DKO were much heavier than WT (DKO = 43.4 ± 1.4 g vs WT = 33.7 ± 1.0 g; $p < 0.0001$). Female DKO mice were also moderately heavier than age-matched WT mice at 40, 70 and 100 days of age ([40 day: DKO = 19.6 ± 0.3 g vs WT = 17.0 ± 0.7 g; $p < 0.01$] [70 day: DKO = 23.6 ± 0.5 g vs WT = 20.8 ± 0.7 g; $p < 0.01$] [100 day: DKO = 26.0 ± 0.5 g vs WT = 24.0

± 0.6 g; $p < 0.05$]). However, by 200 days of age the difference between genotypes in female body weight had disappeared (DKO = 30.9 ± 1.3 g vs WT = 29.7 ± 0.9 g; NS).

Food intake analysis in at 8 weeks of age revealed that male DKO mice ate significantly more than age-matched WT males (+26%; $p < 0.001$) (Table 25). DKO mice also consumed more water at 8 weeks than WT males (+23%; $p < 0.01$). Rectal temperatures were not different between genotypes at this age (Table 25). In adulthood, male DKO mice ate more than did male WT animals (+26%; $p < 0.01$), while food intake in females did not differ between genotypes (Table 25). Analysis of 24 hour food intake patterns showed that male DKO mice ate significantly more than male WT during the dark period (+41%; $p < 0.01$), but intakes were not different between genotypes during the day (Fig. 39). In contrast, while female DKO mice ate more chow during the dark phase than female WT (+18%; $p < 0.05$), they ate less than WT mice during the light phase (-50%; $p < 0.001$), leading to no net difference in 24 hour intake (Fig. 39).

I found that DKO male mice had a higher body mass index than WT males, but no differences were seen between females of each genotype (Table 25). No differences between genotypes were detected in either adult body length or rectal temperatures in either gender (Table 25).

I observed that DKO mice were reproductively competent. Male DKO mice were able to mount and fertilize DKO females, who seemed to carry their young to term normally. Female DKO mice nursed their pups and pup survival rate did not seem to be impaired. Female DKO mice have regular (4-5day) estrous cycles, as assessed by the appearance of cornified vaginal smears. When reproductive organs were dissected and weighed, no gross abnormalities were noted. Both male and female DKO reproductive

organs were of normal weight (**Table 26**).

These results indicate that the lack of both galanin and NPY in DKO mice alters the developmental rate of body weight gain compared to WT mice, and that this difference persists in DKO males during adulthood. Also, circadian feeding patterns in DKO mice are different in both genders when compared to WT, and in males this results in a net increase in total 24 hour food intake. The complete absence of both galanin and NPY does not alter the reproductive capacity of the mutant mice.

B. Experiment 27

Mice lacking galanin have disturbances in both basal and challenged metabolic and reproductive hormone levels, as shown in Part 3. However, NPYKO mice are remarkably normal in this regard. I hypothesized that if galanin and NPY have redundant roles in regulating neuroendocrine systems, some hormonal profiles that are not perturbed in single mutants would be abnormally regulated in DKO mice. To determine whether the altered hormonal profiles in GALKO mice are also evident in DKO animals, I collected blood from both genders of double mutant and WT mice, and measured serum levels of several hormones.

Materials and Methods

Experimental Design: Baseline bloods were collected from male and female DKO and WT mice, either by orbital eye bleed under isoflurane anesthesia, or at sacrifice under isoflurane anesthesia. Details of blood collection procedures are described in Chapter 2. Serum hormone levels were measured by radioimmunoassay, and serum glucose levels

were measured with a hand-held glucometer, as described in Chapter 2.

Statistical Analysis: Student's unpaired t-tests were used to compare hormone levels between WT and DKO animals of each gender. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Metabolic hormone levels were significantly different between DKO and WT mice, with the most dramatic differences being evident in male mice. Compared to WT males, DKO males had much higher fasting glucose (+63%; $p < 0.01$) and insulin levels (+426%; $p < 0.01$) (Fig. 40). Female DKO mice also had higher fasting glucose levels (+66%; $p < 0.05$) than WT females, and while insulin levels were also higher in DKO than in WT females (+47%) the differences did not reach significance (Table 27). Glucagon levels were higher in both male (+22%) and female (+22%) DKO mice than in their respective WT counterparts, but again the differences were not quite statistically significant. Basal leptin levels were much higher in male DKO (+487%; $p < 0.01$) than in male WT (Fig. 41). Basal leptin levels were not different between female DKO and WT mice.

In contrast to the robust changes observed in glucose, insulin and leptin levels in the DKO mice, I found other hormone values to be mostly unchanged between genotypes (Table 27). Serum FSH and LH values were unchanged between genotypes of either sex, and although T levels were significantly lower in DKO males, the mutants had T values well within the normal physiological range. Basal T4 and corticosterone were normal in male and female DKO mice. Serum IGF-1 levels were moderately but significantly

higher in DKO females than in WT females (+22%; $p < 0.05$).

These results show that the complete lack of both galanin and NPY alters the set points of several metabolic hormones, and these differences are more evident in male DKO mice than in females. However, the absence of galanin and NPY does not cause dramatic changes in reproductive hormone levels—despite the putative involvement of both neuropeptides in the reproductive neuroendocrine circuitry.

C. Experiment 28

One possible reason for the increased weight in DKO mice could be lowered basal activity levels, leading to a net decrease in caloric expenditure. I tested this hypothesis by comparing open field activity behaviors of both male and female DKO and WT mice.

Materials and Methods

Experimental Design: Adult male (DKO, $n=8$; WT, $n=7$) and female (DKO, $n=13$; WT, $n=12$) mice were tested individually in activity chambers. Mice were allowed to acclimatize to the chambers for 24 hours before testing, with food and water available ad libitum. Activity levels were then recorded for a further 24 hours, starting in the first few hours of the light phase. The total number of beam breaks for each animal was recorded with automated software. The total number of ambulations was also recorded, which consisted of the breaking of consecutive beams 8.8cm apart within 15 seconds.

Statistical Analysis: Student's unpaired t-tests were used to compare activity levels between WT and DKO animals of each gender. Results of statistical tests were

considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

I found that female DKO mice had significantly fewer ambulations in a 24 hour period than did WT females (DKO female = 730 ± 140 vs WT female = 1430 ± 230 ; $p < 0.05$) (Fig. 42). However, no differences were detected between females of each genotype in total beam breaks (DKO female = 3610 ± 450 vs WT female = 3280 ± 380 ; NS). When male mice were subjected to the same tests, no differences were detected between genotypes in either ambulations (DKO male = 1080 ± 80 vs WT male = 1030 ± 160 ; NS) or beam breaks (DKO male = 3330 ± 620 vs WT = 2710 ± 330 ; NS).

The results of this experiment illustrate that changes in activity levels are unlikely to be the cause of increased body weight in adult male DKO mice. Significant differences were only observed in female mice, who did not differ in adult weight between DKO and WT animals.

D. Experiment 29

The finding that DKO male mice are significantly heavier than WT males, even though they are normally active, suggests that DKO mice may process their caloric intake differently than their WT counterparts. I hypothesized that if galanin and NPY are important regulators of caloric intake, then DKO mice would respond differently to WT mice when faced with dietary changes. One way to test this hypothesis is to measure body weight and food intake in response to a metabolic challenge. In this experiment,

young male mice were placed onto a high-fat diet for several weeks after weaning, and then switched to low-fat chow for another several weeks.

Materials and Methods

Experimental Design: Five week old male DKO (n=3) and WT (n=5) mice were removed from their home cages and group-housed by genotype for one week before the beginning of the experiment. At 6 weeks old, all mice were given a 58% fat diet (Diet D12331, Research Diets Inc New Brunswick, NJ) for 14 weeks. All animals were then switched to an 11% fat diet (Diet D12328 Research Diets Inc, New Brunswick, NJ) for a further 12 weeks. Mice were weighed and food intakes monitored weekly.

Statistical Analysis: Body weight differences over the course of the study were compared by one-way (gender) ANOVA with repeated measures. Individual time points between genotypes were analyzed by Student's unpaired t-tests. Weight differences within each genotype during specific periods were analyzed by Student's paired t-tests. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

At the beginning of the experiment, DKO mice and WT mice were not significantly different in body weight (DKO = 22.6 ± 1.4 vs WT = 18.4 ± 2.1 ; NS). However, DKO mice were significantly heavier than WT mice at all time points during the rest of the study, and after 3 months on a high fat diet were 35% heavier than WT animals (DKO = 45.3 ± 2.6 g vs WT = 33.5 ± 0.6 g; $p < 0.01$) (Fig. 43). When mice were

changed to a low fat diet, DKO mice lost significant amounts of weight (-6.4 ± 0.1 g; $p < 0.05$) before stabilizing to a new set point. WT mice did not lose weight after their diet change (-0.5 ± -0.3 g; NS), and their weights remained stable for the entire 12 week low-fat diet period. Because the animals were housed in social groups, the food intakes of individual animals could not be measured during this study, so significant differences between genotypes could not be assessed. However, DKO mice consumed overall more calories than WT mice during the high fat diet portion of the study (DKO = 19.1 kcal vs WT = 17.2 kcal), while eating less than WT mice during the 2 weeks following a diet change (DKO = 10.9 kcal vs WT = 12.9 kcal) (Fig. 44). During the rest of the low-fat portion of the experiment, DKO mice also consumed more calories than WT mice did (DKO = 17.5 kcal vs WT = 14.5 kcal), reminiscent of the differences in basal food intake patterns observed in Experiment 26.

These results show that DKO mice are not resistant to the body weight-increasing effects of a high fat diet, and in fact DKO mice gained weight robustly in response to this dietary challenge. However, DKO mice were not able to sustain this weight gain when changed from a high-fat to low-fat diet, and unlike WT mice, lost significant amounts of weight when faced with this challenge.

E. Experiment 30

Both GALKO and NPYKO mice are more sensitive to chronic leptin injections than WT controls, losing more weight than WT mice in response to this metabolic challenge. One reason for this could be that both of these neuropeptides are integral parts of systems whose function is to maintain body weight within a narrow homeostatic range.

While single mutants of either galanin or NPY have normal body weights, their inability to respond normally to leptin uncovers a possible defect in the body weight “set-point” of these mutants. I hypothesized that male DKO mice, which are heavier than WT controls, would be less capable of defending their body weight in response to long-term leptin treatment. To test this hypothesis, I treated both DKO and WT mice chronically with leptin, and measured body weight and food intake responses to this challenge.

Materials and Methods

Experimental Design: Young adult male DKO (n=12) and WT mice (n=12) were housed individually prior to the beginning of the study. Mice were separated into weight-matched leptin or saline treatment groups (n=6 of each genotype for each group). For the first 4 days of the study mice were treated ip with 45 μ g leptin (Zymogenetics, Seattle, WA) in 500 μ l 0.9% physiological saline, or with 500 μ l of saline only, twice daily at 1700h and 2200h. On day 5, the dosage was changed to 100 μ g leptin in 500 μ l saline, or with 500 μ l saline only, once daily at 1700h (just before lights out). Food and water intakes and body weights were monitored daily throughout the experiment. At experiments end, mice were sacrificed by cervical dislocation, blood and brains collected, and fat pads dissected out and weighed fresh, as described in Chapter 2. One saline-treated DKO mouse died on day 17, and data from this animal was removed from the study.

Statistical Analysis: Body weight and intake patterns over the course of the study were analyzed by two-way ANOVA (treatment and genotype) with repeated measures. Fat pads weights were analyzed by two-way factorial ANOVA. When significant

differences were found, treatment groups within each genotype were analyzed with Student's unpaired t-tests. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

Leptin treatment led to significant weight loss in both DKO and WT mice (Table 28). Leptin-treated mice of both genotypes lost weight rapidly for the first 4 days, when the WT leptin-treated mice stabilized their weights and maintained a fairly constant weight for the duration of the study. DKO leptin-treated mice continued to slowly lose weight, and by day 10 had lost significantly more weight than WT mice. Repeated measures analysis revealed both a genotype ($p > 0.05$) and a treatment ($p < 0.01$) difference over the entire treatment period, and by the end of the study, DKO leptin-treated mice had lost a greater percentage of their initial body weight than had any of the other groups (Fig. 45).

As I had observed in previous experiments, food intake was only modestly affected by leptin treatment. Mice ate slightly less for the first 5 days of the study, then ate essentially at baseline levels for the rest of the treatment period. Although there was a significant treatment difference over the duration of the study ($p < 0.05$), no genotype differences were detected. Also, within genotype analysis revealed no significant difference between leptin- and saline-treated mice (Table 28). No differences were observed between treatments or genotypes in mean daily water intake (Table 28). When fat pads were dissected and weighed at the end of the study, I found that several of the DKO leptin-treated mice had very small fat pads (Table 28). There was a significant

effect of treatment ($p < 0.001$) and genotype ($p < 0.01$) on fat pad weight, and DKO leptin-treated mice had a lower percentage of measurable fat pad mass than did the other treatment groups (Fig. 45).

These results indicate that DKO male mice are significantly more sensitive to the weight and fat loss effects of leptin than are their WT counterparts, but are not more sensitive to the inhibitory actions of leptin on food intake.

F. Experiment 31

The results of the previous experiments showed that compared to WT mice, DKO male mice are heavier, have altered hormone levels, and are more sensitive to leptin. In contrast, DKO female mice are not heavier than WT females, and have fewer abnormalities in circulating hormones. I hypothesized that if adult DKO females are less perturbed metabolically than DKO males, they would also be relatively less sensitive to leptin. To test this postulate, I treated female DKO and WT mice chronically with leptin, to determine whether, like male DKO mice, they are more sensitive than WT to the weight loss effects of leptin treatment.

Materials and Methods

Experimental Design: Age-matched young adult female DKO (n=12) and WT (n=12) mice were housed individually and allowed to acclimatize for 1 week before the beginning of the experiment. Mice were separated into weight-matched groups (n=6 each group) and treated with either 100 μ g leptin (Zymogenetics, Seattle, WA) in 500 μ l 0.9%

physiological saline, or with 500 μ l saline alone, once daily for 19 days. All treatments were given just prior to lights out. Food intake and body weights were monitored daily throughout the experiment. Mice were allowed to recover for 3 weeks, and then the groups were switched, so that the prior leptin-treated groups were given saline, and the prior saline-treated groups were given leptin. This resulted in all animals of each genotype receiving both treatments. Two DKO mice died during the study, and their data was removed from the results.

Statistical Analysis: Body weight and intake patterns over the course of the study were compared by two-way ANOVA (treatment and genotype) with repeated measures. When significant differences were found, treatment groups within each genotype were analyzed with Student's unpaired t-tests. Results of statistical tests were considered significant at $p < 0.05$. Results are expressed as mean values \pm SEM.

Results

As was seen in Experiment 30, I found that leptin treatment caused a significant decrease in body weight ($p < 0.001$). However, there was no difference between genotypes in percentage body weight loss compared to baseline (**Fig. 46**). Both DKO and WT mice lost increasing amounts of weight for the first 3 days, after which body weights plateaued until day 15. During the last 4 days of the treatment period, both genotypes regained weight to near baseline. When the leptin treatment challenge was removed, both DKO and WT mice rapidly regained weight. There was a significant genotype effect of body weight recovery during the first day after the end of treatments, with DKO mice gaining more weight than WT ($p < 0.05$) (**Table 29**).

In contrast to the slight, yet significant, decrease in food intake I had observed in male mice after leptin administration, this same leptin treatment regimen resulted in no decrease in food intake in either genotype of female mice (Table 29). There was a transient drop in food intake during the first few treatment days, but this decrease did not reach statistical significance. I did find a significant effect of genotype on food intake on the first day after the end of treatment ($p < 0.01$), with both DKO treatment groups eating more than their WT counterparts (Table 29).

These results suggest that unlike male DKO mice, female double mutants are not more sensitive to the weight loss effects of leptin than are WT mice. Moreover, there were no discernible differences between the female DKO and WT controls in the food intake response to leptin. I did observe, however, that there was to be a genotype difference in body weight and food intake responses immediately after the end of treatments.

Experiment 1—Table 1

Relative distribution of galanin mRNA-containing cells (*cell count*) and their levels of galanin expression (*intensity*) in the mouse brain. Each area is qualitatively scored, with + representing lowest levels of expression, and ++++ representing highest levels of expression.

Area	Cell Count	Intensity
<i>Rhinencephalon</i>		
Olfactory bulb		
External plexiform layer	+	+
Glomerular layer	++	+
<i>Telencephalon</i>		
Medial septal nucleus	+	+
Lateral septal nucleus	+	+
Bed nucleus of the stria terminalis	++	++
Bed nucleus of the anterior commissure	++	++
Diagonal band of Broca		
Vertical limb	+	+

Horizontal limb	++	++
Dentate gyrus	++	+
Amygdala		
Central nucleus (medial division)	++	+++
Central nucleus (lateral division)	+	+++
Medial nucleus (posterodorsal division)	++	+
Basal nucleus of Meynert	++	++
 <i>Diencephalon</i>		
Thalamus		
Paraventricular thalamic nucleus	+	+
Central medial thalamic nucleus	+	+
Central lateral thalamic nucleus	++	+
Paracentral thalamic nucleus	+	+
Hypothalamus		
Ventromedial preoptic nucleus	++	+++
Anteroventral periventricular nucleus	+++	+++
Median preoptic nucleus	+	+
Medial preoptic area	+++	+++
Medial preoptic nucleus (median division)	+++	+++
Ventrolateral preoptic nucleus	++	+++
Anterodorsal preoptic nucleus	++	++

Lateral preoptic area	++	++
Periventricular nucleus	+++	+++
Lateral hypothalamic area	++	+
Supraoptic nucleus	++	++
Paraventricular nucleus	++	+
Anterior hypothalamic area (posterior division)	+	++
Dorsomedial hypothalamic nucleus	+++	+++
Perifornical area	++	++
Arcuate nucleus	++	++
Posterior hypothalamic area	+	+
Dorsal tuberomamillary nucleus	+	+
Supramamillary nucleus	+	+

Mesencephalon

Olivary pretectal nucleus	+	+
Edinger-Westphal nucleus	+	++
Oculomotor nucleus	+	++
Rostral linear nucleus (raphe)	+	+
Subbrachial nucleus	+	+
Dorsal lateral periaqueductal gray	+	++

Rhombencephalon

Kolliker-Fuse nucleus (A7 region)	+	+
Subcoeruleus nucleus	+	+
Laterodorsal tegmental nucleus	+	+
Lateral parabrachial nucleus	+	+
Locus coeruleus	+++	+++
A5 area	+	++
Inferior salivatory nucleus	++	++
Lateral paragigantocellular nucleus	++	++
Dorsal cochlear nucleus	+	+
Inferior olive nucleus	++	+
Medial vestibular nucleus	+	+
Solitary tract nucleus	++	++
Dorsal motor nucleus of vagus	+++	+++
Raphe pallidus nucleus	+	+
A1 area	+	++
A2 area	+	+
Lateral reticular nucleus	++	++

Circumventricular

Subfornical organ	++	+
Vascular organ of the lamina terminalis	+	++
Area Postrema	+	+

Experiment 5—Table 2

Major areas of overexpression of galanin in the brain of GALTG mice, showing galanin mRNA-containing cells (*cell count*) and their levels of galanin expression (*intensity*).

Each area is qualitatively scored, with + representing lowest levels of expression, and +++++ representing highest levels of expression.

Area	Cell Count	Intensity
Piriform cortex	++++	++++
Olfactory tubercle	+++	++
Tenia tecta	++	++
Lateral orbital cortex	++	+
Ventral orbital cortex	++	+
Striatum	+	+
Nucleus accumbens	+	+
Cortical layer II (rostral)	++++	+
Cortex-amygdala transition area	+++	+++
Anterior cortical amygdaloid nucleus	+++	+++
Retrosplenial granular cortex	+++	++
Hippocampus	+	++
Subiculum	++++	+/+++++
Parasubiculum	+++	+++
Cerebral aqueductal region	+	++
4 th ventricular region	++	++
Central nucleus inferior colliculus	+++	+

Kolliker-Fuse nucleus	++	++++
Cuneiform nucleus	+	++++
Parabrachial nucleus (all divisions)	++	++++
Subcoeruleus dorsal	++	++++
Subcoeruleus ventral	+	++++
Locus coeruleus	+++++	++++
A5	+++	++++
A2	++	++
Anteroventral cochlear nucleus	++	++

Experiment 6—Table 3

Comparison of galanin concentrations between GALTG and WT mice. Data are expressed as mean values \pm SEM.

region (pmol/gram of wet weight)	WT Male	GALTG Male
Forebrain	3.80 \pm 0.31	7.33 \pm 0.13 ^b
Hindbrain	8.00 \pm 0.76	10.3 \pm 2.8
Cerebellum	2.93 \pm 0.13	2.00 \pm 1.60
Pituitary	433 \pm 100	2160 \pm 610
Adrenals	103 \pm 16	207 \pm 18 ^a
Stomach	11.6 \pm 1.9	22.5 \pm 5.6
Intestine	74.6 \pm 46.0	25.2 \pm 1.7

^a p<0.05 compared to WT

^b p<0.001 compared to WT

Experiment 7—Table 4

Comparison of body weight, food intake and rectal temperature between GALTG and WT mice. Data are expressed as mean values \pm SEM.

	WT Male	GALTG Male	WT Female	GALTG Female
Body weight (grams)	29.1 \pm 0.6	27.4 \pm 0.9	24.8 \pm 0.6	22.8 \pm 0.8
Daily Intake (grams)	4.5 \pm 0.1	4.7 \pm 0.1	3.4 \pm 0.2	3.3 \pm 0.1
Temperature (centigrade)	36.2 \pm 0.1	36.0 \pm 0.1	ND	ND

Experiment 7—Table 5

Comparison of reproductive organ weights between GALTG and WT mice. Data are expressed as mean values \pm SEM.

Organ (wt in mg)	WT Male	GALTG Male	WT Female	GALTG Female
Testes	200 \pm 10	213 \pm 8		
Epididymis	84 \pm 4	85 \pm 3		
Seminal Vesicles	386 \pm 9	376 \pm 23		
Ovaries			12 \pm 1	10 \pm 1
Uterus			79 \pm 10	58 \pm 4

Experiment 8—Table 6

Comparison of serum hormone levels between GALTG and WT mice. Data are expressed as mean values \pm SEM.

	WT Male	GALTG Male	WT Female	GALTG Female
LH (ng/ml)	9.9 \pm 0.6	12.8 \pm 4.5	6.3 \pm 2.4	6.6 \pm 2.3
FSH (ng/ml)	32.0 \pm 2.4	25.2 \pm 2.0 ^a	4.0 \pm 0.5	4.0 \pm 0.8
Testosterone (ng/ml)	9.0 \pm 1.7	6.3 \pm 2.1	ND	ND
Insulin (μ U/ml)	6.1 \pm 1.0	6.1 \pm 0.9	ND	ND
Glucagon (pg/ml)	56.6 \pm 4.2	57.9 \pm 2.7	ND	ND
Glucose (mg/dl)	147 \pm 11	135 \pm 9	ND	ND
Leptin (ng/ml)	0.67 \pm 0.11	0.81 \pm 0.09	ND	ND
T4 (μ g/dl)	2.6 \pm 0.2	3.0 \pm 0.15	ND	ND
Corticosterone	14.9 \pm 2.2	16.3 \pm 2.1	ND	ND

^a $p < 0.05$ compared to WT male

Experiment 10—Table 7

Comparison of organ weights and hormone levels between leptin- and saline-treatments in GALTG and WT. Data are expressed as mean values \pm SEM.

	WT Saline	WT Leptin	GALTG Saline	GALTG Leptin
Adrenals (mg)	11 \pm 1	9 \pm 1	12 \pm 1	8 \pm 1 ^a
White fat (mg)	539 \pm 71	221 \pm 52 ^b	520 \pm 75	230 \pm 39 ^b
Brown fat (mg)	43 \pm 5	47 \pm 3	57 \pm 1	26 \pm 7 ^b
Total fat pad (%body weight)	2.4 \pm 0.3	1.2 \pm 0.2 ^b	2.4 \pm 0.4	1.1 \pm 0.2 ^b
Leptin (ng/ml)	5.3 \pm 1.0	5.0 \pm 1.6	6.1 \pm 1.4	3.5 \pm 0.4
Insulin (ng/ml)	0.8 \pm 0.3	0.1 \pm 0.1	0.4 \pm 0.2	0.3 \pm 0.2

^a p<0.05 compared to saline-treated of same genotype

^b p<0.01 compared to saline-treated of same genotype

Experiment 11—Table 8

Comparison of catecholamine levels between GALTG and WT mice. Data are expressed as mean values \pm SEM.

Area (measurements in ng/ml)	WT	GALTG
NE hypothalamus	37.3 \pm 2.0	36.7 \pm 0.9
NE hippocampus	15.4 \pm 0.6	14.9 \pm 1.0
NE brainstem	26.0 \pm 0.7	24.7 \pm 1.0
DA hypothalamus	11.9 \pm 0.9	11.3 \pm 0.6
DA hippocampus	8.0 \pm 3.0	11.6 \pm 6.8
DA brainstem	3.2 \pm 0.2	3.0 \pm 0.2
DOPAC hypothalamus	1.9 \pm 0.1	1.9 \pm 0.2
DOPAC hippocampus	1.5 \pm 0.2	1.6 \pm 0.1
DOPAC brainstem	1.3 \pm 0.1	1.1 \pm 0.1

Experiment 12—Table 9

Comparison of food intake and hormone levels between 6-OHDA- and aCSF-treatments in GALTG and WT mice. Data are expressed as mean values \pm SEM.

	WT aCSF	WT 6-OHDA	GALTG aCSF	GALTG 6-OHDA
24 hour intake (grams)	3.35 \pm 0.23	3.00 \pm 0.35	2.89 \pm 0.47	2.27 \pm 0.44
Mean daily intake (grams)	3.86 \pm 0.15	3.64 \pm 0.20	3.75 \pm 0.26	3.69 \pm 0.13
LH (ng/ml)	7.2 \pm 3.3	8.0 \pm 2.5	6.9 \pm 1.6	10.0 \pm 1.4
FSH (ng/ml)	14.7 \pm 1.7	14.4 \pm 0.4	13.8 \pm 0.8	15.6 \pm 0.6
Corticosterone (ng/ml)	14.9 \pm 2.2	18.6 \pm 3.1	16.3 \pm 2.1	14.9 \pm 1.0

Experiment 14—Table 10

Comparison of GAL-R2 levels in the hypothalamus between GALTG and WT mice.

Data are expressed as mean values \pm SEM.

Area (ROD signal/background ratio)	WT Female	GALTG Female
Anteroventral periventricular nucleus	1.23 \pm 0.03	1.32 \pm 0.07
Paraventricular nucleus	1.43 \pm 0.04	1.23 \pm 0.02 ^b
Suprachiasmatic nucleus	1.55 \pm 0.09	1.48 \pm 0.05
Ventromedial nucleus	1.50 \pm 0.15	1.38 \pm 0.04
Dorsomedial nucleus	1.39 \pm 0.05	1.33 \pm 0.04
Arcuate nucleus	1.49 \pm 0.04	1.37 \pm 0.01 ^a
Mammillary nuclei	1.33 \pm 0.05	1.41 \pm 0.05
Locus coeruleus	1.26 \pm 0.06	1.30 \pm 0.07

^a $p < 0.05$ compared to WT

^b $p < 0.01$ compared to WT

Experiment 15—Table 11

Relative distribution of GAL-R1 mRNA-containing cells (*cell count*) and their levels of GAL-R1 expression (*intensity*) in the mouse brain. Each area is qualitatively scored, with + representing lowest levels of expression, and ++++ representing highest levels of expression.

Area	Cell Count	Intensity
<i>Telencephalon</i>		
Dorsal peduncular nucleus	++	+
Nucleus accumbens	+	++
Olfactory tubercle	++	++
Caudate putamen	++	++
Lateral septum	++++	+++
Vertical limb of the diagonal band	++	++
Horizontal limb of the diagonal band	++	++
Bed nucleus of the stria terminalis	+++	+++
Ventral Palladium	++	++
Substantia innominata	++	++
Basal nucleus of Meynert	++	++
Central nucleus of amygdala	++	++

Medial amygdaloid nucleus	+++	+++
Basomedial amygdaloid nucleus	+++	+++
Interstitial nucleus of the posterior limb of the anterior commissure	+	++
Hippocampus	+	+
Dentate gyrus (ventral)	++	++
Amygdalohippocampal area	++	++
Retrosplenial granular cortex	+++	+
Medial habenula	++	+++
Lateral habenula	++	++
Pre-subiculum	++	++
Subiculum	++	++
Entorhinal Cortex	+++	+++
 <i>Diencephalon</i>		
Parasubthalamic nucleus	+	++
Reunions nucleus	+	++
Zona incerta	++	++
Xiphoid nucleus	+	++
 Thalamus		
Paraventricular nucleus anterior part	++++	++++
Paraventricular thalamic nucleus	++++	++++

Central medial nucleus	++++	++++
Central lateral nucleus	+++	++++
Mediodorsal nucleus	++++	++++
Paracentral nucleus	+++	++++
Intermediodorsal nucleus	+++	++++
Interanteromedial nucleus	++	++++
Hypothalamus		
Anteroventral periventricular nucleus	++	++
Periventricular nucleus	++	+++
Ventrolateral preoptic nucleus	+	++
Lateral preoptic nucleus	++	++
Medial preoptic area	+++	++
Anterodorsal preoptic nucleus	++	++
Lateral hypothalamus	+++	++
Paraventricular nucleus	++	++
Supraoptic nucleus	+	+++
Lateroanterior hypothalamic nucleus	+++	+++
Anterior hypothalamic area posterior part	++	++
Dorsomedial nucleus	+++	+++
Ventromedial nucleus	+++	++
Arcuate nucleus	+	+

Posterior hypothalamic area	++	++/+++
Premammillary nucleus	++	+++
Mammillary nucleus	++	++
Ventral tuberomammillary nucleus	+	++
 <i>Mesencephalon</i>		
Olivary pretectal nucleus	++	++
Medial pretectal nucleus	++	++
Nucleus of posterior commissure	++	++
Sub-geniculate nucleus	+	++
Geniculate nucleus	++	++
Substantia nigra	++	++
Edinger Westphal nucleus	+	+++
Superior colliculus	+++	++
Periaqueductal gray	+++	++
Sub-brachial nucleus	+	++
Interpeduncular nucleus	+	++
Deep mesencephalic nucleus	+	++

Experiment 15—Table 12

Comparison of GAL-R1 levels in the hypothalamus between GALTG and WT mice.

Data are expressed as mean values \pm SEM.

region (signal/background ratio)	WT Male	GALTG Male
Periventricular nucleus	3.26 \pm 0.18	3.89 \pm 0.16 ^a
Supraoptic nucleus	3.81 \pm 0.40	3.51 \pm 0.35
Lateroanterior nucleus	3.64 \pm 0.44	3.41 \pm 0.44
Anterior hypothalamus	3.53 \pm 0.58	4.20 \pm 0.37
Paraventricular nucleus	3.20 \pm 0.37	2.95 \pm 0.38
Dorsomedial nucleus (anterior)	3.46 \pm 0.35	3.74 \pm 0.15
Dorsomedial nucleus (posterior)	2.99 \pm 0.18	4.33 \pm 0.48 ^a
Ventromedial nucleus	2.05 \pm 0.08	3.15 \pm 0.43 ^a

^a p<0.05 compared to WT.

Experiment 16—Table 13

Comparison of GAL-R1 levels in the hypothalamus between 6-OHDA- and aCSF-treated GALTG mice. Data are expressed as mean values \pm SEM.

Region (signal to background ratio of gray levels)	GALTG aCSF	GALTG 6-OHDA
Periventricular nucleus	3.12 \pm 0.15	3.01 \pm 0.21
Paraventricular nucleus	2.52 \pm 0.28	2.80 \pm 0.11
Dorsomedial nucleus	2.84 \pm 0.31	2.73 \pm 0.26
Ventromedial nucleus	2.32 \pm 0.16	2.46 \pm 0.20

Experiment 17—Table 14

Comparison of NPY mRNA levels between GALTG and WT mice. Data are expressed as mean values \pm SEM.

Area	WT Female	GALTG Female
Arcuate ROD (signal/background ratio)	1.55 \pm 0.25	1.40 \pm 0.10
Arcuate area (pixels)	17900 \pm 3370	15100 \pm 1740
Locus coeruleus (cell count)	22 \pm 12	17 \pm 6

Experiment 18—Table 15

Comparison of baseline physiological indices between GKO and WT mice. Data are expressed as mean values \pm SEM.

	Male WT	Male GKO	Female WT	Female GKO
Body weight (grams)	30.4 \pm 0.4	31.0 \pm 0.4	23.2 \pm 0.5	24.1 \pm 0.2
Daily food intake (grams)	4.53 \pm 0.10	4.77 \pm 0.08	3.74 \pm 0.18	3.87 \pm 0.10
Body Length (centimeters)	10.6 \pm 0.1	10.7 \pm 0.1	8.23 \pm 0.1	8.55 \pm 0.1
Body mass index (wt/l ²)	0.27 \pm .01	0.27 \pm 0.01	0.32 \pm 0.01	0.32 \pm 0.01
Temperature (centigrade)	ND	ND	36.3 \pm 0.1	36.4 \pm 0.1
Adrenals (mg)	6 \pm 1	6 \pm 1	10 \pm 1	10 \pm 1

Experiment 18—Table 16

Comparison of reproductive organ weights between GKO and WT mice. Data are expressed as mean values \pm SEM.

Organ (wt in mg)	WT Male	GKO Male	WT Female	GKO Female
Testis	214 \pm 6	231 \pm 9	ND	ND
Seminal vesicle	295 \pm 10	388 \pm 15 ^a	ND	ND
Uterus	ND	ND	124 \pm 19	158 \pm 18
Ovaries	ND	ND	14 \pm 1	15 \pm 1

^a $p < 0.001$ compared to WT male

Experiment 19—Table 17

Comparison of serum hormone levels between GKO and WT mice. Data are expressed as mean values \pm SEM.

Hormone	WT Male	GKO Male	WT Female	GKO Female
Insulin (μ U/ml)	0.41 \pm 0.15	3.50 \pm 1.35 ^a	0.96 \pm 0.39	1.04 \pm 0.44
Glucose (mg/dl)	85.2 \pm 10.0	87.2 \pm 9.5	75.3 \pm 6.6	108 \pm 7 ^b
Glucagon (pg/ml)	53.5 \pm 4.9	74.8 \pm 8.0 ^a	77.5 \pm 8.2	72.3 \pm 9.7
Leptin (ng/ml)	2.1 \pm 0.3	3.0 \pm 0.5	1.0 \pm 0.2	0.8 \pm 0.3
T4 (μ g/dl)	2.9 \pm 0.1	2.7 \pm 0.2	3.4 \pm 0.3	3.1 \pm 0.2
Corticosterone (μ g/dl)	18.7 \pm 1.3	19.2 \pm 2.8	48.0 \pm 3.7	43.1 \pm 2.5
Prolactin (ng/ml)	24.2 \pm 2.3	20.8 \pm 2.0	ND	ND?
Testosterone (ng/ml)	11.2 \pm 1.6	12.6 \pm 1.8	ND	ND
LH (ng/ml)	9.4 \pm 3.0	13.9 \pm 0.9	7.6 \pm 2.3	10.2 \pm 2.1
FSH (ng/ml)	16.3 \pm 0.7	20.8 \pm 1.0 ^b	4.0 \pm 1.4	2.8 \pm 0.7

^a $p < 0.05$ compared to WT

^b $p < 0.01$ compared to WT

Experiment 20—Table 18

Comparison of catecholamine levels between GKO and WT mice. Data are expressed as mean values \pm SEM.

(ng/ml)	WT Male	GKO Male
NE hypothalamus	30.7 \pm 1.5	26.6 \pm 1.7
NE forebrain	9.2 \pm 0.5	10.7 \pm 1.0
NE brainstem	13.9 \pm 0.8	15.8 \pm 1.2
DA hypothalamus	9.2 \pm 0.9	7.1 \pm 0.6
DA forebrain	54.3 \pm 2.7	54.7 \pm 2.1
DA brainstem	1.6 \pm 0.1	1.8 \pm 0.3

Experiment 21—Table 19

Comparison of fat pads between leptin- and saline-treatments in GKO and WT mice.

Data are expressed as mean values \pm SEM.

	WT Saline	GKO Saline	WT Leptin	GKO Leptin
White adipose (%total weight)	2.2 \pm 0.4	2.0 \pm 0.3	1.1 \pm 0.2 ^a	0.5 \pm 0.1 ^b
Brown adipose (%total weight)	0.24 \pm 0.02	0.19 \pm 0.03	0.21 \pm 0.02	0.20 \pm 0.02

^a p<0.01 from WT saline

^b p<0.001 from GKO saline

Experiment 22—Table 20

Comparison of hormone levels between fasted and fasted with leptin groups in GKO and WT mice. Data are expressed as mean values \pm SEM.

Hormone	WT Fasted	WT Fast/leptin	GKO Fasted	GKO Fast/leptin
LH (ng/ml)	1.0 \pm 0.1	1.8 \pm 0.8	1.1 \pm 0.2	1.6 \pm 0.5
FSH (ng/ml)	7.6 \pm 1.1	9.6 \pm 1.3	6.6 \pm 0.5	10.5 \pm 1.0 ^b
Testosterone (ng/ml)	0.10 \pm 0.10	1.1 \pm 0.1 ^a	0.1 \pm 0.1	0.2 \pm 0.1
T4 (μ g/dl)	0.8 \pm 0.1	1.5 \pm 0.2 ^c	0.9 \pm 0.1	1.5 \pm 0.2 ^b
Corticosterone (μ g/dl)	61.0 \pm 2.8	35.9 \pm 6.3 ^c	64.8 \pm 2.6	20.5 \pm 2.9 ^d
Glucose (mg/dl)	43.0 \pm 3.3	38.2 \pm 2.2	38.9 \pm 2.8	48.5 \pm 4.4 ^a

^a p<0.05 compared to fasted control of same genotype

^b p<0.01 compared to fasted control of same genotype

^c p<0.001 compared to fasted control of same genotype

^d p<0.0001 compared to fasted control of same genotype

Experiment 23—Table 21

Comparison of food intake and body weight change between NPY and saline treatments in GKO and WT mice. Data are expressed as mean values \pm SEM.

	WT aCSF	GKO aCSF	WT NPY	GKO NPY
Food intake 2 hour (g)	0.35 \pm 0.07	0.48 \pm 0.05 ^a	0.90 \pm 0.11	1.01 \pm 0.05 ^a
Food intake 24 hour (g)	4.75 \pm 0.39	5.52 \pm 0.23	5.60 \pm 0.20	5.50 \pm 0.37
Weight change 2 hour (g)	+0.23 \pm 0.19	+0.16 \pm 0.15	-0.15 \pm 0.15	+0.30 \pm .024
Weight change 24 hour (g)	+0.06 \pm 0.25	-0.53 \pm 0.22	-0.44 \pm 0.09	-0.26 \pm 0.24

^a $p < 0.0001$ compared to aCSF-treated control of same genotype.

Experiment 23—Table 22

Comparison of food intake, body weight change, catecholamines and hormone levels between galanin- and aCSF-treatments in GKO and WT mice. Data are expressed as mean values \pm SEM.

	WT aCSF	GKO aCSF	WT Galanin	GKO Galanin
Mean daily food intake (g)	4.86 \pm 0.23	5.06 \pm 0.19	5.74 \pm 0.17 ^b	5.94 \pm 0.16 ^b
Mean body wt change (%)	-0.2 \pm 0.6	-0.2 \pm 0.8	+2.4 \pm .1.0 ^a	+1.7 \pm 0.7 ^a
NE brainstem (ng/ml)	17.3 \pm 0.7	18.9 \pm 0.9	16.9 \pm 0.9	16.3 \pm 0.9
DA brainstem (ng/ml)	1.5 \pm 0.2	1.8 \pm 0.1	1.6 \pm 0.2	1.5 \pm 0.1
Testosterone (ng/ml)	9.5 \pm .0.7	6.4 \pm 1.5	13.2 \pm 2.0 ^a	7.4 \pm 1.8
LH (ng/ml)	10.3 \pm 2.6	11.6 \pm 2.8	11.9 \pm 3.9	8.7 \pm 1.6
FSH (ng/ml)	11.9 \pm 1.0	11.0 \pm 1.2	12.4 \pm .7	11.8 \pm 1.3
Corticosterone (μ g/dl)	39.2 \pm 3.5	35.3 \pm 3.8	37.2 \pm 7.1	24.4 \pm 2.0
Leptin (ng/ml)	0.16 \pm 0.08	0.53 \pm 0.23	0.54 \pm 0.11	0.13 \pm 0.07 ^b

^a p<0.05 compared to aCSF-treated group of same genotype

^b p<0.01 compared to aCSF-treated group of same genotype

Experiment 24—Table 23

Comparison of GAL-R1 mRNA levels between GKO and WT mice. Data are expressed as mean values \pm SEM.

Region (signal/background ratio of gray levels)	WT Male	GKO Male
Periventricular nucleus	3.69 \pm 0.31	4.11 \pm 0.35
Lateroanterior nucleus	3.37 \pm 0.20	3.96 \pm 0.41
Anterior hypothalamic nucleus	3.41 \pm 0.20	3.41 \pm 0.49
Supraoptic nucleus	3.16 \pm 0.14	3.52 \pm 0.42
Paraventricular nucleus	2.65 \pm 0.15	2.94 \pm 0.32
Dorsomedial nucleus (anterior)	3.50 \pm 0.26	3.07 \pm 0.17
Dorsomedial nucleus (posterior)	3.02 \pm 0.11	3.07 \pm 0.13
Ventromedial nucleus	2.69 \pm 0.17	2.73 \pm 0.08

Experiment 25—Table 24

Comparison of NPY mRNA levels between GKO and WT mice. Data are expressed as mean values \pm SEM.

Area	WT Male	GKO Male
Nucleus accumbens (grains/cell)	46 \pm 7	31 \pm 3
Nucleus accumbens (cell count)	85 \pm 21	55 \pm 12
Arcuate nucleus (SBR)	2.77 \pm 0.08	2.87 \pm 0.20
Arcuate nucleus grain area (pixels)	22100 \pm 1900	25300 \pm 2100
Locus coeruleus (grains/cell)	30 \pm 9	29 \pm 8
Locus coeruleus (cell count)	48 \pm 14	40 \pm 4

Experiment 26—Table 25

Comparison of baseline physiological indices between DKO and WT mice. Data are expressed as mean values \pm SEM.

	WT Male	DKO Male	WT Female	DKO Female
Food intake at 8 weeks (g)	2.79 \pm 0.12	3.50 \pm 0.14 ^c	ND	ND
Water intake at 8 weeks (ml)	5.4 \pm 0.3	6.7 \pm 0.3 ^b	ND	ND
Temperature at 8 weeks (C)	36.5 \pm 0.1	36.5 \pm 0.1	ND	ND
Adult 24 hour food intake (g)	5.19 \pm 0.15	6.56 \pm 0.41 ^b	4.18 \pm 0.22	4.04 \pm 0.21
Adult body mass index	0.27 \pm 0.01	0.30 \pm 0.01 ^a	0.32 \pm 0.01	0.32 \pm 0.01
Adult temp (C)	36.4 \pm 0.2	36.1 \pm 0.3	36.3 \pm 0.1	36.5 \pm 0.1
Adult length (cm)	10.2 \pm 0.2	10.5 \pm 0.1	8.2 \pm 0.1	8.1 \pm 0.1

^a p<0.05 compared to male WT

^b p<0.01 compared to male WT

^c p<0.001 compared to male WT

Experiment 26—Table 26

Comparison of reproductive organ weights between DKO and WT mice. Data are expressed as mean values \pm SEM.

Organs (weight in mg)	WT Male	DKO Male	WT Female	DKO Female
Adrenals	ND	ND	10 \pm 1	10 \pm 1
Seminal vesicle	295 \pm 11	333 \pm 41	ND	ND
Testes	214 \pm 6	245 \pm 16	ND	ND
Uterus	ND	ND	124 \pm 19	108 \pm 24
Ovaries	ND	ND	14 \pm 1	14 \pm 2

Experiment 27—Table 27

Comparison of serum hormone levels between DKO and WT mice. Data are expressed as mean values \pm SEM.

Hormone	WT Male	DKO Male	WT Female	DKO Female
Insulin (μ U/ml)	0.8 \pm 0.2	3.5 \pm 0.8 ^b	1.0 \pm 0.4	1.4 \pm 0.4
Glucose (mg/dl)	85.2 \pm 10.0	139 \pm 11.8 ^b	75.3 \pm 6.6	125 \pm 11.7 ^a
Glucagon (pg/ml)	53.5 \pm 4.9	65.4 \pm 5.3	77.5 \pm 8.2	94.7 \pm 22.3
Basal leptin (ng/ml)	4.6 \pm 0.7	22.4 \pm 7.3 ^b	8.4 \pm 1.0	7.0 \pm 1.7
Fasting leptin (ng/ml)	ND	ND	1.0 \pm 0.2	0.8 \pm 0.2
Prolactin (ng/ml)	24.2 \pm 2.3	22.9 \pm 3.9	ND	ND
Corticosterone (ng/ml)	18.7 \pm 1.3	15.5 \pm 1.4	48.0 \pm 3.7	42.9 \pm 3.7
T4 (ng/ml)	2.4 \pm 0.1	2.1 \pm 0.4	3.4 \pm 0.3	3.1 \pm 0.3
LH (ng/ml)	9.4 \pm 3.0	9.4 \pm 2.8	7.6 \pm 2.3	8.4 \pm 2.5
FSH (ng/ml)	16.3 \pm 0.7	15.3 \pm 0.7	4.0 \pm 1.4	5.2 \pm 1.1
Testosterone (ng/ml)	11.2 \pm 1.6	6.0 \pm 1.2 ^a	ND	ND
IGF-1 (ng/ml)	189 \pm 6.4	188 \pm 9.0	144 \pm 11.5	175 \pm 8.0 ^a

^a p<0.05 compared to WT of same gender

^b p<0.01 compared to WT of same gender

Experiment 30—Table 28

Comparison of body weight, food and water intake, and fat pads between leptin- and saline-treatments in male DKO and WT mice. Data are expressed as mean values \pm SEM.

	WT Saline	DKO Saline	WT Leptin	DKO Leptin
Body weight (% initial)	-1.6 \pm 2.0	-4.8 \pm 1.7	-7.7 \pm 1.7 ^a	-14.5 \pm 2.7 ^b
Daily food intake (grams)	4.31 \pm 0.12	4.62 \pm 0.16	4.05 \pm 0.07	4.22 \pm 0.18
Daily water intake (ml)	3.7 \pm 0.1	3.8 \pm 0.2	3.8 \pm 0.2	3.6 \pm 0.3
Fat pads (mg)	464 \pm 24	355 \pm 92	300 \pm 19 ^a	111 \pm 35 ^b

^a p<0.05 compared to saline-treated group of same genotype

^b p<0.01 compared to saline-treated group of same genotype

Experiment 31—Table 29

Comparison of body weight, food and water intake, and fat pads between leptin- and saline-treatments in female DKO and WT mice. Data are expressed as mean values \pm SEM.

	WT Saline	DKO Saline	WT Leptin	DKO Leptin
Body wt change on day 20 (% day 19)	1.0 \pm 0.7	3.3 \pm 0.9	1.9 \pm 0.8	3.7 \pm 1.1
Daily food intake (g)	3.64 \pm 0.14	3.68 \pm 0.13	3.64 \pm 0.13	3.67 \pm 0.15
Food intake on day 20 (g)	5.70 \pm 0.17	6.67 \pm 0.39 ^a	5.92 \pm 0.28	6.93 \pm 0.37 ^a

^a p<0.05 compared to WT

Figure 1: Photomicrograph of galanin mRNA in the preoptic area of a WT mouse.

Experiment 1—Figure 1

Galanin mRNA in the Preoptic Area of the Mouse Brain



Figure 2: Photomicrograph of galanin mRNA in the mid-hypothalamic region of a WT mouse.

Experiment 1—Figure 2

Galanin mRNA in the Mid-Hypothalamus of the Mouse Brain



Figure 3: Photomicrograph of galanin mRNA in the dorsomedial nucleus of a WT mouse.

Experiment 1—Figure 3

Galanin mRNA in the Dorsomedial Nucleus of the Mouse Brain



Figure 4: Photomicrograph of galanin mRNA in the central medial nucleus of a WT mouse.

Experiment 1—Figure 4

Galanin mRNA in the Central Amygdala Nucleus of the Mouse Brain

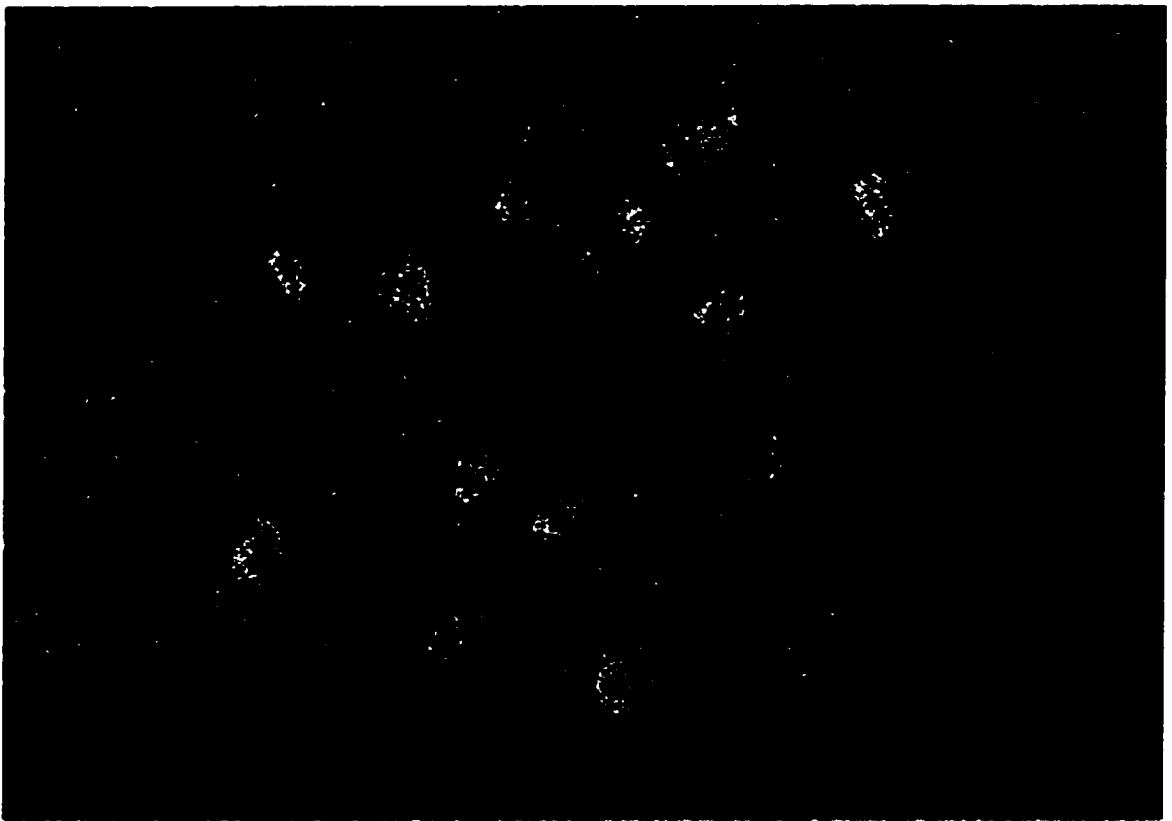


Figure 5: Photomicrograph of galanin mRNA in the locus coeruleus of a WT mouse.

Experiment 1—Figure 5

Galanin mRNA in the Locus Coeruleus of the Mouse Brain

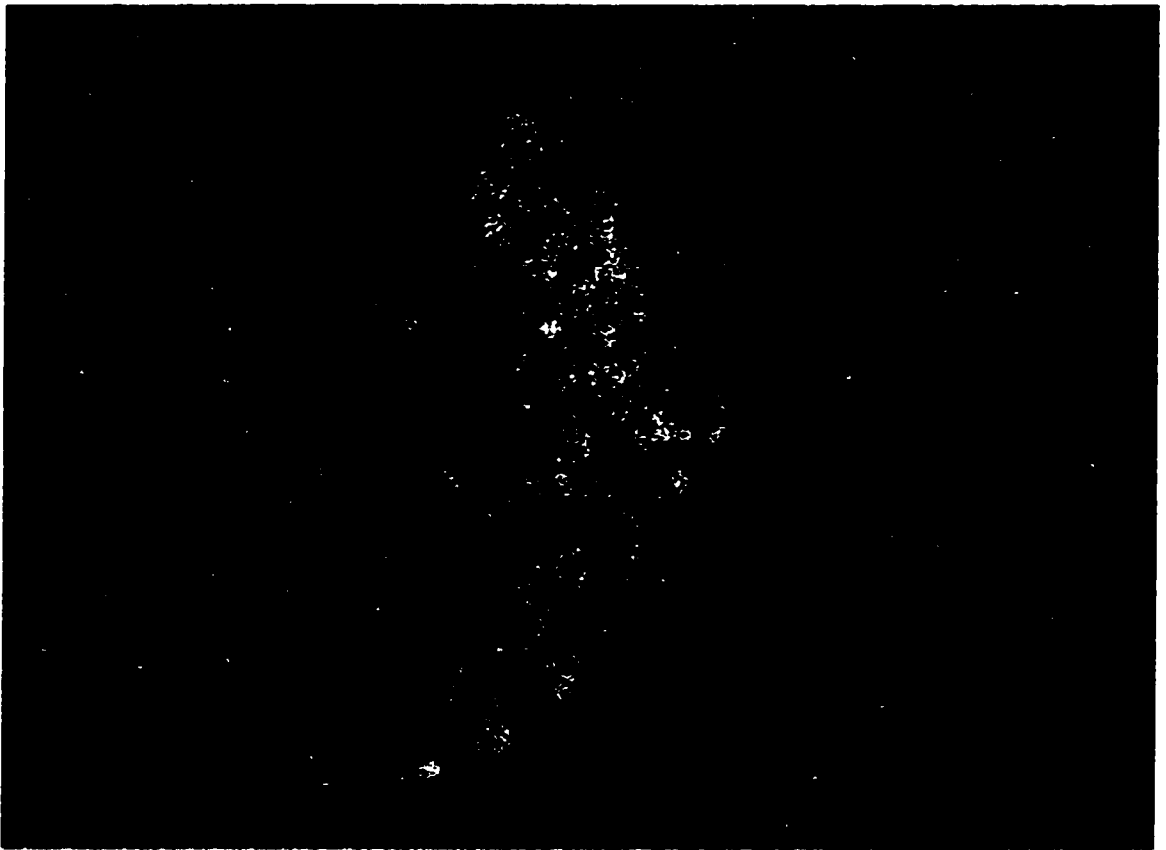


Figure 6: Photomicrograph of galanin mRNA in the dorsal vagal complex of a WT mouse.

Experiment 1—Figure 6

Galanin mRNA in the Dorsal Vagal Complex

of the Mouse Brain



Figure 7: Comparison between WT and NPYKO mice of number of galanin mRNA expressing cells (upper panel) and number of grains per cell in the arcuate nucleus (lower panel). Data are expressed as mean values \pm SEM.

Experiment 2—Figure 7

Galanin mRNA in the Arcuate Nucleus

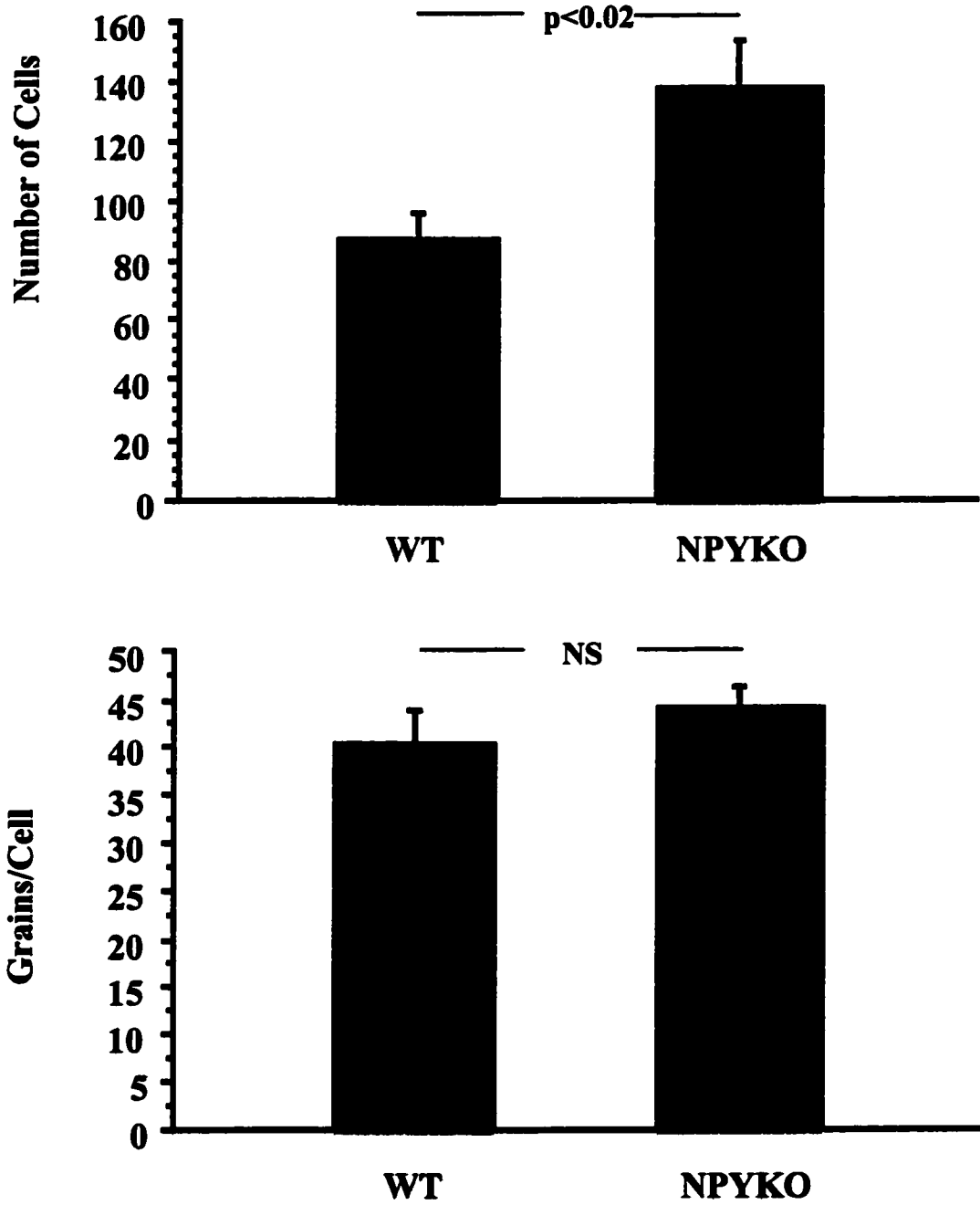


Figure 8: Comparison between WT and NPYKO mice of galanin mRNA in the dorsomedial nucleus of the hypothalamus. Data are expressed as mean proportional grain area \pm SEM.

Experiment 2—Figure 8

Galanin mRNA in the Dorsomedial Nucleus

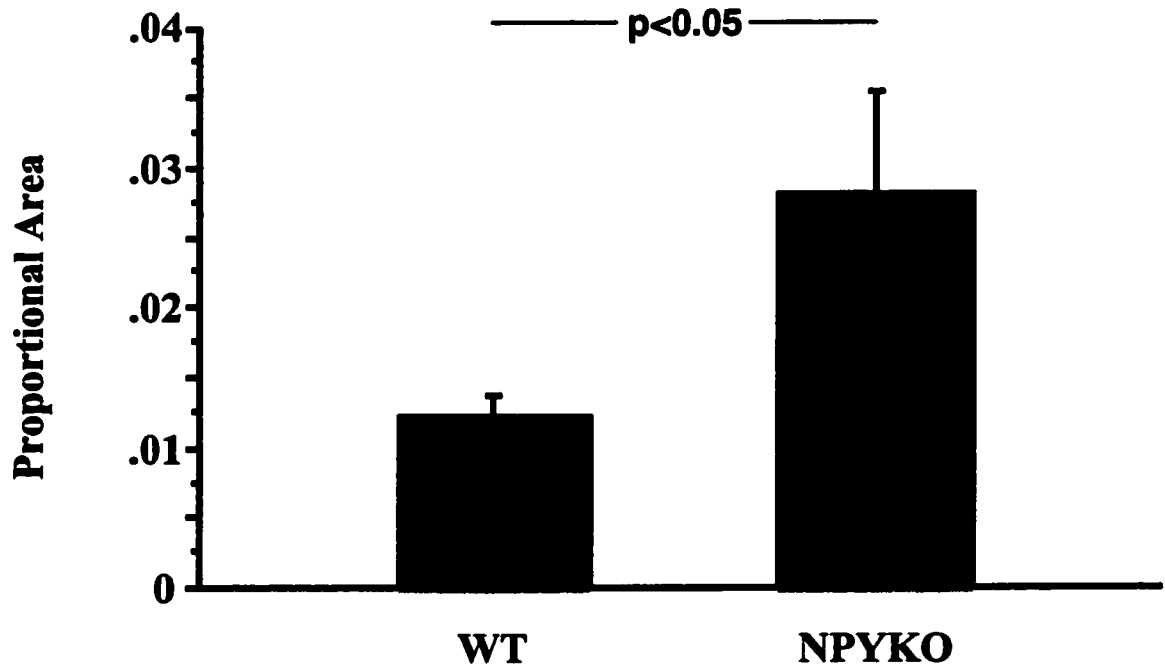


Figure 9: Comparison between WT and NPYKO mice of number of galanin mRNA expressing cells (upper panel) and number of grains per cell in the central nucleus of the amygdala (lower panel). Data are expressed as mean values \pm SEM.

Experiment 2—Figure 9

Galanin mRNA in the Amygdala

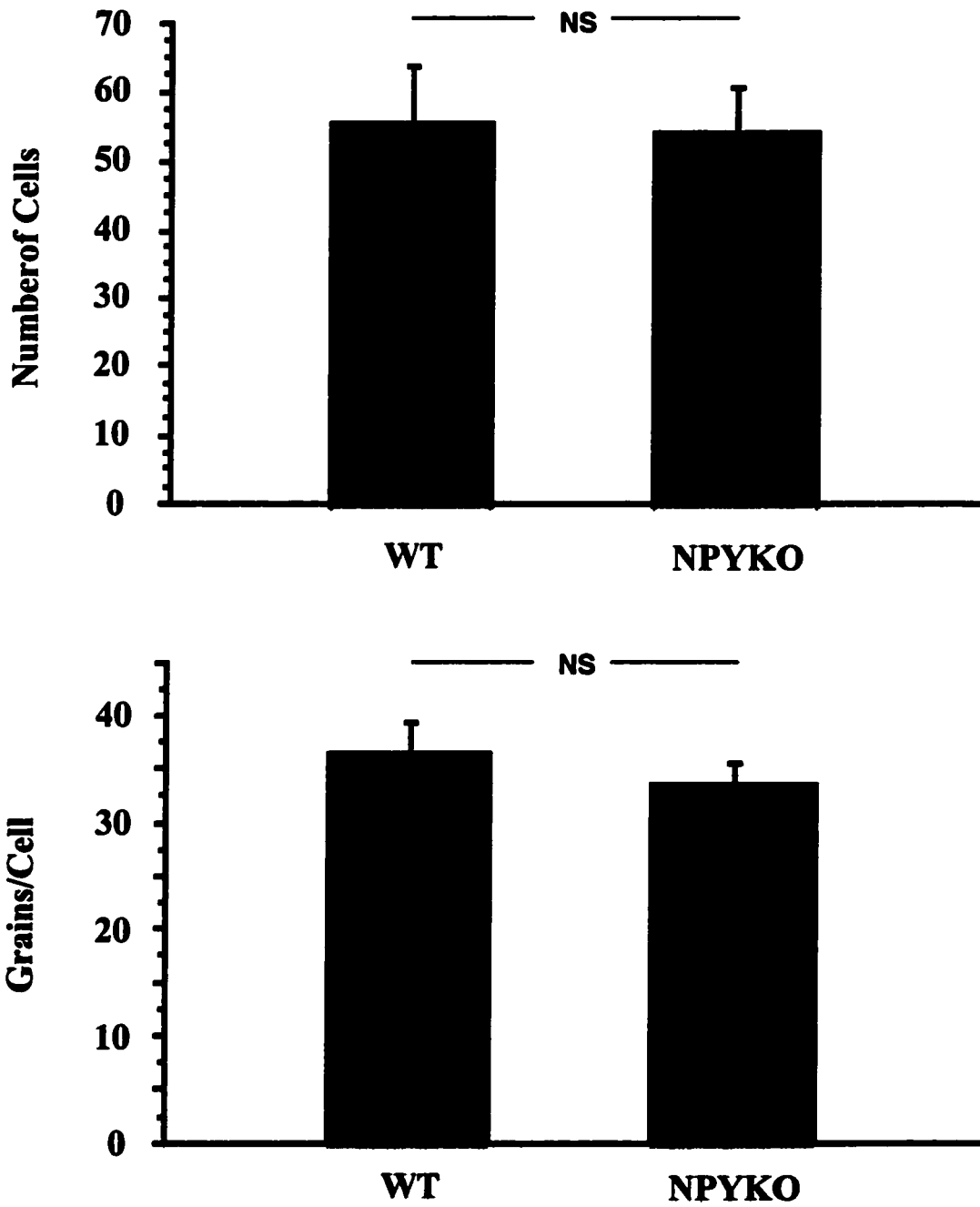


Figure 10: Comparison between WT and NPYKO mice of number of POMC mRNA expressing cells (upper panel) and number of grains per cell in the arcuate nucleus (lower panel). Data are expressed as mean values \pm SEM.

Experiment 3—Figure 10

POMC mRNA in Arcuate Nucleus

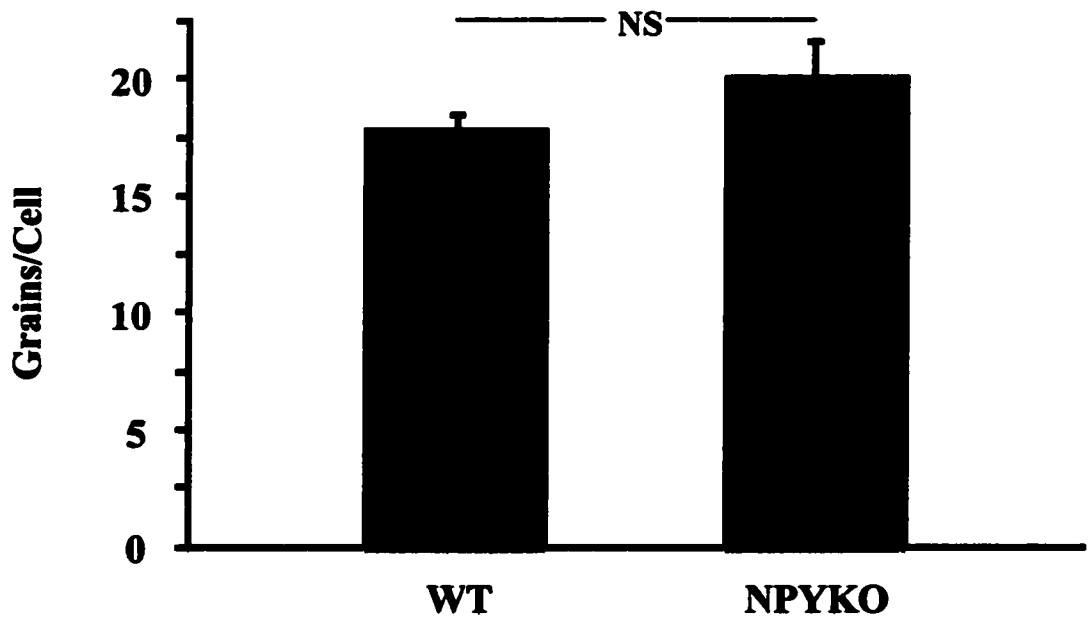
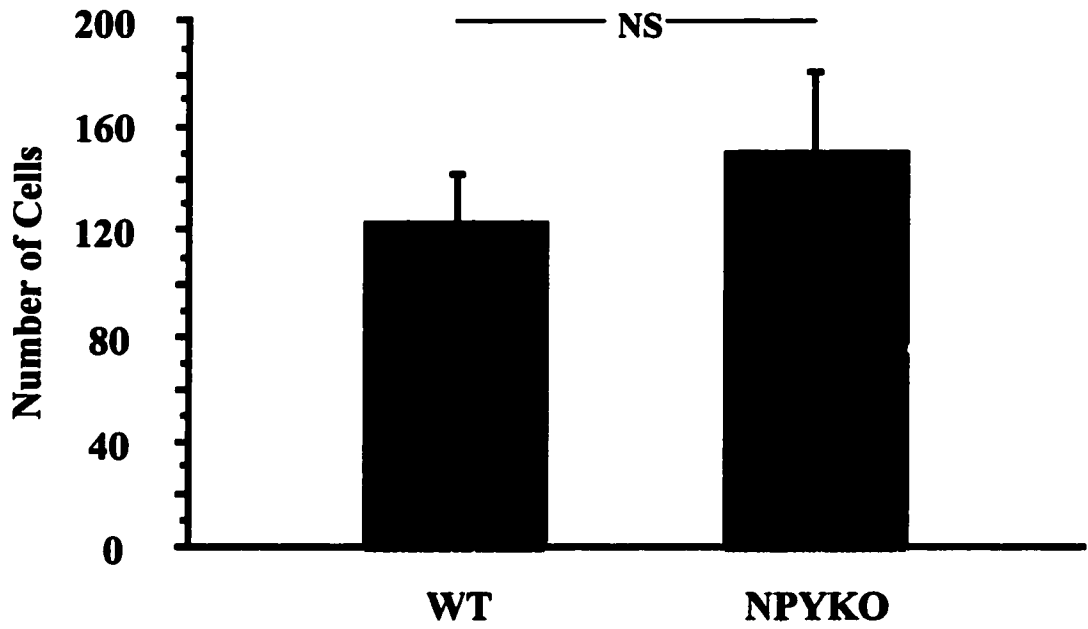


Figure 11: Comparison between WT and GALTG mice of galanin mRNA in the locus coeruleus. Data are expressed as mean relative optical density \pm SEM.

Experiment 4—Figure 11

Galanin mRNA in Locus Coeruleus

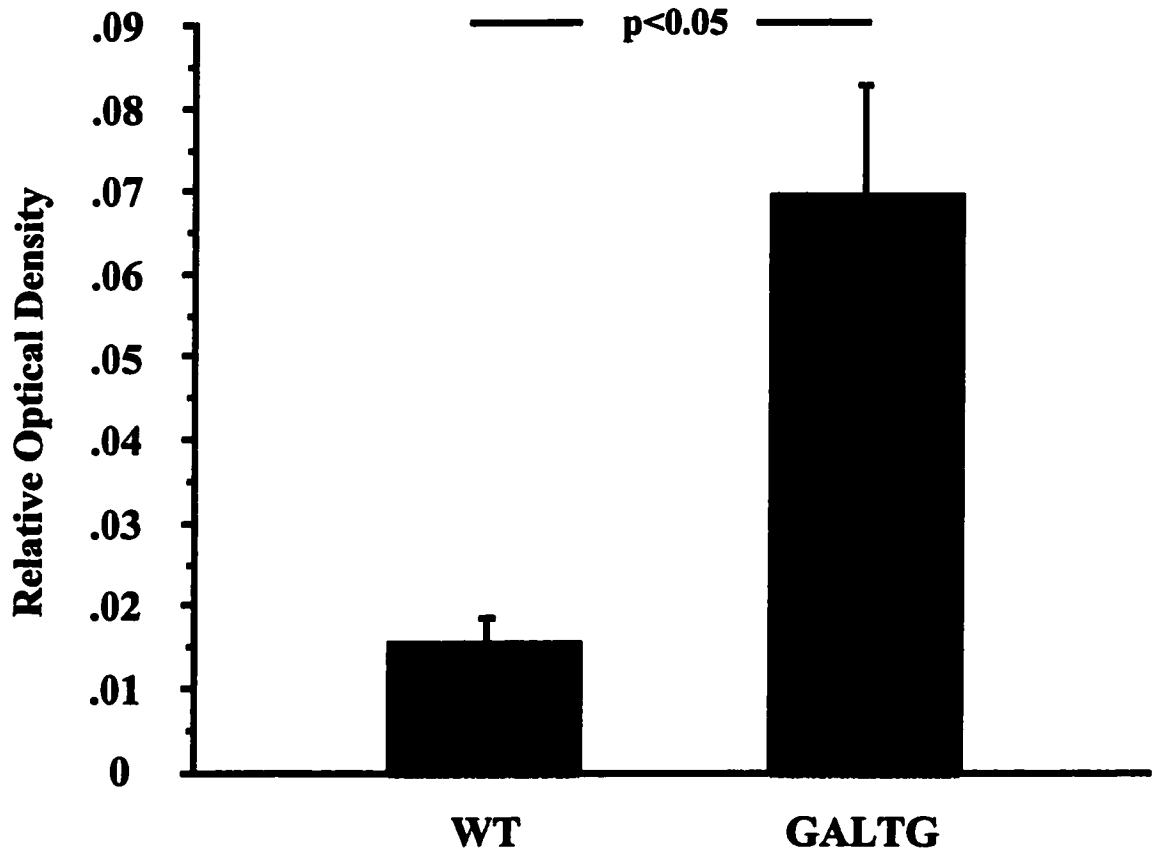


Figure 12: Photomicrograph of galanin mRNA in the piriform cortex of WT (upper panel) and GALTG mice (lower panel).

Experiment 5—Figure 12 (Piriform Cortex)

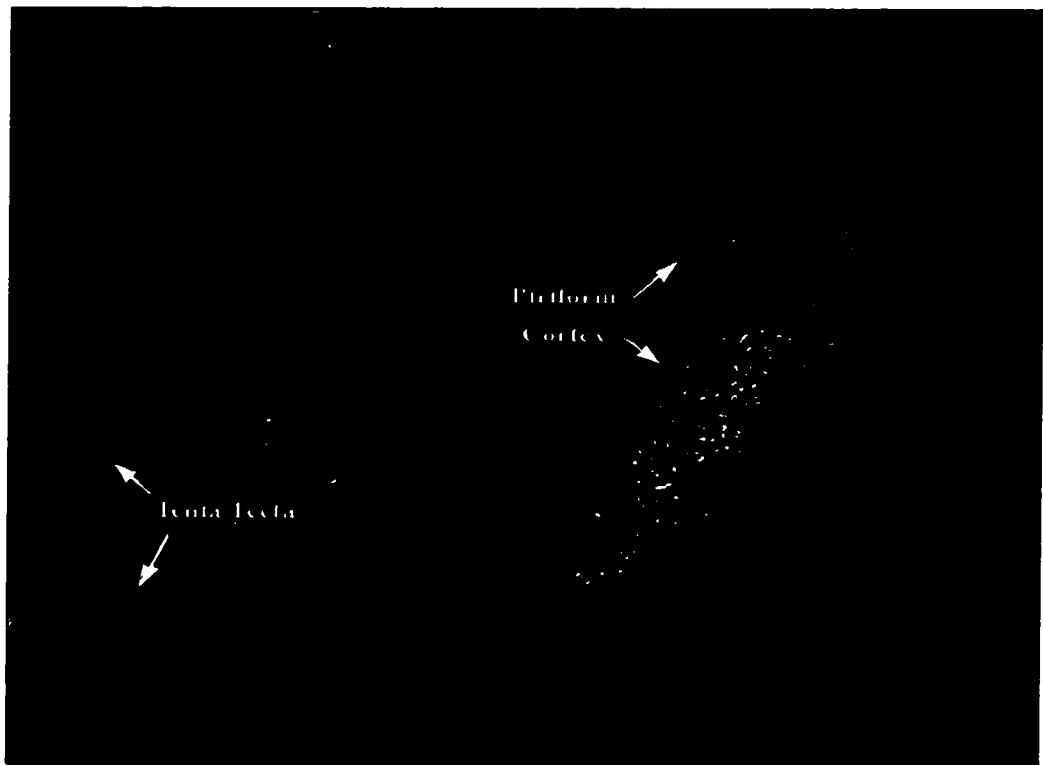
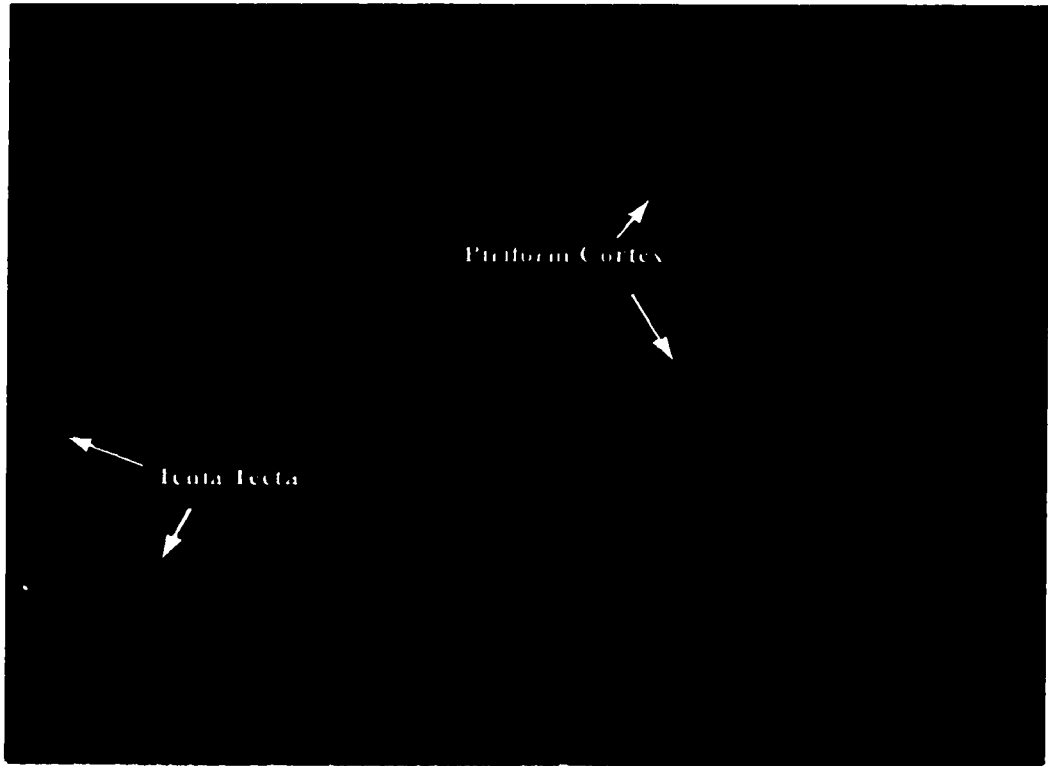


Figure 13: Photomicrograph of galanin mRNA in the entorhinal cortex of WT (upper panel) and GALTG (lower panel) mice.

Figure 14: Photomicrograph of galanin mRNA in the locus coeruleus of WT (upper panel) and GALTG mice (lower panel).

Experiment 5—Figure 14 (Locus Coeruleus)



Figure 15: Photomicrograph of galanin mRNA in the subcoeruleus of WT (upper panel) and GALTG (lower panel) mice.

Experiment 5—Figure 15 (Subcoeruleus)

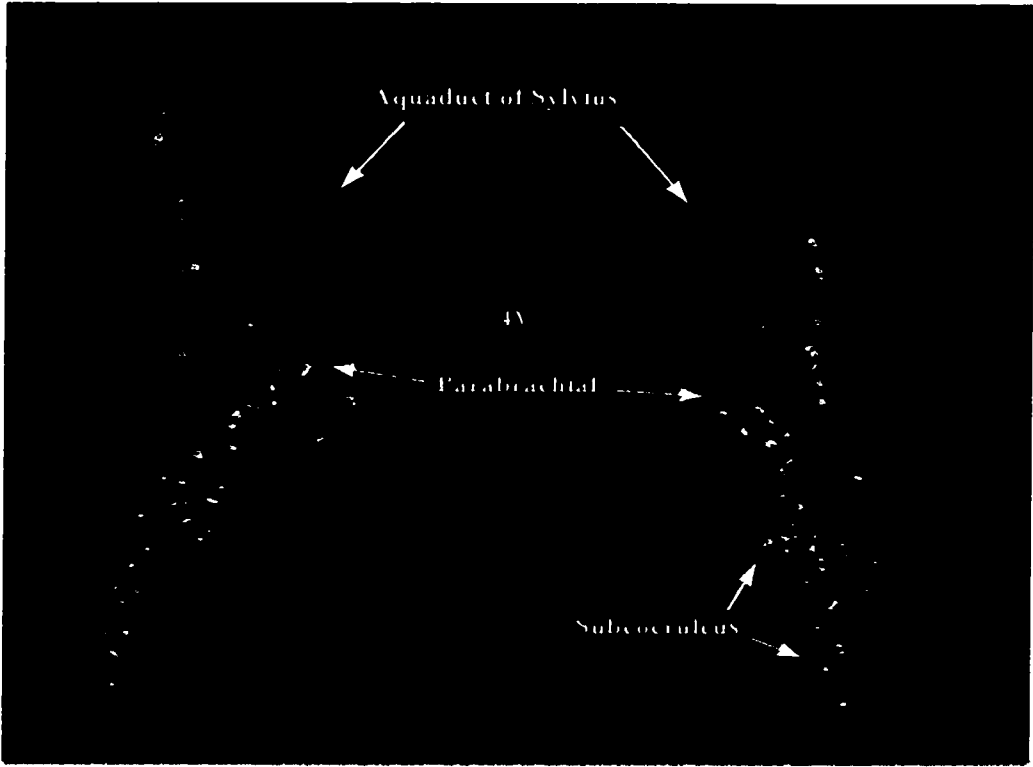
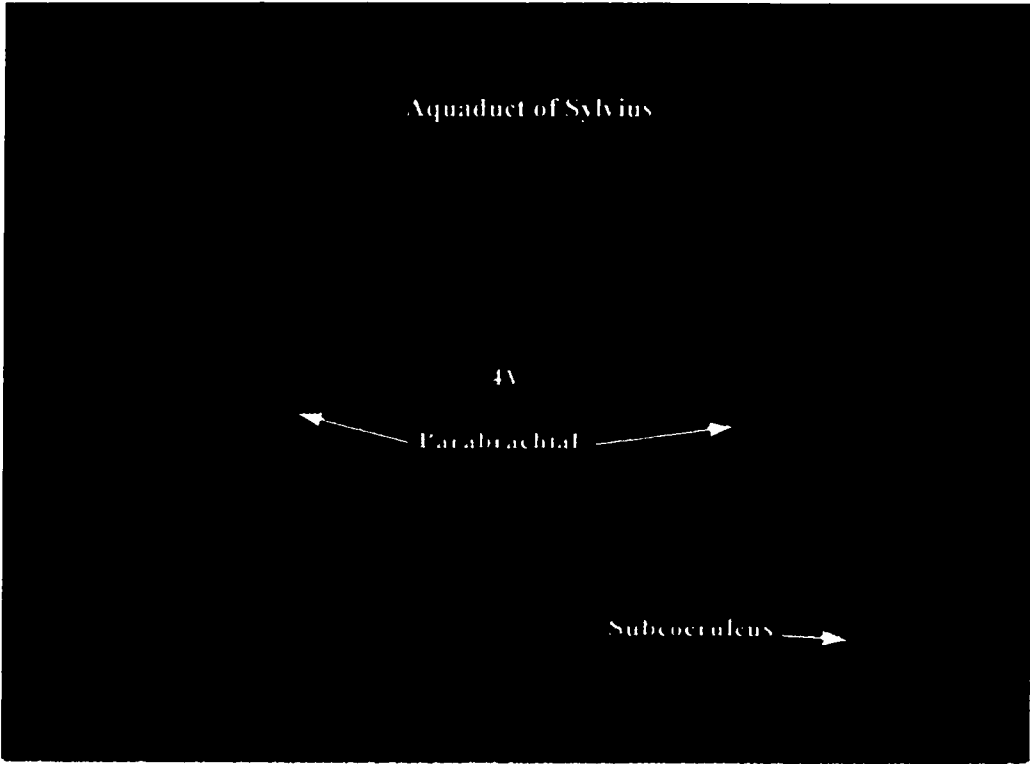


Figure 16: Comparison between WT and GALTG mice of galanin content in various regions of the brain. Data are expressed as mean pmol per gram wet weight \pm SEM.

Experiment 6—Figure 16

Galanin Concentration

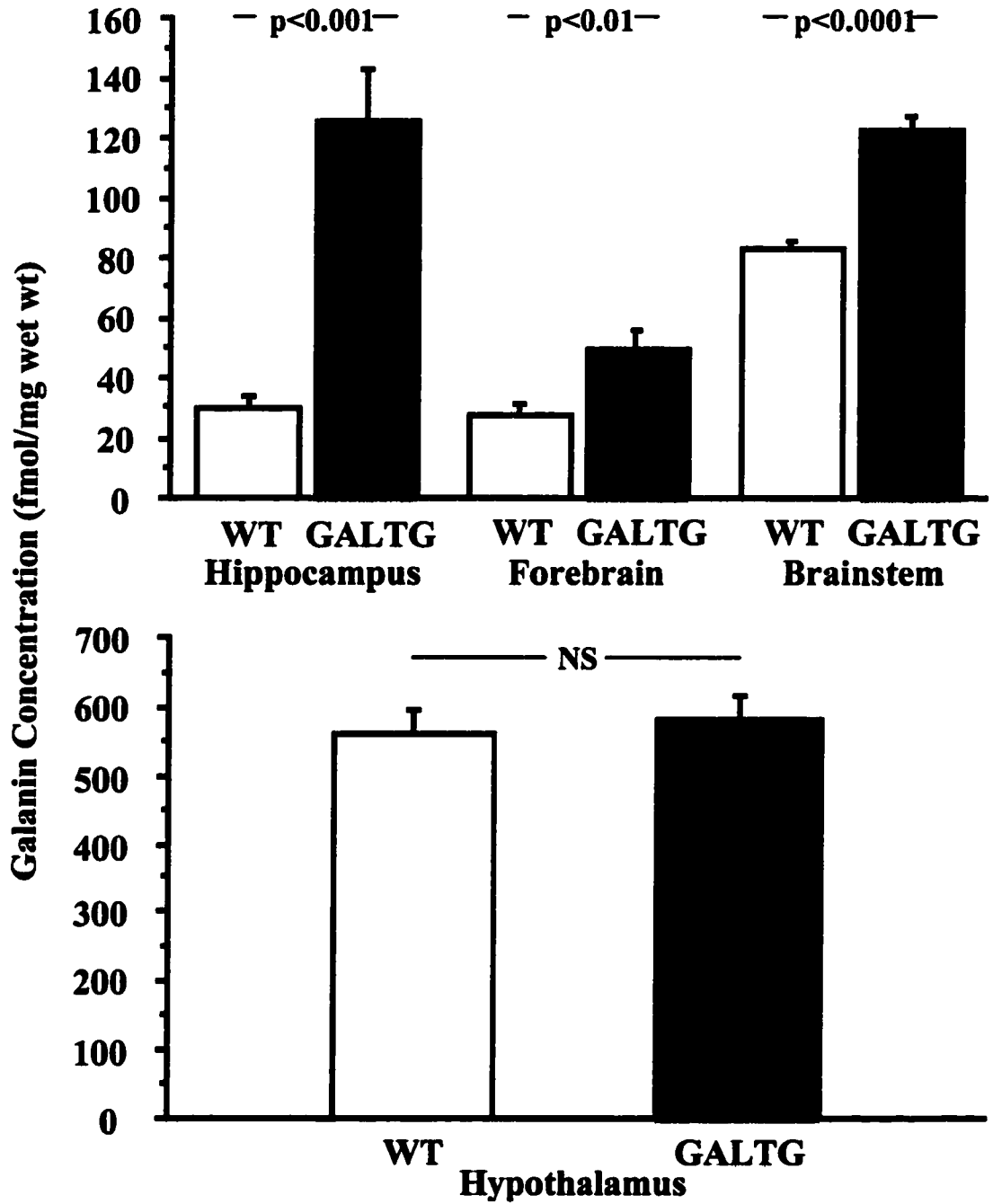
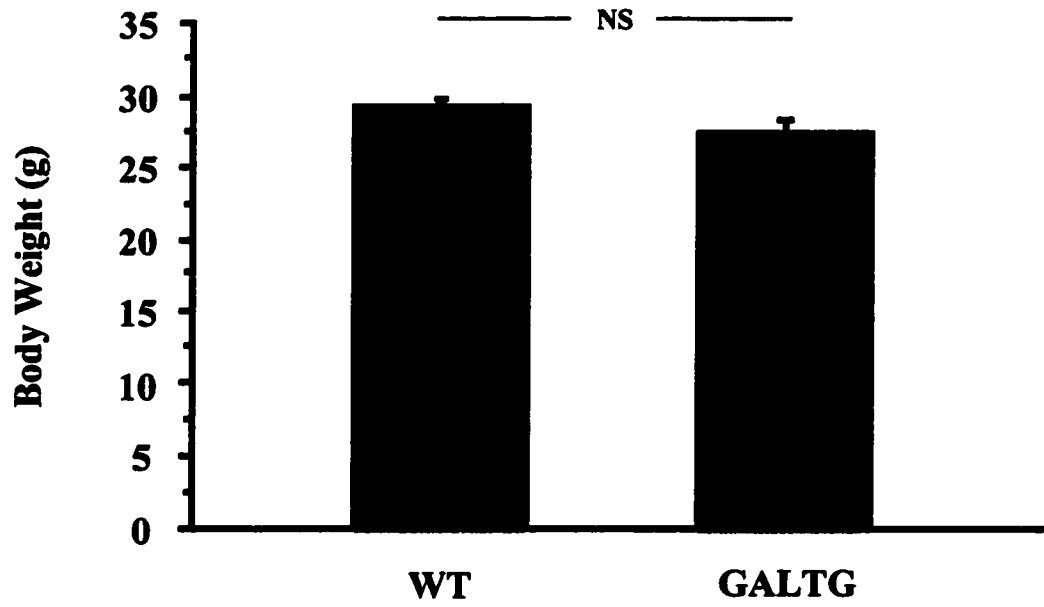


Figure 17: Comparison between WT and GALTG mice of adult body weight in males (upper panel) and females (lower panel). Data are expressed as mean weight in grams \pm SEM.

Experiment 7—Figure 17

Adult Male Body Weight



Adult Female Body Weight

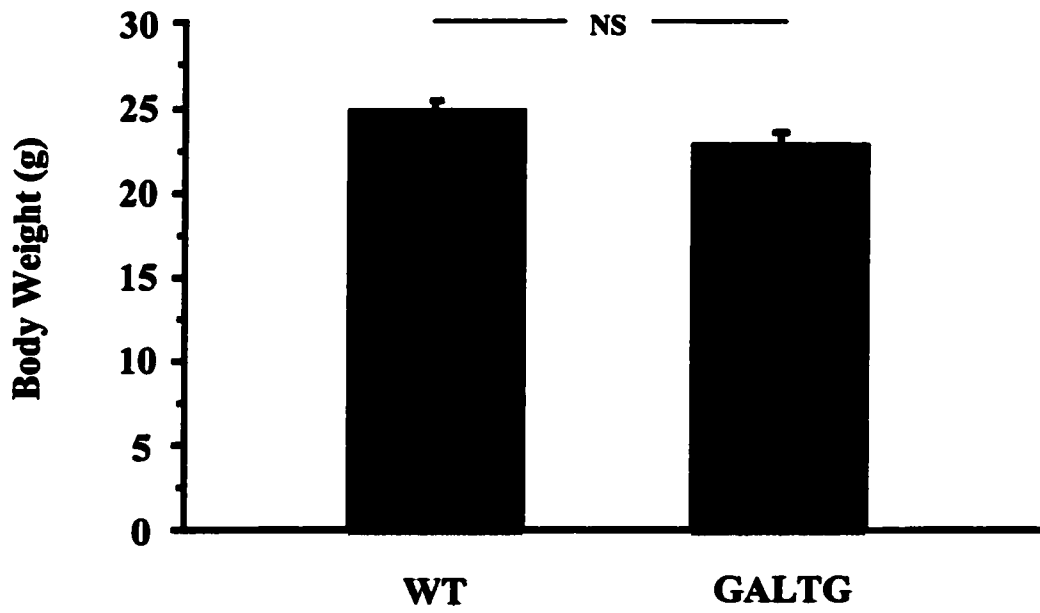
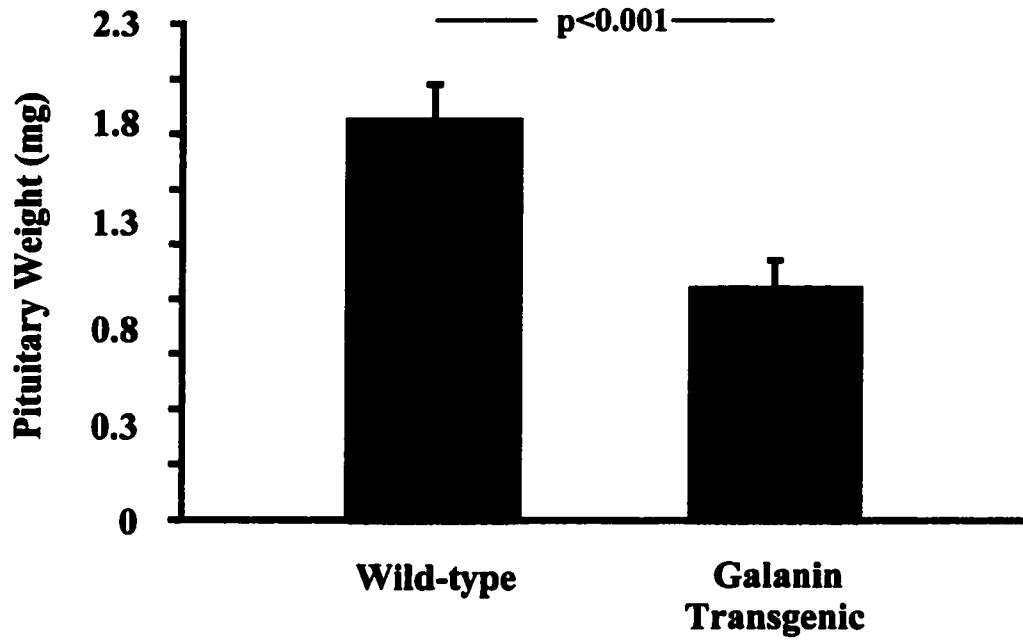


Figure 18: Comparison between female WT and GALTG mice of pituitary weight (upper panel) and adrenal weight (lower panel). Data are expressed as mean weight in milligrams \pm SEM.

Experiment 7—Figure 18

Female Pituitary Weight



Female Adrenal Weight

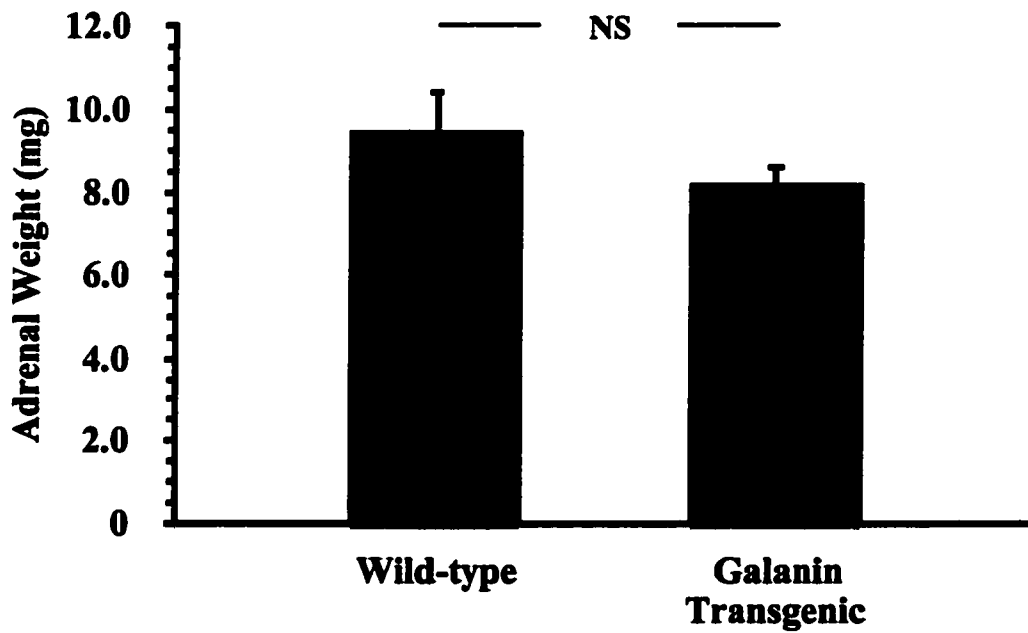
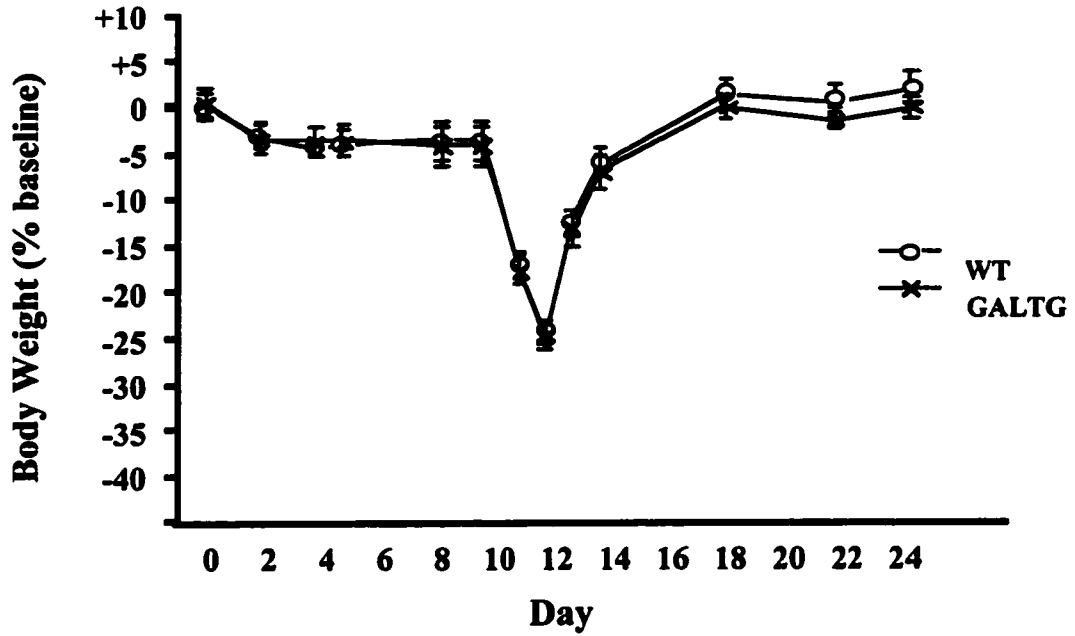


Figure 19: Comparison between WT and GALTG mice of percentage body weight change from baseline (upper panel) and food intake in grams (lower panel) before, during and after a 48 hour fast. Data are expressed as mean values \pm SEM.

Experiment 9—Figure 19

Body Weight Response to 48 Hour Fast



Food Intake Response to 48 Hour Fast

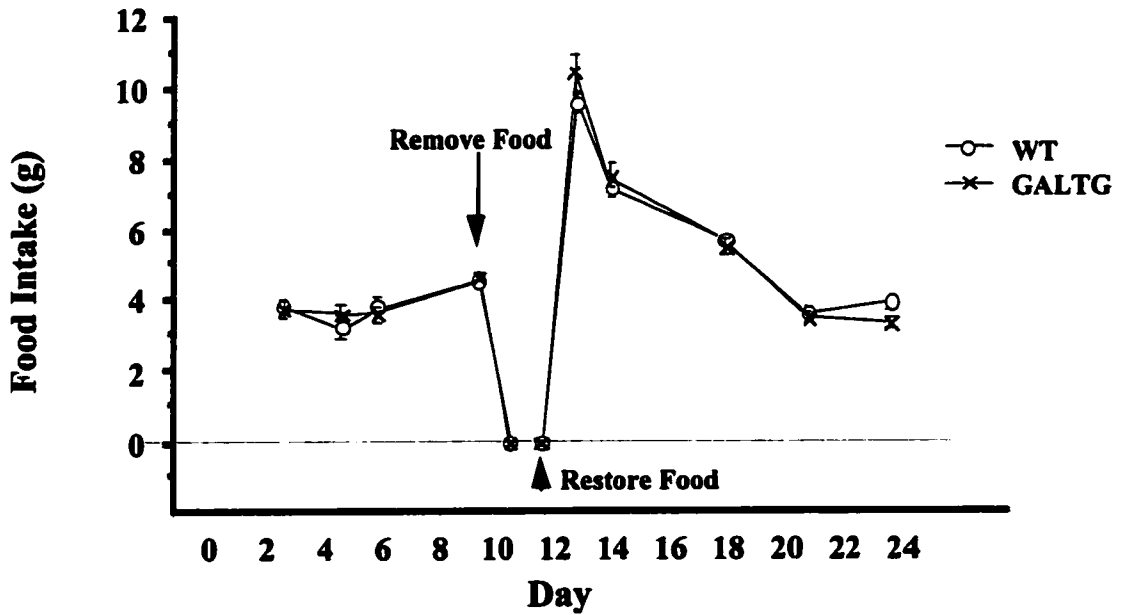
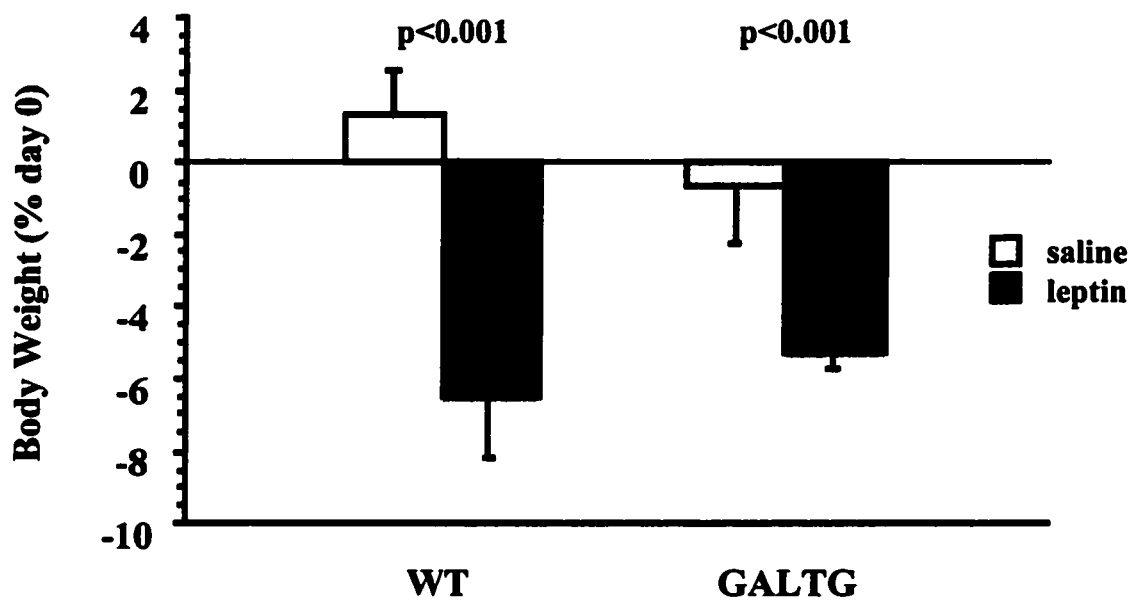


Figure 20: Comparison between WT and GALTG mice of percentage body weight change relative to baseline (upper panel) and food intake in grams (lower panel) in response to chronic injections of leptin. Data are expressed as mean values \pm SEM.

Experiment 10—Figure 20

Body Weight Response to Leptin



Food Intake Response to Leptin

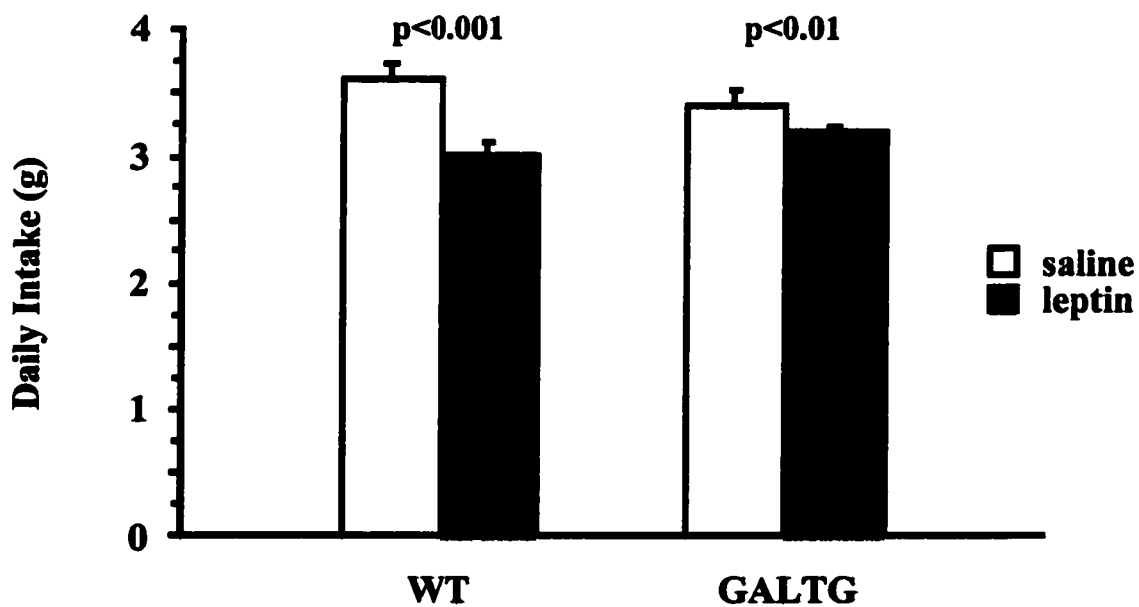
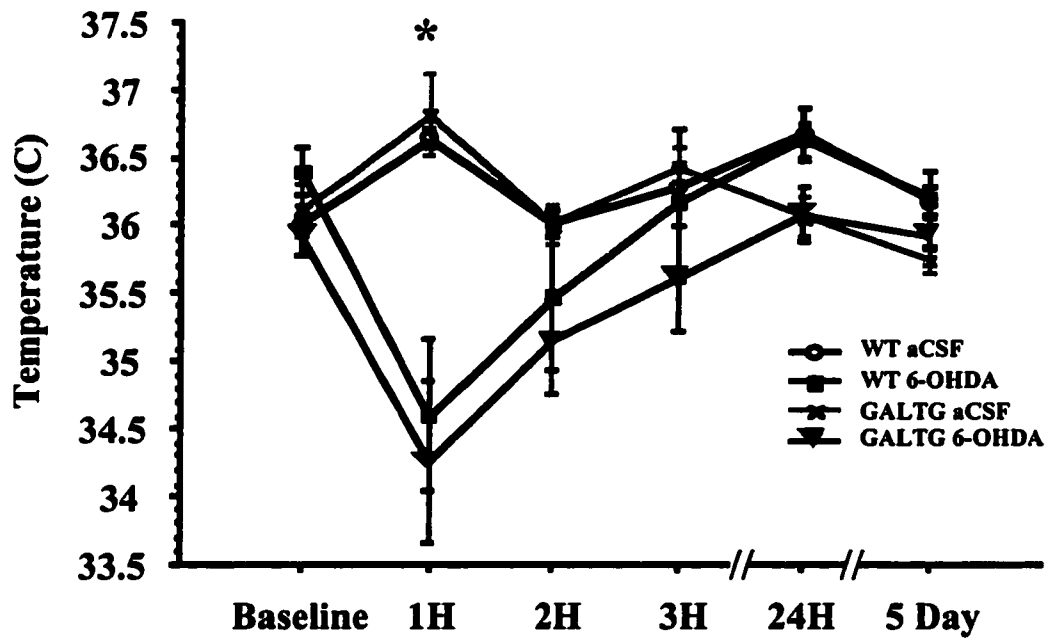


Figure 21: Comparison between WT and GALTG mice of rectal temperatures (upper panel) and body weight change in grams (lower panel) in response to a 6-OHDA lesion. Data are expressed as mean values \pm SEM.

Experiment 12—Figure 21

Temperature Response to 6-OHDA



24 Hour Body Weight Response to 6-OHDA

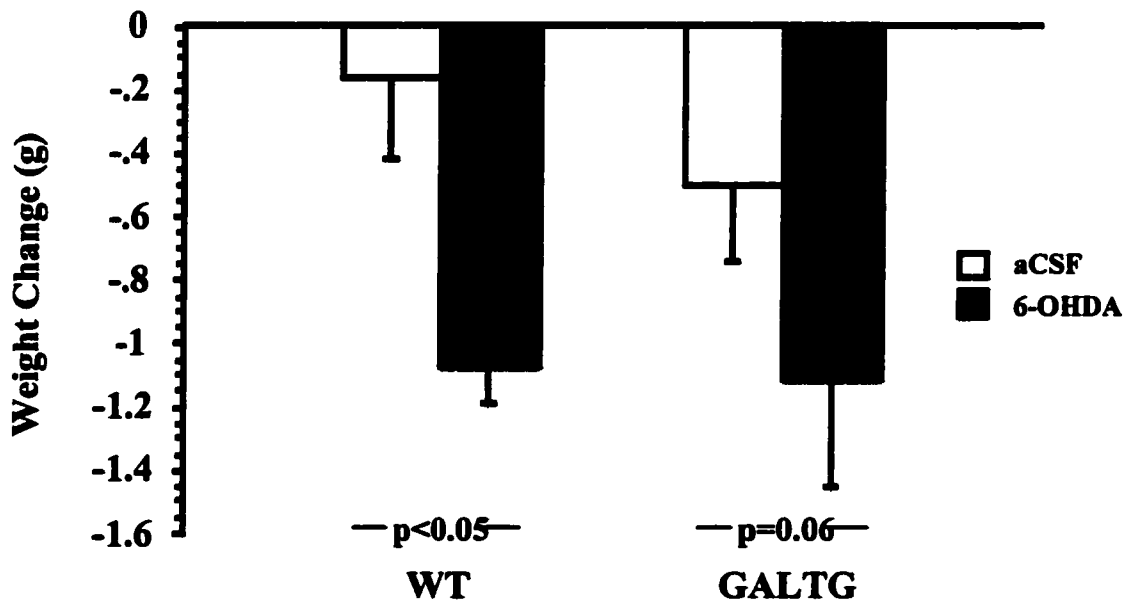
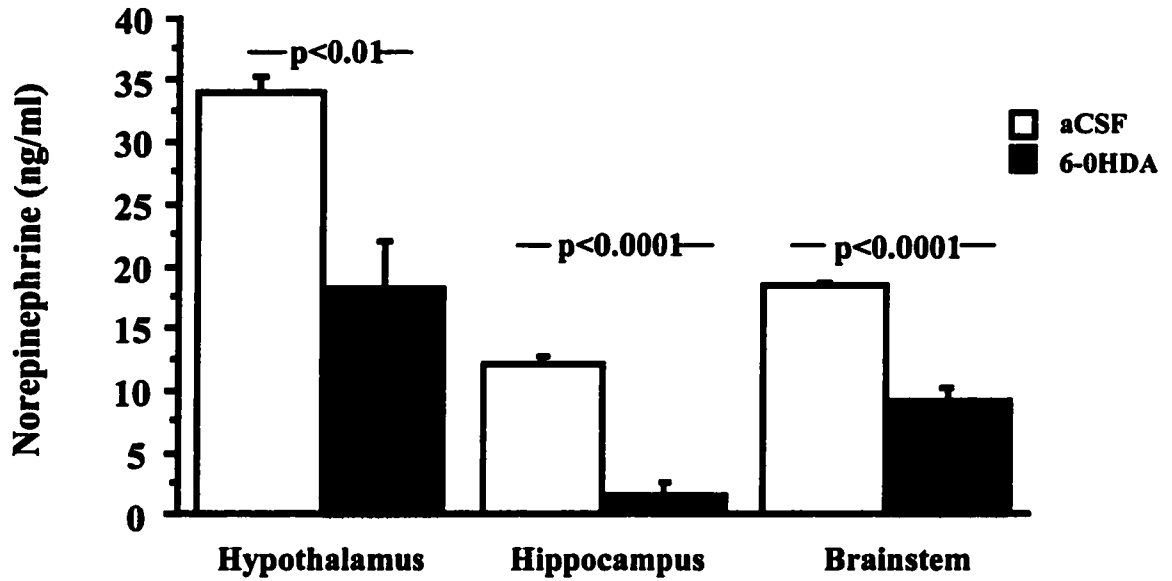


Figure 22: Comparison between aCSF- and 6-OHDA-treated mice of norepinephrine content (upper panel) and dopamine content (lower panel) in various brain regions of WT mice. Data are expressed as mean ng/ml \pm SEM.

Experiment 12—Figure 22

Norepinephrine Levels After 6-OHDA



Dopamine Levels After 6-OHDA

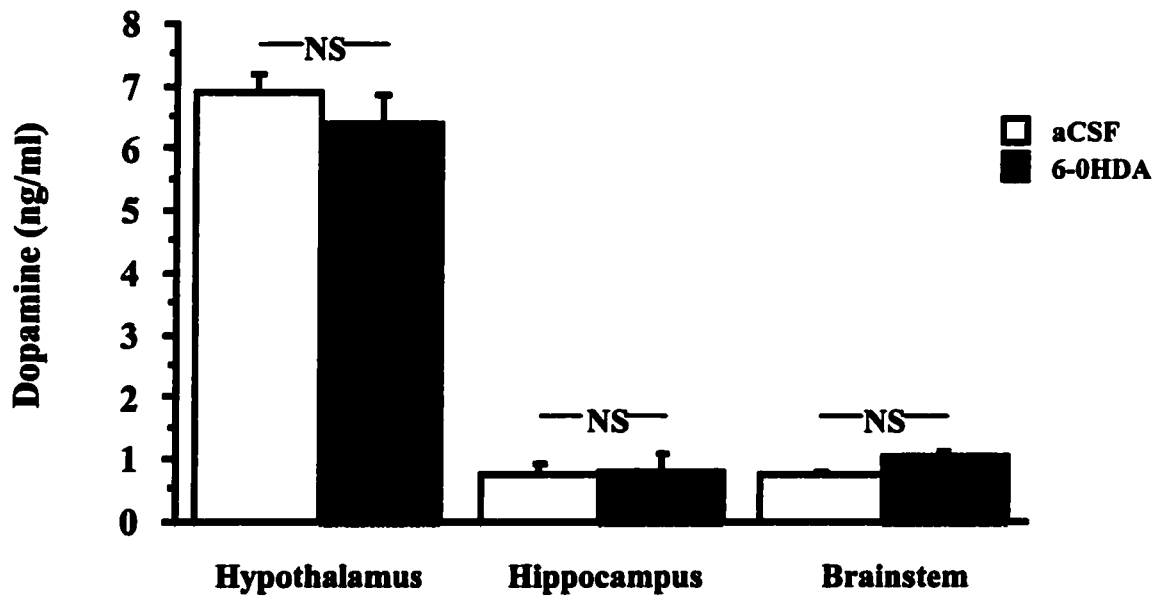


Figure 23: Comparison between female WT and GALTG mice of galanin R-1 receptor mRNA levels in various regions of the hypothalamus. Data are expressed as mean signal to background ratio \pm SEM.

Experiment 13—Figure 23

Galanin R1 mRNA Levels

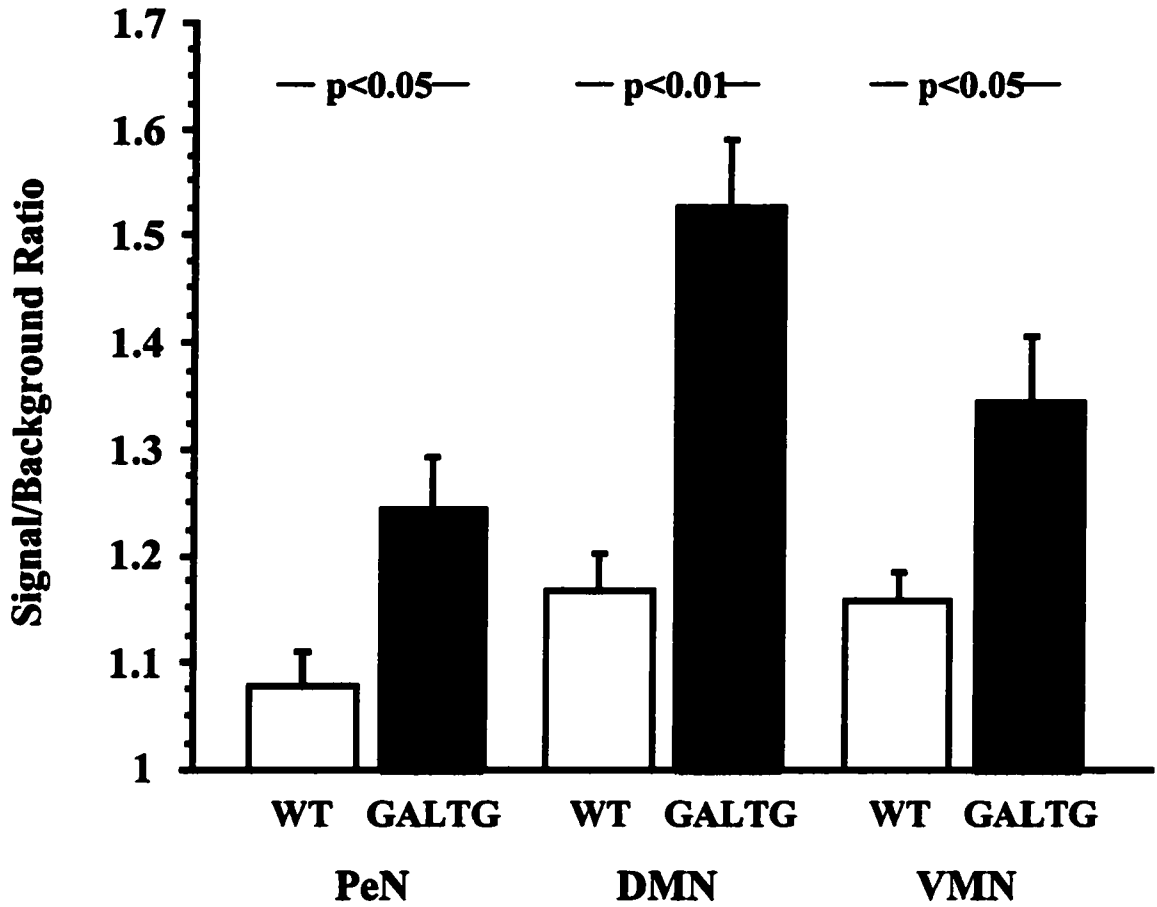


Figure 24: Comparison between female WT and GALTG mice of galanin-R2 receptor mRNA levels in the paraventricular and arcuate nuclei of the hypothalamus. Data are expressed as mean signal to background ratio \pm SEM.

Experiment 14—Figure 24

Galanin-R2 mRNA

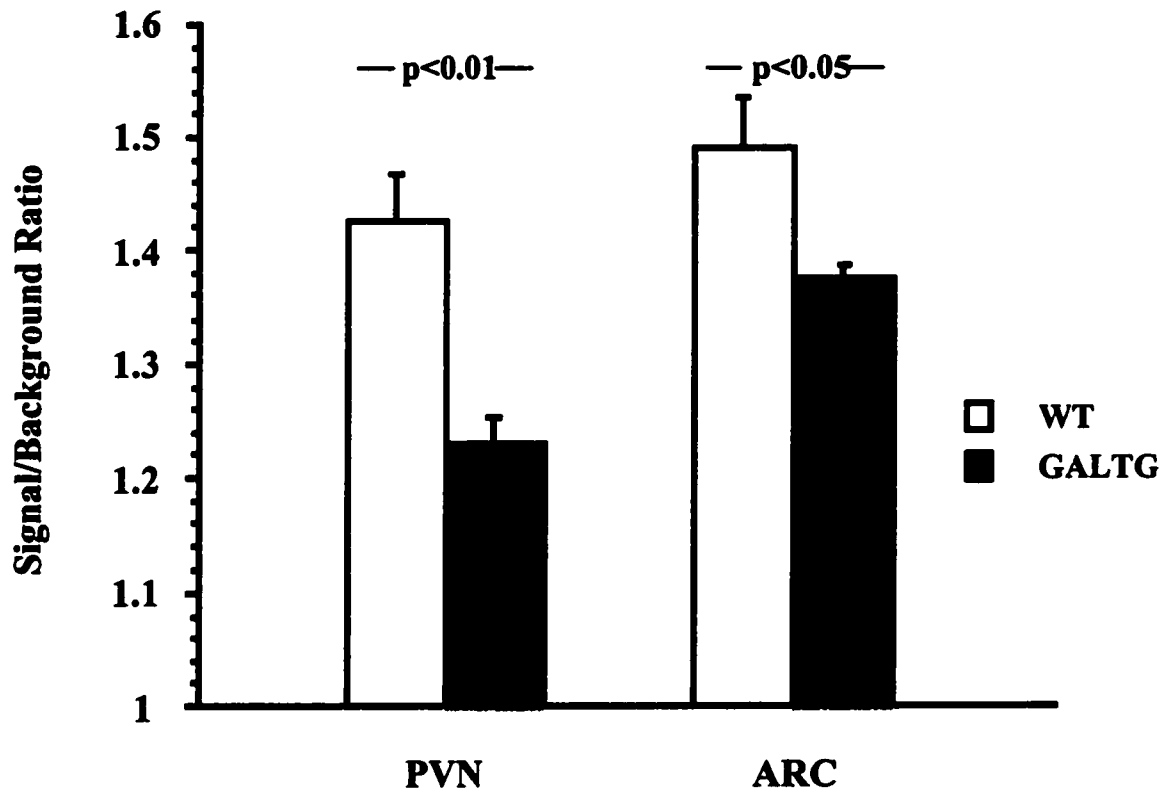


Figure 25: Comparison between male WT and GALTG mice of galanin R-1 receptor mRNA levels in various regions of the hypothalamus. Data are expressed as mean signal to background ratio of gray levels \pm SEM.

Experiment 15—Figure 25

Galanin R1 mRNA Levels

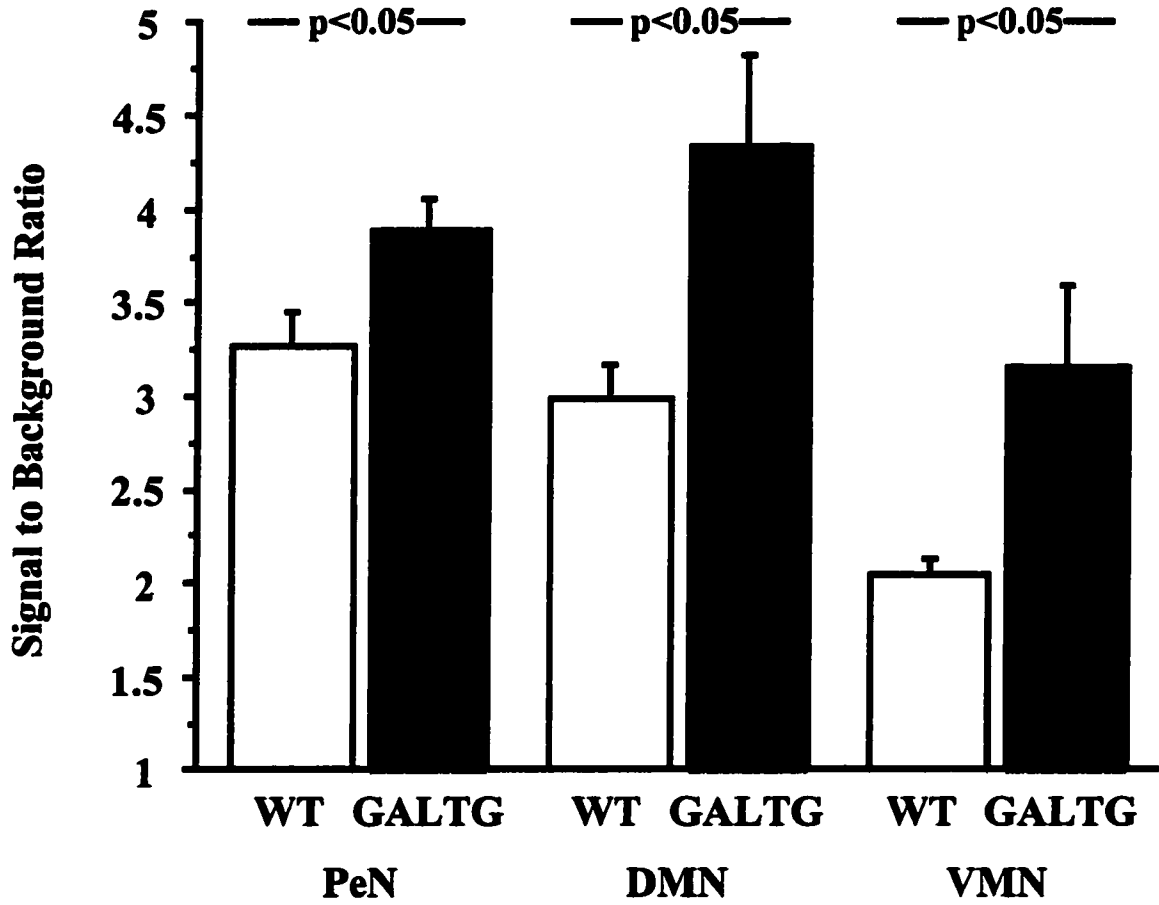


Figure 26: Comparison between male WT and GALTG mice of galanin R-1 receptor mRNA levels in various regions of the hypothalamus, in response to 6-OHDA lesion. Data are expressed as mean signal to background ratio of gray levels \pm SEM.

Experiment 16—Figure 26

Galanin R1 mRNA Levels

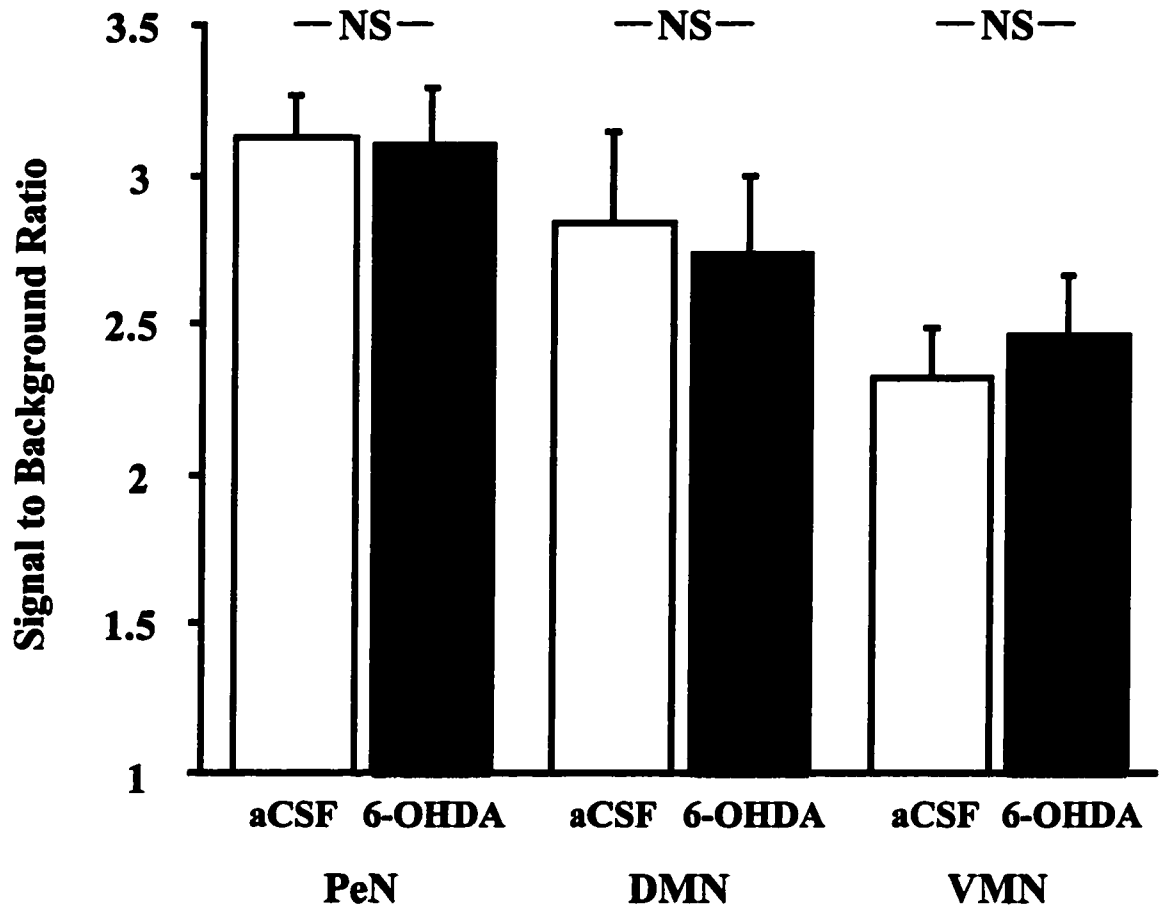


Figure 27: Comparison between WT and GALTG mice of number of NPY mRNA expressing cells (upper panel) and number of grains per cell (lower panel) in the nucleus accumbens. Data are expressed as mean values \pm SEM.

Experiment 17—Figure 27

NPY mRNA

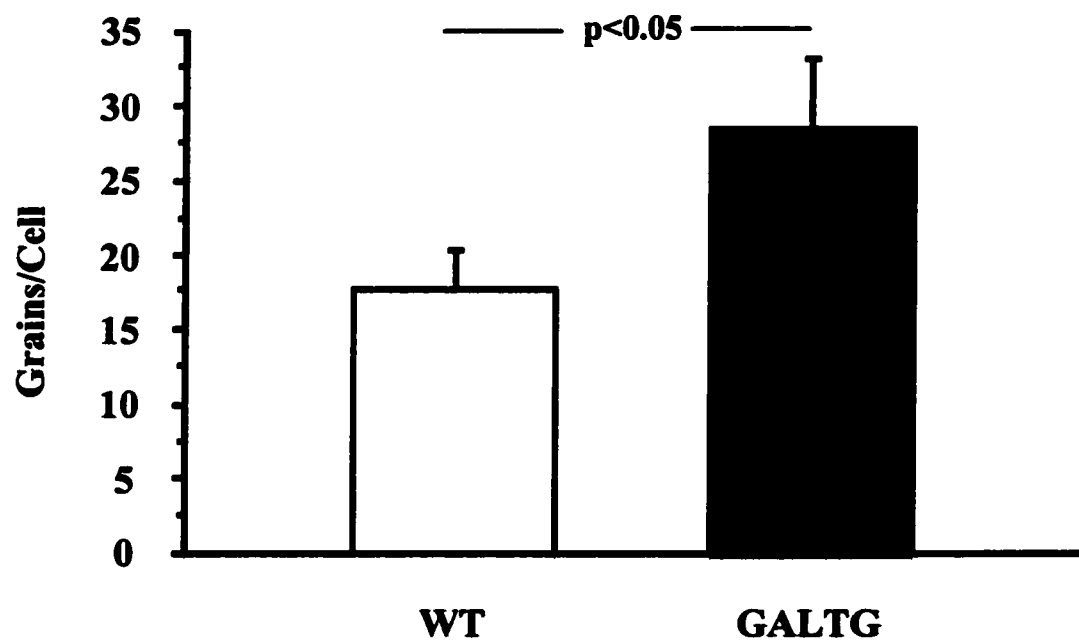
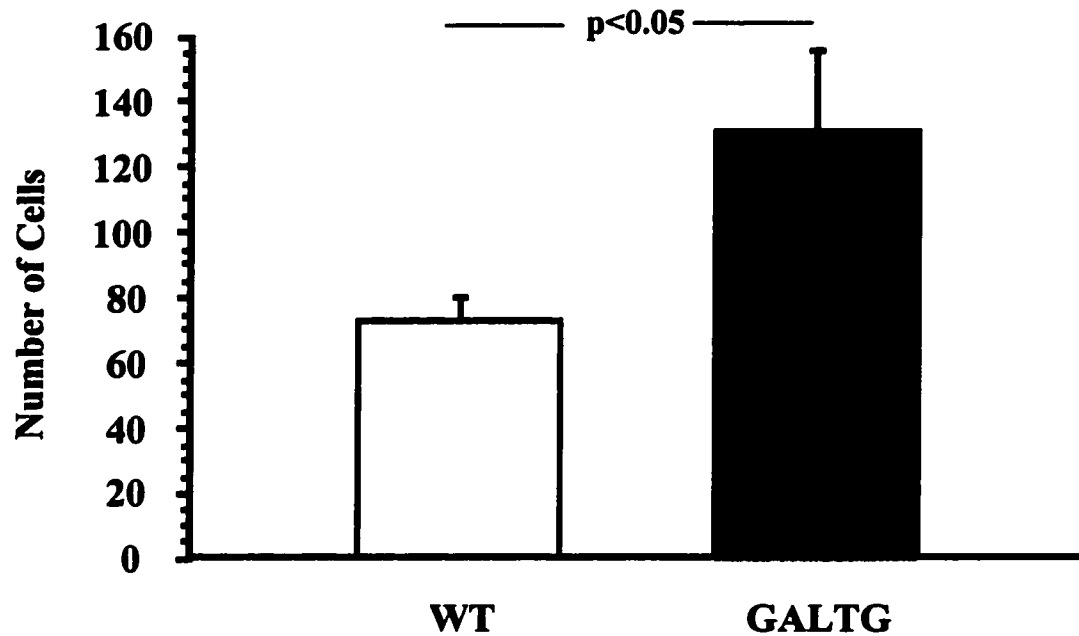
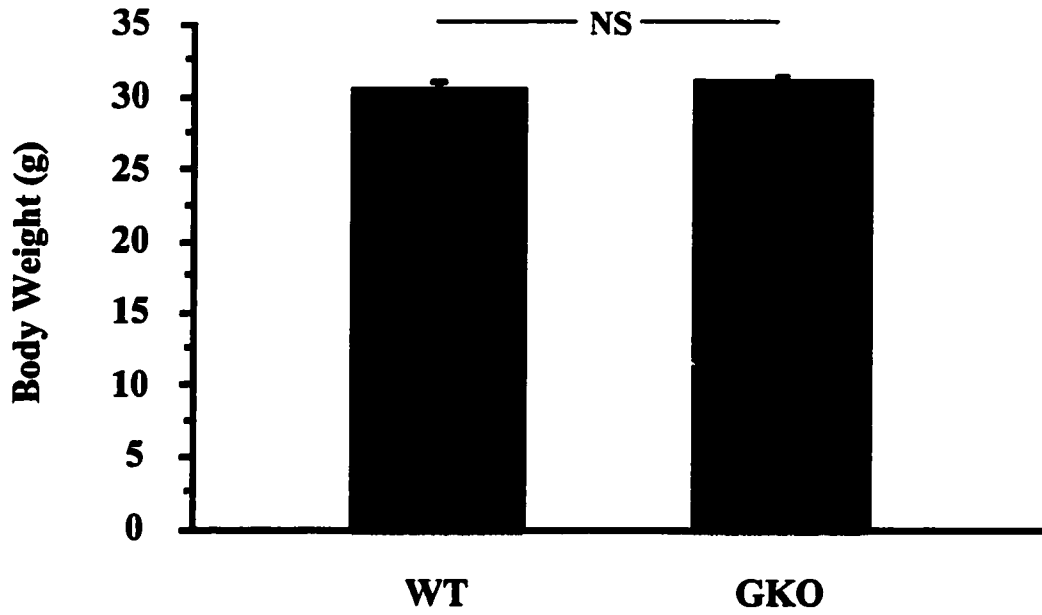


Figure 28: Comparison between WT and GKO mice of adult body weight in males (upper panel) and females (lower panel). Data are expressed as mean weight in grams \pm SEM.

Experiment 18—Figure 28

Adult Male Body Weight



Adult Female Body Weight

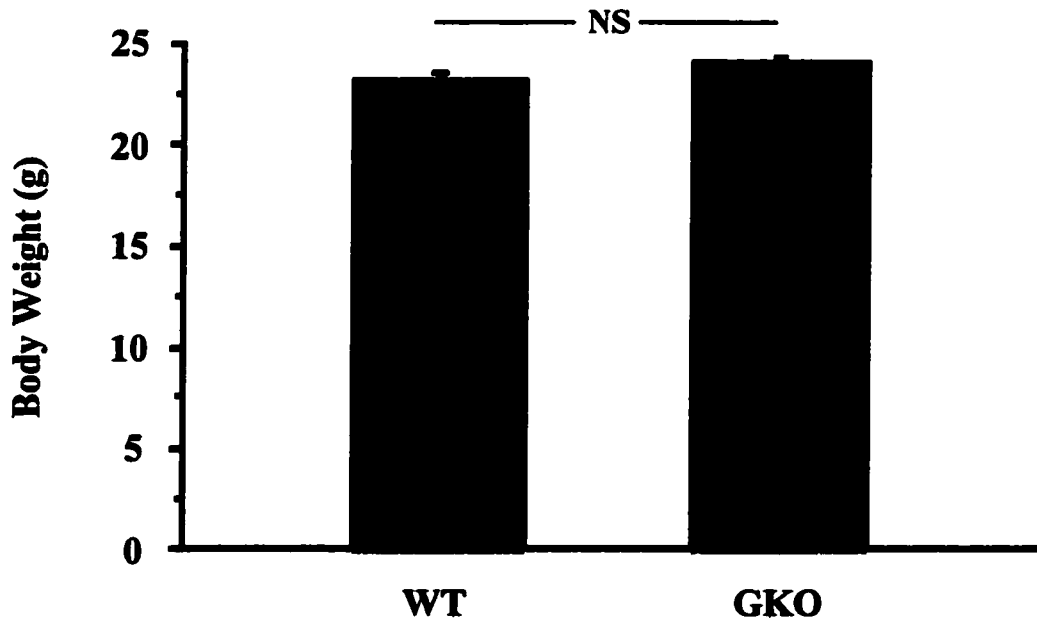
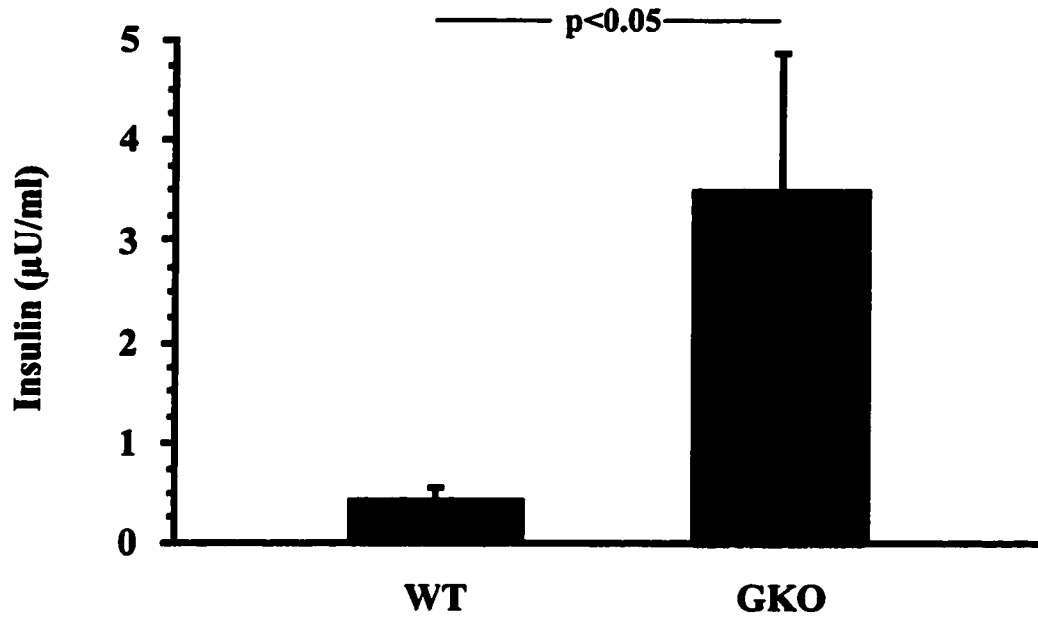


Figure 29: Comparison between male WT and GKO mice of serum insulin (upper panel) and serum glucagon (lower panel). Data are expressed as mean values \pm SEM.

Experiment 19—Figure 29

Insulin in Males



Glucagon in Males

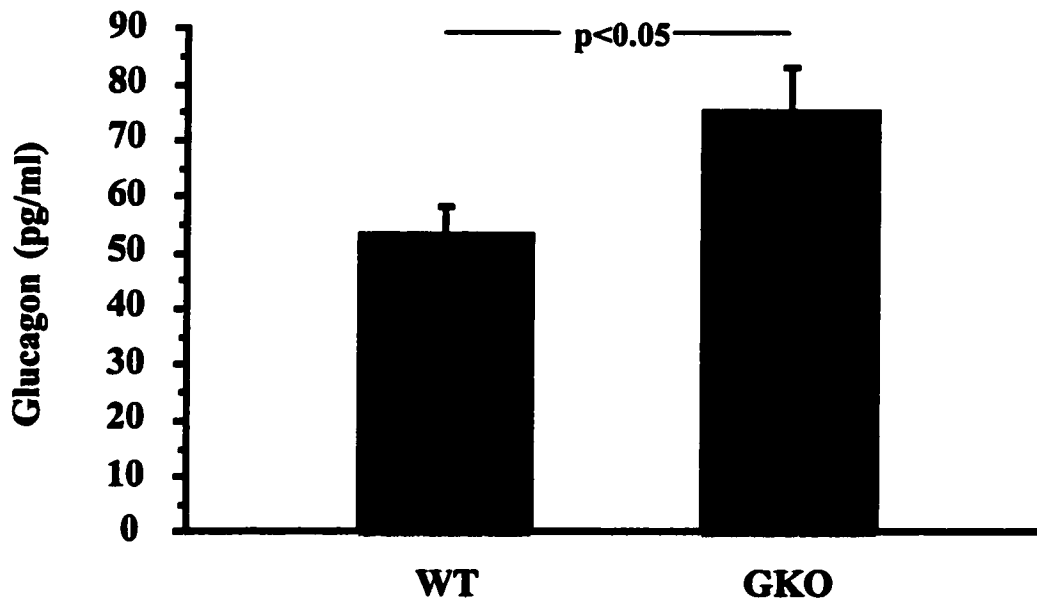
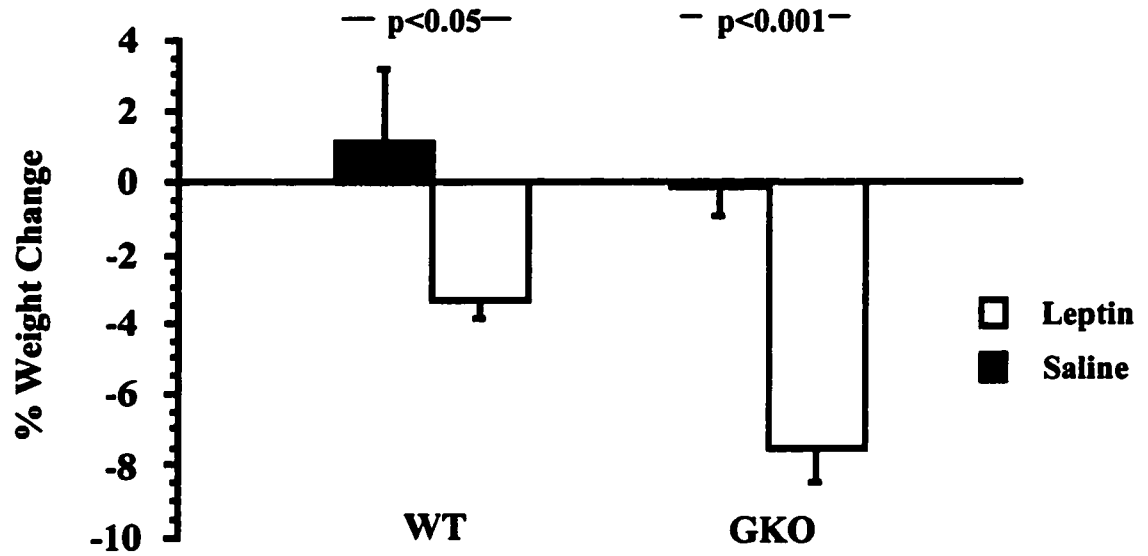


Figure 30: Comparison between WT and GKO mice of percentage body weight change relative to baseline (upper panel) and food intake in grams (lower panel) in response to chronic injections of leptin. Data are expressed as mean values \pm SEM.

Experiment 21—Figure 30

Final Body Weight Change



Mean Daily Food Intake

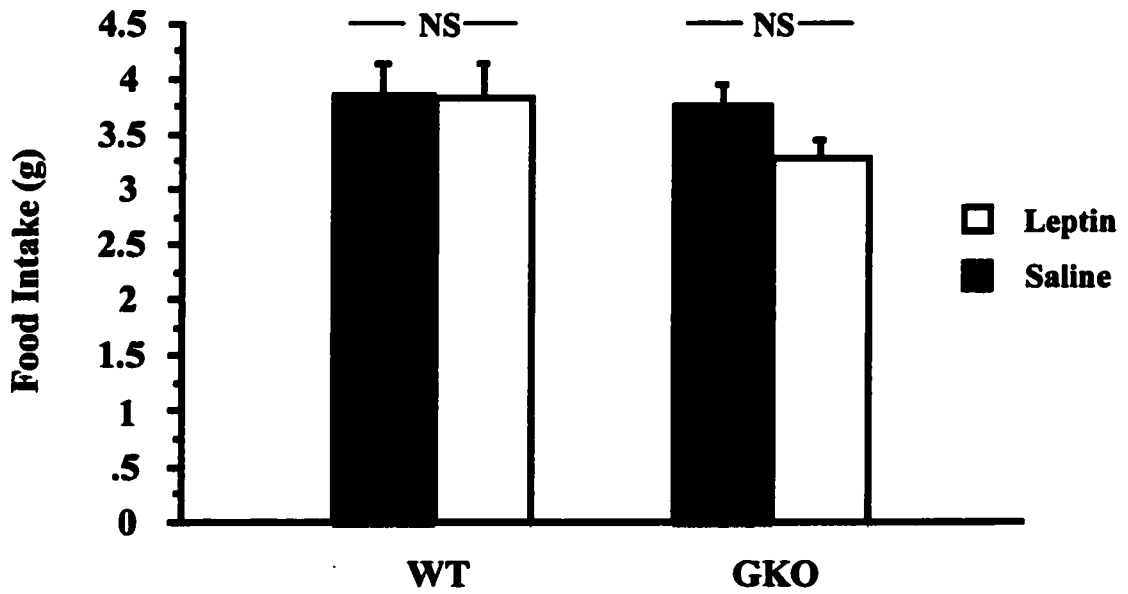
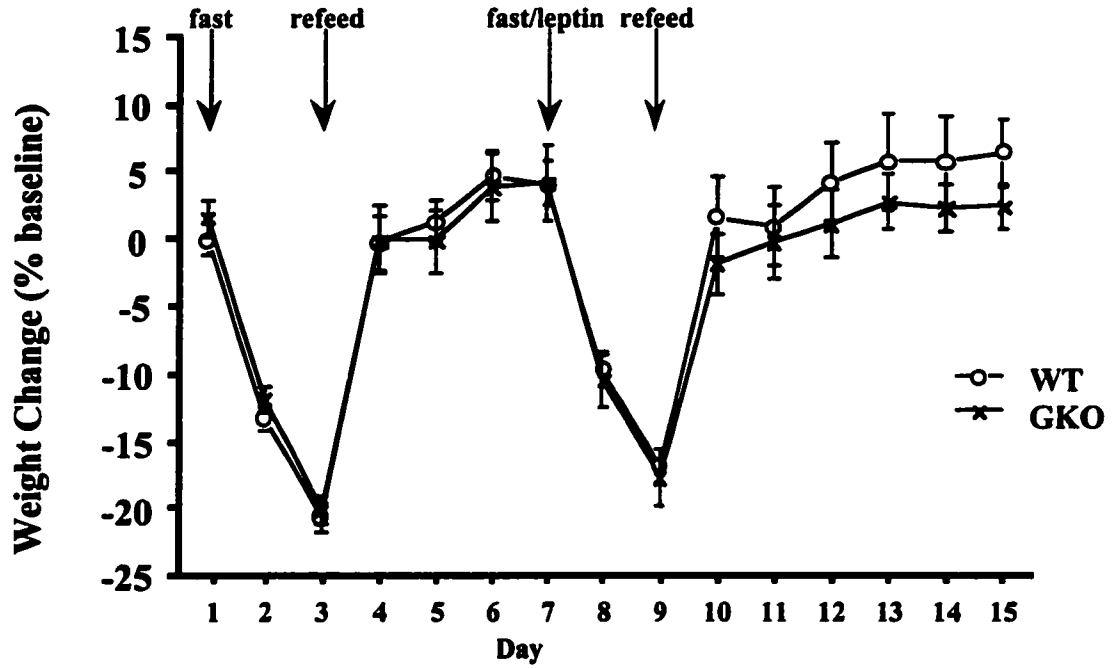


Figure 31: Comparison between WT and GKO mice of percentage body weight change relative to baseline (upper panel) and food intake in grams (lower panel) in response to a 48 hour fast and a 48 hour fast with leptin. Data are expressed as mean values \pm SEM.

Experiment 22—Figure 31

Body Weight



Food Intake

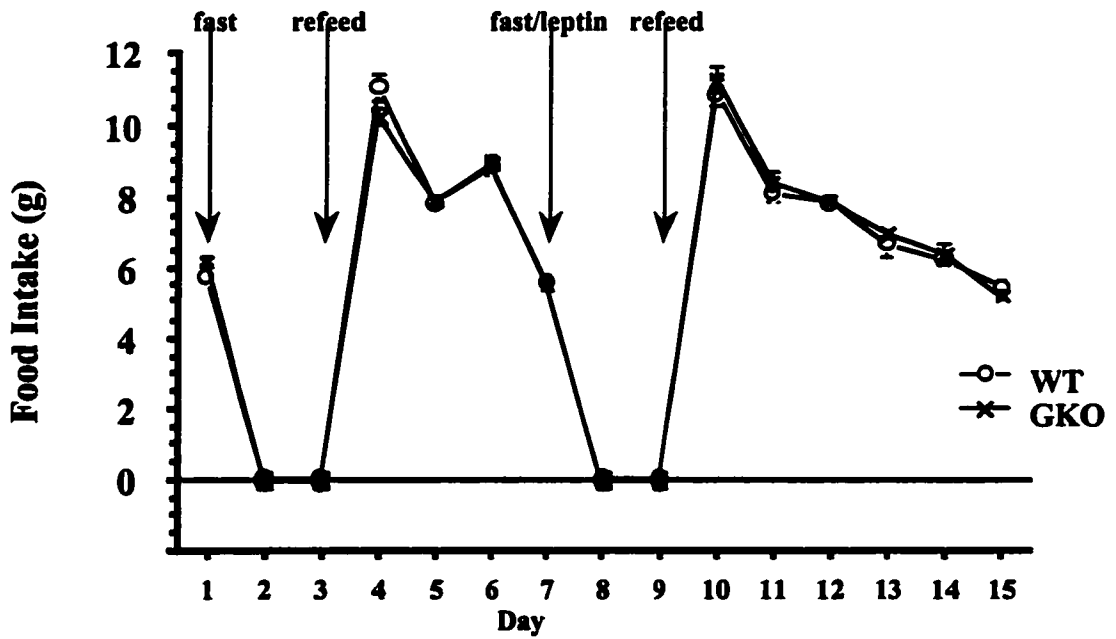
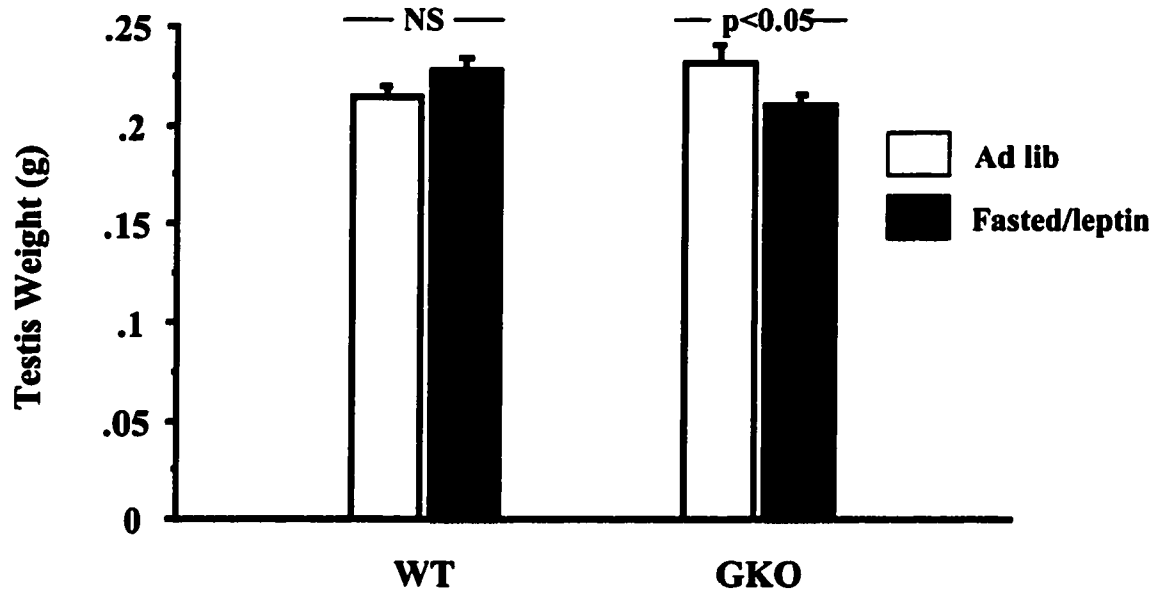


Figure 32: Comparison between ad lib fed and fasted with leptin-treated mice of testis weight (upper panel) and seminal vesicle weight (lower panel) in WT and GKO mice. Data are expressed as mean weights in grams \pm SEM.

Experiment 22—Figure 32

Testis Weight



Seminal Vesicle Weight

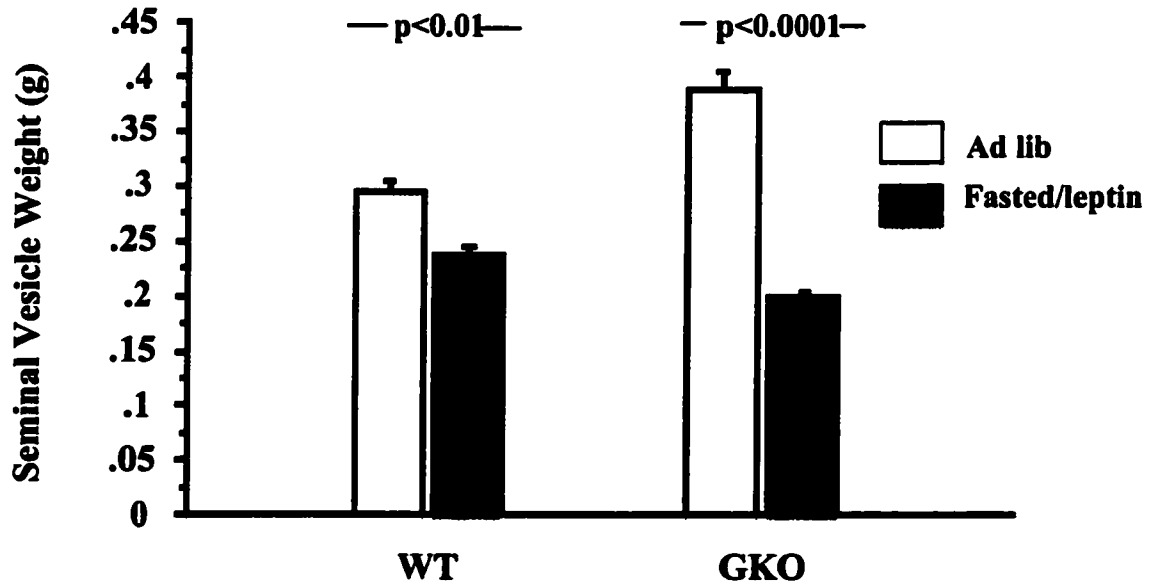


Figure 33: Comparison between fasted and fasted with leptin-treated mice of testosterone (upper panel) and follicle-stimulating hormone (lower panel) in WT and GKO mice. Data are expressed as mean ng/ml \pm SEM.

Experiment 22—Figure 33

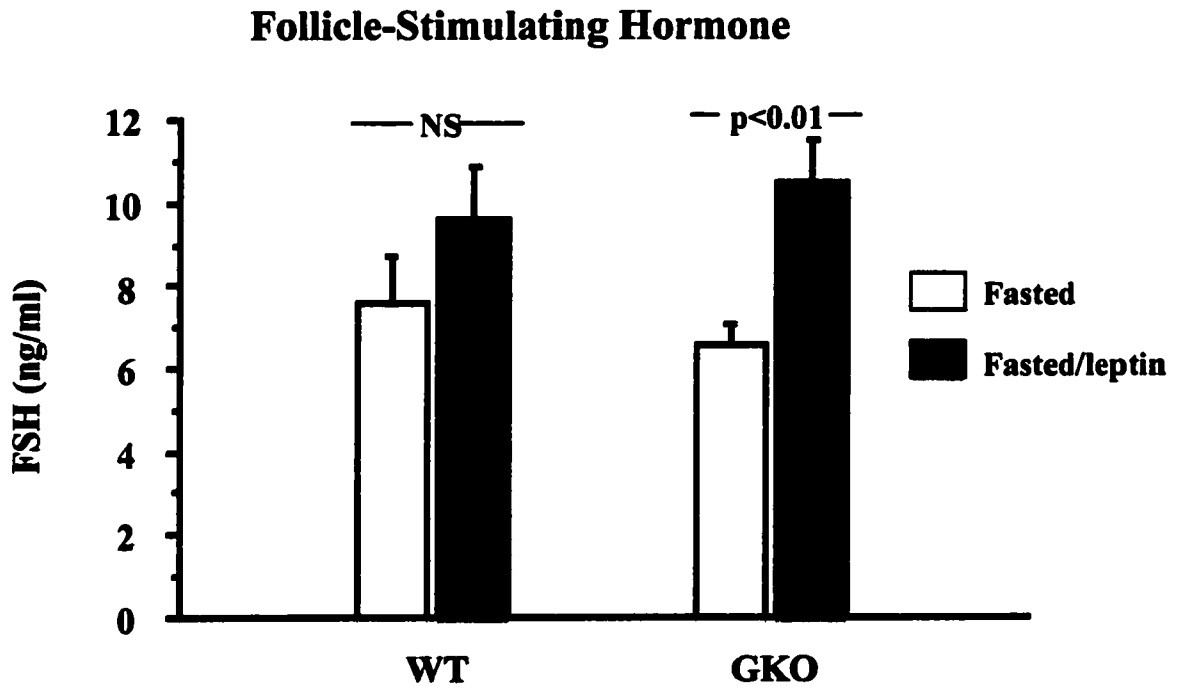
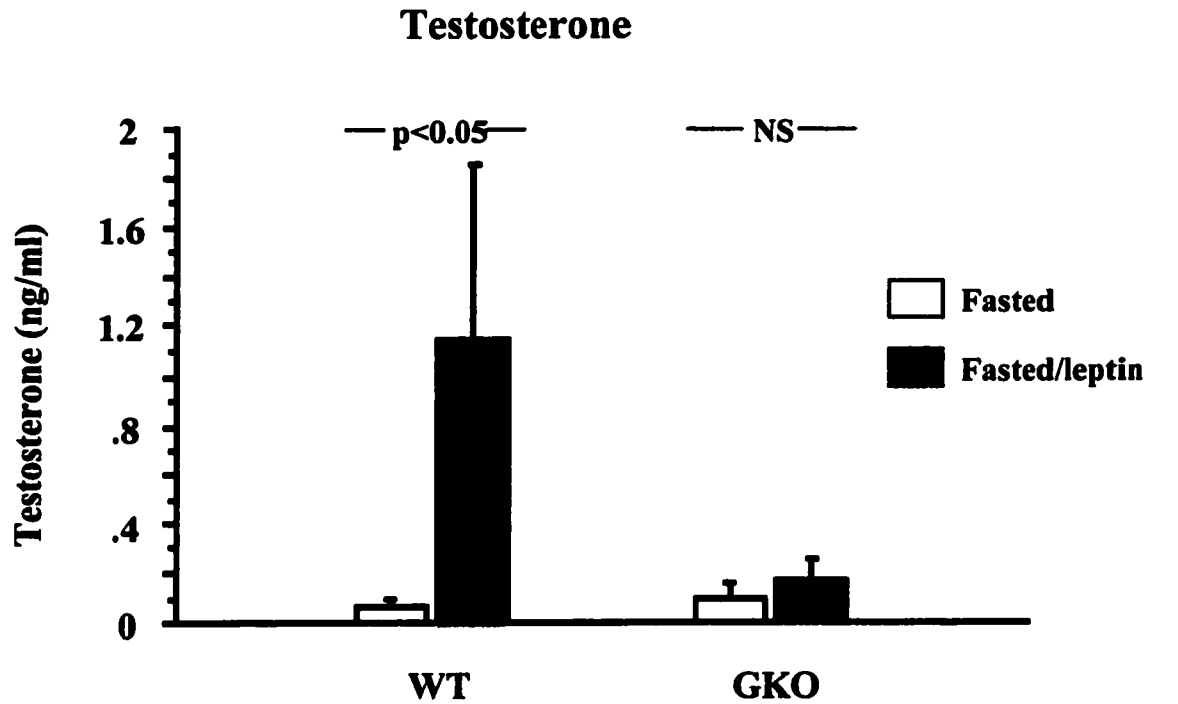
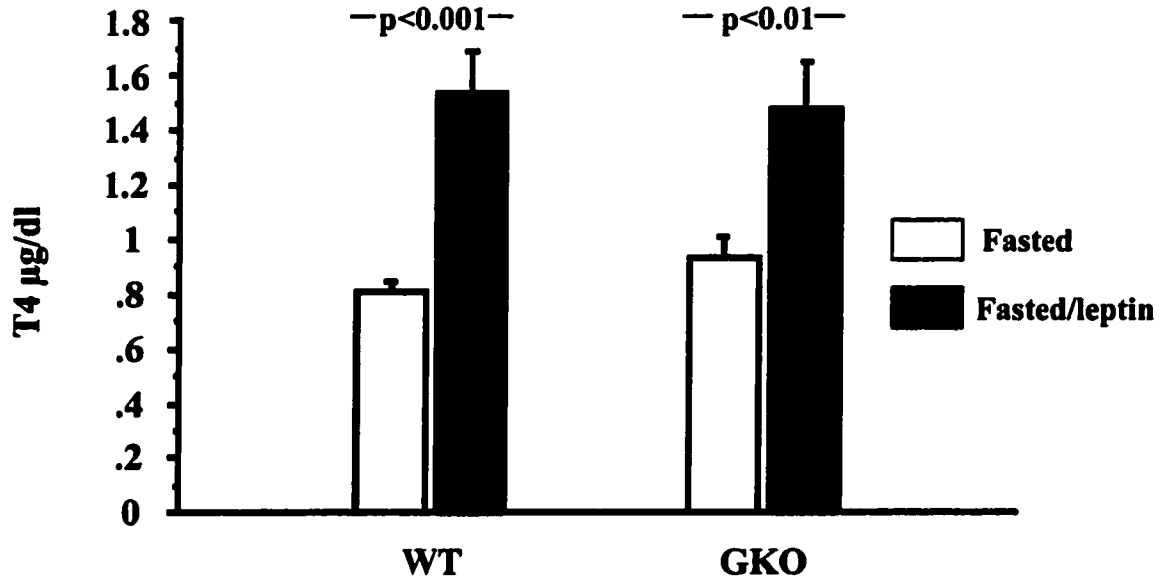


Figure 34: Comparison between fasted and fasted with leptin-treated mice of (upper panel) T4 and corticosterone (lower panel) in WT and GKO mice. Data are expressed as mean $\mu\text{g/dl} \pm \text{SEM}$.

Experiment 22—Figure 34

T4



Corticosterone

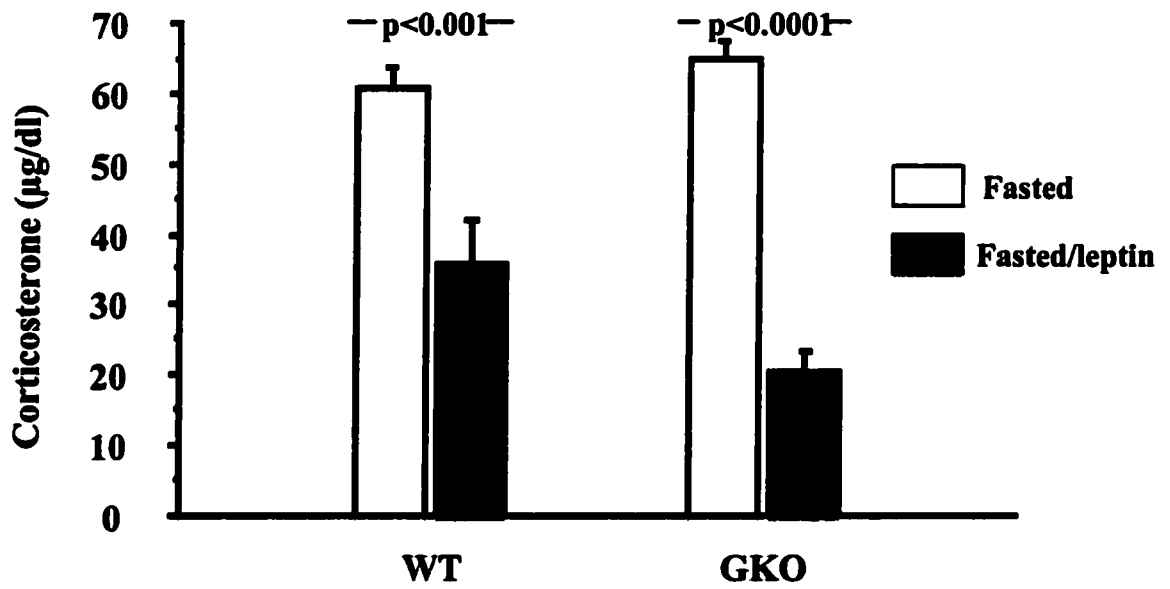
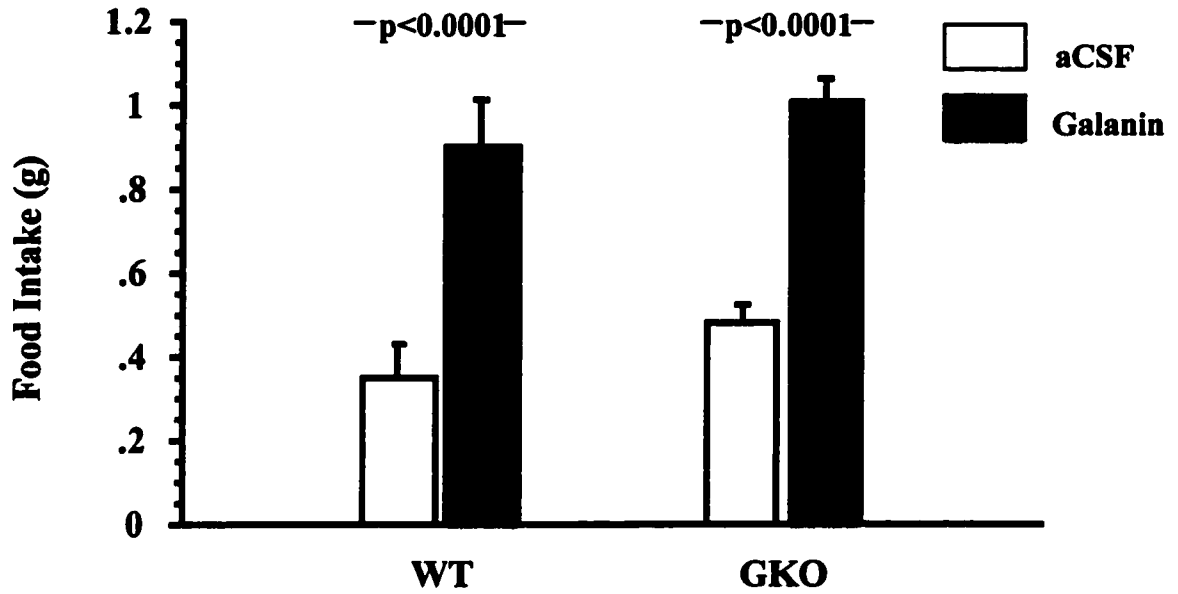


Figure 35: Comparison between aCSF- and NPY-treated mice of food intake in grams in WT and GKO mice (upper panel). Comparison between aCSF- and galanin-treated mice of percentage body weight change relative to baseline in WT and GKO mice (lower panel). Data are expressed as mean value \pm SEM.

Experiment 23—Figure 35

Food Intake 2 Hours After NPY



Mean Body Weight Change After Galanin

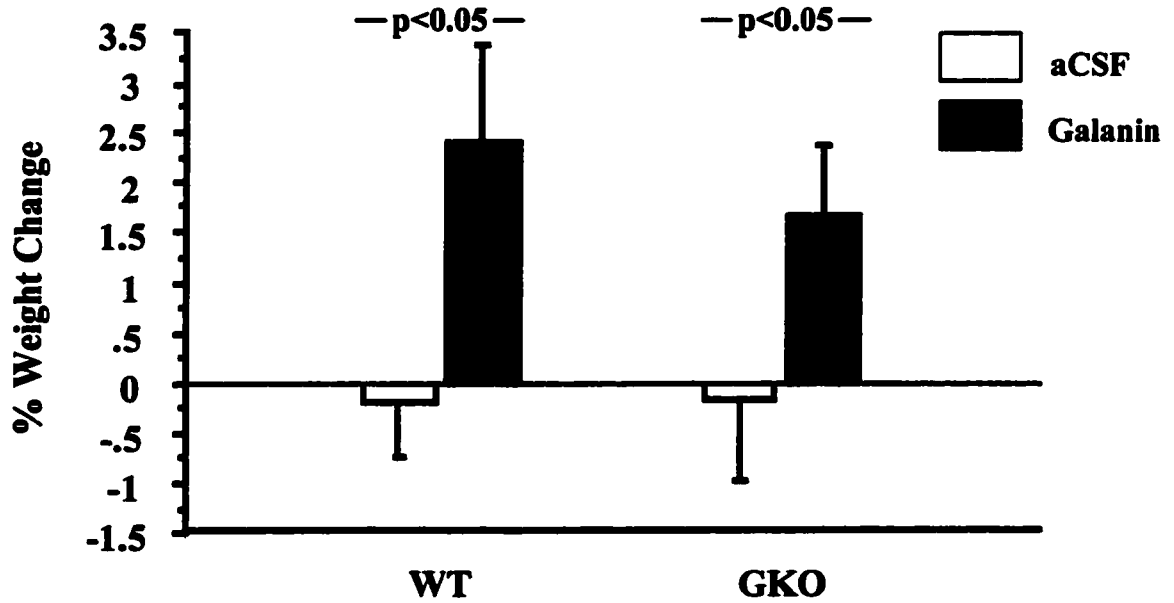


Figure 36: Comparison between WT and GKO mice of galanin-R1 mRNA levels in various regions of the hypothalamus. Data are expressed as mean signal to background ratio of gray levels \pm SEM.

Experiment 24—Figure 36

Galanin R1 mRNA Levels

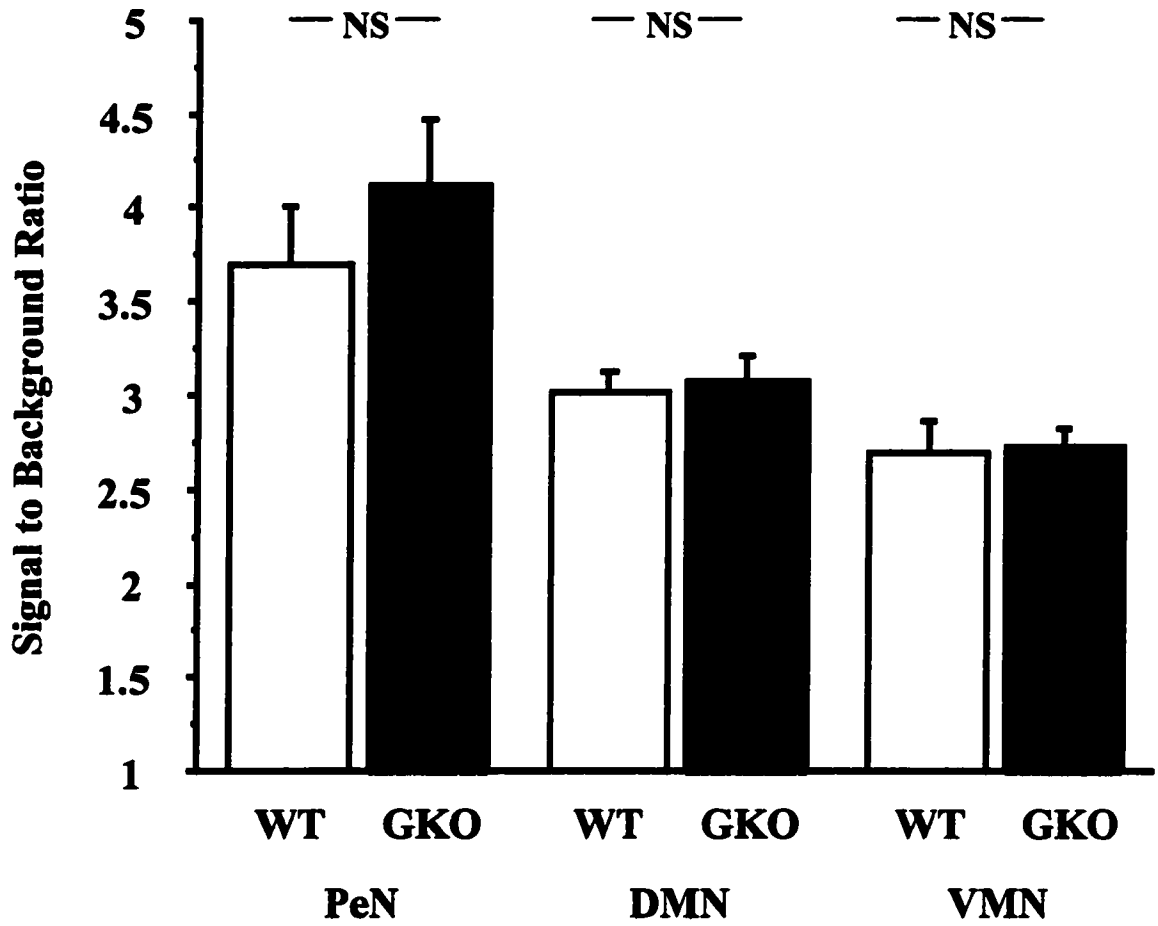


Figure 37: Comparison between WT and GKO mice of number of NPY mRNA expressing cells (upper panel) and number of grains per cell (lower panel) in the nucleus accumbens. Data are expressed as mean values \pm SEM.

Experiment 25—Figure 37

NPY mRNA

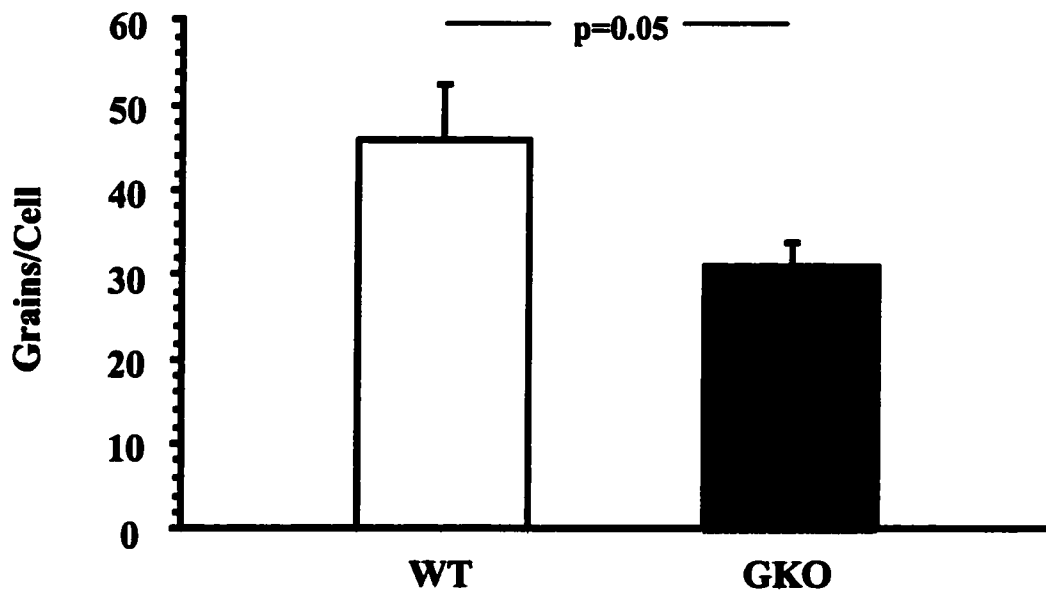
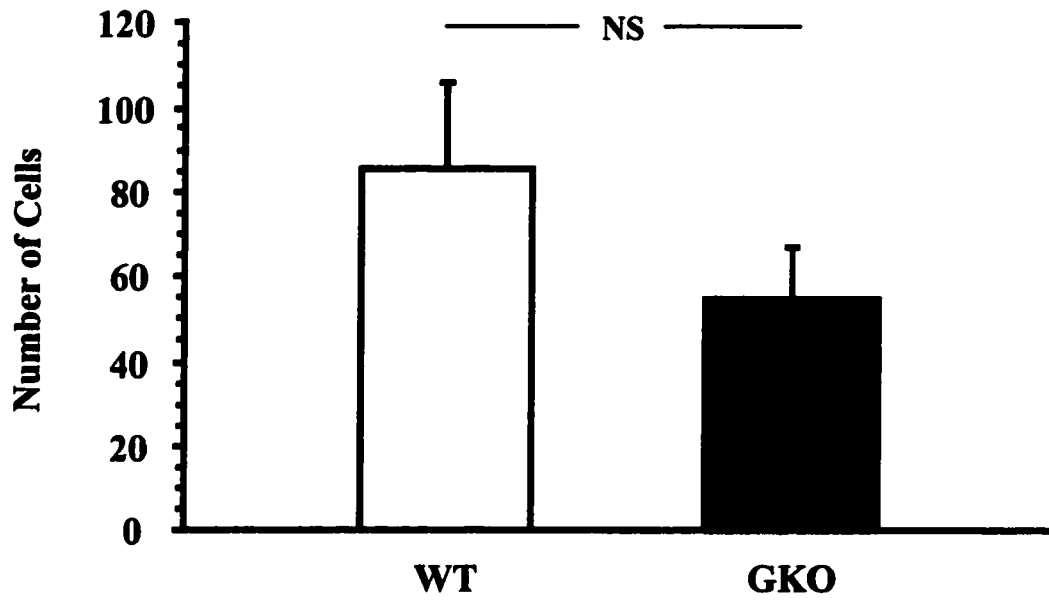


Figure 38: Comparison between WT and DKO mice of body weight in males (upper panel) and females (lower panel). Data are expressed as mean weights in grams \pm SEM.

Experiment 26—Figure 38

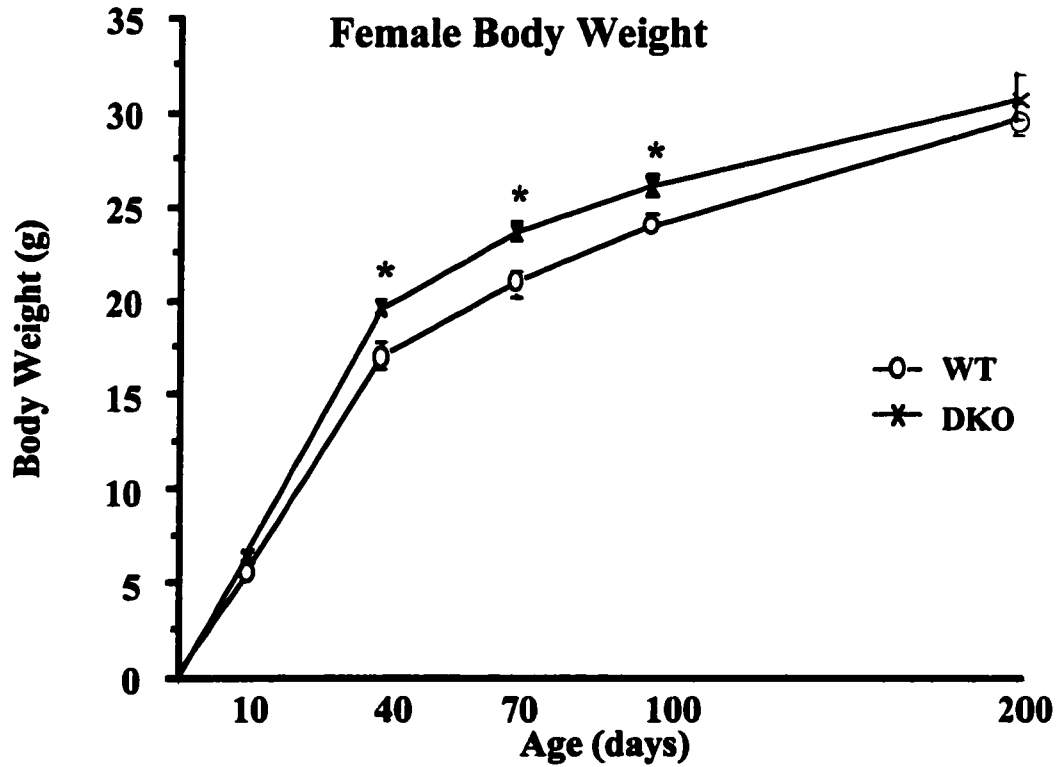
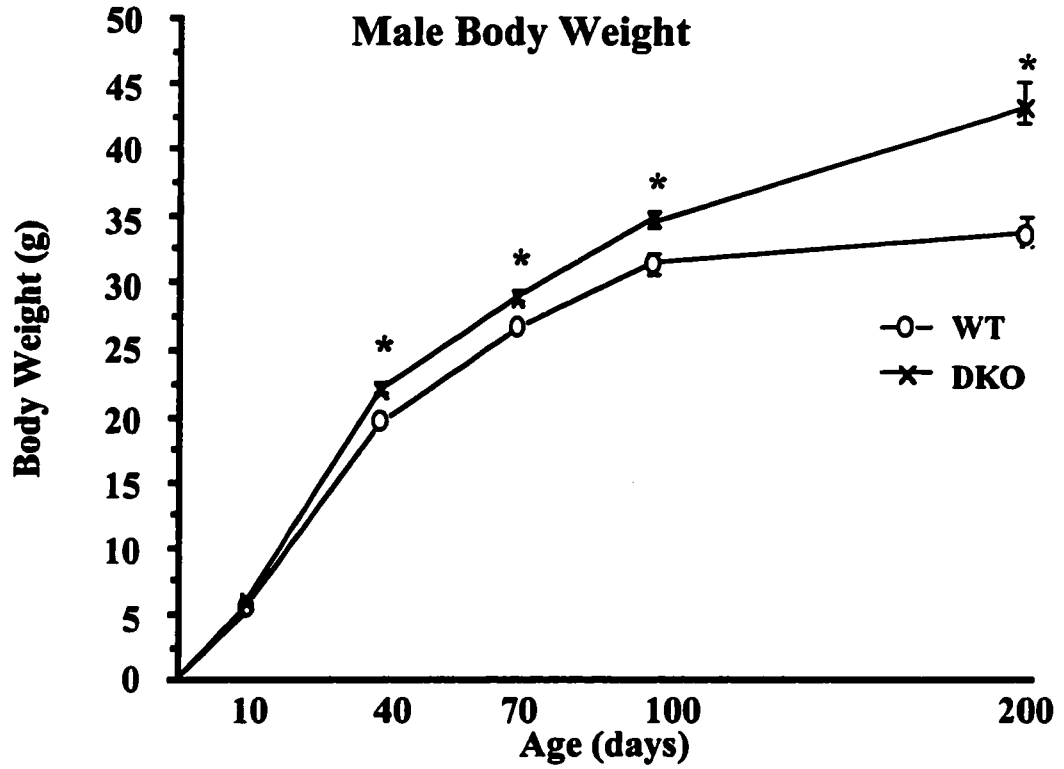


Figure 39: Comparison between WT and DKO mice of dark and light phase food intake in males (upper panel) and females (lower panel). Data are expressed as mean weights in grams \pm SEM.

Experiment 26—Figure 39

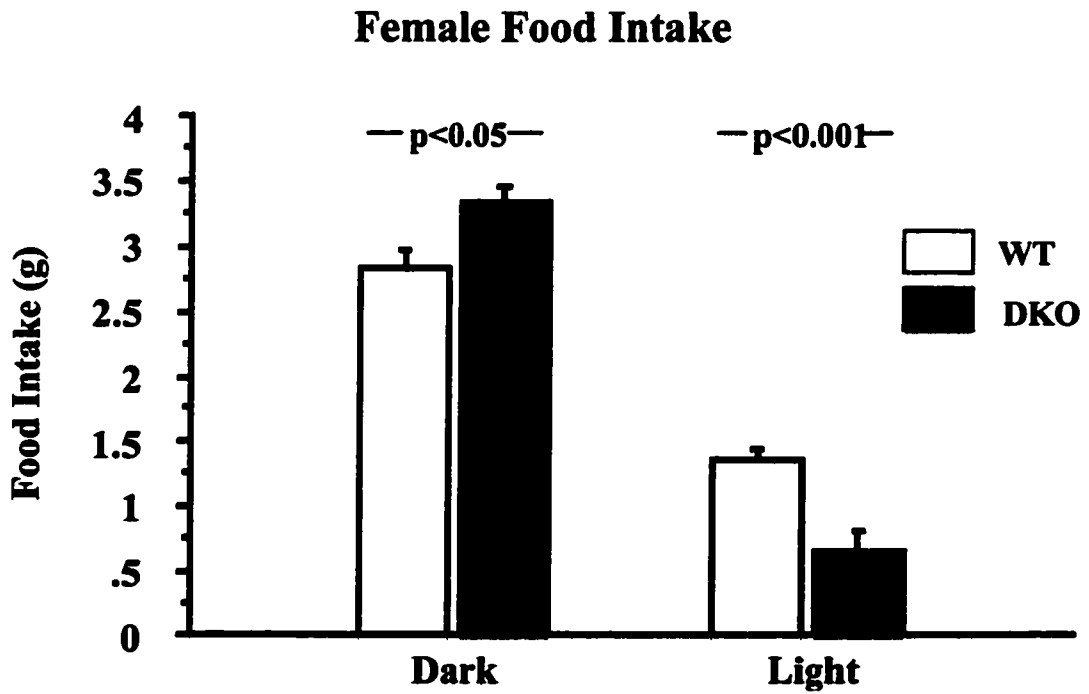
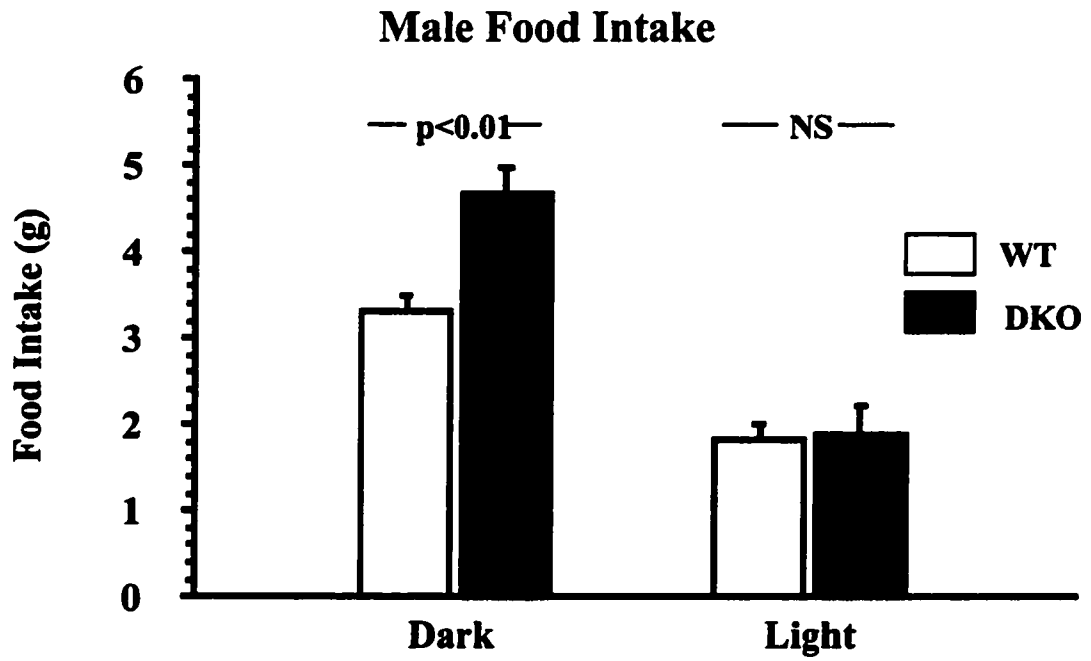
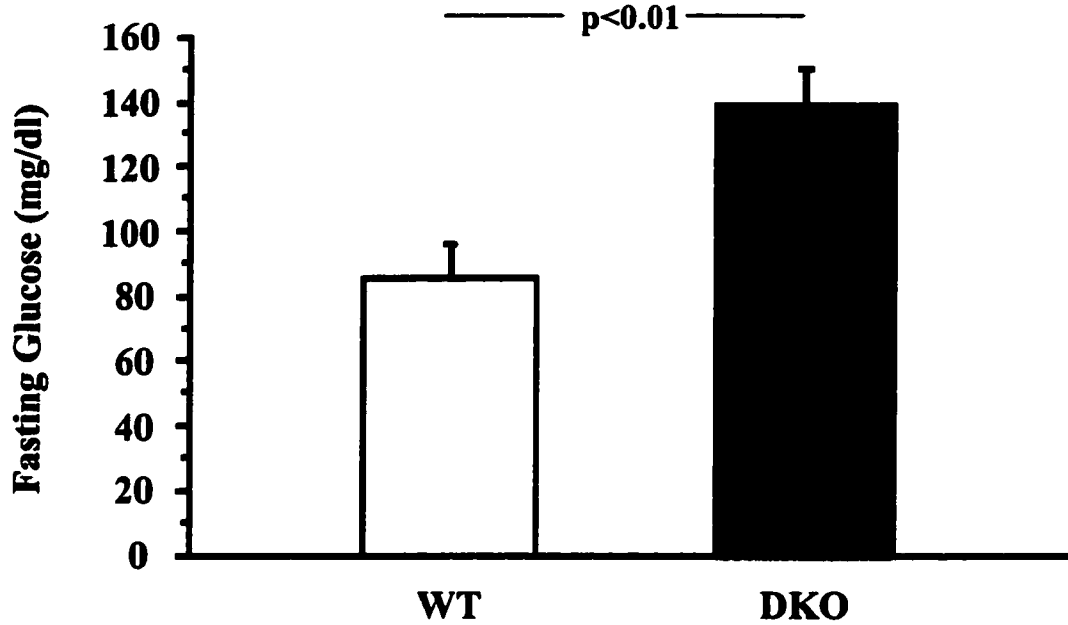


Figure 40: Comparison between male WT and DKO mice of fasting glucose (upper panel) and fasting insulin (lower panel). Data are expressed at mean values \pm SEM.

Experiment 27—Figure 40

Glucose in Males



Insulin in Males

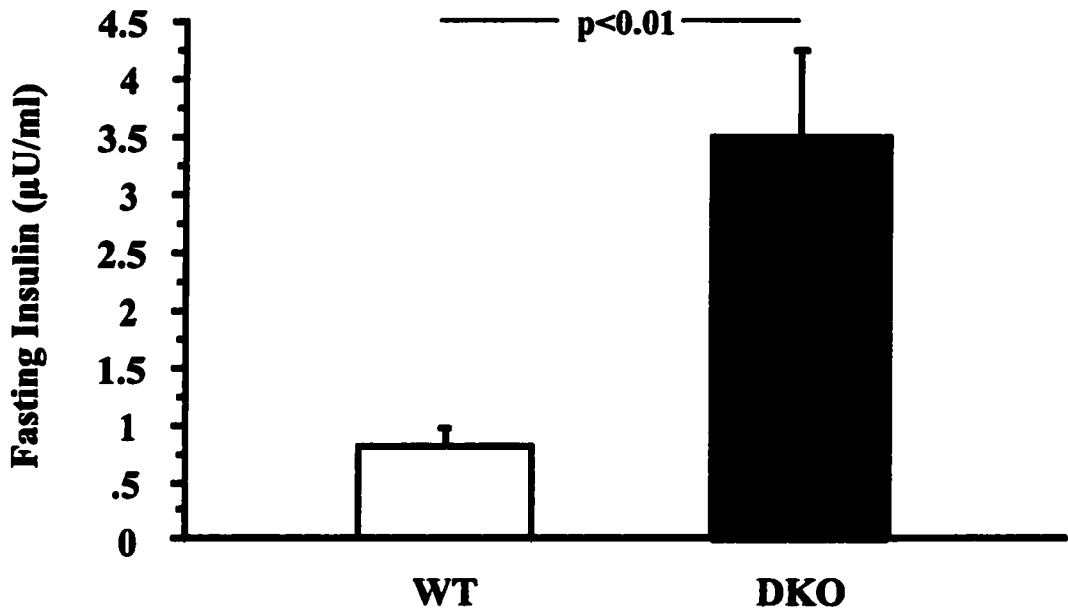
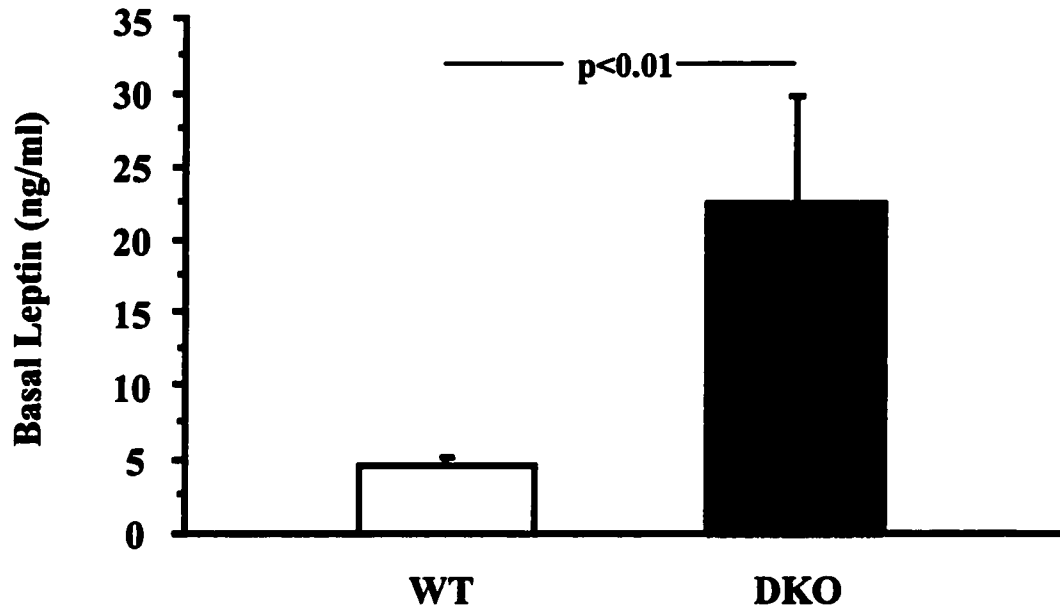


Figure 41: Comparison between WT and DKO mice of serum leptin levels in males (upper panel) and females (lower panel). Data are expressed as mean ng/ml \pm SEM.

Experiment 27—Figure 41

Leptin in Males



Leptin in Females

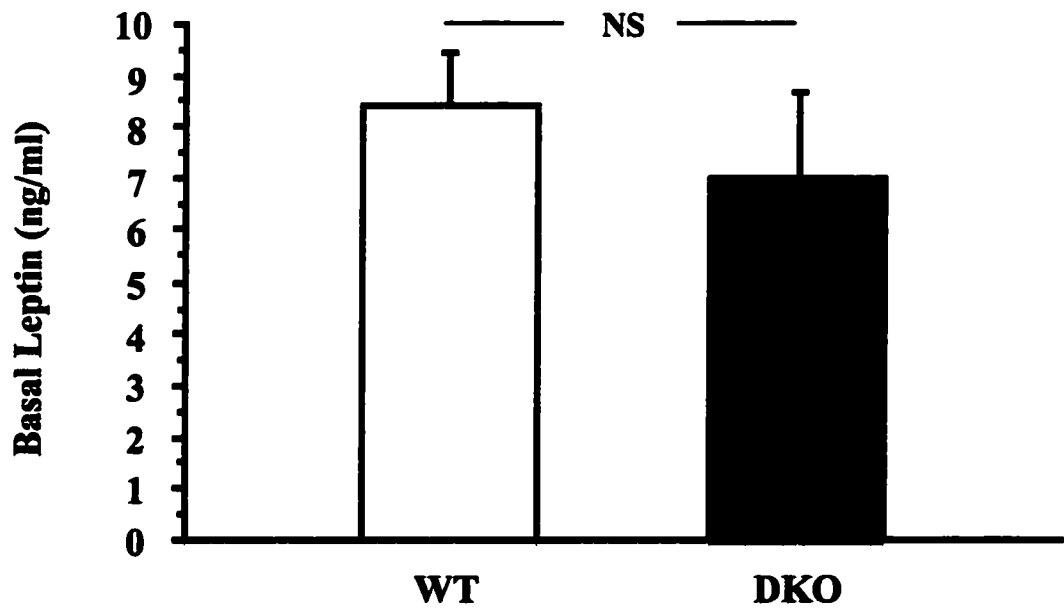
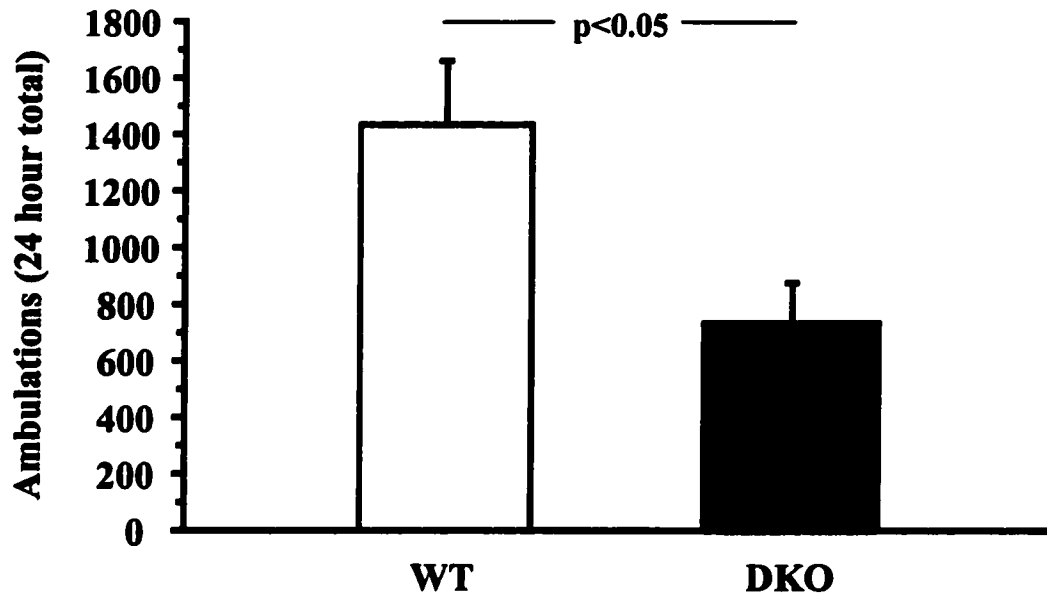


Figure 42: Comparison between WT and DKO mice of activity levels in females (upper panel) and males (lower panel). Data are expressed as mean 24 hour ambulations \pm SEM.

Experiment 28—Figure 42

Ambulations in Females



Ambulations in Males

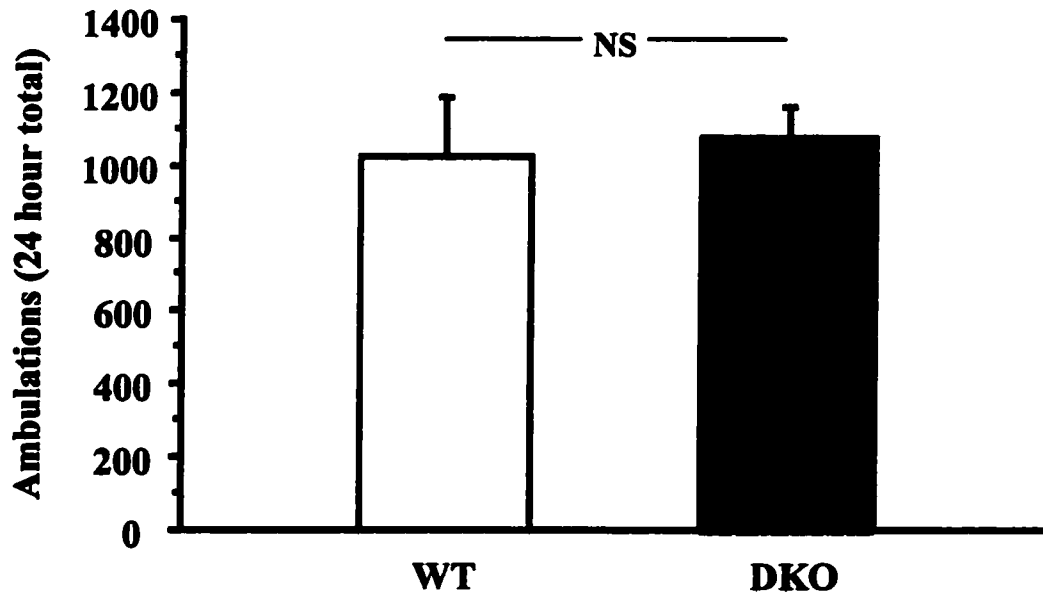


Figure 43: Comparison between WT and DKO mice of body weights in response to both high-fat and low-fat diets. Data are expressed as mean weights in grams \pm SEM.

Experiment 29—Figure 43

Response to High and Low Fat Diet

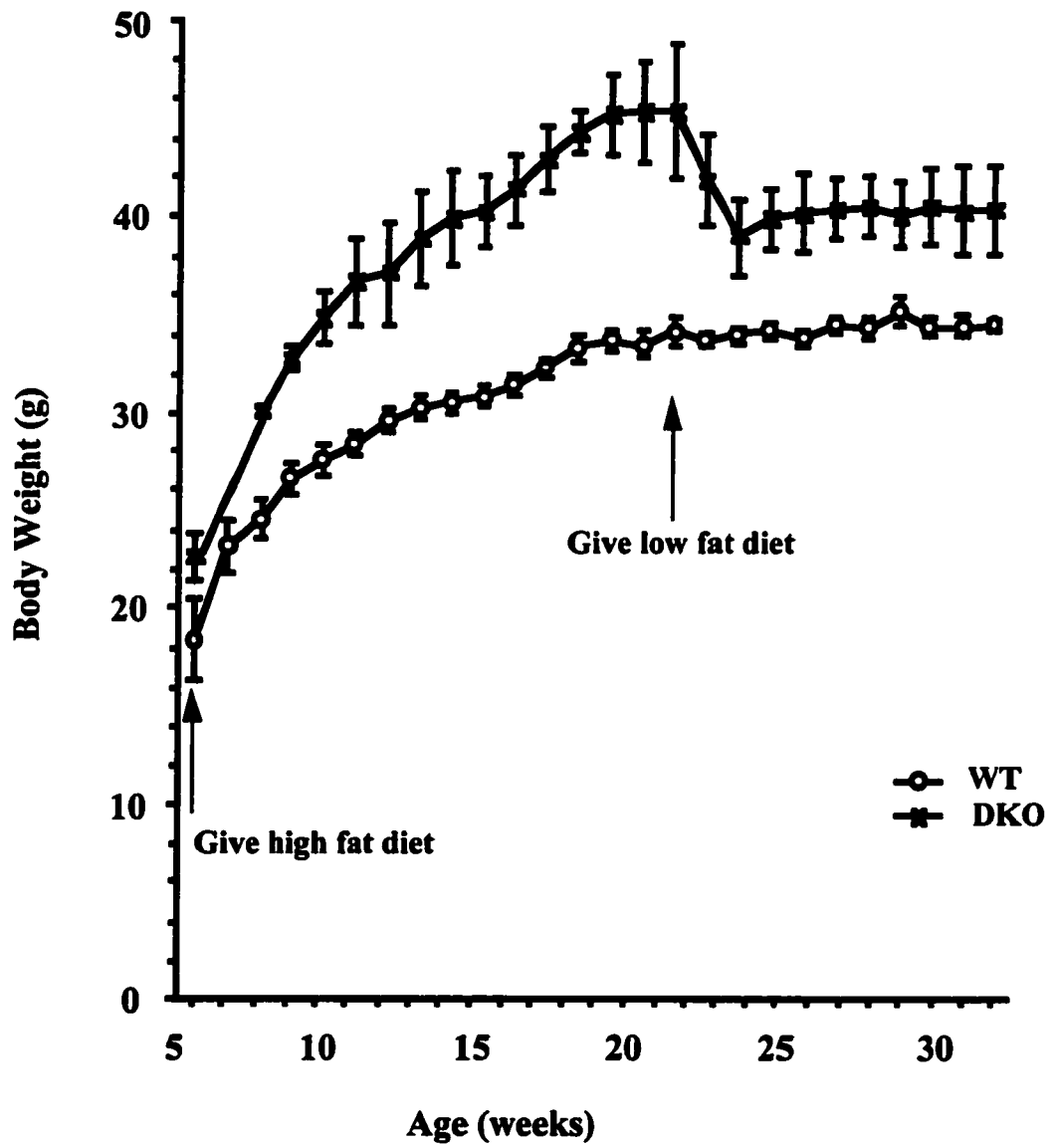


Figure 44: Comparison between WT and DKO mice of food intake in response to both high-fat and low-fat diets. Data are expressed as daily kilocalories for each genotype.

Experiment 29—Figure 44

Response to High and Low Fat Diet

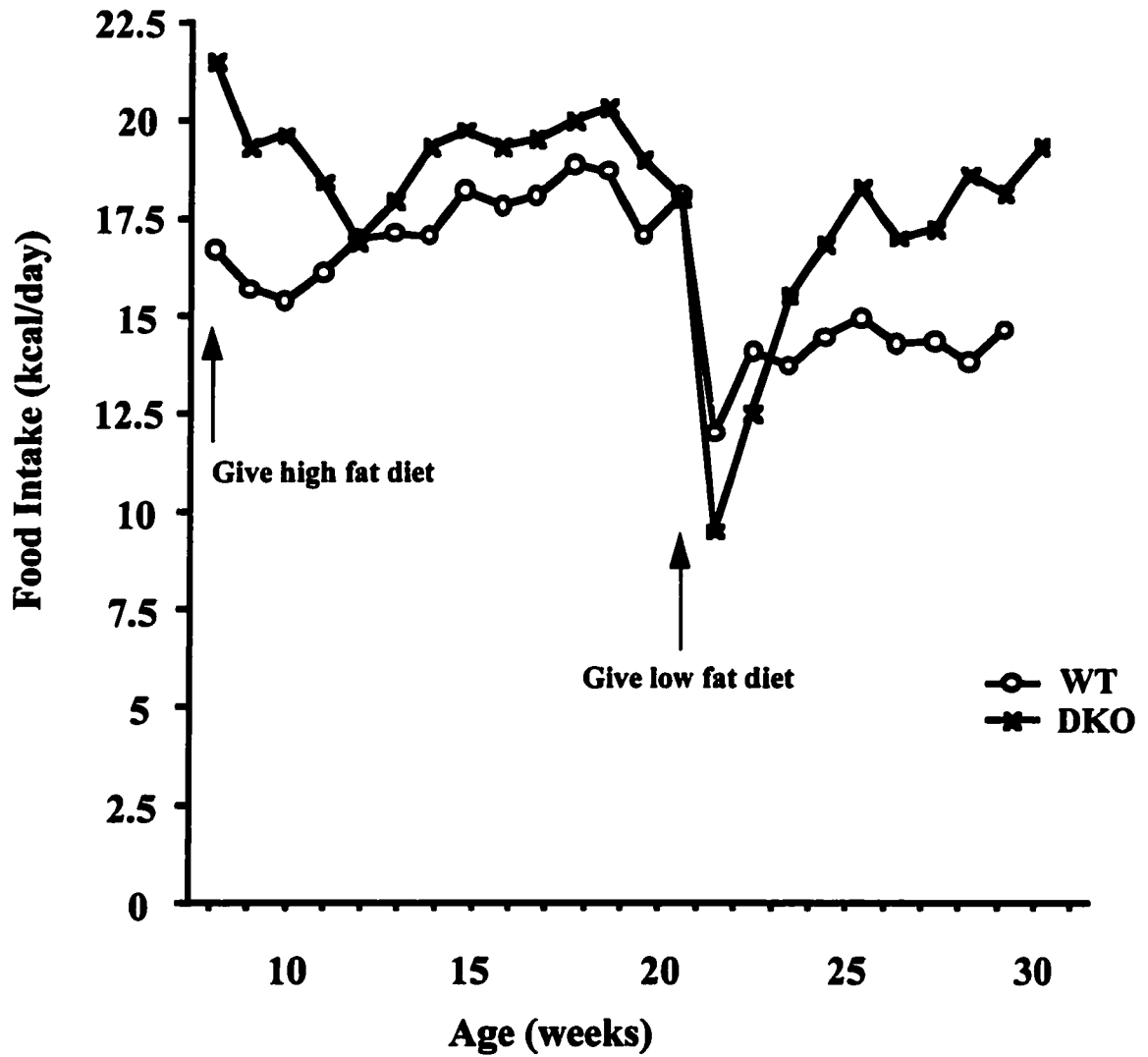
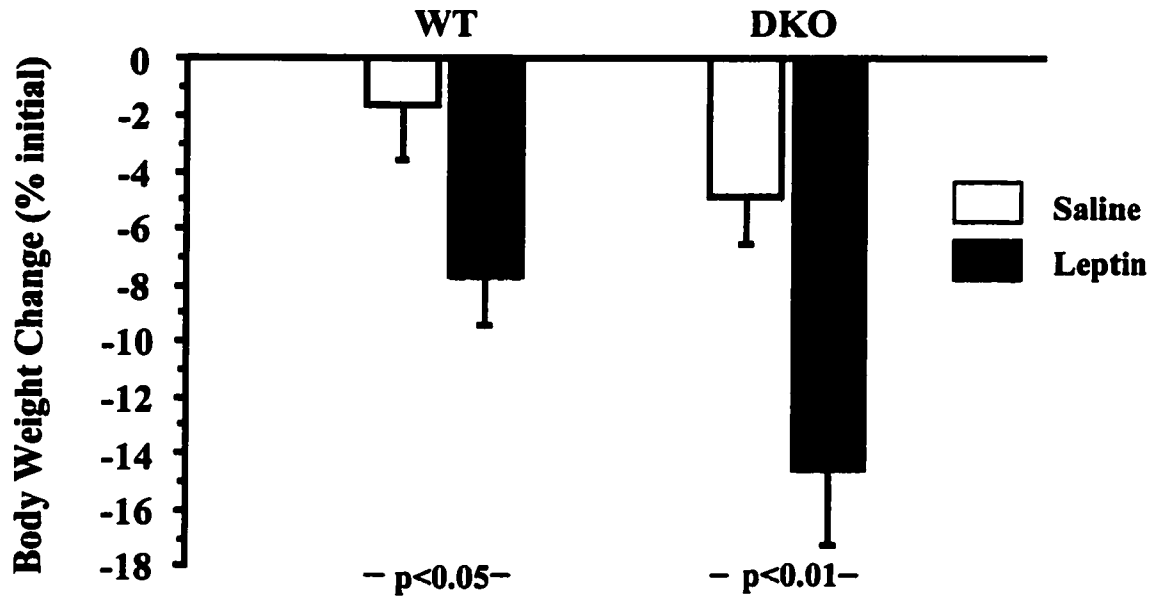


Figure 45: Comparison between saline- and leptin-treatments of percentage body weight change relative to baseline (upper panel) and percentage fat pad weight (lower panel) in male WT and DKO mice. Data are expressed as mean values \pm SEM.

Experiment 30—Figure 45

Body Weight Change



Fat Pads

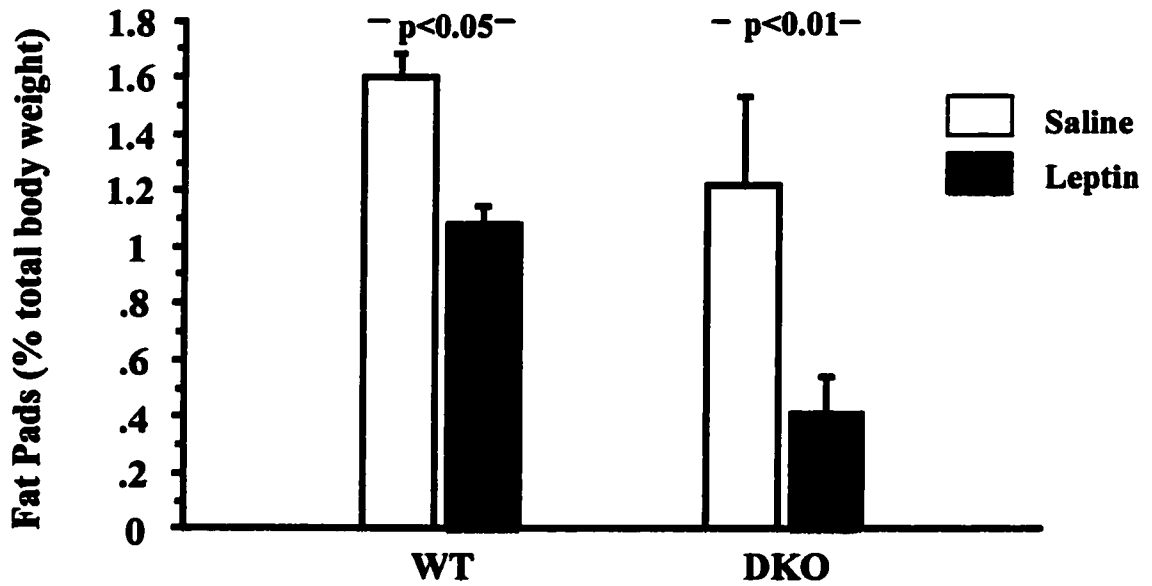
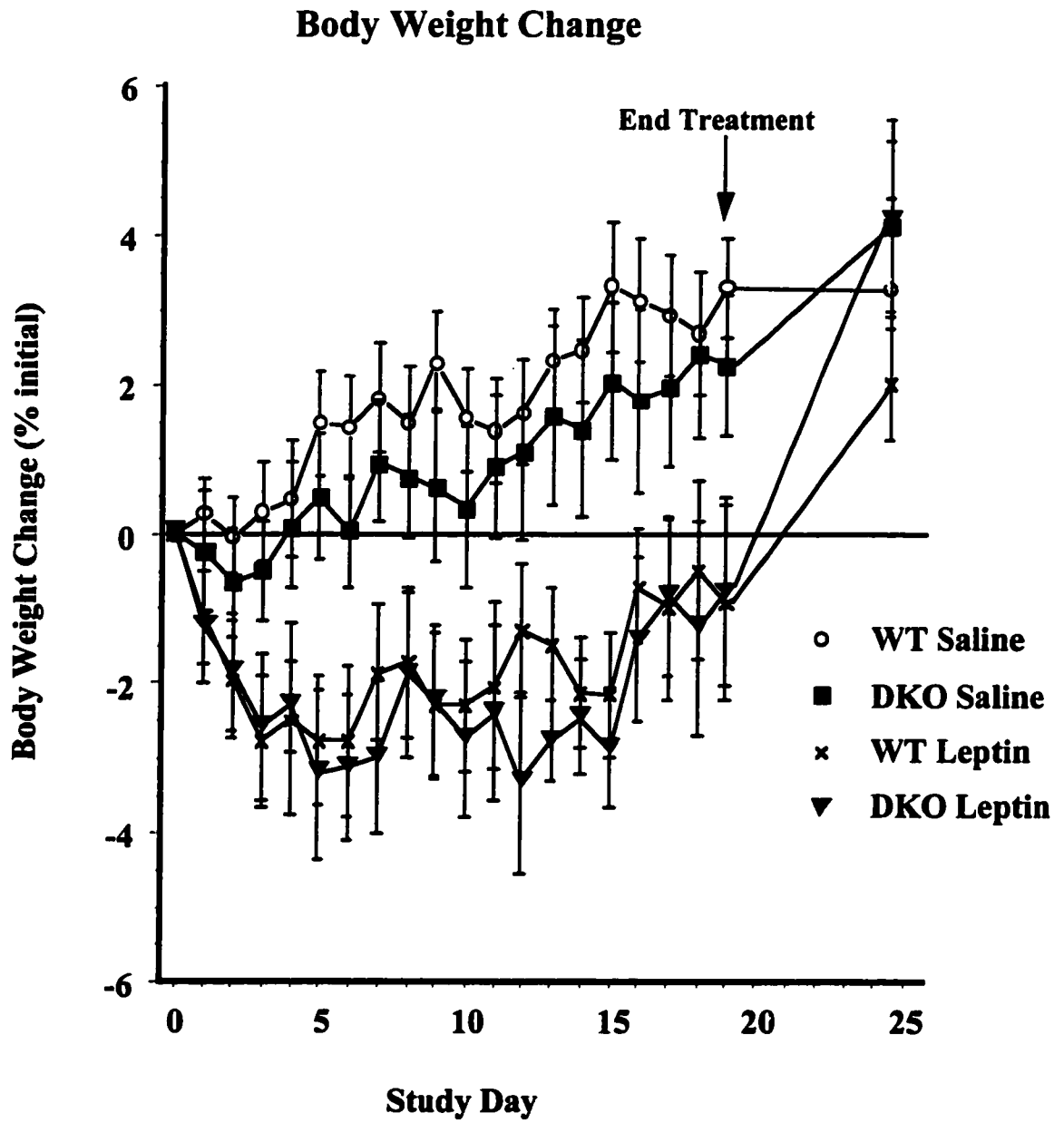


Figure 46: Comparison between saline- and leptin-treatments of percentage body weight change over time in WT and DKO mice. Data are expressed at mean percentage \pm SEM.

Experiment 31—Figure 46



Chapter 4

Discussion

I. The Use of Different Mouse Models to Study the Role of Galanin in the Brain.

A. General Considerations

In my investigation into the functions of galanin in the brain, I used several different mouse models. One important consideration when comparing mice either lacking or overproducing galanin is the appropriate choice of animal type. All experiments with GALTG mice were performed with animals — originally a mix of DBA x C57BL6—that were back-crossed into the C57BL6J strain for at least 6 generations. This strain of mice was chosen for its robust breeding characteristics and the lack of peculiar learning and memory phenotypes (Crawley, et al., 1997). Because my interests were focused on elucidating the physiology of galanin in both neuroendocrine and cognitive functions, I chose the C57BL6J strain as my research model. To minimize genetic variability, all WT and/or saline-treated control animals were litter- or age-matched mice of the same strain.

Although limiting the differences between GALTG and WT mice to the gene of interest (and flanking regions) may help to control for genetic variability between *genotypes*, it does not take into account possible differences in behaviors and neurochemistry between different *strains* of mice. Because many behaviors are strain dependent, the question always remains whether one is truly studying the effects of

alterations in the gene of interest (i.e. galanin) rather than some combination of background modifier genes peculiar to the C57BL6 strain (Crawley, et al., 1997). An ideal situation might be one in which two (or more) strains of mice are maintained, both carrying the same transgene, with each mutant being compared to its respective WT control. Unfortunately, the benefits gained from this approach may not be worth the logistical challenges and costs associated with this strategy. If a robust phenotype were to be discovered, this might necessitate building a stronger proof for the argument that the transgene caused the phenotype. In this case it would be wise to confirm a particular observation in another line of transgenic mice. As the GALTG mice had no clear neuroendocrine phenotype, this may not be an issue here.

As far as neurochemistry is concerned, I have found essentially the same pattern of galanin gene expression patterns in both mixed strain and “pure strain” mouse brains, as have others (Mathieson, et al., 2000). The essential differences here are between *genders*, as expression of galanin is highly sexually dimorphic (Delemarre-van de Waal, et al., 1994; Liposits, et al., 1995; Planas, et al., 1995; Rajendren, et al., 2000). For this reason, all neurochemical comparisons made in these studies employ mutant and control mice of the same gender.

I used a different approach for the generation and maintenance of the GKO and DKO mutants, for several reasons. The original founder mice for the GKO line were on the 129/OLA background, which were crossed with NPYKO mice from a mixed C57BL6J x 129SV colony. The resultant compound heterozygotes were bred together to produce mutants where both the NPY and galanin genes were ablated. Since only 1 in 16 of the progeny of this breeding strategy would have been expected to be either DKO or

WT mice, the expense and time required to pursue this strategy was prohibitive.

Therefore, DKO progeny from several different sets of founder parental sets were crossed together to create a DKO colony, and this same strategy was used to produce a WT control colony. While admittedly not an ideal approach, producing DKO animals from several different breeding sets of founders helped to ensure that no single suite of founder genes predominated in either the DKO or WT colonies

One consequence of using the compound heterozygote approach to create double mutants is the production of single mutants at the same ratio as the doubles. The GKO animals were produced in this way, with the not inconsiderable logistical advantage of being able to use the same colony of WT mice as controls for both GKO and DKO mutants. This approach also would have allowed the direct comparison between DKO mice and single mutants of either galanin or NPY, although this was not the specific objective of my research.

I also developed a “pure” strain line of both GKO and DKO mice, to assess whether the phenotypes revealed on the mixed lines could be confirmed in a separate strain with its own complement of background genes. However, it should be noted that although both GKO and NPYKO founder parental sets were from the 129 strain, they were derived from different sub-strains of 129 ES cells. GKO mice originated from the 129/OLA line and NPYKO mice from the 129SV line. Significant differences exist between these two sub-strains in both genetic complement and behavioral phenotypes (Threadgill, et al., 1997). For this reason, the 129 colony of GKO and DKO mice would more accurately be described as a “semi-hybrid” line, with the some of the attendant genetic variability issues that exist with other mixed-strain mice.

Proponents of both pure-strain and mixed strain approaches to phenotypic analysis of mutations have valid arguments for the advantages and disadvantages of employing either method (Doetschman, 1999; Gerlai, 1996). As stated above, some inbred strains of mice (including most 129 lines) are notoriously poor performers on task of spatial learning and memory, making them less suitable for studies of this nature. However, the undeniable advantage of confining the differences between genotypes to the mutation of interest cannot be denied, favoring the pure strain approach. On the other hand, mixed strain mice have at least one distinct advantage, particularly for behavioral analyses. If sufficient numbers of mice are examined for a specific phenotypic trait, and these mice come from a range of parents with different proportions of strain identity, one could reasonably infer that a consistent phenotype is the result of the mutation in the gene of interest (galanin and/or NPY). The disadvantage of this approach is in the interpretation of phenotypes that vary greatly from animal to animal, which could be the result of incomplete penetrance or strain-dependent modifier genes. Inducible knockouts might partially ameliorate these problems by offering the opportunity to examine a phenotypic trait in mice before the induction of the mutation. The inducible knockout approach still does not solve the conundrum of whether a phenotype is the direct result of either the lack of galanin or compensatory changes or induced by other genes.

B. Galanin Transgenic Mice

Given these caveats, what are the advantages of selectively overexpressing galanin in the noradrenergic brainstem, and not in the entire brain or body? A major benefit of this approach is that it allowed me to study the consequences of manipulating a

discrete subset of galaninergic cells in the central nervous system, without perturbing (at least in a genetic sense) other galanin-producing areas. The galanin-containing neurons in the brainstem project to various areas of the forebrain, including the hypothalamus, hippocampus and cerebral cortex (Holets, et al., 1988; Levin, et al., 1987; Xu, et al., 1998). This makes these neurons attractive candidates as sources for many of the putative roles of galanin in both neuroendocrine and cognitive regulation. Galanin and norepinephrine are co-localized in the majority of neurons in the LC (Holets, et al., 1988; Melander, et al., 1986c), and galanin has inhibitory effects on norepinephrine *in vitro* (Pieribone, et al., 1995; Sevcik, et al., 1993). Overexpressing galanin in the brainstem might serve two objectives. One is to study the function of this particular population of galanin-containing cells in brain-related physiology and behavior. The other is to investigate the interaction of galanin with its co-transmitter norepinephrine. Targeting galanin to noradrenergic neurons does have limitations that may confound the interpretation of some phenotypic outcomes. A major caveat to this approach is that using the DBH promoter to drive galanin gene expression makes this transgene transcriptionally active wherever DBH functions, including many peripheral sites. Previous studies with this same promoter (Mercer, et al., 1991) have shown significant reporter gene expression in non-brain areas such as sympathetic ganglion neurons, adrenal chromaffin cells, and neurons of the enteric system. Since these are all regions that may have effects on neuroendocrine-related physiology and behavior, it may be difficult to ascribe phenotypic differences specifically to one galanin-containing population. The presence of galanin receptors in many parts of the peripheral nervous system suggests that galanin's role outside the brain may be as important as its functions

in the brain.

C. Galanin Knockout Mice

The other primary mutant model used in these studies is the GKO mouse. Originally developed by Dr. David Wynick at the University of Bristol, these mice have the first five exons of the galanin gene replaced by gene-targeting with a *PGK-neo* cassette (Wynick, et al., 1998). As a result, homozygous mutant GKO mice make no galanin, whereas heterozygotes have galanin concentrations that are roughly half those of WT animals. As is the case with selective overexpression, a loss of function mutation resulting in the complete absence of galanin has both advantages and disadvantages. One advantage is that by removing all galanin, both centrally and peripherally, the confounding problem of one galaninergic population possibly compensating for the loss or overexpression of another galanin population is removed.

However, this approach can also be disadvantageous, especially when one is interested primarily in studying the role of galanin in one part of the body, in my case the brain. For neuroendocrine (or endocrine/neuro) phenotypes, the inability to separate central from peripheral effects on phenotypes can make interpretation of anatomically localized functions exceedingly difficult. Although this difficulty can be partially surmounted by selectively reintroducing galanin to various organ systems by injection or mini-pump, the true anatomical specificity in this model must await the creation of an inducible galanin knockout.

D. Galanin/NPY Double Knockout Mice

As part of my research, I also studied mice in which both galanin and NPY have been genetically removed. These DKO mice present a significant challenge to the investigator, from a breeding and colony maintenance perspective and also from an experimental standpoint. In the best of all scientific worlds, every experiment done with double mutant mice would be performed with cohorts of animals that are littermates, gender-matched, and used at similar ages. Furthermore, all mutant combinations (WT, GKO, NPYKO and DKO) would be investigated together in the same studies under the same experimental conditions. In practice, this is either impossible to achieve or so expensive and animal intensive as to make this feasible for only the most well heeled labs. For these reasons, studies with double mutants often compare only the DKO with WT. In my case, this experimental approach made some sense, as I was primarily interested in ascertaining whether galanin and NPY functionally compensate for on another in neuroendocrine-related phenotypes. However, this strategy lends no insight about which missing neuropeptide is responsible for a phenotype in the DKO mice, or whether a phenotype reflects the lack of both galanin and NPY.

The results presented here do not directly compare the various mutant models with each other, but only against their respective WT controls. There are two main reasons for this experimental strategy. The first is related to the issue of strain. The GALTG mice are on a mostly pure C57BL6 background, whereas the GKO and DKO mice are on a mixed C57BL6 x 129 background. Because of this, it did not seem prudent to compare GALTG mice—who are virtual genetic clones of each other—with mixed strain mice having a wide diversity in their genetic makeup. The second reason concerns

the nature of the actual mutations. At first glance it may seem that comparing overexpression of galanin with a null mutant would be a useful approach, as to some degree these two mutations could be considered the converse of each other. However, as previously discussed, this is not really the case, as the overexpression of galanin in the GALTG mice is selective, whereas in the GKO galanin is absent in the brain and throughout the body. If the GALTG were a globally overexpressing animal, then a direct comparison might have been more useful. Also, since hypothalamic galanin content is apparently normal in the GALTG mice, this would undermine the rationale for seeking hypothalamic phenotypic differences between GALTG and GKO mice.

Regarding the GKO and DKO mice, although their mutations are certainly related to each other, the lack of suitable cohorts of NPYKO mice made direct comparisons between genotypes seem unwise. As previously mentioned, the lack of the second single mutant (NPYKO) magnifies the difficulty in interpreting phenotypic differences between genotypes. Thus, it seemed easier to decipher (and logistically much less complex) to only compare each mutant with its own WT control group.

II. Neuroendocrine Phenotypes

Galanin is thought to mediate neuroendocrine function in the hypothalamus. Using the current literature as a reference, we might infer what phenotypes would result from targeted deletion or overexpression of the galanin gene. Perhaps the best studied aspect of galanin as a hypothalamic neuropeptide is its putative role in regulating food intake and body weight. Several investigators have shown, at least in rats, that centrally administered galanin induces feeding (Koegler, et al., 1999; Kyrkouli, et al., 1990; Rada, et al., 1998;

Schick, et al., 1993; Smith, et al., 1996). In many of these studies, the most robust feeding effects were seen within 2 hours of galanin treatment, and overall weight-gains compared to saline-treated controls were small. Notwithstanding, a plausible prediction for the GALTG mice was that they would exhibit some alteration in feeding behaviors and body weight control. Also, if galanin were an important regulator of ingestive behaviors, one would expect GKO mice to exhibit some perturbations in feeding patterns and body weight regulation. Remarkably, I could find no evidence of feeding-related abnormalities in either the overexpressors or the null-mutants. Both developmentally and as adults, GALTG and GKO mice were of normal weight, and consumed amounts of food equivalent to their respective WT controls.

Again to my surprise, DKO mice were discovered to have the unexpected phenotype of *increased* body weight and food intake, especially in the male double mutants. The appearance of this phenotype was unanticipated and difficult to reconcile, considering that both galanin and NPY are thought to be orexigenic factors. It would have seemed more plausible that deleting both genes would result in a lean, hypophagic animal. This increased body weight was first observed around the time of weaning, and by 8 weeks of age DKO males ate approximately 26% more, and drank 23% more water than age-matched WT controls. This phenotype is especially remarkable considering the robust feeding effects of NPY (Bray and York, 1998; Schwartz, et al., 1993), although it is perhaps instructive that single mutants of NPY have normal body weights and feeding behaviors (Erickson, et al., 1997; Erickson, et al., 1996b).

The reasons for this unusual mild-obesity phenotype in DKO mice are unknown, but may involve alterations in circulating hormones, which in turn act on the body weight

control circuitry in the hypothalamus in some unusual manner. Another plausible explanation for increased feeding and weight gain in DKO animals could be that alterations exist in other components of the ingestive regulatory circuitry. While beyond the scope of this thesis, an examination of gene expression patterns of other molecules associated with these pathways is certainly warranted. Among likely candidates for investigation are AgRP, MCH, POMC, the orexins, and CRF. Significantly altered signaling by any of these molecules could conceivably tilt the balance away from tightly controlled homeostatic weight control to an aberrantly regulated state.

Another neuroendocrine function for which both galanin and NPY have been implicated is the regulation of the hypothalamic-pituitary-gonadal axis. Galanin in particular has been shown to have stimulatory effects on GnRH and LH release (Lopez, et al., 1993; Lopez and Negro-Vilar, 1990a; Sahu, et al., 1987; Sahu, et al., 1994). Coupled with galanin's coexistence in the same neurons as GnRH (Marks, et al., 1992), and its robust regulation over the estrous cycle (Marks, et al., 1993b), it would seem plausible that genetically altered levels of galaninergic signaling would lead to a significantly perturbed reproductive axis. Surprisingly, neither GALTG nor GKO mice have any apparent deficits in reproductive behaviors or fertility. Reproductive hormones were relatively normal in both mutant models, with the exception of FSH, which was lower in GALTG mice and higher in GKO mice compared to their respective WT controls.

Although this may seem unusual in light of galanin's generally stimulatory influence on gonadotropin release, at least the FSH result is consistent with a recent study (Todd, et al., 1998), where it was reported that galanin dose-dependently reduces GnRH-

stimulated FSH release in dispersed anterior pituitary cells. Galanin concentrations are very high in pituitaries of GALTG mice, and it may well be that at high exogenous levels, galanin acts as a paracrine inhibitor of FSH release. Conversely, when galanin is completely absent in the GKO mice, the high serum FSH levels may reflect a removal of tonic galanin inhibition of gonadotropin release. The moderately higher seminal vesicle weights in GKO mice may be related to this phenomenon, although seminal vesicle weight in GALTG mice is not different from WT controls.

NPY has also been closely linked to the regulation of the HPG axis, although its action appears to be steroid-dependent. NPY has been shown to be inhibitory to LH release in castrated and intact rats, while being stimulatory in OVX steroid-primed rats (McDonald, et al., 1985; Raposinho, et al., 1999; Reznikov and McCann, 1993; Sahu, et al., 1987). We hypothesized that compensatory changes in the expression of NPY in GALTG or GKO mice might be responsible for maintaining physiological levels of LH observed in both these mutants. If this were the case, then the removal of NPY in DKO mice should unmask this compensatory mechanism. However, LH levels were completely normal in both genders of DKO mice, and in contrast to what was observed in the single mutants, FSH levels were also normal. DKO mice did have significantly lower levels of T than their WT counterparts, showing that the HPG axis in the double mutants is not completely normal. Nevertheless, levels of T were still in the low physiological range in DKO mice, and this mild perturbation had no apparent effect on male fertility. Although these results do not rule out a role for either galanin or NPY as an important mediator of the brain's control of reproduction, they do point out that neuropeptide regulation of this process is likely to be *modulatory* rather than *mandatory*. Also, as with

the feeding axis, it is entirely likely that other molecules—up- or down-regulated as a result of the lack of both galanin and NPY—may take over their roles in what is likely to be a highly redundant circuit.

Many hormones other than LH and FSH are regulated by acute and chronic administration of galanin. Among these are insulin, growth hormone, thyroid hormone, and corticosterone. Insulin in particular is regulated by, and regulates galanin. As a co-transmitter with NE, galanin inhibits the release of glucose-stimulated insulin in mouse pancreas (Lindskog and Ahren, 1987). In turn, central infusions of insulin inhibit galanin (and NPY) gene expression and release in the hypothalamus of rats (Wang and Leibowitz, 1997). Curiously, this effect would appear to be hypothalamic specific, since no effect of ICV insulin has been observed on neuropeptide levels in other brain areas (Wang and Leibowitz, 1997). It was not surprising then, that in both the GKO and DKO mice, metabolic hormone levels were significantly perturbed, with both insulin and glucagon levels being higher than in WT mice. Glucose levels were also higher in both mutants, but further experiments will need to be performed to determine the mechanism underlying this phenomenon. In contrast to the findings in GKO and DKO mice, metabolic hormone levels were completely normal in GALTG mice. If the effects of galanin on insulin (and visa versa) were exclusively hypothalamic in nature, then this result might be expected, as indeed galanin levels in the hypothalamus of GALTG mice are completely normal.

One consequence of high insulin levels, at least in the DKO mice, may be altered metabolic signaling in the brain, contributing to the obesity evident in these mice. Insulin has been proposed as an adiposity signal in the brain, acting in the hypothalamus to

inform the animal of its state of metabolic reserves (Baskin, et al., 1999b). As previously mentioned, one action of insulin in the hypothalamus is to reduce the production and release of galanin and NPY. The removal of both of these insulin targets in the DKO mice may perturb the homeostatic mechanisms that regulate body weight, leading to moderate obesity.

Leptin levels were much higher in the DKO males compared to WT controls, whereas there were no differences in leptin levels between genotypes in females. Whether higher leptin levels in male DKO mice leads to obesity in these animals, or is simply a result of increased adiposity, is unknown. The normal leptin levels in GALTG, GKO and NPYKO mice (Erickson, et al., 1997) suggest that the overexpression or removal of either galanin or NPY alone is not sufficient to perturb serum levels of leptin. The increased sensitivity of GKO, NPYKO (Erickson, et al., 1996b) and DKO mice to chronic injections of leptin would suggest that both galanin and NPY may be substrates for leptin's actions in the brain, and may act normally to counterbalance the anorectic effects of leptin. Neurochemical evidence exists for a direct interaction between leptin and NPY, where leptin receptors have been shown to colocalize with NPY in arcuate nucleus neurons ((Baskin, et al., 1999a). The occasional leptin receptor-positive immunoreactive cell has been observed colocalizing with galanin in the arcuate (Hakansson, et al., 1998) but Cheung et al were unable to find conclusive evidence of this by *in situ* hybridization (Cheung, et al., 2001). GALTG mice have a completely normal response to chronic leptin treatment, which may be the result of the normal concentrations of galanin in the hypothalamus of these animals.

Further evidence that galanin mediates part of leptin's actions on the reproductive

system comes from the results of the fasting study comparing the responses of GKO and WT mice to leptin-treatments during a 48h starvation challenge. In a seminal study, Ahima et al reported that treatment with leptin during a 48h fast reversed many of the effects of starvation on reproductive indices and metabolic hormone levels (Ahima, et al., 1996). For the most part, I was able to repeat the finding of this study. As expected, a 48h fast caused a severe drop in body weight in both genotypes, and upon refeeding GKO and WT mice overate until regaining their baseline weights. The lack of galanin here apparently had no direct effect on the animal's weight control regulation machinery.

There were, however, significant genotype differences in reproductive responses to this challenge, suggesting that some of leptin's protective effects in maintaining normal reproductive function during starvation may be mediated through galanin. As in the Ahima study, a 48 h fast reduced T levels to almost below detectability, but when leptin was given during the fast this inhibition was partially relieved in WT mice. In GKO mice leptin treatment was completely without effect, with T levels staying very low. This result was confirmed by measuring seminal vesicle weights, which is often used as a morphological bioassay for T activity. In WT mice, seminal vesicle weights were only modestly lower in fasted mice treated with leptin when compared to ad-lib fed animals. In contrast, GKO fasted with leptin mice had seminal vesicle weights which were only half those of their ad-lib fed counterparts. Another difference between genotypes was observed in testis weights. In WT mice, fasted mice treated with leptin showed no reduction in testis weight compared to ad-lib fed mice. However, in GKO mice leptin treatment during fasting was unable to keep testis weights normal, showing a significant reduction when compared to ad-lib fed mice.

These genotype differences in response to leptin treatment during fasting were apparently unique to the reproductive axis. In WT and GKO animals that were fasted for 48h without leptin, T4 levels were significantly reduced, but when leptin was administered during fasting T4 levels returned nearly to those of ad-lib fed animals in both genotypes. Another general consequence of fasting is the activation of the HPA axis, with corticosterone levels reaching values much higher than those seen in ad-lib fed mice. In this study leptin treatment during fasting caused dramatic reductions in serum corticosterone levels, dropping significantly when compared to mice that were fasted without leptin. Again, there were no differences in this effect between GKO and WT mice, suggesting that galanin's role as a mediator of the "leptin effect" during fasting may be specific to the HPG axis.

It was of interest to determine whether in mice, as in rats, galanin would be an orexigenic molecule when administered chronically into the brain. To examine this question, I administered galanin to both WT and GKO mice for several days via a subcutaneous-implanted Alzet mini-pump, coupled to a brain infusion kit. Galanin was infused into the 3rd ventricle at a dose that I previously had found to induce a bout of feeding when acutely injected ICV. The modest increases in food intake and body weight in the galanin-treated mice compared to aCSF-treated animals show that this molecule is in fact orexigenic, and chronic exposure leads to weight gain. It must be stressed that, as in rats, the effects of galanin do not approach those of NPY. When both peptide were administered acutely via ICV injection, a 3 µg dose of NPY led to a much higher food intake over a two hour period than did a 6-fold higher dose (20 µg) of galanin.

I could find few differences in the response to galanin in GKO mice compared to

WT mice. Both genotypes ate and gained more weight than did their respective saline-treated controls, and hormone levels in either genotype were mostly unaffected by galanin treatment. Curiously, galanin treatment led to a modest but significant increase in T levels compared to aCSF-treated controls, but only in WT mice. The lack of an effect of galanin on T levels in GKO mice suggests that galanin stimulates the reproductive axis through different pathways than those through which it stimulates feeding, given the normal food intake response of GKO mice to galanin treatment.

The fact that GKO mice also respond normally to acute injections of NPY suggests that—at least for feeding—the NPYergic system is not functionally upregulated in the absence of galanin. Because only single acute ICV injections of NPY were given in this study, it is conceivable that different doses, methods of administration, or durations of treatment may yet reveal differences between GKO and WT mice in response to NPY or other orexigenic molecules.

As previously discussed, galanin coexists with NE in a large percentage of neurons in the locus coeruleus (Holets, et al., 1988; Melander, et al., 1986b), and has inhibitory effects on these neurons in brain slice preparations (Pieribone, et al., 1995; Sevcik, et al., 1993). Therefore, it seemed plausible that permanent overexpression or removal of galanin would lead to abnormal catecholamine activity in GALTG and GKO mice. To my surprise, NE (and dopamine) levels were completely normal in the hypothalamus, hippocampus and brainstem of both mutant models. It is likely that galanin, as a neuromodulator of classical transmitter activity, may be important in this context only during times of high neuronal demand, either to enhance or reduce transmitter signaling. As our baseline catecholamine measurements were taken in

unstressed animals at sacrifice, this technique may not have uncovered a genotype difference in NE activity. Perhaps a better approach would have been to induce a form of stress known to increase catecholamine production and release, such as induced hypothermia. Also, a more sensitive method of measuring NE release such as microdialysis might be preferable to whole tissue method employed here, which does not distinguish between vesicular and extracellular catecholamine.

One way to study the effects of NE in mice is to pharmacologically remove it from certain areas of the brain, and then observe the consequences. Using 6-OHDA as a neurotoxin, I lesioned the noradrenergic neurons of GALTG and WT mice by injecting this toxin ICV into the lateral ventricles. This method reliably lesions only NE containing neurons, and leaves levels of other monoamines such as dopamine and serotonin relatively unchanged [(Erwin and Cornell, 1986; Suaudeau, et al., 1995)]. Using this method, I sought to determine if reducing NE content in the brain would affect GALTG mice differently than in WT animals. Since this lesion should also reduce the excess input of overexpressed galanin coming from the brainstem to the hypothalamus in GALTG mice, a secondary goal was to determine whether this lesion would have a neurochemical consequence on the hypothalamic galanin system. This issue will be discussed in the next section.

I confirmed that 6-OHDA delivered by this method selectively lesioned NE neurons. NE content was greatly reduced in the hypothalamus, hippocampus and brainstem after treatment, whereas dopamine concentrations were not different from those of saline-treated animals. One primary consequence of this lesion was the dramatic, yet transient hypothermia induced by the toxin as the contents of NE nerve terminals are

released into the hypothalamus, deregulating temperature control mechanisms. As NE is cleared from the synapse or diffuses away, temperatures began to normalize, and returned to baseline within 3 hours. GALTG mice and WT mice exhibited almost identical temperature loss and recovery profiles, suggesting that both the available pool of vesicular NE and the appropriate thermogenic-related NE receptor systems are similar between mutant and WT. Since no differences were observed between 6-OHDA-treated and saline-treated mice in measured hormone levels, I was unable to assess whether NE may affect neuroendocrine functions differently in GALTG compared to WT. However, food intake and body weights were equally reduced in both genotypes 24 h after treatment with the toxin. Collectively, these observations and the normal baseline NE levels observed in GALTG mice suggest that noradrenergic function is largely unperturbed by overexpression of the cotransmitter galanin.

The lack of detectable noradrenergic-related phenotypes in galanin mutants, while unpredicted, is consistent with the fact that many other systems in which galanin has an implicit role are also apparently normal in GALTG and GKO mice. In fact, while modest phenotypes exist, both of these mutants are remarkably unaffected by either the absence or overabundance of galanin. There are several explanations for this. One potential reason would be that galanin is neither essential nor particularly important for neuroendocrine functions. Given the redundant mechanisms of the hypothalamus, any purely neuromodulatory role for galanin in this region of the brain might be compensated for by other, complementary molecules. However, pharmacological evidence does suggest that galanin regulates the release of hormones such as gonadotropins, prolactin and GH. Also, molecular studies show that hypothalamic galanin gene expression is robustly regulated

under different physiological circumstances. Taken together, this implies that galanin may indeed have an important role in neuroendocrine regulation.

Another plausible explanation for the subtle phenotypes of GALTG and GKO mice could be that galanin's role is actually *very* important in maintaining physiological homeostasis of some systems. For this reason, compensatory systems *must* be up- or down-regulated to counteract to effects of either too much or not enough galanin. Hints of this emerge from the findings in the DKO mice, where most of the phenotypes examined are more severe in the double mutants compared to single knockouts of galanin or NPY, suggesting a possible functional redundancy between these two molecules. The fact that NPY and galanin appear to have overlapping roles in the hypothalamus (Kalra and Kalra, 1996), combined with the anatomical linkage between NPY and galanin (Horvath, et al., 1996) strengthens the functional compensation argument. Other prime candidates for compensatory regulation in the face of altered levels of galanin signaling would be its own receptors, GAL-R1 and GAL-R2. One could imagine receptor regulation to be the most direct method of compensation, with the brain responding either developmentally or acutely to changes in levels of ligand.

III. Neurochemical Phenotypes

A. Galanin Gene Expression in WT Mice

One experimental approach to determine whether compensation by other molecules exists in galanin mutants is to study gene patterns and levels of gene expression. As a first step towards this goal, and to set a foundation for further

neurochemical studies, I performed a comprehensive survey of the expression patterns of galanin in WT mice,

Results of the distribution analysis for galanin mRNA-expressing cells in the brain of the mouse reveal a pattern of galanin expression similar—but with several important differences—to that previously described for the rat (Cortes, et al., 1990a; Gundlach, et al., 1990; Ryan and Gundlach, 1996). As in the rat, the highest numbers of galanin mRNA-expressing cells were observed in the diencephalon and rhombencephalon, with moderate labeling in the telencephalon and mesencephalon. In the forebrain, the presence of galanin mRNA within all of the major basal forebrain areas that contain cholinergic neurons suggests that galanin plays a similar role in modulating acetylcholine synthesis and release in the mouse as has been postulated for the rat and primate (reviewed in (Mufson, et al., 1998; Ogren, et al., 1998)). A notable and curious finding in the mouse is the apparent lack of galanin message in the cortex and striatum—areas that have been reported to contain both galanin mRNA and peptide in primates (Cebus monkeys, baboons and humans) (Kordower, et al., 1992a; Kordower and Mufson, 1990) and rats (Cortes, et al., 1990a; Melander, et al., 1986a).

One major difference between several of these studies and ours is that the mice used in our study did not receive colchicine pretreatment, whereas the majority of rat studies have used colchicine to enhance galanin peptide levels. Colchicine has been shown to induce the expression of neuropeptide genes, including galanin (Cortes, et al., 1990a). Since the studies in the rat where galanin has been reported to be expressed in the cortex and striatum entailed colchicine as a pretreatment, any direct comparison between the results we have obtained in the mouse and these earlier published studies in the rat

must be viewed cautiously. Studies in the rat examining the distribution of galanin mRNA in non-colchicine treated animals report little if any galanin gene expression in these areas (Gundlach, et al., 1990; Ryan and Gundlach, 1996). However, in the primate studies, where colchicine was not used, galanin appears to be expressed robustly in the striatum (Kordower, et al., 1992a), suggesting an unequivocal difference in distribution of galanin mRNA-containing cells between primate and mouse.

Another notable observation in the mouse is the modest, yet highly reproducible, presence of galanin mRNA within the dentate gyrus of the hippocampus. This contrasts with studies of this region in the rat (without colchicine pretreatment), where galanin mRNA is apparently not expressed and the peptide is detected only with the use of certain antibodies (Cortes, et al., 1990a; Gundlach, et al., 1990; Ryan and Gundlach, 1996; Skofitsch and Jacobowitz, 1986). In primate studies, galanin-containing neurons are found throughout the hippocampal formation, in contrast to the discrete localization in the dentate gyrus found in our study of the mouse (Kordower, et al., 1992a).

Within the hypothalamus, of the mouse most nuclei exhibited at least moderate labeling, with notable exceptions being the ventromedial and suprachiasmatic nuclei, which had no detectable signal for galanin mRNA. Relatively few cells with modest intensity of labeling were observed in both the parvocellular and magnocellular regions of the PVN, which contrasts to the reports of high levels of galanin mRNA and peptide seen in the PVN of the rat ((Melander, et al., 1986a; Ryan and Gundlach, 1996; Skofitsch and Jacobowitz, 1986). However, in complete accord with observations in the rat, I observed very high numbers of labeled cells in the DMN, PeN, and POA, underscoring the general importance of these nuclei as major sources of hypothalamic galanin. As a

step toward understanding the functional significance of these galaninergic cells, it would be interesting to learn whether these cells coexpress other peptides. Rajendren et al. have recently shown in the mouse that GnRH neurons within the POA contain significant amounts of galanin and that this co-localization phenomenon is sexually dimorphic and sex-steroid dependent (Rajendren and Gibson, 1999), corroborating earlier findings in the rat (Merchenthaler, et al., 1991). The results of my analysis of galanin mRNA expression in the hypothalamus of the mouse are in good agreement with a recent report of galanin immunoreactivity in the murine basal forebrain (Rajendren, et al., 2000), although the relative abundance of galanin peptide reported is at slight variance with our mRNA findings. It's conceivable that this variance results from methodological differences between the two studies; alternatively, it's possible that it simply reflects the fact that levels of mRNA and peptide are regulated differentially in some brain regions. This has been reported in the hypothalamus for cells that are actively secreting peptides (Silverman, et al., 1994).

The midbrain of the mouse contained very little galanin mRNA, which is consistent with findings in primates where no galanin-containing cells are observed (Kordower, et al., 1992a). My results in the midbrain of the mouse also concur with those in the rat, except in the dorsal raphe. In the rat (but not the mouse), there are abundant galanin-producing cells in the dorsal raphe, suggesting that the putative interactions between galanin and the serotonergic system might be highly species specific (reviewed in (Fuxe, et al., 1998)).

In the brainstem of the mouse, galanin mRNA was particularly abundant in two areas—the LC and dorsal vagal complex. In the LC, galanin mRNA was nearly as

abundant as in the DMN and POA of the hypothalamus, in agreement with previous studies in the rat. Many scattered cell groups were found in other parts of the brainstem, but the only other large population of galanin mRNA-expressing cells was in the vagal complex, notably in the dorsal NTS and DMV. The distribution of galanin mRNA-containing cells in the NTS of the mouse is consistent with previously published reports in other species (Kordower, et al., 1992b; Skofitsch and Jacobowitz, 1985). However, the robust population of galanin mRNA-expressing cells I observed in the DMV is apparently unique to the mouse. Studies in the rat and primates report only low numbers of galanin-containing cell bodies in this region, despite the appearance of abundant fiber tracts (Skofitsch and Jacobowitz, 1985). Although the functional significance of this observation is unknown, one plausible hypothesis would be that in the mouse, galanin modulates pre-ganglionic parasympathetic neurons.

The absence of detectable levels of galanin mRNA in areas such as the cerebellum, cortex and striatum of the mouse, which in other species have been reported to contain galanin, may reflect genuine species differences. However, it is also possible that differences among laboratories in the relative sensitivity in the methods used for detecting galanin mRNA account for the apparent differences in the distribution profile among species.

B. Overexpression of Galanin in GALTG Mice

The coupling of galanin to the DBH promoter in GALTG mice resulted in the expected overexpression of galanin in all noradrenergic cell groups of the brainstem. In particular, the LC was robustly labeled with galanin mRNA-expressing cells throughout its rostral to caudal extent. Unexpectedly, I could find no clear evidence for galanin

overexpression in the C1-C3 population of epinephrine-producing neurons. Notably, in rats, galanin does not coexist with phenylethanolamine-N-methyltransferase (PNMT), the synthetic enzyme for epinephrine (Melander, et al., 1986c), and the expression of galanin in adrenergic cells has not been reported in WT mice. However, with the presence of DBH in these neurons as a requisite enzyme in the adrenergic biosynthetic pathway one would have logically predicted that galanin expression would be evident here in GALTG mice. Another curious pattern of expression in GALTG mice is the continuum of galanin expression extending ventrally from the cuneiform nucleus through the medial parabrachial nucleus down to the ventral subcoeruleus. Although NE has been reported to be present in scattered cells throughout this region in rats (Swanson and Hartman, 1975), the surprising extent of galanin expression observed in GALTG mice may well represent a species difference in the distribution of DBH in this region.

The most surprising finding regarding galanin expression in the GALTG mice was the extensive labeling in all regions of the piriform and entorhinal cortices. Early studies of TH-like immunoreactivity in the rat (Kosaka, et al., 1987) and monkey (Kohler, et al., 1983) showed TH-positive cells in cortical areas corresponding to those where galanin is overexpressed in the GALTG mice. Specifically, TH immunopositive cells were found throughout the cortex in layer II/III. This agrees closely to my findings in the GALTG mice, where galanin mRNA was found exclusively in layer II/III in a continuum from the piriform cortex caudally through the rostral entorhinal cortex. Galanin-expressing cells were also seen in this layer of the retrosplenial granular cortex, and in other cortical layer II/III in some animals. It could be that the same cells that have been shown to contain TH also express galanin in GALTG mice.

No dopamine or norepinephrine has been found in this band of cortical cells, and DBH itself has not been identified in the piriform and entorhinal cortices. However, it is clear from the very robust expression of galanin seen in the GALTG mice, coupled with a complete absence of galanin expression in this region of WT mice, that the DBH promoter is selectively active in layer II/III. Determining whether this is directly related to the reported TH-positive cells in this band of cells was beyond the scope of these studies. It is very interesting though that the cells in this layer are made up of both GABAergic interneurons and glutamate pyramidal neurons, and that both types are directly modulated by NE and DA (Gellman and Aghajanian, 1993). Galanin may be contained in the same projection fields bringing NE and DA to layer II/III, and GAL-R2 is expressed in both the piriform and retrosplenial cortices (O'Donnell, et al., 1999). Thus, the same cells in this area that overexpress galanin in GALTG mice may well be neurons that normally receive galaninergic input.

Other areas of the forebrain also exhibited apparent modest increases in galanin gene expression in the GALTG compared to WT, including the olfactory nucleus, caudate putamen, nucleus accumbens, hippocampus, and subiculum. These patterns of expression are almost certainly due to ectopic activity of the DBH promoter, as has been previously shown to be the case with this 5.8kb promoter fragment (Hoyle, et al., 1994; Mercer, et al., 1991). Hoyle et al. found that fragments of the DBH promoter ranging from 0.6 to 5.8 kb 5' to the DBH transcription start site produced variable patterns of expression in the central nervous system. Out of 5 different fragments coupled to lacZ as a reporter gene, only one, the 1.5 kb segment, produced noradrenergic specific expression. Shorter fragments were not sufficient to produce any DBH transcription, and

all longer fragments directed DBH expression to various ectopic sites. This suggests that multiple positive and negative regulatory elements are responsible for limiting its expression to noradrenergic neurons. In particular, these researchers found that the same 5.8 kb fragment that we used to drive galanin in the GALTG mice, also directed lacZ expression to forebrain areas such as the cerebral cortex, hypothalamus and substantia nigra. Although this pattern of lacZ expression is somewhat different than the apparent ectopic expression of galanin seen in GALTG mice, it is clear that this fragment of the DBH promoter does not specifically target brainstem noradrenergic neurons. In another study, it was found that this 5.8 kb upstream promoter fragment drove lacZ into several forebrain areas, including in the same layer II of the piriform and entorhinal cortices where galanin is overexpressed in GALTG mice (Mercer, et al., 1991). Using TH as a marker for catecholamine-containing neurons, these investigators found coexistence of TH and the transgene marker lacZ in regions of the forebrain. This finding lends further credence to the notion that the cortical neurons that ectopically express galanin may indeed be the same ones observed in early studies to produce TH in the rat (Kosaka, et al., 1987).

C. GAL-R1 Gene Expression in WT Mice.

The distribution of GAL-R1 in the brain has been well described in the rat (Burazin, et al., 2000; Burgevin, et al., 1995; Gustafson, et al., 1996; Landry, et al., 1998; Mitchell, et al., 1997; O'Donnell, et al., 1999; Parker, et al., 1995; Waters and Krause, 2000). No description of GAL-R1 has been reported to date in the brain of the mouse, and only a partial distribution has been reported for humans (Deecher, et al., 1998;

Mufson, et al., 2000; Sullivan, et al., 1997). To map the pattern of GAL-R1 mRNA distribution in the brain of the mouse, and to set the foundation for gene regulation studies with this receptor, I qualitatively assessed the locations and intensity of GAL-R1 gene expression in the mouse forebrain and midbrain.

I found GAL-R1 message to be widely distributed in the mouse brain, and for the most part, the patterns of gene expression were generally similar to those reported in the rat, with some exceptions. In the telencephalon, all cholinergic regions of the basal forebrain expressed GAL-R1 mRNA, with the exception of the medial septum. This agrees with findings in the rat, where little or no GAL-R1 message is present in the medial septum, except in one isolated report, where weak labeling in this region was seen (Burazin, et al., 2000). I found robust GAL-R1 expression in the lateral septum, where both the number of cells and intensity of labeling were the highest of any telencephalic nucleus.

Both the central and medial divisions of the amygdala were intensely labeled, suggesting a role for galanin in these regions of the amygdala most closely associated with autonomic and neuroendocrine function. The medial amygdaloid nucleus exhibited a dense pattern of GAL-R1 mRNA signal. This region projects to several areas of the hypothalamus, and is thought to be an area that sends higher order signals to the defensive, ingestive and reproductive regions of the hypothalamus (Swanson and Petrovich, 1998). GAL-R1 expression was also found to be high in the bed nucleus of the stria terminalis, another region that receives limbic system input, and projects strongly to the neuroendocrine hypothalamus. This further strengthens the concept that galanin has a role in modulating cortical and limbic system output to the hypothalamic nuclei that

coordinate appropriate physiological responses to emotional signals.

Several significant differences were observed between the pattern of GAL-R1 mRNA expression in the mouse compared to that previously reported in the rat. For example, I found many GAL-R1 mRNA-positive cells scattered throughout the rostral to caudal extent of the caudate nucleus. In contrast, with the exception of one study (Burazin, et al., 2000), all other published distributions in the rat brain report no GAL-R1 mRNA in the caudate. Also, in the rat a robust expression of GAL-R1 message is consistently reported in the piriform cortex, throughout its rostral to caudal extent. In mice, I found no expression of GAL-R1 mRNA in any piriform cortex region, but did observe labeling in other cortical areas such as the retrosplenial granular and entorhinal cortices. Interestingly, these latter two areas also exhibit ectopic expression of galanin in GALTG mice, but whether the same cells that express galanin also express GAL-R1 in these regions is not known. Another area in the rat where all previously published studies report strong GAL-R1 signal is the ventral CA1 region of the hippocampus, an region that has been associated with the effects of galanin on classical neurotransmitter release (reviewed in (Ogren, et al., 1999)). In the mouse, only a few weakly-labeled and scattered cells are seen in this area, suggesting that the role of galanin in hippocampal function may differ between rodent species.

In the diencephalon, there is a remarkably good agreement between the distribution of GAL-R1 message reported in the rat and my results in the mouse. Without a doubt, the most robustly labeled region in the mouse forebrain was found in the thalamus, especially the midline and intralaminar nuclei. In the hypothalamus GAL-R1 message—like galanin itself—is distributed widely in all major nuclear groups. All

regions of the preoptic area exhibited robust labeling for GAL-R1 message, underscoring the putative role of galanin as a mediator of sexual and reproductive behaviors. Nearly every nucleus known to be important for neuroendocrine functions had at least moderate amounts of GAL-R1 message, including the PeN, PVN, DMN, LH and VMN. The striking exception to this was the Arc, where only scattered labeled cells were seen in any part of this nucleus.

The mesencephalon was the region of the brain where I found the most striking differences in GAL-R1 expression between the mouse and rat. In the midbrain of the rat, relatively little receptor message is seen, except for fairly consistently reports in the superior colliculus, central gray, and (in some studies) the substantia nigra (Burazin, et al., 2000; Parker, et al., 1995). In contrast, I found GAL-R1 message to be widely distributed in the midbrain of the mouse, including robust labeling in some structures where no positive signal has been reported in the rat. Areas such as the nucleus of the posterior commissure, geniculate nucleus and sub-brachial nucleus all had distinct populations of GAL-R1 mRNA-containing cells. The expression of GAL-R1 in the geniculate, pretectal and superior collicular areas suggests that in the mouse, galanin has a role in visual system function, as these are all relay nuclei for input to visual cortex. Because none of these regions apparently express GAL-R1 in the rat, this may be a function specific to the mouse.

When comparing the expression patterns of galanin with those of GAL-R1, it is worth noting that almost without exception, wherever the peptide is expressed in the forebrain and midbrain, the receptor mRNA is present in the same nuclei. The one area where this is not the case is the medial septum, which shows a moderate degree of

galanin message labeling but is devoid of receptor mRNA. The extensive overlap between galanin and GAL-R1 implies this receptor subtype may have a role as an autoreceptor in the mouse, as has been previously suggested for the rat. In one study, galanin and GAL-R1 were found to coexist in a sub-population of basal forebrain neurons (Miller, et al., 1997), and in another report galanin and its receptor were colocalized in the DMN of the hypothalamus. Although I found that GAL-R1 nearly always exists in regions where galanin is made, the reverse is not always the case. In many areas, where galanin message was not present at all, GAL-R1 was highly expressed, showing that although this receptor may indeed serve as an autoreceptor, its function is certainly not limited to this role. Areas where GAL-R1 is expressed in significant amounts (and where galanin is not expressed) include the subiculum, entorhinal cortex, ventromedial hypothalamic nucleus, and most of the GAL-R1 expressing midbrain structures. GAL-R1 has recently been shown to colocalize with GnRH in the preoptic area (Mitchell, et al., 1999b) and with POMC in the arcuate (Bouret, et al., 2000), suggesting that this receptor is involved in the mediation of galanin's downstream effects on neuroendocrine-related targets.

D. GAL-R1 Gene Expression in the Hypothalamus of GALTG

Mice

Like galanin, the GAL-R1 receptor is significantly regulated in response to physiologic or chemical challenges (Bouret, et al., 2000; Faure-Virelizier, et al., 1998a; Gorbatyuk and Hokfelt, 1998; Landry, et al., 1998; Mitchell, et al., 1999b; Ortego and

Coca-Prados, 1998; Zachariou, et al., 2000; Zhang, et al., 1998) . Since GAL-R1 has been postulated to be an autoreceptor on galanin-containing neurons, I hypothesized that its expression would be altered in the face of chronically high galaninergic tone in GALTG mice. I found that this receptor subtype was upregulated in the hypothalamus, but in a selective manner. In three specific nuclei, the—PeN, DMN and VMN,—GAL-R1 was significantly upregulated in GALTG mice compared to WT in both males and females. In several other areas, such as the PVN, LA, and SON, GAL-R1 message was not different between genotypes, and in fact its expression seemed to be slightly reduced in GALTG when compared to WT.

If GAL-R1 is an autoreceptor, it should be upregulated in GALTG mice in regions where galanin gene expression is high, as part of a mechanism that keeps galanin levels within the physiological norm. Thus, it makes sense for GAL-R1 levels to be higher in the PeN and DMN, which are two of the major hypothalamic populations of galanin-containing neurons. Both these regions are major centers of neuroendocrine integration, and project widely to other regions of the hypothalamus. The PeN is richly endowed with both peptide and sex steroid receptors, including those for GH, α -MSH, leptin, estrogen and progesterone. Cells in this nucleus project widely to other neuroendocrine regions, such as the PVN, MPOA, and septal areas, and the PeN receives projections from these regions as well (Gu and Simerly, 1997). Thus, if galanin were an important molecule for maintaining neuroendocrine homeostasis, then keeping galanin levels normal in the PeN of GALTG mice may well be necessary to maintain neuroendocrine homeostasis. One possible mechanism for ensuring this would be to increase levels of galanin's autoregulating receptor, GAL-R1.

The increased expression of GAL-R1 message in the DMN of GALTG mice was also not a surprise, as this is one of the highest expressing regions in the entire brain for both galanin and GAL-R1 (Burazin, et al., 2000; Cheung, et al., 2001; Mitchell, et al., 1997). The DMN is one of the principal neuroendocrine integrative and relay nuclei, and is highly interconnected with other hypothalamic regions (Thompson, et al., 1996; Thompson and Swanson, 1998). This nucleus receives a major input from the arcuate nucleus, and provides a major input to the PVN, placing it in a pivotal position to mediate galanin's roles in regulating the hypothalamic-hypophysial axis. The DMN also receives a significant input from the noradrenergic brainstem, particularly the LC (Palkovits, 1999), and from telencephalic areas such as the subiculum and lateral septum (Thompson and Swanson, 1998). The brainstem afferents presumably also contain large amounts of galanin in the GALTG mice, which would theoretically result in a larger than normal galaninergic input to the DMN in GALTG mice compared to WT.

GAL-R1 may be upregulated in the DMN of GALTG mice for several reasons. One reason for induction of GAL-R1 could be to reduce the amount of galanin being produced endogenously in the hypothalamus, to counteract the increased galanin being released by noradrenergic/galaninergic nerve terminals originating in the brainstem of GALTG mice. Another possible role for GAL-R1 could be as a presynaptic receptor on galanin-containing axons projecting from the brainstem. Here, the upregulation of GAL-R1 could serve to limit the release of galanin into the hypothalamus, again assisting in keeping galanin concentrations clamped within the physiological range. This hypothesis, while beyond the scope of these studies, could be tested by assaying the expression of GAL-R1 in LC or other brainstem neurons that also contain galanin and project to the

DMN. A third possibility is that GAL-R1 is upregulated in the DMN as a presynaptic receptor at DMN terminal fields, in such areas as the PVN. It is interesting that galanin-containing neurons provide a major input to the parvocellular PVN, where they are thought to regulate the release of hormones from the anterior pituitary (Ceccatelli, et al., 1992; Levin, et al., 1987). This same region of the PVN also receives a significant input from the A1 and A6 noradrenergic cell groups, which also overexpress galanin in the GALTG mice. This being the case, it would be important for galanin being released into the PVN from these dual afferents (DMN and brainstem) to be tightly controlled, perhaps in part by GAL-R1.

It is clear that GAL-R1 is not only an autoreceptor, because it is robustly expressed in the VMN, where little or no expression of its ligand galanin can be detected, in either the rat or the mouse (Burazin, et al., 2000; Cheung, et al., 2001; Mitchell, et al., 1997). It is puzzling that GAL-R1 would be upregulated in this nucleus, and not in others more closely associated with galanin, such as the SON, LA and PVN. However, although galanin is not produced in the VMN, it clearly has an effect on several physiological systems mediated by this nucleus. Galanin injected into the VMN induces a bout of feeding (Schick, et al., 1993), and colchicine-induced lesions of the VMN lead to increased body weight, which is associated with higher galanin levels in nuclei involved in feeding (Pu, et al., 1999). One curious aspect of VMN anatomy is that this nucleus is one of the few regions in the hypothalamus that does not receive a strong projection from the noradrenergic brainstem (Swanson and Hartman, 1975), although it does project to the brainstem (Canteras, et al., 1994). The lack of a direct NE/galanin input from the brainstem could suggest that, at least in this nucleus, the upregulation of GAL-R1 is *not* a

response to abnormally high galanin input. Also, the lack of galanin production in this area argues against GAL-R1 working as an autoreceptor to limit endogenous peptide concentrations in the VMN. One plausible explanation for increased GAL-R1 signaling in the VMN of GALTG mice may be that this nucleus has an abundance of steroid and hormone receptors, and galanin has a role in modulating the actions of the ligands to these receptors (Bennett, et al., 1998; Mufson, et al., 1999; Scott, et al., 2000).

Interestingly, although galanin-containing projections from the brainstem do not innervate the VMN, a direct projection does exist from the subiculum to this area of the hypothalamus (Bennett, et al., 1998; Fahrbach, et al., 1989; Kita and Oomura, 1982; Mufson, et al., 1999; Scott, et al., 2000). Since the subiculum overexpresses galanin ectopically in GALTG mice, it is conceivable that GAL-R1 is selectively upregulated in this region to compensate for the effects of increased galanin input from subicular areas.

Given these differences in GAL-R1 gene expression between GALTG and WT mice, how might these changes fit in with what is known about how this receptor is regulated in a fashion that might support its role as a regulator of galanin's actions? Several published reports may lend insight into this. For example, Landry et al. found that during times of neuronal insult—such as after hypophysectomy or colchicine treatment—galanin expression is upregulated to act in a what has been postulated as a neuroprotective role (Landry, et al., 1998). Concomitantly, GAL-R1 is downregulated after these treatments, which makes sense if the compensatory need after neuronal insult were for increased galanin signaling, and if GAL-R1 were an inhibitory autoreceptor. Also, in peripheral nerves, galanin mRNA is increased after either axotomy or inflammation in dorsal horn neurons (Zhang, et al., 1998). This is accompanied by a

significant decrease in GAL-R1 message levels, which again would corroborate with the notion that when high galanin concentrations are required as a neuroprotectant, GALR-1 levels should decrease, to amplify the overall effectiveness of galanin.

Furthermore, galanin and GAL-R1 are regulated in opposite directions by sex steroids. Galanin expression is increased by estrogen, which may act directly on an estrogen response element in the galanin promoter (Rokaeus, et al., 1998). In contrast, levels of GAL-R1 negatively correlate with circulating levels of estrogen and progesterone. In a recent report, levels of GAL-R1 in the preoptic area decrease in OVX animals treated with estradiol and progesterone, and in cycling rats, the highest levels of GAL-R1 mRNA coincide with the period when progesterone levels are lowest (Faure-Virelizier, et al., 1998a). In another study, the expression of GAL-R1 in GnRH neurons was assessed at various times during the estrous cycle, and in OVX animals (Mitchell, et al., 1999b). Up to 21% of GnRH neurons also expressed GAL-R1 during estrus, but no double-labeling could be detected at 1800 on proestrus, when estrogen-stimulated LH levels (and galanin levels) are very high. In addition, while double-labeled cells could be observed in OVX animals, the coexistence of GAL-R1 and GnRH is apparently abolished after EP treatment. In a final study, administration of NE into the human ocular ciliary epithelium decreases galanin content and concomitantly increases GAL-R1 message (Ortego and Coca-Prados, 1998).

A caveat to the hypothesis that GAL-R1 acts primarily as an inhibitory receptor for the production and/or release of galanin is that both receptor and ligand are often regulated in the same direction in magnocellular neurons of the hypothalamus. In contrast

to changes in opposite directions after hypophysectomy or colchicine, salt-loading or lactation leads to parallel changes in both receptor and ligand in the SON and PVN (Landry, et al., 1998). Also, injections of 2-deoxy-D-glucose or sodium mercaptoacetate, which are thought to increase the sensitivity of SON and PVN neurons to galanin, lead to a significant upregulation of GAL-R1 in these nuclei (Gorbatyuk and Hokfelt, 1998). It seems likely that in the magnocellular neurosecretory neurons, where galanin coexists with vasopressin and oxytocin, GAL-R1 may facilitate the actions of galanin in controlling the release of peptides in the posterior pituitary. Nevertheless, the results of my studies, combined with previous reports by others, supports the concept that one important role of GAL-R1 is as a brake on excess galanin activity, restraining the impact of galanergic circuits when their activity becomes chronically exaggerated.

One important question regarding the upregulation of GAL-R1 in the hypothalamus of GALTG mice is whether this is a developmental phenomenon or happens in response to changing levels of galanin in the adult animal. While the mechanisms for desensitization and *downregulating* receptors are beginning to be unraveled (reviewed in (Bohm, et al., 1997)), little is known about how receptors are chronically or acutely *upregulated*. It may be that the brain compensates for continually high levels of galanin during development by “resetting” the basal levels of GAL-R1 in regions of the brain critical for galanin signaling, such as the DMN, PeN and VMN. Although it is poorly understood, this is certainly not without precedent in mutant mice, as receptor levels have been shown to be altered basally in response to both permanently high and low levels of peptide(Kobayashi, et al., 1995; Trivedi, et al., 1999).

It may also be that levels of GAL-R1 respond dynamically in the adult mice to

acute alterations in peptide levels. This has not been tested directly with galanin and its receptors, but a considerable literature exists regarding the regulation of adrenergic receptor mRNAs in response to ligand exposure (reviewed in (Hadcock and Malbon, 1993)). Almost without exception, this literature describes mechanisms that desensitize and downregulate receptor expression and production, and does not consider cellular strategies for transcribing *more* receptor message.

I tested the hypothesis that message levels of GAL-R1 would be decreased in GALTG mice when galaninergic input to the hypothalamus is reduced by lesioning the NE/galanin projections with 6-OHDA. Surprisingly, no changes in GAL-R1 mRNA were observed in any hypothalamic region in response to this neuronal insult. There are several possibilities for why this is the case. One possibility would be that GAL-R1 message levels are determined developmentally, and as such do not respond to acute changes in ligand. Another potential explanation might be that the 6-OHDA lesion was not successful in reducing galanin levels in the hypothalamus to a degree that would be recognized by the GAL-R1 regulatory machinery. Although this was not directly tested in this study, the fact that NE levels were dramatically reduced in the hypothalamus of the lesioned mice would argue that the projection fields, which presumably contain both NE and galanin, had been damaged by the neurotoxin. A third scenario is that in GALTG mice, there is no increased input of galanin into the hypothalamus from the overexpressing galanin neurons in the brainstem. This possibility is suggested by the lack of a difference in galanin concentrations in the hypothalamus between WT and GALTG mice (when clearly other brain regions do contain increased amounts of peptide). This seems unlikely, however, since hypothalamic expression of the GAL-R1 gene is clearly

differentially regulated in these mutants, as is the expression of GAL-R2 (see next section), and by far the most plausible candidate for causing these changes is galanin itself. It seems more likely that galanin levels are kept normal in GALTG mice *because* of compensatory changes in receptor gene expression. Further studies will need to be performed to define the developmental and adult regulatory mechanisms that are responsible for altering receptor gene expression in GALTG mice. Even so, the evidence from the 6-OHDA lesion studies hints that basal receptor message levels may not primarily influenced by acute changes in galanin levels, but rather by some as yet unknown developmental mechanism.

Contrary to the findings on GAL-R1 mRNA in the hypothalamus of GALTG mice, I could ascertain absolutely no difference in GAL-R1 message levels between GKO and WT mice in any region examined. As was the case with WT controls for GALTG mice, GAL-R1 receptor message was widely distributed among many different hypothalamic areas, from the rostral extent of the preoptic area to the caudal portions of the mammillary nuclei. Because this study was performed with pure 129 strain mice, and the GALTG study utilized pure C57BL6 animals, the concordance in receptor expression patterns would suggest that the differences observed in distribution patterns between rat and mouse are true *species* differences, rather than simply variations between *strains* of mice.

GKO mice exhibited identical patterns of GAL-R1 receptor expression as WT mice in the hypothalamus, suggesting strongly—at least in this region of the brain—that a certain level of receptor expression is genetically and developmentally programmed, and is not dependent on ligand exposure either during development or in adulthood. This is

quite different from the situation in GALTG mice, where the brain is responding to the chronic “insult” of high galanin levels, and must take actions to counteract this.

Apparently, in the absence of ligand in the GKO, there is nothing to be gained by changing receptor expression levels, and it may make better sense physiologically to alter levels of other molecules that could functionally compensate for the loss of galanin. It would be interesting to drive galanin levels up chronically in GKO mice, to see whether levels of GAL-R1 respond dynamically to alterations in hypothalamic galanin in this model.

Of course, a major caveat to these receptor studies is that I have examined only one component of the receptor regulatory process—control at the message level. It may be that GAL-R1 expression is altered in GKO mice at other levels, such as peptide content, receptor insertion into the membrane, internalization rates, turnover, or myriad alternative possibilities. However, since levels of mRNA reflect one important aspect of functional regulation, it seems reasonable use this approach as a starting point for a more detailed analysis of the mechanisms that control the signaling capabilities of a receptor, in response to changes in its ligand.

E. GAL-R2 Gene Expression in GALTG and WT mice

I also found that expression of GAL-R2 mRNA in the hypothalamus was different between GALTG and WT mice. In WT animals, GAL-R2 was fairly widely distributed in the hypothalamus, although not as widely or abundantly as GAL-R1 mRNA. I observed significant numbers of GAL-R2 expressing cells in several hypothalamic nuclei, including the AVPe, PVN, SCN, Arc, DMN, VMN and MM. While many of the areas

that exhibit GAL-R2 message also express GAL-R1, the overlap in distribution was not complete between the 2 receptor subtypes. Little or no expression of GAL-R2 mRNA was observed in either the SON or LA, regions that exhibit robust labeling for GAL-R1. Also, the SCN, which is completely devoid of both galanin and GAL-R1 message, showed moderate expression levels of GAL-R2. In the Arc, where GAL-R1 is only moderately expressed, GAL-R2 mRNA signal is evident throughout the rostral to caudal extent of this nucleus. Other areas of the hypothalamus, including the MPOA, LH and PH had faint labeling for GAL-R2 message, but due to the relatively high background which is characteristic of the riboprobe used, it was not possible to assess levels of expression or make comparisons between genotypes in these areas. A complete analysis of GAL-R2 gene expression in the mouse hypothalamus was not possible with this riboprobe, but the areas that could be recognized as above background signal levels generally agreed with the published reports of GAL-R2 message in the hypothalamus of the rat (Burazin, et al., 2000; Fathi, et al., 1997b; Kolakowski, et al., 1998b; Mitchell, et al., 1999a; O'Donnell, et al., 1999).

I found that in two areas of the hypothalamus, the PVN and Arc, GAL-R2 mRNA levels were significantly lower in GALTG mice than in WT controls. No differences between genotypes in GAL-R2 message were seen in the areas where I found GAL-R1 mRNA to be upregulated in GALTG mice, including the PeN (anteroventral part) DMN and VMN. I also measured GAL-R2 message levels in the noradrenergic LC, but was able to detect no difference between genotypes in this brainstem region. This latter finding shows that the massive overexpression of galanin in the LC has no effect on levels of gene expression of one of galanin's major receptor subtypes, suggesting that

perhaps GAL-R2 is not directly involved in controlling the production and/or release of galanin. This postulate is also strengthened by the lack of apparent alteration in the regulation of GAL-R2 in any region where galanin is produced in large amounts in the hypothalamus, such as the DMN or preoptic area.

It is interesting that the two areas where GAL-R2 mRNA is differentially regulated between GALTG and WT mice are the two “powerhouse” neuroendocrine nuclei, but which actually express (in the mouse) relatively little galanin relative to some other hypothalamic regions (Cheung, et al., 2001). In the rat, however, galanin is abundantly produced in both these areas, and is coexpressed with many different transmitters and peptides in both of these regions (Everitt, et al., 1986; Merchenthaler, et al., 1993; Palkovits, et al., 1987). Galanin-containing fibers originating in the Arc terminate in the parvocellular region of the PVN (Levin, et al., 1987) and as this region of the PVN in turn sends projections back to the Arc, it is conceivable that these fibers also contain galanin, since this peptide is abundantly produced here (in rats) (Toth and Palkovits, 1998). Both of these nuclei receive major afferent projections from the noradrenergic brainstem, so they are likely to be subjected to increased galaninergic tone in GALTG mice (Levin, et al., 1987; Palkovits, 1999). Also, both the PVN and Arc receive significant projections from the DMN, where galanin is highly expressed (Levin, et al., 1987; ter Horst and Luiten, 1986).

Because GAL-R2 is downregulated in regions of GALTG mice that *receive* a large galaninergic input instead of in areas that *produce* galanin abundantly, it would seem plausible that GAL-R2 is primarily a post-synaptic receptor that mediates some of the downstream effects of its ligand. Thus, it would make sense for expression of this

receptor subtype to be diminished in GALTG mice, as a mechanism for reducing galaninergic tone in the hypothalamus. Several other studies in the literature would support this hypothesis. Following experimental nerve crush in facial motor nerves, galanin mRNA is upregulated 8-10 fold, reflecting galanin's putative role in a neuroprotective or regenerative capacity (Burazin and Gundlach, 1998). At the same time, while GAL-R1 is downregulated, GAL-R2 is upregulated 4-fold, possibly to enhance the actions of galanin during a period of high demand.

At first glance, the *positive* correlation between levels of galanin and GAL-R2 message after nerve injury may seem contradictory to the findings in GALTG mice, where *high* levels of galanin lead to *lowered* levels of GAL-R2. However, the physiological situations are very different in the two models. In the case of the nerve crush, the nervous system may be taking steps to enhance the actions of galanin, and one mechanism for achieving this would be to increase levels of its post-synaptic, activating receptor. On the other hand, in GALTG mice, the brain may be responding to chronically high levels of galanin by attempting to reduce galanin signaling, through lowered levels of GAL-R2 expression. In both cases, the regulation of GAL-R2 may be a primary strategy for altering galanin tone to the level required by the system. Another example where GAL-R2 is regulated concomitantly with galanin during a period when higher than normal peptide levels may be required is after peripheral inflammation (Sten Shi, et al., 1997). Galanin is dramatically upregulated in dorsal root ganglia following inflammation, and this is accompanied by a 4-fold increase in GAL-R2 message. Curiously, these same investigators found that after axotomy, GAL-R2 mRNA moderately decreases, suggesting that the regulation of this receptor is not always concordant with that of its

ligand.

To summarize, both receptor subtypes are differentially expressed in mutant mice compared to WT, but in different areas and in different directions. GAL-R1 mRNA is upregulated in some hypothalamic areas, whereas GAL-R2 is downregulated in other regions. Given that each of these galanin receptor subtypes is coupled to a different signal transduction pathway (reviewed in (Branchek, et al., 2000)), and each has a unique distribution in the brain, this differing pattern of regulation may not be so surprising. Also, in a comprehensive study examining the developmental distribution of both GAL-R1 and GAL-R2 mRNAs in the rat brain, it was found that each subtype has a unique pattern of expression early in life (Burazin, et al., 2000). While GAL-R1 mRNA distributions are much the same in early post-natal life as in adulthood, GAL-R2 expression is much more widespread and abundant during development than at P70. Based on this observation, the authors suggest that GAL-R1 is involved in normal synaptic transmission, and as such its expression needs to be developmentally set at an early age. This finding agrees with my results, which suggest that GAL-R1 levels may be determined in GALTG mice during development, and may be insensitive to perturbations in galaninergic tone in adulthood. On the other hand, the patterns of GAL-R2 mRNA expression seem to change dramatically from post-natal to adult ages. This suggests that the GAL-R2 subtype may play a role in synaptic development and maturation, a function that might be related to galanin's actions—through GAL-R2—as a neuroprotective or regenerative agent after neuronal insult.

F. Galanin Gene Expression in the Hypothalamus of NPYKO

Mice

The observation that galanin was selectively upregulated in the hypothalamus of NPYKO mice suggests that in the absence of NPY, galanin may compensate for some of the missing molecule's functions. This may not be too surprising, considering the evidence for anatomical and functional linkages between these two molecules. The two areas where I found altered galanin mRNA in NPYKO mice, the Arc and DMN, are both important areas for galanin and NPY signaling. Galanin is present in all sections of the Arc, where it colocalizes with DA and GHRH (Everitt, et al., 1986). This population of galanin-containing cells projects both to other parts of the hypothalamus, and also to the median eminence (Palkovits, et al., 1987). This widespread projection of galaninergic projections from the Arc makes these cells well-placed to modulate neuroendocrine processes. The Arc is the primary site of NPY synthesis in the hypothalamus, and tightly-clustered, robustly-expressing cells are situated in the medial portions of this nucleus. Curiously, although NPY is thought to be an important mediator of hypothalamic-hypophysial hormone release, few NPY Arc axons project to the median eminence (Everitt, et al., 1986). Instead, the majority of NPY efferents are directed to other hypothalamic nuclei, such as the PVN and DMN, both of which receive a dense innervation by NPYergic fiber systems (Sawchenko and Pfeiffer, 1988). In turn, these nuclei may respond to NPY signaling by influencing activity of releasing hormones such as TRH, CRF, and GnRH.

It seems reasonable to infer that if galanin and NPY were to have some

overlapping functions, that the Arc would be a site where increased galanin signaling might be evident. NPYKO mice are completely normal in such endocrine functions as reproduction, body weight control, serum hormone levels, and response to fasting (Erickson, et al., 1997)—all processes in which galanin has been implicated. The link between galanin and NPY in the Arc is further strengthened by the demonstrated anatomical interconnections in this nucleus (Horvath, et al., 1996). In the Arc (and PVN) NPY-containing boutons surround galanin-positive cells and dendrites, and electron microscopic analysis has revealed that NPY terminals make synaptic contact with galanin-expressing neurons. Taken together, the evidence suggests that increased galanin signaling in the Arc of NPYKO mice is a prime candidate for a compensatory mechanism allowing maintenance of a normal neuroendocrine phenotype in NPYKO mice.

The potential reasons for the increase in galanin mRNA in the DMN of NPYKO mice are slightly more speculative. It may be that as this is a major site of galanin production in the neuroendocrine hypothalamus, this population of neurons responds to the need for increased galaninergic tone by generally upregulating message levels here. A telling anatomical connection is that the galanin-containing neurons in the DMN project strongly to the parvocellular PVN ((Fodor, et al., 1994; Levin, et al., 1987). This region of the PVN also receives a massive input from NPY neurons in the Arc (Broberger, et al., 1999). As this latter projection system is completely missing in NPYKO mice, it may be that the galaninergic projections from the DMN to the PVN increase their activity to compensate for the loss of NPY signaling here. Also, while NPY is only weakly expressed in the DMN of normal rats, in certain physiological states NPY gene expression increases dramatically here. For example, in two models of murine obesity,

obese yellow mice and MC4-R knockout mice, late-onset weight gain is accompanied by increased expression of NPY in the DMN (Kesterson, et al., 1997). Also, increased NPY mRNA has been observed in lactating rats, another energetically draining physiological state (Li, et al., 1998). It may be that this population of NPY neurons—normally undetectable—plays an important role in certain neuroendocrine circumstances, and that in the absence of NPY the galanin population in the DMN takes over part of this role. An interesting further experiment would be to measure galanin gene expression in the DMN during energetically stressful circumstances, such as during fasting or lactation.

G. NPY Gene Expression in GALTG and GKO mice

Considering that the galanin gene was significantly upregulated in the hypothalamus of NPYKO mice, I was surprised to find that no altered regulation was evident for hypothalamic NPY gene expression in either GALTG or GKO mice. This is particularly surprising considering that levels of both galanin and NPY are often changed after experimental manipulations or challenges, albeit not always in the same direction. For example, insulin injected centrally reduces both galanin and NPY gene expression and immunoreactivity in the PVN, Arc and DMN, and insulin application to isolated hypothalamic fragments reduces the release of both these peptides (Wang and Leibowitz, 1997). Leptin administered centrally also reduces both galanin and NPY gene expression in the hypothalamus of ad lib-fed rats (Sahu, 1998a). Metabolic challenges such as chronic zinc deficiency also alters the expression of both galanin and NPY mRNAs, but in this case galanin message levels are decreased and NPY message levels increased

(Selvais, et al., 1997). In the peripheral nervous system, this coordinate regulation is also evident. Axotomy of the sciatic nerve leads to a dramatic appearance of both NPY and galanin in dorsal root ganglion where neither peptide is expressed in appreciable amounts under normal circumstances (Landry, et al., 2000). In sympathetic neurons of the superior cervical ganglion, the story is somewhat different. In an elegant series of studies, Zigmond and co-workers have elucidated several of the key interactions between various peptide system that occur after peripheral nerve injury (reviewed in (Zigmond, et al., 1998; Zigmond and Sun, 1997) 1997). After nerve damage, NPY gene expression is reduced, and galanin gene expression is increased. Both of these changes are dependent on the presence of another peptide, leukemia inhibitory factor, and perhaps also on nerve growth factor.

In all of these manipulations, the constant is that *both* galanin and NPY levels are altered, rather than just one peptide system. This suggests a functional interaction or redundancy between the two systems, and therefore it would seem logical that the complete lack of galanin in GKO mice might lead to a compensatory upregulation of NPY in the Arc and perhaps DMN. I only examined expression of the NPY gene under basal conditions, and it may well be that a compensatory upregulation might be most evident under circumstances of energetic stress, such as starvation or lactation. It has been postulated that NPY is most important as a molecule that mediates systems that require activation during times of physiological duress.. As such, there may be a baseline amount of hypothalamic NPY gene expression needed as a reserve, ready for use “on demand”, which under basal conditions might not be different between GKO and WT mice. It is also possible that NPY may be differentially regulated between mutant and

control mice, but not at the level of its mRNA. It would be most interesting to measure NPY peptide content in these animals, both at the cell body and at the nerve terminal, to get estimates of peptide synthesis and possible turnover rates.

The lack of a change in hypothalamic NPY gene expression levels between GALTG and WT mice is easier to understand. The most plausible explanation for this finding is that NPY neurons are not sensing that any altered state exists in the hypothalamus, as the brain has put into place mechanisms to ensure that galanin levels are normal in this part of the brain. If this were the case, then most peptide system in the hypothalamus, including NPY, would be regulated at a level similar to that seen in WT animals. The lack of a change in NPY in this model, despite significant changes in GAL-R2 in the Arc of GALTG mice, suggests that either GAL-R2 is not resident on NPY neurons, or it does not participate in the regulation of the NPY gene.

The last piece to consider in the still very incomplete puzzle of altered gene expression regulation in the galanin mutant mice is that of NPY in the Acb. In contrast to the very limited spatial expression pattern of NPY in the hypothalamus, this neuropeptide is widely expressed in many areas of the telencephalon. In fact, the entire cortical mantle of the mouse is punctuated by highly-expressing discrete NPY cells which are not clustered in any type of apparent grouping (J. Hohmann, personal observation). Whether these represent interneurons or projections neurons is not known. The Acb also exhibits this punctate patterning of NPY mRNA positive cells, in both the shell and core regions of the nucleus, and in all rostral to caudal portions.

I found that in GALTG mice, both the number of NPY-expressing cells and the mean grains per cell in this region were lower than in the same area of WT control mice.

If galanin and NPY have overlapping or redundant roles in this nucleus, then perhaps the overabundance of galanin in the Acb would lead to a compensatory reduction in NPY message levels here. NPY concentrations are known to be much higher in the Acb of obese Zucker rats than in WT controls (Beck, et al., 1990), suggesting that the obesity of these animals might involve an accumbens component. Also, several studies have demonstrated that NPY injected into the Acb induces a conditioned place preference in rats, suggesting that this peptide in this region may be involved in some of the reward aspects of feeding (Brown, et al., 2000; Brown, et al., 1998). Interestingly, galanin has also been associated with Acb-linked feeding behaviors, through its regulation of both DA and ACh. Injections of galanin into the PVN induces a bout of feeding in a subset of animals that also exhibit a concomitant increase in Acb release of DA and decrease in release of ACh (Rada, et al., 1998). The authors of this work suggest that the increase in food intake is directly linked to the regulation of transmitters in the Acb, since another subset of animals, that do not eat in response to injections of galanin, show no alterations in DA or ACh release in the Acb. However, when the same experiment was performed with NPY, food intake was significantly increased without a change in levels of either DA or ACh in the Acb, suggesting that NPY and galanin may act through different orexigenic pathways.

I did not measure galanin concentrations in the Acb, but anatomical evidence from the literature would support the idea that levels of this peptide could be higher in this nucleus of GALTG mice, providing a neurochemical reason for the compensatory lowering of NPY message here. DBH-containing fibers coming from the brainstem innervate the shell region of the Acb (Berridge, et al., 1997), and these same fibers

presumably contain greater than normal amounts of galanin in GALTG mice.

Furthermore, both the shell and core regions of the Acb receive strong projections from the entorhinal and piriform cortices, as revealed by retrograde labeling with fluoro-gold (Brog, et al., 1993). Both of these areas massively overexpress galanin in the GALTG mice, which combined with the noradrenergic/galaninergic afferents from the LC, would make it seem likely that the Acb receives a triple “overdose” of galanin in these mutant animals.

The decreased expression of NPY message in this region of GALTG mice shows that there is a clear neurochemical consequence in the forebrain as a result of galanin overexpression, apart from the regulation of galanin receptors. It would be of interest to measure DA and ACh release by microdialysis in the Acb and other areas to see if the overabundance of galanin leads to abnormalities in transmitter release here. In contrast to other neuropeptide systems known to be involved in the control of ingestive behaviors, such as the POMC/CART and NPY/AgRP circuits, the neurochemical mechanisms that mediate galanin’s effects on feeding are still poorly understood. It may well be that the galaninergic ingestive circuitry involves extrahypothalamic regions such as the Acb, and the future elucidation of these pathways may well have to take into account this nucleus.

The final surprise regarding NPY regulation in galanin mutant mice was the finding that instead of the expected *rise* in NPY mRNA in the Acb of GKO mice, message levels of this peptide tended to actually be *lower* as a consequence of the complete lack of galanin. Although the differences did not quite reach significance, both the number of NPY-expressing cells and the mean grains per cell trended lower in GKO mice, mirroring the previously described results in GALTG animals. This curious result,

while not yet understandable in terms of physiological importance, underscores the concept that GKO and GALTG mice are not the “opposites” of one another. In fact, a similar confounding result has been obtained when assessing impact on the cholinergic system in both these models. In GALTG mice, cognitive deficits are correlated with the selective reduction in ChAT-positive cells in the basal forebrain, suggesting that overexpression of galanin inhibits the production and release of ACh (Steiner, et al., 2001). A remarkably similar result has been obtained in GKO mice (O'Meara, et al., 2000). In mice wherein galanin is completely absent, a striking reduction in ChAT-positive cells—although in a different basal forebrain region than in GALTG mice—is also associated with a behavioral cognitive deficit.

One possible explanation for the similar neurochemical phenotypes in certain regions of both GALTG and GKO mice may be that what is required for *normal* expression of molecules such as NPY or ChAT is *normal* expression of galanin. Thus, either the overabundance or lack of this neuromodulator may be sufficient to partially “shut-down” molecular systems that are sensitive to changes in galanin concentrations. Another possible reason for the similarities observed between the two mutants in NPY expression is that although the end result may be nearly the same, the reasons for getting there could be quite different. Galanin has been implicated in neuroprotective and neurogenerative roles, and may be involved in developmental processes that effect neuronal survival. It has been suggested that the lack of galanin in GKO mice leads to the death of cholinergic neurons, resulting in the diminishment of ChAT-containing cells in the basal forebrain (O'Meara, et al., 2000). On the other hand, the reduction in ChAT-containing neurons in GALTG mice is more likely to be a result of reduced ChAT

production rather than the complete loss of cells (Steiner, et al., 2001). In fact, the number of cells in the basal forebrain that express ChAT message in GALTG mice is only slightly reduced compared to WT, lending support to the notion that these neurons are still present and active. This may also be the case with NPY in the Acb. It could be that in the GALTG mice, the neurons that express NPY are all present and functioning, but are simply reduced in their complement of NPY in response to increased amounts of galanin in the Acb. In GKO mice, however, it is possible that due to the lack of developmental trophic effects of galanin, some of the neurons that contain NPY may actually be missing.

I have attempted in these behavioral, physiological and neurochemical studies to gain a further understanding of the role of galanin as a participant and regulator of neuroendocrine functions in the brain. At best, I have only been partly successful in this endeavor. As a neuromodulator, the important actions of this widely distributed, highly conserved molecule are difficult to elucidate. The mammalian brain is an astonishingly complex organ, with a bewildering array of seemingly redundant overlays; thus studying the roles of a molecule whose functions may be subtle in nature is challenging. It seems that many more questions have resulted from these studies than have been answered, but perhaps this is the desired nature of a fruitful scientific education. For me, the beauty is in loving the questions as much as the answers. Perhaps, if enough carefully crafted questions are pursued, the answers to galanin's functional significance in the brain will eventually be fully revealed

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