INFLAMMATORY REGULATION OF HYPOTHALAMIC NEURONS IN SICKNESS BEHAVIOR

by

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CERTIFICATE OF APPROVAL

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5HT	Serotonin (5-hydroxytryptamine)
AAV	Adeno-associated virus
α-MSH	α-melanocyte stimulating hormone
aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotopic hormone
AD	Adenosine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AgRP	Agouti-related peptide
ARC	Arcuate nucleus of the hypothalamus
BAT	Brown adipose tissue
BBB	Blood-brain barrier
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CPP	Conditioned place preference
CRH	Corticotrophin releasing hormone
CSF	Cerebrospinal fluid
DA	Dopamine
db	Diabetic gene
DMH	Dorsomedial nucleus of the hypothalamus
DMSO	Dimethyl sulfoxide
DR	Dorsal raphe nucleus

DVC	Dorsal vagal complex
EEG	Electroencephalogram
EGFP	Enhanced green fluorescent protein
FAA	Food anticipatory activity
FR	Food restriction/restricted
GABA	γ-aminobutyric acid
GC	Glucocorticoid
GFP	Green fluorescent protein
GHSR	Growth hormone secretagogue receptor
GHSR KO	GHSR knockout mouse
GIRK	G-protein regulated inwardly rectifying potassium channel
HPA	Hypothalamic-pituitary-adrenal
i.c.v.	Intracerebroventricular
IHC	Immunohistochemistry
IκBα	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-1R	Interleukin-1 receptor
IL-1RI	Type 1 interleukin-1 receptor
IL-1ra	Interleukin-1 receptor antagonist
IL-1RAcP	IL-1R accessory protein
IL-2	Interleukin-2
IL-6	Interleukin-6
Indo	Indomethacin

i.p.	Intraperitoneal
IR	Immunoreactive
JAK	Janus kinase
LBM	Lean body mass
LC	Locus coeruleus
LDT	Laterodorsal tegmental nucleus
Lep-R	Leptin receptor
LHA	Lateral hypothalamic area
LIF	Leukemia inhibitory factor
LIF-R	LIF receptor
LMA	Locomotor activity
LPS	Lipopolysaccharide
MC3-R	Type 3 melanocortin receptor
MC4-R	Type 4 melanocortin receptor
MC4-RKO	MC4-R knockout mouse
MCH	Melanin-concentrating hormone
MnR	Median raphe nucleus
mPGES-1	Microsomal prostaglandin E synthase-1
mPGES-1 KO	mPGES-1 knockout mouse
mPGES-2	Microsomal prostaglandin E synthase-2
MTII	Melanotan-II
NF-κB	Nuclear factor-kappa B
NE	Norepinephrine
NO	Nitric oxide
NPY	Neuropeptide Y
NREM	Non-rapid eye movement

NTS	Nucleus of the solitary tract
Nts	Neurotensin
NTX	Naltrexone
ob	Obese gene
OSM	Oncostatin-M
Ox	Orexin
OX1R	Type 1 orexin receptor
OX2R	Type 2 orexin receptor
OxA	Orexin-A
OxB	Orexin-B
OxKO	Orexin knockout mouse
PCR	Polymerase chain reaction
PFA	Perifornical area of the hypothalamus
PG	Prostaglandin
PI3K	Phosphoinositide-3 kinase
PKB/Akt	Protein kinase B
POMC	Proopiomelanocortin
PPT	Pedunculopontine nucleus
PTP1B	Protein-tyrosine phosphatase 1B
PVN	Paraventricular nucleus of the hypothalamus
REM	Rapid eye movement
RT-PCR	Reverse transcriptase polymerase chain reaction
SCN	Suprachiasmatic nucleus of the hypothalamus

SOCS-3	Suppressor of cytokine signaling-3
SPZ	Subparaventricular zone
STAT3	Signal transducer and activator of transcription 3
TMN	Tuberomammillary nucleus of the hypothalamus
TNF-α	Tumor necrosis factor-α
VLPO	Ventrolateral preoptic nucleus of the hypothalamus
VMH	Ventromedial nucleus of the hypothalamus
VTA	Ventral tegmental area
WT	Wild-type
ZT	Zeitgeber time

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ABSTRACT

Illness is associated with a constellation of symptoms including weakness, malaise, anorexia, weight loss, and inability to concentrate. Motivation and arousal are suppressed, and patients report hypersensitivity to pain and a lack of interest in normally enjoyable activities. Remarkably, these features are present in a wide spectrum of diseases, regardless of etiology. Perhaps because these symptoms are so common, they have in the past been underestimated as being an uncomfortable side effect of the illness process. However, this response represents an evolved adaptive strategy with important survival implications. Known collectively as "sickness behavior", this coordinated set of physiologic changes represents a critical element of the host response to disease. By suppressing an animal's motivational drive, metabolically expensive activities are restricted, allowing energy to be diverted into fueling heat generation (fever) and immune system mobilization. When viewed in the context of chronic illness, the resulting metabolic state is not only debilitating, but strongly predicts both morbidity and mortality.

Neurons in the hypothalamus regulate homeostatic behavior and physiology in accordance with environmental, emotional, and physiologic cues. This region is also susceptible to inflammatory signaling in response to disease. Thus, the hypothalamus is well positioned to mediate illness-induced changes in behavior. Recent work has confirmed that neurons comprising the central melanocortin system, a critical regulator of metabolic homeostasis, are sensitive to pro-inflammatory cytokines. Manipulation of central melanocortin signaling restores feeding in several anorectic animal models of disease. A causative role for cytokine regulation of these neurons in mediating anorexia

and wasting has not been confirmed, however. Another population of neurons in the hypothalamus that express the neuropeptide orexin (Ox) regulates arousal and stabilizes wakefulness. Genetic ablation of this peptide in mice leads to suppression of volitional locomotor activity (LMA) and feeding and an increase in somnolence—a phenotype closely resembling sickness behavior. The regulation of these neurons by disease and inflammation is unknown.

The focus of this thesis is to characterize the hypothalamic neural populations underlying two aspects of sickness behavior – anorexia & lethargy. First, the response of the central melanocortin system to the pro-inflammatory cytokine leukemia inhibitory factor (LIF) was investigated to examine the mechanism of LIF-induced anorexia. We then explored the involvement of Ox neurons in mediating sickness-associated lethargy and anorexia. The interaction between the melanocortin and Ox systems was evaluated to determine whether a common mechanism mediates the effects of inflammation on both systems. These studies have advanced our understanding of the role of melanocortin signaling in sickness behavior and identified a neural mediator of inflammation-induced lethargy. This work posits a role for Ox replacement in the treatment of lethargy and further supports the therapeutic value of melanocortin antagonism in reversing anorexia during disease.

CHAPTER 1

INTRODUCTION

1. Sickness Behavior Is An Adaptive Response to Disease

1.1 Fever

The maintenance of energy homeostasis is one of the most crucial physiological tasks that animals perform to ensure long-term survival. Under normal conditions, energy intake and energy expenditure are precisely controlled, providing remarkable stability of body weight over an animal's lifetime. Because infectious illness is an acute threat to life, systems have evolved that sacrifice energy homeostasis in order to provide the greatest chance of survival. Indeed, nearly all of the behaviors and metabolic changes typical of sickness can be linked directly to a highly organized strategy of the organism to fight infection (1, 2). One of the primary features of the host response to acute infection is fever. Increased body temperature is strongly linked to survival from infection in numerous species, and is one of the most critical non-specific defense systems animals employ to fight pathogens (3). The increase in body temperature during fever favors proliferation of immune cells and is unfavorable to the growth of many pathogens. Although it is generally an adaptive strategy for survival, the generation of fever comes at a high metabolic cost. The increase in metabolism per 1ºC of fever is estimated to be 13 percent (4). Therefore, it is reasonable to view fever as an adaptive, but metabolically expensive, febrile response. In neurophysiological terms, fever represents an increase in the hypothalamic thermoregulatory set point. In this instance, animals feel cold at previously normal body temperatures, and will seek out warmer environments to compensate. Furthermore, thermogenesis is increased (e.g. by shivering and activation of sympathetic outflow), and heat loss is reduced both behaviorally (e.g. by curling up, huddling, seeking warm environments) and physiologically (e.g. by shifting blood flow away from the skin and improving insulation through piloerection).

1.2 Anorexia in Acute Illness

Although the inhibition of volitional activity is relatively straightforward to justify from a teleological perspective, it is somewhat more difficult to understand how anorexia that is typical of acute disease provides a survival benefit. In fact, one could argue that the increased metabolic demands imposed by fever and immune system activation should lead to relative hyperphagia. On the other hand, an animal that is not hungry will not spend time foraging, thereby preserving energy reserves and reducing heat loss. In addition, prey animals are particularly vulnerable to predation when they are ill because of impaired locomotion, potentially providing another survival advantage of anorexia. It has also been suggested that decreased food intake during fever helps to maintain relatively low circulating levels of iron and zinc, both of which are known to be critical trace elements for bacterial growth (for review, see (5)). Overall, it appears that relative anorexia is an adaptive response to acute infection. This idea is reinforced by the observation that maintaining basal food intake by force-feeding infected mice doubles their mortality, although it is unclear whether this is a general phenomenon (6). Coincident with the decrease in food intake, an overall hypermetabolic, catabolic state is maintained, thereby providing a ready fuel supply for the immune system, particularly in the form of muscle-derived amino acids.

1.3 Cachexia of Chronic Disease

Whereas acute infection has provided a steady evolutionary pressure throughout most of human evolution, relatively recent medical developments have produced large numbers of individuals who are living with chronic inflammatory insults. Although

undoubtedly an oversimplification, the metabolic effects of chronic diseases can be viewed as an extension of the acute response to infection that, although potentially beneficial in the acute setting, becomes pathological when prolonged. Cachexia, from the Greek *kakos* (bad) and *hexis* (condition), or disease-associated wasting, is a common occurrence in cancer, renal failure, and infectious disease. Hippocrates wrote about the relationship between dropsy (congestive heart failure) and cachexia more than 2400 years ago: “The flesh is consumed and becomes water…the abdomen fills with water, the feet and legs swell, the shoulders, clavicles, chest and thighs melt away…This illness is fatal” (7). This devastating catabolism is brought about by a synergistic combination of a dramatic decrease in appetite and an increase in metabolism of fat and lean body mass. The severity of cachexia in many illnesses is the primary determining factor in both quality of life and in eventual mortality (8, 9). Other illness-induced morbidities, including lethargy, also directly compromise the ability of patients to recover from potentially life-saving or extending interventions, including surgery and cytotoxic chemotherapy, and can diminish the motivational drive to aggressively battle the condition. Although cachexia in chronic disease was described more than two thousand years ago, the central mechanisms underlying this disorder of energy homeostasis were until recently quite poorly understood. However, with recent developments in understanding of appetite and body weight control, investigators have begun to unravel the fundamental aspects of the pathophysiology of cachexia.

1.4 Therapeutic Approaches to Cachexia

Unfortunately, despite our increased understanding of cachexia, there is currently no safe and effective treatment for this condition. Extensive trials with anti-inflammatory agents have shown modest benefit, and significant gastrointestinal and renal side effects

are common (10). Perhaps the most promising agents in early trials were the progestational agents (e.g., megestrol acetate), but more recent trials have shown that the modest weight gain produced by these agents is primarily due to gains in body fat and water retention, with little beneficial effect on lean body tissue (10, 11). Glucocorticoids (GC) produce weight gain and improvement in quality of life scores, but they do not improve strength or lean body mass and are associated with numerous unwanted side effects typical of adrenocortical excess, i.e. iatrogenic Cushing's syndrome (12). Growth hormone has also received attention as a potential anti-cachexia agent, but its usefulness is limited by cost, difficulty of delivery, worsened insulin resistance, potential to promote the growth of tumors in individuals with cancer, and growth hormone resistance in most forms of cachexia (11, 13). Our work and that of others has demonstrated that melanocortin antagonists and drugs that diminish endogenous melanocortin activity (e.g. ghrelin) show great promise in preclinical and early clinical trials (14-19). However, there remains a critical need for the development of new agents with efficacy in treating various features of cachexia brought about by a variety of diseases.

1.5 Defining Cachexia

Given its clinical importance it is not surprising that a great deal of effort has gone into developing therapeutics for cachexia. Until recently, there was no unifying definition of cachexia, nor were there any accepted guidelines for demonstrating efficacy of new therapeutic modalities. In December of 2007, a group of scientists, clinicians, and representatives of the Food and Drug Administration of the USA met to establish an operational definition of cachexia, as well as to begin discussions of possible therapeutic endpoints for clinical trials. The definition that emerged from this consensus conference

was: "*Cachexia is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity"* (20). Like any consensus statement, numerous details and nuances were debated, and the relative complexity of this definition reflects the real complexity of this metabolic derangement. Nonetheless, this definition provided a starting point for discussion of meaningful clinical trial endpoints.

2. Inflammation Coordinates the Behavioral Response to Illness

2.1 Sickness Behavior is Associated with Inflammation

A shared characteristic of chronic diseases associated with the development of sickness behavior and cachexia is increased production of pro-inflammatory cytokines. Cells of the innate immune system, such as macrophages and dendritic cells, act as sentinels that detect microbial infection and tissue damage by recognizing common molecular markers. In response, these cells release pro-inflammatory cytokines, which amplify the immune response both locally (paracrine) and at distant sites in the body (endocrine). In this way, a local signal of damage or danger is augmented and distributed systemically, resulting in common immunological and behavioral responses to sickness. For example, elevations in interleukin-1 beta (IL-1β, LIF, interleukin-6 (IL-6),

and tumor necrosis factor alpha (TNF-ą are found in rats, mice, and sheep following peripheral administration of lipopolysaccharide (LPS), a potently inflammatory bacterial endotoxin known to induce the full spectrum of sickness behavior (6, 21-25). Shortly after the identification and cloning of the first cytokines, in the 1980s, clinical trials began evaluating interleukins and interferons as potential anti-tumor therapeutics. These studies were rapidly abandoned because of safety concerns. However, the side-effect profiles on these cytokines included all the cardinal features of sickness behavior, including malaise, anorexia, lethargy, and anhedonia (26). It appears that these cytokines also mediate disease-associated wasting syndromes. Indeed, elevated circulating levels of TNF-α and IL-6 in patients with cardiac cachexia are the strongest predictor for pathological weight loss (27, 28). IL-1β, IL-6, LIF and TNF-α are produced by human tumor cells both in tissue culture and patients (29, 30). This rise in circulating cytokines has been implicated in the physiologic and behavioral responses to inflammation in rodents, including anorexia (31), HPA axis activation (32), fever (33), increased non-REM sleep (34), and anhedonia (35). Indeed, intracerebroventricular (i.c.v.) administration of several pro-inflammatory cytokines in rodents is effective in recapitulating the cardinal features of cachexia including: anorexia, weight loss, increased energy expenditure and catabolism of fat and lean body mass (LBM) (36, 37). Further, patients in clinical trials for treatment of malignant glioma frequently reported severe lethargy in response to intrathecal recombinant pro-inflammatory cytokine therapy (38). These findings suggest that systems within the brain promote the behavioral response to cytokines.

2.2 Peripheral Inflammation and Muscle Wasting

Pro-inflammatory cytokines act directly on muscle cells to disrupt the normal balance between anabolic and catabolic pathways (39). Activation of nuclear factor-kappa B (NF-κB) in muscle is increased by IL-1β and TNF-α (40). Increased NF-κB activation in turn promotes muscle degeneration by accelerating protein breakdown through ubiquitin-dependent proteolysis (41) and prevents muscle repair by decreasing the expression of the transcription factor MyoD (42). Further, muscle explants, when treated with inflammatory cytokines, exhibit dysregulated protein turnover leading to catabolism (43). These studies indicate that direct cytokine signaling can mediate lean tissue wasting. However, activation of the hypothalamic-pituitary-adrenal (HPA) axis and the subsequent release of GCs is necessary for active muscle catabolism (44-46). Simultaneously, insulin signaling must be diminished for GC-induced muscle wasting to occur (47). Both activation of the HPA axis and suppression of insulin signaling by inflammation are mediated by direct inflammatory signaling in the central nervous system (CNS) (as discussed below). Although muscle wasting eventually leads to weakness, sick patients and animals exhibit lethargy long before significant catabolism occurs. Collectively, these data support the hypothesis that the coordinated behavioral and metabolic responses in sick animals involve the synergistic action of cytokines acting in both the brain and periphery.

2.3 Central Inflammation

In the absence of infection or tissue damage, cytokine expression in the brain remains low, though basal cytokine levels may play a role in sleep and energy balance (48-51). Infection of the brain or meninges, brain tissue damage, or seizure results in elevations of cytokine expression in the CNS (52-55). Cytokines are also found in the cerebrospinal fluid (CSF) from patients with cancer, HIV, or acute infection (56). Cytokines are large, polar molecules and do not diffuse well through the blood brain

barrier (BBB). Rather, cytokines are actively transported across the BBB and are also expressed locally within the CNS. Peripheral injections of LPS increase the expression of IL-1α, IL-1β, IL-6, TNF-α and LIF in the rodent brain (57-59)(Figure 1). Central administration of cytokines including IL-1β, TNF-α, and LIF stimulates their own production while simultaneously inducing the synthesis of other cytokines in the brain (60-62). Thus, inflammatory signaling is locally amplified within the brain in response to peripheral immune challenge. The importance of central inflammation was recently supported by studies that delete MyD88, the adaptor protein that transduces IL-1 and LPS signals into the cell, which completely prevented anorexia following intraperitoneal injection of IL-1 or LPS (63). Although one must always consider developmental adaptation when interpreting knockout data, this finding suggests that amplification of inflammation within CNS is necessary to promote the behavioral response to disease.

It is important to note that doses and routes of administration of endotoxin and cytokines used in these and subsequently cited animal studies vary considerably between experiments. Because different doses or injection sites of immune-modulating agents may recruit distinct inflammatory mechanisms, these data must be cautiously interpreted. In lieu of standardizing dosing regimens, evaluation of existing studies can identify important common mediators of sickness behavior. The induction of cytokine expression in the brain in response to peripheral immune challenge appears to be conserved through mammalian evolution, affording us the opportunity to integrate observations from several species into a common understanding of sickness behavior. Taken together, these studies imply an important role for cytokine signaling, both in the CNS and the periphery, in mediating the sickness response.

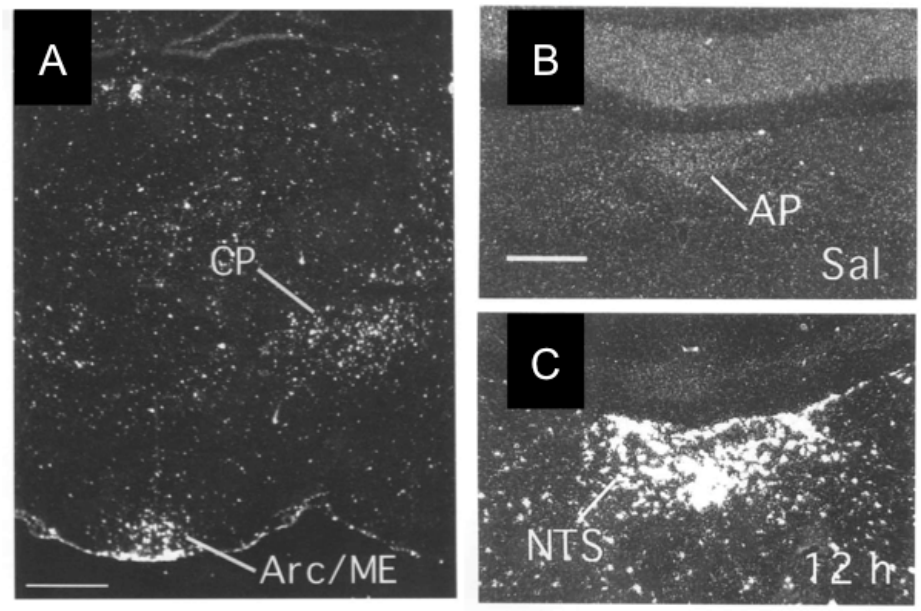

Figure 1. *LPS induces IL-1β expression in the hypothalamus and brainstem.*
(A) Low power darkfield photomicrographs showing IL-1β mRNA labeling in ARC/ME, 12 h after i.p. injection with LPS (2.5 mg/kg). (B,C) Low power darkfield photomicrograph showing IL-1β mRNA in the brainstem, at the level of the area postrema (AP) and solitary tract nucleus (NTS). Tissue harvested 12 h after animals injected i.p. with saline (B) or LPS (C). *Arc, arcuate nucleus; ME, median eminence; CP, cerebral peduncle.* Scale bars = 2 mm (A), 1 mm (B,C). Reprinted with modifications from *Neuroscience*, 83 (1). N. Quan, M. Whiteside, M. Herkenham. “Time course and localization patterns of interleukin-1beta messenger RNA expression in brain and pituitary after peripheral administration of lipopolysaccharide.” pp. 281-93, with permission from Elsevier.

3. Hypothalamic Involvement in Cachexia

3.1 The Hypothalamus and Brainstem Respond to Inflammation

Inflammation within neuronal tissue, as represented by markers for NF-κB activation, is localized exclusively to the hypothalamus and brainstem following systemic immunologic challenge (64). Here neurons and glia respond to inflammatory stimuli by locally producing cytokines (65-68). These findings point toward the hypothalamus and brainstem as the site of inflammatory amplification within the CNS. Furthermore, feeding centers in the hypothalamus, including the paraventricular (PVN) and arcuate nuclei (ARC), express receptors for these cytokines (69-72). The immediate early gene, *c-fos*, a marker of neuronal activation, is expressed in the ARC and PVN in response to peripheral inflammatory stimuli, suggesting that cytokines may exert their cachexigenic effects by influencing the activity of hypothalamic centers controlling energy balance (73-75)(Figure 2). Feeding nuclei in the brainstem, such as the nucleus of the solitary tract (NTS), also show strong *c-fos* induction and NF-kB pathway activation in response to peripheral inflammatory challenges (Figure 2). The relationship of acute inflammation-induced *c-fos* to the chronic cachexia syndrome is not clear, however these studies identify candidate inflammation-responsive loci in the CNS.

3.2 The Role of Central Melanocortins in Cachexia

The ARC is critical in regulating energy homeostasis. This nucleus is linked with other hypothalamic and brainstem metabolic regulatory centers by a complex web of reciprocal innervation (76). The ARC contains two populations of neuropeptide-expressing neurons with opposing actions on energy balance. One population expresses the anorexigenic peptide, alpha-melanocyte-stimulating hormone (α-MSH), a cleavage

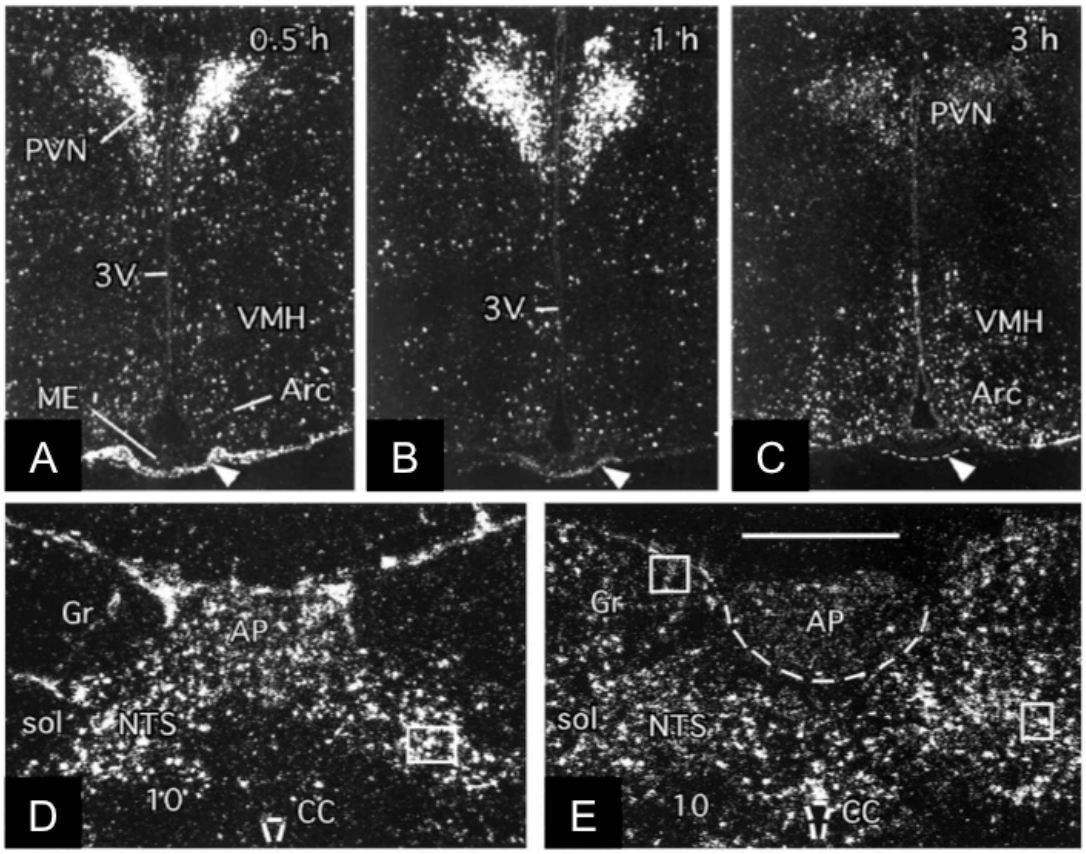

Figure 2. *Induction of c-fos mRNA in hypothalamus and brainstem following intravenous IL-1β administration.*

Low power darkfield photomicrographs showing *c-fos* mRNA labeling in the hypothalamus at the level of the PVN, ARC, and ME, 0.5 (A), 1 h (B), and 3 h (C) after intravenous injection with IL-1β (2-10 μg/kg). Low power darkfield photomicrographs depicting *c*-fos mRNA expression in the brainstem, at the level of the AP and NTS, 0.5 h (D) or 3 h (E) after intravenous IL-1β (2-10 μg/kg) administration. Arc*, arcuate nucleus; ME, median eminence; VMH, ventromedial nucleus; 3V, third ventricle; NTS, solitary tract nucleus; CC, central canal, AP, area postrema; Gr, gracile nucleus; sol, solitary tract; 10, dorsal motor nucleus of the vagus.* Scale bars = 0.5 mm. Reprinted with modifications from *J Comp Neurol*, 400 (2). M. Herkenham, H.Y. Lee, R.A. Baker. “Temporal and spatial patterns of c-fos mRNA induced by intravenous interleukin-1: a cascade of non-neuronal cellular activation at the blood-brain barrier.” pp. 175-96, with permission from Elsevier.

product of the pro-opiomelanocortin (POMC) precursor, which is also expressed by brainstem neurons of the NTS (77). ARC POMC neurons are opposed by adjacent neurons expressing the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide-Y (NPY) (78). α-MSH derives its anorectic effects by binding to type-3 melanocortin receptors (MC3-R) located on POMC neurons and within other areas of the hypothalamus, and the type-4 melanocortin receptors (MC4-R) distributed widely throughout the brain (79-82). AgRP neurons project to a majority of MC4-R expressing neurons throughout the brain and act as an endogenous antagonist at the receptor (83). Together, these populations of POMC- and AgRP-expressing neurons in the ARC and NTS and the neurons that express their receptors make up the central melanocortin system. This neural network plays a critical role in regulating feeding behavior, linear growth, metabolic rate, and insulin sensitivity (recently reviewed in (84)). Although the role of the MC3-R in energy balance remains somewhat obscure, a great deal is now known about the MC4-R. MC4-R is expressed in hypothalamic and brainstem feeding centers as well as on parvocellular neurons in the medial PVN, which project to brainstem nuclei controlling the activity of the sympathetic nervous system. MC4-R agonists, such as α-MSH or the small molecule agonist melanotan-II (MTII) dramatically decreases food intake and increases energy expenditure, through resting thermogenesis (85). Conversely, melanocortin antagonists trigger positive energy balance by increasing food consumption and reducing energy expenditure and physical activity (77, 86-88). The relative activity of each of these neuronal populations is modulated by circulating signals of energy status as well as synaptic input from the brainstem and other hypothalamic areas. Thus, the central melanocortin system orchestrates energy balance in accord with the metabolic demands of the organism.

The remarkably similar physiologic signature of increased melanocortin signaling and cachexia--anorexia, increased metabolic rate, and alterations in tissue mass--suggest a potential role for MC4-R in mediating some aspects of sickness behavior. Indeed, blockade of MC4-R signaling by genetic and pharmacologic means prevents many of the features of cachexia that would normally occur in both acute and chronic models of inflammation (17, 18, 89-91). Marks and colleagues showed that mice with genetic or pharmacologic blockade of melanocortin signaling (*MC4-RKO* and AgRP-treated mice, respectively) hads attenuated feeding responses to non-specific inflammation (LPS) as well as to tumor implantation (Figure 3). Similar results were found in tumor-bearing rats and LPS-treated sheep treated with AgRP (19, 92). Alternatively, analogues of the orexigenic hormone ghrelin, which stimulates appetite through AgRP neuron activation, improve food intake and body weight in rat models of cancer cachexia and chronic renal failure (14, 15). These observations led to the development of drugs that dampen or block central melanocortin signaling as potential therapeutics for cachexia. Although these observations provided an exciting therapeutic target, the mechanistic details of cytokine feedback to central melanocortin signaling is largely unexplored.

3.3 Neuropeptide Y in Cachexia

NPY is a potent orexigenic neuropeptide produced in the ARC of all animals and the DMH of lactating rats (93). Expression and release of NPY are stimulated in fasting conditions, where it helps restore body weight by increasing food intake and decreasing metabolic rate. NPY mRNA expression is diminished in several inflammatory states, providing an alternative mechanism for inflammation-induced negative energy balance

(reviewed in (94)). Central administration of IL-1β can block NPY-induced feeding (95). Conversely, NPY can restore feeding after IL-1β treatment, suggesting that altered NPY signaling may underlie sickness-induced anorexia (96). Megestrol acetate is thought to derive its orexigenic effects by increasing NPY production, though this leads to a gain primarily in fat mass and little lean mass retention (10, 97).

However, tumor studies have illustrated a more complex role interaction between illness and the NPY system. NPY is less effective in stimulating feeding in tumor bearing animals compared to controls. The implantation of a glucagonoma into rats increases the expression of NPY despite inducing profound anorexia (98). Hypothalamic NPY mRNA is increased in other catabolic tumor models as well, suggesting an intact NPY response to negative energy balance at the transcriptional level (99). The reason for this paradoxical effect is unclear, but two explanations include a divergence between expression and peptide release in tumor bearing animals; or an overriding influence of other anorectic circuits induced by tumor implantation (100, 101). Further, in contrast to AgRP administration, treatment of glucagonoma bearing rats with i.c.v. NPY worsens their anorexia, suggesting that cachexia does not result from a selective reduction in NPY release (102). These data suggest dysregulation of NPY system in inflammatory states. The nature of this process is uncertain, though the response to NPY is clearly diminished in animal models of disease. The interaction of the NPY system with inflammation is further complicated by the large number and divergent expression patterns of NPY receptors. Therefore, targeting the NPY system in cachexia may not be of therapeutic value.

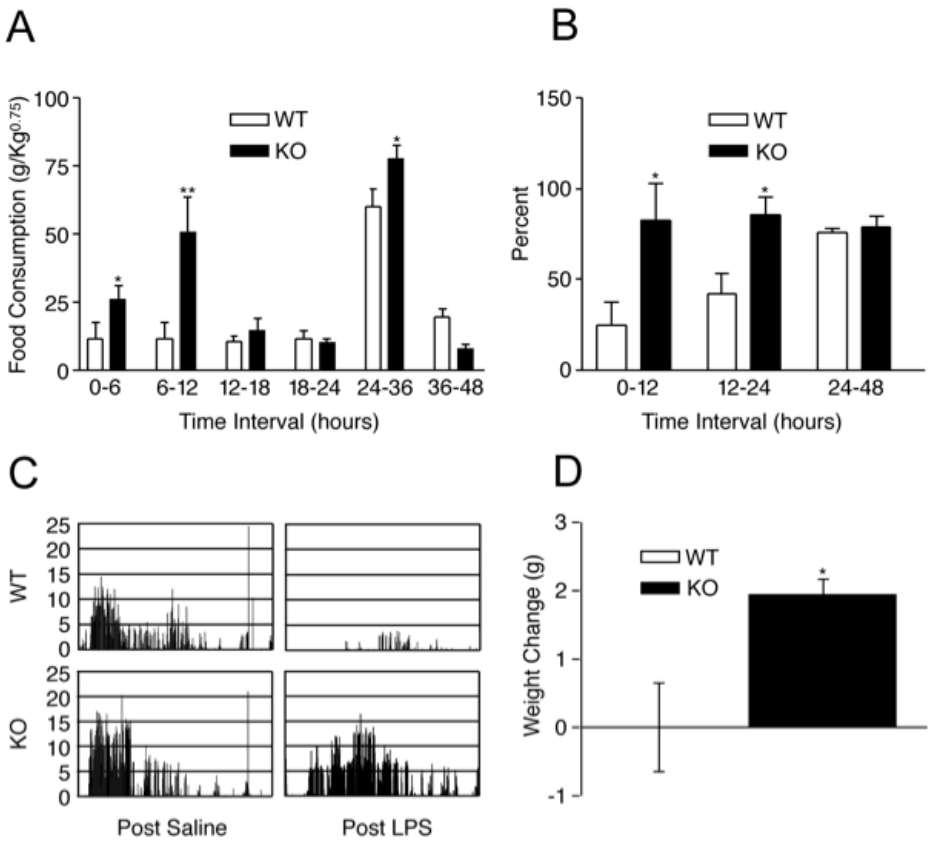

Figure 3. *MC4-RKO mice are resistant to LPS-induced cachexia and illness behavior.* LPS reduces food intake for approximately 36 h in WT mice, but not MC4-RKO mice, when expressed as a normalized value (A) or percent basal intake (B). LPS reduces wheel running activity below baseline in WT mice, but not MC4-RKO (C). Normal nocturnal increase in wheel running activity is maintained in MC4-RKO mice despite LPS-challenge. LPS-induced growth failure in young WT mice, while young MC4-RKO mice are resistant (D). *, $P < 0.05$, ** $P < 0.01$ v. WT control. Adapted with permission from Marks, D.L.; Ling, N.; and Cone, R.D. Role of the central melanocortin system in cachexia. Cancer Res, 2001. 61: 1432–8. Figure 2.

4. Neurohumoral Link

4.1 The Hypothalamus Responds to Circulating Signals of Energy Status

Although early ablation studies identified the hypothalamus as a critical regulator of energy balance (103, 104), it was not until parabiosis experiments between two spontaneously obese strains of mice, the *obese* (*ob/ob*) mouse and diabetic (*db/db*) mouse, that the existence of a neurohumoral link regulating food intake and adiposity was confirmed. In these experiments, *ob/ob* partners of *db/db* mice displayed fatal metabolic exhaustion, characterized by hypophagia, hypoglycemia, and weight loss, within four weeks of surgery (105). This seminal experiment demonstrated that excess of a circulating satiety factor can subvert normal energy homeostasis and lead to a fatal wasting disorder. Subsequently the *ob* gene encoding this factor was cloned and the protein was named leptin from the Greek *leptos*, meaning thin (106). The leptin receptor (Lep-R) was cloned from the *db* locus, and Lep-R was highly expressed in the hypothalamus, particularly in feeding and satiety centers (107). This finding, when taken together with lesion studies, revealed that the hypothalamus was the critical site for the integration of neural and humoral factors controlling energy balance. Subsequent studies have confirmed that the active isoform of Lep-R is expressed by POMC and AgRP neurons in the ARC, and that leptin specifically activates POMC neurons while inhibiting AgRP neurons (108-110). These studies describe an elegant neurohumoral network that is capable of supporting normal energy homeostasis, while being susceptible to changes in circulating factors.

4.2 Transmission of Signals Across the Blood Brain Barrier

Although leptin is secreted by adipocytes, structurally it resembles class I helical cytokines, such as IL-6, LIF, oncostatin-M (OSM), and ciliary neurotrophic factor (CNTF). Therefore, studies of leptin offer potentially valuable insights into the mechanism of cytokine transfer into the CNS and its actions there. Leptin levels in the CSF are strongly correlated to plasma leptin levels indicating that the molecule crosses the blood brain barrier (BBB) in proportion to its circulating concentration (111). The ARC is located immediately adjacent to the median eminence, a circumventricular organ with an attenuated BBB. It has long been thought that this anatomic location allows leptin and other cytokines access to ARC neurons. The microvasculature in the mediobasal hypothalamus has a specialized fenestrated endothelium, which could explain the passage of cytokines from the circulation to the CNS (112). Brain endothelial cells also directly participate in the transfer of inflammatory and metabolic signals into the CNS. The circulating satiety factors leptin and insulin exhibit saturable transport into the CNS, suggesting that passive diffusion is not solely responsible for their actions in the brain (reviewed in (113)). Similarly, active transport of several inflammatory cytokines into the CNS, including IL-1β, IL-6, and TNF-α, and LIF has been described, and these cytokines facilitate the transfer of each other across the BBB (114-117).

Endothelial cells are further known to release cytokines and diffusible inflammatory signals, such as prostaglandins (PG) and nitric oxide (NO) into the CNS in response to circulating cytokines. These small molecules readily cross the BBB and are sufficient and under some conditions necessary to provoke several aspects of the physiological response to sickness (118), including the induction of cytokine expression, fever, and short-wave sleep (119). Of particular interest is the synthesis of the prostanoid, PGE_2 by endothelial cells and perivascular phagocytes in immune-sensitive

brain nuclei in response to inflammation. PGE_2 is necessary for the induction of fever by LPS (120), and plays a role in the induction of anorexia by central or peripheral IL-1β(121). PGE_2 synthesis requires both arachadonic acid cleavage by the rate-limiting cyclooxygenase enzymes (COX-1 and COX-2) common to the production of all prostanoids, as well as microsomal prostaglandin E synthases (mPGES-1 and mPGES-2) (122). COX-2 and mPGES-1 are co-expressed and induced by inflammatory stimuli (123, 124). mPGES-1 KO mice exhibit attenuated cFos induction by LPS in immunosensitive CNS nuclei (125) and are relatively resistant to anorexia in a murine cancer model (126). Serrats and colleagues have eloquently described the role for endothelial cells and perivascular macrophages in transducing inflammatory signals from the circulation to the CNS (127). In inflammatory conditions, endothelial cell activation induces the production of PGE_2 by perivascular macrophages, which is in turn necessary for the induction of the hypothalamic-pituitary-adrenal (HPA) response. However, this cascade does not appear to be responsible for the induction of fever or hypomotility by circulating IL-1β or LPS. The HPA response can be blocked by inhibition of either COX-1 or COX-2, suggesting that both enzymes are involved in HPA activation by LPS (127, 128). Together these data support an important role for PG, particularly PGE_2, in relaying and amplifying inflammatory information in the CNS. However, the central mechanisms of prostaglandin action remain poorly understood. Specifically, it is unclear whether these molecules directly influence neuronal activation or whether they act as an intermediate signal. Interestingly, in cachectic patients COX inhibition has shown limited effectiveness at ameliorating anorexia or lethargy, indicating that either this mechanism is not conserved in humans or that COX inhibition alone is insufficient to block transmission of the inflammatory signals into the CNS.

4.3 The Vagus Communicates Inflammation Into the CNS

Several studies have examined a role for the vagus nerve in transducing inflammatory signals from the periphery. Blockade of endothelial IL-1R signaling prevents the induction of fever or lethargy by IL-1βadministered centrally or intravenously, but not intraperitoneally (129). Subdiaphragmatic vagotomy attenuates IL-1β expression and cFos immunoreactivity (IR) in the hypothalamus, brainstem and amygdala resulting from immune challenge, though treated animals still display significantly different cFos IR and cytokine expression compared to controls (130, 131). The role of the vagus in mediating the sickness response is controversial. Several investigators showed that vagotomy attenuates behavioral depression (131-133) and also anorexia (134). Others report that vagotomy does not block the anorectic component of sickness behavior (135). Given the different doses and routes of administration used in these studes, the vagus appears to be more important in mediating the behavioral response to small doses of immune modulating agents delivered into the peritoneal cavity. This may be due to the unique relationship of the abdominal vagus with adjacent immune cells, in which it increases its discharge rate in response to locally released cytokines. This leads to altered firing of excitatory catecholeminergic neurons originating in the NTS and projecting to several hypothalamic nuclei (136, 137). These studies reinforce the importance of brainstem participation in the development of sickness behavior and hypothalamic inflammation. How subdiaphragmatic vagotomy alters CNS cytokine expression in response to inflammation remains unclear, but it may remove an essential co-factor for cytokine production by neural cells. Unfortunately, there is no experimental evidence for differential cytokine expression in specific brain areas in vagotomized animals, so the influence of ascending autonomic tone on the hypothalamus is not well defined. Collectively, these data

illustrate the complex exchange from systemic inflammation to cytokine induction in the brain and further support a critical role for hypothalamic inflammation in the development of sickness behavior.

5. Interaction of Cytokines with the Central Melanocortin System

Although the primary cytokine signals producing cachexia during illness have been studied in detail, we have limited understanding of the specific hypothalamic cell groups involved in processing these signals. The success of melanocortin antagonism in ameliorating the catabolic features induced by several disease models suggests that POMC and AgRP neurons are plausible targets for CNS cytokine action (Figure 4). This idea is supported by data showing that ARC POMC mRNA was increased following peripheral LPS and TNF-ą whereas AgRP mRNA expression was simultaneously decreased by TNF-α (138, 139). Subsequent work, reviewed below, indicates that POMC and AgRP neurons are directly responsive to inflammatory cytokines.

5.1 IL-1β Regulates the Central Melanocortin System

IL-1β is often considered the “prototypic” pro-inflammatory cytokine because it is sufficient to drive the majority of the immune response. In a healthy brain, trace expression of IL-1β is detected in glia, neurons, cerebrovascular cells and circulating immune cells [reviewed in (140)]. Expression of IL-1β in the brain, mainly in the

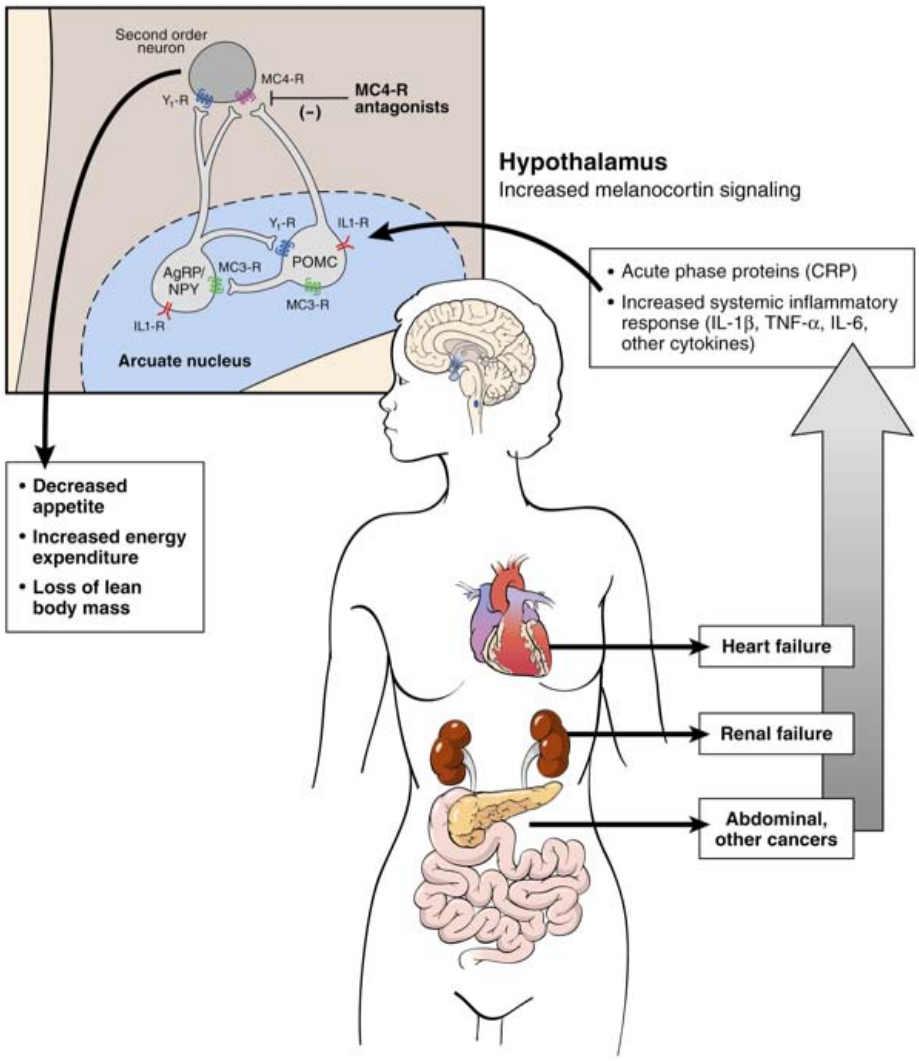

Figure 4. *Model for the role of the central melanocortin system in the production of cachexia during disease.*

During disease states, such as heart failure, renal failure, or cancer, levels of circulating inflammatory cytokines are increased. These circulating cytokines, including IL-1β, TNF-α, and IL-6, initiate inflammatory signaling within the ARC. Here, cytokines induce negative energy balance and wasting by increasing the activity of anorexigenic POMC neurons and inhibiting the activity of orexigenic NPY/AgRP neurons. Reprinted with permission from DeBoer, M.D. and Marks, D.L. Therapy insight: Use of melanocortin antagonists in the treatment of cachexia in chronic disease. *Nat Clin Pract Endocrinol Metab*. 2006. 2: 459-66.

hypothalamus, is markedly increased in response to disease and inflammation both in the periphery and within the CNS itself (141-144). Inflammation-induced brain IL-1β is primarily produced by microglia and perivascular and meningeal marcrophages (23). Two isoforms of IL-1, IL-1α and IL-1β, act as agonists at the IL-1 receptor (IL-1R), but only IL-1β is cleaved and secreted whereas IL-1αremains mainly intracellular (145, 146). Another member of the IL-1 family, the IL-1 receptor antagonist (IL-1ra), binds the IL-1RI, but does not initiate signaling (147).

The IL-1R is a membrane-bound receptor complex formed of two proteins, the type 1 IL-1R (IL-1RI) and the IL-1R accessory protein (IL-1RAcP), both of which are necessary for the cellular response to ligand (148). Binding of IL-1β to IL-1R initiates a signaling cascade involving the adaptor protein, MyD88, and several subsequent phosphorylation steps leading to nuclear translocation of NF-κB, a transcription factor that plays key role in initiating the cellular response to inflammation (148). Importantly, non-MyD88-dependent signaling can also be induced by IL-1R activation, which initiates distinct signaling cascades, including phosphorylation of phosphoinositide-3 kinase (PI3K) and subsequently protein kinase B (PKB or Akt) (149). In the brain, IL-1RI is expressed primarily by non-neuronal cells including endothelial cells, glial cells, and macrophages (69). Only a few neuronal cell groups express IL-1RI, however these groups are predominately found in areas associated with energy homeostasis and behavior including the ARC, NTS, area postrema, basolateral nucleus of the amygdala, hippocampus, and the trigeminal and hypoglossal motor nuclei (69, 150). Inflammatory challenge by LPS (151), IL-1β (95), or by chronic inflammation due to tumor growth (144) increases IL-1RI expression in the hypothalamus and hindbrain.

I.c.v. administration of IL-1β induces fever by altering the activity of thermosensitive neurons in the anterior and preoptic hypthalamus (152). This effect is driven locally by PGs, as fever is attenuated by COX inhibition (153). IL-1β activates the hypothalamic-pituitary-adrenal (HPA) axis leading to elevated plasma levels of ACTH and GCs (154). Central IL-1β also regulates feeding behavior and energy homeostasis. IL-1β potently suppresses food intake when injected peripherally and centrally (155, 156) and unlike IL-1β-mediated fever, the anorexic effect of IL-1β is not completely blocked by inhibition of PG synthesis (157, 158). Further, administration of IL-1ra into the lateral ventricle abrogates anorexia induced by peripheral IL-1βwithout affecting the febrile response (159). In the ARC, IL-1β induces cFos IR in both POMC and NPY neurons, suggesting involvement of the melanocortin system in mediating the feeding and metabolic effects of IL-1β (160).

Scarlett et al. investigated the nature of the relationship between IL-1β and the central melanocortin system (161), demonstrating that ARC POMC and AgRP neurons both express IL-1RI (Figure 5). Central IL-1β administration induces cFos IR in both of these neuronal types in the ARC, and this activation was only modestly attenuated by PG synthesis inhibition. Brainstem POMC neurons, however, do not appear to be specifically activated by i.c.v. IL-1β̃Activation of ARC POMC neurons was confirmed by whole-cell slice electrophysiology, which measured an increase in POMC neuron firing rate in response to IL-1β. IL-1β also increases the release of α-MSH from hypothalamic explants. This effect was blunted, but not reversed by inhibiting PG synthesis (161). IL-1β also significantly reduced AgRP release from hypothalami *in vitro*. Surprisingly, rat models of peripheral inflammation, including LPS, tumor, and chronic renal failure all increased AgRP mRNA expression above control, though i.c.v. IL-1β did not (162). The

in vitro and electrophysiology data strongly suggest that IL-1β like leptin, increases central melanocortin tone in the ARC by increasing the α-MSH/AgRP ratio. The paradoxically increased transcription of AgRP in inflammatory states may serve to prepare the animal for rebound hyperphagia, to recover energy stores lost during the acute illness. Taken together, these data demonstrate the existence of a neuroanatomic framework capable of supporting direct cytokine regulation of POMC and AgRP activity. These data conflict with a previous study, which showed worsened hypophagia following ablation of the medial ARC and suggested that the role of the ARC in cachexia may be to limit the extent of the anorexia (160). These ablation studies killed off the entire AgRP population as well as the medial POMC population. Lateral POMC neurons, which are thought to be more important in the control of body weight and feeding, were spared (78, 163, 164). Therefore, the worsened anorexia reported by this group may be explained by unopposed α-MSH at the MC4-R.

Central administration of IL-1β initiates a cachexia-like metabolic phenotype, which is reversed by melanocortin antagonism. Although IL-1β increases melanocortin signaling by acting directly on POMC and AgRP neurons, the relative contribution of this mechanism in disease states remains uncertain. However, the low EC_{50} for IL-1β-mediated changes in neuropeptide release and the anatomic location of IL-1β expression in inflammatory states, suggest that this mechanism is likely to play a significant role. Cachexia becomes overtly maladaptive only when wasting persists as in late-stage chronic disease. Although IL-1β potently induces acute anorexia, animals rapidly desensitize to continuous i.c.v. administration (165). Therefore, IL-1β may not be sufficient on its own to produce sustained catabolism. For this reason, other pro-

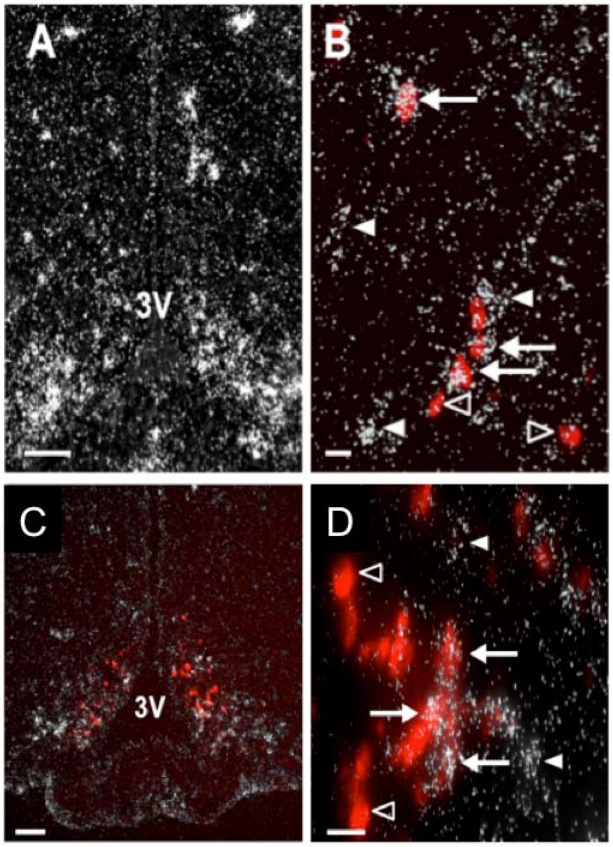

Figure 5. *POMC and AgRP neurons in the ARC express IL-1RI.*
(A) Low power dark field photomicrograph showing distribution of IL-1RI mRNA in the ARC. (B) Double-label *in situ* hybridization demonstrating expression of IL-1RI mRNA (*silver grains*) by cells expressing POMC (*red precipitate*). (C) Double-label *in situ* hybridization demonstrating label for IL-1RI mRNA (silver grains) and AgRP mRNA (red precipitate) in the ARC. (D) High power photomicrograph showing AgRP neurons express IL-1RI. *Arrows* represent cells that co-express POMC or AgRP and IL-1RI. *Open arrowheads* represent POMC or AgRP neurons that do not co-express IL-1RI mRNA. *Arrowheads* represent silver grain clusters not overlying POMC or AgRP neurons. Scale bars, 300 μm (A), 70 μm (B), 100 μm (C) and 25 μm (D). *3V*, Third ventricle. Adapted with permission from Scarlett, J.M.; Jobst, E.E.; Enriori, P.J.; Bowe, D.D.; Batra, A.K.; Grant, W.F.; Cowley, M.A.; and Marks, D.L. Regulation of central melanocortin signaling by interleukin-1 beta. *Endocrinology*, 2007. 148: 4217–25. Figure 7. and Scarlett, J.M.; Zhu, X.X..; Enriori, P.J.; Bowe, D.D.; Batra, A.K.; Levasseur, P.L.; Grant, W.F.; Meguid, M.M.; Cowley, M.A.; and Marks, D.L. Regulation of Agouti-related protein messenger ribonucleic acid transcription and peptide secretion by acute and chronic inflammation. *Endocrinology*. 2008. 149: 4837-45. Figure 5.

inflammatory cytokines must contribute to the persistent wasting syndrome.

5.2 Leukemia Inhibitory Factor (LIF) Chronically Suppresses Feeding

LIF was initially cloned and named for its ability to induce differentiation of the murine leukemic M1 cell line; it also plays an important role in inflammatory signaling, neuro-immune function, and development (166). Depending on alternative mRNA splicing, LIF is synthesized as either a secreted protein or a matrix-associated protein from a single gene locus (167). LIF is variably glycosylated, which directly influences its bioactivity in a cell-specific manner (168, 169). The effects of LIF further depend on the cell type and developmental stage of the organism. In the developing nervous system, LIF is neuropoietic and involved in the differentiation of autonomic nerves and astrocytes in several brain locations. In the adult animal, LIF is thought to be involved predominately in the inflammatory response to tissue damage or infection (reviewed in (170)).

LIF binds to a heterodimeric receptor complex consisting of the ligand-specific LIF receptor (LIF-R) and the signal transducing gp130 subunit, which is common to all IL-6 family cytokines, including IL-6, CNTF, and OSM (reviewed in (171)). Receptor binding stimulates gp130-mediated activation of the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway (172), the same pathway activated by the binding of leptin to its receptor (173). Importantly, STAT3 phosphorylation by any of these cytokines induces transcription of an autoregulatory gene product, suppressor of cytokine signaling-3 (SOCS-3), which inhibits further JAK/STAT signaling in response to ligand binding. This feedback inhibition is thought to play a role in leptin resistance (174).

Previous studies linking leptin-induced STAT3 phosphorylation to its anorectic effects suggest that IL-6 family cytokines could also be used to inhibit feeding (175). This hypothesis is supported by the observation that activators of gp130 acutely reduce food intake when injected in the CNS (176). Though there is consensus on the anorectic activity of LIF and CNTF in the brain, the ability of IL-6 to induce negative energy balance remains somewhat controversial (165). Targeted deletion of gp130 from POMC neurons prevents the anorectic effects of CNTF, demonstrating that direct cytokine signaling on POMC neurons alters energy balance (177). Central injection of adeno-associated viruses (AAV) expressing CNTF or LIF demonstrate that both of these cytokines are capable of sustaining negative energy balance in animals, in contrast with similar studies using chronic leptin administration (178). These studies suggest that despite initiating similar signaling cascades, gp130 activation must fundamentally differ from Lep-R signaling. A recent study indicates that differential activation of protein-tyrosine phosphatase 1B (PTP1B), an autoihibitory phosphatase induced in neurons by leptin but not by gp130 activation, may underlie these differences in sustained anorectic efficacy (179). A role for PTP1B in cachexia is further supported by research demonstrating that its expression is diminished in tumor-bearing rats and that silencing of the gene in the brain using antisense oligonucleotides can cause fatal anorexia (180). In adipocytes, it has been shown that some, but not all, members of the gp130 cytokines exhibit cross desensitization, effectively suppressing the activation of JAK/STAT signaling by other family members. This phenomenon has been attributed to differential abilities of these cytokines to induce phosphorylation of LIF-R, which leads to subsequent receptor degradation (181). This difference explains the divergent abilities of

leptin, LIF, and CNTF to induce sustained anorexia as well as to inhibit the activity of each other.

Serum LIF is elevated in chronic disease and malignancy (182-184), an observation that correlates with poor prognosis (185). Hypothalamic expression of LIF is induced by acute LPS administration (32, 58, 62). In contrast to IL-1β or LPS, animals do not desensitize to chronic LIF-induced anorexia (186-188). This lack of tachyphylaxis more closely resembles clinical cachexia, indicating that LIF could be an essential CNS mediator of chronic inflammation-induced anorexia. Studies by Shlomo Melmed and colleagues demonstrated that LIF expression in the pituitary is necessary to drive the increased POMC mRNA expression and ACTH release by pituitary corticotrophs in response to inflammation (32, 189). This process is dependent on gp130-mediated activation of JAK2/STAT3 signaling, which is known to underlie the induction of POMC mRNA expression by leptin, suggesting that LIF may activate hypothalamic POMC neurons in a similar manner (175, 190).

6. Sickness Induced Lethargy and Inactivity

For the reasons outlined above, anorexia is the most well understood feature of sickness behavior. No less significant is the induction of profound lethargy and inactivity as a result of illness. Like eating, locomotor activity is a complex behavior resulting from the summation of many motivational, emotional, circadian, and physiologic inputs. Unlike feeding behavior, however, decreases in activity are not inherently linked to a subsequent negative outcome. Rather, measurements of ambulation provide insight into

the overall well being of an individual. Several studies in humans have demonstrated that LMA correlates tightly with self-reports of quality of life and psychological well being; increasing daily activity exhibits a dose-response with overall health in elderly populations (191-194). Furthermore, increased daily activity provides therapeutic benefit in a range of chronic illnesses (195). Thus, improving LMA in the chronically ill may favorably impact quality of life, mental health, and response to treatment.

Ameliorating lethargy and improving attention may have utility in acutely ill patients, as well. Patients with mildly symptomatic self-limiting infections report difficulties in completing daily tasks, focusing, and maintaining wakefulness (196). Though suppressing volitional activity is the result of a concerted effort by the immune system to redirect energy expenditure to fight the infection, it is not always possible to take the necessary time to recover. High stress occupations, such as military personnel, commercial pilots, and healthcare professionals, often must work through illness, despite some loss of faculties. Therefore, understanding and blocking the central mechanisms involved in lethargy induction could also have beneficial effects among the acutely ill.

The onset of lethargy in response to illness occurs prior to the development of limiting physical debilitation. Thus, lethargy appears to be an active process that occurs in response to inflammation. Inactivity is reported in cancer patients without any corresponding increase in sleep or decrease in exercise capacity, indicating that their physical activity is specifically suppressed (193). Little research has directly examined the mechanisms of inflammation-induced lethargy. However, several groups have examined the effects of inflammatory cytokines on neurons in nuclei that are critical for the regulation of arousal. Furthermore, the neural circuits and factors that underlie the

control of arousal and wakefulness have been heavily investigated. The regulation of sleep has been investigated most thoroughly. Although peripheral to the focus of this thesis, sleep and arousal studies define neuronal populations that may be susceptible to inflammatory signaling, as discussed below.

7. Central Regulation of Arousal

Investigation of the brain regions involved in regulating arousal began with the observations of a Viennese neurologist, Baron Constantin von Economo, during the early 20th century. During this period, a disorder of sleep and wakefulness regulation known as encephalitis lethargica spread throughout Europe and North America. Most patients slept excessively—often more than 20 hours per day—until the resolution of the encephalitis. Though he never identified the pathogenic virus, von Economo observed that nearly all patients had lesions where the midbrain meets the diencephalon (197). This finding led him to postulate the existence of an arousal system in which the brainstem modulates the alertness of the forebrain. Subsequent research confirmed the existence of such a neural pathway and specifically described components including the rostral pons and the midbrain reticular formation (198).

This “ascending reticular activating system” was later shown to have two branches: an upper pathway consisting of cholinergic neurons in the pedunculopontine and laterodorsal tegmental nuclei (PPT/LDT), which activate the thalamic relay nuclei that transmit information to the cortex; and a lower pathway consisting of monoaminergic neurons in the upper brainstem which project to the hypothalamus, basal forebrain, and

directly to the cortex (199)(Figure 6). These brainstem nuclei include the noradrenergic locus coeruleus (LC), the serotoninergic dorsal (DR) and median raphe (MnR) nuclei, and the dopaminergic ventral periaqueductal grey matter. Also included in this pathway are histaminergic neurons in the tuberomammillary nucleus (TMN), located in the caudal hypothalamus. In general, the neurons in each of the aforementioned components of the ascending arousal system are active during wakefulness and activity and suppressed during rest and sleep. Thus, the somnolence of von Economo's patients could be explained by a loss of ascending arousal tone. However, a smaller percentage of patients with encephalitis lethargica presented with the opposite complaint. Rather than excessive sleepiness, these patients reported debilitating insomnia, which persisted despite reports of extreme tiredness (200). These patients exhibited lesions in the anterior hypothalamus and basal ganglia, indicating that the forebrain targets of the arousing brainstem nuclei send reciprocal connections responsible for suppressing arousal during rest and sleep. Indeed, as discussed below, research during the 1980s and 1990s confirmed the existence of such a pathway.

7.1 Anatomy of the Ascending Arousal System

The majority of research on the ascending arousal system and its target circuitry has focused on the promotion of sleep and wakefulness, and on the control of the transitions between these states. Given that wakefulness can generally be defined as the maintenance of a requisite level of physiologic and cognitive arousal and sleep as the suppression or lack of such arousal, this body of work identifies possible contributors to inflammation-induced suppression of arousal. Both the upper and lower pathways of the ascending arousal system contain wake-active, arousal-inducing neurons. PPT/LDT neurons send excitatory projections to the thalamus, where this input functions as a

critical gating mechanism (201). Cholinergic tone here prevents thalamic neuron hyperpolarization, thereby allowing thalamocortical sensory transmission (202). PPT/LDT neurons are most active during both wakefulness and rapid eye movement (REM) sleep, and inactive during non-rapid eye movement (NREM) sleep (203). A similar role is performed by cholinergic neurons in the basal forebrain, which receive excitatory inputs from the lower monoaminergic ascending brainstem pathway (199). Acetylcholine-expressing neurons here also project to the thalamus, presumably playing a similar gating role, and send direct excitatory projections to the cortex (204). A critical role for basal forebrain inputs in maintaining wakefulness is supported by studies demonstrating that lesions of this region produce coma (205). Interestingly, pharmacologic antagonism of cholinergic receptors has little effect on overall sleepiness, but potently inhibits REM sleep, demonstrating that cholinergic signals are not required for sleep induction (206).

The lower brainstem pathway of monoaminergic neurons also exhibits activity during wakefulness, with less during NREM sleep and, in contrast to PPT/LDT neurons, virtually none during REM sleep (207-209). The primary targets of these neurons are nuclei within the hypothalamus, including the preoptic nuclei in the rostral hypothalamus and the lateral hypothalamus (LHA), more caudally (199). These hypothalamic nuclei relay ascending monoaminergic tone to the basal forebrain and cerebral cortex, and share reciprocal connections with the brainstem arousal centers (202).

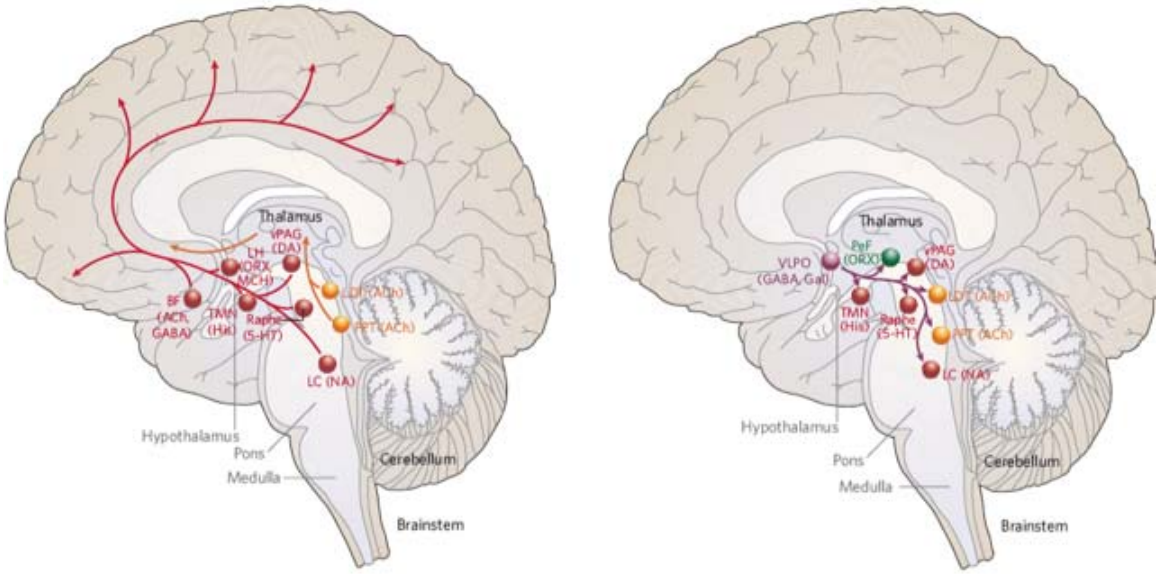

Figure 6. *Diagram showing projections of ascending arousal system and reciprocal inhibitory projections from VLPO.*

(Left panel) Thalamocortical transmission is facilitated by input to the relay and reticular nuclei of the thalamus (yellow pathway) from the cholinergic (ACh) pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDT). The monoaminergic arousal pathway (red) activates the cerebral cortex to facilitate the processing of inputs from the thalamus. This arises from neurons in the tuberomammillary nucleus (TMN) containing histamine, the A10 cell group containing dopamine (DA), the dorsal and median raphe nuclei containing serotonin (5-HT), and the locus coeruleus (LC) containing norepinephrine. This pathway also receives contributions from peptidergic neurons in the lateral hypothalamus (LHA) containing orexin (ORX) or melanin-concentrating hormone (MCH), and from basal forebrain (BF) neurons that contain γ-aminobutyric acid (GABA) or ACh. (Right panel) The somnogenic VLPO sends reciprocal inhibitory projections to each of the nuclei comprising the ascending arousal system. Reprinted with permission from Elsevier from Saper, C.B.; Scammell, T.E.; Lu, J. Hypothalamic regulation of sleep and circadian rhythms. *Nature*. 2005. 437: 1257-63.

7.2 VLPO Neurons Promote Sleep

Although loss of ascending excitatory transmission is sufficient to induce sleep, normal sleep-wake transitions require a rapid and reliable “switch”. As von Economo’s insomniac patients demonstrated, the anterior hypothalamus contains a vital region for sleep induction (200). Examination of the inputs to brainstem monoaminergic nuclei confirmed the existence of a region of sleep-active neurons in the ventrolateral preoptic (VLPO) nucleus (210). These neurons contain the inhibitory neurotransmitters γ-amino butyric acid (GABA) and galanin and project to all of the monoaminergic nuclei in the brainstem and hypothalamus that participate in arousal (210-213). Lesions of the VLPO lead to severe reductions in both REM and NREM sleep, though the specific location of the lesion alternatively inhibits NREM (VLPO cluster) v. REM (extended VLPO) sleep (214-216). These findings correlate with innervation patterns, as the VLPO cluster primarily innervates the tuberomammillary nucleus of the hypothalamus (TMN), whereas the extended VLPO sends projections to the locus coeruleus (LC) and raphe nuclei (217, 218). The VLPO receives projections from each of these monoaminergic nuclei and neurons in this nucleus are inhibited by both serotonin (5HT) and dopamine (DA) (219, 220). VLPO neurons do not express histamine receptors, but neurons projecting from the TMN also contain inhibitory peptides, and may exert an inhibitory influence on the VLPO via one of these neurotransmitters (221).

7.3 Regulation of Sleep/Wake Consolidation and Transitions

Two paradoxical features characterize sleep. The first is the phenomenon of consolidation--that is, humans and other animals tend to concentrate sleep into one or more long periods of rest. Several studies have demonstrated that consolidation of sleep is critical for its restorative properties. Animals and humans that undergo fragmented

sleep exhibit decreased attention and cognitive ability, increased anxiety, and depressed mood (222, 223). The second is that sleep-wake transitions occur rapidly, regardless of how long an individual has been in either state. Not only is this a salient feature, it is essential to survival. Animals must be able to react quickly to threats that present themselves during periods of slumber. The existence of a transitional state of reduced arousal would create periods of waking during which an animal's alertness is insufficient, placing that animal at increased risk. How does arousal circuitry establish two states of relative stability, while ensuring a rapid transition between these states?

Saper and colleagues describe the regulation of sleep-wake by hypothalamic and brainstem nuclei as a "flip-flop switch", producing two opposing states with sharp transitions (199)(Figure 7). In this model, monoaminergic nuclei activate hypothalamic, basal forebrain, and cortical regions while inhibiting VLPO neurons, leading to a wakeful animal. In this state the ascending arousal signal is both inciting downstream neural activity as well as relieving reciprocal inhibition of the monoaminergic cells by the VLPO. In the sleep state, VLPO neuron firing inhibits the monoaminergic cells, blocking both their arousal-inducing effects and their inhibition of the VLPO neurons. It is this mutual inhibition that underlies the instability of transition states, as factors that favor one state or another will rapidly alter the direction of the switch. For this system to effectively execute rapid changes, mathematical modeling predicts that weakening either side would not only alter the relative stability of each state, but also result in more frequent transitions between these two states (199). Accordingly, animals with VLPO lesions sleep less, wake up more often, and have a marked decrease in sleep bout duration (215). Therefore, consolidation of sleep and wakefulness requires the influence of stabilizing factors that are not direct participants in the flip-flop switch.

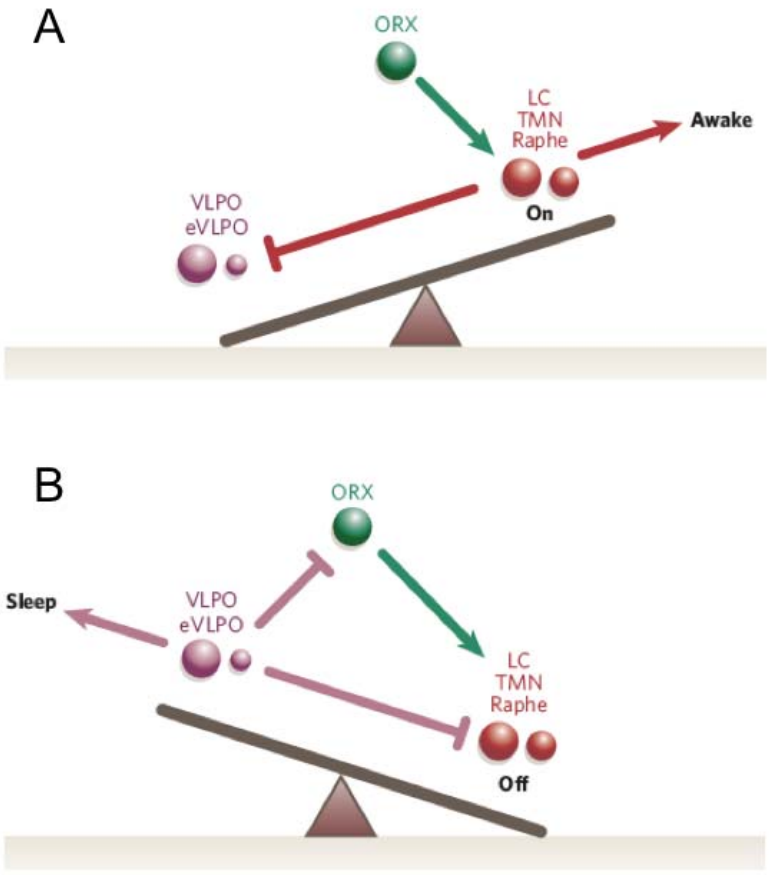

Figure 7. *Schematic diagram of flip-flop switch model of sleep-wake regulation.*
During wakefulness (A), the monoaminergic brainstem nuclei (red) inhibit the sleep-promoting VLPO (purple), thereby relieving the inhibition of the monoaminergic cells, and that of the orexin (ORX) neurons (green). Because the VLPO neurons do not have orexin receptors, the orexin neurons serve primarily to reinforce the monoaminergic tone, rather than directly inhibiting the VLPO. During sleep (B), the firing of the VLPO neurons inhibits the monoaminergic cell groups, thereby relieving their own inhibition. This also allows it to inhibit the orexin neurons, further preventing monoaminergic activation that might interrupt sleep. The direct mutual inhibition between the VLPO and the monoaminergic cell groups forms a flip-flop switch, which produces sharp transitions in state, but is relatively unstable. The addition of the orexin neurons stabilizes the switch. eVLPO, extended ventrolateral preoptic nucleus; LC, locus coeruleus; TMN, tuberomammillary nucleus. Reprinted with permission from Elsevier from Saper, C.B.; Scammell, T.E.; Lu, J. Hypothalamic regulation of sleep and circadian rhythms. *Nature*. 2005. 437: 1257-63.

7.4 Circadian Regulation of Arousal

Throughout their 24 h cycle, animals show remarkable consistency in their patterns of activity and sleep, suggesting a strong circadian influence on arousal. This periodicity is maintained, even when animals are removed from normal light/dark patterns. Studies on rodents maintained in constant darkness, as well as human forced desynchrony studies using a 28 h day have confirmed a strong 24 h cycle in both sleep drive and LMA patterns (224-227). Recent animals studies have demonstrated that the suprachiasmatic nucleus (SCN) of the hypothalamus acts as a master clock. The SCN contains a set of neurons that fire in a 24 h cycle and, through synaptic and circulating factors (GC and melatonin), synchronize the cellular clocks throughout the body (228). The firing cycle of these neurons is reset daily by light inputs from the retina such that the animal's circadian rhythm remains in accordance with its environment (229). The SCN does not directly innervate any of the ascending arousal system nuclei. The majority of the projections from the SCN are sent into the adjacent subparaventricular zone (SPZ) and the dorsomedial hypothalamus (DMH) (230). The SPZ also sends projections to the DMH, which in turn sends projections to the VLPO, LHA, and other regions implicated in the control of circadian physiology, including GC release and feeding (219, 231-233). Thus, the SCN indirectly communicates with arousal centers through intermediaries in the DMH. The importance of these DMH neurons in maintaining circadian control is illustrated by lesion studies demonstrating decreased LMA, wakefulness, GC release, and feeding in DMH-ablated animals (234).

Further, DMH neurons can supersede SCN clock neurons in altering circadian control of arousal. Several studies have described an adaptive phenomenon whereby restricting an animal's feeding to a fixed time each day, during the animal's normal

sleeping period, can alter circadian rhythms such that the animal exhibits peak arousal during the period immediately preceding feeding (235, 236). The resulting peak in activity, referred to as food-anticipatory activity (FAA), develops independent of the SCN, which remains phase-locked to the light/dark cycle (237-239). Neurons in the DMH express cFos IR in synchrony with daily feeding, suggesting this region is a crucial component of the food-entrainable oscillator (240). In response to food restriction (FR) DMH neurons alter the expression pattern of clock genes, synchronizing with the feeding schedule rather than the light/dark cycle. Mice null for the clock gene *Bmal1* do not respond to FR by increasing preprandial LMA, but anatomically specific restoration of *Bmal1* in the DMH of *Bmal1-KO* mice restores FAA in these animals (241, 242). Therefore, neurons in the DMH can integrate circadian signals from the SCN with metabolic signals to coordinate arousal. Recent work has shown, however, that lesions of the DMH do not prevent the development of FAA in response to FR, illustrating that these neurons are sufficient, but not necessary for food entrainment of biological rhythms (243, 244).

7.5 Homeostatic Regulation of Arousal

Like all homeostatic systems, sleep accrual is closely regulated such that periods of sleep deprivation lead to subsequent compensatory increases in proportion to the sleep loss. Therefore, it has been posited that a sleep-promoting factor accumulates during wakefulness and dissipates with sleep, acting as a “sleep homeostat” (202). The existence of such a factor is supported by electroencephalogram (EEG) observations in sleep-deprived humans. The emergence of delta waves, or slow wave activity (1-3 Hz), defines stage 3-4 sleep, also known as deep sleep. It is during these stages that NREM sleep predominates and that transitions into REM sleep occur. As sleep deprivation

accumulates, delta wave power, indicating greater sleep intensity, increases, suggesting that sleep debt is measured by a biological substrate (245). The identity of this sleep homeostat is unknown, but it is known that VLPO neurons do not independently accumulate a need to sleep (210). These neurons exhibit no change in activity during wakefulness regardless of sleep debt amassed. They only increase firing when the animal falls asleep, but then discharge at an increased rate, suggesting that they are sensitive to homeostatic factors (214).

This homeostatic sleep-promoting substrate may interact with circadian factors to promote sleep consolidation. During wakefulness, circadian arousal-promoting factors must directly oppose the wake-dependent increase in sleep propensity. As the animal shifts into its resting period, this circadian drive for wakefulness is removed at the zenith of homeostatic sleep promotion, leading to sleep initiation. It is thought that consolidated sleep results from a circadian sleep drive opposing a decrease in homeostatic sleep drive. Basal forebrain adenosine (AD) is one candidate homeostatic regulator of sleep (203). During wakefulness and increased brain activity, the brain depletes adenosine triphosphate (ATP) levels, hydrolyzing it to adenosine diphosphate (ADP), and eventually AD. AD then accumulates, exerting a somnogenic influence on VLPO neurons (246-248). Supporting a possible role in sleep homeostasis, central AD induces sleep, and extracellular levels of AD increase throughout wakefulness and decrease throughout sleep (249, 250). These circadian and homeostatic models can explain the phenomena of periodicity and consolidation of arousal or rest, but do not address rapid or short-term changes in behavior that occur as a result of stress or illness.

7.6 Allostatic Regulation of Arousal

It is well described that restraint, feeding, and inflammation have potent effects on LMA and sleep, and that such perturbations alter the circadian rhythm. Thus, arousal systems are also controlled in accordance with complex and transient environmental signals. Allostatic drive was first coined to describe systems designed to fluctuate to meet perceived and anticipated demands from external forces rather than to maintain constancy (251). Although we know little about how external forces regulate the arousal system, several important relationships between environmental perturbations and arousal state have been described. Feeding, stress, and inflammation all influence arousal, often overcoming both homeostatic and circadian regulation. The vagus may relay this information to arousal centers--it has long been recognized that alterations in vagus activity correlate with sleep/wake state (252, 253). Feeding inhibits arousal through the coordinated actions of visceral inputs to the NTS from gastric stretch sensors as well as the effect of circulating satiety hormones, including insulin, glucose, and cholecystokinin (254-256). Conversely, undernutrition augments arousal and LMA, putatively through opposing signals that indicate energy deficit (257, 258).

Emotional and cognitive states both exert strong influences on arousal, though the mechanisms of this effect are somewhat less clear. Recent work has identified inputs from corticolimbic sites into key areas of the hypothalamus, including the SCN, VLPO, and LHA (219, 232, 259, 260). Further, PET studies in patients with insomnia report an increase in activity in corticolimbic sites during sleep compared to non-insomniac controls (261). Emotional or physiologic stress has a potent activating effect on neurons in the PVN that express corticotrophin-releasing hormone (CRH) (262). These neurons are known to send excitatory inputs into the LHA, which could thereby promote arousal,

though a role for these neurons in the allostatic control of vigilance has not been confirmed (263).

8. The Orexin System

8.1 Orexin Peptides and Receptors

In 1998 two separate groups reported the discovery of hypothalamic neurons that express a neuropeptide called orexin (Ox) (264) or hypocretin, by the other (265). Expression of Ox in the CNS is limited to neurons in the lateral hypothalamic area (LHA), and parts of the DMH, perifornical area (PFA), and posterior hypothalamus. A single preproorexin (*Ox*) precursor transcript is processed into two peptides, orexin-A (OxA) and orexin-B (OxB), which appear to colocalize in the terminals of all Ox neurons (264, 266). Currently, little is known about how the specific production or release these two proteins is regulated, though little evidence supports alternative regulation or transport of the two isoforms (267). Ox neurons project to diverse regions throughout the CNS, including the cerebral cortex, brainstem, thalamus, and hypothalamus. Dense Ox IR projections are found in several nuclei involved in arousal regulation, including the LC, the raphe nuclei, the TMN, the PPT/LDT, and the VLPO (268). Orexins are excitatory peptides and histologic data demonstrate that many Ox-containing terminals also release glutamate (269).

The receptors for the Ox peptides, the type-1 (OX1R) and type-2 (OX2R) Ox receptors are expressed in neuroanatomically discrete regions matching the location of Ox IR terminals (270). Although these G-protein coupled receptors are widely

distributed, few regions express both receptor types (271). OX1R mRNA is expressed in the hypothalamus, the LC, the cerebral cortex, the ventral tegmental area (VTA), and several brainstem nuclei (271). OX2R mRNA is expressed in the TMN, brainstem cholinergic nuclei, including the PPT and LDT; and shows overlapping expression with OX1R in the hypothalamus and VTA (271). The Ox receptors exhibit different binding affinities for the two peptides, with OX1R being 100-1000 times more selective for OxA than OxB (264). Both Ox peptides appear to have equal activity at the OX2R, however. OX1R is thought to signal via $G_{\alpha 11}$ class of G-protein, leading to increases in phospholipase C and subsequent phosphatidylinositol signaling, resulting in Ca^{2+} influx. OX2R is coupled to both the $G_{\alpha 11}$ excitatory and the G_i inhibitory G-protein families (272). The Ox receptors are located post-synaptically, and the excitatory effects of orexins persist in the presence of tetrodotoxin, suggesting direct activation of post-synaptic neurons (273).

8.2 Orexins and Narcolepsy

Initial pharmacologic studies indicated that these peptides are important for energy homeostasis. Central administration of orexins increases food intake in rodents and fasting induces an increase in *Ox* mRNA expression (264). Mice null for *Ox* expression are hypophagic, but obese, suggesting that orexins play a vital role in both feeding behavior and energy expenditure (274, 275). Further examination of the behavior of these mice determined that the animals exhibit excessive sleepiness throughout the light and dark phase, and that their decreased food intake could largely be attributed to their inactivity.

Approximately one year following the discovery of Ox neurons, two groups reported that lack of either orexins or OX2R could cause narcolepsy-like symptoms in experimental animals (274, 276). Narcolepsy is a sleep-wake disorder characterized by attacks of uncontrollable daytime sleepiness and cataplexy, complete loss of muscle tone during emotional situations causing patients to collapse (277). Subsequent research into the role of Ox in narcolepsy determined that humans with narcolepsy have few chemically identifiable Ox neurons in the LHA and depressed levels of Ox peptides in the CSF (278-280). Mutations in either Ox or its receptors have been identified in very few narcolepsy patients (277, 279). The disease is late-onset, with most patients presenting in their second or third decade of life. It also appears to be a very specific ablation of Ox neurons, as neighboring neurons in the LHA that express melanin-concentrating hormone (MCH) are unaffected (278, 279). Current consensus suggests that narcolepsy is caused by autoimmune destruction of otherwise normal Ox neurons, though little evidence exists to directly support this conclusion (277). Importantly, Ox peptide replacement prevents cataplexy and improves wakefulness in an Ox neuron-ablated model of narcolepsy in mice, demonstrating that peptide replacement is a viable treatment modality for patients with narcolepsy and spurring pharmaceutical interest in the design of Ox agonists (281). Because specific agonists and antagonists of the Ox receptors have not been developed, the physiologic functions of these receptors are not well differentiated. However, narcolepsy appears to be due to a specific defect in OX2R signaling, as OX1R deficient mice only exhibit increased sleep fragmentation and not narcolepsy/cataplexy (282). OX1R likely plays some role in sleep regulation, however, because OX1R/OX2R double knockout mice exhibit a more severe narcolepsy phenotype than OX2R single knockout mice (283).

8.3 Neurotransmitters Regulating Orexin Neuron Activity

Electrophysiologic studies, using mice that express green fluorescent protein (GFP) under the control of the Ox promoter, identified neurotransmitters that activate or inhibit Ox neuron activity. In slice culture, the firing rate of orexin neurons is primarily modulated by changes in glutamatergic tone (284). The inhibitory neurotransmitter, GABA, hyperpolarizes Ox neurons and reduces activity via $GABA_B$ receptors expressed on Ox neurons, though the importance of tonic GABA-mediated inhibition has not been evaluated (285). Ox neurons exhibit varied responses to cholinergic input, though more neurons were activated than inhibited by the cholinergic agonist carbachol (286). Monoaminergic neurotransmitters implicated in arousal, including DA, 5HT, and norepinephrine (NE), all inhibit Ox neuron activity. Both 5HT and NE hyperpolarize and inhibit Ox neurons by activating G-protein-regulated inwardly rectifying K^+ channels (GIRK). These effects are mediated by the α_2-adrenoreceptors and $5HT_{1A}$ receptors, each of which is expressed by Ox neurons (286, 287). This finding indicates that these monoaminergic systems, which are activated by Ox, exert feedback inhibition on the Ox system. Ox neurons do not express DA receptors, but DA can inhibit Ox neurons via the α_2-adrenoreceptor (286, 287). Histamine, however, does not activate or inhibit Ox neurons.

8.4 Orexins Stabilize Wakefulness

Recent research validated a critical role for Ox neurons in maintaining arousal. Ox neurons are active during wakefulness and quiescent during sleep, though they exhibit some burst activity during REM sleep (288-290). The concentration of OxA in the CSF of rats exhibits circadian variation such that levels increase throughout the active phase, reaching a peak at the end of the dark period. CSF OxA concentration then falls

throughout the light phase, with a nadir just before lights off (291, 292). Given that no enzyme or transporter that clears orexins from the synapse has been described, these levels presumably reflect the dissipation of orexins from the synaptic cleft and their accumulation in the CSF. Central administration of OxA or OxB increases time spent awake and decreases the time spent in either NREM or REM sleep (293, 294). Additionally, the Ox system is required for emergence from general anesthesia (295). Together, these data led many investigators to hypothesize that orexins are the key brain substrate controlling wakefulness. In a 2005 review, Saper et al. propose that orexins function to stabilize wakefulness by reinforcing arousal systems, “like a ‘finger’ on the switch that might prevent unwanted transitions into sleep.” (199). Thus, according to the flip-flop switch hypothesis, removal of an arousing factor (orexins) should destabilize the sleep-wake switch, leading to more frequent shifts in arousal state. Indeed, patients with narcolepsy experience more frequent sleep-wake transitions but do not spend more time sleeping than normal individuals (277). Similarly, *OxKO* mice exhibit normal circadian rhythm and normal amounts of sleep, but their sleep is markedly fragmented, characterized by significantly more sleep-wake transitions than control animals (296). Transgenic mice, in which Ox neurons express the light-sensitive cation channel, channelrhodopsin-2, were generated to test whether Ox neurons are the key neural mediator of wakefulness (297). In these animals, depolarization of Ox neurons increased the probability of and decreased the latency for sleep-wake transitions. However, they did not immediately rouse, and there was considerable variability in the latency to waking. This elegant work confirms a direct role for Ox neurons in reinforcing arousal, but demonstrates that the firing of these neurons is insufficient to independently drive the transition to wakefulness. Accordingly, the pharmaceutical industry has pursued the utility of Ox antagonism as a treatment for insomnia. A new orally administered, non-specific reversible OXR antagonist, ACT-078573 (“Almorexant”), is

currently in clinical trials and appears to induce sleep without inciting cataplexy, reinforcing the important role of Ox signaling in regulating arousal (298).

Consistent with these physiologic findings are electrophysiologic observations that Ox peptides excite neurons in nuclei that regulate arousal. *In vitro* slice preparations confirmed an excitatory role for orexins on noradrenergic LC neurons (273, 299), serotoninergic DR neurons (300, 301), histaminergic TMN neurons (302, 303), dopaminergic VTA neurons (304), and cholinergic neurons in the basal forebrain (305). Ox neurons also project to cholinergic neurons in the PPT/LDT. Direct injection of OxA into the LDT induces wakefulness in cats and causes long-lasting activation of cholinergic neurons there (306, 307). Actions of OxA in the PPT are more complex, as OxA appears to indirectly inhibit some cholinergic neurons there by activating GABAergic interneurons (308). Because PPT neurons are thought to promote both wakefulness as well as REM sleep, these paradoxical observations could reflect the functional divergence of cholinergic neurons in the regulation of arousal. Ox neurons project to, and receive projections from, inhibitory sleep-promoting neurons in the VLPO (219, 232, 259). Whereas VLPO neurons act directly on Ox neurons to inhibit their firing, they do not themselves express Ox receptors (309). Thus, any effect of Ox on the activity of these neurons is likely mediated by a local inhibitory circuit. The interactions between Ox neurons and other arousal regulating regions are summarized in (Figure 8).

8.5 Orexins Promote Arousal in Response to Depleted Energy Status

The physiologic effects of orexins extend beyond promoting wakefulness. Arousal is also characterized by increased LMA and heightened responses, and orexins

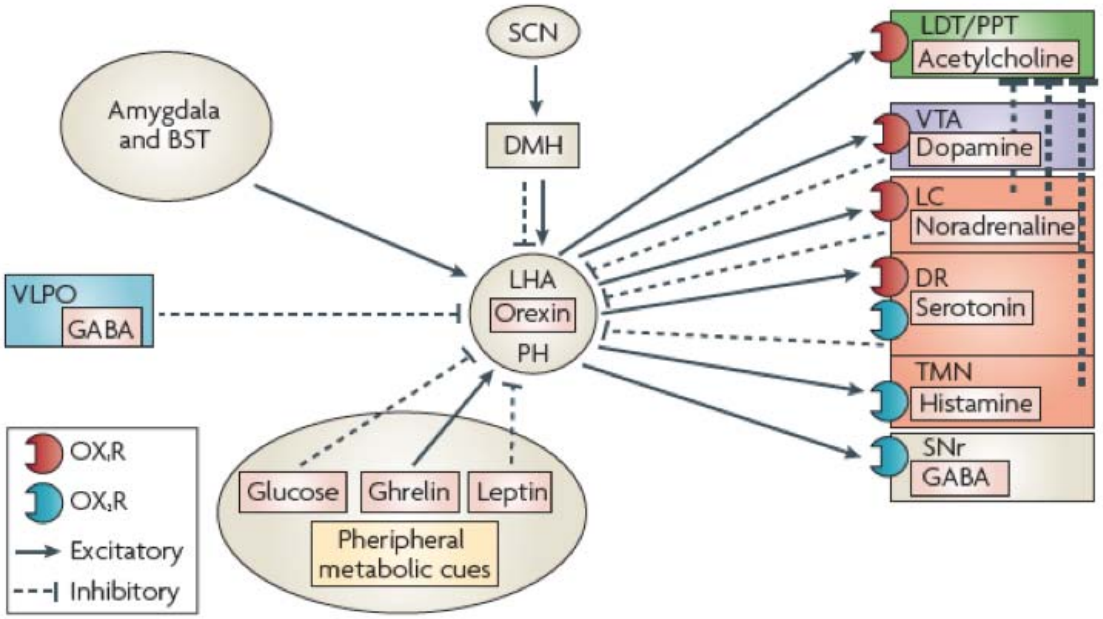

Figure 8. *Interactions between orexin neurons and other brain regions implicated in the regulation of arousal.*

Orexin neurons in the lateral hypothalamic area (LHA) provide a link between the limbic system, systems involved in energy homeostasis and arousal-associated neurons in the brainstem. Solid arrows show excitatory projections, and broken lines inhibitory ones. Wake-active regions, sleep-active regions and REM-active regions are shown by red, blue and green boxes, respectively. Orexin neurons activate monoaminergic nuclei that are engaged during wakefulness. Orexins stimulate dopaminergic systems, which mediate effects on locomotion and reward (purple). Peripheral metabolic signals such as leptin, ghrelin and glucose influence orexin neuronal activity to coordinate arousal and energy homeostasis. The nucleus suprachiasmaticus (SCN), the central body clock, sends signals to orexin neurons via the dorsomedial hypothalamus (DMH), which also acts as a food-entrainable oscillator, and influences orexin neuronal activity. Input from the limbic system (amygdala and bed nucleus of the stria terminalis (BST)) might regulate the activity of orexin neurons upon emotional stimuli to evoke emotional arousal or fear-related responses. *VLPO*, ventrolateral preoptic area; *DR*, dorsal raphe; *GABA*, γ-aminobutyric acid; *LC*, locus coeruleus; *LDT*, laterodorsal tegmental nucleus; *PPT*, pedunculopontine tegmental nucleus; *SNr*, substantia nigra pars reticulata; *TMN*, tuberomammillary nucleus.

exhibit their largest effects when arousal is necessary to respond to physiologic, emotional, or environmental challenges. Indeed, Ox neurons are active especially during periods of increased LMA, such as exploration of a novel environment or foraging (288-290, 310). Similarly, Ox antagonists decrease LMA and performance on cognitive tasks, such as the Morris water maze, and promote sleep (298, 311-314). As discussed above, a fundamental challenge for all organisms is the maintenance of energy homeostasis. Animals adapt to periods of undernutrition by increasing their LMA and time spent awake, presumably to forage for food. This relationship between nutrient availability and arousal is a defining feature of Ox physiology. Ox neuron activity and CSF OxA peptide concentration are acutely increased during periods of fasting, and Ox neurons can be entrained to fire during the preprandial period in animals undergoing FR (315). Orexins are required for the behavioral adaptation to FR, as *OxKO* animals do not food-entrain their circadian rhythms and fail to exhibit FAA (310). Ox neurons are apparently activated to promote arousal and foraging such that the animal can maintain energy balance during periods of food scarcity.

The activity of Ox neurons is regulated by circulating signals of energy status, including glucose and leptin, both of which reflect energy repletion and independently inhibit Ox neurons. In contrast to glucose-excited cells, Ox neurons sense extracellular glucose levels independent of intracellular glucose metabolism and ATP levels (316). Rather, glucose as well as the non-metabolizable glucose analogue, 2-deoxyglucose, hyperpolarizes Ox neurons by activating an outward K^+ current via K_{2P} channels (317, 318). This mechanism shows remarkable sensitivity, altering Ox neuron excitablilty in response to very small changes in extracellular glucose concentration. This effect of glucose on Ox neuron activity is modified by food intake, however. Fasted, hypoglycemic

rats exhibit increased cFos IR in Ox neurons compared to normoglycemic rats, but *ad libitum* feeding blocks this effect, suggesting that Ox neurons integrate other indicators of energy status (319). Furthermore, Ox mRNA is increased in both hypoglycemic as well as fasted rats, but this effect does not persist in chronically FR animals (320).

The effects of leptin on Ox neurons are quite distinct from glucose. Several studies have demonstrated that leptin inhibits Ox neurons (315). Though initial reports suggested that Ox neurons expressed both LepRb and its downstream transcription factor, STAT3, recent work demonstrates that the inhibition of Ox neurons by leptin is indirect (321). Mice expressing Cre recombinase under the control of the LepRb promoter were generated and crossed with GFP-expressing reporter mice to identify all LepRb-expressing cells (marked by GFP-IR) in the CNS. The authors found many GFP-IR cells in the LHA, throughout both the Ox neuron and MCH neuron populations. Staining for Ox or MCH demonstrated no colocalization between LepRb and either Ox or MCH (322). Subsequent tract tracing studies determined that LHA LepRb-expressing neurons project within the LHA and synapse with Ox neurons (323). A significant percentage of these LHA LepRb-expressing neurons were later found to express both GABA and the neuropeptide neurotensin (Nts). A circuit was thus described in which leptin activates LHA LepRb-/GABA-/Nts-expressing neurons, which then inhibit the activity of Ox neurons (324). Paradoxically, removal of LepRb from Nts-expressing neurons not only blocked leptin sensing by Ox neurons, but also diminished overall LMA and energy expenditure. Leinninger et al. also found that leptin increases *Ox* expression in both WT and leptin deficient *ob/ob* mice, though previous work demonstrated that leptin blocks the fasting-induced rise in Ox expression. The discordant results regarding the role of leptin in regulating Ox neuron may reflect the complexity of inputs into the Ox

system or the non-specific effects of administering pharmacologic doses of the adipokine. Ox neurons are most active during the dark phase in rodents, but Ox mRNA expression is highest during the inactive period, suggesting that Ox stores are replenished during rest in preparation for the next period of Ox neuron activity (325). Thus, leptin, which is often associated with increasing energy expenditure, may enhance Ox mRNA expression to increase the animal's "arousal stores" for future periods of peak activity. Indeed, leptin-deficient mice both exhibit significantly less locomotor activity as well as deficient arousal responses to threatening stimuli (326).

Conversely, ghrelin, an orexigenic hormone released from the fundus of the stomach prior to meals and in response to fasting, activates Ox neurons during periods of hunger. Ghrelin binds the growth hormone secretagogue receptor (GHSR) and increases feeding behavior by activating ARC NPY/AgRP neurons and LHA Ox neurons (327). Superfusion of ghrelin solution onto dissociated Ox neurons caused depolarization and increased firing in ~67% of neurons tested, and in a hypothalamic slice preparation, ghrelin depolarized Ox neurons in a dose-dependent manner (328). Blockade of central Ox signaling, using neutralizing antibodies to the peptides, attenuates ghrelin-induced feeding, illustrating an essential role for Ox signaling in the orexigenic effects of ghrelin (329). The effects of ghrelin on LMA are somewhat controversial. Central ghrelin administration associates with both increased feeding and decreased LMA, similar to AgRP (330). Conversely, ghrelin administration causes an increase in LMA in goldfish (331). Mice with targeted mutations of the ghrelin receptor gene (*GHSR KO*) exhibit a decrease in FAA, and daily ghrelin injections induce a pattern of hypothalamic cFos IR closely resembling that observed during FR (332). Furthermore, ghrelin antagonism attenuates the increase in LMA induced by cocaine or

methamphetamine administration (333). Together, these studies suggest that ghrelin is necessary for reward-associated increases in LMA but insufficient to induce this behavior in the absence of other motivating factors.

8.6 Orexins Associate Stress and Arousal

Orexins appear to link increased arousal to stressful situations. Central administration of high dose OxA elicits stress-related behaviors, including hyperlocomotion and grooming (299, 334) and physiologic responses, including increased body temperature, blood pressure, heart rate, sympathetic nerve activity and gastric acid secretion (335-340). Stress stimulates the expression of CRH in hypothalamic neurons located in the PVN. CRH acts on pituitary corticotrophs to release adrenocorticotrophic hormone (ACTH), which circulates through the blood and stimulates the secretion of glucocorticoids, the primary biomarker of the stress response, from the adrenal glands (341). Several studies indicate that Ox neurons and CRH neurons constitute a feedback loop that engages arousal systems in response to stressors. Ox neurons receive stimulatory projections from PVN CRH-expressing neurons and send reciprocal excitatory projections to this region (263). Electrophysiologic and histologic studies confirm that Ox neurons express CRH-R1 and that CRH depolarizes a subset of Ox neurons. Further, PVN neurons express OX2R and are depolarized by the addition of Ox in a tetrodotoxin-insensitive manner (342, 343). Pituitary corticotrophs and cells in the adrenal cortex and medulla also express Ox receptors and are responsive to orexins (344-347). Blockade of the CRH-R1 prevents several of the stress-related responses to central OxA, including grooming and GC release (348-350). There is also some *in vitro* evidence that OxA can inhibit CRH-induced ACTH release from corticotrophs, though this effect has not been observed *in*

vivo (351). Not all stressful stimuli induce Ox release—CSF Ox levels are only increased during perturbations that provoke a locomotor response. Other stressful interventions, including immobilization, sleep deprivation, or cold exposure had no such effect, supporting a close link between LMA and Ox signaling (352). *OxKO* mice show reduced defense responses to acute stressors, such as in the resident intruder test. Furthermore, physiologic stress measures evoked by this challenge, including increased heart rate, were reduced in *OxKO* mice, supporting a critical role for Ox signaling in mounting an integrated stress response (353).

8.7 Orexins, Reward, and Addiction

Ox neurons have recently been implicated in reward seeking and addiction. Patients with narcolepsy are often treated with highly addictive amphetamines, but show little evidence of addiction to the treatment (354, 355). Similarly, *OxKO* mice exhibit reduced preference for morphine and a diminished withdrawal response, supporting an association between orexins and addiction (356, 357). Ox neurons are activated following exposure to cues associated with drug or food reward. Conditioned place preference (CPP), a measure of reward preference and learning, for morphine is completely absent in *OxKO* mice. Also, cFos IR in Ox neurons correlates with the expression of CPP for drugs or rewards, while blockade of OX1R with SB-334867 prevents the acquisition of morphine preference (358). Ox signaling is required for the reinstatement of previously extinguished drug-seeking behavior (358, 359).

Orexins modulate the activity of DA neurons in the VTA, providing a mechanism for their effects on reward. As mentioned above, Ox neurons project to the VTA, and

intra-VTA OX1R antagonism blocks the acquisition of a morphine CPP, indicating that Ox signaling in the VTA is essential for associating this behavior with morphine (358). OX1R blockade does not, however, impact cocaine-associated CPP, though it does block cue-induced reinstatement of extinguished cocaine seeking (359). Because cocaine independently manipulates the DA system by inhibiting DA reuptake in the VTA, Ox signaling may be unnecessary in that setting. Chronic cocaine or morphine exposure followed by forced abstinence causes a change in hedonic processing such that the animal's preference for drug is increased, but its preference for natural reward, such as food, is decreased (360). When exposed to the preferred drug, cFos in Ox neurons is activated, in proportion to the expression of preference. However, when food is presented to these animals, cFos IR is reduced in Ox neurons, compared to control animals or drug-presentation, suggesting an association between Ox neuron activity and hedonic processing (361). Subsequent studies have confirmed the link between Ox neurons and reward processing (362).

8.8 Orexin Regulation of Feeding and Metabolism

Ox infusion increases food intake in animals, and central OX1R inhibition causes a reduction in food intake and partially rescues obesity in leptin-deficient *ob/ob* mice (264, 363). Central blockade of Ox signaling, using an OX1R antagonist or Ox neutralizing antibody, also reduces food intake, and the transgenic mice lacking Ox peptide or neurons exhibit marked hypophagia (274, 275, 364, 365). These feeding effects have been attributed to a nonspecific effect of eliciting wakefulness and arousal. However, microinjections of Ox into the ARC stimulate orexigenic GABAergic neurons and inhibit anorexigenic POMC neurons, suggesting a direct appetite-stimulating effect. Neurons in the ventromedial nucleus (VMH), a satiety center, are also inhibited by Ox

(366). Recent work suggests that the effects of orexins on food intake reflects enhancement of reward pathways as well. Leptin signaling alters Ox neuron activity and subsequently changes the dynamics of mesolimbic DA transport, an important intermediate in reward pathways (324). Furthermore, OxA infusions into the shell of the nucleus accumbens, which receives dopaminergic input from the VTA, stimulates feeding behavior (367). Given the clear role for Ox signaling in wakefulness and LMA, Ox-induced increases in food intake may maintain energy balance by replenishing energy stores that are consumed during periods of heightened activity.

Ox neurons project to nuclei in the hypothalamus and brainstem that regulate sympathetic nervous system activation. Centrally administered orexins influence autonomic physiology, including increasing blood pressure and heart rate by recruiting adrenergic signaling (338). *OxKO* mice exhibit a resting blood pressure 10-15 mmHg lower than WT littermates, demonstrating that Ox is required for normal sympathetic outflow (353, 368). OxA injection into the VMH leads to an increase in glucose utilization by muscle, but not white adipose tissue, also by engaging the sympathetic nervous system (369). These findings suggest that some of the obesity seen in Ox deficient mice and humans are due to insufficient sympathetic tone and energy utilization in combination with increased food intake. Disease states are often associated with an increase in basal metabolic rate and sympathetic tone and a decrease in food intake, leading to negative energy balance (370), though this pathophysiology is generally attributed to cytokine-induced activation of pre-autonomic PVN neurons.

8.9 Orexins and Depression

Ox signaling is implicated in modulating behavior in depression. The diagnostic criteria for major depressive episodes include perturbations in sleep, appetite and motivation--behaviors that are associated with orexins. Acutely and chronically ill individuals also report many of the same emotional and behavioral characteristics, leading researchers to investigate a role for inflammatory signaling in the pathogenesis of major depression (371). Ox concentrations in the CSF from suicidal patients diagnosed with major depressive disorder are lower than in patients with dysthymia or adjustment disorder (372). In a rat model of depression induced by neonatal clomipramine administration, treated animals exhibited altered Ox protein levels in several brain regions (373). Depression is further associated with a reduction in the arousal response evoked by stressful or hedonic stimuli. As a result, patients with depression often report difficulty in mounting motivational drive and a loss of interest in normally enjoyable activities. These behaviors are conserved in rodent models of depression, where they are measured by shorter latency to immobilization and greater total immobility in a forced swim test and by deficits in social interaction. These responses can be induced by chronically stressing the animals using a chronic social defeat paradigm. This intervention involves housing the mice with aggressive strains, allowing a limited amount of defeat interaction, then separating them with a porous plastic divider, allowing the animals to safely interact and reinforcing the learned social defeat. FR animals perform better in each of the depression measures compared to *ad-libitum* fed mice following chronic social defeat conditioning. This effect is not seen in *OxKO* mice, and the number of cFos positive Ox neurons induced by FR correlates strongly with the improved performance (374). These data demonstrate that Ox neurons

are necessary for the anti-depressant effects of FR and that activation of these neurons by environmental stressors, such as calorie restriction, can overcome learned behavioral inhibition. Although the link between Ox neurons and depression is not clearly defined, these studies are most consistent with a role for Ox neurons in mediating the motivational component of depressed behavior.

8.10 Functional Dichotomy in Ox Neurons

Orexins modulate both arousal and reward pathways, but recent evidence implicates separate populations of Ox neurons in each of these effects. During peak wakefulness, forced activity, or footshock stress, medial Ox neurons, located in the DMH and PFA, are preferentially activated over lateral Ox neurons, located in the LHA (358). Conversely, using a CPP paradigm, cues conditioned with food or drug reward elicit an increase in cFos in the LHA, but not the DMH or PFA Ox neuron population (375). This anatomic division in Ox neuron function is supported by tract tracing studies showing LHA Ox neurons receive afferents from reward centers, including the nucleus accumbens and the VTA, but the DMH and PFA populations do not (376). DMH and PFA Ox neurons receive projections from arousal-related neurons in the pre-optic area and the posterior hypothalamus, as well as from the periaqueductal grey matter, whereas the majority of brainstem afferents target LHA Ox neurons (232). Unfortunately, the efferent projections from each of these Ox neuron subpopulations are not as well described as the afferent inputs to each region. Putatively, unique projection sites of the DMH, PFA, and LHA neurons correspond to their divergent effects.

8.11 Orexins Are Allostatic Regulators of Arousal

Taken together, these findings advocate two important roles for Ox in regulating arousal. First, Ox neurons are vital for regulating allostasis and arousal. This is supported by studies showing that Ox signaling mediates a generalized arousal response to stressors including food deprivation, drug availability, and forced activity, independent of circadian and homeostatic cues. Second, orexins regulate motivated behavior both directly, by altering feeding circuits and reward pathways, as well as indirectly, by reinforcing the arousal necessary to mount the behavioral responses. Importantly, these motivated behaviors— volitional activity, feeding, sexual behavior, reward-seeking, and social exploration—are all significantly attenuated in sick animals and humans. The connection between leptin, the activity of Ox neurons, and behavior raises the possibility that Ox neurons respond to other circulating signals of physiologic status, including pro-inflammatory cytokines, and serve as a common intermediate for the allostatic control of arousal.

9. Inflammation and Arousal

9.1 Sleep

The effects of illness on sleep depend greatly on the infective pathogen, host immune response, and route of infection. Generally, sickness is associated with more time spent in NREM sleep, an increase in NREM sleep fragmentation, and a decrease in the amount of REM sleep (377). Data from human studies report sleep disturbances ranging from an encephalitis lethargica-like syndrome secondary to streptococcal

infection to modest alterations in sleep patterns following infection with rhinovirus or influenza (196, 378, 379). Human patients with HIV have reported sleep disturbances antecedent to any other detectable AIDS pathology (380). It is believed that the actions of pro-inflammatory cytokines in the CNS underlie these alterations in sleep. Indeed, peripheral administration of bacterial-derived immunogens or central cytokine injection recapitulate the hypersomnolence and sleep fragmentation seen in disease states, and these effects have been linked to CNS IL-1β and TNF-α {Krueger, 1982 #5162;Krueger, 1984 #5158;Olivadoti, 2008 #5155;Opp, 1991 #5160;Krueger, 1994 #5168}. Specifically, blockade of IL-1 signaling completely abrogates both LPS- and muramyl dipeptide-induced increases in NREM sleep (381, 382).

Interestingly, IL-1β and TNF-α appear to have an important role in the normal physiologic regulation of sleep. The expression of these cytokines varies diurnally, with a peak in production at the onset of the dark phase (in humans) or the light phase (in nocturnal rodents) (383-385). Administration of antibodies, antagonists, or soluble receptors to these cytokines reduces NREM sleep, and mice lacking IL-1R or TNF-R exhibit less NREM sleep (381, 386, 387). This sleep-promoting effect of CNS cytokines is the result of altered neuronal activity throughout neurons comprising the ascending arousal system, leading to a generalized suppression of arousal and wakefulness. The effects of cyokines on the activity of the specific nuclei involved in arousal are explored in more detail below.

9.2 Inflammatory Regulation of the VLPO and Basal Forebrain

Recent research demonstrates that inflammatory cytokines act directly on sleep-active neurons in the VLPO and wake-active neurons in the basal forebrain to disrupt sleep patterns. Specifically, IL-1β directly inhibits wake-promoting neurons and stimulates a subset of GABAergic VLPO sleep-promoting neurons (388). Both central IL-1β and peripheral LPS increase the number of cFos IR neurons in the preoptic hypothalamus and adjacent basal forebrain (75, 389). The relative induction of cFos IR in each of these regions correlates positively with the amount of NREM sleep during the period preceding sacrifice, suggesting that these neurons were active during sleep.

Cytokines may also act in the anterior hypothalamus and basal forebrain by modulating the local release of AD (371). *In vitro* studies show that IL-1β and LPS each increase the concentration of AD in the media of neuroendocrine PC12 cells (390). Pharmacologic studies reveal that blockade of the adenosine A_{2A} receptor prevents LPS-induced behavioral depression in the forced swim test and tail suspension test (391). AD production in the brain is regulated by PG synthesis and signaling. Peripheral LPS and muramyl dipeptide are both known to increase the production of PGD_2 from the choroid and meninges (392, 393). PGD_2, when administered i.c.v., promotes NREM sleep and increases cFos IR in VLPO neurons (394, 395). Blockade of A_{2A} receptors blocks the sleep-promoting effects of PGD_2, suggesting that AD is an important intermediate linking inflammation and somnolence (396). As AD is known to be somnogenic, it is possible that cytokine-induced increases in local AD concentration underlie some sickness behavior. Unfortunately, no studies to date specifically test this hypothesis, so the contribution of AD signaling to sickness behavior remains unclear.

9.3 Dopaminergic Neurons Exhibit Biphasic Responses to Inflammation

The majority of studies investigating the impact of inflammation on DA neurons focus on the pathogenesis of Parkinson's Disease, which is caused by selective degeneration of dopaminergic cell bodies in the substantia nigra. As such, the activity of DA neurons, particularly those that mediate arousal, under inflammatory conditions has not been well studied. Several studies show that intraparenchymal administration of immunogenic agents into the substantia nigra induces DA neuron loss (397, 398). Further, prenatal exposure to endotoxin leads to selective loss in DA neurons, revealing that some DA neurons are sensitive to inflammatory cytokines (399).

The impact of inflammation on the activity of DA neurons may underlie sickness-induced anhedonia. Peripheral LPS increases cFos IR in VTA DA neurons that are associated with motiviation, incentive and reward (400). LPS treatment correlates with an increase in DA efflux from the nucleus accumbens (401) and a decrease in intracranial self-stimulation behavior. However, LPS pretreatment decreases the firing rate of VTA DA neurons 7-10 days following treatment, which is associated with increased alcohol intake in mice (402). Evidence of increased DA turnover in both the NTS and the LC was observed following LPS treatment, which would predict increased arousal, not decreased (403). It appears that VTA DA neurons exhibit a biphasic response to inflammation that impacts reward processing, but it is unclear to what degree DA contributes to sickness-induced lethargy.

9.4 Cytokines Suppress Serotonin Neuron Activity

The sleep-promoting effect of inflammatory challenges is linked to changes in the activity of serotoninergic neurons in the DR. Serotoninergic signaling is important for both initiating sleep and maintaining wakefulness. These divergent actions of 5HT are associated with differential activation of DR 5HT neurons projecting to different nuclei. Intraperitoneal LPS causes a PG-dependent increase in cFos IR in tryptophan hydrolase-IR neurons in the DR, though no change in plasma L-tryptophan, a metabolite of 5HT, was observed (404). Subdiaphragmatic vagotomy only slightly reduces brain 5HT and its turnover in response to LPS and IL-1β. However, sickness behavior remains unaltered, confirming that the vagus is not the major mechanism by which inflammation impacts 5HT signaling (405).

Peripheral inflammation may also act on the 5HT system via local increases in cytokine production. The interactions between IL-1β and the 5HT system have been extensively studied during the last decade. IL-1β injection into the DR increases NREM sleep in rats (406). Electrophysiologic studies demonstrate that IL-1β reduces the firing rate of DR 5HT neurons by potentiating GABA-induced inhibitory postsynaptic potentials (406, 407). IL-1 enhances GABAergic hyperpolarization in other brain regions as well, including the anterior hypothalamus and hippocampus, though this effect is mediated, at least in part, by the induction of other inhibitory factors, such as AD in the preoptic area (408-410). IL-1β also modulates 5HT release in the preoptic region of the anterior hypothalamus. Here, tonic 5HT release from axon terminals is stimulated by local application of IL-1β, but state dependent release is not altered (411). Thus, the increased 5HT release during wakefulness and decrease during sleep are superimposed

upon an elevated baseline level of 5HT release in the presence of IL-1β. Although increases in 5HT release are typically associated with increased arousal, increasing serotoninergic signaling in the preoptic area/basal forebrain restores physiological sleep in insomniac cats (412). In this region 5HT hyperpolarizes and inhibits wake-active cholinergic neurons, thereby stimulating NREM sleep. Therefore, inflammation suppresses arousal through local actions of IL-1β to both inhibit arousal-associated DR neurons and stimulate of 5HT release from axon terminals in the preoptic area and basal forebrain. Given that PG blockade ameliorates DR neuron responses to LPS but does not reverse sickness behavior, it is likely that 5HT is not the only player in inflammation-induced lethargy.

9.5 Inflammation Dissociates Noradrenergic Tone from Arousal Behavior

In contrast to the DR, inflammation appears to have an activating effect on noradrenergic neurons in the LC. Peripheral LPS administration increases the firing rate of LC neurons and induces IL-1β expression in the LC (413, 414). LPS treatment also increases both NE content and DA turnover in the LC, suggesting activation of both of these monoaminergic systems (403). The excitation of LC neurons by LPS appears to be mediated by vagal afferents, as this effect is not seen following subdiaphragmatic vagotomy (415). Independently, local injection of IL-1 into the LC increases firing rate of LC neurons in live, anesthetized rats (413). Specifically, burst firing, which normally occurs after an excitatory stimulus, is increased in these neurons. This effect appears to be mediated by activity of IL-1 within the LC, as injection just outside the LC had no effect on firing rate and the effects of IL-1 within the LC could be blocked by IL-1ra administration. These authors also demonstrated that very low concentrations of IL-1 reduces firing of LC neurons and that this effect is mediated by local CRF signaling

(416). Because the aforementioned electrophysiology studies were performed in anesthetized animals, behavior could not be assessed. A subsequent study demonstrated that motivated behaviors, including LMA, grooming, eating, and drinking, correlate with extracellular 5HT and NE in the hippocampus in normal rats. However, following LPS treatment, these behaviors were dissociated from the amount of 5HT and NE measured in the hippocampus—each of these behaviors was suppressed, but levels of the neurotransmitters were increased compared to control animals (417). Co-treatment with indomethacin partially blocked the increase in hippocampal NE and 5HT, but did not ameliorate sickness behavior. Thus, neurons in the LC are paradoxically excited by inflammation, despite suppression of behaviors normally associated with increased noradrenergic signaling. These findings indicate that LC neurons are not the mediators of inflammation-induced lethargy. Rather, the recruitment of other arousal circuits by inflammatory stimuli can suppress the arousal-stimulating effects of increased NE. *In vitro* NE reduces the inflammatory response to LPS in cortical microglia, indicating that the increase in NE release by inflammation may be important for feedback inhibition of central inflammatory signaling (418).

9.6 TMN Histaminergic Neurons Are Inhibited by Inflammation

Histaminergic neurons in the TMN are critical for maintaining wakefulness and arousal. These neurons receive inhibitory projections from VLPO neurons, which are activated during sleep and inflammatory challenge via PGD_2 and AD (211, 247, 395). Consistent with this relationship, subarachnoid administration of an A_{2A} receptor agonist blocked HA release and wakefulness in freely moving rats. In anesthetized rats, administration of A_{2A} agonist blocks HA release in the preoptic area and increases local GABA concentration in the TMN. Blockade of $GABA_A$ signaling with picrotoxin blocks the

inhibition of HA release by the AD agonist, consistent with inhibition by VLPO sleep-active neurons (396).

Inflammatory signaling also has direct effects on HA neuron activity and associated behavior. Peripheral IL-1 administration increases the expression of enzymes regulating the synthesis of HA in a PGE_2-dependent manner. Blocking HA synthesis attenuates peripheral IL-1-induced anorexia, but has no effect on fever or decreased water intake (419). Although inflammation increases the synthesis of HA, the activity of TMN HA neurons is suppressed after peripheral LPS treatment (420). These authors demonstrated that the TMN receives projections from the NTS and and prevented suppression of cFos in the TMN by LPS reversibly blocking nerve conduction through the dorsal vagal complex (DVC), which includes the NTS. TMN neurons are inhibited by GABAeric projections from the VLPO and NTS during both physiologic (sleep) and pathophysiologic (inflammation) suppression of arousal. Though the activity of these neurons is necessary for the maintenance of wakefulness (217), there is no known association between the suppression of TMN neuron activity and wakefulness during inflammatory challenge.

9.7 Effects of Inflammation on the LHA and Orexin Neurons

Given the dysregulation of several homeostatic processes during disease, the activity of neurons in hypothalamic nuclei following immune challenge has been extensively studied. Most studies have investigated nuclei that exhibit an increase in cFos IR in response to immunogenic stimuli, but recently a reduction in cFos IR in the LHA following LPS treatment was observed (421). Bath application of IL-1β or TNF-α

onto hypothalamic slice preparations reduces the firing rate of glucose-sensitive neurons in the LHA, which may include Ox or MCH neurons (422). During periods of normal feeding, LPS causes a selective loss of cFos IR in LHA Ox neurons (423). Given their known orexigenic properties, the authors of these studies postulate that suppression of Ox neuron activity by LPS plays a key role in sickness-associated anorexia. However, Ox neurons are thought to primarily mediate wakefulness and arousal. The biology and connectivity of Ox neurons are consistent with the effects of inflammation on other nuclei involved in arousal. NE, DA, and 5HT, all of which are stimulated by LPS treatment, inhibit Ox neurons. They receive input from the brainstem arousal nuclei, hypothalamic nuclei involved in circadian and metabolic control, and corticolimbic emotion centers, allowing them to integrate distinct environmental stressors into a single response. Orexins stabilize arousal states, and sickness is associated with both somnolence and reduced sleep quality, indicative of destabilized arousal states. Further, *OxKO* mice exhibit not only hypophagia, but also hypoactivity and hypersomnolence, all of which resemble sickness behavior (274, 275). As discussed above, Ox neurons are known to mediate allostasis in arousal behavior. Sickness is a physiologic challenge that elicits non-homeostatic behavioral and physiologic responses. Thus, Ox neurons are well positioned to mediate one or more aspects of sickness behavior.

Hypothesis and Specific Aims of this Thesis

The long-term goal of our research is to elucidate the central mechanisms underlying the metabolic and behavioral alterations common to inflammatory conditions. The objective of the research presented in this thesis was to *determine the neural mechanisms of inflammation-induced anorexia and lethargy*.

Specific Aims:

1. Determine whether direct activation of POMC neurons by LIF causes anorexia and weight loss.

2. Evaluate whether inflammation-induced lethargy is mediated by suppression of Ox neurons.

CHAPTER 2

Manuscript #1

Arcuate Nucleus Proopiomelanocortin Neurons Mediate the Acute Anorectic Actions of Leukemia Inhibitory Factor via gp130

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Abstract

The pro-inflammatory cytokine LIF is induced in disease states and is known to inhibit food intake when administered centrally. However, the neural pathways underlying this effect are not well understood. We demonstrate that LIF acutely inhibits food intake by directly activating POMC neurons in the arcuate nucleus of the hypothalamus. We show that arcuate POMC neurons express the LIF-R, and that LIF stimulates the release of the anorexigenic peptide, alpha-melanocyte stimulating hormone from *ex vivo* hypothalami. Transgenic mice lacking gp130, the signal transducing subunit of the LIF-R complex, specifically in POMC neurons fail to respond to LIF. Furthermore, LIF does not stimulate the release of alpha-melanocyte stimulating hormone from the transgenic hypothalamic explants. These findings indicate that POMC neurons mediate the acute anorectic actions of central LIF administration and provide a mechanistic link between inflammation and food intake.

Introduction

Cachexia is a wasting syndrome common to the late stages of many chronic diseases, including HIV, heart failure, uremia, and cancer (8). Cachexia is marked by paradoxical responses to a starved state, including 1) anorexia, 2) increased basal metabolic rate, and 3) preferential loss of lean body mass. Indeed, the severity of this synergistic catabolic state is associated with poor clinical outcomes, increased mortality, and reduced quality of life (8, 9). A growing body of evidence shows that increases in central inflammatory cytokine signaling are sufficient to cause anorexia and increased basal metabolic rate (36, 37, 422). Administration of the bacterial endotoxin LPS induces an acute cachexia syndrome associated with increases in central and peripheral pro-inflammatory cytokines, including IL-1β, TNF-α, and LIF (6, 21-25, 424). The finding that central administration of each of these cytokines individually recapitulates acute cachexia, strongly suggests a site of cytokine action within the CNS.

An identified target for central cytokine action is the arcuate nucleus of the hypothalamus. The ARC is known to be a key regulator of energy homeostasis and a major site for the integration of metabolic signals (76, 425). The ARC includes two populations of neuropeptide-expressing neurons with opposing actions on energy balance. One population expresses the anorexigenic peptide, α-MSH, a cleavage product of the POMC precursor (77). POMC is also expressed by brainstem neurons of the NTS as well as pituitary corticotrophs (77). ARC POMC neurons are opposed by adjacent neurons expressing the orexigenic neuropeptides AgRP and NPY (78). α-MSH derives its anorectic effect via activation of MC4-R (85), whereas AgRP acts as an endogenous antagonist and inverse agonist at the same receptor (83). POMC post-translational processing also results in the production of β-endorphin, which is putatively

co-released with α-MSH from axon terminals (426). The role of β-endorphin in energy homeostasis remains controversial, as exogenous administration acutely increases food intake while endogenous release may act to inhibit food intake (427). Energy balance is tightly regulated by the relative activity of each of these neuronal populations, which, in turn, are responsive to numerous circulating signals of energy status. Importantly, the blood brain barrier in the ARC is relatively permissive, allowing the neurons access to circulating macromolecules (425). For example, recent work in our laboratory has shown that both POMC and NPY/AgRP neurons express the IL-1RI and are oppositely regulated by this cytokine to promote negative energy balance (161, 162). Previous studies indicate that the ARC may act as an inflammatory amplifier within the CNS, suggesting that neurons in the ARC are likely subject to much higher concentrations of pro-inflammatory cytokines than found in the circulation (67, 428). Further, this central inflammatory response is necessary for the induction of anorexia by LPS (63).

The pro-inflammatory cytokine LIF is an essential component in inflammatory signaling and neuro-immune function (170, 429, 430). Serum LIF is elevated in chronic disease and malignancy (182-184), an observation that has been correlated to poor prognosis (185). Hypothalamic expression of LIF is induced in animal models of acute inflammation (431, 432). The potential role of LIF in cachexia is further exemplified by the finding that, in contrast to IL-1β or LPS-induced anorexia, animals do not desensitize to chronic LIF-induced anorexia in either LIF-overexpressing tumor models (186, 188) or following i.c.v. administration of a LIF-expressing viral vector (187). This lack of tachyphylaxis more accurately represents clinical cachexia, indicating that LIF could be an essential CNS mediator of chronic inflammation.

The mechanism of LIF-mediated anorexia is still unknown. One possibility is that LIF, like IL-1β, alters melanocortin signaling in the ARC. LIF is a member of the IL-6 cytokine family, along with CNTF, which share a common gp130 signal transducing subunit (170). LIF derives target specificity via binding to a heterodimeric receptor, consisting of LIF-R and gp130 (172). Binding stimulates gp130-mediated activation of the Janus kinase 2 (JAK2)/STAT3 pathway (172), the same pathway activated by the binding of leptin to its receptor (173). LIF orchestrates the pituitary response to inflammation by inducing POMC gene expression and ACTH release in corticotrophs (189, 190, 433), an effect dependent on JAK2/STAT3 signaling and mediated by gp130 (190, 434-436). Although pituitary and neuronal POMC expression are differentially regulated (437), it is possible that LIF may also directly influence ARC POMC activity.

In the present work, we explore the role of POMC neurons in the acute anorectic effects of LIF. We first examine the effects of central LIF on feeding behavior and neuronal activation. We further investigate the neuroanatomic framework and the molecular mechanism to show that LIF induced anorexia is dependent upon functional expression of gp130 in POMC neurons. Our results also demonstrate that LIF acutely induces anorexia by direct activation of ARC POMC neurons.

Materials and Methods

Animals and Surgical Procedures

Male Sprague Dawley rats (300 –350 g; Charles River Laboratories, Wilmington, MA), wild-type C57BL/6J mice (6–9 wk of age; Jackson Laboratory, Bar Harbor, ME), transgenic C57BL/6J POMC-enhanced green fluorescent protein (EGFP) mice [6–9 wk

of age; genotyping and breeding of mice were as described previously (438)], and transgenic PomcCre-gp130$^{flox/flox}$ mice (6-9 wk of age; genotyping and breeding described below) were maintained on a normal 12-h light, 12-h dark cycle at 22-22°C with *ad libitum* access to food (Purina rodent diet 5001; Purina Mills, St. Louis, MO) and water, unless otherwise noted for an experiment. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Oregon Health and Science University.

Generation of PomcCre-gp130$^{flox/flox}$ mice

PomcCre mice (gift of Dr. Gregory Barsh) were generated and maintained on a mixed FVB/N, C57BL/6J, and 129 background as previously described (439). PomcCre heterozygotes were mated with gp130$^{flox/flox}$ mice (gift of Dr. Beth Habecker) (440) and the colony was maintained by mating PomcCre-gp130$^{flox/flox}$ mice with gp130$^{flox/flox}$ mice. gp130^{flox} mice were backcrossed on a C57BL/6J background for at least 5 generations prior to mating with PomcCre mice. Comparisons involving these animals were only conducted between mice of mixed background (FVB, 129, and C57BL/6). PomcCre mice were genotyped as previously reported (174), and no homozygotes were used for breeding or experiments. gp130$^{flox/flox}$ mice were genotyped using previously described primers (441).

Peripheral LPS injections in rats and mice for LIF and CNTF mRNA expression study

On the day of the experiment at 0900 h, animals received i.p. injections of LPS [100 μg/kg (Sigma-Aldrich Corp., St. Louis, MO) dissolved in 0.5% BSA (Sigma-Aldrich)

in 0.9% saline] or 0.5% BSA in 0.9% saline alone, and were placed in clean cages without food. At 1, 4, or 8 h after treatment, animals were anesthetized with isoflurane and killed by decapitation. The brains were immediately removed, and a hypothalamic block was dissected out, preserved in RNAlater solution (Ambion, Inc., Austin, TX), and stored at 4°C overnight. RNA was extracted the next day and used for RT-PCR analysis.

Implantation of lateral ventricle cannulae

C57BL/6J, POMC-EGFP, or PomcCre-gp130$^{flox/flox}$ mice were anesthetized with 1-2% isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A sterile guide cannula with obturator stylet was stereotaxically implanted into the lateral ventricle. The coordinates used were: 1.0-mm posterior to bregma, 0.5-mm lateral to midline, and 2.35-mm below the surface of the skull (442). The cannula was then fixed in place with dental cement. The animals were individually housed after surgery for a minimum of 1 wk, and were handled and administered 1 μl i.c.v. injections of commercial aCSF (Harvard Apparatus, Holliston, MA) daily.

Central IL-1β injection in mice for LIF and CNTF mRNA expression study

On the day of the experiment at 0900 h, mice received i.c.v. injections of 10 ng murine IL-1β (R&D Systems) dissolved in 1 μl aCSF, or aCSF alone, and were placed in clean cages without food. At 8 h after treatment, mice were anesthetized with isoflurane and killed by decapitation. The brains were immediately removed, and a hypothalamic block was dissected out, preserved in RNAlater solution, and stored at -80°C until RNA extraction and RT-PCR analysis.

RNA preparation and RT-PCR

Hypothalamic blocks were dissected by making coronal cuts at the rostral extent of the optic chiasm and caudal to the mammillary bodies; sagittal cuts were made along the optic tracts. Cortex was then removed at the level of the corpus callosum. Tissue was homogenized for 60 s prior to extraction. Total RNA was extracted from hypothalamic blocks using QIAGEN RNeasy kits (QIAGEN, Inc., Valencia, CA). DNA was removed from total RNA using ribonuclease (RNase)-free deoxyribonuclease (QIAGEN). Reverse transcriptase (RT) reactions were prepared using a TaqMan Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA). For each reaction cDNA synthesis was prepared using 400 ng RNA in a reaction containing 4 ml 10X RT buffer, 8.8 µl 25 mM MgCl2, 8 µl 10 mM dNTPs, 2 µl 50 mM random hexamers, 0.8 µl RNase inhibitor, 2 µl MultiScribe RT, Q.S. to 40 µl with nuclease-free water. RT reactions were performed on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) programmed for 25°C for 10 min, 37°C for 1 h, and 95°C for 5 min. Samples were stored at 4°C until RT-PCR was performed. Real-time RT-PCR was performed on an ABI 7300 Real-Time PCR System using mouse-specific primer probe sets obtained from Applied Biosystems. Each RT-PCR contained 10 µl TaqMan Universal PCR MasterMix, 1 µl Assays-on-demand Gene Expression Assay Mix, and 9 µl cDNA. Samples and endogenous controls (eukaryotic 18S rRNA) were run in duplicate to ensure reproducibility. Auto comparative threshold values were calculated using 7300 Relative Quantity (RQ) Study Software version 1.3 and manually verified. Raw C_t values from 18S endogenous controls were compared between groups to validate observed changes in target genes.

Central LIF injection in mice for feeding studies

Animals were transferred to clean cages and food was removed at lights off, the night before the experiment. On the day of the experiment at 0830 h, mice received i.p injections of ketorolac [40 mg/kg (Sigma-Aldrich) dissolved in 0.9% saline]. At 0900 h, mice received i.c.v. injections of 50 ng murine LIF (Santa Cruz Biotechnology, Santa Cruz, CA) dissolved in 1 µl 0.5% BSA in 0.9% saline, or 0.5% BSA alone, and given a weighed quantity of food. At 1, 2, 4, 8, and 12 h after treatment, food was weighed and returned to the cage. For the PomcCre-gp130$^{flox/flox}$ feeding study, animals were given one week to recover, and the experiment was repeated with all animals switching i.c.v. treatments. All animals were randomly assigned to treatment group.

Central LIF injection in mice for immunohistochemistry

On the day of the experiment at 0830 h, mice received i.p injections of ketorolac [40 mg/kg (Sigma-Aldrich) dissolved in 0.9% saline]. At 0900 h, mice received i.c.v. injections of 10 ng or 100 ng murine LIF (Santa Cruz Biotechnology, Santa Cruz, CA) dissolved in 1 µl 0.5% BSA in 0.9% saline, or 0.5% BSA alone, and placed in clean cages with food removed. For the cFos and EGFP IHC, 90 min following i.c.v. injection, POMC-EGFP mice were anesthetized with a ketamine cocktail and transcardially perfused with 0.01 M PBS followed by 4% paraformaldehyde for fixation. For the pSTAT3 and ACTH IHC, PomcCre-gp130$^{flox/flox}$ mice and WT littermates were sacrificed and fixed as described above 30 min following i.c.v. treatment. Brains were removed and post-fixed 2-4 h, cryopreserved in 20% sucrose, and stored at -80°C until sectioning and staining. All animals were randomly assigned to treatment group.

Immunohistochemistry

Dual-immunofluorescence histochemistry (IHC) was performed as previously described (443). Briefly, free-floating sections were cut at 30 µm from perfused brains using a sliding microtome (Leica Microsystems, Bannockburn, IL). Four sets of sections were generated from the hypothalamus of each brain. Sections were collected from the diagonal band of Broca (bregma 1.0 mm) caudally through the mammillary bodies (bregma -3.00 mm). For the cFos and EGFP IHC, the sections were incubated for 1 h at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS and 0.3% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-c-Fos antibody (PC38; EMD Biosciences, Inc., San Diego, CA) diluted 1:75,000 in blocking reagent for 48 h at 4 C, followed by incubation in 1:500 donkey anti-rabbit Alexa 594 (Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature. For the pSTAT3 and ACTH IHC, sections were pretreated for 20 min in 1% NaOH, 1% H_2O_2 in 0.01 M PBS; followed by 10 min in 0.3% glycine in 0.01 M PBS; then incubated 10 min in 0.3% SDS in 0.01 M PBS. Sections were then incubated for 1 h in blocking reagent (5% normal donkey serum in 0.01M PBS and 0.3% Triton X-100). The sections were then incubated in rabbit anti-pSTAT3 antibody (Cell Signaling Technology, Danvers, MA) diluted 1:5000 in blocking reagent for 48 h at 4°C, followed by incubation in 1:1000 donkey anti-rabbit Alexa 594 (Molecular Probes, Inc.) for 1 h at room temperature. Sections were then incubated in a second blocking reagent (5% normal goat serum in 0.01M PBS and 0.3% Triton X-100) for 3 h at room temperature. Following this second blocking step, guinea pig anti-ACTH antibody (Peninsula Laboratories, San Carlos, CA) was applied diluted 1:500 in blocking reagent and incubated 72 h at 4°C, followed by

incubation in 1:200 FITC-conjugated goat anti-guinea pig secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Between each stage, the sections were washed thoroughly with 0.01 M PBS. Incubating the sections in the absence of primary antisera was used to ensure specificity of the secondary antibodies. Sections were mounted onto gelatin-coated slides, coverslipped using Vectashield mounting media (Vector Laboratories, Burlingame, CA), and viewed under a fluorescence microscope (Leica 4000 DM).

Cell counting

The number of cFos- or pSTAT3-immunoreactive cells and double-labeled cells was counted by eye in sections representing the ARC by investigators blinded to the treatments as previously reported (161). Results are expressed as the number of cells per section as well as the percentage that are double-labeled. Each set of ARC sections contained seven to nine sections expressing immunopositive cells. A cell was determined to be single-labeled when visible only under the fluorescence filter corresponding to the emission wavelength of the primary/secondary antibody complex used (*e.g.* 594 nm and not 488 nm for cFos). Cells were examined at multiple focal planes within the section and at multiple magnifications to ensure that the cell was indeed representative of a single-labeled cell. When the cell was visible at both 594- and 488-nm filters, it was deemed to be double-labeled. Double-labeled cells were examined at multiple focal planes within the section and at multiple magnifications to ensure that the cell was indeed representative of a single cell labeled with both antibody complexes and not two single-labeled cells in close proximity within different levels of the optical section. The cells were also examined under a third wavelength (350 nm) not

corresponding to the emission wavelength of either of the secondary antibodies to ensure that the immunoreactivity was specific.

Hypothalamic peptide secretion

Male C57BL/6J mice or PomcCre-gp130$^{flox/flox}$ mice and WT littermates were anesthetized with isoflurane and killed quickly by decapitation. The brain was removed (with care taken to ensure that there was no contamination of the hypothalamic portion with residual pituitary), and a 2-mm slice was prepared using a vibrating microtome (Leica VS 1000) to include the paraventricular and arcuate nuclei. Individual hypothalami received a 1 h equilibration period with aCSF [126 mM NaCl, 0.09 mM Na_2HPO, 6 mM KCl, 4 mM $CaCl_2$, 0.09 mM $MgSO_4$, 20 mM $NaHCO_3$, 8 mM glucose, 0.18 mg/ml ascorbic acid, and 0.6 trypsin inhibitory unit (TIU) aprotinin/ml] at 37°C. Hypothalami were then incubated for 45 min at 37°C in 700 µl aCSF (basal period) before being challenged with a single concentration of murine LIF (Santa Cruz Biotechnology) (0.1–50 nM) in 700 µl aCSF for 45 min at 37°C. Tissue viability was verified by a 45-min exposure to 700 µl aCSF containing 56 mM KCl. At the end of each treatment period, supernatants were removed and frozen at -80°C until assayed by RIA. Hypothalamic explants that failed to show peptide release above that of basal in response to aCSF containing 56 mM KCl were excluded from data analysis. Animals were randomly assigned to treatment groups.

α–MSH RIA

α–MSH immunoreactivity was measured with a rabbit anti- α–MSH specific for α–MSH (Phoenix Pharmaceuticals, Inc., Belmont, CA). The antibody cross-reacts fully with the mature α–MSH (*N*-acetylated α–MSH), and partially (46%) with desacetylated α–MSH, but not with NPY or AgRP. ^{125}I-labeled α–MSH was prepared by the iodogen method and purified by high-pressure liquid chromatography (University of Mississippi Peptide Radioiodination Service Center, University, MS). All samples were assayed in duplicate. The assay was performed in a total volume of 350 µl 0.06 M phosphate buffer (pH 7.3) containing 1% BSA. The sample was incubated for 3 d at 4°C before the separation of free and antibody-bound label by goat anti-rabbit IgG serum (Phoenix Pharmaceuticals). 200 µl of supernatant were assayed. The lowest detectable level that could be distinguished from the zero standard was 0.22 fmol/tube. The intraassay coefficient of variation was determined by replicate analysis (n = 6) of two samples at α-MSH concentrations of 2 and 10 fmol/tube, and the results were 6.7 and 9.7%, respectively. The interassay coefficients of variation were 22.7 and 12.5% for the range of values measured.

Double-label in situ hybridization histochemistry

Simultaneous visualization of POMC and LIF-R mRNA in the rat brain (n = 3) was performed as previously reported (161), with slight modifications. Coronal sections (20 µM) were cut on a cryostat and thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Hypothalamic sections were collected in a 1:6 series from the diagonal band of Broca (bregma 0.50 mm) caudally through the mammillary bodies (bregma -5.00 mm). Antisense ^{33}P-labeled rat LIF-R riboprobe (corresponding to bases 785-1645 of rat LIF-R; GenBank accession no. NM_031048) (0.2 pmol/ml) and

antisense digoxigenin-labeled rat POMC riboprobe (corresponding to bases 49–644 of rat POMC; GenBank accession no. AF_510391) (concentration determined empirically) were denatured, dissolved in hybridization buffer along with tRNA (1.7 mg/ ml), and applied to slides. Controls used to establish the specificity of the LIF-R riboprobe included slides incubated with an equivalent concentration of radiolabeled sense LIF-R riboprobe or radiolabeled antisense probe in the presence of excess (1000X) unlabeled antisense probe. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55°C. The following day, slides were treated with RNase A and washed under conditions of increasing stringency. The sections were incubated in blocking buffer and then in Tris buffer containing antidigoxigenin fragments conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN), diluted 1:250, for 3 h at room temperature. POMC cells were visualized with Vector Red substrate (SK-5100; Vector Laboratories) according to the manufacturer's protocol. Slides were dipped in 100% ethanol, air dried, and then dipped in NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed 6 d later and coverslipped. Determination of cells expressing both LIF-R and POMC mRNA was performed using criteria previously described (161). Briefly, POMC-mRNA-containing cells were identified under fluorescent illumination, and Grains 2.0.b (University of Washington, Seattle, WA) was used to count the silver grains (corresponding to radiolabeled LIF-R mRNA) over each cell. Signal-to-background ratios for individual cells were calculated; an individual cell was considered to be double-labeled if it had a signal-to-background ratio of 2.5 or more. For each animal, the amount of double-labeling was calculated as a percentage of the total number of POMC-mRNA-expressing cells and then averaged across animals to produce mean ± SEM.

Metabolic Phenotyping of PomcCre-gp130$^{flox/flox}$ mice

Food intake and body weight of PomcCre-gp130$^{flox/flox}$ mice (n=10) and littermate controls (n=9) were measured daily from 6 wks to 8 wks after birth to confirm there were no differences in either parameter during the ages used in reported experiments. Refeeding behavior was assessed by removing food from the cages of 6 week-old male, individually-housed PomcCre-gp130$^{flox/flox}$ mice (n=8) and littermate controls (n=8) at lights off (1800 h). Food was returned at 0900 h and food intake was measured at 1, 2, 4, 8, 12, and 24 h. Body weight was also measured daily at 0900 h beginning the morning before food was removed from the cages. Feeding behavior and voluntary activity were measured using metabolic cages equipped with a running wheel and an infrared feeding monitor (Mini-Mitter, Sunriver, OR). Feeding frequency and duration were quantified by measuring the number and duration of infrared beam breaks caused by the animals' heads while feeding. Activity was recorded as the number of wheel revolutions. of Seven week-old male PomcCre-gp130$^{flox/flox}$ mice (n=10) and littermate controls (n=9) were individually housed in these cages for 5 days. Data from the first two days of adaptation were discarded and data from the remaining three days were averaged across the 24 h period to compare feeding and activity patterns. Metabolic rate was evaluated by indirect calorimetry (Oxymax, Columbus Instruments, Columbus, OH). Mice were housed in separate chambers at 24 ± 1°C. Eight week-old male PomcCre-gp130$^{flox/flox}$ mice (n=10) and littermate controls (n=9) were first acclimatized to the chambers for two days. VO_2 and VCO_2 were simultaneously recorded for 4–5 h during the middle of the light cycle (1100–1600 h). Samples were recorded every 3 min with the room air reference taken every 30 min and the air flow to chambers at 500 ml/min. Basal oxygen consumption was determined for individual curves as the average

of the lowest two intervals corresponding to resting periods. Total oxygen consumption was the average of all measurements during periods of activity and inactivity.

Peripheral LPS injections in WT and PomcCre-gp130$^{flox/flox}$ mice for feeding study

On the day of the experiment at 0900 h, 7 week old PomcCre-gp130$^{flox/flox}$ mice (n=4 per treatment) or littermate controls (n=4 per treatment) received i.p. injections of LPS [100 µg/kg (Sigma-Aldrich Corp., St. Louis, MO) dissolved in 0.5% BSA (Sigma-Aldrich) in 0.9% saline] or 0.5% BSA in 0.9% saline alone, and were placed in clean cages with weighed quantities of food. Food intake was measured at 1, 2, 4, 8, 12, and 24 h post-treatment by experimenters blinded to condition and genotype.

Central LIF injection for LIF mRNA study

Eight week old male C57BL/6J mice had stainless steel cannulae placed in their lateral ventricle as previously described (161). After one week of recovery and restraint handling, animals received i.c.v. injections of LIF (50 ng, n=4) or vehicle (n=6) twice daily at 600 h and again at 1800 h for 5 days. On the morning of the sixth day, animals again received an i.c.v. injection at 600 h and were sacrificed 90 min later. Hypothalami were isolated and RNA extracted for quantitative RT-PCR analysis as described above.

Results

Induction of hypothalamic LIF mRNA expression following i.p LPS and i.c.v. IL-1β administration

Both systemic LPS and central IL-1β are commonly used to model inflammation-induced anorexia. Though IL-1β is sufficient to inhibit feeding acutely, it is possible that other inflammatory mediators are employed in anorectic animals. We used each of these models to assess whether LIF mRNA expression is acutely induced in hypothalami from cachectic animals. For the LPS experiment, male C57BL/6J mice were randomly assigned to receive i.p. LPS (100 μg/kg) or saline and sacrificed 1, 4, or 8 h later. Hypothalami were harvested and assessed for LIF mRNA content by RTPCR. We found that LIF mRNA was increased 10.67 ± 2.79-fold in LPS -treated mice 1 h following treatment, but that this effect was transient, as LIF was induced only 4.34 ± 2.01-fold at 4 h, and no increase was observed by 8 h (Figure 9A).

To evaluate the LIF-inducing capacity of central IL-1β, male C57BL/6J mice had stainless steel cannulae placed unilaterally in the lateral ventricle. After one week of recovery and restraint conditioning animals were randomly assigned to receive i.c.v. injections of either IL-1β (10 ng) or vehicle and sacrificed 8 h later. IL-1β treatment increased hypothalamic LIF mRNA by 54.89 ± 15.16-fold compared to vehicle (Figure 9B). Together these data demonstrate that both peripheral and central anorectic inflammatory conditions potently induce hypothalamic LIF mRNA expression and support a potential role for central LIF signaling in inflammation-induced anorexia. Hypothalamic IL-6 mRNA expression was also transiently induced following both LPS and IL-1β treatment, though no elevation in CNTF mRNA was observed (Figure 10).

Intracerebroventricular LIF induces anorexia associated with increased ARC POMC neuron activity

Decreased food intake in rodents has been reported following multiple modalities for LIF administration, including i.p. injection (444), implantation of LIF overexpressing tumor (186, 188), viral delivery (178, 187), and i.c.v. LIF in rats (445). We tested whether i.c.v. LIF also inhibits food intake in mice. Male C57BL/6J mice were fasted overnight and injected i.p. with ketorolac (40 mg/kg) after lights on. One hour later, mice were injected i.c.v. with 100 ng LIF (n=13) or vehicle (n=12). LIF treated mice showed reduced food intake at all time points and ate significantly less at 2 and 4 h post-treatment than vehicle treated mice, though by 8 h no statistical differences in food intake were found (Figure 11A and 11B).

To evaluate the role of ARC POMC neurons in mediating LIF-induced anorexia, we repeated this experiment in POMC-EGFP mice, and POMC neuron involvement was assessed using cFos-IR as a marker for neuronal activation (446, 447) (Figures 12A-I). I.c.v. LIF administration elicited significant increases in the number of cFos-IR neurons per section in the ARC compared to vehicle (10 ng LIF, 49.51 ± 2.73 cells per section; aCSF, 9.69 ± 0.81 cells per section; $P<0.0001$) (Figures 12A, D, G, and J). LIF also elicited significant increases in cFos-IR in POMC-EGFP neurons (10 ng LIF, 30.48 ± 1.33 cells per section; aCSF, 5.97 ± 0.88 cells per section; $P<0.001$) (Figures 12C, F, I, and K). No additional increase in cFos-IR in POMC neurons was seen when higher doses of LIF (100 ng) were used (Figures 12J-K). We also looked for changes in cFos-IR in NTS POMC neurons. Though an increase in total cFos-IR per section was observed, there was no change in the percentage of NTS POMC neurons showing cFos-IR (Figure 13).

LIF-R is expressed by POMC neurons in the ARC

To determine whether a neuroanatomical framework exists for direct LIF activation of POMC neurons, we examined the expression of LIF-R in the rat brain using *in situ* hybridization. LIF-R expression was noted in several brain regions, including cortex, hippocampus, and hypothalamus. Within the hypothalamus, expression was densest in the ARC (Figure 14A). Double label *in situ* hybridization showed clusters of silver grains representing LIF-R mRNA overlying POMC neurons (red precipitate)(Figure 14B). Semiquantitative analysis using a signal to background ratio cutoff of 2.5 indicated that radiolabeled LIF-R was expressed by 20.37 ± 1.26% of digoxigenin-labeled POMC neurons. We also performed double label *in situ* hybridization for gp130 expression by POMC neurons (data not shown). gp130 labeling in the ARC was too dense to resolve individual neuron clusters, as the glycoprotein is likely expressed by numerous glia and neurons in the region.

LIF increases α-MSH release from ARC POMC neurons

We used a murine hypothalamic explant model to assess whether central LIF stimulates the release of the anorexigenic peptide, α-MSH, from ARC POMC neurons. Hypothalami were harvested from male C57BL/6J mice and incubated in LIF dissolved in aCSF (50 nM, n=10) or aCSF alone (n=9). LIF significantly increased α-MSH release from the explants by 240.77 ± 25.4% compared to aCSF (Figure 15). This result demonstrates that increases in local LIF concentration, as seen in inflammatory states, are able to acutely induce α-MSH release from ARC POMC neurons. No increase in α-MSH release in response to lower concentrations of LIF (0.1 nM, 1.0 nM, 5.0 nM, n=5-6 per concentration) was observed (data not shown).

Generation of mice lacking gp130 in POMC-expressing cells

To evaluate the role of intact LIF-R/gp130 signaling in POMC neurons, we generated a transgenic mouse in which gp130 was functionally deleted specifically in POMC neurons (PomcCre-gp130$^{flox/flox}$). This mouse was generated by crossing a mouse in which exon 16 of gp130, which encodes the transmembrane region of the protein, is flanked by loxP sequences with a mouse expressing Cre recombinase under the control of the POMC promoter (Figure 16). This mouse has been reported previously in the literature, and was shown to have no changes in number of POMC neurons, stress response, or POMC neuron activation by leptin (177). We also verified that the transgenic animals show no differences in feeding behavior, body weight, refeeding following fast, wheel running activity, or metabolic rate as measured by oxygen consumption (Figure 17). To verify functional removal of gp130 from POMC neurons, PomcCre-gp130$^{flox/flox}$ mice and wild type (WT) littermates were pretreated with i.p. ketorolac (40 mg/kg) 30 min. before i.c.v. injection of LIF (50 ng, WT n=6; PomcCre-gp130$^{flox/flox}$ n=5) or vehicle (WT n=5; PomcCre-gp130$^{flox/flox}$ n=4). Double-label IHC for pSTAT3 and ACTH revealed that LIF increased pSTAT3 IR cells per section in the ARC in WT mice compared to control (LIF, 180.5 ± 13 cells/section; vehicle, 72.4 ± 11 cells/section ; $p<0.001$). Increased pSTAT3 signal was also observed in POMC neurons in WT mice (LIF, 64.86 ± 1.6%; vehicle, 35.55 ± 3.9%; $p<0.001$). Notably, though PomcCre-gp130$^{flox/flox}$ mice also showed an increase in pSTAT3 IR neurons per section following LIF administration (LIF, 164.3 ± 14 cells/section; vehicle, 87.1 ± 15 cells/section; $p<0.001$), no increase in POMC neurons containing pSTAT3 IR was observed compared to vehicle (LIF, 32.03 ± 2.7%; vehicle, 33.00 ± 2.0%; n.s.)(Figures 18A-R). This result indicates that LIF directly stimulates POMC neurons via the LIF-R/gp130 receptor complex. Further, the lack of a response in the transgenic mice provides a functional confirmation of gp130 removal from these neurons.

PomcCre-gp130$^{flox/flox}$ hypothalami do not increase α-MSH release in response to LIF

We repeated the hypothalamic explant study in the transgenic mice to determine whether functional gp130 signaling was necessary for LIF induced increases in α-MSH release *in vitro*. Hypothalami were harvested from PomcCre-gp130$^{flox/flox}$ mice and littermate controls and cultured in 50 nM LIF or aCSF. Though WT mice again increased α-MSH release in response to LIF, this response was absent in PomcCre-gp130$^{flox/flox}$ hypothalami (WT, 207.70 ± 39.2% control; PomcCre-gp130$^{flox/flox}$, 120.80 ± 17.8%; $p<0.05$)(Figure 19A). No differences were observed in basal or aCSF-induced α-MSH secretion between genotypes.

PomcCre-gp130$^{flox/flox}$ mice display a diminished anorectic response to LIF

To evaluate whether gp130 expression in POMC neurons is necessary for LIF-induced anorexia, we conducted a feeding study in the PomcCre-gp130$^{flox/flox}$ mice and litter matched controls. Overnight fasted mice were pretreated with i.p. ketorolac (40 mg/kg) 30 min. prior to i.c.v. injection with LIF (50 ng) or vehicle. Animals were given a week to recover before the experiment was repeated with the opposite treatment for each animal. LIF-treated WT mice significantly reduced food intake by ~30% at 2 h and 4 h post-injection compared to vehicle, but LIF-treated PomcCre-gp130$^{flox/flox}$ mice showed a blunted anorectic response at both 2 h ($p<0.01$ compared to WT, LIF-treated) and 4 h post-injection ($p<0.05$ compared to WT, LIF-treated), neither of which was significantly different from vehicle treated mice (Figure 19B). There was no significant difference observed between genotypes at any other time points, though LIF-treated WT

mice had eaten less than LIF-treated PomcCre-gp130$^{flox/flox}$ mice at all time points to 24 h.

Discussion

In acute and chronic disease, central expression of inflammatory cytokines is significantly elevated. Recent work has demonstrated that CNS amplification of inflammatory signaling is necessary for the induction of disease-associated anorexia (63). Understanding how this process inhibits food intake is essential to develop effective treatment for cachexia. Disease models in animals have been shown to induce LIF expression in the hypothalamus (58, 428), and central LIF administration is known to be anorectic both acutely (445) and chronically (187). Prior to these studies little was known, however, about which neuronal populations mediate LIF's anorectic effects. We show here that activation of POMC neurons by LIF is sufficient to inhibit food intake and that POMC neuron-specific ablation of LIF signaling restores food intake.

The results of these experiments represent the first time that the anorectic action of an inflammatory cytokine has been directly linked to the activation of POMC neurons. This work extends previous work in our laboratory, which showed that IL-1β regulates the activity of ARC POMC and NPY/AgRP neurons (161, 162), though those studies did not directly link those molecular findings to physiology. We demonstrated that both central IL-1β and peripheral LPS, given in doses that induce an anorexic response, increase hypothalamic LIF mRNA transcripts up to 55-fold over control levels. Though these findings do not confirm that LIF is responsible for reducing food intake in either model, they do support a potential role for LIF in transducing the inflammatory signals within the hypothalamus. We postulate LIF is being expressed and released by cells in

the ARC such that it can act in a paracrine or autocrine manner upon POMC neurons there, though the cell type responsible for LIF production remains unidentified. LIF, in contrast to IL-1β or LPS induces a pervasive anorexia when administered chronically, which more closely recapitulates cachexia. Elevations in circulating LIF have been observed cross-sectionally in humans with chronic disease, but it is unknown whether this finding accurately reflects long-term cytokine levels (182-184). Our own observations have shown that central LIF exposure itself can potently induce hypothalamic LIF expression (Figure 20) suggesting the possibility of positive feedback sustaining central LIF signaling and leading to protracted anorexia in chronic disease. We have demonstrated that the acute anorectic actions of LIF are dependent on central melanocortin signaling, but the role of this system in mediating chronic LIF-induced anorexia has not yet been investigated. Further studies investigating the neurophysiology of chronic LIF exposure are necessary to evaluate the importance of the present findings in cachexia.

The mRNA levels of another anorectic IL-6 family cytokine, CNTF, were indeed decreased in both inflammatory conditions tested. CNTF is also known to reduce food intake chronically, though at a far higher dose than LIF (177). This distinction is significant because both cytokines have been investigated as potential therapeutics for leptin resistance and obesity, but any effective treatment should avoid replicating the central inflammation seen in cachectic states. The observation that chronic LIF treatment induces cachexia and increased mortality whereas CNTF and leptin do not (178, 186, 188) indicates divergent physiologic roles in metabolic regulation. Despite initiating apparently identical intracellular signaling cascades, these three cytokines do not exhibit cross-desensitization; this finding has been attributed to differential induction of phospho-tyrosine phosphatase 1B, which can act as a feedback inhibitor of these

cascades (179). Further exploration of this phenomenon could yield important pathways separating cachexia from weight homeostasis.

Our observation that LIF-induced acute anorexia was almost completely abrogated in the gp130-deficient animals was unexpected. Numerous parallel and intersecting pathways converge to result in the final metabolic response, and it was expected that LIF could act on several pathways in concert to inhibit feeding. Given the dense expression of LIF-R and gp130 in the ARC, it is possible that other ARC neuron populations involved in energy homeostasis, such as those expressing NPY/AgRP play a role as well, but, based on our observations, inhibition of these neurons alone is not sufficient for LIF to induce anorexia. Furthermore, the animals were fasted overnight before LIF was administered, which should increase the activity of NPY/AgRP neurons. We can therefore posit that the increase in melanocortin tone is sufficient to inhibit feeding, even if LIF treatment inhibits NPY/AgRP neurons in parallel. This conclusion may not be that surprising, given that AgRP and NPY knock out mice show a limited metabolic phenotype (88). Recent studies have shown that though NPY and AgRP peptides may not be necessary to maintain food intake, the GABAergic activity of these neurons is essential to prevent starvation. Further, this starvation has been shown to be non-melanocortin dependent (448, 449). The role of this inhibitory tone in cachexia has not yet been evaluated, though the success of melanocortin antagonism in ameliorating anorexia associated with inflammation but not AgRP-neuron ablation suggests disparate pathways. Further work investigating the role of NPY/AgRP neurons in acute and chronic cachexia is necessary to understand the contribution of these neurons to LIF-induced anorexia.

Taken together, our double label *in situ* hybridization data indicate that at least 20% of POMC neurons express LIF-R. This estimate somewhat underrepresents the percentage of POMC neurons found to respond to LIF by IHC (~30-40%). Given the high background signal in the ARC and the absolute abrogation of pSTAT3 induction by LIF in the PomcCre-gp130$^{flox/flox}$ mice, it is likely that the *in situ* hybridization assay provides a conservative underestimate of receptor expression. Interestingly, activation of this minority of POMC neurons by LIF is sufficient to inhibit food intake, demonstrating the incredible potency of POMC neuron activation. Having demonstrated a neuroanatomical basis for direct LIF activation of POMC neurons, we then directly assessed the capacity of LIF to increase the release of α-MSH *in vitro*. Advantages offered by measuring secreted peptide in solution over measuring total peptide in tissue, include the quantitation of released, not stored, peptide, as well as the ability to evaluate the acute effects of our compound on these neurons, which total peptide may not be sensitive enough to detect. We showed that LIF induces a 2-2.5-fold increase in α-MSH release over control. This increase in melanocortinergic tone is consistent with the acute inhibition of food intake we observed. Previous studies using this technique have noted increases of similar magnitude in α-MSH secretion following treatment with leptin or IL-1β, suggesting physiological relevance (161, 450). We found that hypothalamic explants from mice lacking gp130 in POMC neurons were resistant to increased α-MSH release, supporting our hypothesis that LIF acts directly on POMC neurons to acutely elicit anorexia.

We were concerned that LIF may induce non-specific local CNS inflammation that could ultimately mediate acute anorexia following LIF treatment. In each of the whole-animal physiology studies, high dose ketorolac was administered before LIF treatment to block prostaglandin production. Studies by our laboratory and others have

confirmed that prostaglandins have a moderate inhibitory effect on food intake, which may be at least partially melanocortin-dependent (161, 451). Nonspecific cyclooxygenase inhibition was utilized to control for any confounding effect of prostaglandins. Importantly, ketorolac administration alone does not have any effect on food intake or hypothalamic neuropeptide expression levels at the dose used in these experiments (data not shown). We also observed induction of IL-6 mRNA in hypothalami from cachectic animals. Though central IL-6 has been shown to be acutely anorectic, this effect appears to be significantly smaller in magnitude than for LIF (445). Further, we found no evidence of IL-6R mRNA expression in the ARC, which is required for gp130 signaling (171), suggesting that any central IL-6 effects on feeding are mediated via an alternative neural circuit. Further, to minimize non-specific inflammation, minimal doses of LIF were used to elicit anorexia and neuron activation. Our studies used approximately 20-fold less LIF than previous studies investigating the anorectic effect of class I helical cytokines, such as CNTF and leptin (177, 452). If this lower concentration of cytokine is capable of non-selectively activating gp130-deficient POMC neurons, it is possible that the much higher concentrations of CNTF and leptin used in these studies are also non-selectively activating these neurons, possibly through inflammatory pathways. Indeed, it has been shown that the anorectic effects of pharmacologic doses of leptin require IL-1β signaling, whereas physiologic levels do not (453, 454).

Our finding that LIF-induced anorexia is mediated by POMC neurons provides the first direct evidence for melanocortin antagonism as a specific cachexia therapy. Treatment with MC4-R antagonists has been successful in retaining lean mass and increasing food intake in several animal models of cachexia (17-19, 89, 455). Though animals retained mass, it had not been conclusively shown that antagonism at the MC4-

R was not simply masking the underlying pathology. As molecular and genetic tools, such as the gp130$^{flox/flox}$ mouse, become more available, the role of melanocortin signaling in other models of cachexia can be better delineated. Also, the successes of melanocortin antagonism in attenuating cachexia suggest that modulation of this system by cytokines such as LIF and IL-1β is of physiologic significance in inflammatory states. We have shown that IL-1β induces the expression of LIF in the hypothalamus. It is possible that LIF mediates some of the acute anorectic effect of IL-1β, though we did not test this in the present study. These two cytokines are known to activate distinct intracellular signaling pathways and likely induce unique inflammatory profiles. Elucidation of these pathways and the downstream mediators of LIF-induced inflammation in chronic models could greatly augment our understanding of the sustained negative energy balance exhibited by cachectic patients and identify other neural populations that contribute to cachexia.

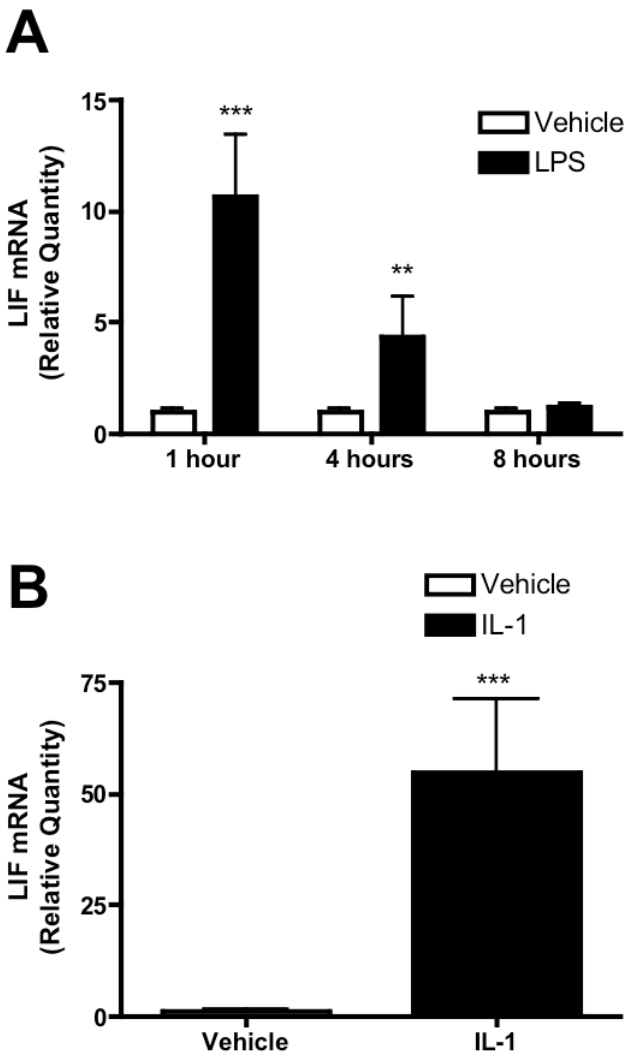

Figure 9. *Hypothalamic LIF expression in response to LPS and IL-1β.*
(A) Relative quantity of hypothalamic LIF mRNA following i.p. LPS (100 μg/kg) compared to vehicle in mice. LPS transiently induces hypothalamic LIF expression at 1 and 4 h following injection, though this effect is abolished at 8 h. (B) Central (i.c.v.) IL-1β (10 ng) potently induces hypothalamic LIF expression 8 h following administration. Data normalized to vehicle at each time point. Results expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by *post hoc* analysis using a Bonferroni corrected *t* test (A) or two-tailed Student's *t* test (B)(**p<0.01 vs. vehicle; ***p<0.001 vs. vehicle).

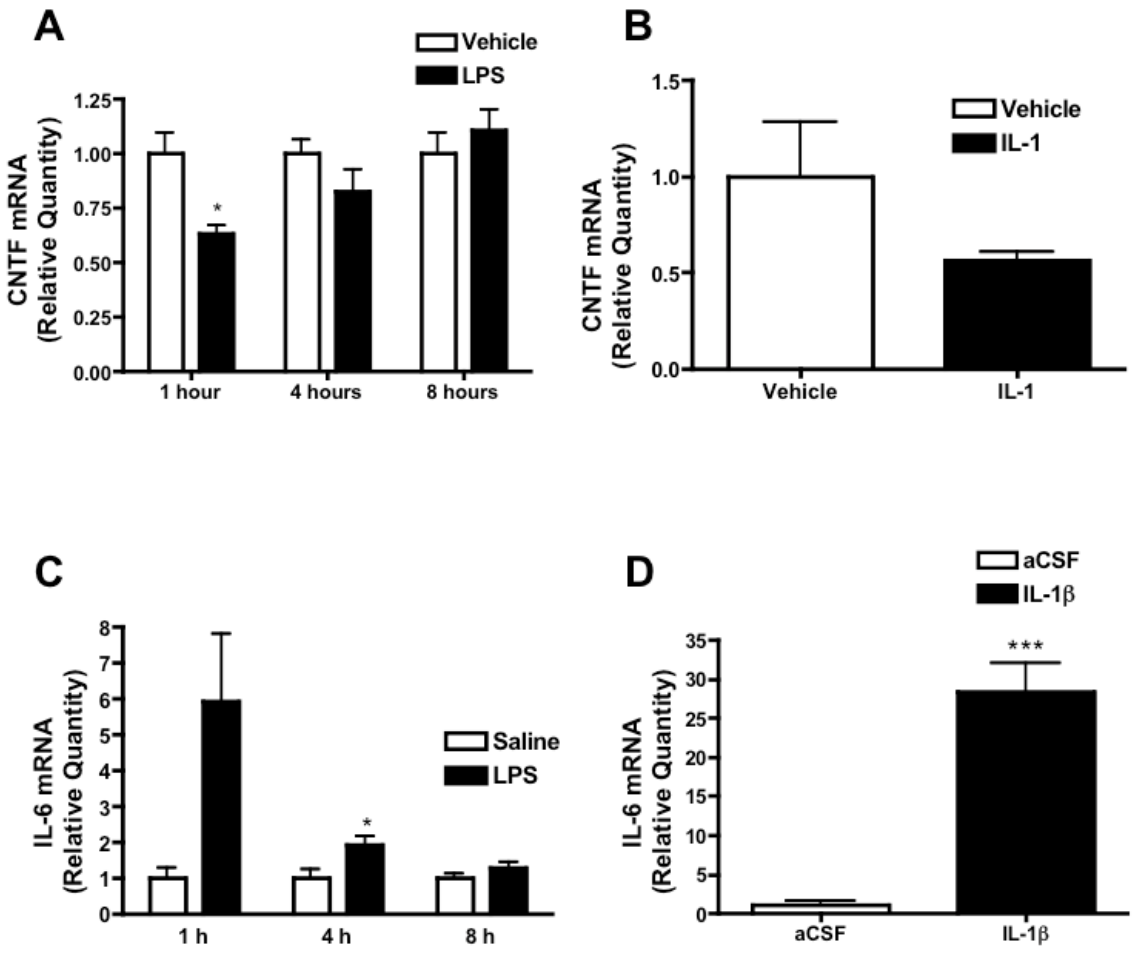

Figure 10. *Hypothalamic CNTF and IL-6 expression in response to LPS and IL-1β.*
(A) Relative quantity of hypothalamic CNTF mRNA following i.p. LPS (100 µg/kg). LPS suppresses CNTF expression at 1 h following injection, though CNTF expression is not significantly altered at 4 or 8 h. (B) Central (i.c.v.) IL-1β (10 ng) does not significantly alter CNTF expression 8 h after administration. (C) LPS induces IL-6 expression 4 h following i.p. injection, but IL-6 expression is not significantly altered at 1 or 8 h. (D) IL-1β potently induces IL-6 expression 8 h after i.c.v. administration. Data normalized to vehicle at each time point. Results expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by a *post hoc* analysis using a Bonferroni corrected *t* test (A, C) or by two-tailed Student's *t* test (B, D)(*p<0.05, ***p<0.001 vs. vehicle).

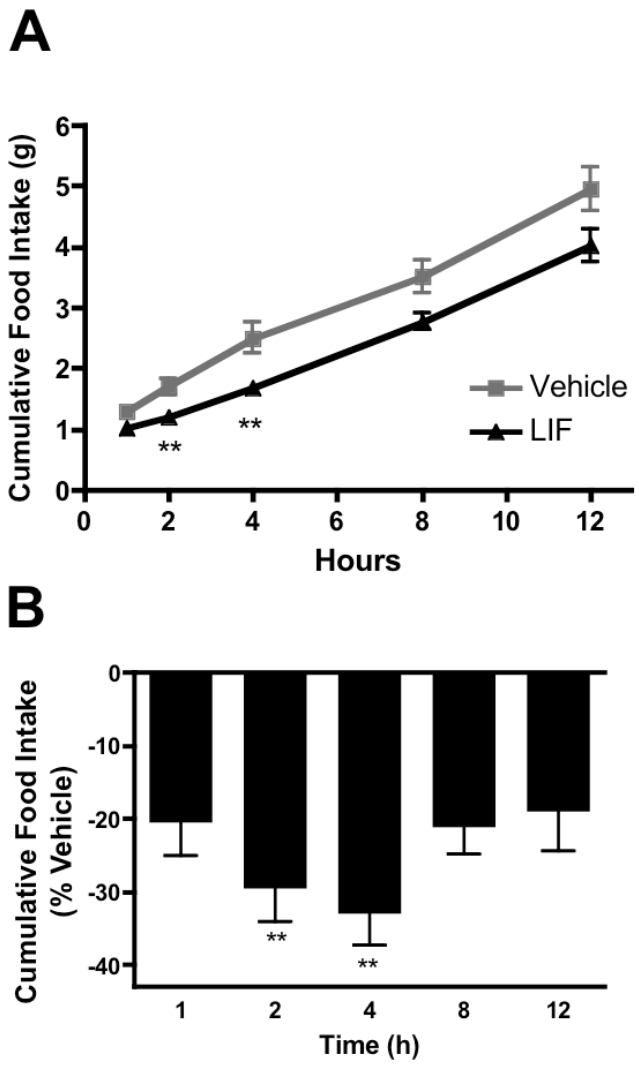

Figure 11. *Effects of central LIF on food intake in wild type mice.*
(A) Cumulative food intake following i.c.v. bolus injection of LIF (100 ng) vs. vehicle in wild type mice. Mice fasted overnight and pretreated with i.p. ketorolac (40 mg/kg). (B) Cumulative food intake in LIF-treated mice expressed as percent reduction compared to vehicle. Inhibition of food intake was significant at 2 and 4 h post-treatment. Data expressed as mean ± SEM. Statistics calculated two-way ANOVA followed by *post hoc* analysis using a Bonferroni corrected *t* test (**p<0.01 vs. vehicle).

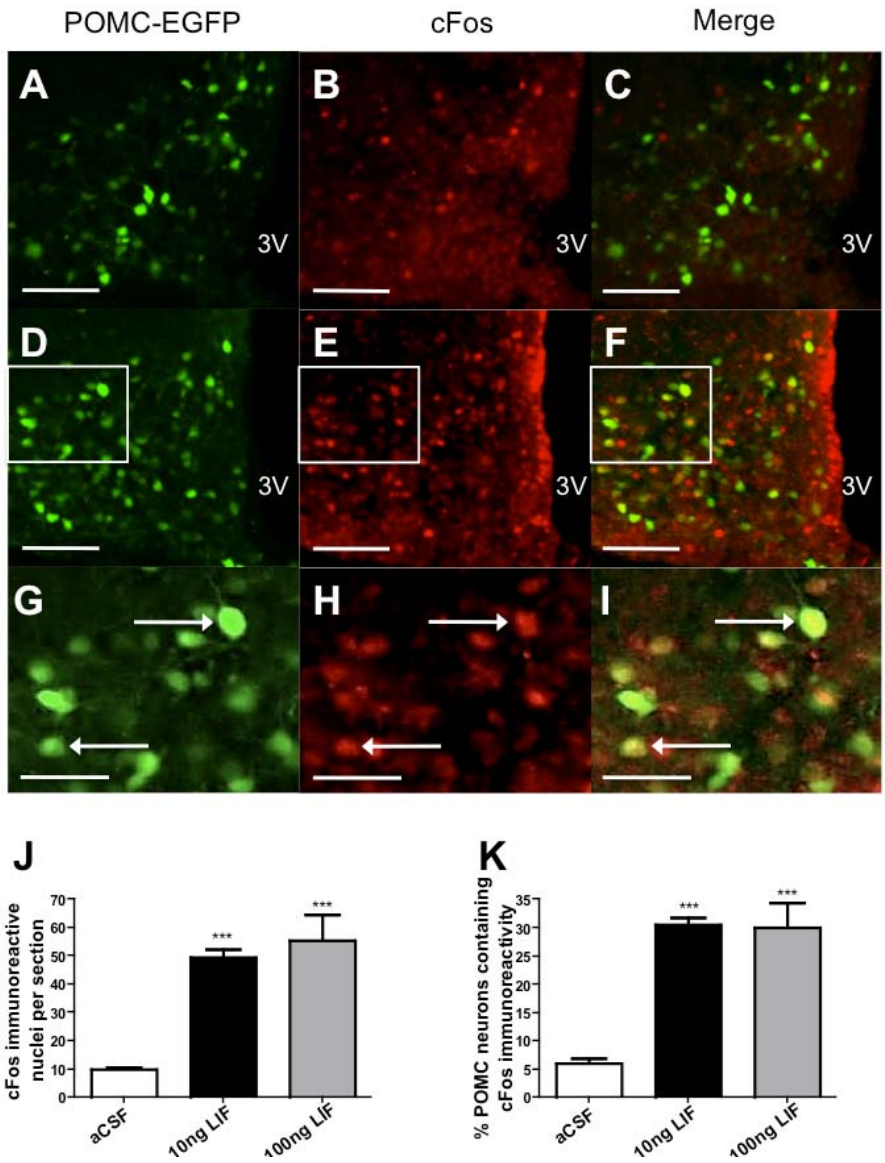

Figure 12. *LIF activates POMC neurons in the hypothalamus.*
(A, D, G) POMC-EGFP expression in the arcuate nucleus. (B) Low cFos IR (*red*) in vehicle-treated mice (n=3). (C) Few POMC neurons show nuclear cFos IR after vehicle treatment. (E, H) cFos IR is increased in LIF-treated (10 ng, n=4) mice. (F, I) LIF increases cFos IR in POMC neurons. (G, H, I) Enlargement of D-F shown for clarity (*white boxes* indicate regions of enlargement). (J) LIF increases cFos IR per section. (K) Approximately 30% of POMC neurons contain cFos immunoreactivity after either 10ng or 100 ng LIF treatment. Data expressed as mean ± SEM, and statistics calculated by one-way ANOVA followed by a *post hoc* analysis using a Bonferroni corrected *t* test (***p<0.001 vs. vehicle). *Scale bars,* 100 µm (A-F) and 50 µm (G-I). *3V*, Third ventricle.

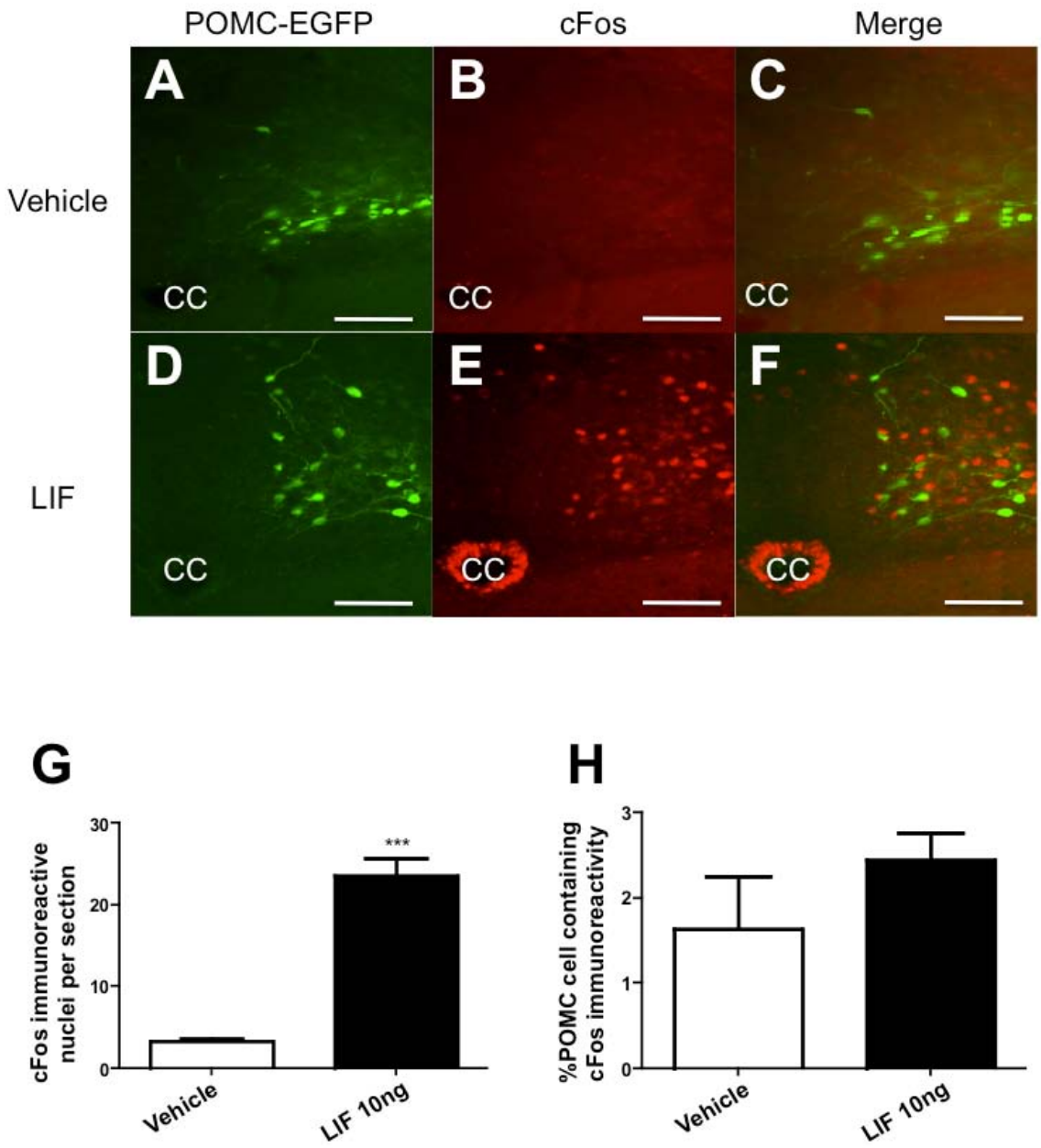

Figure 13. *LIF does not activate brainstem POMC neurons.*

(A, D) POMC-EGFP expression in the NTS. (B) Low cFos IR (*red*) in the NTS from vehicle-treated mice (n=4). (C) Few POMC neurons exhibit nuclear cFos IR following vehicle treatment. (E, G) Central (i.c.v.) LIF treatment (10 ng) increases total cFos expression (n=5). (F, H) NTS POMC neurons do not exhibit increased nuclear cFos IR following LIF administration. Results expressed as mean ± SEM. Statistics calculated by two-tailed Student's *t* test (***p<0.001 vs. vehicle).

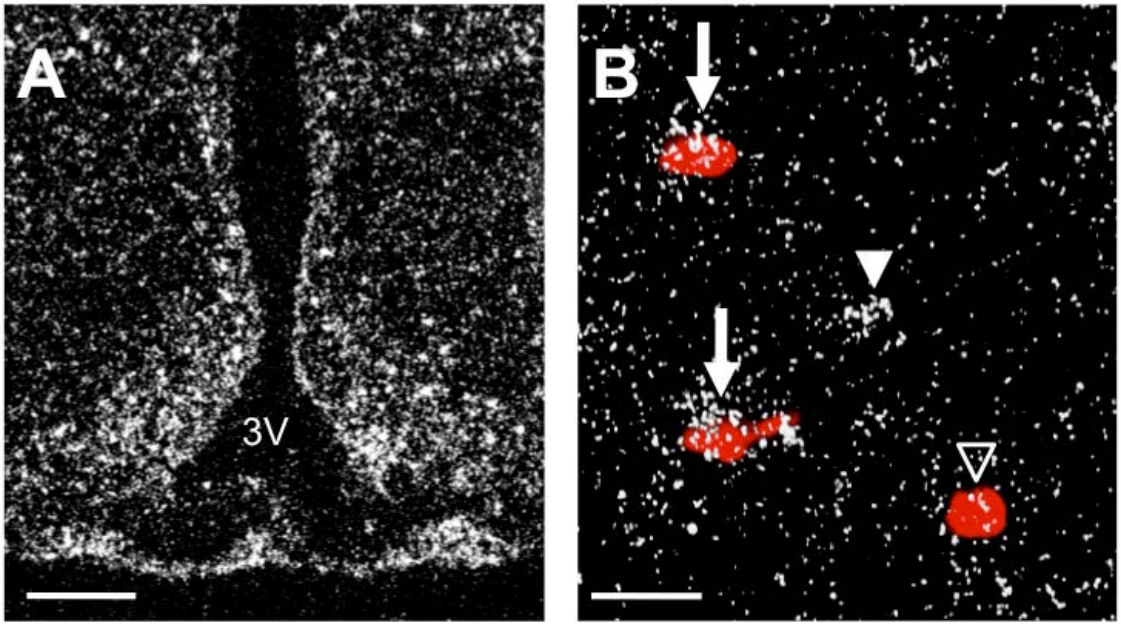

Figure 14. *Expression of LIF-R in the hypothalamus.*
(A) Representative dark-field photomicrograph showing expression of LIF-R mRNA (*silver grain clusters*) in the ARC of wild-type rats. (B) Double-label *in situ* hybridization showing expression of LIF-R (*silver grain clusters*) by cells expressing POMC mRNA (*red precipitate*). *Arrows* point to POMC neurons that coexpress LIF-R mRNA. *Open arrowheads* signify POMC neurons that do not express LIF-R mRNA. *Filled arrowheads* denote cells that express LIF-R mRNA but not POMC mRNA. *Scale bars,* 100 μm (A) and 25 μm (B). *3V*, Third ventricle.

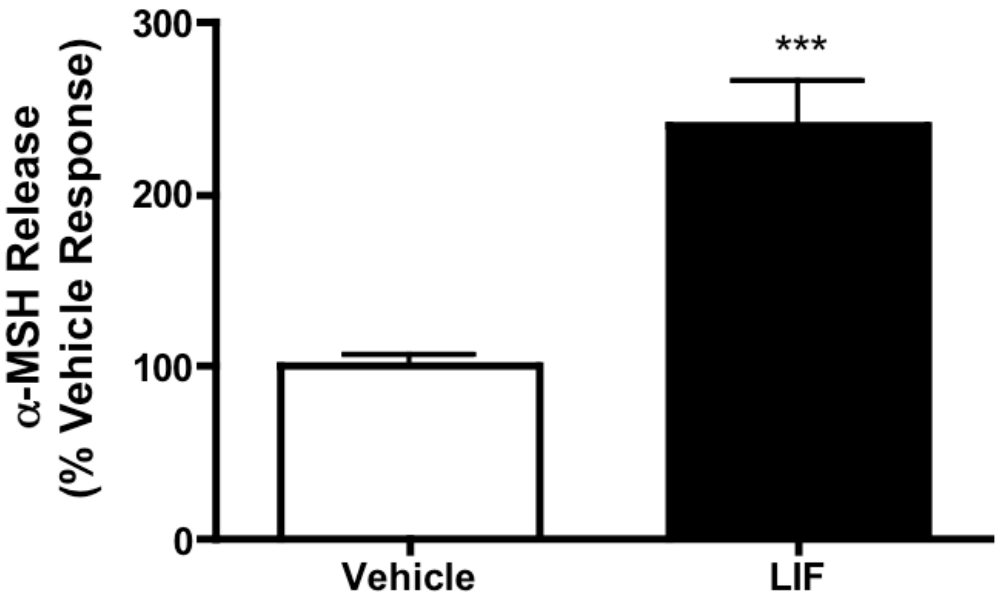

Figure 15. *LIF stimulates α-MSH release from murine hypothalamic explants.*
LIF (50 nM, n=10) increased α-MSH release *in vitro* by approximately 2.5-fold over vehicle (n=9). Results expressed as mean ± SEM. Statistics calculated by two-tailed Student's *t* test (***p<0.001 vs. vehicle).

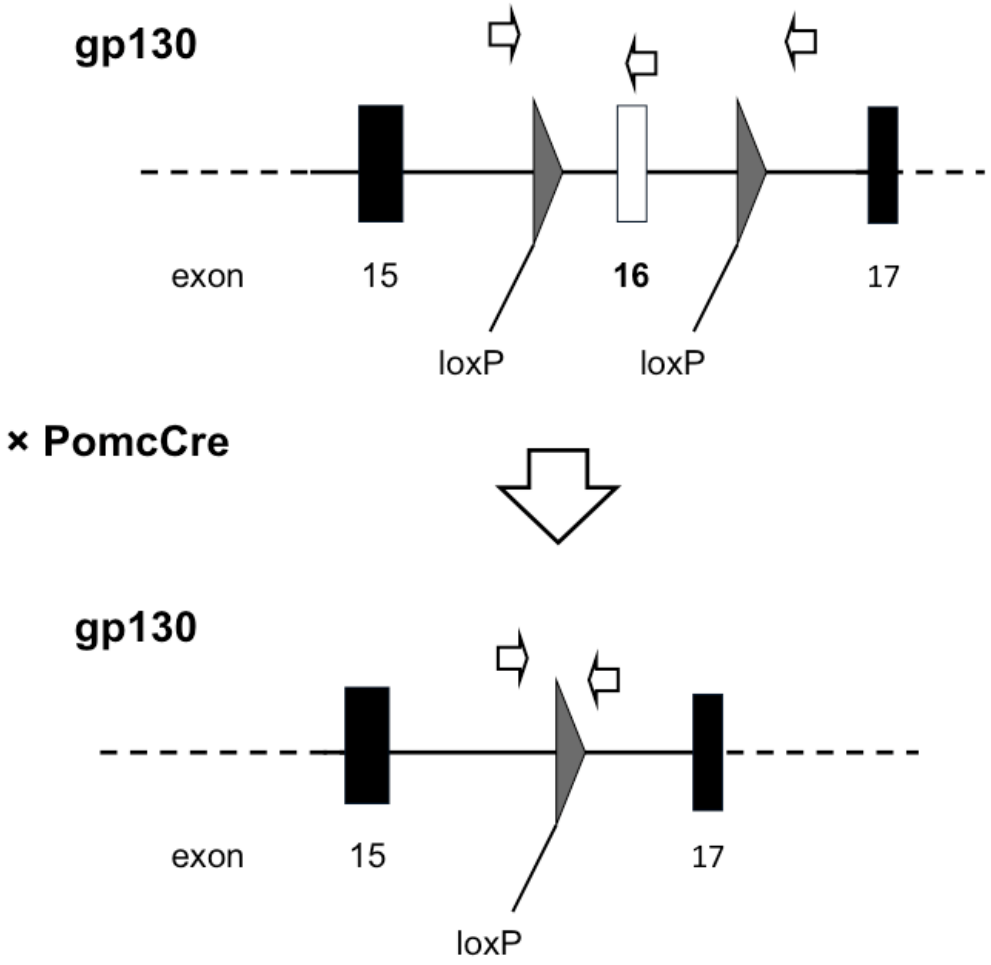

Figure 16. *Diagram of PomcCre-gp130$^{flox/flox}$ mouse genetics.*
gp130$^{lox/lox}$ mice were generated with *loxP* sites flanking exon 16, which encodes the transmembrane region of the receptor. These mice were crossed with PomcCre mice, which express the viral *Cre* recombinase under the control of the POMC promoter. In POMC neurons from PomcCre-gp130$^{flox/flox}$ mice, exon 16 is removed from gp130, specifically inactivating the receptor.

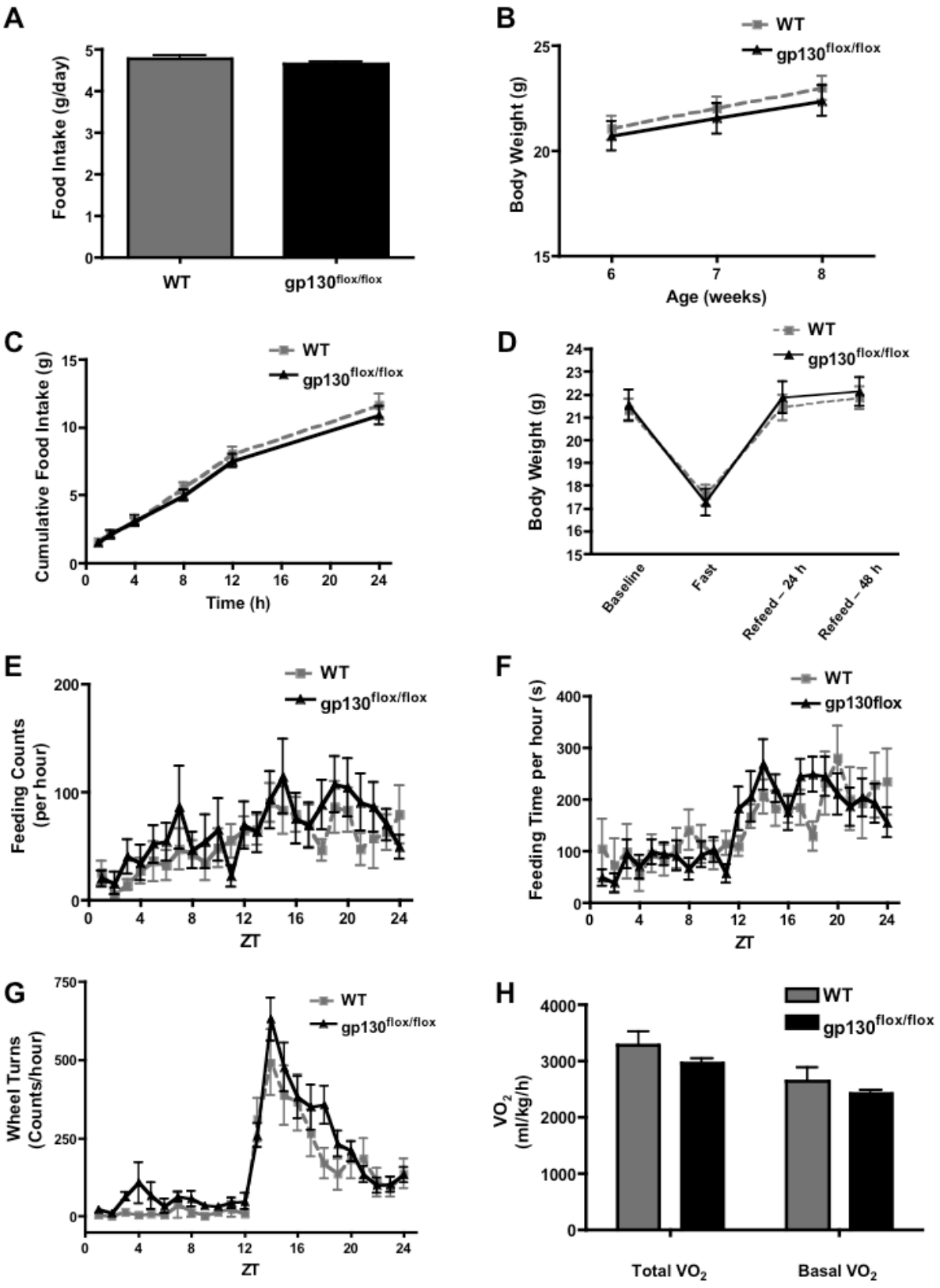
A
Food Intake (g/day)
WT
gp130flox/flox
B
Body Weight (g)
Age (weeks)
C
Cumulative Food Intake (g)
Time (h)
D
Body Weight (g)
Baseline
Fast
Refeed – 24 h
Refeed – 48 h
E
Feeding Counts (per hour)
ZT
F
Feeding Time per hour (s)
gp130flox
G
Wheel Turns (Counts/hour)
H
VO2 (ml/kg/h)
Total VO2
Basal VO2

Figure 17. *Metabolic phenotype of PomcCre-gp130$^{flox/flox}$ mice.*
(A) Average daily food intake did not differ between PomcCre-gp130$^{flox/flox}$ mice (n=10) and control littermates (n=9). (B) No changes in body weight were observed between PomcCre-gp130$^{flox/flox}$ mice (n=10) and control littermates (n=9) during the ages of animals used in the experiments described in this manuscript. (C) No changes in refeeding following an overnight fast, measured as cumulative food intake, were observed between transgenic (n=8) and control (n=8) mice. (D) Changes in body weight due to fasting and refeeding did not differ between genotypes. No differences in feeding frequency (E) or duration (F) were observed between PomcCre-gp130$^{flox/flox}$ mice (n=10) and control littermates (n=9). (G) Voluntary activity, measured by running wheel turns, did not differ between PomcCre-gp130$^{flox/flox}$ mice (n=10) and control littermates (n=9). (E, F, G) Measurements were taken for three days and averaged. Cumulative values for each hour were then averaged across animals in each group. (H) No significant differences in basal or total metabolic rate were observed between transgenic (n=10) and control (n=9) animals. Data are expressed as mean ± SEM. Statistics calculated by Student's *t* test (A, H) or two-way ANOVA (B-G).

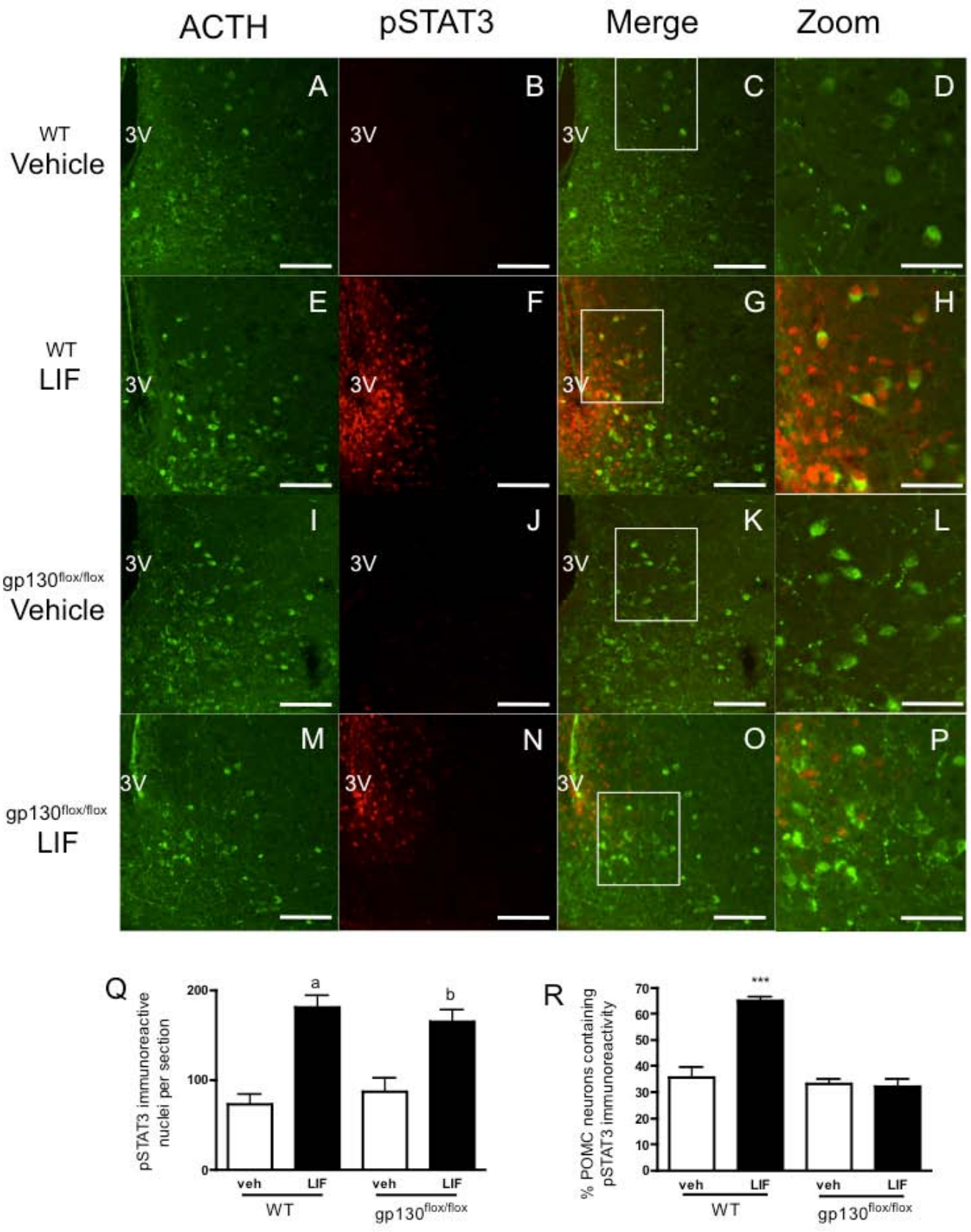
ACTH
pSTAT3
Merge
Zoom
WT
Vehicle
WT
LIF
gp130flox/flox
Vehicle
gp130flox/flox
LIF
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
3V
Q
pSTAT3 immunoreactive nuclei per section
200
100
0
a
b
veh
LIF
veh
LIF
WT
gp130flox/flox
R
% POMC neurons containing pSTAT3 immunoreactivity
70
60
50
40
30
20
10
0
***
veh
LIF
veh
LIF
WT
gp130flox/flox

Figure 18. *LIF-induced pSTAT3 IR in WT and gp130$^{flox/flox}$ POMC neurons.*
(A, E, I, M) ACTH IR in the ARC of WT and gp130$^{flox/flox}$ mice was used to identify POMC neurons. (B, J) pSTAT3 IR is low in vehicle treated WT (n=5) and gp130$^{flox/flox}$ (n=4) mice. (C, D, K, L) Few POMC neurons show pSTAT3 IR in vehicle treated mice of both genotypes. (F, N) LIF treatment (50 ng) increased pSTAT3 IR in WT (n=6) and gp130$^{flox/flox}$ (n=5) mice 30 min following i.c.v. injection. (G, H) LIF treatment increases pSTAT3 IR in WT POMC neurons. (O, P) Despite increased total pSTAT3 IR, few gp130$^{flox/flox}$ POMC neurons show pSTAT3 IR following LIF treatment. (D, H, L, P) Enlargement of C, G, K, & O for clarity (*white boxes* denote area of enlargement - area chosen to maximize # of POMC neurons in panel). (Q) pSTAT3 IR per section is increased by LIF treatment in both genotypes. (R) LIF treatment increases the number of POMC neurons containing pSTAT3 IR in WT but not gp130$^{flox/flox}$ mice. Data expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by a *post hoc* analysis using a Bonferroni corrected *t* test (a, $p<0.001$ vs. WT/vehicle & gp130$^{flox/flox}$/vehicle; b, $p<0.001$ vs. WT/vehicle, $p<0.01$ vs. gp130$^{flox/flox}$/vehicle). *Scale bars*, 100 µm (A-C, E-G, I-K, M-O) and 50 µm (D, H, L, P). *3V*, Third ventricle.

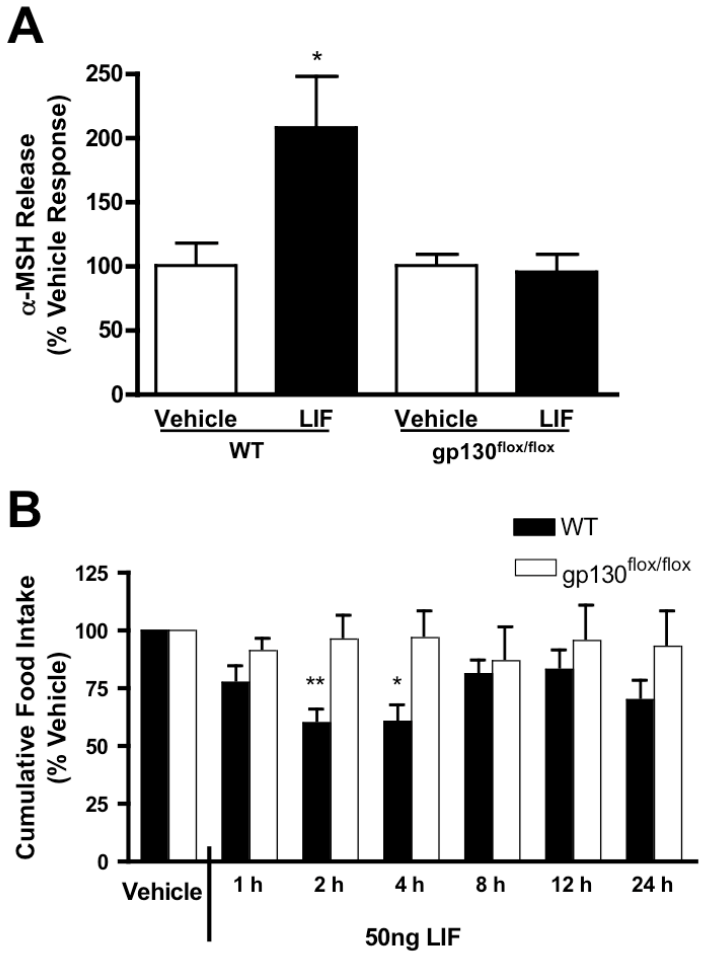

Figure 19. *gp130$^{flox/flox}$ mice are resistant to both LIF-induced α-MSH release and anorexia.*
(A) LIF-induced α-MSH release from WT and gp130$^{flox/flox}$ murine hypothalamic explants. LIF (50 nM, n=5) increases α-MSH release from WT hypothalami compared to vehicle (n=7). LIF does not increase α-MSH release from gp130$^{flox/flox}$ hypothalami (n=8) compared to vehicle (n=5). Data shown as mean ± SEM and statistics calculated by two-way ANOVA followed by a *post hoc* analysis using a Bonferroni corrected *t* test (*p<0.05 vs. LIF-treated gp130 $^{flox/flox}$).
(B) LIF-induced anorexia is attenuated in gp130$^{flox/flox}$ mice. I.C.V. LIF (50 ng, n=7) reduced food intake in fasted, ketorolac (40 mg/kg) treated mice at 2 and 4 h post-injection compared with vehicle (n=5). This effect was diminished in gp130$^{flox/flox}$ mice (LIF, n=6; vehicle, n=6). Results normalized to percentage of vehicle food intake and expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by a *post hoc* analysis using a Bonferroni corrected *t* test (*p<0.05; **p<0.01 vs. gp130$^{flox/flox}$). No statistically significant differences between genotypes were found at any time following vehicle treatment (p>0.05).

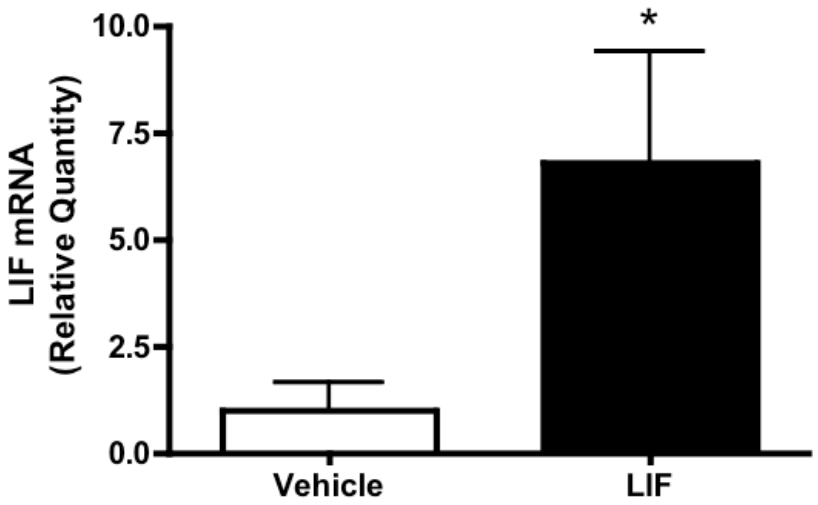

Figure 20. Hypothalamic LIF expression induced by chronic central LIF administration. Animals were injected i.c.v. twice daily with LIF (50 ng/injection, n=4) or vehicle (n=6) for 5 days. Chronic LIF exposure induced LIF mRNA expression in the hypothalamus compared to vehicle. Data expressed as mean ± SEM. Statistics calculated by Student's *t* test (*p<0.05 vs. vehicle).

CHAPTER 3

Manuscript #2

Inflammation-induced lethargy is mediated by suppression of orexin neuron activity

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Abstract

The hypothalamus and brainstem regulate circadian patterns of arousal and activity, corresponding to the sleep-wake cycle as well as other stereotypic behavior, such as feeding. During disease states, LMA and vigilance are suppressed, even under conditions that normally stimulate physiologic arousal. LHA Ox neurons are active during periods of waking, stress, and calorie deprivation in healthy animals. Deficiencies in Ox signaling lead to hypersomnolence, hypoactivity, and abnormal responses to stressful stimuli. In the present work, we examine the effect of endotoxin-induced inflammation on Ox neuron biology and LMA in rats. We then investigate the mechanisms of Ox neuron inhibition and lethargy induction by inflammation. Our results demonstrate a vital role for diminished Ox signaling in mediating inflammation-induced lethargy. This work defines a specific population of inflammation-sensitive, arousal-associated Ox neurons and identifies a proximal neural target for inflammatory signaling to Ox neurons, while eliminating several others.

Introduction

The regulation of arousal is essential for mounting sufficient drive to carry out basic activities such as waking, feeding, copulation, and escape. These processes are regulated by a circuit including hypothalamic neurons that integrate circadian, metabolic, limbic, and physiologic signals with ascending arousal information from the brainstem. Together, this pathway modulates central monoaminergic signaling and peripheral autonomic tone, as well as cortical and thalamic activity (199). The circadian influence of clock neurons in the SCN of the hypothalamus plays a prominent role, as movement, feeding, and sleeping patterns exhibit remarkable consistency across a 24 h period in a given animal. Yet, animals must also be able to modulate vigilance in response to their environment, particularly to avoid imminent threat. Recently, neural populations that are sensitive to environmental stressors, but that also influence arousal, have been identified (456).

One set of neurons that has been implicated in the allostatic control of arousal is the Ox-expressing neurons of the perifornical LHA (271). Ox neurons are activated during waking, stress, exposure to novel environments, and undernutrition (268). Ox neurons project widely throughout the brain, and orexins excite arousal-mediating noradrenergic neurons in the locus coeruleus, serotonergic neurons in the dorsal raphe, dopaminergic neurons in the ventral tegmental area, and histaminergic neurons in the tuberomammillary nucleus (270, 299, 302, 357, 457). A role for Ox neurons in mediating sleep/wake transitions has been described, supported by multiple lines of evidence linking deficient Ox signaling to narcolepsy, a condition marked by excess daytime sleepiness (276, 281, 297). Ox neurons are also responsive to circulating signals of energy status, including glucose, insulin, and leptin, and mediate the increase in foraging

behavior exhibited by FR animals (268, 310, 315). Ox neurons have been linked to other motivated behaviors as well, including drug-seeking and sexual behavior (356, 358, 458). Taken together, this work suggests that orexins serve as a crucial intermediate inciting arousal in response to metabolic needs, stress, and reward-oriented stimuli.

Inflammation and disease are states of physiologic stress that are associated with dysregulation of homeostatic neural networks and paradoxical behavioral responses to environmental and physiologic cues. This sickness behavior includes anorexia, lethargy, increased daytime somnolence, increased metabolic rate, and activation of the HPA axis (155). Central inflammation is commonly associated with these hallmark responses and is sufficient to drive this behavior in the absence of peripheral inflammation or disease (459). Recent research by our laboratory has demonstrated that local elevations in inflammatory cytokines can alter the activity of hypothalamic neurons involved in homeostatic control of feeding and metabolism (62, 161, 162). The inflammation-sensitive population of neurons that mediates the lethargic response to sickness has not yet been identified, however.

Several lines of evidence suggest that Ox neurons mediate this behavior. First, Ox knockout mice exhibit marked hypophagia, hypolocomotion, hypersomnolence, and an inability to adapt to restricted feeding, a phenotype resembling sickness behavior (274). Second, studies have demonstrated a localized reduction in cFos IR in the LHA following inflammatory insults, suggesting reduced neuron activity in this region (421, 423). Third, LHA glucose-sensitive neurons show reduced activity in the presence of IL-1β or TNF-α (422). In the present work we use LPS- and tumor-induced models of inflammation to investigate whether Ox neurons are inhibited during inflammation and if

the suppression of this system mediates the lethargic response to sickness. We also evaluate the proximal neural input by which inflammation inhibits Ox neurons.

Materials and Methods

Animals and surgical procedures

Male Sprague Dawley (250–450 g; Charles River Laboratories, Wilmington, MA) or F344/NTacfBR rats (200-250 g; Taconic Farms, Inc., Hudson, NY) and Nts-cre/GFP mice (324) were maintained on a normal 12 h light/dark period with lights on 0600 –1800 h; corresponding to ZT 0-12 at 22-24°C with *ad libitum* access to food (Purina rodent diet 5001; Purina Mills, St. Louis,MO) and water, unless otherwise noted. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Oregon Health and Science University or the University of Michigan.

Generation of Nts-cre/GFP mice

The Nts-cre mice were generated as detailed by Leinninger et al. (324). Briefly, the Nts IRES-Tau-GFP targeting sequence (460) was modified to create mice that express cre recombinase in Nts-expressing cells. The Tau-GFP coding sequence was replaced with an IRES-cre cassette (461) downstream of the 3' non-coding region of the Nts mRNA. The Nts-cre construct was then linearized and electroporated into mouse ES cells. 293 clones were obtained and screened by qPCR for loss of homozygosity, of which, 8 putative positives were re-screened by Southern Blot for final confirmation. One clone was positive by both analyses, and was linearized and injected into mouse

blastocysts to generate chimeras, which were bred to C57BL/6 animals. Targeted allele in offspring was confirmed by qPCR for Nts and conventional PCR for neo.

Locomotor Activity and Temperature

Voluntary home cage LMA and brown adipose tissue (BAT) temperature were measured using implantable telemetric transponders (MiniMitter, Bend, OR). Animals were anesthetized using 2% isoflurane, a small midline incision was made just anterior to the BAT, and transponders were implanted beneath the BAT, with care taken to leave innervation unperturbed. For rats receiving i.c.v. injections, transponders were implanted during the lateral ventricle cannulation surgery. Rats were individually housed and allowed to acclimate for at least 5 days before temperature and net movement in *x*-, *y*-, and *z*-axes was recorded in 1 min intervals (Vital View, MiniMitter, Bend, OR).

Implantation of lateral ventricle cannulae

Sprague Dawley or F344/NTacfBR rats were anesthetized with 2% isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A sterile guide cannula with obturator stylet (Plastics One, Inc., Roanoke, VA) was stereotaxically implanted into the lateral ventricle. The coordinates used were: 1.0 mm posterior to bregma, 1.5 mm lateral to midline, and 4.1 mm below the surface of the skull (462). The cannula was fixed in place with dental cement. The animals were individually housed after surgery for a minimum of 1 wk, and were handled daily for at least 3 d before each treatment.

Dark phase LPS injections

For evening experiments, on the day of the experiment at 1630 h (ZT 10.5), individually housed animals received i.p. injections of LPS [250 µg/kg (Sigma-Aldrich Corp., St. Louis, MO)] dissolved in 0.5% low-endotoxin BSA (Equitech-Bio, Inc., Kerrville, TX) in 0.9% saline or 0.5% BSA in 0.9% saline alone, and were placed in their home cage without food. Behavioral studies were conducted with a crossover design, with activity and temperature monitored throughout the evening. The following morning at 0530 h (ZT 23.5), rats were anesthetized with 2% isoflurane and CSF was collected by percutaneous cisterna magna puncture (291). Protease inhibitor cocktail (Roche Diagnostics, GmbH, Mannheim, Germany) was added to the CSF before snap freezing on dry ice and storage at -80°C. Following 5 d of recovery treatment groups were switched and the experiment was repeated. For histology experiments, rats or mice were sacrificed at 1930 h (ZT 13.5) and processed for IHC as described below. Experiments were performed on serial days to minimize variation in time-of-sacrifice between animals.

Central OxA replacement experiments

For the acute OxA replacement experiments, male Sprague Dawley rats were implanted with lateral ventricle cannulae and telemetric transponders. After 5 d of recovery and handling, LPS was administered at 1630 h (ZT 10.5) and food was removed, as described above, followed by i.c.v. injection of OxA (1 µg; California Peptide Research, Inc, Napa, CA) dissolved in aCSF or aCSF alone. In the lethargy prevention experiment, i.c.v. injections were conducted at 1730 h (ZT 11.5); in the lethargy reversal experiment, i.c.v. injections were performed at 2100 h (ZT 15).

Following injection, rats were returned to their cages and activity and temperature were measured. For the subchronic Ox replacement experiment, rats were implanted with Alzet i.c.v. brain infusion kits connected to 3-day osmotic minipumps [model 1003D, 1 µl/h (Durect Corp., Cupertino, CA)] on the day before the experiment using the above coordinates for lateral ventricle cannulation. Pumps were filled with OxA dissolved in aCSF (3 µg/µl) or aCSF. During pump assembly and priming, the catheters connecting the minipumps to the cannulae were filled with saline with a small bubble to separate the saline from the drug. These catheters were measured such that the drug began delivery into the brain approximately 1-2 h before onset of the dark phase on the experimental day to ensure that no G-protein coupled receptor desensitization to Ox occurs (463). During minipump implantation, the distance from the bubble to the cannula was confirmed to ensure appropriate treatment start time. On the day of the experiment at 1630 h (ZT 10.5), rats were injected i.p. with LPS or veh, as described above, except that food was weighed and returned to the cage. Activity, temperature, and food intake were measured during the subsequent 24 h period.

Peripheral LPS and central IL-1β injections in food-entrained rats

Sprague Dawley rats were implanted with lateral ventricle cannulae and subcutaneous telemetric transponders and allowed to recover for 5 d before the beginning of food entrainment. FR rats were individually housed, handled, and allowed access to food from 1000 h – 1200 h (ZT 4-6) each day for 8 days. Food intake, body weight, activity, and temperature were recorded daily. On day 9 rats were injected i.p. with LPS (250 µg/kg) or veh (LPS experiments) or injected i.c.v. with IL-1β (100 ng) dissolved in aCSF + 0.5% BSA or veh (IL-1β experiments) at 0730 h (ZT 1.5). In the

behavioral studies, all animals then received i.c.v. injections of OxA (1 µg) or aCSF at 0900 h (ZT 3). Food was placed in the cages from 1000 h – 1200 h (ZT 4-6) and food intake, body weight and activity were recorded. FAA was measured during the 60 min preceding food presentation. For histology studies, rats were sacrificed at 0930 h (ZT 3.5) by transcardial perfusion fixation as described below. Experiments were performed on serial days to minimize variation in time of treatment and sacrifice between animals.

RNA preparation and qRT-PCR in FR rats

Male Sprague Dawley rats were allowed *ad libitum* access to food or FR for 8 d as described in main text. On experiment day, rats were injected i.p. with LPS (250 µg/kg) v. vehicle at 0730 h (ZT 1.5). One hour later (ZT 2.5) the animals were deeply anesthetized using sodium pentobarbital (65 mg/kg), the thoracic cavity was opened and blood was collected by cardiac puncture of the right ventricle. Blood was stored on ice until the end of the experiment, then plasma was isolated and frozen at -80°C. Rats were then perfused with RNase-free DEPC 0.01M PBS + heparin sodium (15,000 U/L) to flush the vasculature in the hypothalamus. Hypothalamic blocks with median eminence attached were isolated, preserved in RNAlater solution (Ambion, Inc., Austin, TX), and stored at 4°C overnight. RNA was extracted the next day and used for qRT-PCR analysis as described previously (162). cDNA was synthesized and RT-PCR reactions were run using prevalidated TaqMan master mix and primer-probes (Applied Biosystems, Foster City, CA). Statistical analysis using 2-way ANOVA followed by *post hoc* analysis using Bonferroni's corrected *t* test performed on dC_t values. Raw C_t values from 18S endogenous controls were compared between groups to validate observed changes in target genes.

Plasma IL-6 ELISA

Plasma was collected as described above. IL-6 ELISA (R&D Systems, Minneapolis, MN) was performed according to manufacturer instruction. All samples were run at 1:2 dilution as well as 1:200 dilution to ensure concentration was within the dynamic range of the assay.

Central anti-inflammatory treatments

Blockade of central IL-1 signaling and PG synthesis was performed using the dark phase LPS injection model detailed above with slight modifications. Sprague Dawley rats were implanted with lateral ventricle cannulae and subcutaneous telemetric transponders and allowed to recover for 5 d before the beginning of these experiments. Peripheral LPS treatments were performed as described in the main text with slight modifications. In the central IL-1 blockade experiment, rats were co-treated with i.c.v. IL-1ra (4 µg) dissolved in aCSF + 0.5% BSA v. vehicle and i.p. LPS (250 µg/kg) v. vehicle injection at ZT 10.5 (1630 h). Food was weighed and returned to the cages as a positive control for central drug action. Food intake, body weight, temperature, and activity were recorded. Following 5 d of recovery, rats were crossed-over such that all animals received a different i.p. and i.c.v. treatment and the experiment was repeated. For nonspecific, systemic PG blockade, rats were pretreated with i.p. indomethacin (indo; 4 mg/kg) dissolved in DMSO v. vehicle at ZT 10 (1600 h). Rats were subsequently treated with i.p. LPS (250 µg/kg) v. vehicle injection at ZT 10.5 (1630 h). For central COX-2 inhibition, rats were co-treated with i.c.v. NS-398 (5 µg) dissolved in 50% DMSO/50%

0.01 M PBS v. vehicle and i.p. LPS (250 µg/kg) v. vehicle injection at ZT 10.5 (1630 h). In both PG blockade experiments, rats had *ad-libitum* access to food and water and data were collected as described in the IL-1ra experiment. Body temperature was monitored as a positive control for PG inhibition.

Central opioid blockade

Sprague Dawley rats were implanted with lateral ventricle cannulae and subcutaneous telemetric transponders and allowed to recover for 5 d before the beginning of the experiment. Peripheral LPS treatments were performed as described in the main text with slight modifications. Rats were co-treated with i.c.v. naltrexone (NTX; 5 µg) dissolved in normal saline v. vehicle and i.p. LPS (250 µg/kg) v. vehicle injection at ZT 10.5 (1630 h). Food intake, body weight, temperature, and activity were recorded. Following 5 d of recovery, rats were crossed-over such that all animals received a different i.p. and i.c.v. treatment and the experiment was repeated.

Tumor implantation

Male F344/NTacfBR rats were individually housed, and divided into four age and weight-matched groups: sham-operated controls treated with aCSF; tumor-bearing rats treated with aCSF; tumor-bearing rats treated with AgRP; and sham-operated rats that were pair fed with the tumor-bearing/aCSF group. Seven days prior to tumor implantation, animals had lateral ventricle cannulae and biotelemetry devices implanted in a single surgery. On d 0, the animals were anesthetized with 2% isoflurane, and fresh tumor tissue (0.2–0.3 g) from a rat donor was implanted subcutaneous into the flank of

the rat as previously described (162). The tumor is a methylcholanthrene-induced sarcoma that does not metastasize and induces anorexia, cachexia, and lethargy (464). Beginning on d 10 after surgery, rats received 5 μl i.c.v. injections of aCSF or AgRP (1 nmol) daily at 1600 h. On d 13 after tumor implantation, tumor growth had fallen within the predetermined endpoints of the study, according to the Oregon Health & Science University Institutional Animal Care and Use Committee Policy on Tumor Burden, and the animals were killed. The brains were immediately removed and frozen on dry ice. Brains were stored at -80°C until *in situ* hybridization analysis.

In situ hybridization histochemistry

Fresh frozen brains were collected and processed for *in situ* hybridization as previously described (161). Coronal sections (20 μm) were cut on a cryostat and thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Hypothalamic sections were collected in a 1:6 series from the diagonal band of Broca (bregma 0.50 mm) caudally through the mammillary bodies (bregma -5.00 mm). Antisense ^{33}P-labeled rat preproorexin (*Ox*) riboprobe (corresponding to bases 18-420 of rat *Ox*; GenBank accession no. NM_ 013179) (0.1 pmol/ml) was denatured, dissolved in hybridization buffer along with tRNA (1.7 mg/ ml), and applied to slides. Controls used to establish the specificity of the *Ox* riboprobe included slides incubated with an equivalent concentration of radiolabeled sense *Ox* riboprobe or radiolabeled antisense probe in the presence of excess (1000X) unlabeled antisense probe. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55°C. The following day, slides were treated with RNase A and washed under conditions of increasing stringency. Slides were dipped in 100% ethanol, air dried, and then dipped in NTB-2 liquid emulsion

(Eastman Kodak Co., Rochester, NY). Slides were developed 6 d later and coverslipped. Grains 2.0.b (University of Washington, Seattle, WA) was used to count the number of silver grain clusters (corresponding to radiolabeled *Ox* mRNA) in each hypothalamic nucleus (user-defined) as well as the number of silver grains in each cell.

Double-label *in situ* hybridization was used for simultaneous visualization of *Ox* mRNA with IL-1RI, TNF-R, LIF-R, IκBα, MC3-R and MC4-R in the rat brain (n=3) was performed as previously reported (161), with slight modifications. Briefly, tissue was prepared as described above. Antisense digoxigenin-labeled rat *Ox* riboprobe and antisense ^{33}P-labeled IL-1RI (bases 207–930 of rat *Il1r1*; GenBank accession no. M95578) (0.2 pmol/ml), IκBα (bases 112-922 of rat *Nfbia*; GenBank accession no. NM_001105720) (0.1pmol/ml) TNF-R (bases 63-1129 of rat *Tnfrsf1a*; GenBank accession no. NM_013091) (0.2 pmol/ml), LIF-R (bases 785-1645 of rat *Lifr*; GenBank accession no. NM_031048) (0.2 pmol/ml), MC3-R (bases 90-486 of rat *Mc3r*; GenBank accession no. NM_001025270) (0.3 pmol/ml), or MC4-R (bases 557-1006 of rat *Mc4r*; GenBank accession no. NM_013099) (0.3 pmol/ml). Co-expression of radiolabeled and digoxigenin-labeled mRNA was assessed using criteria previously described (465). Signal-to-background ratios for individual cells were calculated; an individual cell was considered to be double-labeled if it had a signal-to-background ratio of 2.5 or more. For each animal, the amount of double-labeling was calculated as a percentage of the total number of *Ox* -expressing cells and then averaged across animals to produce mean ± SEM.

Perfusion and IHC

Histology experiments were performed as previously described with modifications (62). For histology experiments, rats or mice were deeply anesthetized using sodium pentobarbital (65 mg/kg), and sacrificed by transcardial perfusion fixation with 150 mL (rats) or 15 mL (mice) ice cold 0.01 M PBS + heparin sodium (15,000 U/L) followed by 200 mL (rats) or 25 mL (mice) 4% paraformadehyde (PFA) in 0.01 M PBS. Brains were post-fixed in 4% PFA overnight at 4°C and cryoprotected in 20% sucrose for 24 h at 4°C before being stored at -80°C until used for IHC. Dual-immunofluorescence histochemistry was performed as described below. Free-floating sections were cut at 30 µm from perfused brains using a sliding microtome (Leica SM2000R; Leica Microsystems, Bannockburn, IL). Three (rats) or four (mice) sets of sections were generated from the hypothalamus and hindbrain of each brain. Hypothalamic sections were collected from the division of the optic chiasm (bregma -2.0 mm, rats; bregma -1.0 mm, mice) caudally through the mammillary bodies (bregma -5.00 mm, rats; bregma -3.0 mm, mice). The sections were incubated for 1 h at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS and 0.1% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-c-Fos antibody (PC38; EMD Biosciences, Inc., San Diego, CA) diluted 1:50,000, rabbit anti-cFos antibody (SC-52; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:25,000, and goat anti-OxA (SC-8070; Santa Cruz Biotechnology) diluted 1:1,500 in blocking reagent for 72 h at 4°C, followed by incubation in donkey anti-rabbit Alexa 594 (1:500; Invitrogen) and donkey anti-goat Alexa 488 (1:500; Invitrogen) for 2 h at room temperature. Triple-IF histochemistry was performed as described above with the following modifications. Nes-cre/GFP sections were incubated in blocking reagent as described above, then washed and incubated in the anti-cFos and anti-OxA antisera as well as chicken anti-GFP (Abcam, Cambridge, MA) diluted 1:1000 for 72 h @ 4°C. Sections were then incubated in donkey anti-rabbit Alexa 594, donkey anti-chicken-FITC (Jackson Immunoresearch,

West Grove, PA), and donkey anti-goat-DyLight 649 (Jackson Immunoresearch) for 2 h at room temperature. Between each stage, the sections were washed thoroughly with 0.01 M PBS. Incubating the sections in the absence of primary antisera was used to ensure specificity of the secondary antibodies. Sections were mounted onto gelatin-coated slides, coverslipped using Vectashield mounting media (Vector Laboratories, Burlingame, CA), and viewed under a fluorescence microscope (Leica 4000 DM; Leica Microsystems, Bannockburn, IL, or Zeiss LSM710; Carl Zeiss, Inc.).

OxA radioimmunoassay (RIA)

OxA concentration was measured in rat CSF samples, collected as described above, using a commercially available RIA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA). RIA was performed in duplicate on 25 µl CSF samples according to manufacturer instructions. The lowest detectable level was 22.47 pg/mL. The interassay variability (assessed by replicate analysis of a 250 pg/mL standard) was 0.31%.

Image processing and statistical analysis

Confocal photomicrographs between matched brain sections were taken using a Zeiss LSM710 (Carl Zeiss, Inc., Oberkochen, Germany) under identical microscope conditions. Images were merged with NIH ImageJ software. Photomicrographs were only collected to show representative images. Data were graphed and analyzed using GraphPad Prism 5 (La Holla, CA). Comparisons between two groups at a single time point were performed using two-tailed Student's *t* test. Comparisons between three or more groups used a one-way ANOVA with post-hoc Bonferonni corrected *t* test. All comparisons involving two treatments or multiple time points used two-way ANOVA

followed by post-hoc Bonferonni corrected *t* test. Differences between groups were considered significant when $p < 0.05$.

Results

LPS-induced inhibition of home cage LMA is associated with suppressed dark phase Ox signaling

Diminished voluntary home cage LMA is a hallmark behavior associated with both sickness and insufficient Ox signaling. To investigate a possible connection, male Sprague-Dawley rats were injected with LPS (250 µg/kg, n = 7) or vehicle (veh, n = 10) 90 min prior to lights off (Zeitgeber Time [ZT] 10.5). We observed nearly complete inhibition of the normal dark phase LMA in LPS-treated animals (Figure 21A). To evaluate the involvement of Ox signaling, this experiment was repeated with the addition of CSF collection either at lights off or just before lights on, at the zenith of CSF Ox concentration (291). We measured a significant decrease in CSF OxA concentration in LPS-treated animals (n=6) compared to veh (n=5) at ZT 0, suggesting reduced Ox release throughout the dark phase in these animals (LPS, 887 ± 78 pg/mL; veh, 1720 ± 130 pg/mL; $p < 0.001$), though no significant change was observed at the onset of the dark phase, during the nadir of OxA concentration (Figure 21B). This is unlikely a transcriptional effect, as no change in *Ox* mRNA expression was observed 1 h or 8 h after LPS administration (Figure 22).

In nocturnal rodents, the activity of Ox neurons increases during the dark phase, coincident with increased Ox release and increased wakefulness and activity (289, 466, 467). We examined whether LPS administration just before onset of the dark phase

suppresses the vespertine induction of cFos IR in Ox neurons. In veh treated animals (n=3), cFos IR colocalized with 37.6 ± 1.8% of Ox-IR neurons, whereas in LPS-treated animals (n=4), 22.8 ± 1.8% of Ox neurons showed cFos IR ($p < 0.01$, Figures 21C-I). Because previous investigators proposed unique roles for anatomically distinct populations of Ox neurons (375), colocalization was quantified in DMH, perifornical area (PFA), and lateral LHA subpopulations (anatomy described in Figure 21J). No change in cFos was observed in DMH Ox neurons (veh, 65.87 ± 4.1%; LPS, 52.6 ± 5.3%), however a significant decrease in cFos IR was observed in both PFA (veh, 41.8 ± 1.8%; LPS, 21.0 ± 2.3%; $p < 0.01$) and LHA (18.7 ± 2.4%; LPS, 10.5 ± 1.9%; $p < 0.05$) Ox neurons (Figures 21C-I). No effect of rostrocaudal location on Ox neuron cFos IR was observed.

Central (i.c.v.) OxA replacement prevents the onset of LPS-induced lethargy

To test the hypothesis that a specific inhibition of Ox signaling underlies the reduced LMA exhibited by LPS-treated rats, we undertook a series of i.c.v. OxA replacement studies. During dose-finding studies we found that central OxA administration influences LMA and arousal for approximately 90 min after injection (Figure 23A), in accordance with previous studies (299). We also found that a 1 µg bolus i.c.v. injection of OxA had no significant effect on LMA in healthy male Sprague Dawley rats (Figure 23B). Because the duration of Ox action is relatively short compared to the behavioral effects of LPS, we tested the effect of Ox replacement in three paradigms: (1) acute LPS-induced lethargy prevention, (2) acute dark phase lethargy reversal, and (3) subchronic (overnight) OxA infusion. In the acute lethargy prevention experiment (1), rats treated with i.p. LPS (250 µg/kg) or veh at ZT 10.5 were subsequently administered

i.c.v. OxA (1 µg) or aCSF at ZT 11.5, 30 min before onset of the dark period (n = 6/group). Although OxA had no effect on LMA in veh-treated rats, OxA restored LMA in LPS-treated rats to near-control levels during the subsequent 90 min (Figures 24A and 24B). Following this period, LPS/OxA rats reduced their activity to the level of LPS/aCSF rats for the remainder of the dark phase.

In the dark phase lethargy reversal experiment (2), rats were treated with LPS v. veh, as described above, and subsequently centrally injected with OxA (1 µg, veh/OxA n=4; LPS/OxA n=6) v. aCSF (veh/aCSF n=3; LPS/aCSF n=3) at ZT 15, 3 h after the onset of the dark phase. We found that LPS inhibited LMA compared to veh and that this was reversed by OxA administration during the 90 min following treatment (Figures 24C and 24D). *Post hoc* analysis demonstrated significant differences between veh/aCSF and LPS/aCSF groups ($p < 0.001$) as well as between LPS/OxA and LPS/aCSF groups ($p < 0.05$).

In the subchronic OxA administration experiment (3), rats were implanted with i.c.v. minipumps to deliver OxA (3 µg/h) or aCSF throughout the dark phase, with *ad-libitum* access to food. Pumps were constructed to ensure drug delivery coincided with the onset of the dark phase. This dose elicits no acute or long-term effects on body weight, food intake, brown adipose temperature, plasma corticosterone, or fat depot mass in rats (468). Rats were treated with LPS or veh at ZT 10.5, as described above, yielding 3 groups – veh/aCSF (n=10), LPS/aCSF (n=7), and LPS/OxA (n=7). Though both LPS-treated groups moved significantly less than veh-treated controls, LPS/OxA-treated rats showed an amelioration of this effect throughout the dark phase (Figures 25A and 25B). *Post hoc* analysis of dark phase and total 24 h LMA demonstrated a

significant effect of OxA treatment on LPS-treated rats ($p < 0.05$). OxA treatment reinstated normal crepuscular LMA peaks, which are suppressed in the LPS/aCSF rats. OxA treatment had no effect on either food intake or body weight in LPS-treated rats (Figures 25C and 25D), suggesting that the increase in LMA was not due to increased feeding behavior.

LPS blocks FAA and Ox neuron activity in food-entrained rats

24 h cyclic FR paradigms induce entrainment of biological rhythms to adapt to the period of food availability. This behavior is marked by an increase in LMA in the period immediately preceding food availability (FAA) that is dependent on Ox signaling but independent of SCN master clocks, which remain phase locked to the light/dark cycle (239, 310). Therefore, we undertook several studies investigating the effect of inflammation on FAA and the concomitant increase in Ox neuron activity. We first evaluated whether i.p. LPS could inhibit FAA, and if central OxA administration could restore this behavior. As the animals entrained to FR, LMA maxima shifted from two crepuscular peaks to a single activity peak during the hour preceding feeding (Figure 26A). FR rats were injected i.p. with LPS (250 µg/kg) or veh at ZT 1.5 and subsequently treated i.c.v. with OxA (1 µg) or aCSF at ZT 3, yielding 4 groups: veh/aCSF (n=9), veh/OxA (n=7), LPS/aCSF (n=7), LPS/OxA (n=7). We found that LPS potently inhibited FAA compared to veh treatment, but that OxA treatment restored activity during this period to control levels. OxA treatment had no significant effect on veh treated rats (Figures 27A and 27B). LPS inhibited food intake, and this effect was not reversed following OxA treatment (veh/aCSF, 15.1 ± 2.0 g; veh/OxA, 12.6 ± 1.9 g; LPS/aCSF, 9.6 ± 3.4 g; LPS/OxA, 10.6 ± 2.8 g) (Figure 27C). To determine whether Ox neurons are

inhibited in this paradigm, the above experiment was repeated without i.c.v. injection and rats were sacrificed at ZT 3.5, during the peak of FAA. As a control for the effects of FR, a group of *ad-libitum* fed, veh-treated rats were sacrificed at the same time (n=4/group). We found that FR significantly increased cFos IR in Ox neurons compared to *ad-libitum* fed animals, but that LPS treatment significantly reduced the percentage of total Ox neurons exhibiting cFos IR compared to veh (ad-lib, 3.9 ± 1.4%; veh, 31.2 ± 2.0%; LPS 15.9 ± 2.6%) (Figures 27D and 27E). When counted by anatomic location, we found that FR specifically activated Ox neurons in the DMH and PFA, but not in the LHA. The LPS-induced suppression of Ox neuron activity was localized to the PFA regions of neurons (ad-lib, 3.2 ± 1.3%; veh, 29.1 ± 2.0%; LPS, 11.3 ± 1.4%); no difference was observed between veh- or LPS-treated FR rats in the DMH or LHA populations. No difference in the total number of Ox IR neurons was observed between groups. Previous studies demonstrated that calorie restriction can diminish cytokine responses to pro-inflammatory stimuli (469). Two hours following LPS treatment, we found that the induction of serum IL-6 and hypothalamic pro-inflammatory markers (IL-1β, TNF-α, SOCS-3, IκBα) remained intact in FR rats (Figure 26B).

Central IL-1β blocks FAA and Ox neuron activity in food-entrained rats

To test whether central inflammation in the absence of peripheral inflammation is sufficient to suppress Ox neuron activity and FAA, we measured LMA and food intake in FR rats following i.c.v. administration of the inflammatory cytokine, IL-1β (100 ng). Rats were then treated with central OxA (1 µg) or veh as described above (n=5/group). IL-1β treatment potently blocked FAA compared to veh, and this effect was ameliorated by subsequent OxA administration, with no effect on food intake (Figures 27F-27I). We

observed a significant reduction in immunohistochemical (IHC) colocalization of cFos and OxA in IL-1β-treated FR rats compared to veh, as seen following LPS administration (ad-lib, 7.0 ± 1.5%; veh, 34.7 ± 2.5%; IL-1β, 18.5 ± 3.9%) (Figure 27J). Again, FR induced cFos IR in DMH and PFA Ox neurons, while inflammation specifically reduced cFos IR in PFA Ox neurons, with no significant effect on either DMH or LHA populations (PFA; ad-lib, 7.2 ± 1.9%; veh, 43.9 ± 4.3%; IL-1β, 18.2 ± 5.3%). These data demonstrate that central inflammation is sufficient to suppress FAA in an Ox-dependent manner. They further suggest that central and peripheral inflammatory challenge may share a common pathway for suppressing PFA Ox neuron activity.

Ox neurons are not direct targets for inflammatory signaling

We previously demonstrated that inflammatory cytokines directly modulate the activity of hypothalamic melanocortin neurons. We therefore tested whether Ox neurons also express cytokine receptors. Using dual-label *in situ* hybridization, we did not observe colocalization of IL-1RI, LIF-R, or TNF-R mRNA with preproorexin (*Ox)* mRNA (Figures 28A-28C). Further, we assessed whether Ox neurons receive direct inflammatory input by measuring the expression of IκBα, which is induced in response to activation of the inflammatory NF-κB signaling pathway. Though we observed IκBα expression in the LHA of LPS-treated rats, we found no colocalization of this mRNA with cells expressing *Ox*, suggesting that Ox neurons do not directly respond to pro-inflammatory signals (Figure 28D).

Because i.c.v. IL-1β is sufficient to suppress FAA in FR rats, we tested whether central IL-1 signaling is necessary for the induction of lethargy by LPS using

pharmacologic antagonism of IL-1 receptor. Co-administration of i.c.v. IL-1 receptor antagonist (IL-1ra; 4 µg) with i.p. LPS (250 µg/kg) at ZT 10.5 (veh/veh, n=6; veh/LPS, n=5; IL-1ra/veh, n=6, IL-1ra/LPS n=5) had no acute effect on vespertine LMA in LPS- or veh-treated rats, despite ameliorating LPS-induced anorexia (Figures 28E and 29B). We observed that IL-1ra partially restored LMA in LPS-treated rats approximately 8 h following treatment, suggesting a role for IL-1 signaling in propagating the acute behavioral response to LPS (Figure 29A).

Several studies have further implicated that PG signaling is an essential step in the induction of sickness behavior (127, 470). To test the involvement of PG in LPS-induced lethargy, we blocked PG synthesis using two cyclooxygenase (COX) inhibitors: the blood brain barrier permeable, non-specific COX-1/-2 inhibitor indomethacin; and the COX-2 specific NS-398. Systemic (i.p.) pretreatment with indomethacin (4 mg/kg) (veh/veh, n=6; veh/LPS, n=5; indo/veh, n=6, indo/LPS n=5) at ZT 10 potently blocked LPS-induced pyresis, but no significant effect on LMA was observed (Figures 28F, 29C, and 29D). Statistical analysis by 2-way ANOVA demonstrated a significant effect of LPS treatment ($P<0.05$), but no effect of indomethacin and no interaction between treatments. Central pretreatment with NS-398 (5 µg) also suppressed the febrile response to LPS, but, like indomethacin, had no significant effect on LMA (Figures 29E and 29F). Together, these data demonstrate that neither central IL-1 signaling nor PG signaling is required to produce lethargy in response to LPS.

LPS-induced lethargy is not melanocortin dependent

These results indicate that the LPS-induced inhibitory input onto Ox neurons is mediated by a proximal, inflammation-sensitive neuron population. We tested whether

inflammation-induced activation of central melanocortin signaling could relay this information by assessing the expression of melanocortin receptors on Ox neurons using dual-label *in situ* hybridization. We found no coexpression of either MC4-R or MC3-R in cells expressing *Ox* (Figures 30A and 30B).

We tested the potential role of increased melanocortinergic tone on the suppression of LMA by administering the non-specific MC3-R/MC4-R antagonist SHU-9119 (1 nmol) in animals treated with LPS v. veh (Veh/aCSF, n=6; Veh/SHU, n=5; LPS/Veh, n=4; LPS/SHU, n=6). Food was removed from the cages to control for feeding-induced suppression of LMA. Pharmacologic blockade of melanocortin receptors in LPS-treated animals did not increase LMA, and SHU treatment alone suppressed LMA to levels approximating those of LPS (Figures 30C and 30D). We also tested the effects of melanocortin blockade on LMA and food intake in a tumor-bearing rat model of chronic inflammation. Rats were received a subcutaneous implantation of a syngenic sarcoma or a sham surgery and were centrally treated with the endogenous MC3-R/MC4-R antagonist, AgRP (1 nmol) or aCSF during the final 4 d of the experiment. The sham animals were either fed *ad-libitum* or pair-fed to tumor-bearing animals and were treated with aCSF, yielding 4 groups (Sham/ad-lib, Sham/pair-fed, Tumor/aCSF, Tumor/AgRP; n=3/group). Nightly administration of AgRP did not ameliorate lethargy in tumor bearing rats, despite restoring food intake (Figures 30E and 30F). Again, melanocortin antagonism suppressed LMA in healthy (sham-operated) rats.

Arcuate POMC neurons release both alpha-melanocyte stimulating hormone (α-MSH), which binds MC3-R & MC4-R, and β-endorphin, which binds the opioid receptors. Opioids have been shown to inhibit Ox neuron activity via the mu-opioid receptor (μOR),

which is expressed by Ox neurons (356, 471). Blockade of central opioid signaling by i.c.v. pretreatment with the non-selective OR antagonist naltrexone (10 µg) had no effect on LMA or food intake in LPS- or veh-treated rats (n=6/group) (Figures 31A-31C).

LHA neurotensin (Nts) neurons are activated during LPS-induced inflammation

Recent work demonstrated that LHA Nts-expressing neurons, which also express the long form of the leptin receptor (LepRb), provide an inhibitory input on Ox neurons when activated by leptin (324). Because leptin is closely structurally related to class I helical cytokines (472) and shares intracellular signaling pathways with IL-6 family cytokines (473), we tested whether these neurons are also activated during inflammatory challenge. We injected male Nts-cre/GFP mice with LPS (250 µg/kg, n=4) or veh (n=4) at ZT 10.5 and looked at cFos IR in Nts- and Ox-IR neurons at ZT 12.5. Because many cells in multiple nuclei express Nts, we only counted those cells that overlap with the Ox neuron distribution, where the LepRb+ cells are located. We found a significant increase in the total number of Nts neurons with cFos-IR (veh, 6.8 ± 2.3 cells/section; LPS, 17.1 ± 3.8 cells/section) as well as the percentage of total Nts neurons co-labeled with cFos in LPS-treated rats (veh, 3.9 ± 1.2%; LPS, 8.1 ± 2.0%), but no difference in total Nts neurons per section between groups (veh, 174.3 ± 33.2 cells/section; LPS, 206.8 ± 24.3 cells/section) (Figures 32A-32H). Further, this corresponded with a significant decrease in cFos IR in Ox neurons following LPS treatment (veh, 34.2 ± 6.7%; LPS, 12.9 ± 4.6%) (Figures 32F, 32G, and 32I). A small percentage of GFP-IR cells were also labeled for Ox, (< 1%) and cFos IR was not counted in these cells. This finding is consistent with the hypothesis that LHA Nts neurons are activated during inflammatory challenge and may contribute to the inhibition of Ox neuron activity.

Tumor-bearing rats exhibit decreased LMA and fewer medial Ox-expressing neurons

Sickness-induced behavioral inhibition has also been described in cancer patients and tumor-bearing animals (155, 474). We implanted male rats F344/NTacfBR with a syngenic sarcoma (n=12) or performed a sham surgery (n=6). Tumor-bearing animals exhibit a steady decline in total daily LMA during days 10-13, when tumor growth is maximal. On the final day of the experiment (d 13 after implantation) tumor-bearing rats significantly decreased their dark phase activity compared to their own baseline activity (average of days 3-5 post-implantation)(Figure 33A). They also exhibit a significant decrease in 24 h LMA on d 13 compared to sham-operated controls (Figure 33B). Single label *in situ* hybridization on brains from tumor-bearing rats using an antisense *Ox* riboprobe showed a significant decrease in the number of Ox-expressing cells specifically in the medial Ox neuron distribution (sham, 59.8 ± 12 cells/section; tumor, 27.8 ± 3 cells/section; $p < 0.01$), but not in the lateral, though no difference in grains/cell was observed (Figures 33C-33F). These data indicate that suppression of Ox signaling may play a role in chronic disease, as well.

Discussion

Despite the nearly ubiquitous suppression of physical activity and arousal in response to illness, the neural mediators of this response are unknown. We now demonstrate that inflammation-induced lethargy is mediated by suppression of normal Ox signaling. We show that dark phase associated increases in Ox neuron activity and OxA release are suppressed by perhipheral inflammatory challenge with LPS. Central

replacement of OxA can restore LMA in LPS-treated rats at doses that have no effect on LMA in healthy animals. In FR animals, we also observe an LPS- and IL-1β-induced decrease in Ox neuron activity and LMA during the preprandial period, the latter of which is rescued by subthreshold i.c.v. OxA replacement. These effects are unlikely mediated by direct inflammatory or melanocortin signaling on Ox neurons, but we show here evidence that Nts-expressing GABAergic interneurons may play a key role in mediating Ox neuron inhibition.

Though LPS challenge may not directly reflect the pathophysiology of a specific illness, it reliably induces peripheral and central cytokine responses and sickness behavior, resembling changes observed in several disease models (66, 475). Despite robust movement suppression, i.c.v. OxA replacement restored LMA in LPS-treated rats in both circadian- and FR-conditions of increased arousal. This effect is likely OxA-specific, as the dose of i.c.v. OxA administered did not increase LMA in veh-treated rats.

Previous investigators have suggested that the medial (DMH/PFA) and lateral (LHA) Ox neuron populations exhibit distinct effects on behavior (375). Our cFos IR data support an anatomically based functional dichotomy for Ox neurons, as we observed increases in cFos IR in DMH and PFA, but not LHA Ox neurons during both vespertine and food anticipatory peaks in LMA. We also observed a specific suppression of cFos IR in PFA Ox neurons, more than DMH, in response to LPS or central IL-1β. This finding separates the medial arousal-associated Ox neuron population into inflammation-sensitive (PFA) and inflammation-insensitive (DMH) populations and indicates that the activity of the PFA Ox neuron population is necessary for normal LMA responses to both circadian and non-circadian cues. Tract tracing has identified differences in innervation

between medial (DMH/PFA) and lateral (LHA) Ox neurons that are consistent with previously reported roles for medial Ox neurons in mediating arousal and lateral Ox neurons influencing reward (232). Similar examination of afferents to and projections from DMH and PFA subpopulations may define the circuits underlying arousal modulation and inflammation sensitivity.

Initially, we hypothesized that inflammatory cytokines inhibit Ox neurons by directly binding to these neurons. We found no evidence for the expression of cytokine receptors or the expression of NF-κB response genes following LPS treatment in Ox neurons. Previous reports investigating the pathogenesis of sickness behavior have suggested a prominent role for PG signaling in pyresis (120), anorexia (118), cFos induction (125), and HPA activation (127). However, in accordance with Serrats et al. (2010), we found no effect of COX inhibitors on LMA, indicating that LPS-induced lethargy is PG-independent. We were, however, able to demonstrate that central inflammation, induced by i.c.v. IL-1β, is sufficient, but not necessary to suppress LMA and Ox neuron activity. Further, blocking nerve conduction through the dorsal vagal complex (DVC) prevents LPS-induced social withdrawal and cFos IR in multiple nuclei (476), identifying the brainstem as a crucial mediator of sickness behavior. Given that the DVC and central IL-1β can each independently mediate lethargy, we postulate that a population of inflammation-sensitive neurons that receive afferent input from the DVC integrates these two inflammatory signals and relays this signal to Ox neurons.

Recently, Leinninger et al. demonstrated that leptin activates GABAergic Nts neurons in the LHA, which then inhibit Ox neuron activity (324). That LHA Nts neurons can respond to leptin indicates that, like arcuate nucleus POMC neurons, these neurons

may be cytokine-sensitive (62, 161). Though Nts is expressed by neurons in various nuclei, the highest concentration of Nts-expressing neurons is centered over the PFA Ox neuron population. The LPS-induced decrease in cFos IR in Ox neurons was associated with an increase in cFos IR in LHA Nts neurons, indicating that these interneurons play a common role in the allostatic suppression of arousal by both overnutrition and inflammation. Furthermore, modulation of Ox neuron activity by LPS and leptin both alter the behavioral response to amphetamine (304, 324, 477). This suggests exploitation of similar circuitry and supports a role for VTA DA signaling in mediating the LMA effects of LPS. However, the effects of leptin and LPS are not completely congruent. In contrast to leptin, LPS does not acutely induce *Ox* mRNA expression. This may be explained by recruitment of unique, but overlapping Ox-regulating pathways by leptin and LPS. Further anatomic and functional studies are needed to elucidate the role of LHA Nts neurons in mediating LPS-induced suppression of Ox neurons.

Importantly, restoration of LMA in LPS-treated rats by i.c.v. OxA did not influence food intake, despite the well-documented orexigenic properties of OxA. After i.c.v. injection, LPS/OxA-treated animals exhibit normal home cage exploratory behavior, including investigation of their food hopper, but, unlike veh-treated rats, these animals seldom consume food. Therefore, the increase in LMA induced by OxA replacement was not simply an increase in feeding behavior, but rather represented a more active animal in lieu of depressed feeding. We previously reported that inflammation induces anorexia by increasing central melanocortin signaling, and that melanocortin antagonism restores feeding (18). We found no evidence that Ox neurons receive direct input from melanocortinergic neurons, though we observed MC4-R expression in non-Ox neurons throughout the LHA. Melanocortins may, via MC4-R, regulate the activity of LHA neurons that influence the Ox system, such as Nts neurons. Specific activation of AgRP

neurons using designer receptors exclusively activated by designer drugs (DREADD) potently induces foraging behavior in the absence of food (478). However, AgRP treatment failed to restore LMA in LPS-treated rats suggesting that either an alternative neurotransmitter released by AgRP neurons (e.g. NPY, GABA) coordinates feeding and LMA, or that non-synaptically delivered AgRP acts ectopically to suppress LMA. Thus, in the setting of increased melanocortin tone following LPS administration, OxA provides an insufficient orexigenic stimulus to induce food intake. Coversely, blockade of MC4-R has no effect on Ox-dependent suppression of LMA. This marks the first time these two features of sickness behavior have been mechanistically separated.

Though the present work focuses primarily on acute mechanisms of illness-induced lethargy, our TB rat studies suggest that Ox may play a role in the lethargy of chronic disease, as well. TB rats exhibited a marked decrease in the number of *Ox* mRNA-expressing neurons, indicating either specific degeneration of medial Ox neurons or transcriptional repression of Ox synthesis in these cells. Either phenomenon, if responsible for cancer-associated lethargy, should be sensitive to Ox replacement. Subchronic OxA replacement increased LMA throughout the dark phase in LPS-treated rats, demonstrating that Ox replacement is a viable therapeutic avenue for sickness-induced lethargy. Though allostatic suppression of LMA and food intake by inflammation is an important, and reversible, part of the response to acute infection, chronic dysregulation of metabolism leads to wasting, debilitation, and death. We propose that Ox agonists would be useful in chronically ill patients to improve quality of life and independent living. In the setting of acute illness or injury, reduced arousal increases the risk of mistakes and personal harm while performing tasks that demand attention. Better understanding of the allostatic control of arousal will lead to both preemptive behaviors and direct treatment to improve vigilance in the face of inflammatory challenge.

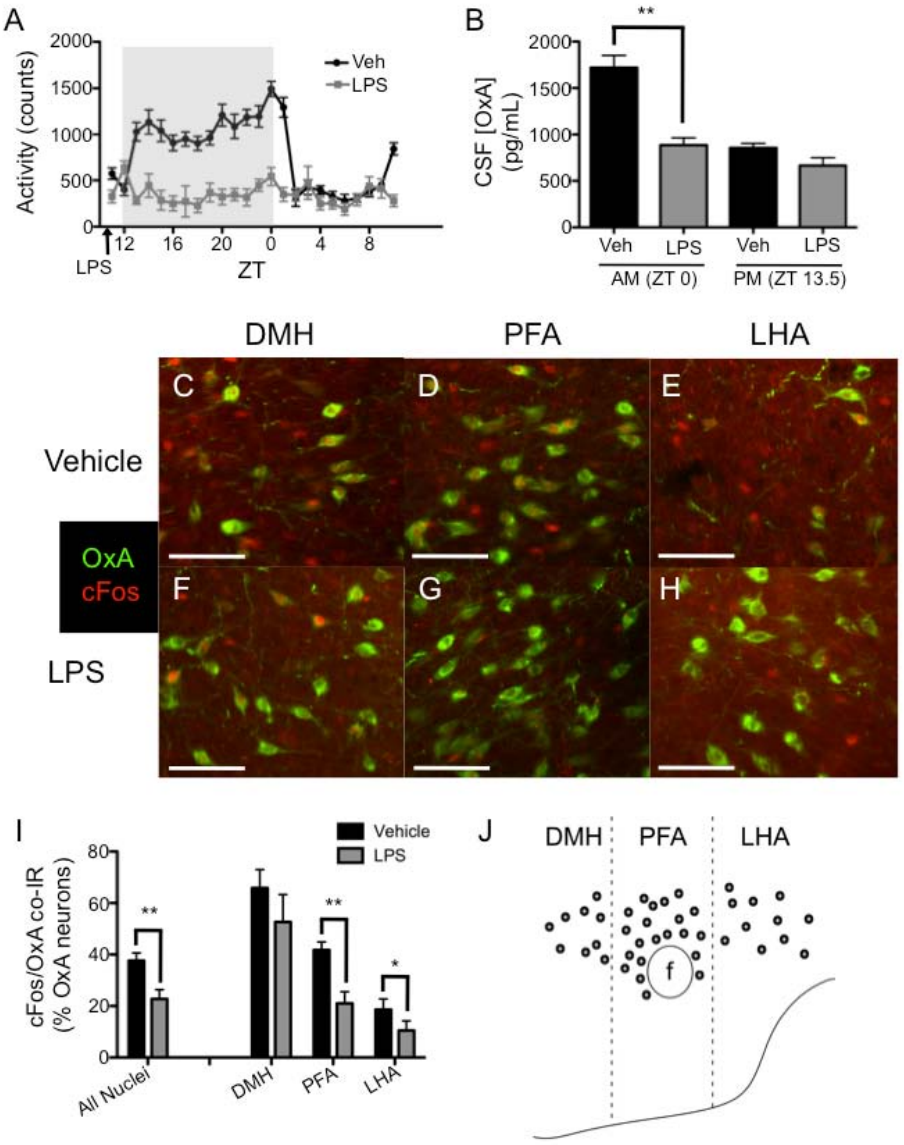

Figure 21. *LPS -induced inhibition of home cage LMA is associated with reduced vespertine activation of Ox neurons and reduced dark phase accumulation of OxA in the CSF.*
(A) Home cage LMA in rats receiving i.p. LPS v. veh just before the onset of the dark phase. (B) [OxA] in the CSF collected either at ZT 0 or ZT 13.5 from LPS or veh-treated rats. (C-H) High power representative photomicrographs of cFos IR in Ox neurons in DMH (C,F), PFA (D,G), and LHA (E,H) in veh- and LPS-treated rats. (I) Percentage of Ox IR neurons exhibiting cFos IR following i.p. treatment with LPS or veh. Percent colocalization expressed as total Ox neurons and by anatomic location. (J) Schematic illustrating division between DMH, PFA, and LHA Ox neuron populations. Data are expressed as mean ± SEM (*, $P < 0.05$; **, $P < 0.01$). Scale bars, 100 μm; *f*, fornix.

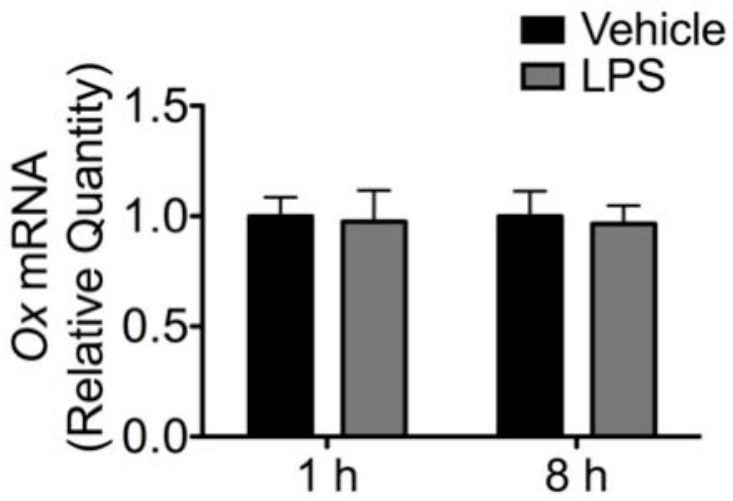

Figure 22. *LPS does not alter Ox mRNA expression.*

Male Sprague Dawley rats were treated with LPS (250 µg/kg) v. veh (n=5-7/group) and *Ox* mRNA expression in total hypothalamus was measured 1 or 8 h after injection. LPS treatment did not alter *Ox* mRNA levels at either time point. Data are expressed as mean ± SEM and statistics calculated by two-way ANOVA followed by *post hoc* analysis using Bonferroni's corrected *t* test.

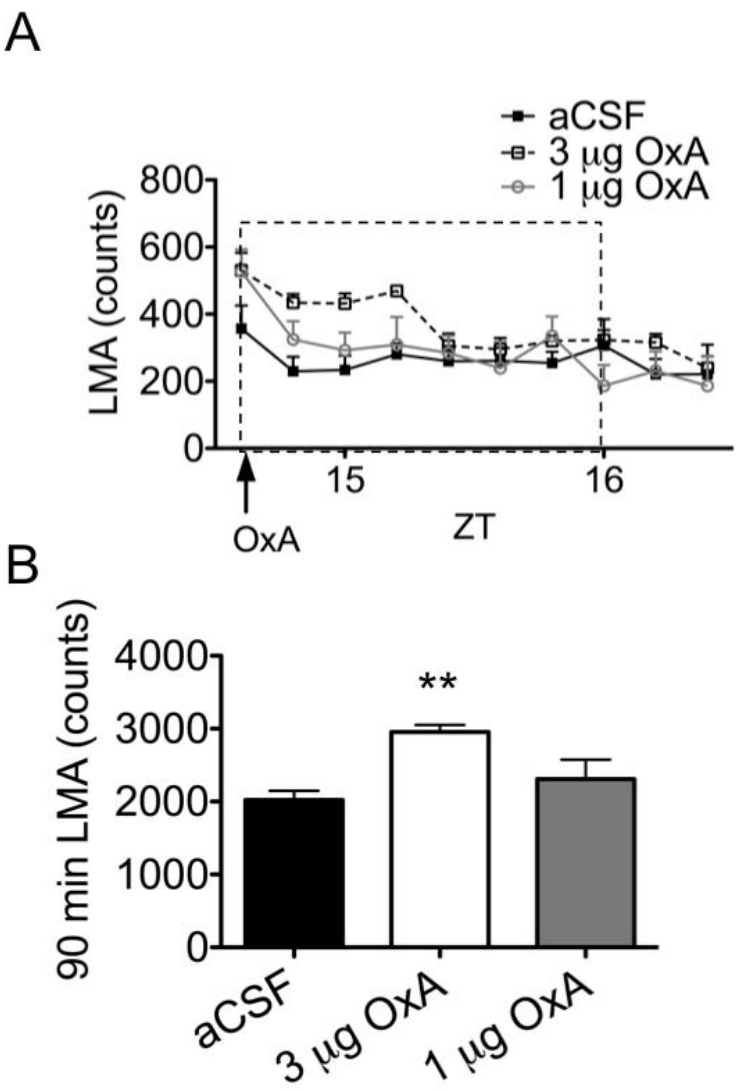

Figure 23. *OxA dose-response in aCSF- and LPS-treated rats*

(A) Effect of i.c.v. OxA (1 µg or 3 µg) v. aCSF administration on voluntary LMA in rats 2.5 h after the onset of the dark phase. Movement data expressed in 12 min intervals. (B) Sum total of voluntary home cage movement in rats during 90 min following i.c.v. OxA (1 µg or 3 µg) or vehicle injection. Period of summed data outlined by dotted line in (A). Data are expressed as mean ± SEM, and statistics calculated by one-way ANOVA followed by a post hoc analysis using a Bonferroni corrected *t* test (**, $P < 0.01$).

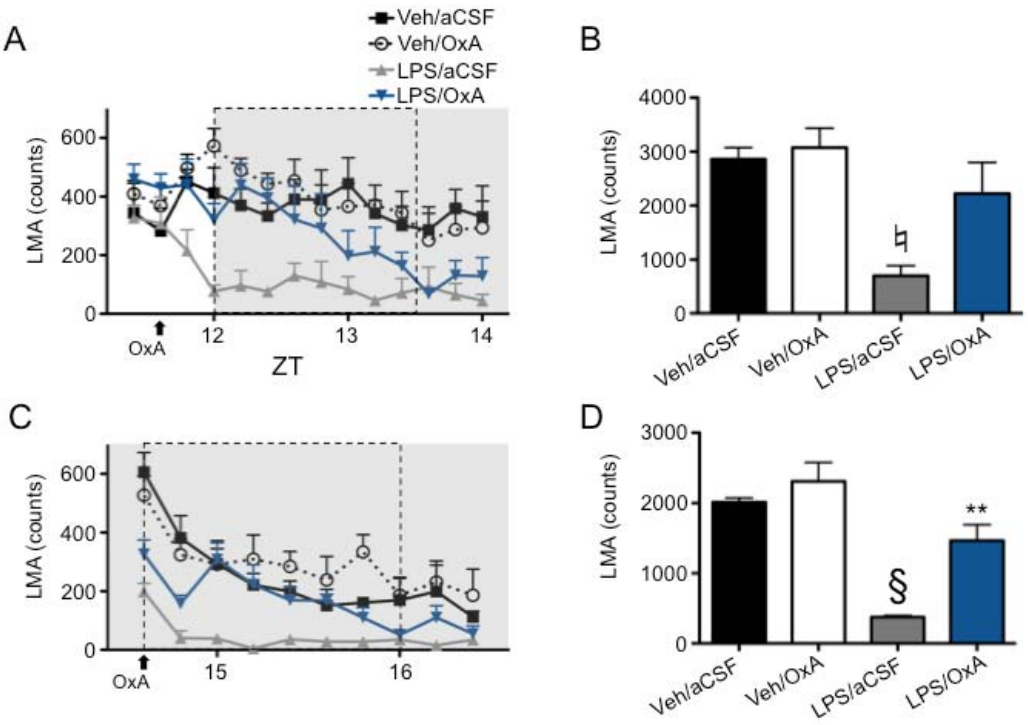

Figure 24. *Central OxA replacement prevents and reverses LPS-induced lethargy.*
(A) Effect of i.c.v. OxA v. aCSF administration on voluntary LMA in LPS v. veh-treated rats during transition from light phase to dark phase. Movement data expressed in 12 min intervals. (B) Sum total of voluntary home cage movement during first 90 min of dark phase in rats treated with i.p. LPS v. veh and i.c.v. OxA or veh. Period of summed data outlined by dotted line in (A). (C) Effect of central OxA v. aCSF administration on voluntary LMA in LPS- v. veh-treated rats during dark phase, after onset of lethargy. LMA expressed in 12 min intervals. (D) Sum total of LMA in rats treated with i.p. LPS (250 µg/kg) during 90 min following i.c.v. OxA v. veh. Period of summed data outlined by dotted line in (C). Shaded region in (A) and (C) indicates dark phase; arrows indicate time of i.c.v. injection. Data are expressed as mean ± SEM (ϟ, $P < 0.05$ v. LPS/OxA & Veh/aCSF; §, $P < 0.01$ vs. LPS/OxA & Veh/aCSF; **, $P < 0.01$ v. Veh/OxA & LPS/aCSF).

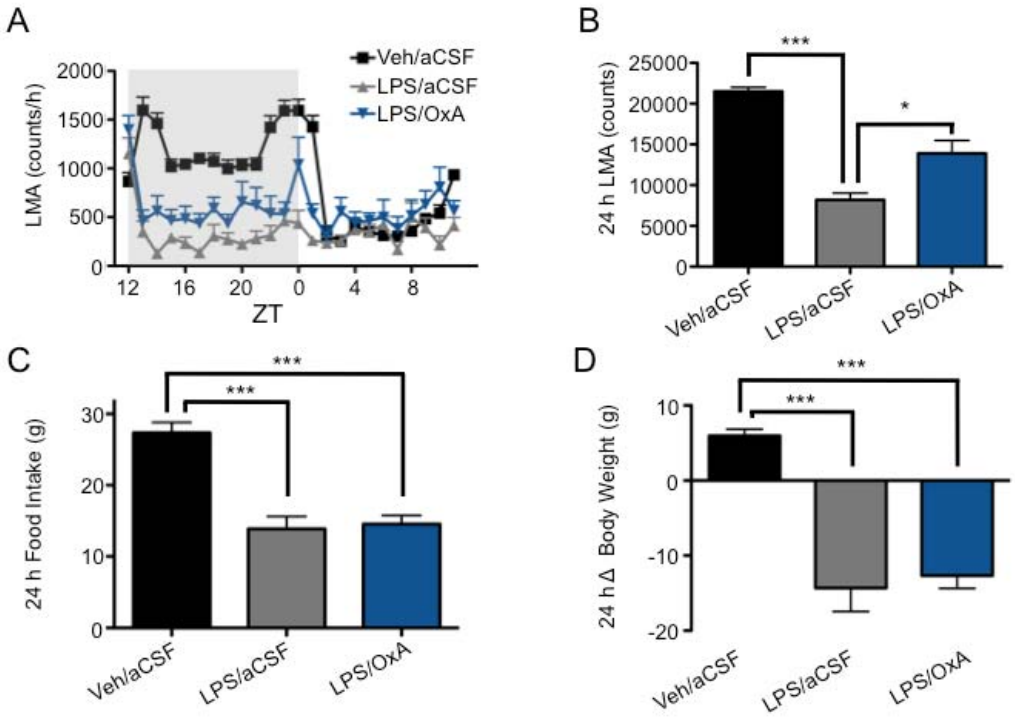

Figure 25. *Continuous central OxA administration ameliorates LPS-induced lethargy throughout the active phase.*

(A) Effect of 24 h of continuous central OxA or veh infusion on voluntary LMA in LPS- or veh-treated rats. Movement data expressed as hourly sums. LPS administered at ZT 10.5. OxA onset between ZT 8 and ZT 10. Shaded region indicates dark phase. (B) Sum total of voluntary LMA during 24 h following LPS administration (ZT 12 – ZT 12). (C) Effect of LPS and OxA on 24 h food intake. (D) Changes in body weight during 24 h following treatment with LPS and Ox. Data are expressed as mean ± SEM (*, $P < 0.05$; ***, $P < 0.001$).

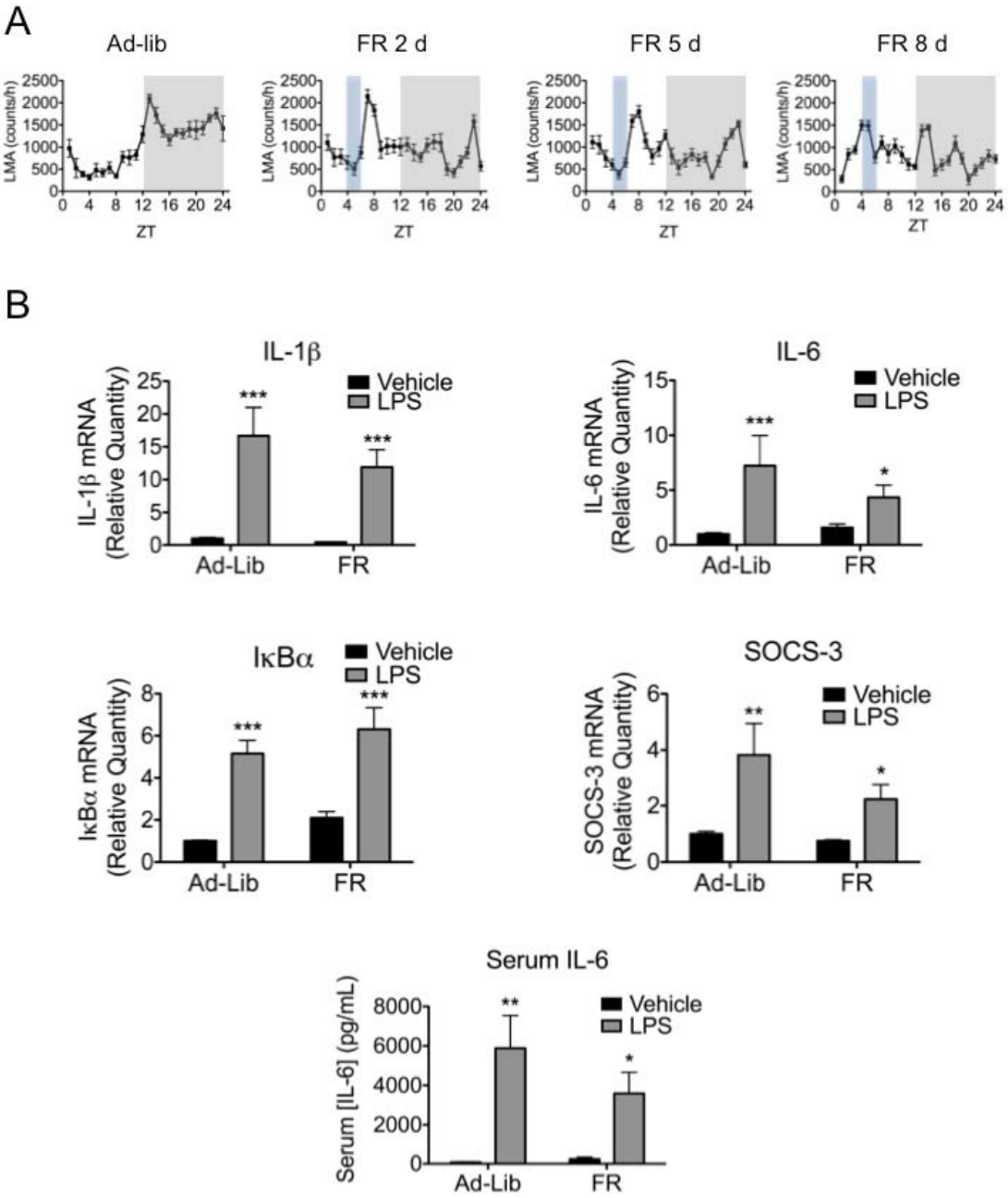

Figure 26. *Effects of food entrainment on circadian LMA and inflammatory response to LPS.* (A) Hourly LMA in rats undergoing food entrainment. Animals allowed access to food from ZT 4-6 each day (blue bar). FAA experiments performed on Day 9. Shaded area denotes dark phase. (B) Hypothalamic inflammatory cytokine mRNA expression and plasma IL-6 concentration in FR rats v. *ad-libitum* fed animals treated with LPS v. veh. Hypothalami and plasma collected 1 h after LPS administration. Data are expressed as mean ± SEM and statistics calculated by two-way ANOVA followed by *post hoc* analysis using Bonferroni's corrected *t* test (*, P<0.05; **, P<0.01; ***, P<0.001).

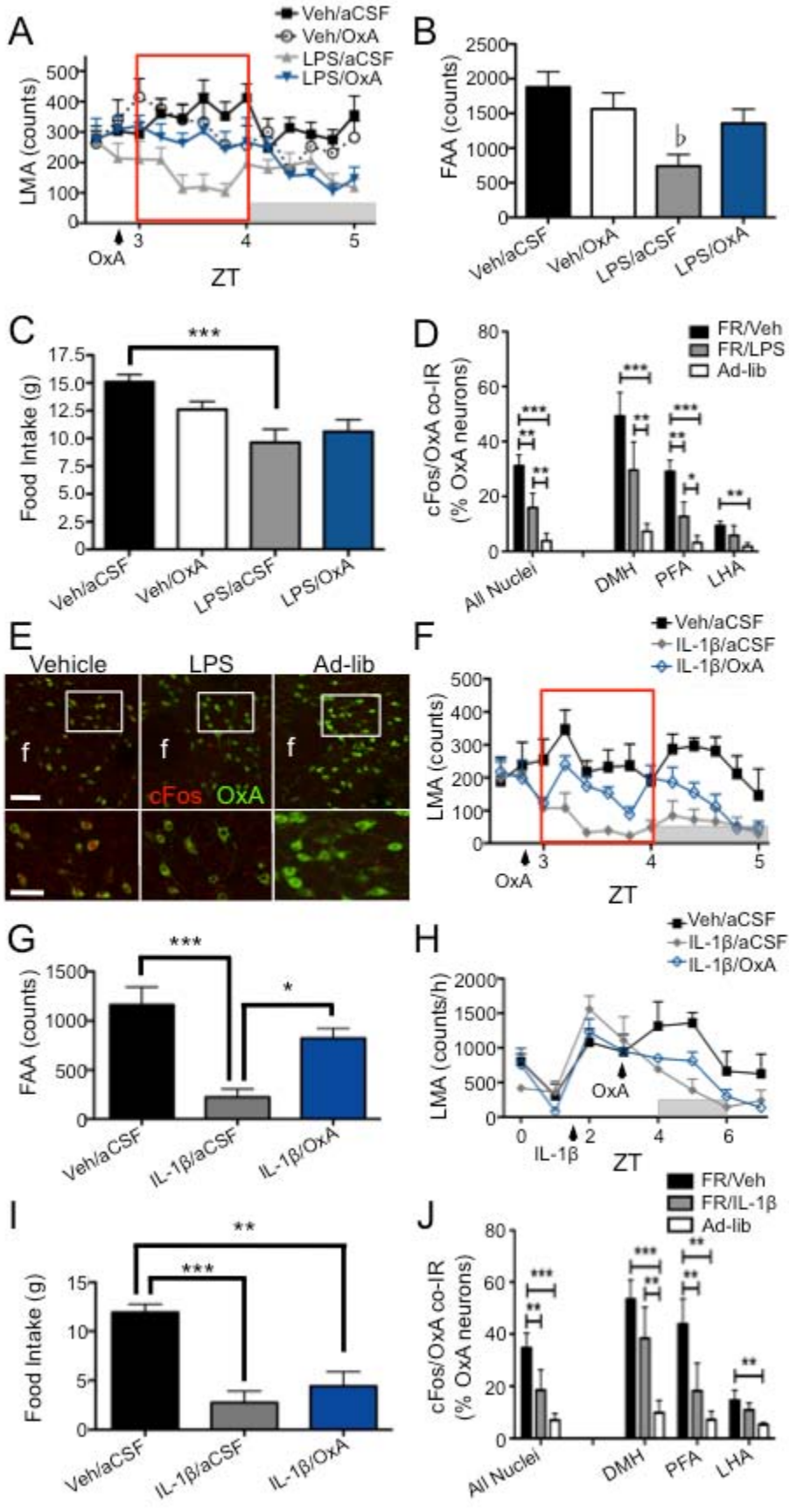
A
Veh/aCSF
Veh/OxA
LPS/aCSF
LPS/OxA
LMA (counts)
OxA
ZT
B
FAA (counts)
Veh/aCSF
Veh/OxA
LPS/aCSF
LPS/OxA
C
Food Intake (g)
D
cFos/OxA co-IR (% OxA neurons)
FR/Veh
FR/LPS
Ad-lib
All Nuclei
DMH
PFA
LHA
E
Vehicle
LPS
Ad-lib
cFos
OxA
F
Veh/aCSF
IL-1β/aCSF
IL-1β/OxA
LMA (counts)
OxA
ZT
G
FAA (counts)
Veh/aCSF
IL-1β/aCSF
IL-1β/OxA
H
LMA (counts/h)
IL-1β
OxA
ZT
I
Food Intake (g)
J
FR/Veh
FR/IL-1β
Ad-lib
cFos/OxA co-IR (% OxA neurons)
All Nuclei
DMH
PFA
LHA

Figure 27. *Central OxA administration restores FAA in FR rats treated with i.p. LPS or i.c.v. IL-1β.*

(A) Inhibition of FAA by i.p. LPS and restoration with central OxA in food-entrained rats. Movement is plotted in 12 min intervals. Grey bar indicates introduction of food into cages. Arrow denotes treatment with OxA or aCSF. (B) Sum of FAA (home cage activity during the hour prior to food presentation) in LPS- v.veh-treated FR rats treated administered i.c.v. OxA or aCSF. Period of FAA indicated by red box in (A). (C) Food intake from ZT 4-6 following LPS v. veh and OxA v. aCSF treatment. (D) Percentage of Ox IR neurons exhibiting cFos IR in FR rats following treatment with i.p. LPS or veh. Ad-lib, veh-treated rats also counted as a control for FR. Percent colocalization expressed as total Ox neurons and by anatomic location. (E) Low (top) and high power (bottom) representative photomicrographs of cFos IR (red) in Ox neurons (green) in PFA from veh- and LPS-treated FR rats and *ad-libitum* fed controls. (F) FAA in FR rats treated with i.c.v. IL-1β v. veh and restoration with OxA. Movement is plotted in 12 min intervals. Grey bar indicates introduction of food into cages. Arrow denotes treatment with OxA or aCSF. (G) Sum of FAA (home cage activity during the hour prior to food presentation) in IL-1β- v.veh-treated FR rats and OxA-replaced, IL-1β-treated rats. Period of FAA indicated by red box in (F). (H) Hourly home cage LMA on treatment day. Arrows indicate time of central treatment with IL-1β or aCSF and OxA or aCSF. Grey bar indicates food access. (I) Food intake during 2 h of food access following treatment with IL-1β or veh and OxA or aCSF. (J) Percentage of Ox IR neurons exhibiting cFos IR in FR rats following treatment with i.c.v. IL-1β or veh. Ad-lib, veh-treated rats also counted as a control for FR. Percent colocalization expressed as total Ox neurons and by anatomic location. Data are expressed as mean ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Scale bars, 100 μm (E, top panels), 50 μm (E, bottom panels).

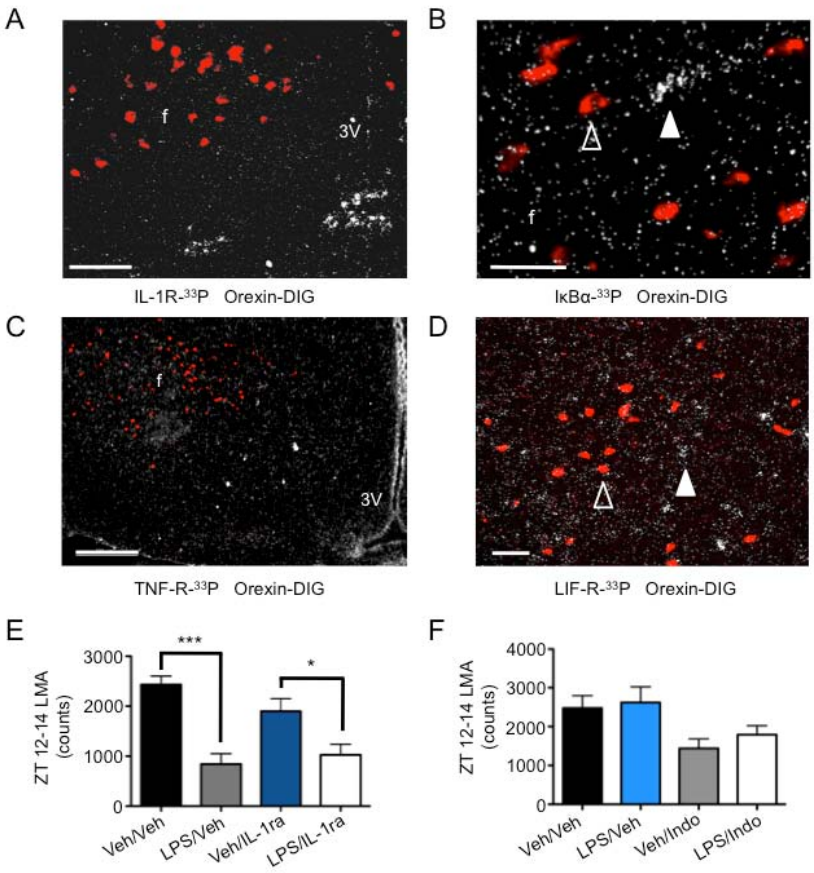

Figure 28. *Ox neurons are not direct targets for inflammatory signaling. LPS-induced lethargy is not dependent on central IL-1 or PG signaling.*

(A) Representative low-power photomicrograph of a double-label *in situ* hybridization showing that IL-1RI mRNA (silver grains) is not detected in the region of *Ox* mRNA expression (red precipitate). (B) IκBα mRNA (silver grains) is expressed in the region of Ox neurons (red precipitate) 1 h following treatment with i.p. LPS, however colocalization of the two messages was not observed. *Filled arrowhead* denotes cell expressing IκBα but not Ox. *Open arrowhead* signifies cell that expresses *Ox* but not IκBα. (C) Low power photomicrograph demonstrating that TNF-R mRNA (silver grains) is not expressed by Ox neurons (red precipitate). (D) LIF-R mRNA (silver grains) is expressed in LHA, but does not colocalize with *Ox* mRNA (red precipitate). *Filled arrowhead* denotes cell expressing LIF-R but not Ox. *Open arrowhead* signifies cell that expresses Ox but not LIF-R. (E) Sum of home cage LMA during first 120 min of dark phase in rats treated with i.p. LPS or veh and i.c.v. IL-1ra or veh. (F) Sum of home cage LMA during first 120 min of dark phase in rats treated with i.p. LPS or veh and indomethacin (indo) or veh. Data are expressed as mean ± SEM (*, P<0.05; ***, P<0.01). Scale bars, 100 μm (A), 50 μm (B,D); 200 μm (C); 3V, Third ventricle; f, fornix.

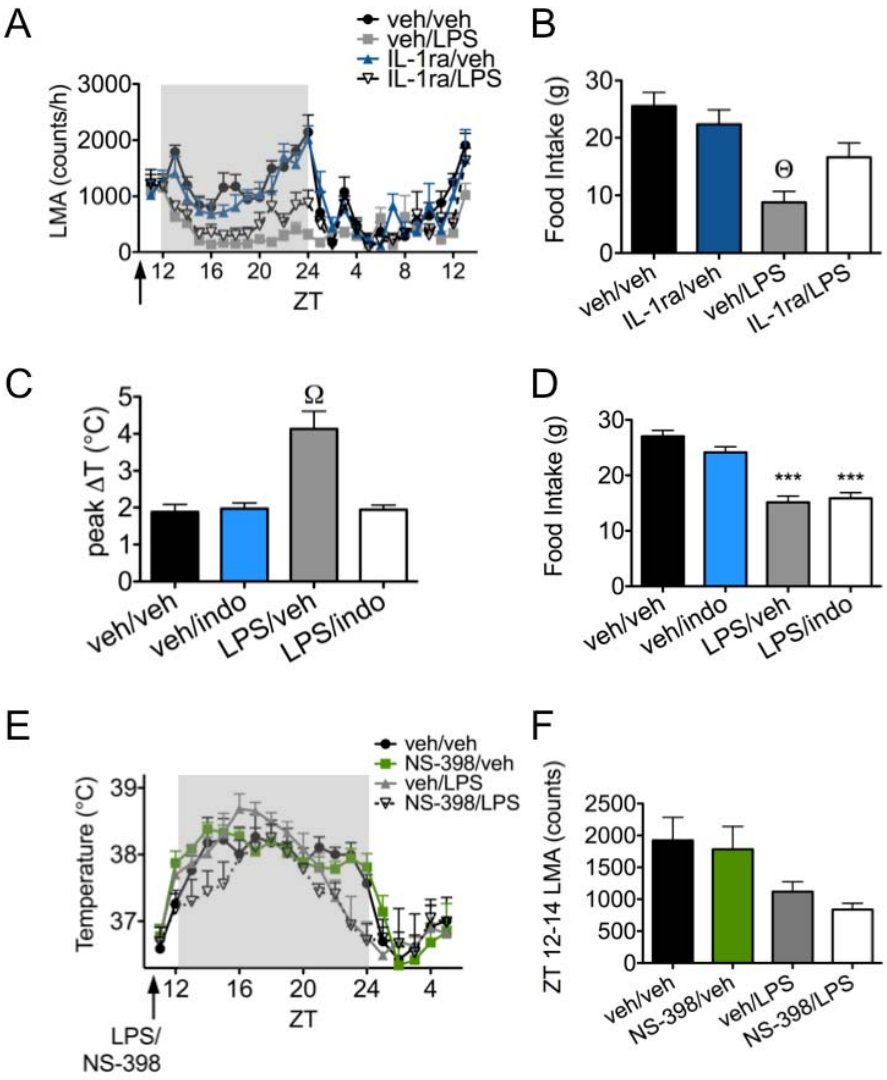

Figure 29. *Inhibition of prostaglandin synthesis blocks LPS induction of fever but does not affect LMA.*

(A) Hourly LMA in rats co-treated with i.p. LPS v. veh and i.c.v. IL-1ra v. veh. IL-1ra does not impact short term LPS-induced lethargy, but ameliorates the suppression of LMA ~8 h after treatment. (B) 24 h food intake in rats co-treated with i.p. LPS v. veh and i.c.v. IL-1ra v. veh. Blockade of central IL-1 signaling attenuated LPS-induced anorexia. (C) Systemic indomethacin administration attenuates LPS-induced fever. (D) Indomethacin does not prevent anorexia in response to LPS. (E) Central COX-2 inhibition with NS-398 reduces the hyperthermic response to LPS. (F) NS-398 has no effect on LPS-induced suppression of LMA. Analysis by 2-way ANOVA demonstrates a significant effect of LPS ($P<0.05$), but no effect of NS-398 and no significant interaction. Data are expressed as mean ± SEM and statistics calculated by two-way ANOVA followed by *post hoc* analysis using Bonferroni's corrected *t* test (Θ, $P<0.05$ v. all other groups; ***, $P<0.001$ v. veh/veh and veh/indo).

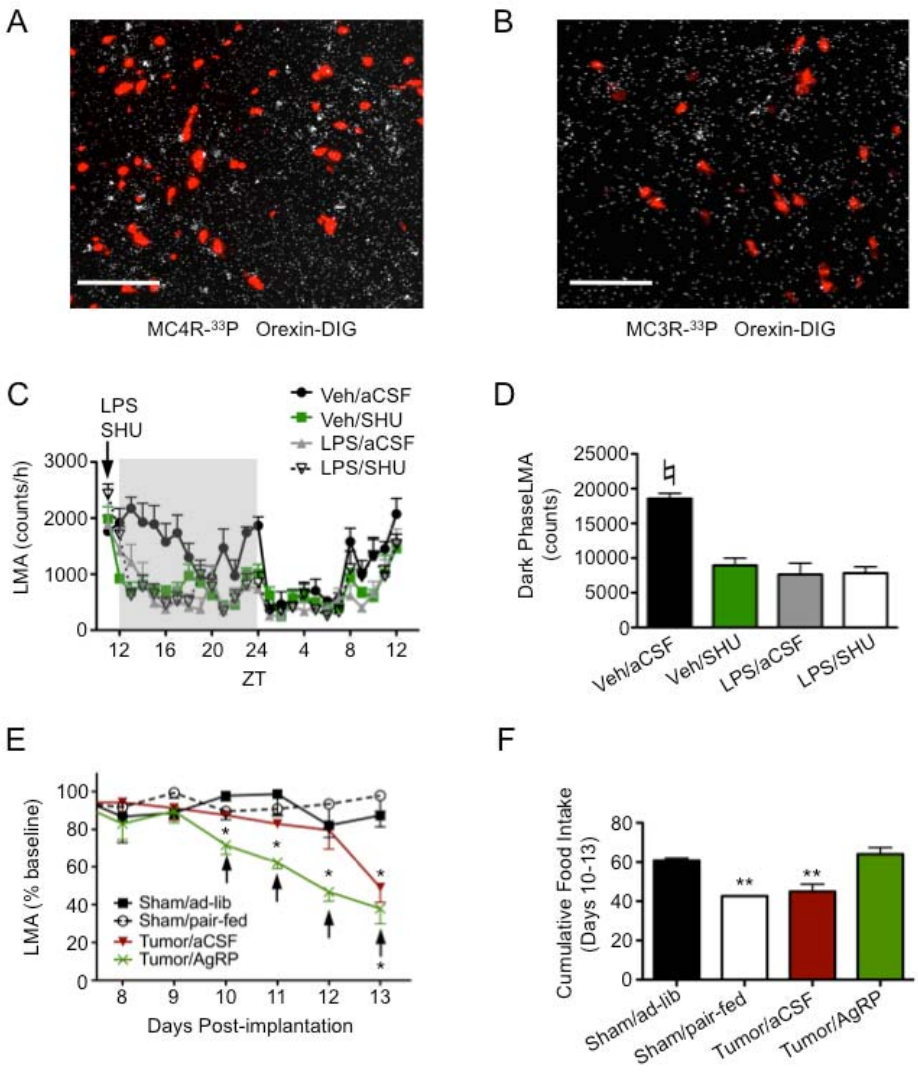

Figure 30. *LPS-induced lethargy is not melanocortin-dependent*.

(A, B) Double label *in situ* hybridization showing no colocalization of *Ox* mRNA (red precipitate) with either MC4R (A, silver grains) or MC3R (B, silver grains). (C) Hourly LMA from animals co-treated with i.p. LPS or veh and i.c.v. SHU-9119 or aCSF. Arrow denotes treatment time. (D) Summed overnight LMA from study depicted in (C). (E) Daily LMA, measured as a percentage of each animal's baseline daily LMA, from tumor bearing and sham-operated rats. Tumor-bearing rats were treated with i.c.v. AgRP or aCSF. Sham-operated animals were fed *ad-libitum* or pair-fed to tumor and treated with aCSF. Arrows denote i.c.v. treatment days. (F) Cumulative food intake during i.c.v. treatment period from study in (E) demonstrating that AgRP restores feeding in tumor-bearing animals to sham/pair-fed levels. Data are expressed as mean ± SEM (♮, $P<0.001$ v. Veh/SHU, LPS/aCSF, & LPS/SHU; *, $P<0.05$ v. Sham/ad-lib & Sham/pair-fed; **, $P<0.01$ v. Sham/ad-lib & Tumor/AgRP). Scale bars, 100 μm (A,B).

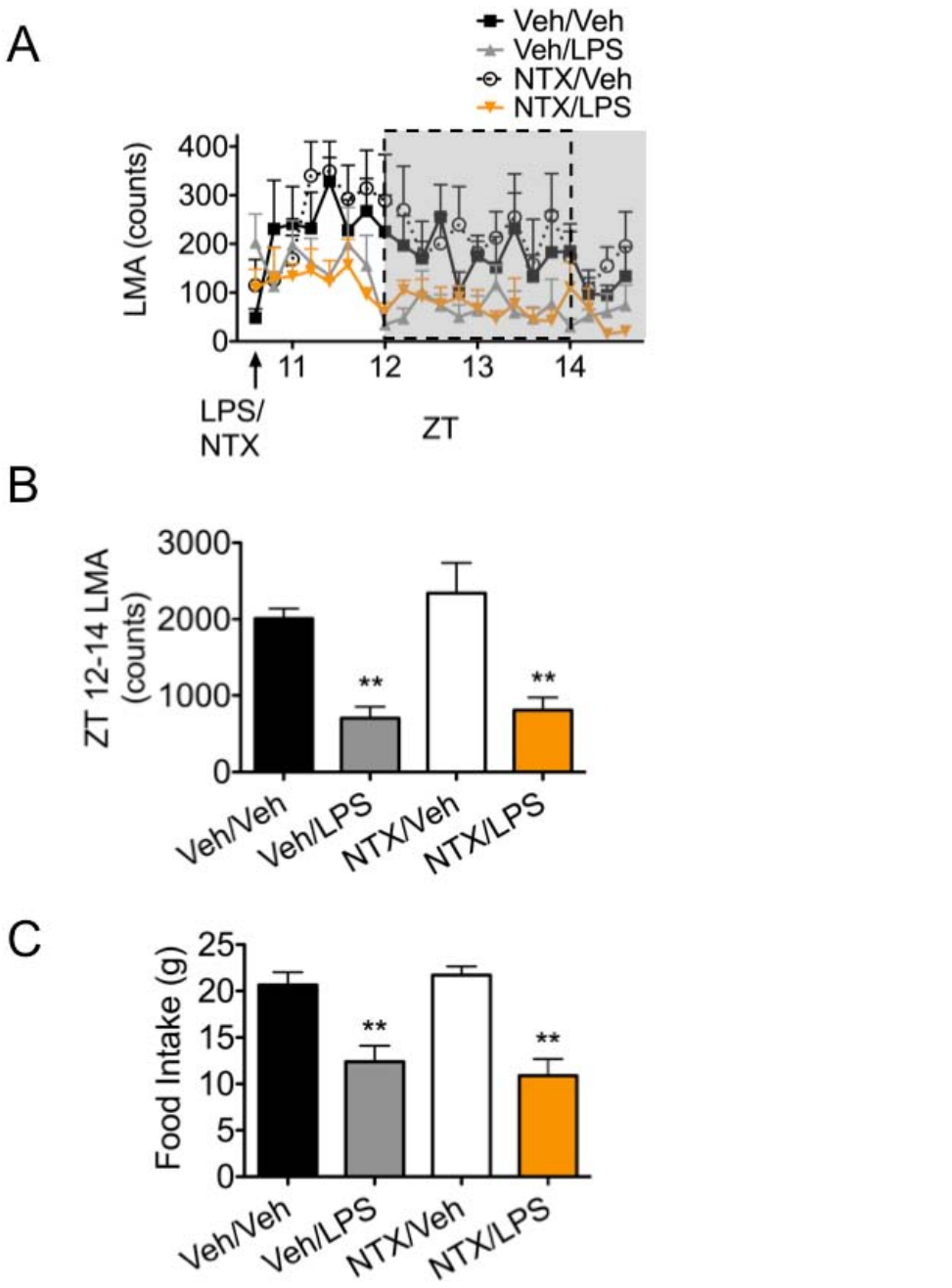

Figure 31. *LPS inhibition of orexin neuron activity is not dependent on central opioid signaling.* (A) LMA in rats co-treated with i.p. LPS v. veh and i.c.v. NTX v. veh. (B) Sum of LMA during first 2 h of dark phase following co-administration of i.p. LPS v. veh and i.c.v. NTX v. veh. NTX treatment has no effect on LMA in LPS- or veh-treated rats. (C) 24 h food intake in rats co-treated with i.p. LPS v. veh and i.c.v. NTX v. veh. Central opioid blockade does not affect food intake in LPS- or veh-treated rats. Data are expressed as mean ± SEM, and statistics calculated by two-way ANOVA followed by a post hoc analysis using a Bonferroni corrected *t* test (**, P<0.01 v. veh/veh and NTX/veh).

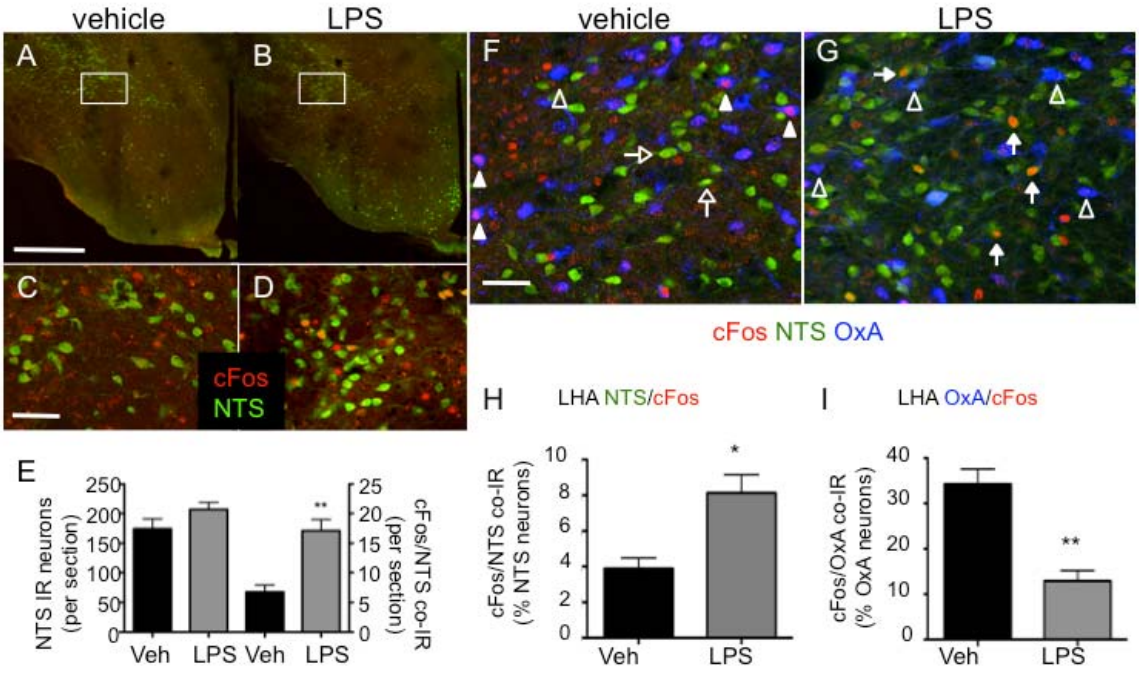

Figure 32. *LPS treatment induces cFos IR in LHA NTS neurons while suppressing vespertine activation of Ox neurons in NTS-cre/GFP mice.*

(A, B) Low power photomicrographs showing distribution of GFP IR (*green*) and cFos IR (*red*) in veh (A) and LPS-treated (B) NTS-cre/GFP mice. (C,D) High power photomicrographs of regions outlined in (A) and (B). (E) Total number of LHA NTS-expressing neurons and number of neurons exhibiting GFP and cFos IR per section. (F, G) Representative high power images of sections triple labeled for GFP, cFos, and OxA from veh- and LPS-treated mice. (H, I) Quantification of co-staining for cFos (red) and GFP (green) (H) or OxA (blue) (I) expressed as a percentage of neuropeptide-expressing cells. *Filled arrows* signify cells labeled with cFos and GFP*;open arrows* denote cells labeled with GFP alone. *Filled arrowheads* denote cells IR for both cFos and Ox; *open arrowheads* point to cells stained only for Ox. Data are expressed as mean ± SEM (*, $P < 0.05$; **, $P < 0.01$ vs. veh). Scale bars, 500 µm (A,B), 50 µm (C,D), 50 µm (F,G); 3V, Third ventricle; f, fornix.

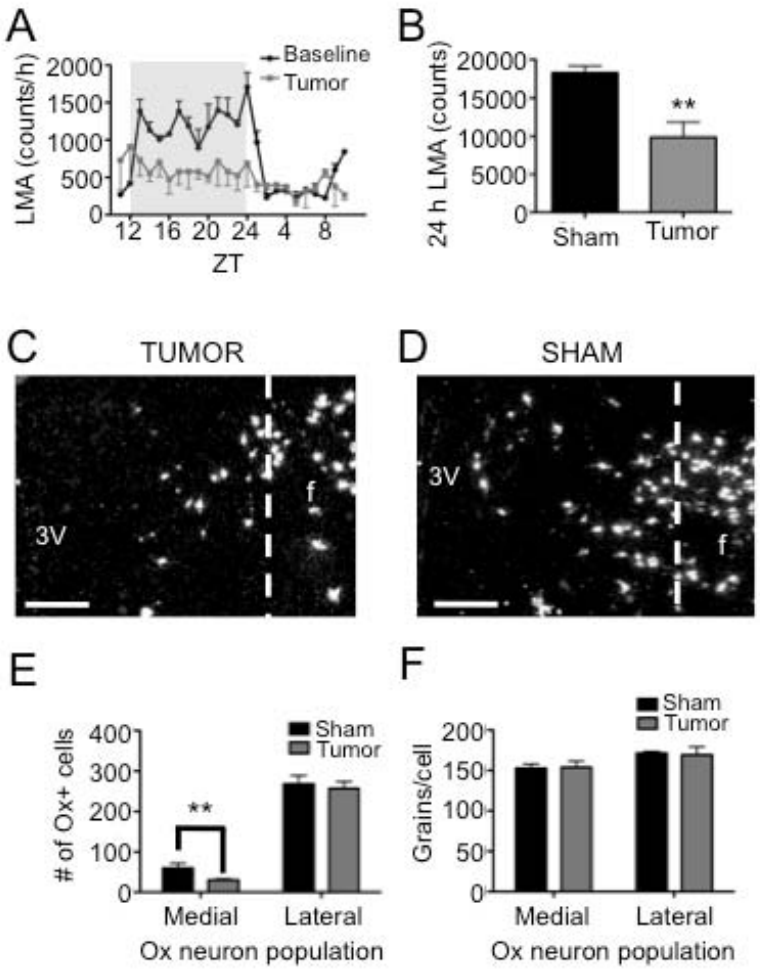

Figure 33. *Tumor-induced inflammation inhibits home cage LMA and reduces Ox mRNA expression in the medial hypothalamus.*

(A) Hourly home cage LMA in tumor-bearing rats 13 days after tumor implantation compared to baseline activity. (B) 24 h home cage LMA in tumor-bearing rats compared to sham-operated, pair-fed controls during final day before sacrifice. (C, D) Representative dark field photomicrographs of *in situ* hybridization showing *Ox* mRNA expression (*silver clusters*) in tumor-bearing (C) and sham-operated (D) rats. Dotted line represents division between medial and lateral Ox neuron fields. (E) Quantification of the number of *Ox* mRNA-expressing cells in medial and lateral Ox neuron fields. (F) *Ox* mRNA expression, represented as silver grains per cell, in medial and lateral Ox neurons from tumor-bearing and sham-operated rats is compared. Data are expressed as mean ± SEM (**, $P < 0.01$). Scale bars, 200 μm; 3V, Third ventricle; f, fornix.

CHAPTER 4

SUMMARY AND CONCLUSIONS

During the last 20 years, research has demonstrated that 1) the hypothalamus regulates homeostatic behavior and physiology, 2) inflammatory signals are produced within the hypothalamus during disease, and 3) local inflammation within the hypothalamus can alter the activity of neurons controlling homeostasis. Few studies directly tested the causality of these interactions and the relevance to sickness behavior. The work described in this thesis explores the neural mechanisms underlying two aspects of sickness behavior—anorexia and lethargy. These studies were predicated on the hypothesis that hypothalamic inflammation during disease underlies sickness behavior and that local inflammatory signaling within the hypothalamus disrupts normal homeostatic and circadian circuits that modulate feeding behavior and physical activity. The results presented in the two previous chapters support a causative relationship between hypothalamic inflammation and the behavioral response to illness and suggest that the behavioral components of sickness can be separated from the peripheral immune response.

1. LIF suppresses food intake by directly activating ARC POMC neurons

Previous work in our laboratory demonstrated that ARC POMC and AgRP neurons express the IL-1RI, and IL-1β potently activates anorexigenic POMC neurons while inhibiting the release of the orexigenic AgRP (161, 162). These results follow a series of studies illustrating that IL-1β is anorectic (479) and that melanocortin antagonism restores feeding in IL-1β treated animals (480). Together, these studies suggest that IL-1β inhibits food intake by shifting activation of MC3-R/MC4-R toward an anorectic response. However, no studies evaluated whether this relationship was either causative or physiologically relevant during disease. The development of conditional

genetic models enabled us to ask whether cytokines binding to their receptors on POMC neurons could induce anorexia. We observed that acute i.c.v. LIF injection activates ARC POMC neurons, but brainstem POMC neurons were not activated (62), despite significant activation of neighboring cells and widespread expression of the LIF-R throughout the brainstem (including the NTS)(Figures 13A-13H and 35A). ARC POMC neurons also express the LIF-R and hypothalamic explants increase α-MSH release in response to LIF. When the gp130 subunit is deleted specifically from POMC neurons, the ability of LIF to induce α-MSH release and inhibit food intake is completely abolished, indicating that the acute anorectic effects of LIF are mediated by direct activation of gp130 signaling in POMC neurons. Importantly, we found that though LIF and IL-6 expression were increased in the hypothalami of LPS or IL-1β treated animals, CNTF expression was reduced by inflammation, suggesting that this cytokine is not involved in inflammation-induced anorexia. Furthermore, this cytokine is not secreted, so a role in inflammatory anorexia is not likely. We did not observe any detectable IL-6 receptor mRNA expression by POMC neurons, strongly supporting a unique role for LIF in activating gp130 signaling on POMC neurons in inflammatory states. This is the first study that directly implicates inflammation-induced changes in melanocortin signaling with the anorectic component of cachexia and provides mechanistic support for therapeutic use of melanocortin antagonists.

1.1 The Role of LIF in Cachexia

However, the importance of LIF in the behavioral response to illness remains unconfirmed. Although we observed increases in LIF mRNA in hypothalamic homogenates from LPS- or IL-1β-treated animals, perfusion of LPS-treated rats with RNase-free PBS completely eliminated the rise in LIF mRNA, but not IL-1β or IL-6

(Figures 9A and 36). This suggests that LIF is being primarily expressed intravascularly, perhaps by circulating immune cells. Although studies have confirmed that LIF is actively transported across the BBB during inflammatory states, it is unknown whether this cytokine is biologically relevant in the ARC under these conditions. Single label *in situ* hybridization demonstrates a distinctly vascular and meningeal pattern of LIF expression 1 h following LPS, at the zenith of hypothalamic LIF expression (Figure 37). Furthermore, deletion of gp130 from POMC neurons does not attenuate LPS-induced anorexia (Figure 38). Although peripheral LPS induces widespread upregulation of several anorectic cytokines, including IL-1β, LIF, and TNF-α, this study indicates that the activation of POMC neurons by LIF is not a necessary component of the anorectic response to LPS.

LIF is distinct from other anorectic, pro-inflammatory cytokines in that the anorectic response does not diminish in the setting of chronic administration, a feature shared with clinical cachexia (186, 187). IL-1β is a much more potent and efficacious inhibitor of feeding behavior, but it does not sustain an anorectic response when chronically infused into the brain (165). This contrast in tachyphylactic responses indicates that LIF may be more important for maintaining negative energy balance for long periods. Although IL-1β infusion does not induce sustained anorexia, animals never refeed as normal homeostatic control of food intake would predict, and catabolism is maintained for at least three days (165). Recent unpublished studies in our laboratory have established that central IL-1β is sufficient to induce muscle catabolism, independent of changes in food intake, in a GC-dependent manner (Braun et al., *in preparation*). LIF is known to be an important regulator of inflammation-induced HPA axis activation, and may also induce catabolism when administered centrally, though this

hypothesis has not been tested. Central IL-1β potently induces LIF expression in the hypothalamus, although the extent to which LIF mediates the catabolic effects of IL-1 is unknown. That LIF can chronically suppress food intake, whereas IL-1 cannot indicates that the induction of LIF by IL-1 is likely transient and subject to the same desensitization that characterizes the feeding response to IL-1. Thus, it is uncertain whether the acute actions of LIF on central melanocortin signaling are important in the chronic disease process, and studies looking into this are vital to establish a role for LIF in cachexia.

1.2 LIF Regulation of the Central Melanocortin System

Our study demonstrated that POMC neurons are necessary for the acute inhibition of food intake by LIF. This finding was somewhat surprising, given recent work suggesting that AgRP neurons are the primary drivers of ingestive behavior. Specifically, activation of AgRP neurons using optogenetics or designer receptors exclusively activated by designer drugs (DREADD) demonstrates that AgRP neuron activity is sufficient to rapidly drive feeding (478, 481). Conversely, targeted ablation of AgRP neurons in adult animals is sufficient to block feeding to the point of fatal starvation. This effect is mediated by loss of GABAergic release from AgRP neuron terminals in the brainstem parabrachial nucleus (448, 449). Current examination of ARC circuitry posits that POMC neurons may be more important for regulating other aspects of energy homeostasis, including glucose homeostasis (482) and LMA (483), than for feeding. In our studies, gp130 signaling in AgRP neurons and other non-POMC-expressing cells presumably remained intact, as pSTAT3 IR in the ARC was not significantly different between WT and PomcCre-gp130$^{flox/flox}$ animals. Yet, absence of LIF signaling in POMC neurons was sufficient to reverse the anorectic response. Given that we observed similar LIF-R mRNA signal on AgRP neurons as POMC neurons (Figure 35B) this finding

demonstrates that POMC neuron activation is necessary to block feeding following LIF administration, and supports previous data suggesting an important role for POMC neurons in suppressing food intake, independent of AgRP neuron activity (484, 485).

This interpretation is subject to several potential limitations of Cre-lox transgenic studies. First, several investigators have reported transgenic models in which either Cre is expressed or the lox-flanked allele is deleted ectopically. This recombination event occurs following the mating of F_1 (or later) generations of Cre/lox + mice and only in mice that were developed using the random insertion of a Cre-encoding transgene (486, 487). An alternative approach, in which an internal ribosome entry site (IRES)-Cre transgene is inserted under the control of the endogenous promoter has successfully circumnavigated this problem (488). In our PomcCre-gp130$^{flox/flox}$ studies, the PomcCre line was derived by random transgene insertion (439), raising the possibility of ectopic gp130 deletion. Unfortunately, we could not assess whether ectopic deletion occurred, as our gp130 riboprobe exhibited too much background expression to specifically define cells that do not express the transcript. Second, a recent report indicates that several neuronal cells transiently express POMC during development, including approximately 25% of AgRP/NPY neurons (489). Thus, during development, these AgRP neuron precursor cells may express Cre at sufficient levels to drive deletion of floxed alleles. We observed similar induction of pSTAT3 in WT and transgenic animals, indicating that deletion of gp130 did not affect total ARC response to LIF. Because reliable antibodies for the detection of NPY/AgRP neuron cell bodies are not commercially available, we were not able to directly assess the responsiveness of these neurons. Thus, although POMC neuron activation is necessary for the anorectic effects of LIF, we cannot exclude a critical role for AgRP neurons. However, mimicking leptin or LIF signaling by

constitutive activation of STAT3 in AgRP neurons does not alter food intake, but does increase energy expenditure by increasing LMA (490). Like many inflammatory interventions, i.c.v. LIF administration leads to a reduction in LMA. Although it is not known whether this feature is mediated directly by LIF or by nonspecific effects of inflammation, it suggests that AgRP neurons are not involved in LIF-induced changes in energy balance.

We have shown that inflammation increases α-MSH tone at central melanocortin receptors and that blocking MC4-R signaling restores feeding and lean mass in several disease models (17, 18, 62, 89, 161, 162). However, it is possible that this increase in melanocortin tone is not solely responsible for the anorexia of chronic disease and that melanocortin antagonism may be outcompeting the endogenous anorectic circuitry. Indeed, the doses HS014 used to treat cachexia in animal models potently stimulate food intake in healthy animals (491). Central administration of either AgRP or SHU-9119 at a dose that did not affect food intake in healthy animals restored feeding in tumor-bearing or LPS-treated rats, respectively, indicating that melanocortins are the major physiologic signal inducing anorexia in these models (18, 19). Additional non-melanocortinergic anorexic signals may derive from amino acid neurotransmitter release from cytokine-responsive melanocortin neurons. As recently described, reductions in the release of GABA from AgRP neuron terminals depresses food intake and threatens survival (448). Following complete AgRP neuron ablation, this process supersedes rescue by melanocortin antagonism, but a less dramatic loss of inhibitory tone in the parabrachial nucleus may be amenable to reversal by MC4-R blockade (449). Alternatively, other non-melanocortinergic anorectic circuitry may also be engaged during inflammation. As described above, increased activity of brainstem neurons in the

parabrachial nucleus is associated with reduced food intake and starvation, even in the face of undernutrition (448). Also, early studies examining patterns of cFos IR in response to LPS found that the parabrachial nucleus is among the most highly activated brain regions (75). Surprisingly, little work has followed up on this finding, so the relative contribution of neurons in this nucleus to inflammation-induced anorexia is undetermined.

1.3 The Central Melanocortin System and Locomotor Activity

Our studies and others have conclusively established that melanocortin neurons in the ARC respond to inflammation and alter feeding behavior. Though several studies also report attenuation of inflammation-induced suppression of LMA by genetic or pharmacologic blockade of MC4-R, closer examination of these data reveals that baseline LMA is reduced by MC4-R blockade or deletion (18, 492, 493). Thus, in the fed state, melanocortin antagonism suppresses physical activity to a similar degree that LPS or IL-1 does, despite restoring food intake in these animals. We hypothesized that this might be an artifact of the central location of the food hopper in animal cages, which allows the animals to eat without foraging or searching. However, when we repeated this study but removed food from the cages, we saw an equivalent suppression of LMA in all SHU-9119-treated animals, regardless of inflammatory status. Accordingly, in tumor-bearing animals, i.c.v. AgRP treatment potently suppresses LMA while increasing food intake. Restoration of POMC neuron activity in obese, hypoactive *db/db* mice by re-expression of LepRb in POMC neurons, potently stimulates LMA in these animals (483), and constitutive activation of STAT3 in AgRP neurons, presumably reducing their activity, increases LMA (490). Paradoxically, in the absence of food, specific activation of AgRP neurons using DREADD is sufficient to drive foraging behavior, though this

behavior is absent in the presence of food (478). These studies demonstrate a clear role for melanocortinergic neurons in the regulation of LMA by both MC4-R-dependent and independent mechanisms. However, in both cases, elevated POMC (and decreased AgRP) neuron activity is associated with increased LMA, rather than the decrease in movement observed during disease. This suggests that a separate neural process mediates the suppression of physical activity during inflammatory challenge.

2. Orexin Neurons Mediate Inflammation-Induced Lethargy

The data presented in this thesis demonstrate that the inhibition of Ox neurons underlies this reduction in LMA during inflammation. We found that peripheral LPS treatment suppresses the normally elevated Ox neuron activity and arousal associated with both onset of the active period and the introduction of food to FR animals. Central OxA replacement, at levels that have no effect on control animals, transiently restores LMA in each of these models. Central inflammation, induced by IL-1β, was also sufficient to inhibit Ox neurons and LMA. Although sufficient, neither IL-1 nor PG signaling is necessary for the induction of lethargy by LPS. We found no evidence that Ox neurons are responding directly to cytokines or to melanocortin peptides. However, we did observe activation of LHA Nts-expressing neurons by LPS. Some LHA Nts neurons provide an inhibitory influence on Ox neurons in response to activation by leptin (324), suggesting that inflammation may be inhibiting Ox neurons in a similar manner.

2.1 Perifornical Orexin Neurons Are Inflammation-Sensitive

Previous authors have posited a functional divergence in anatomically distinct populations of Ox neurons (375). Our data support this hypothesis, as we found that

medial (DMH/PFA) Ox neurons are preferentially activated during periods of peak arousal, whereas lateral Ox neurons are less affected. This medial Ox neuron population can be divided according to inflammation-sensitivity: DMH Ox neurons were not inhibited by LPS or IL-1β treatment, while PFA Ox neurons were. Furthermore, inhibition of the PFA Ox neuron population was sufficient to suppress LMA. To our knowledge this is the first time that this medial, arousal-associated population has been functionally separated. The distribution of the inflammation-sensitive Ox neurons closely overlapped with the highest concentration of LHA Nts neurons in the dorsal, perifornical LHA. This co-distribution supports our hypothesized role of Nts neurons in mediating the suppression of Ox neurons by LPS. This circuit requires additional defining, however. First, it is not known how inflammatory signals are transmitted to LHA Nts neurons. These neurons may be cytokine responsive, as they are leptin responsive. Alternatively, they may receive excitatory input from inflammation-responsive neurons in the brainstem or elsewhere within the hypothalamus. Recent data demonstrates that blocking nerve transmission through the DVC can attenuate LPS-induced LMA suppression (476). Administration of voltage-gated Na^+ channel blockers to the DVC also reverses LPS-induced suppression of cFos IR in TMN HA neurons during periods of wakefulness and activity (420). Orexins potently activate TMN neurons via the OX2R, and removal of this stimulation independently suppresses normal HA neuron activity (283, 302). Thus, it is possible that inflammation sensitive neurons in the brainstem project to the LHA, where they activate Nts neurons, which then inhibit Ox neurons and remove this excitatory input onto TMN neurons. Inflammation activates catecholaminergic neurons in the caudal brainstem that project to the hypothalamus (136, 494). Norepinephrine inhibits Ox neurons, indicating that brainstem catecholaminergic neurons may alternately directly suppress Ox neuron activity. The existence and function of these circuits is purely hypothetical and requires further investigation.

Second, no studies have directly evaluated the efferent projections from perifornical Ox neurons. Because Ox neurons project widely to areas associated with arousal and locomotion, and orexins excite arousal associated neurons in each of these regions, it is uncertain which downstream neurons mediate the increased LMA induced by the activity of PFA inflammation-sensitive Ox neurons. Our data demonstrate that both vespertine and FAA peaks in LMA require the activity of this Ox neuron subpopulation. Therefore, identifying the targets of these circuits will lend insight into circuits mitigating the behavioral expression of increased arousal. Strong evidence supports the involvement of the mesolimbic DA system in mediating Ox-induced elevations in LMA during periods of peak activity. Ox neurons project densely into both the VTA and the nucleus accumbens. Pharmacologic administration of OxA induces DA-dependent hyperlocomotion, and OxA directly induces Ca^{2+} influx in VTA dopaminergic neurons *in vitro* (304). Both LPS and leptin induce alterations in DA transporter function via the activity of Ox neurons, as evidenced by reduced behavioral responses to amphetamine (324, 477). Further, we effectively restored LMA in LPS-treated animals using OxA, which binds OX1R with 100-1000X greater affinity than OX2R (264). Because our dose induced no noticeable side effects in either healthy or LPS-treated rats, it is likely that this effect was OX1R specific, though this interpretation should be confirmed in future studies. Both OX1R and OX2R are expressed in the VTA, where OX1R activity may permit motivated behaviors during periods of appropriate arousal (495). Thus, during disease, when the behavioral priorities of the animal are shifted to conserve energy, suppressing Ox release in the VTA could inhibit motivated behaviors in response to conditioned stimuli and circadian cues. Other regions that express OX1R include the DR and the LC, both of which influence arousal and vigilance. It is unclear

whether these regions are innervated by PFA Ox neurons, but our preliminary tract tracing data show very few retrogradely labeled OxA-IR neurons in the PFA of rats injected in the DR with cholera toxin B (Figure 39).

2.2 Locomotor Activity and Feeding Are Independently Regulated by Inflammation

Several studies identify Ox neurons as the link between negative energy balance and LMA. Our data demonstrate that Ox replacement influenced arousal and LMA independent of any effects on feeding. Animals explored their cage, groomed, and investigated their food hoppers, but seldom ate. OxA is known to be orexigenic, and ablation of Ox signaling decreases food intake, but the loss of Ox signaling associated with LPS does not appear to impact feeding behavior. This observation corroborates recent work suggesting that the impairment in feeding seen in *OxKO* mice is primarily due to insufficient arousal (268). Orexins may be necessary to coordinate motivated behaviors in response to multiple conditioned and unconditioned stimuli, but insufficient to drive these behaviors in the absence of specific cues directing these behaviors. In sick animals circadian biology remains intact but orexigenic signals are blocked. The concomitant decrease in Ox neuron activity induces a hypoactive, lethargic state. Replacement of OxA restores normal exploratory and grooming behavior associated with the active period, presumably because many of the signals directing this behavior persist throughout the inflammatory challenge. However, inflammation is associated with increased stimulation of the MC4-R and impaired NPY signaling, thereby removing the drive to eat. Thus, Ox replacement does not restore feeding (Figure 34). Future experiments in which melanocortin antagonists and Ox agonists are co-administered could verify this relationship.

2.3 Inflammation, Orexin, and Sleep

Because orexins stabilize wakefulness, an increase in LMA might be expected solely from increased time spent awake. Indeed, sick animals spend more of the dark phase sleeping than healthy animals. This sleep is predominately NREM in type and is characterized by increased fragmentation (377). We did not evaluate the effects of OxA replacement on total time awake or sleep/wake transitions, but we observed increased activity after i.c.v. injection. When LPS-treated animals are awake, they exhibit noticeably less activity and home cage exploration than healthy animals or OxA-treated animals. We believe that OxA is having arousal-promoting effects that extend beyond increasing or consolidating wakefulness. Correspondingly, Ox neurons are more active when an animal is actively moving or exploring their environment than during quiescent wakefulness. Daytime (active phase) somnolence and nighttime (inactive phase) sleep disruptions are also common features of sickness behavior that may result from reduced Ox signaling. Based on data derived from *OxKO* mice and patients with narcolepsy, decreased Ox would be expected to impact the consolidation of sleep and wakefulness by destabilizing the flip-flop switch (199, 274, 277). Although this relationship has not been evaluated in disease models, restoration of circadian Ox oscillations in the CSF may be a viable therapeutic strategy for illness-associated somnolence and, perhaps, insomnia, as well.

2.4 Therapeutic Potential of Orexin Agonists

Several issues must be explored before Ox agonists can be considered as a therapeutic for illness-associated lethargy. First, the involvement of Ox neurons in

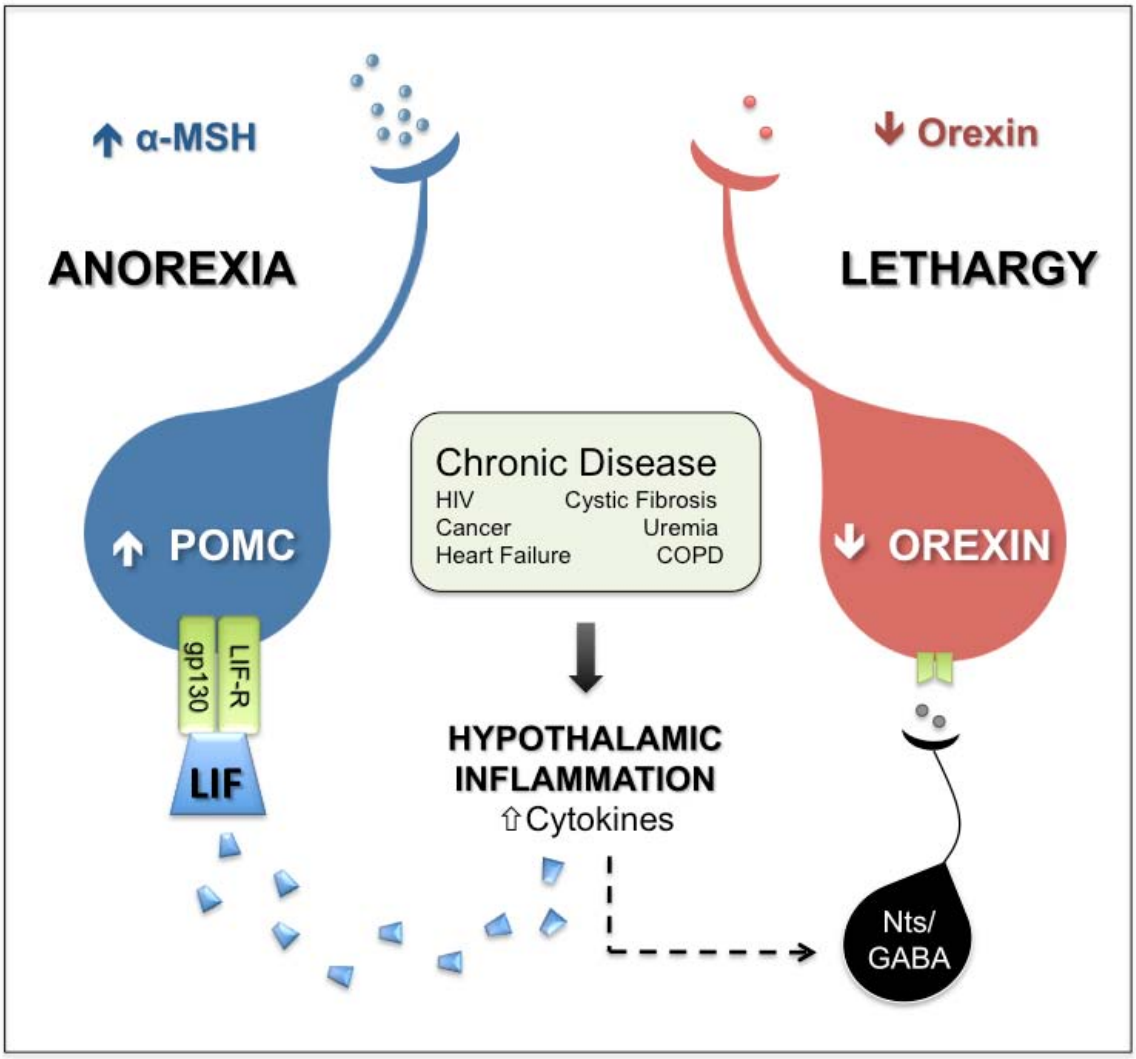

Figure 34. *Schematic diagram depicting the hypothesized mechanisms by which hypothalamic inflammation induces sickness-associated anorexia and lethargy.*

Acute and chronic disease are associated with hypothalamic inflammation and increased cytokine production. (Left) Hypothalamic LIF expression is induced during inflammatory challenge and chronic disease. LIF inhibits food intake by binding to the LIF-R/gp130 receptor complex expressed on ARC POMC neurons and increasing the release of α-MSH from POMC neuron terminals. (Right) Inflammation inhibits the activity of orexin neurons during periods of wakefulness and arousal, leading to diminished locomotor activity and lethargy. Putatively, LHA Nts-expressing GABAergic neurons mediate the suppression of orexin neuron activity during disease.

mediating lethargy of chronic disease must be confirmed. Our studies demonstrated a decrease in the number of medial (arousal-associated) *Ox*-expressing neurons in hypoactive tumor-bearing rats. This represents a different population of Ox neurons than we saw inhibited during acute inflammatory challenge with LPS. Thus, the physiologic relevance of this finding as well as the afferent signals that generate this response must be evaluated. It is unknown whether this represents transcriptional repression or neuronal cell death, however. A previous study reported that 30 d infusion of LPS into the LHA resulted in a decrease in the number of OxA-IR neurons (496). Though the relevance of this study to disease states is questionable, it supports the notion that chronic inflammation can reduce the number of Ox neurons.

Additionally, the use of Ox agonists poses several pharmacological concerns. Ox receptors are G-protein coupled, and thus subject to desensitization in the sustained presence of agonist. Therefore, the overall utility of these drugs in maintaining arousal during disease may be limited by downregulation of the receptor or one of its signaling adaptors. During our trial with overnight OxA infusion, we observed a sustained improvement in LMA over vehicle-infused animals. Importantly, OxA-treated rats maintained their matutinal peak in activity, which is present in healthy rats and otherwise absent following LPS challenge. This occurs at the end of the dark phase treatment period and indicates persistent response to treatment for at least 12 h. Because Ox-mimetics would not be administered during the inactive (sleeping) period, presumably any loss in Ox sensitization could be restored between treatment periods. Such dosing regimens are currently used to effectively deal with desensitization to vasodilating nitrates in patients with angina pectoris. Route of administration is also of concern, as maintenance of an intrathecal port poses potentially great risk to patients. Orexins are biologically active in the periphery, so systemic administration would likely be associated

with a wide spectrum of digestive and metabolic side effects. Intranasal Ox agonists are being developed for the treatment of narcolepsy and may evade these dosing issues.

Finally, treatment with Ox agonists poses a significant risk for side effects that could be incompatible with clinical use. As described above, central administration of Ox is associated with increased temperature and sympathetic nervous system activity. Both of these effects could increase energy utilization and exacerbate fever in already sick individuals that are likely to be in negative energy balance. Presumably, if Ox dosing closely mimics endogenous levels during wakefulness, these effects should be minimal. Indeed, we saw no significant rise in body temperature following OxA treatment compared to vehicle in either LPS- or vehicle-treated animals. However, given that Ox may be acting at different locations for its LMA-inducing effect and its sympathomimetic effects, heart rate, blood pressure, body temperature, and adrenal activity should be carefully monitored in subsequent studies. Recent literature has suggested that OxA and OxB have anxiogenic effects mediated by activity in the paraventricular nucleus of the thalamus (497). Augmenting anxiety is clearly contraindicated in patients that are already dealing with the psychological implications of chronic disease and lethargy or anhedonia. Importantly, anxiety only occurred in response to pharmacologic doses of Ox peptides to levels 3-10-fold higher than used in our studies. Regardless, the serious nature of these effects demands careful evaluation if Ox agonists are to be used in patient treatment.

3. Summary

The work reported in this thesis has defined two hypothalamic neuropeptide-expressing neuronal populations that mediate the anorexia and lethargy associated with inflammation. These results support modulation of neuropeptide signaling as a therapeutic approach to sickness behavior and identify parts of a complex circuit involving the immune system and the brain. Our results suggest that parallel pathways underlie these two features of sickness behavior, yet the afferent and efferent contributors to this neuroimmune circuit remain undefined. Rather than disparate circuits, both of these behaviors may branch from a common upstream integrator of inflammatory signals. The brainstem is the most likely location for this common sensor of inflammation, given the robust response of several brainstem nuclei to inflammation and the multiple projections to both the ARC and LHA. The contribution of this region has been more difficult to study as a result of fewer molecular markers that identify unique neuronal populations. Aiding in the obfuscation of common intermediaries is that no cytokine or signaling molecule has been shown to be both sufficient and necessary to drive anorexia and lethargy in the face of inflammatory challenge. Numerous cytokines are associated with sickness behavior and sufficient to drive specific inflammation-induced behavioral changes, but none has been isolated as the causative agent. Part of this is due to the amplifying nature of immune stimulation—that a single pro-inflammatory cytokine rapidly induces the production of several other cytokines. However, without knowing the identity of the essential inflammatory signal, it is difficult to investigate which regions are primarily responsive to this signal. Having defined neural mediators of these responses, future work can expand this circuit, both to define the downstream neural mediators of sickness behavior as well as to identify common upstream intermediates.

In summary, we have shown that modulation of hypothalamic neuron activity by inflammatory cytokines mediates the anorectic and lethargic responses to sickness. This work provides the first conclusive evidence that direct cytokine activation of ARC POMC neurons is necessary for the reduction of food intake by LIF. We have also identified a population of perifornical Ox neurons that are inhibited during inflammatory challenge by peripheral LPS or i.c.v. IL-1β, resulting in lethargy. Collectively, this work supports a vital role for the hypothalamus in mediating the behavioral responses to sickness and identifies new drug targets for the treatment of disease-associated anorexia and physical inactivity.

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APPENDIX

APPENDIX A

Supplemental Figures referred to in main text.

Figure 35. *LIF-R mRNA expression in brainstem and by ARC AgRP neurons in rat.*

Figure 36. *qRT-PCR examination of cytokine and neuropeptide mRNA in hypothalamic homogenates from FR v. ad-lib rats after LPS treatment.*

Figure 37. *LIF mRNA expression throughout forebrain 1 h after LPS administration.*

Figure 38. *Feeding response to LPS in PomcCre-gp130$^{flox/flox}$ mice.*

Figure 39. *Retrograde tract tracing in rats from DR to LHA/PFA.*

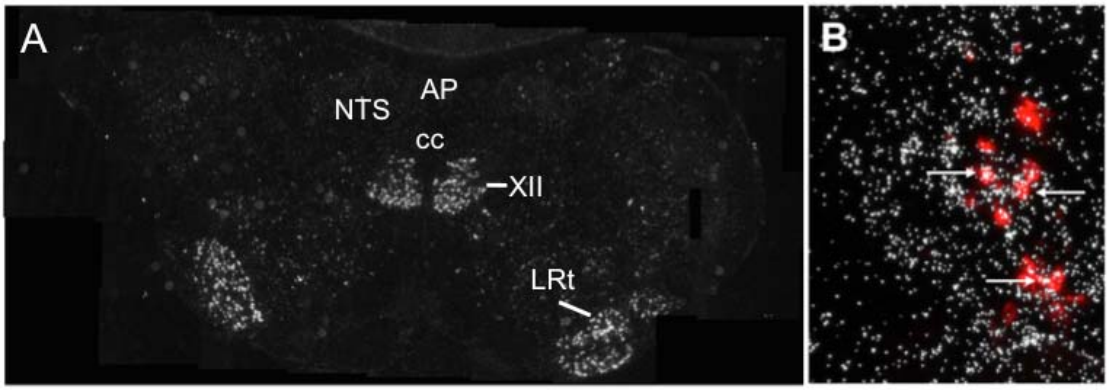

Figure 35. *LIF-R mRNA expression in brainstem and by ARC AgRP neurons in rat.*
(A) Single label *in situ* hybridization demonstrating LIF-R expression throughout rat brainstem, particularly in the nucleus of the hypoglossal nerve (XII) and the lateral reticular nucleus (LRt). The nucleus of the tractus solitarus (NTS) also exhibits LIF-R expression, though at a lower intensity. (B) High power photomicrograph of dual label *in situ* hybridization depicting the expression of LIF-R (*silver grains*) by ARC AgRP neurons (*red precipitate*). *Arrows* indicate cells expressing both LIF-R and AgRP*. AP,* area postrema; *cc,* central canal*.*

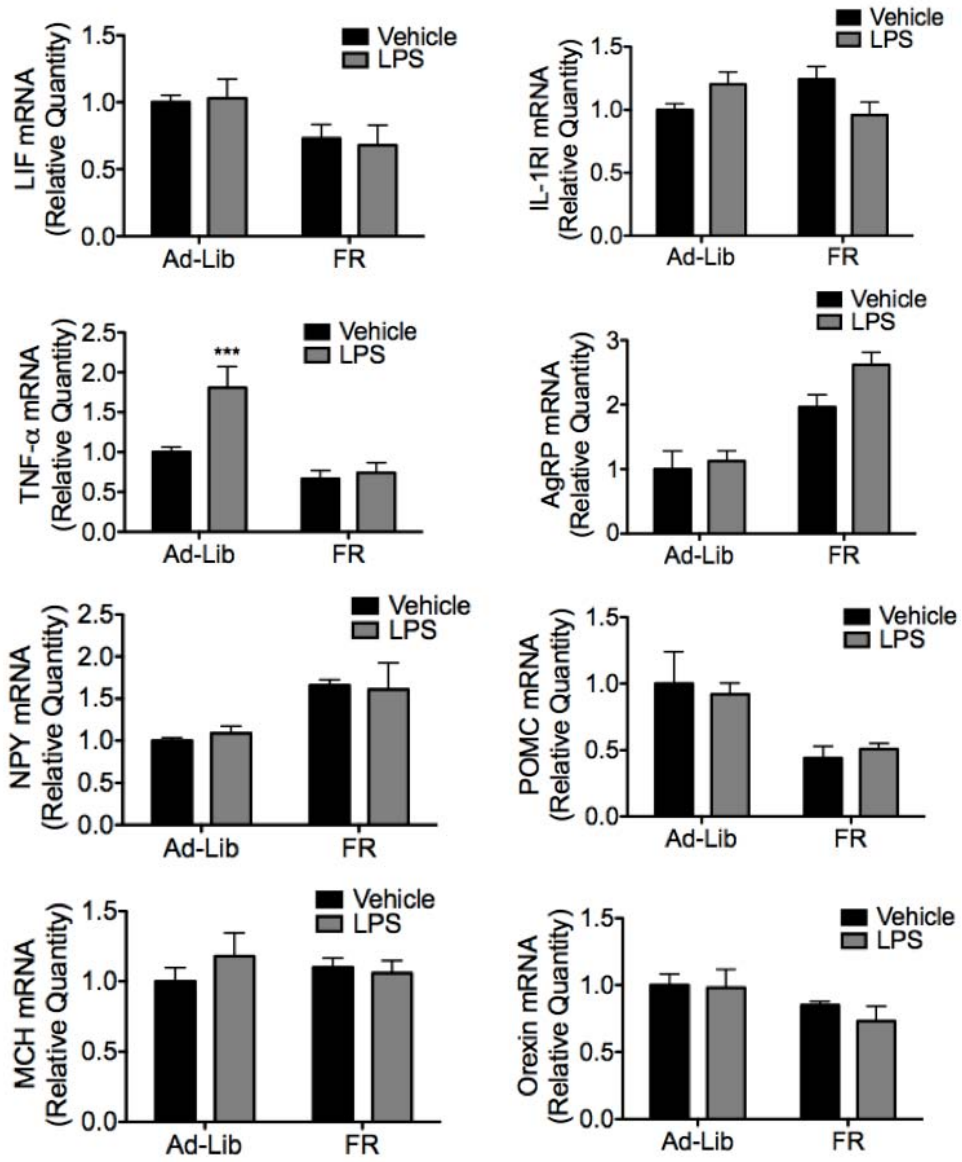

Figure 36. *qRT-PCR examination of cytokine and neuropeptide mRNA in hypothalamic homogenates from FR v. ad-lib rats after LPS treatment.*

Male Sprague Dawley rats were either fed *ad-libitum* or FR for 8 days prior to experiment. On experimental morning, rats were treated with i.p. LPS (250 μg/kg) v. vehicle and sacrificed 1 h later. All animals were perfused with RNase free PBS to remove blood before hypothalamic dissection. Data expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by post hoc analysis using a Bonferroni corrected *t* test (***, $P < 0.001$).

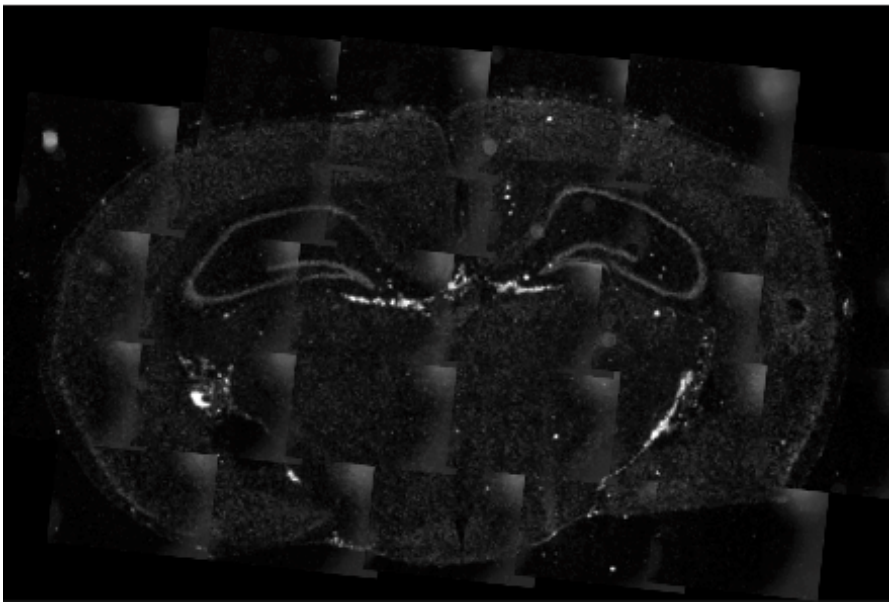

Figure 37. *LIF mRNA expression throughout forebrain 1 h after LPS administration.*
(A) Low power photomicrograph of single label *in situ* hybridization demonstrating LIF (*silver grains*) expression in rat forebrain following i.p. treatment with LPS (250 µg/kg). Expression is highest in the median eminance, meninges, and blood vessels throughout the forebrain. Little parenchymal LIF mRNA is observed.

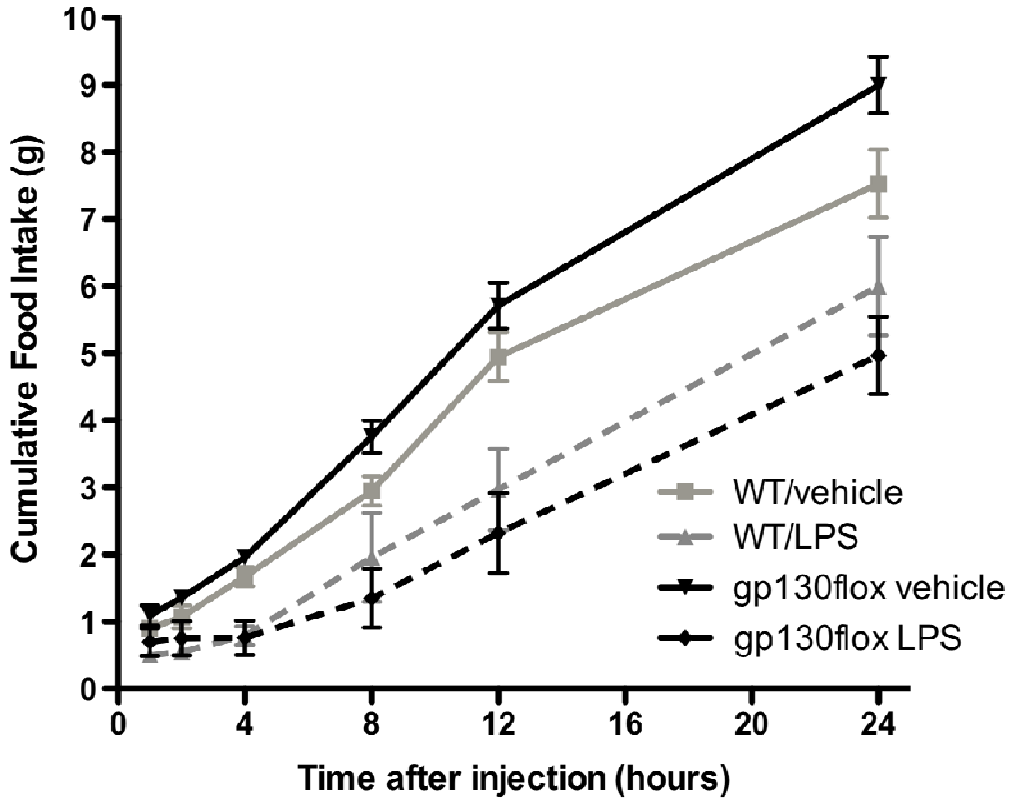

Figure 38. *PomcCre-gp130$^{flox/flox}$ mice show a normal anorectic response to LPS-induced systemic inflammation.* Animals were injected i.p. with LPS (100 µg/kg, n=4 per genotype) or vehicle (n=4 per genotype) and food intake was measured at 1, 2, 4, 8, 12, and 24 h post-injection. LPS reduced food intake significantly in both groups, though no effect of genotype was observed at any time point. Data are expressed as mean ± SEM. Statistics calculated by two-way ANOVA.

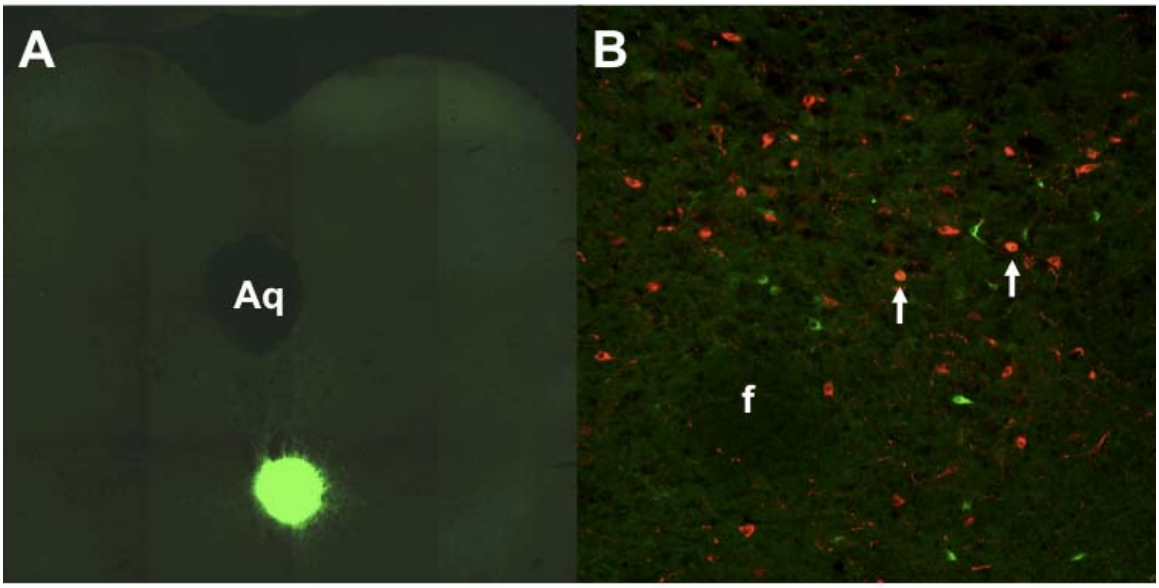

Figure 39. *Retrograde tract tracing in rats from DR to LHA/PFA.*
Male Sprague Dawley rats were iontophoretically injected with cholera toxin, subunit B (CTB) conjugated to AlexaFluor 488 in the DR (X=0; Y = -7.75; Z= - 6.3 mm from bregma). Glass pipettes pulled to a tip diameter of 15-20 µm. All injections were 45 min of +5.0 µA (5 s on/off) followed by pipette withdrawal with -0.5 µA for 1 min. Rats sacrificed 7 d after injection and processed for immunofluorescence. (A) Low power photomicrograph depicting injection site in DR. (B) Dual label immunofluorescence showing CTB-containing neurons (*green*) and OxA IR neurons (*red*) in the perifornical LHA. Few cells co-labeled for OxA and CTB. *Arrows* denote CTB+ cells that exhibit OxA IR. *Aq*, cerebral acqueduct; *f*, fornix.

APPENDIX B

Studies exploring the effects of chronic central LIF administration.

Background

Multiple studies demonstrate that chronic increases in central or circulating LIF result in a sustained anorexia and negative energy balance (186, 187). Our recent work showed that the direct activation of ARC POMC neurons mediates the acute anorectic effects of LIF (62). We therefore sought to develop a model of chronic LIF-induced anorexia and weight loss to evaluate whether the melanocortin system mediates the sustained metabolic effects of LIF, as well.

Study 1 – *Serial intracerebroventricular injection of LIF into WT mice.*

Materials & Methods

Twenty male WT mice had cannulae placed in their lateral ventricles (X=1.0; Y=-0.5; Z = -2.35 mm from bregma). Mice were allowed to recover for at least a week and were handled twice daily (0800 h and 1700 h) for 5 d before the experiment began. Starting on day 1 mice were randomly assigned to receive 1 µl i.c.v. injections of vehicle (PBS + 0.5% BSA; n = 10) or LIF (25 ng, n = 10; Santa Cruz Biotechnology) twice daily –

at 0800 h and 1700 h—for 7 d. Food intake was recorded daily. On the 7th day, mice were sacrificed by decapitation and hypothalami were dissected and stored in RNAlater for qRT-PCR analysis.

Results

Serial injections were associated with a very high risk of infection—4/10 vehicle treated animals and 7/10 LIF-treated animals had to be dropped from the study due to cannula infection. Of the remaining animals, LIF reduced cumulative food intake compared to vehicle (Figure 40A). Though the cumulative feeding effect was significant, LIF only significantly reduced daily intake on one day (Figure 40B). qRT-PCR analysis revealed a significant increase in the expression of POMC in LIF treated animals compared to control (Figure 40B). No change in AgRP, Ox, or MCH expression was observed. LIF induced hypothalamic expression of SOCS-3, IL-1β, and LIF.

Conclusions

Our data support previous reports suggesting that anorexia is sustained in response to chronic LIF administration. As previously illustrated, the magnitude of feeding inhibition is quite small (187), thus differences in cumulative food intake are likely a better measure of LIF-induced anorexia than daily food intake. This anorexia was associated with an increase in hypothalamic POMC expression, supporting our hypothesis that chronic LIF-induced anorexia is melanocortin dependent. This also suggests that LIF must activate the neuronal enhancers of POMC expression, in addition to the pituitary promoter (437, 498). Though we made every effort to dissect only hypothalami, we cannot rule out the possibility of pituitary contamination. LIF also

chronically induces the expression of itself, as well as IL-1β. Importantly, LIF retains its anorectic efficacy despite induction of SOCS-3. It is unclear whether this is due to the recruitment of other anorectic factors (such as IL-1β) or because LIF inhibits food intake in a SOCS-3 insensitive manner. We conclude that, although our data suggest that LIF may chronically induce anorexia by altering melanocortin signaling, serial injection is not a viable experimental paradigm to test this hypothesis. We suggest the central infusion of LIF using osmotic minipumps connected to brain infusion cannuale.

Study 2 – *Intracerebroventricular infusion of LIF into WT& MC4-R-KO mice.*

Materials & Methods

Fifteen WT (C57BL/6J) and 16 MC4-R-KO mice (7-10 weeks old) were implanted with brain infusion cannulae connected to osmotic minipumps implanted subcutaneously between the scapulae (Alzet Corp.). Animals were randomly assigned to receive LIF (1 μg/d; n=8 WT, 8 MC4-R-KO) vs. vehicle (n=7 WT, 8 MC4-R-KO). Food intake, body weight, and body composition were measured daily for 6 days. Mice were then sacrificed and hypothalami, gastrocnemius muscle, and brown adipose tissue were harvested and stored in RNAlater for subsequent qRT-PCR analysis.

Results and Conclusions

We failed to see any significant LIF-induced changes in body weight or food intake in either genotype, despite preliminary studies suggesting that this dose of LIF is effective at suppressing food intake for 6 d (Figure 41A). Our pilot study also demonstrated that LIF significantly induces hypothalamic SOCS-3, LIF, and IL-1β

expression. In the current study, we observed a statistically significant increase in SOCS-3 expression in both genotypes, but could not recapitulate the increased POMC expression measured using the serial injection paradigm in either genotype (Figure 41B). Importantly, our feeding data suggested that the animals were densensitizing to LIF infusion after 4 days. However, minipumps were implanted on three serial days following LIF dilution. Animals receiving LIF on each subsequent day exhibited a return to baseline food intake one day sooner (Figure 41C). It appears that LIF loses its efficacy over time when stored in solution at 37°C. Therefore, we suggest the central injection of LIF-expressing lentivirus or AAV to chronically administer the cytokine.

Study 3 – *Intra-3V injection of LIF-expressing lentivirus into WT mice.*

Materials & Methods

Twelve 9-week old male C57BL/6J mice will receive two (2) third ventricular injections of lentivirus expressing a murine LIF-IRES-ZsGreen construct or saline two weeks apart. Food intake and body weight monitored daily for 2 weeks, then weekly until 6 weeks. Animals sacrificed by perfusion/fixation with 4% paraformaldehyde and tissue processed for pSTAT3 IHC as described previously (62). Virus manufactured by OHSU West Campus Virus Core. mLIF virus titer 2.40 x 10^9 TU/ul, by RTPCR (~10^6 TU/ul by FACS). Pilot study using 1 injection found no difference in feeding behavior or body weight. Pilot study had two problems: 1) many injections missed 3V; 2) single 3V injection rather than 2 injections to boost virus dose.

Results

No difference in food intake or body weight between treatments was observed at any time point (Figure 42A). IHC demonstrated many infected periventricular ependymal cells and an increase in pSTAT3 IR in regions adjacent to infected cells. Virus did not infect cells distal to injection site, and no significant difference in pSTAT3 IR in the ARC was observed (Figure 42B).

Conclusions

Though we were able to infect some ependymal cells with 3V injections of our mLIF-expressing lentivirus, we were unable to induce anorexia. Very few cells were infected away from the injection site, indicating low viral titer or poor infection. Although pSTAT3 IR near infected cells suggests the production of functional LIF, the lack of increased pSTAT3 in the ARC suggests that the cytokine is not affecting neurons in this region. Given that previous studies have successfully induced chronic anorexia using central administration of high titer ($>10^{12}$) LIF-expressing AAV, we believe that our results stem from insufficient LIF generation in response to our lentivirus. Further studies should investigate chronic LIF using a similar model.

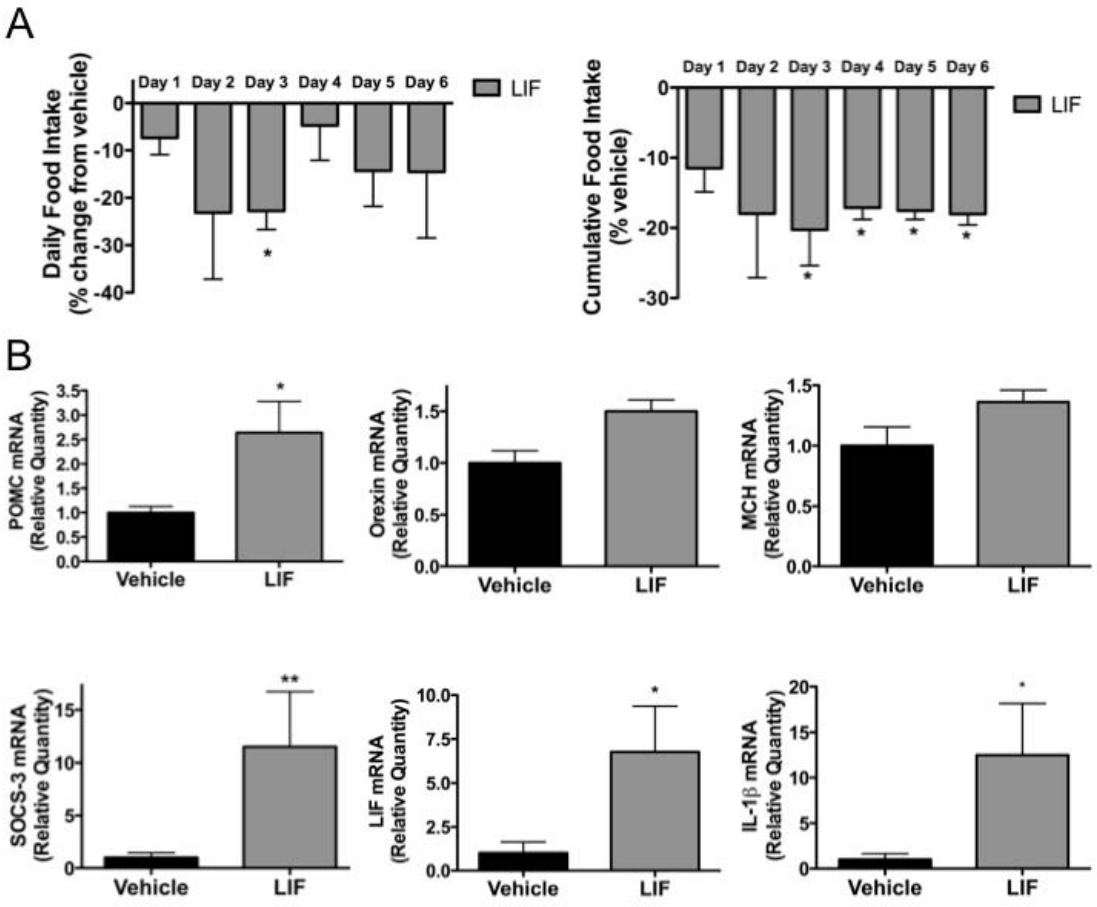

Figure 40. *The effects of twice daily i.c.v. LIF bolus injection on feeding behavior and hypothalamic gene expression in C57BL/6J mice.*

(A) Percent reduction in food intake each day or cumulatively in mice treated with LIF twice daily for 6 days compared to vehicle. (B) Relative mRNA levels of neuropeptides and cytokines following 6 days of LIF treatment compared to vehicle. Data expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by post hoc analysis using a Bonferroni corrected *t* test (A) or Student's *t* test (B) (*, $P < 0.05$; **, $P < 0.01$).

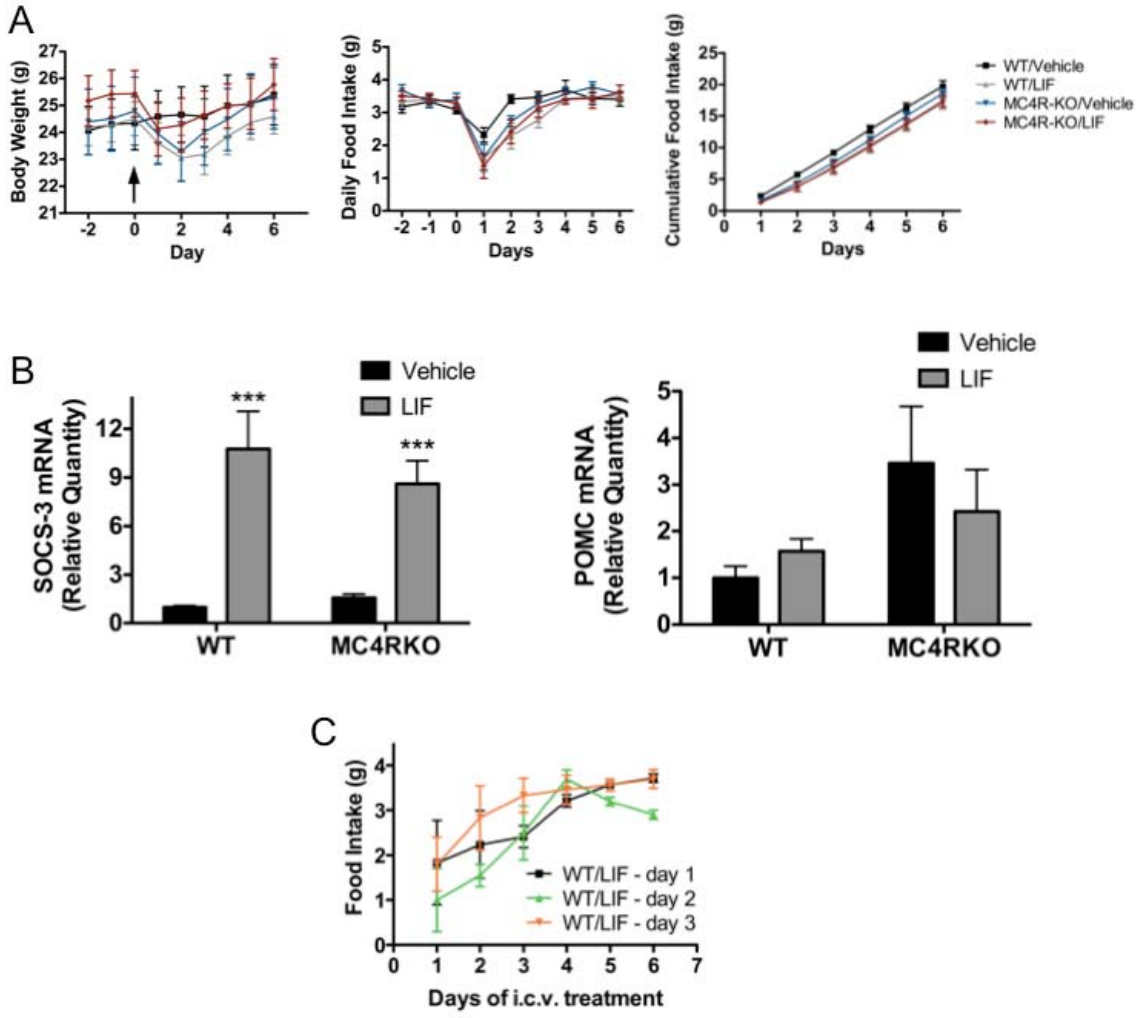

Figure 41. *The effects of chronic i.c.v. LIF infusion on feeding behavior, body weight, and hypothalamic gene expression in C57BL/6J and MC4R-KO mice.*

(A) Body weight, daily food intake, and cumulative food intake in WT and MC4R-KO mice receiving i.c.v. infusion of LIF v. vehicle. (B) Relative mRNA levels of SOCS-3 and POMC following 6 days of LIF treatment compared to vehicle. (C) Food intake in LIF-infused WT mice receiving pumps on first, second, and third day. Animals receiving pumps each subsequent day exhibit apparent desensitization one day sooner, indicating degradation of LIF. Data expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by post hoc analysis using a Bonferroni corrected *t* test (***, $P < 0.001$).

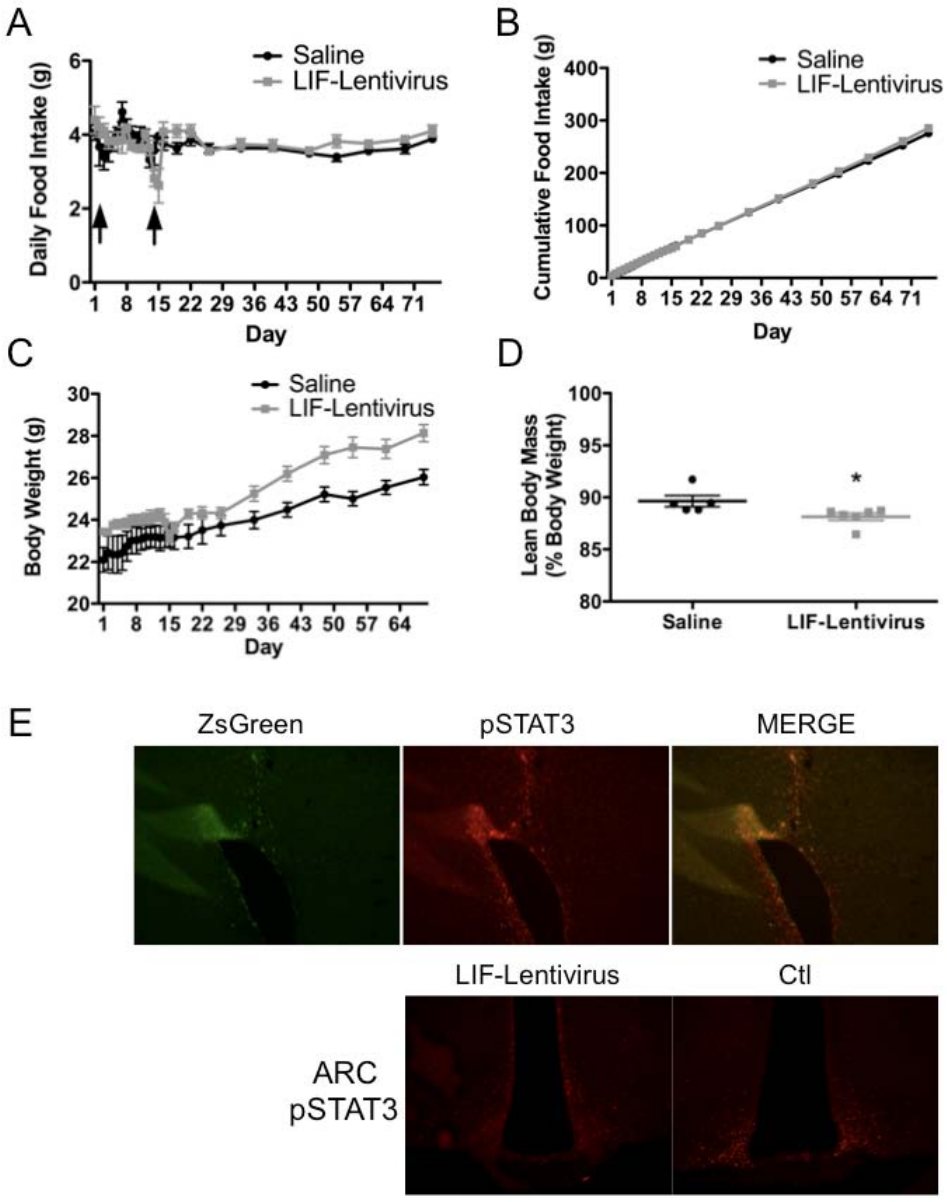

Figure 42. *Intra-3V administration of a LIF-expressing lentivirus failed to induce anorexia, loss of body weight or pSTAT3 IR in the ARC of WT mice.*

(A) Daily and (B) cumulative food intake in mice receiving LIF-lentivirus or saline injection. (C) Body weight and (D) lean body mass in WT mice injected with LIF-lentivirus or saline. (E) IHC demonstrating increased pSTAT3 IR in region near injection and in some ependymal cells. No difference in pSTAT3 IR was observed in the ARC of LIF-lentivirus v. saline-treated animals. Data expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by post hoc analysis using a Bonferroni corrected *t* test (A-C) or Student's *t* test (D) (*, $P < 0.05$).

APPENDIX C

Studies investigating the mechanisms of tumor-induced cachexia.

Background

Our laboratory and others have characterized a dramatic cachexia induced by the subcutaneous implantation of a syngenic methylcholanthrene-induced (MCA) sarcoma into F344/NTacfBR rats (464). Others and we have demonstrated that melanocortin antagonists can prevent cachexia in multiple disease models, suggesting that melanocortin signaling mediates the wasting response. This study will test whether MC4R blockade can ameliorate MCA sarcoma induced cachexia, as well.

Several studies report that prostaglandins mediate various aspects of the behavioral response to sickness (reviewed in (499)). Further, blockade of PG synthesis with indomethacin attenuates both tumor growth and the cachectic response to the murine MCG-101 sarcoma (500, 501). Our laboratory has focused on behavioral and metabolic effects of CNS inflammatory cytokines. In previous studies using the MCA sarcoma, we failed to observe increases in circulating IL-1β or TNF-α or hypothalamic inflammatory cytokine mRNA (unpublished data). However, in addition to severe anemia, these animals exhibited excessive extracellular fluid at sacrifice, which may be due to PG-

induced vascular permeability. This study was designed to investigate whether PG also mediate MCA sarcoma-induced cachexia.

Study 1: Evaluating whether melanocortin antagonism can reverse MCA sarcoma-induced anorexia and wasting.

Materials & Methods

Thirty male F344/NTacfBR rats were implanted with cannulae in the lateral ventricle (X=1.5 mm; Y=-1.0 mm; Z=-4.0 mm from bregma). One week after cannulation surgery, rats were implanted subcutaneously with ~1.2 g of MCA sarcoma (harvested fresh from a donor animal; n=16) or received a sham surgery (n=14). Tumor-bearing rats were randomly assigned to receive 4 nightly injections of AgRP (1 nmol; n=8) or aCSF (n=8) on d 10-13 just before lights-out. All sham animals received aCSF injection. All tumor-bearing rats and half of the sham animals (n=7) were allowed *ad-libitum* access to food. The remaining sham animals were pair-fed to the average food intake of the tumor bearing/aCSF-treated animals on the previous day. Body composition (by NMR), food intake, and body weight were recorded daily after surgery. At sacrifice, 14 d after tumor implantation, tumors were removed and weighed, blood was collected and hematocrit was assessed, and BAT was collected and stored in RNAlater for qRT-PCR analysis of UCP-1 expression.

Results

Both tumor-bearing groups gained significantly more weight during the experiment that sham-operated animals, though this weight gain was primarily

due to tumor growth (Figure 43A and 43D). Tumor implantation significantly reduced food intake on d 11-14 in aCSF-treated animals compared to ad-lib fed sham controls. AgRP treatment significantly restored food intake in tumor bearing animals on d 11-13, before AgRP lost its feeding effect on d 14 (Figure C1B). Lean body mass was reduced in both Tumor/aCSF and Sham/Pair-Fed animals, but not Sham/aCSF or Tumor/AgRP animals, though there were no significant differences between groups (Figure 43C). AgRP treatment improved food intake, but did not augment tumor size compared to aCSF treatment (Figure 43D). the expression of UCP1 in the BAT was slightly elevated in Tumor/aCSF animals compared to both groups of sham controls, though this effect did not reach statistical significance (Figure 43E). However, AgRP potently suppressed UCP1 expression in the BAT. Tumor bearing animals exhibited markedly lower hematocrit than sham operated controls (Figure 43F).

Conclusions

Melanocortin antagonism effectively restored food intake in tumor-bearing rats. On the final day of life, this effect was lost, however. It is important to note that these animals had reached end-stage disease at this point, as evidenced by their critically low hematocrit, and their lack of food intake may reflect their moribund state rather than tumor-induced cachexia. Though not statistically significant, the BAT UCP1 expression data, in concert with the feeding data and change in lean body mass suggest that the MCA sarcoma potentiates wasting in a melanocortin-dependent manner. Confirmation of this relationship should be conducted with a more conservative sacrifice point, as the health of tumor-bearing animals declined during the final day of the experiment. Despite restoring

energy intake, melanocortin antagonism did not augment tumor growth, refuting the hypothesis that increasing feeding will increase tumor burden.

Study 2: Determining whether MCA sarcoma-induced cachexia is PG dependent.

Materials & Methods

Eight male F344/NTacfBR rats were implanted with ~1.2 g of MCA sarcoma (harvested fresh from a donor animal). During tumor surgeries, rats were randomly assigned for subcutaneous implantation with osmotic minipumps (Alzet Corp.) filled with saline (n=4), or ketorolac (n=4), a COX inhibitor. All animals also were implanted with telemetry transponders (Mini-mitter, Inc.) subcutaneously between the scapulae. Body composition was measured by NMR on surgery day, and body weight, food intake, body temperature, and LMA were monitored for 13 d after tumor implantation. On d 13, animals were sacrificed and carcass body weight and composition was recorded. Tumors were dissected and weighed.

Results

Both saline- and ketorolac-treated tumor-bearing rats gained weight throughout the study, primarily during the logarithmic phase of tumor growth (d 10-13; Figure 44A). All animals exhibited marked reductions in food intake from d 11-13 (Figure 44B). Ketorolac did not attenuate the anorexia—rather ketorolac-treated rats ate less on their final day. This may not be due to any effect of ketorolac on food intake, as ketorolac-treated rats grew modestly larger tumors (Figure 44C; P=0.058), and previous studies demonstrate an inverse correlation between tumor size and food intake. Ketorolac did not attenuate muscle loss (Figure 44D) or significantly attenuate loss of lean body mass

(Figure 44E). No difference in loss of fat mass was observed between groups (Figure 44F). We did not observe any differences in LMA or body temperature between saline- or ketorolac-treated rats (Figures 44G and 44H).

Conclusions

Ketorolac does not attenuate any features MCA sarcoma-induced cachexia. Thus, PG unlikely mediate the catabolic actions of this tumor. The trend toward increased tumor size in ketorolac-treated animals may be due to a decrease in the synthesis of PG-dependent anti-tumor factors, but may simply be an irreproducible artifact of this experiment. Ketorolac treatment does improve food intake during the recovery from surgery, but does not improve tumor-induced anorexia. This is likely due to suppression of PG-mediated inflammatory factors in response to syngenic tissue transplant. Finally, this tumor model exhibits a dramatic decrease in food intake and LMA on the final 2 days of the experiment. This phenomenon is replicated in other experiments using this tumor and coincides with severe anemia, organ edema, and rapid decline in overall health. Therefore, it is difficult to assess whether the “chronic” changes in physiology and behavior that are elicited by this model are representative of sickness behavior in cancer patients. Alternative tumor models should be investigated for both the ability to induce sustained cachexia and sickness behavior.

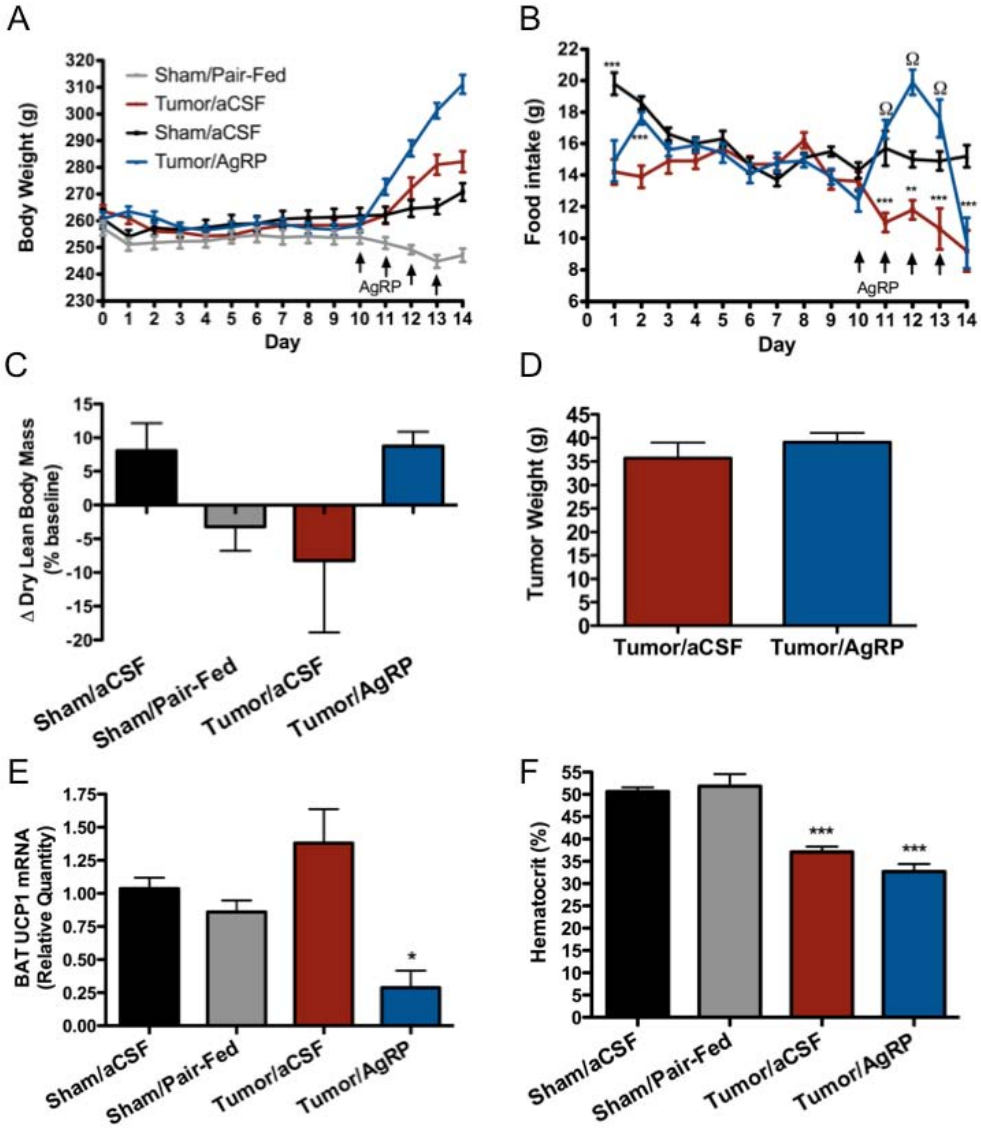

Figure 43. *AgRP reverses MCA sarcoma induced anorexia and lean body wasting.*
(A) Changes in body weight following implantation of MCA sarcoma. (B) Food intake in tumor-bearing and sham operated rats receiving i.c.v. injections of AgRP or aCSF. (C) Change in lean body mass, as a percent of baseline lean mass, in tumor-bearing and sham operated animals. (D) Tumor weight in AgRP v. aCSF treated rats. (E) BAT UCP1 mRNA levels in sham operated and tumor bearing rats following treatment with central AgRP or aCSF. (F) Hematocrit following sacrifice in tumor bearing and sham operated rats. *Arrows* denote the evenings with AgRP injections. Data expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by post hoc analysis using a Bonferroni corrected *t* test (B) or one-way ANOVA (C-F) (*, P<0.05 v. all other groups; **, P<0.01 v. Sham/aCSF; ***, P<0.001 v. Sham/aCSF; Ω, P<0.001 v. Tumor/aCSF).

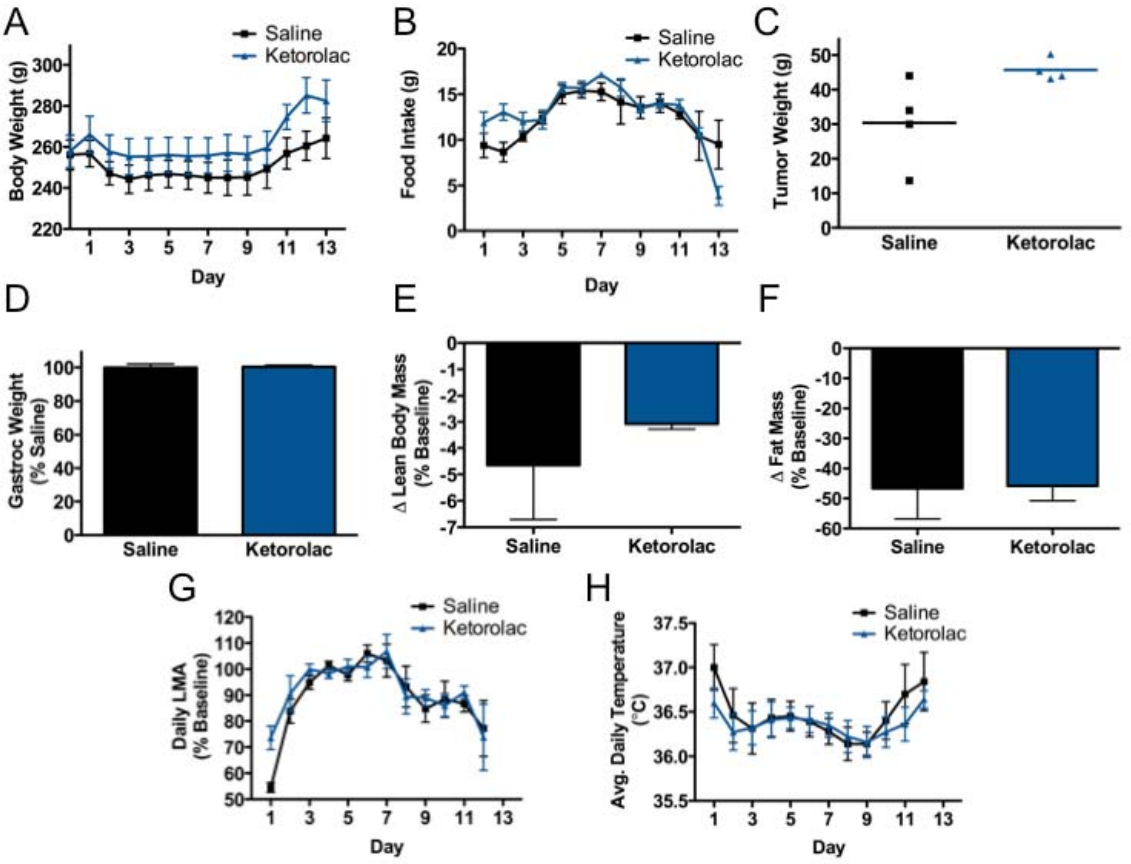

Figure 44. *Effect of COX inhibition on the sickness response to MCA sarcoma.*
(A) Ketorolac does not improve BW in tumor bearing rats. (B) Ketorolac treatment does not alter food intake in tumor bearing rats compared to control. (C) Tumor weight from ketorolac v. saline-treated tumor bearing rats. (D) Gastrocnemius weight of ketorolac v. saline-treated tumor bearing rats (expressed as % of saline-treated gastrocnemius weight). (E) Ketorolac does not significantly improve loss in lean body mass in MCA-sarcoma bearing rats. (F) Ketorolac does not alter loss in fat mass caused by tumor. (G) Ketorolac does not improve decrease in LMA due to MCA tumor burden. (H) Ketorolac-treated rats exhibited generally lower (but not significantly) average body temperature than saline treated animals. Data expressed as mean ± SEM. Statistics calculated by two-way ANOVA followed by post hoc analysis using a Bonferroni corrected *t* test (A, B, D, H) or Student's *t* test (C-F).