LESSONS FROM SLEEPY MICE: NARCOLEPSY AND THE OREXIN NEUROPEPTIDE SYSTEM

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DEDICATION

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LESSONS FROM SLEEPY MICE: NARCOLEPSY AND THE OREXIN NEUROPEPTIDE SYSTEM

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LESSONS FROM SLEEPY MICE: NARCOLEPSY

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The hypothalamic orexin neuropeptide system is a neuronal pathway regulating behavioral vigilance states and metabolic functions. Orexins activate the orexin receptors type 1 and type 2 (OX2R). Melanin-concentrating hormone (MCH) is an anatomically-related peptide that may have complementary functions. Orexin null (orexin$^{-/-}$) mice exhibit a behavioral and electroencephalographic phenotype similar to narcolepsy-cataplexy, a neurological disorder caused by orexin deficiency. Narcolepsy-cataplexy consists of inability to maintain wakefulness, abnormal intrusions of rapid-eye-movement (REM) sleep and related phenomena (i.e. cataplexy) into wakefulness, and poorly characterized metabolic

abnormalities. I demonstrate that both *OX2R*$^{-/-}$ and *orexin*$^{-/-}$ mice are unable to maintain wakefulness normally. In contrast, *OX2R*$^{-/-}$ mice are only mildly affected with abnormalities of REM sleep, whereas *orexin*$^{-/-}$ mice are severely affected. Thus, the profound dysregulation of REM sleep control unique to the narcolepsy-cataplexy syndrome emerges from loss of signaling through both OX2R-dependent and OX2R-independent pathways. Transgenic mice, in which orexin neurons are ablated, fail to respond normally to fasting with increased wakefulness and activity indicating that orexin neurons provide a crucial link between energy balance and arousal. *Orexin*$^{-/-}$ and *MCH*$^{-/-}$ mice have increased and decreased adiposity and susceptibility to diet-induced obesity, respectively. While *orexin*$^{-/-}$ mice exhibit sleepiness and cataplexy, *MCH*$^{-/-}$ mice are more wakeful than wild-type mice. *Orexin*$^{-/-}$;*MCH*$^{-/-}$ exhibit exacerbation of the narcolepsy phenotype, indicating that orexin and MCH complementarily regulate behavioral stabilty. Mice that ectopically overexpress orexin in the brain exhibit reduced body weight and resistance to diet-induced obesity, inability to maintain sleep, and reduced REM sleep with abnormal myoclonic activity relative to wild-type controls, providing further evidence that orexin alters homeostatic set-points of both energy metabolism and sleep/wakefulnuss. Modafinil, a stimulant used to treat narcoleptics, effectively increases wakefulness but does not suppress cataplexy in *orexin*$^{-/-}$ mice, indicating that orexin is not required for the wake-promoting action of the drug. By contrast, expression of an orexin transgene in the brain completely prevented cataplectic arrests and other abnormalities of REM sleep in the absence of endogenous orexin neurons. Thus, orexin neuron-ablated mice retain the ability to respond to orexin neuropeptides and orexin receptor agonists would likely contribute to treating human narcolepsy.

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Ascending cortical activating system (ACAS) – System of neurons in the brainstem and basal forebrain that are most active during wakefulness and drive desynchronized cortical activity directly and via thalamic relays.

Arousal – See Wakefulness.

Brainstem muscle tone suppression system – Set of neurons in pons and medulla that mediate inhibition of spinal cord alpha-motor neurons during sleep and, under some condidions, in response to emotional stimuli.

Cataplexy – Abrupt onset of muscle atonia during wakefulness. Typically triggered by emotion and conceptualized as an abnormal manifestation of REM sleep atonia. Cataplexy is a very specific symptom of narcolepsy-cataplexy.

Electroencephalography – Neurophysiologic examination of electrical activity of the brain by the application of electrodes to the scalp or brain surface. The resulting traces are known as an electroencephalogram (EEG) and represent so-called brainwaves. The characteristics of the EEG can be used to discriminate normal behavioral states such as stages of sleep and wakefulness or to discriminate abnormal patterns associated with seizures.

Hypnogogic hallucinations – Intrusion of dream-like REM sleep phenomena at the onset of sleep with resultant hallucination. It is a relatively non-specific feature of narcolepsy-cataplexy that may occur at the same time as sleep paralysis.

Hypnopompic hallucinations – Intrusion of dream-like REM sleep phenomena at the offset of sleep with resultant hallucination. It is a relatively non-specific feature of narcolepsy-cataplexy that may occur at the same time as sleep paralysis.

Hypocretins – See Orexins.

Knockout mice – Line of genetically engineered laboratory mice in which a gene of interest has been deleted or replaced with a mutant gene, resulting in loss of function of the gene of interest.

Lateral hypothalamic area (LHA) – Reticular region of the dorsal and lateral regions of the hypothalamus that is classically associated with regulation of feeding behavior, metabolic rate, and arousal in response to various stimuli. Intrinsic neurons project throughout the neuroaxis, and it also contains important fibers of passage including the median forebrain bundle and the fornix. The perifornical nucleus is often considered to be part of the LHA.

Limbic system – System of forebrain and brainstem structures with complex interactions that play an important role in emotion, memory, and behavior.

Melanin-concentrating hormone (MCH) – Neuropeptide product of the *prepro-mch* gene that is expressed in the lateral hypothalamic area and contiguous zona incerta and that activates a single G protein-coupled receptor, melanin-concentrating hormone receptor, in mice. It was originally purified from the skin of teleost fish.

Narcolepsy-cataplexy – Primary neurological sleep disorder with the symptom tetrad of inability to maintain wakefulness (sleep attacks), cataplexy, sleep paralysis, and

hypnogogic/hypnopompic hallucinations. It typically results from absence or disfunction of orexin (hypocretin) neuropeptides or orexin receptors.

Neuropeptides – Peptides acting as neurotransmitters or hormones that are released by neurons or neuroendocrine cells and mediating slow activity via activate G-protein coupled receptors.

Non-rapid-eye movement (non-REM) sleep – Restful stage of sleep associated with synchronized cortical activity, low muscle tone, and delta rhythms in the electroencephalogram (EEG).

Orexins – Neuropeptides (orexin A and orexin B, also called hypocretins A and B) expressed in the lateral hypothalamic area, especially the perifornical nucleus. Products of the *prepro-orexin* gene activate two G protein-coupled receptors, orexin receptors type 1 and type 2, in mice and humans. Orexins (hypocretins) were originally deduced from genes expressed specifically in hypothalamus and purified biochemically as agonists of orphan G protein-coupled receptors.

Perifornical nucleus – Cluster of neurons in the LHA that surround the fornix and have been implicated functionally in arousal and autonomic regulation. The majority of orexin neurons are found in the perifornical nucleus in mice and humans.

Pontine REM sleep generator – Poorly characterized set of neurons that are active during REM sleep and drive desynchronized cortical activity during this state.

Rapid-eye-movement (REM) sleep – Also called paradoxical sleep. Stage of sleep associated with desynchronized cortical activity, vivid dreaming, muscle atonia, and theta rhythms in the electroencephalogram (EEG).

Sleep attack – Manifestation of inability to maintain wakefulness in narcolepsy-cataplexy or other sleep disorders. It is percieved by the subject as an uncontrollable onset of sleep, and it may be related to either non-REM sleep or REM sleep.

Sleep paralysis –A relatively non-specific symptom of narcolepsy-cataplexy and occurring at the onset of offset of sleep in which a person is alert to surroundings but unable to move due to residual REM sleep atonia. It may occur at the same time as hypnogogic or hypnopompic hallucinations.

Transgenic mice – Line of genetically engineered laboratory mice in which additional copies of a gene of interest have been inserted at a random location in the genome, resulting in gain of function of the gene of interest.

Wakefulness – Also referred to as arousal. State of alert cortical activity associated with maximal responsivity to external stimuli and maximal muscle tone with locomotor activity.

CHAPTER ONE
Introduction and Historical Perspective

HOMEOSTATIC FUNCTIONS OF THE LATERAL HYPOTHALAMIC AREA

Feeding behavior is dependent upon the integration of metabolic, autonomic, endocrine and environmental factors coordinated with an appropriate state of cortical arousal (wakefulness). Historically, the hypothalamus has been recognized to play a critical role in maintaining energy homeostasis by integrating these factors and coordinating the behavioral, metabolic, and neuroendocrine responses. In mammals, the neurons of the lateral hypothalamic area (LHA) have been historically recognized as particularly important for feeding and behavioral arousal (Bernardis and Bellinger, 1993; Bernardis and Bellinger, 1996). Animal models with lesions of the LHA exhibit hypophagia, an increased metabolic rate and decreased arousal that frequently leads to death by starvation. Furthermore, they consistently fail to respond to homeostatic challenges such as fasting with appropriate adaptive behavioral and physiologic responses. Therefore, the LHA has classically been regarded as the hypothalamic "feeding center" and as an important component of the autonomic nervous system with extensive projections within the hypothalamus and throughout the entire neuroaxis (Saper, 2002; Saper et al., 2002). With the ability to influence nuclei throughout the central nervous system, the LHA appears to be anatomically well placed to coordinate the metabolic, motivational, motor, autonomic, and arousal processes necessary to elicit environmentally appropriate feeding-related behaviors.

Until recently, the neuropeptide melanin-concentrating hormone (MCH) was the only signaling factor implicated in feeding regulation known to be produced almost exclusively within the LHA. MCH dose-dependently increases food consumption when administered centrally in rodents. Genetic disruption of the MCH gene in mice results in hypophagia and reduced body weight compared with wild-type littermates (Shimada et al., 1998). With the discovery of orexin neuropeptides A and B (Greek: *Orexis* = appetite), two more neuropeptides from the LHA have been identified that mediate feeding behavior and are likely to play a significant role in energy homeostasis, as well as other increasingly recognized lateral hypothalamic functions, such as arousal and behavioral state control.

THE OREXIN SIGNALING PATHWAY

Orexin Neuropeptides

Our group isolated orexin-A and orexin-B while screening high-resolution high-performance liquid chromatography fractions from brain extracts for stimulation of signal transduction in cell lines expressing orphan G-protein-coupled receptors (GPCRs) (Sakurai et al., 1998a). Mammalian orexin-A is a 33 amino-acid peptide of 3562 Da with an N-terminal pyroglutamyl residue, C-terminal amidation (both typical of neuropeptides), and two sets of intrachain disulfide bonds (**Figure 1-1**). The primary structure of orexin-A is completely conserved among human, rat, mouse, cow, and pig genera (Dyer et al., 1999; Sakurai et al., 1998a). Mammalian orexin-B is a 28-amino acid, C-terminally amidated peptide of 2937 Da with

46% (13/28) amino acid identity to the orexin-A sequence. Mouse and rat orexin-B peptides are identical, but human orexin-B has two amino acid substitutions compared with the rodent sequences. Orexins have also been cloned in the amphibian *Xenopus laevis* and found to have a high amino acid identity with their mammalian counterparts, especially at the carboxyl terminus (**Figure 1-1**) (Shibahara et al., 1999).

A single gene composed of two exons and an intervening intron encodes the orexin neuropeptides. This structure is conserved within rodent and human genomes (Sakurai et al., 1998b; Sakurai et al., 1999). *prepro-orexin* cDNAs encode 130-residue and 131-residue polypeptides in rat and human neurons, respectively. These polypeptides have typical secretory signal sequences and are cleaved to form mature orexin-A and orexin-B peptides that are post-translationally modified as neuropeptides.

A messenger RNA encoding the same neuropeptide precursor was independently isolated from a hypothalamus-enriched cDNA library by using a differential cloning approach, and the putative encoded peptides were named hypocretins (de Lecea et al., 1998). Nucleotide sequence alignment shows that the base-pair sequences of hypocretins-1 and -2 are the same as orexins-A and -B, but that the mature peptides predicted in this report had additional amino acids not found in native orexins. The hypocretin peptides, synthesized according to sequences predicted by de Lecea and co-workers (de Lecea et al., 1998), are markedly less potent agonists compared with orexins on transfected cells expressing human orexin receptors (Smart et al., 2000). The hypocretins were named for the limited identity of hypocretin-2 (orexin-B) with the gut hormone secretin, but native orexin peptides are, in fact, distantly similar to the bombesin neuropeptide

family and not the secretin family (**Figure 1-1**). Nevertheless, both names are used interchangeably in the literature.

Orexin Receptors

Our group identified two orexin receptor subtypes, named orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R) that are structurally similar to other G- protein-coupled neuropeptide receptors (Sakurai et al., 1998b). The OX1R is the orphan G-protein-coupled receptor used during ligand hunting to first identify and then purify the orexins. A search of the GenBank dbEST database with the OX1R amino acid sequence revealed two candidate ESTs. Using PCR, with primers designed from these ESTs, the OX2R receptor was discovered and found to have a 64% amino-acid identity with OX1R. Competitive radio-ligand binding assays reveal that the orexin receptors have different binding profiles for the respective orexin peptides. The OX1R has a 1-order-of-magnitude greater affinity for orexin-A [50% Inhibitory Concentration (IC_{50} = 20 nM)] compared with orexin-B (IC_{50} = 250 nM). In contrast, orexins-A and -B bind the OX2R with equal affinity (IC_{50} = 20 nM). Therefore, it appears that the OX1R is moderately selective for orexin-A, whereas OX2R is a non-selective receptor for both neuropeptide agonists. Evidence from receptor-transfected cell lines and isolated receptor-expressing hypothalamic neurons suggest that the OX1R is coupled exclusively to the G_q subclass of heterotrimeric G proteins, whereas OX2R may couple to $G_{i/o}$, and/or G_q (Sakurai et al., 1998b; van den Pol et al., 1998). To date, orexin peptides have exhibited mostly directly neuroexcitatory activities in a variety of reports utilizing different methods (Sutcliffe and de Lecea, 2002).

Neuroanatomy of the Orexin System

In the rodent CNS, orexin-producing cells (**Figure 1-2**) are a small group of neurons restricted to the lateral and posterior hypothalamus and perifornical areas (Peyron et al., 1998; Sakurai et al., 1998b). Despite their highly restricted origin, immunohistochemistry studies using orexin antibodies have shown that orexin neurons project widely throughout the entire neuroaxis. Particularly abundant projections are those found in the cerebral cortex, olfactory bulb, hippocampus, amygdala, septum, diagonal band of Broca, bed nucleus of the stria terminalis, thalamus, anterior and posterior hypothalamus, midbrain, brainstem, and spinal cord (Date et al., 1999; Nambu et al., 1999; Peyron et al., 1998; van den Pol, 1999). Orexin immunoreactivity is also reported in the enteric nervous system and pancreas (Kirchgessner and Liu, 1999), and *orexin* mRNA expression has been found in the testes (Sakurai et al., 1998b).

In situ hybridization studies with orexin receptor riboprobes demonstrate that orexin receptors are expressed in a pattern consistent with orexin projections, but that they have a marked differential distribution (Marcus et al., 2001; Trivedi et al., 1998). *OX1R* mRNA is highly expressed in the prefrontal cortex, hippocampus, paraventricular thalamus, ventromedial hypothalamus (VMH), arcuate nucleus (ARC), dorsal raphe nucleus, and locus coeruleus (LC). *OX2R* mRNA is found in the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, dorsal and median raphe nuclei, and many hypothalamic nuclei including the tuberomammillary nucleus (TM), dorsomedial hypothalamus (DMH), paraventricular hypothalamic nucleus (PVN), and ventral premammillary nucleus. Orexin receptor mRNA expression has also been reported in the adrenal gland (Malendowicz et al., 1999), enteric nervous system, and pancreas (Kirchgessner and Liu, 1999).

Intracerebroventricular (i.c.v.) injection studies of orexin neuropeptide using Fos as an immunohistochemical marker of neuronal activation demonstrated that the distribution of neurons activated by either orexin-A or orexin-B is similar (Date et al., 1999). The pattern of Fos immunoreactivity is consistent with orexin immunohistochemistry studies and orexin receptor in situ hybridization studies. Areas with the strongest activation include the arcuate nucleus (ARC), PVN, supraoptic nucleus, paraventricular thalamic nucleus, LC, central gray, dorsal raphe, nucleus of the solitary tract, dorsal motor nucleus of the vagus, and suprachiasmatic nucleus. However, no conclusions regarding direct receptor-specific activation can be derived from this study. Furthermore, these studies should be interpreted cautiously since nuclei in close proximity to the ventricular space would be preferentially activated from ventricular delivery, and no conclusions regarding possible orexin-mediated inhibition of neuronal pathways were obtained. The potential importance of orexin-mediated inhibition, at least within parts of the hypothalamus, is confirmed by the work of van den Pol et al. (van den Pol et al., 1998). These investigators found that orexins increase the release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), as well as the excitatory neurotransmitter glutamate, by acting directly on axon terminals of neuroendocrine cells in the ARC nucleus.

Orexin neurons produce glutamate and may also express the orexigenic opioid dynorphin (Chou et al., 2001), the secretory marker secretogranin II (Risold et al., 1999), and angiotensin II (Hara et al., 2001); and the biosynthesis of some of these peptides may be similarly regulated (Griffond et al., 1999). Immunoreactivity for the appetite stimulating neuropeptide galanin has also been identified in orexin neurons (Hakansson et al., 1999). MCH neurons, like orexin neurons, are found in the LHA and project diffusely throughout the entire neuroaxis. However,

definitive immunohistochemistry studies have demonstrated that orexin and MCH neurons are distinct and independent neuronal populations within the LHA (Broberger et al., 1998; Elias et al., 1998). Notably, however, the similarity of projection patterns of orexin and MCH neurons as well as the overlapping distributions of the expression of receptors for these neuropeptides indicates that these systems may be functionally related (Kilduff and de Lecea, 2001).

OREXINS MAINTAIN WAKEFULNESS

While a few neuroanatomical studies had previously predicted a potential role of orexins in modulation of arousal or attentional processes (among other disparate functions), recognition of the primary importance of the orexin system in maintenance of wakefulness came after the publication of results described in part in **Appendix A**. Due to the rapid development of the the field and the numerous publications exploring this primary function of orexins since that time, I review many of these studies in detail below as they are of critical importance to the directions pursued in later chapters. Experiments presented in **Chapters 2-7** further dissect the genetic and physiological regulation of sleep and wakefulness processes by orexins.

The Neurobiology of Mammalian Sleep and Wakefulness States

The need for sleep remains a fundamental mystery of neuroscience. While the cortical activity associated with wakefulness is clearly prerequisite for life-sustaining behaviors such as the acquisition and consumption of food, sexual reproduction, etc., the need for rest or sleep in

animals has provoked many hypotheses about its ultimate function. Sleep could be said to be " by the brain, of the brain,and for the brain", an idea summarizing many pututive functions of sleep and its central role in processes of learning and memory, synaptic plasticity, and brain energy homeostasis. Yet sleep plays important complementary roles in peripheral physiological functions such as growth, energy, and endocrine homeostasis as well. The regulation of sleep-wakefulness cycling within the context of circadian and environmental influences is critical for the efficient maintenance of both peripheral and central energy homeostasis.

In mammals, the sleep-wake cycle is traditionally divided into three states based on distinct behavioral and neurophysiologic characteristics (Jones, 1998). Wakefulness is characterized as a behaviorally interactive state, with full consciousness that is associated with low-voltage mixed-frequency fast activity on electroencephalography (EEG), purposeful eye movements, and high muscle tone with phasic motor activity. It is associated with high neuronal activity of the ascending cortical activating system (ACAS), a diffuse collection of specific monoaminergic and cholinergic structures in the basal forebrain, hypothalamus, and brainstem that drive cortical desynchrony directly and via thalamic relays (McGinty and Szymusiak, 2001; Saper et al., 2001; Steriade and McCarley, 1990)..

Decreased activity of this system and increased activity in the ventral lateral preoptic hypothalamus (VLPO) is associated with gradual progression from wakefulness to non-REM sleep. Non-REM or slow-wave sleep is behaviorally quiet with progressively reduced mental activity and responsiveness to environmental stimuli associated with high-voltage "slow wave" activity on EEG (including delta waves of 1-4 Hz and sleep spindles of 12-14 Hz), generally absent eye movements, reduced muscle tone and phasic motor activity.

In contrast, REM sleep, a behaviorally active state, normally occurs after a prolonged period of non-REM sleep. In addition to rapid eye movements of unknown purpose, REM sleep is associated with absence of tonic postural muscle tension (REM sleep atonia) despite frequent muscle twitches in the extremities. REM sleep is further associated with vivid dreaming, and the EEG is similar to that recorded during attentive wakefulness with low-voltage fast activity rhythms of low amplitude and mixed frequency. These may be distinguished from wakefulness by a particular dominance of hippocampal theta rhythms in some species (especially rodents in which the hippocampus is located in close proximity to the cerebral cortical surface). Despite EEG similarities to the wakefulness state, responsiveness to environmental stimuli is even further reduced as compared with non-REM sleep. Maintenance of REM sleep is associated with activity of subsets of VLPO neurons and cholinergic and cholinoceptive neurons in the mesopontine tegmentum (MPT), as well as inactivity of wake-related monoaminergic structures. Normal sleep is characterized by an orderly progression from wakefulness to non-REM sleep and then to REM sleep.

Orexin, Sleep, and Narcolepsy

A role for LHA in sleep regulation was suggested by early animal experiments that showed electrical stimulation of the LHA promoted wakefulness while destruction of the LHA caused somnolence, and disorganized sleep/wakefulness patterns (Bernardis and Bellinger, 1996; Danguir and Nicolaidis, 1980; Devenport and Balagura, 1971; Levitt and Teitelbaum, 1975). While most sleep-wakefulness studies have centered on the anatomically-related posterior hypothalamic nuclei including the histaminergic neurons of the tuberomammillary nucleus

(TMN), more recent studies have identified posterior lateral hypothalamic neurons whose firing rates vary with the sleep-wakefulness cycle (Steininger et al., 1999). Sleep studies in rats found that Fos immunoreactivity in orexin neurons is positively correlated with wakefulness and negatively correlated with the amount of non-REM and REM sleep, suggesting that these neurons are "waking-active" (Estabrooke et al., 2001). They further demonstrated that orexin neuronal activity appears to be under strong circadian control since the temporal relationship with wakefulness and the usual onset of the dark phase is preserved even under conditions of constant darkness. Fos is also increased after mild sleep deprivation produced by gentle handling, and after administration of wake-promoting stimulants such as modafinil and amphetamine (Chemelli et al., 1999; Estabrooke et al., 2001). I.c.v. administration of orexin-A in rodents dose-dependently increases wakefulness and suppresses non-REM and REM sleep providing further evidence that orexins play a causative role in sleep-wakefulness regulation (Hagan et al., 1999). Orexin i.c.v. injection in rats is also associated with behavioral changes indicative of an aroused state including increased locomotor activity, rearing, grooming, burrowing, searching behaviors, and food consumption (discussed below) (Hagan et al., 1999; Ida et al., 1999; Sakurai et al., 1998b).

Two ground-breaking reports highlight the importance of orexin signaling in the promotion and consolidation of wakefulness and linked this system to human disease. Our group discovered that orexin neuropeptide knockout mice have a phenotype remarkably similar to the human sleep disorder narcolepsy-cataplexy (Chemelli et al., 1999). Several results from this critical study are presented in **Appendix A**. And, in a complementary but independent study, Mignot and colleagues found that the genetic defect in a narcoleptic dog model is found in the *OX2R* gene (Lin et al., 1999).

Primary (idiopathic) narcolepsy-cataplexy is a debilitating neurological disorder that provides a unique perspective on the mechanisms of sleep/wake control. Narcolepsy-cataplexy is a lifelong neurologic disease characterized by excessive daytime sleepiness, cataplexy and other pathologic manifestations of REM sleep. Narcoleptic patients experience sleepiness that is constant and severe, often complaining of involuntary or irresistible daytime "sleep attacks" that can occur while talking, standing, walking, eating, or driving. Cataplexy consists of attacks of sudden bilateral skeletal muscle weakness, often provoked by strong emotion, without impairment of consciousness or memory, that last no more than a few minutes (Honda, 1988). Sleep paralysis and sleep-associated hallucinations are also experienced by some. The full tetrad of daytime somnolence, cataplexy, sleep paralysis and sleep-associated hallucinations is present in only about 15% of patients (Aldrich, 1998). Of these symptoms, cataplexy is the most specific marker of the syndrome in humans. The symptoms of narcolepsy-cataplexy are believed to result from two underlying problems: (1) inability to maintain wakefulness, and (2) intrusion of features of REM sleep into wakefulness or at sleep onset (Taheri et al., 2002). Clinically, the diagnosis is confirmed by polysomnography, a technique that employs simultaneous recording of EEG and the electromyogram (EMG). This procedure reveals "sleep-onset REM periods," REM sleep occurring at sleep onset or within 15 min of sleep onset (Aldrich, 1998). Treatment regimens, which are of limited effectivenesscurrently rely upon amphetamine-like stimulants or the atypical stimulant modafinil to treat excessive daytime sleepiness. Additionally, tricyclic antidepressants are utilized to reduce cataplexy.

Narcolepsy affects males and females equally with an estimated prevalence of 0.02%-0.18% within caucasian populations (Mignot, 1998). Familial narcolepsy, although rare, is reported in the literature, with risk to first degree relatives estimated at 1-2%. Studies of monozygotic twins using strict diagnostic criteria have found concordance rates of only 25-31%, leading most authors to conclude that undefined environmental factors act on a susceptible genetic background to produce the disease. Several studies have reported a strong association between certain class II HLA haplotypes on human chromosome 6 and narcolepsy. HLA DQB1*0602 and DQA1*0102 are found in up to 90% of affected populations, compared with 12-38% in the general population, suggesting that autoimmunity plays a role in the disorder (Kadotani et al., 1998). However, extensive studies over the past two decades have failed to find convincing evidence of a systemic autoimmune process or one restricted to the central nervous system in narcoleptic patients. Moreover, patients with familial narcolepsy usually lack these HLA risk factors (Mignot, 1998). Extensive gross and microscopic examination of narcoleptic brains as well as sophisticated in vivo proton spectroscopy studies have failed to identify evidence of a structural or biochemical brainstem lesion in idiopathic narcolepsy (Ellis et al., 1998).

Several other studies have since confirmed the contribution of disrupted orexin signaling to the etiology of human narcolepsy. After a preliminary report in which it was found that orexin-A was undetected in the cerebrospinal fluid (CSF) of 7 of 9 narcoleptic patients, but readily and consistently detected in normal controls (Nishino et al., 2000b), other clinical studies followed. It is now clear that >95% of cases of narcolepsy-cataplexy, but not narcolepsy without cataplexy or other sleep and neurological disorders, is associated with low or undetectable levels of orexin-A in CSF and in LHA cells, confirming the specific role that loss of orexin-producing neurons plays in

this syndrome (Kanbayashi et al., 2002; Mignot et al., 2002a; Peyron et al., 2000; Ripley et al., 2001b; Thannickal et al., 2000). Furthermore, an unusually severe, early onset case of human narcolepsy-cataplexy is associated with a mutation in the secretory signaling sequence of the *orexin* locus (Peyron et al., 2000). In support of a critical function of orexins being to maintain wakefulness, it was found that detection of extracellular orexin peptide levels in brains of normal animals by microdialysis indicates that orexin levels accumulate during wakefulness and decline with the occurrence of sleep (Kiyashchenko et al., 2002; Yoshida et al., 2001).

Prior to the discovery of orexins, investigations into the neurologic basis of narcolepsy-cataplexy had been greatly facilitated by the discovery of canine narcolepsy. Although sporadic cases have been reported in a variety of breeds, only two colonies of narcoleptic Doberman Pinschers and Labrador retrievers were successfully established to transmit a candidate narcolepsy gene, previously designated *canarc-1*, as an autosomal recessive trait with full penetrance (Baker et al., 1982). Similarities between human and canine narcolepsy include emotionally triggered cataplexy, increased daytime sleepiness, pathological manifestations of REM sleep, sleep-onset REM periods on polysomnography, an early age of onset, and a familial tendency. Extensive studies of the neuropharmacology and neurochemistry of affected Dobermans and humans have found a subtle disturbance involving hyperactivity of cholinergic, and hypoactivity of monoaminergic, neurotransmitter systems (Nishino and Mignot, 1997). These studies had collectively led to the hypothesis that "the pathophysiology of narcolepsy involves minute abnormalities of the neurochemical mechanisms regulating sleep rather than a localized lesion or an obvious developmental abnormality in the CNS" (Nishino et al., 1995).

A ten-year positional cloning effort to find the canine narcolepsy gene identified defects in the *OX2R* gene as the cause of autosomal recessive narcolepsy phenotypes in Dobermans and Labradors (Lin et al., 1999). Emmanuel Mignot's group at Stanford indentified intron deletions in the canine gene that cause defective mRNA splicing and consequent production of non-functional OX2R receptors. This would appear to suggest that the orexin-OX2R pathway is paramount in the regulation of sleep-wake states and the prevention of symptoms of narcolepsy-cataplexy, but conclusive evidence would still rely on careful comparisons of animals deficient in discrete components of the orexin system under defined genetic and environmental conditions. To address such questions, our lab has investigated the effects of targeted disruptions of the *OX1R* and *OX2R* receptor genes in mice. In **Chapter 2**, I address this issue and present results in which the behavioral, pharmacological, and electrophysiological phenotypes of orexin and OX2R knockout mice are directly compared. In **Chapter 6** I examine whether orexin plays a role in the action of modafinil, an orphan drug. In **Chapter 7**, I address the critical question of whether the narcolepsy phenotype can be rescued by genetic or pharmacologic therapy with orexins.

The unique phenomenon of cataplexy is observed in dogs as well as mice with disrupted orexin signaling. Strong, generally positive**,** emotional stimuli such as laughter are known to trigger cataplexy in humans with narcolepsy. This implies that orexin neurons may play a role in the physiologic responses associated with emotions. Intense innervations of orexin neurons to components of the limbic system including nucleus accumbens, amygdala, arcuate nucleus of the hypothalamus, dopaminergic ventral tegmental area, and the basal forebrain cholinergic centers are consistent with this suggestion (Date et al., 1999; Nakamura et al., 2000; Peyron et al., 1998; Sakurai et al., 1998b). Furthermore, neurons controlling cardiovascular responses to emotion map

to the perifornical nucleus (Smith et al., 1990), an area of the LHA rich in orexin and MCH neurons. Centrally administered orexin-A potently stimulates grooming behavior in rats, a behavior that is often associated with a stress response. Therefore, orexin-A may also promote emotional arousal. Orexin-induced grooming can be partially inhibited by a corticotropin-releasing-factor (CRF) antagonist further strengthening the assertion that central stress and orexin signaling pathways may be related (Ida et al., 2000a; Ida et al., 2000b). Conversely, orexin signaling may not specifically increase anxiety as one report suggests that rats exhibit normal exploratory behavior after orexin-A administration (Hagan et al., 1999). The role of the orexin and MCH systems in modulation of emotionality and cataplexy is explored in **Chapter 4**.

Neuroanatomical Correlates

Dense projections from orexin neurons to the ascending cortical activating system (described above) including the histaminergic TMN, noradrenergic LC, serotonergic dorsal raphe, cholinergic pedunculopontine nucleus, and cholinergic nucleus basalis provide further anatomic evidence of these peptides' important roles in sleep-wakefulness regulation (Chemelli et al., 1999; Date et al., 1999; Peyron et al., 1998). Slice electrophysiology studies of the LC, an important vigilance-promoting nucleus, found that application of orexin-A dose-dependently increases its intrinsic neuronal firing rate (Hagan et al., 1999; Horvath et al., 1999b). Taheri and co-workers found that orexin-A immunoreactivity varies diurnally in the pons, the location of LC, and peaks during the dark phase in rats (Taheri et al., 2000). This finding appears consistent with a significant role in promoting wakefulness since orexin is most abundant in the region of the LC during the normal waking period in rats. Orexin neurons also innervate the "sleep-active"

ventolateral preoptic area (VLPO) (Chou et al., 2002). The VLPO is thought to regulate the transition between non-REM and REM sleep by inhibiting the TMN, LC, and median raphe nuclei (Sherin et al., 1998; Sherin et al., 1996). Orexin-A immunoreactivity in the preoptic/anterior hypothalamus, the location of VLPO, also exhibits diurnal variation with the peak occurring during the light phase when VLPO neurons are most active (Taheri et al., 2000). Direct innervation of ventral tegmental dopaminergic neurons (Nakamura et al., 2000) and orexin receptor expression in the substantia nigra (Marcus et al., 2001) suggest that orexin may modulate dopaminergic involvement in motivational and emotional pattern generators. Interestingly, dopamine D1 and D2 antagonists dose-dependently suppress orexin-induced hyperlocomotor and grooming behaviors (Nakamura et al., 2000).

OREXINS INFLUENCE INGESTIVE BEHAVIORS AND METABOLISM

The original report describing the orexin neuropeptides and their receptors emphasized evidence of a role for orexins in promoting feeding behavior (Sakurai et al., 1998b). Were accounts of increased feeding behavior simply a secondary pharmacological effect of orexins? How do orexins link sleep-wakefulness regulation with that of feeding and metabolism? This subject is introduced here, and experiments undertaken to address these questions are presented in **Chapter 3-5, and Appendix C**.

Feeding

Early lesioning experiments of the LHA consistently caused a syndrome of decreased food and water intake that lowered body weight set-point to about 75%-80% that of sham-operated controls in several species (Bernardis and Bellinger, 1996). Complementary electrical stimulation studies of the LHA found that acute stimulation causes hyperphagia and that chronic stimulation can cause obesity. Electrical self-stimulation studies of the LHA demonstrate that the LHA participates in a dopaminergic transmission dependent reward system that is markedly facilitated by food deprivation. This suggests that energy balance may significantly influence the excitability of the LHA feeding circuitry.

Acute injection of orexin-A into the lateral ventricles of fed rats, during the early light phase, significantly and dose-dependently stimulates food consumption (Edwards et al., 1999; Haynes et al., 1999; Sakurai et al., 1998b; Yamanaka et al., 2000). Similar experiments with orexin-B were inconsistent, but positive studies found the feeding effects to be shorter-lived than those of orexin-A. This suggests that orexin-A may be more resistant to inactivating peptidases due to its disulfide bonds or that only a subset of the orexin-A feeding pathways (presumably OX1R and OX2R mediated) may be activated by orexin-B (presumably OX2R mediated). It should also be noted that orexin-A is significantly less potent at stimulating food consumption than NPY under the same conditions. However, its duration of action is apparently longer than that of NPY (Sakurai et al., 1998b), and the magnitude of the maximum effect of orexins is similar to that of other appetite stimulating peptides such as MCH and galanin (Edwards et al., 1999). The physiologic relevance of feeding effects of orexin is supported by the finding that central administration of a neutralizing anti-orexin antibody significantly and dose-dependently

suppresses spontaneous feeding in fasted rats (Yamada et al., 2000). In addition, a selective OX1R antagonist can inhibit natural feeding over several days as well as feeding stimulated by fasting or i.c.v. injection of orexin-A (Arch, 2000). Furthermore, orexin-A dose-dependently increases gastric acid secretion only when given centrally and with an intact vagus nerve, suggesting a role for orexin in autonomic functions such as the cephalic phase of digestion (Takahashi et al., 1999). A confounding factor in orexin-feeding studies to date is the potential that these effects may be partly or completely secondary to changes in other unmeasured variables, such as wakefulness.

Circadian processes have a marked influence on feeding behavior and disruptions of normal circadian feeding patterns are well-described effects of LHA lesions (Bernardis and Bellinger, 1996). Interestingly, Haynes and co-workers found that the feeding response of rats to acute i.c.v. injection of orexin-A is highly dependent on the time of day (Haynes et al., 1999). They found the largest increase in exogenous orexin-stimulated food intake occurred in the early light phase and 6 hr into the dark phase when normal food intake is at a nadir or slowing in normal rats. Injection of orexin-A at the beginning of the dark phase, when the normal feeding rate is at its highest, had no effect. Similarly, orexin-A was ineffective at increasing intake in the first hour of refeeding after a fast. It seems reasonable to speculate that at the beginning of the dark cycle and immediately after a fast, orexin-stimulated feeding pathways are already maximally activated and therefore unresponsive to additional pharmacologic stimulation by orexin-A.

Chronic infusion of orexin-A over several days disrupts the normal circadian feeding pattern in rats by increasing daytime and decreasing nighttime food intake (Haynes et al., 1999; Yamanaka et al., 1999). These studies showed no effect on total daily food intake, adiposity, or body weight. The inability of chronic orexin-A infusions to increase overall food consumption or

body weight may be due to a circadian variation in the relative responsiveness to exogenous orexin. Food intake is increased during the day when orexin responsiveness is high (and endogenous orexin activity is low), but this daytime hyperphagia may result in counter-regulatory measures that later reduce the drive to eat during periods of relative orexin-insensitivity (when endogenous orexin activity is high) (Haynes et al., 1999). Increased wakefulness is another possible confounding factor: in continuously infused rats, that increased daytime wakefulness has been observed that per se may have resulted in increased feeding (Yamanaka et al., 1999).

Metabolic, Autonomic, and Endocrine Effects

The LHA is also important in controlling metabolic rate. Animal models with lesions in the LHA consistently become hypercatabolic and remain so, even after they reach a lower body weight set-point (Bernardis and Bellinger, 1993). The hypermetabolic phenotype of MCH knockout mice is consistent with these findings. Conversely, the finding of decreased food intake and normal body weight suggests that *orexin* knockout mice are likely to be hypometabolic. This is supported by experiments that determined the metabolic effects of i.c.v. orexin injection using indirect calorimetry (Lubkin and Stricker-Krongrad, 1998). They found that orexin-A injection during the light phase increases oxygen consumption and the respiratory quotient by an apparent increase in carbohydrate metabolism. Interestingly, the effect on respiratory quotient is dependent on circadian phase because, although oxygen consumption increases, the respiratory quotient decreases when orexin-A was given in the dark phase.

Orexin projections to the nucleus of the solitary tract, dorsal motor nucleus of the vagus, and sympathetic neurons in the intermediolateral column of the spinal cord suggest that orexins

may modulate autonomic function and participate in the stress response (Date et al., 1999; van den Pol, 1999)**.** Both central and peripheral administration of orexin increase plasma levels of corticosterone, and orexins can stimulate corticosterone release directly from adrenocortical cells in vitro (Hagan et al., 1999; Malendowicz et al., 1999). Furthermore, *OX1R* and *OX2R* mRNAs are expressed in the adrenal medulla, suggesting that orexins may modulate systemic epinephrine release and therefore influence vascular tone (Lopez et al., 1999). This proposal is consistent with studies by several investigators who found that injection of orexin dose-dependently increases heart rate and blood pressure (Chen et al., 2000; Samson et al., 1999; Shirasaka et al., 1999). The sympathomimetic effects of orexin may also indirectly influence orexin-mediated changes in oxygen consumption (VO_2) and substrate utilization. VO_2 and substrate utilization in genetically modified mice is explored further in **Chapters 4 and 5**.

REGULATION OF OREXIN NEURONS

The LHA receives direct as well as indirect innervation from much of the neuroaxis and can also be influenced by actively transported peripheral factors, such as leptin, insulin, and other hormones, as well as diffusible factors including glucose, electrolytes, amino acids, and peptides (Bernardis and Bellinger, 1996). Therefore the regulation of orexin neurons is potentially complex.

Circadian Influences and Interaction with Sleep/Wake Centers

Orexin neurons are significantly influenced by circadian processes because Fos studies indicate that orexin neurons are primarily "waking active" (Estabrooke et al., 2001), and circadian feeding patterns are disrupted by chronic central orexin-A injection (Haynes et al., 1999). The suprachiasmatic nucleus (SCN), the CNS circadian oscillator, appears to play a prominent role in controlling diurnal feeding and activity patterns, and SCN lesions eliminate the circadian patterns of sleep-wakefulness and food intake as well as the circadian electrical activity of many LHA neurons (Bernardis and Bellinger, 1996; Edgar et al., 1993; Zucker and Stephan, 1973), and daily rhythms of orexin-A release into CSF (E. Mignot, personal communication). Notably, SCN neurons project directly to LHA neurons producing orexins as well as those producing MCH (Abrahamson et al., 2001), and orexin in turn activates SCN neurons, at least indirectly (Date et al., 1999). The SCN might also indirectly influence orexin neurons through other hypothalamic and thalamic nuclei that in turn project to the LHA. It is unclear at the behavioral and physiological level whether orexin signaling pathways mediate circadian feeding and sleep patterns based on SCN signals, or if circadian orexin activity is just an epi-phenomenon of these processes.

Recent electrophysiolgical evidence suggests that local glutamate interneurons within the lateral hypothalamus orchestrate activation of orexin neurons but that orexin neurons are hyperpolarized by norepeinephrine and serotonin (Li et al., 2002) and depolarized by the cholinergic agonist carbachol (Yamanaka et al., 2003b). These studies may suggest that orexin neurons are the recipients of positive and negative feedback interactions from several arousal-related nuclei that mediate the effects of orexin upon sleep-wakefulness.

Glucose, Leptin, and Other Signals Reflecting Energy Balance

Orexin neurons are activated under conditions of acute hypoglycemia. Insulin, given to acutely lower blood glucose levels in rats, caused a marked increase in Fos-immunoreactivity in LHA neurons now known to express orexin (Bahjaoui-Bouhaddi et al., 1994a; Moriguchi et al., 1999). When insulin and glucose were given together so that euglycemia was maintained, no increase above baseline Fos levels was found. This, in combination with the report that insulin receptor mRNA is not expressed in the LHA (Marks et al., 1990), suggest that insulin is unlikely to act directly on these neurons. Also, it is unlikely that reduced plasma osmolarity is a confounding factor in these experiments because there were no significant differences in plasma osmolality between the control and experimental groups. Insulin-induced hypoglycemia does not increase Fos in MCH neurons (Bahjaoui-Bouhaddi et al., 1994b), suggesting that the mechanisms regulating orexin and MCH neurons are different.

Cai and co-workers performed a detailed study of *orexin* mRNA expression in rats after physiologic and pharmacologic manipulations designed to stimulate food intake through different mechanisms (Cai et al., 1999). These authors identified subnormal plasma glucose levels and absence of food intake as key factors associated with increased *orexin* mRNA expression.

Convincing neuroanatomic evidences of extensive reciprocal innervation between the leptin-sensitive feeding pathways of the ARC nucleus and orexin neurons have been reported (Broberger et al., 1998; Elias et al., 1998), and leptin receptor immunoreactivity has been identified in orexin neurons (Hakansson et al., 1999; Horvath et al., 1999a).

In a striking confirmation of the preceding predictions, our group has recently demonstrated that hypothalamic orexin neurons directly monitor indicators of energy balance and mediate adaptive augmentation of arousal in response to fasting (Yamanaka et al., 2003a). Activity of isolated orexin neurons is stimulated by reductions in glucose and reciprocally inhibited by elevations of glucose within physiological concentrations. Orexin neurons are inhibited by leptin (an anorectic protein produced by and secreted in proportion to adipocyte fat stores), and stimulated by the novel peptide ghrelin which is released from the stomach in response to nutritional depletion (Cummings et al., 2002; Lee et al., 2002; Nakazato et al., 2001). Furthermore, *orexin* expression of normal and *ob*/*ob* mice correlates negatively with changes in blood glucose, leptin, and food intake. To explore the physiological relevance of these observations further, I examine the role of orexin and MCH in behavioral responses to negative energy balance in **Chapters 2 and 3**.

DISPLAY ITEMS

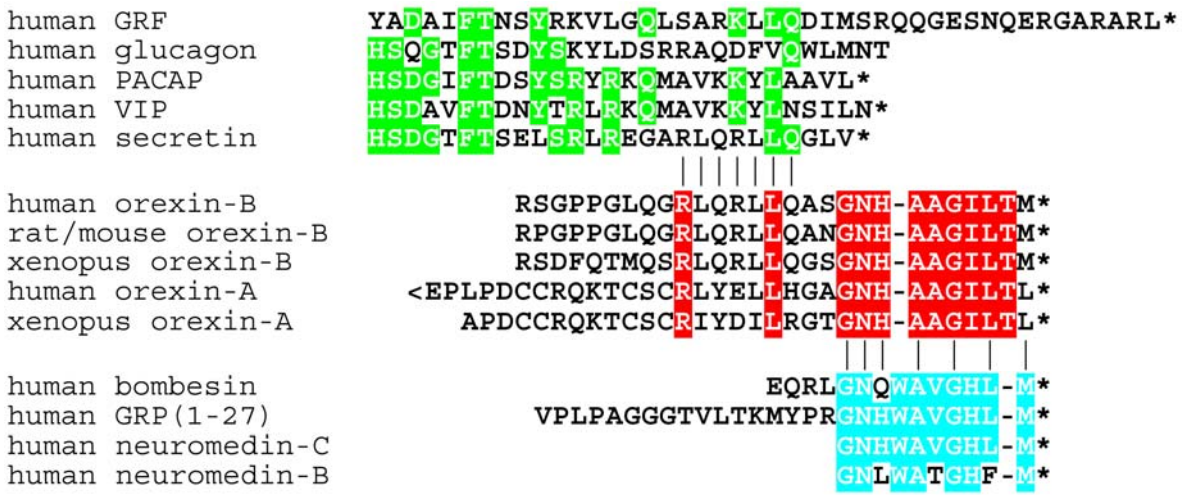


Figure 1-1. Comparison of orexin sequences with the secretin and bombesin families.

"Signature" peptide sequences characteristic of secretins (green highlights) are found primarily at the amino-terminus. Orexin-B is similar to the carboxy-terminus of secretin, but neither orexin peptide shares significant identity with the other members of the secretin family. In contrast, characteristic sequences of the bombesin family (blue highlights) reside in the carboxy-terminus. In this region, both orexin peptides share significant identity with all bombesin family members. Red highlights depict absolute interspecific and inter-isopeptide identity among orexins. Note that there are two intrachain disulfide bonds in orexin-A (Cys6-Cys12 and Cys7-Cys14) but none in orexin-B. Abbreviations and Symbols: GRF, growth-hormone-releasing factor; PACAP, pituitary adenylyl cyclase-activating peptide; VIP, vasoactive intestinal peptide; GRP, gastrin-releasing peptide; *, C-terminal amide; <E, pyroglutamyl residue.

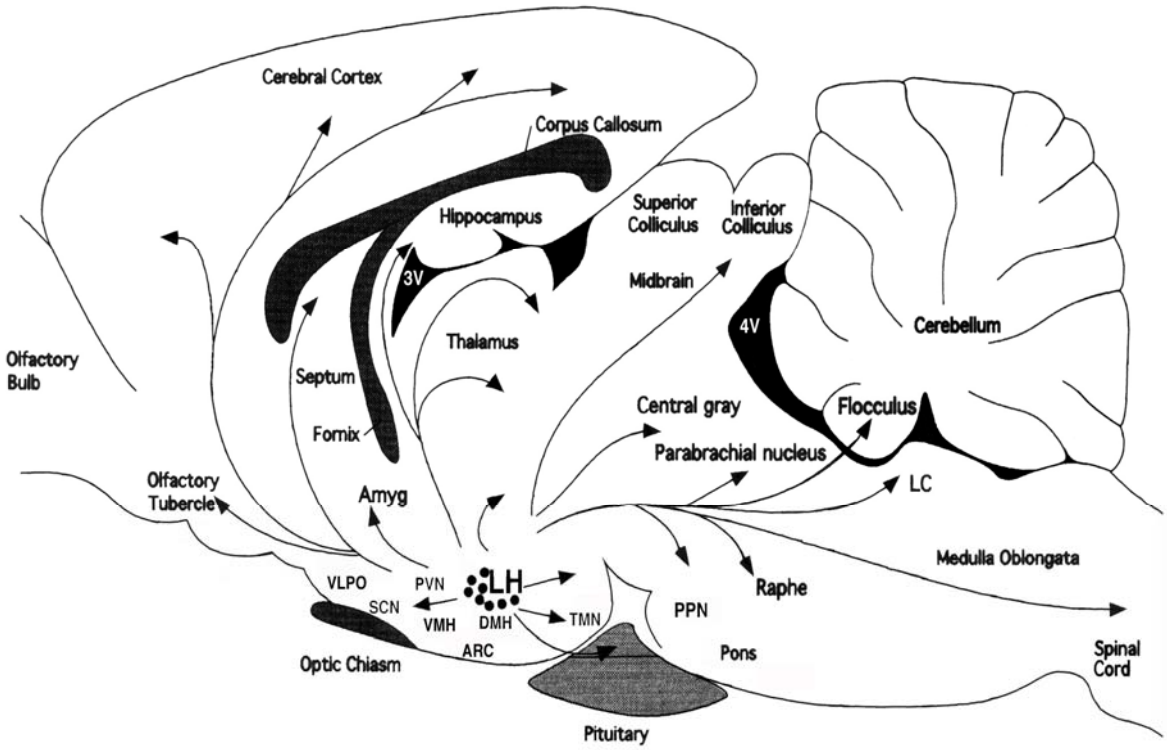


Figure 1-2. Neuroanatomy of the Orexin Neuropeptide System.

Orexin neurons are found in the lateral hypothalamic area and project to the entire central nervous system. Schematic drawing of a sagittal section through the rat brain summarizes the organization of the orexin neuronal system. Abbreviations: 3V, third ventricle; 4V, fourth ventricle; Amyg, amygdala; VLPO, ventrolateral preoptic area; SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; Arc, arcuate nucleus; DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; TMN, tuberomamillary nucleus; PPN, pedunculopontine nucleus; LC, locus coeruleus. Modified with permission from T. Sakurai (1999).

CHAPTER TWO
Distinct Narcolepsy Syndromes in Orexin Receptor Type 2 and Orexin Knockout Mice: Molecular Genetics of Non-REM and REM Sleep Regulation

SUMMARY

The narcolepsy-cataplexy syndrome consists of both inability to maintain wakefulness and intrusion of REM sleep into wakefulness. I hypothesize that disruption of independent orexin-receptor mediated pathways result in the symptom complex. I document, using behavioral, electrophysiological, and pharmacological criteria, two distinct classes of behavioral arrests exhibited by mice deficient in orexin-mediated signaling. Both *OX2R*$^{-/-}$ and *orexin*$^{-/-}$ mice are similarly affected with behaviorally abnormal attacks of non-REM sleep (equivalent to "sleep attacks" in human patients) and show similar degrees of disrupted wakefulness. In contrast, *OX2R*$^{-/-}$ mice are only mildy affected with cataplexy-like attacks of REM sleep and other REM sleep abnormalities, whereas *orexin*$^{-/-}$ mice are severely affected. While normal regulation of wake/non-REM sleep transitions depends critically upon OX2R activation, the profound dysregulation of REM sleep control unique to the narcolepsy-cataplexy syndrome emerges from loss of signaling through both OX2R-dependent and OX2R-independent (presumably OX1R-mediated) pathways. These findings represent the first molecular genetic dissection of the regulatory pathways of non-REM versus REM sleep,

and expand the existing neuroantomical/neurochemical models of sleep/wake regulation with new insights.

INTRODUCTION

Following the revelatory implication of orexin signaling in animal models of narcolepsy (reviewed in **Chapter 1**; see also **Appendix A**), human narcolepsy-cataplexy was also recognized to result from loss of hypothalamic neurons containing orexin (hypocretin) neuropeptides, possibly by an autoimmune process, renewing interest in the hypothalamus as a critical participant in sleep/wake control (Mignot et al., 2002b; Saper et al., 2001; Sutcliffe and de Lecea, 2002; Willie et al., 2001).

Orexin peptides are endogenous ligands for the cell-membrane receptors, OX1R and OX2R (Sakurai et al., 1998a). OX1Rs exhibit selective affinity for orexin-A while OX2Rs exhibit equal affinity for both orexin-A and orexin-B. Both receptors are expressed in neurons of the hypothalamus and MPT as well as more widespread regions of the central nervous system (Marcus et al., 2001). The differential distribution of orexin receptors suggests distinct roles of each receptor in aspects of vigilance state control, muscle tone, and energy homeostasis. In particular, *OX2R* expression is prominent in the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei, and many hypothalamic nuclei including the histaminergic tuberomammillary nucleus (TM). Notably, recent studies have indicated an important role for histamine signaling as a down-stream mediator of

wakefulness produced by intracerebroventricular (i.c.v.) administration of orexin (Huang et al., 2001; Yamanaka et al., 2002).

A variety of studies together suggest that a function of orexin signaling might be to stabilize prolonged periods of wakefulness by opposing homeostatic sleep propensity (Saper et al., 2001). However, mechanisms by which absence of orexin signaling causes symptoms of narcolepsy-cataplexy are unknown. Despite the association of *OX2R* mutations with narcolepsy in dogs, the differential roles of orexin receptors remain uncertain. While this animal model has been used extensively in studies investigating the neurochemical substrates of narcolepsy (Nishino and Mignot, 1997), it differs biochemically from the vast majority of human narcolepsy cases in which orexin peptides are lacking.

In this chapter, I systematically compared the phenotypes resulting from OX2R- and orexin-deficient states in mice (*orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice). I used behavioral, pharmacological, and electroencephalographic/ electromyelographic (EEG/EMG) methods to compare symptoms of narcolepsy in *OX2R*$^{-/-}$ and *orexin*$^{-/-}$ mice in detail.

RESULTS

Production of OX2R Knockout Mice

The targeting of the mouse mouse *OX2R* gene by replacement of exon 1 in-frame with nuclear lac-Z and neo cassettes by homologous recombination in embryonic stem cells by R.M.Chemelli is as reported (Willie et al., 2003). Heterozygous F1 mice were

phenotypically normal, and progeny of F1 heterozygote crosses survivied until weaning in a ratio consistent with Mendelian inheritance (homozygosity did not cause significant lethality in utero or up to the time of weaning). Homozygous mice were anatomically and histologically normal except that in situ hybridization of brain sections from *OX2R*$^{-/-}$ mice (performed by S.C. Williams) using a riboprobe specific for exon 3 revealed no significant signal above background in brain regions where *OX2R* is normally expressed.

Northern blots of whole-brain RNA, performed by S. Tokita, revealed attenuation of *OX2R* mRNA expression in *OX2R*$^{+/-}$ mice and complete absence of signal in *OX2R*$^{-/-}$ mice. Notably, *orexin* mRNA expression, attenuated in *orexin*$^{+/-}$ mice and absent in *orexin*$^{-/-}$ mice, was elevated about 2-fold in *OX2R*$^{-/-}$ mice. In contrast, *OX1R* mRNA expression was not affected by the loss of either the *orexin* or *OX2R* genes. Radioimmunoassays specific for orexin-A and orexin-B on whole-brain homogenates, performed by S. Tokita, failed to detect significant changes in amounts of these peptides in and *OX2R*$^{-/-}$ mice compared to wild-type mice. These findings indicate that the targeted *OX2R* allele is functionally null, but despite increased expression of *orexin* mRNA, whole-brain orexin peptide levels are not impacted by loss of OX2Rs.

Characterization of Two Classes of Behavioral Arrests

Behavioral arrests characteristic of narcoleptic mice are observed frequently during the dark phase when mice are most active (Chemelli et al., 1999; Hara et al., 2001). These episodes in narcoleptic *orexin*$^{-/-}$ mice have been characterized previously using the following criteria: (1) an abrupt cessation of purposeful motor activity; (2) a sustained postural collapse

maintained throughout the episode; and (3) an abrupt end to the episode with resumption of purposeful motor activity (see **Appendix A**). Preliminary screening of *OX2R*$^{-/-}$ mice during the dark phase by infrared video photography only rarely identified behavioral arrests that fulfilled these strict requirements (**Figure 2-1A** and corresponding video footage under "Supplemental Movies" at http://www.neuron.org/cgi/content/full/38/5/715/DC1). Instead, examination of *OX2R*$^{-/-}$ mice revealed the occurrence of a distinct variety of behavioral arrests with onsets that were more gradual in nature (**Figure 2-1B** and "Supplemental Movies" at http://www.neuron.org/cgi/content/full/38/5/715/DC1) than those typically observed in *orexin*$^{-/-}$ mice. Such gradual arrests typically began during quiet wakefulness (see below) and could be easily distinguished from the normal onset of resting behavior by: (1) the absence of stereotypic preparation for sleep (e.g., nesting and/or assumption of a curled or hunched posture with limbs drawn under the body) and (2) a characteristic ratchet-like "nodding" of the head over a period of several seconds with a transition to a collapsed posture. I therefore adopted these different criteria for judging onset of this type of behavioral arrest, but I maintained requirements for sustained postural change and abrupt termination (see Experimental Procedures below). I refer to these two distinct types of behavioral arrests as "abrupt" and "gradual" arrests throughout this chapter.

In collaboraton with S. Tokita and R.M. Chemelli, I characterized the behavioral arrests of male mice of mixed genetic background (12-13 weeks of age) elicited during exploration of standard open field arenas. Fourteen *OX2R*$^{-/-}$, 12 *orexin*$^{-/-}$, and 20 wild-type littermates were filmed for 4 h each from the onset of dark phase, and video records were scored by observers, blinded to genotype. No behavioral arrests of any kind were observed in

records of wild-type mice. All homozygous knockout mice exhibited some form of arrest with the exception of 2 *OX2R*$^{-/-}$ mice (**Figure 2-2A, B**). In general, mutant mice of both groups exhibited variability in the frequency of arrests, highlighting in part the possible influence of mixed genetic background. In contrast to *orexin*$^{-/-}$ mice, all of which exhibited abrupt arrests, only about half of *OX2R*$^{-/-}$ mice exhibited abrupt arrests. Notably, despite similar mean durations of abrupt arrests, *orexin*$^{-/-}$ mice displayed a 31-fold higher mean frequency of abrupt arrests per mouse than did *OX2R*$^{-/-}$ mice ($p=0.0048$) (**Figure 2-2B**). As a result, total time consumed by abrupt arrests was much greater in *orexin*$^{-/-}$ mice in this experiment ($p=0.0021$).

In contrast, similar proportions of *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice exhibited gradual arrests, the frequency of which varied widely in individuals of each group (**Figure 2-2B**). Overall, gradual arrests affected both genotypes with similar frequency, but the mean duration of arrests in *orexin*$^{-/-}$ mice was longer than in *OX2R*$^{-/-}$ mice ($p=0.0034$).

To seek insight into the emotional states that may precede arrests, S. Tokita and I categorized normal specific behaviors immediately (<5 sec) prior to arrests in a manner similar to that previously reported (Chemelli et al., 1999). Abrupt arrests in both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice were highly associated with "emotive" motor activity such as vigorous grooming, ambulation , and climbing; very few abrupt arrests were preceded by less conspicuous behaviors including quiet wakefulness (**Tables 2-1** and **2-2**). In contrast, gradual arrests in both genotypes were most commonly preceded by quiet wakefulness (**Tables 2-3** and **2-4**). Although ambulation was associated with a smaller number of gradual arrests, only very rarely were such arrests preceded by vigorous grooming. In addition, gait disturbances,

a possible indicator of partial cataplexy (Chemelli et al., 1999), were exclusively associated with *orexin*$^{-/-}$ mice in this study and immediately preceded 42% (98/235) of abrupt arrests in these mice. Gait disturbances never preceded the gradual arrests in either genotype (0/56 in *orexin*$^{-/-}$ mice and 0/70 in *OX2R*$^{-/-}$ mice.

Pharmacological Responses of Behavioral Arrests

To probe in murine models the effects of drugs that are used in the treatment of human narcolepsy-cataplexy (Aldrich, 1998; Honda, 1988; Shneerson, 2000), I treated matched cohorts of *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice with doses of caffeine and clomipramine. Over a dose range reported to increase wakefulness in mice (Wisor et al., 2001), the psychostimulant caffeine dose-dependently suppressed gradual arrests (**Figure 2-3**) as well as normal resting behavior (data not shown) in mutant mice. Similar results were achieved in both genotypes. Rather than inhibiting abrupt arrests, caffeine tended to produce a mild exacerbation of abrupt arrest frequency in both knockout strains, but these changes did not reach statistical significance.

In contrast to caffeine, the anti-cataplectic agent clomipramine, administered at a dose selected to suppress REM sleep without altering motor activity in mice (Eschalier et al., 1988; Kitahama and Valatx, 1980), suppressed the frequency of abrupt arrests in *orexin*$^{-/-}$ mice compared to vehicle control without affecting the frequency of gradual arrests in these mice. Due to the low baseline frequency of abrupt arrests in *OX2R*$^{-/-}$ mice, no effect of clomipramine was observed on behavioral arrests in these mice when administered alone. Importantly, simultaneous administration of both caffeine and clomipramine resulted in

suppression of both arrest types in *orexin*$^{-/-}$ mice, suggesting that the effects of these agents upon arrests are independent.

EEG/EMG Correlates of Behavioral Arrests

Cerebral cortical activity and postural muscle tone, monitored by EEG/EMG, are useful for discriminating sleep/wake abnormalities. To examine vigilance states during abrupt and gradual arrests, I performed concurrent EEG/EMG/video recording in electrode-implanted animals. About 80% (61/76) of abrupt arrests in *orexin*$^{-/-}$ mice, and 40% (4/10) in *OX2R*$^{-/-}$ mice were accompanied by a rapid transition from wakefulness to an EEG pattern indistinguishable from normal REM sleep with atonia (**Figure 2-4A**, **Table 2-5**, and "Supplemental Movies" at http://www.neuron.org/cgi/content/full/38/5/715/DC1). Spectral analysis of EEG during abrupt arrests in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice by fast Fourier transform (FFT) revealed that the micro-architecture of the EEG during these episodes appeared normal, without any evidence of seizure-related activity. EEG spectral patterns during abrupt arrests in *orexin*$^{-/-}$ mice confirmed strong correlation with normal REM sleep (r=0.987) rather than with non-REM sleep (r=0.623) or wakefulness (r=0.744) (**Figure 2-5**). Similar spectral patterns were observed from the much smaller sample of REM sleep-like abrupt arrests in *OX2R*$^{-/-}$ mice (r=0.989 compared to REM sleep, r=0.687 compared to non-REM sleep, and r=0.722 compared to wakefulness). The remaining 20% (15/76) of abrupt arrests observed in *orexin*$^{-/-}$ mice and 60% (6/10) in *OX2R*$^{-/-}$ mice resembled EEG patterns of mouse non-REM sleep in the pre-REM stage (high-amplitude spindle-oscillations superimposed on a non-REM sleep background) with atonia in the EMG (**Table 2-5**). EEG power spectra from such

episodes exhibited lower correlations with REM sleep (r=0.843 in *orexin*$^{-/-}$ mice, r=0.955 in *OX2R*$^{-/-}$ mice), non-REM sleep (r=0.819 in *orexin*$^{-/-}$ mice, r=0.747 in *OX2R*$^{-/-}$ mice), and wakefulness (r=0.596 in *orexin*$^{-/-}$ mice, r=0.833 in *OX2R*$^{-/-}$ mice).

In contrast, EEG/EMG correlates of gradual arrests in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice invariably revealed onset of attenuated muscle tone, but not atonia, and an EEG transition from wakefulness to non-REM sleep (**Figure 2-4B, C, Table 2-5**; and "Supplemental Movies" at http://www.neuron.org/cgi/content/full/38/5/715/DC1). In *orexin*$^{-/-}$ mice, 38% (20/53) of gradual arrests initiated with non-REM sleep but progressed rapidly to REM sleep with a latency of less than 1 min (**Figure 2-4C**). Notably, gradual arrests recorded in *OX2R*$^{-/-}$ mice correlated exclusively (40/40) with characteristics of non-REM sleep (**Figure 2-4B**, **Table 2-5**). FFT analysis confirmed that the EEG spectra during the non-REM phase of gradual arrests conformed to those of normal non-REM sleep in both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice (**Figure 2-5**).

Unexpectedly, I also observed that gradual arrests were occasionally accompanied by apparent automatic behavior (continuation of semi-purposeful motor activities after the onset of light sleep) in this experiment. Both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice, but not wild-type mice, exhibited such dissociated states in which stereotypic chewing of food continued after the onset of non-REM sleep as judged by EEG/EMG. Although the brevity of many episodes (typically less than 10 sec) precluded detailed analysis, a particularly long (42 sec) episode demonstrates that the EEG features of this example conform unambiguously to the spectral features of non-REM sleep (**Figure 2-6** and "Supplemental Movies" at http://www.neuron.org/cgi/content/full/38/5/715/DC1).

Dysregulation of Non-REM versus REM Sleep Transitions

Narcolepsy-cataplexy is characterized by abnormalities in the frequencies, durations, and amounts of vigilance states over the 24-h day. These abnormalities indicate instability of sleep/wake states that include more frequent waking during the rest phase, inability to maintain wakefulness during the active phase, and intrusions of REM sleep into wakefulness or at sleep onset. To compare such abnormalities in *orexin*$^{-/-}$, *OX2R*$^{-/-}$, and wild-type littermate mice, C.M. Sinton and I examined spontaneous sleep/wake patterns by concurrent EEG/EMG recording of electrode-implanted mice in home cages. Results obtained from wild-type mice (**Figure 2-8**, **Table 2-6**) closely resemble parameters previously published for C57BL/6J, 129Sv, and C57BL/6J-129SvEv hybrid strains (Chemelli et al., 1999; Parmentier et al., 2002; Tafti et al., 1997).

Inability to maintain sleep and wakefulness is indicated in rodents by increased fragmentation of vigilance states. Such fragmentation is illustrated by hypnograms of *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice (**Figure 2-7**). Indeed, features of wakefulness and non-REM sleep were disrupted to a similar degree in both genotypes. Compared to wild-type controls, both knockout groups exhibited statistically insignificant tendencies toward reduced amounts of awake and increased amounts of non-REM sleep during the dark phase (**Figure 2-8B, Table 2-6**). More importantly, both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice exhibited higher frequencies of awake episodes during the light ($p=0.044$ and $p=0.0050$, respectively) and dark ($p=0.0002$ and $p=0.0002$, respectively) phases as well as higher frequencies of non-REM sleep episodes during the dark phase ($p=0.0013$ and $p=0.0006$, respectively).

To further characterize the fragmentation of vigilance states, I plotted the hourly mean durations of each state. Importantly, episode durations of wakefulness in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice were severely diminished (p=0.037 and p=0.0015, respectively) compared to wild-type mice over the entire dark phase (**Figure 2-8A**, **Table 2-6**). In fact, throughout most of the dark phase, *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice retained reduced hourly durations characteristic of the light phase. Fragmentation of vigilance patterns is also indicated by the consistently reduced durations of non-REM sleep in mutant strains, especially at night (*orexin*$^{-/-}$, p=0.0007; *OX2R*$^{-/-}$, p=0.0020) (**Figure 2-8B**, **Table 2-6**). These results demonstrate that neither *orexin*$^{-/-}$ nor *OX2R*$^{-/-}$ mice are able to maintain long bouts of wakefulness or non-REM sleep, especially during the active phase. Overall, with respect to parameters of wakefulness and non-REM sleep, *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice are indistinguishable.

In contrast, *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice are distinct from each other in the expression of abnormalities of REM sleep. *Orexin*$^{-/-}$ mice exhibited a striking 75% increase in amount of time in REM sleep (p=0.0002) over the entire dark phase (**Figure 2-8A, Table 2-6**). Hourly plots of amounts and durations of REM sleep illustrate abnormalities of REM sleep that peak during the early part of the dark phase in *orexin*$^{-/-}$ compared to *OX2R*$^{-/-}$ and wild-type mice (**Figure 2-8**). Only *orexin*$^{-/-}$ mice showed significant alterations in the circadian frequencies of REM sleep episodes: an increase over the entire dark phase (p=0.012) and a compensatory reduction during the light phase (p=0.017). Only *orexin*$^{-/-}$ mice had a reduced mean interval between REM sleep episodes during the dark phase (p=0.028; **Table 2-6**) and reduced latencies to REM sleep during both phases (dark, p=0.0002; light, p=0.0069). While in comparison to *orexin*$^{-/-}$ and wild-type mice, *OX2R*$^{-/-}$ mice tended to exhibit intermediate

values for some of these same parameters of REM sleep, only a reduced REM sleep latency during the dark phase differed significantly from wild-type mice (p=0.0006; **Figure 2-8**, **Table 2-6**). Indeed, REM sleep amounts of *OX2R*$^{-/-}$ mice were significantly less than those of *orexin*$^{-/-}$ mice over the dark phase (p=0.0076).

Unlike wild-type mice, all *orexin*$^{-/-}$ mice displayed the hallmark of rodent narcolepsy, spontaneous transitions from the waking state directly to REM sleep, on every day of recording, without exception (**Figure 2-7B**). Such spontaneous transitions were far less frequent or completely absent during the same period in individual *OX2R*$^{-/-}$ mice. Overall, the mean frequency of spontaneously occurring transitions from awake to REM sleep during each 24-h period was 13-fold greater in *orexin*$^{-/-}$ mice compared to *OX2R*$^{-/-}$ mice (p=0.010). With respect to characteristics of REM sleep, *OX2R*$^{-/-}$ mice displayed an intermediate phenotype at most, with statistically insignificant changes in most parameters of REM sleep compared to wild-type mice. Overall, the general patterns of REM sleep amounts and durations of *OX2R*$^{-/-}$ mice more closely resemble those of wild-type mice than those of orexin-deficient mice.

DISCUSSION

Based on features of observed behavior, EEG/EMG, and pharmacological responses, I discriminated at least two distinct forms of behavioral arrest in narcoleptic mice. I believe these arrests to be analogous to cataplexy and sleep attacks observed in narcoleptic patients. I

demonstrated that *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice exhibit an equivalent dysregulation of wakefulness and non-REM sleep. In contrast, *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice differ in the expression of abnormalities of REM sleep control.

Discrimination of Cataplexy and Sleep Attacks in Humans and Mice

The phenomenon of cataplexy has been conceptualized either as a fragmentary manifestation of REM sleep or, alternatively, as a transitional state between wakefulness and REM sleep (Hishikawa and Shimizu, 1995). Accepted criteria for the definition of cataplexy in humans include: sudden bilateral weakness involving skeletal muscles, provocation by sudden strong emotions, lack of impairment of consciousness and memory, short duration (less than a few minutes), and responsiveness to treatment with clomipramine or imipramine (Honda, 1988). Studies performed during cataplectic attacks have produced conflicting results in both humans and dogs, with some authors reporting wakefulness and others reporting REM sleep characteristics in the EEG, despite preservation of consciousness (Dyken et al., 1994; Dyken et al., 1996; Guilleminault et al., 1974; Kushida et al., 1985; Mitler and Dement, 1977).

The abrupt arrests that I observed in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice appear to fulfill criteria used for human cataplexy. Abrupt arrests are discrete phases of postural atonia of short duration (seconds to minutes) that are often preceded by gait disturbances due to propogating atonia. Such arrests are triggered during active waking periods with emotional content such as running, climbing, vigorous grooming, or even social interaction (see also **Appendix A**), and they are specifically suppressed by clomipramine. I have also occasionally

documented apparent consciousness shortly after arrest onset by evaluating the responsiveness of paralyzed mice to a potential threat (a pen or similar object waved in front of the open eyes of an immobilized mouse typically elicited slight jerking motions; see "Supplemental Movies" at http://www.neuron.org/cgi/content/full/38/5/715/DC1).

During REM sleep occurring in direct succession to an attack of cataplexy, patients often experience sleep paralysis and hypnagogic hallucinations. On such occasions, it is very difficult to differentiate transitions from cataplexy to sleep paralysis (Hishikawa and Shimizu, 1995). Because I have also observed REM sleep EEG/EMG in succession to the onset of arrests, it is possible that a correlate of sleep paralysis may also occur in these mice. This is consistent with the observation that abnormal behavioral features seen during arrests, such as rocking or slight jerking activities, always accompany REM sleep EEG patterns.

In contrast to cataplexy, the sleep attacks of human narcolepsy are associated with impaired consciousness and memory, but not with strong emotions or abrupt muscle weakness. The identification of sleep attacks is based upon behavioral context: patients report sudden irresistible sleepiness and intrusion during unusual circumstances (e.g., meals, conversations, driving), and such attacks may also be associated with automatic behavior (Aldrich, 1998; Zorick et al., 1979). Sleep attacks are generally associated with the onset of early stages of non-REM sleep, reflect a compression of the normal process of entering sleep, and mimic the effects of sleep deprivation in normal humans (Alloway et al., 1999; Dement et al., 1966). In the context of narcolepsy-cataplexy, but not other disorders of excessive sleepiness, sleep attacks may also give way rapidly to REM sleep periods (so called "sleep-onset" REM or SOREM periods) that can be accompanied by sleep paralysis or

hallucinations (Hishikawa and Shimizu, 1995; Nishino and Mignot, 1997; Roth et al., 1969). Unlike cataplexy, sleepiness is reduced by psychostimulants such as amphetamines, modafinil, and caffeine (Shneerson, 2000).

Gradual arrests in both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice resemble human sleep attacks in several respects. Unlike abrupt arrests, gradual arrests most frequently occur following inconspicuous activities and quiet waking periods rather than vigorous motor activities with a higher potential for emotional content. Not once were onsets of gradual arrests observed in association with preceding gait disturbances or muscle atonia suggestive of cataplexy. Unlike normal sleep, however, gradual arrests lack mouse-specific behavioral features of rest and exhibit a loss of head and neck posture reminiscent of "nodding off" in sleep-deprived humans and narcoleptic dogs (see below); such events are not observed in wild-type mice under the same conditions. Like sleep attacks, onset of gradual arrests is consistently accompanied by non-REM sleep EEG/EMG, and arrests are specifically suppressed by caffeine. In both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice, gradual arrests are occasionally accompanied by semi-purposeful automatic behavior (chewing of food) during non-REM sleep, providing further evidence of the behavioral abnormality of these arrests and underscoring the subjective sleepiness of these mice (Guilleminault et al., 1975). In *orexin*$^{-/-}$ mice, a large proportion of sleep attacks transitioned prematurely from non-REM sleep to REM sleep. Such rapid transitions to REM sleep, which resemble the SOREM episodes of narcoleptic patients, were observed only rarely in *OX2R*$^{-/-}$ mice.

Distinct Narcolepsy Syndromes in Various Animals Models

Corresponding to that which I have described above in mice, at least two phenotypic forms of narcolepsy have been reported in canines. Familial narcolepsy has been maintained in lines of Doberman pinschers and Labrador retrievers, while sporadic non-familial narcolepsy has been studied to a lesser extent in poodles, beagles, and other small breeds. Doberman and Labrador lines have recently been found to carry functionally equivalent inactivating mutations of the *OX2R* gene (Hungs et al., 2001; Lin et al., 1999), while sporadically narcoleptic dogs, like the majority of narcoleptic-cataplectic humans, are deficient in orexin peptides (Ripley et al., 2001a). *OX2R*$^{-/-}$ and *orexin*$^{-/-}$ mice are the direct biochemical correlates of these two forms of narcolepsy. By examining the effects of inactivating these two genes in mice, I have produced, for the first time, a comprehensive analysis of the resulting phenotypes under controlled genetic conditions of species and strain, allowing the contribution of the orexin-OX2R pathway to symptoms of narcolepsy-cataplexy to be dissected at the molecular genetic level.

Combining behavioral, pharmacological, and electrophysiological means, I determined that narcolepsy syndromes in orexin- and OX2R-deficient mice appear identical with respect to fragmentation of vigilance states and inability to sustain wakefulness due to interruption by sleep periods including non-REM sleep attacks. Similarly, both sporadic and genetic forms of canine narcolepsy exhibit sleepiness based on increased tendencies to fall asleep and more fragmented wakefulness patterns (Lucas et al., 1979; Nishino et al., 2000a), although no direct experimental comparison of sleep in these models has been reported. Corresponding to the gradual arrests I report in mice, behavioral attacks of drowsiness

associated with "stop motion" and nodding of the head and neck are observed in genetically narcoleptic Dobermans, although these have not been reliably distinguished from cataplexy by EEG/EMG due in part to the more staged onset of non-REM sleep appearance in canines (S. Nishino, personal communication). Together, these facts suggest that excessive sleepiness is a consistent feature of orexin- and OX2R-deficient forms of narcolepsy in both dogs and mice. Thus, activation of the *OX2R* gene regulates gating of transitions from wakefulness to non-REM sleep and plays a predominant role in preventing the emergence of sleepiness and sleep attacks (**Figure 2-9**).

In contrast, I found important differences in both REM sleep control and pathological manifestations of REM sleep between orexin- and OX2R-deficient forms of narcolepsy. Unlike *orexin*$^{-/-}$ mice, individual *OX2R*$^{-/-}$ mice, if at all, only infrequently exhibit cataplexy-like arrests or direct transitions from wakefulness to REM sleep, and sleep attacks in *OX2R*$^{-/-}$ mice were not associated with transitions to REM sleep or atonia. This finding is not without precedent in dogs: early studies of narcoleptic Dobermans and Labradors found these dogs to be 30-80 fold less severely affected with cataplexy than poodles with sporadic narcolepsy that showed literally hundreds of attacks a day (Baker et al., 1982) (see especially Table 2 of the reference), an effect previously attributed solely to differences in breed and breed size. Notably, Dobermans and Labradors differ from each other in severity of phenotype despite the functional equivalence of their mutations, with Labradors exhibiting cataplexy only very rarely. In terms of other abnormalities of REM sleep, studies of narcoleptic Dobermans showed relatively normal patterns of REM sleep cylicity (Nishino et al., 2000a), a finding seemingly at odds with the long accepted view that human narcolepsy-cataplexy is primarily

a disorder of REM sleep (Dement et al., 1966), but consistent with our findings in *OX2R*$^{-/-}$ mice.

Together, studies of dogs and mice suggest that OX2R inactivation leads to a spectrum of intermediate narcolepsy phenotypes in which some animals have only sub-threshold abnormalities with respect to detectable changes in REM sleep control. If so, OX2R-deficiency in mice may also resemble features of less understood human syndromes including narcolepsy without cataplexy and idiopathic hypersomnia, in which CSF levels of orexin are not absent (Kanbayashi et al., 2002; Mignot et al., 2002a).

Our findings offer genetic evidence that the processes that sustain wakefulness by gating transitions to non-REM sleep versus gating transitions to REM sleep are at least partly distinct (**Figure 2-9**). While stable regulation of transitions between wakefulness and non-REM sleep largely depends upon OX2R activation, appropriate control of REM sleep involves both OX2R-dependent and OX2R-independent mechanisms. An intact OX1R signaling pathway in *OX2R*$^{-/-}$ mice provides the simplest explanation for the distinct narcolepsy phenotypes observed within a given species of the same genetic background. Indeed, an important role of OX1R activation in REM sleep control is supported by a recent report demonstrating that a selective OX1R antagonist prevents orexin-A-induced suppressions of REM sleep in rats (Smith et al., 2003). Furthermore, analysis of *OX1R*$^{-/-}$ and double receptor knockout (*OX1R*$^{-/-}$;*OX2R*$^{-/-}$) mice supports this hypothesis as well. Interestingly, *OX1R*$^{-/-}$ mice do not have any overt behavioral abnormalities, but exhibit a reduced REM sleep latency (Kisanuki et al., 2000). *OX1R*$^{-/-}$;*OX2R*$^{-/-}$ mice appear to be a phenocopy of the ligand knockout mice (Kisanuki et al., 2000). This suggests that despite the

lack of an overt *OX1R* phenotype, loss of signaling through both receptor pathways is necessary for the severe narcoleptic characteristics of the *orexin*-null mice.

EXPERIMENTAL PROCEDURES

Breeding and Maintenance of Mice

The generation of OX2R knockout mouse lines by R.M.Chemelli is described (Willie et al., 2003). Experiments with 2 independent *OX2R* and 4 independent *orexin* knockout lines (Chemelli et al., 1999), including littermate controls, were concurrently performed using male F2 and F3 generations (produced from crosses of F1 and F2 heterozygotes) on a C57BL/6J-129/SvEv mixed background. Mice were provided food and water ad lib (except where noted below), maintained on a 12-h light:dark cycle at all times, and were housed under conditions that controlled temperature and humidity. All procedures were approved by the appropriate institutional animal care and use committees, and were carried out in strict accordance with NIH guidelines.

Infrared Video Characterization of Narcoleptic behavior

CCD video cameras (8-mm format Sony TRV-CCD66 and mini-DV format DCR-TRV8) with infrared and digital time recording capabilities were used to document behavior (4 h/mouse) using a standard open field apparatus (Opto-Varimex-3, Columbus Instruments,

Columbus, OH) as described previously (Chemelli et al., 1999). Mice had free access to food and water throughout experiments.

Criteria for scoring abrupt arrests included (1) an abrupt (less than 1-2 sec) transition from obvious, generally robust, purposeful motor activity; (2) a sustained collapsed posture maintained throughout the episode; and (3) an abrupt end to the episode with resumption of obvious purposeful motor activity. Criteria for gradual arrests are identical except for a gradual (>2 sec) onset with transition from obvious, generally quiet, purposeful motor activity. Gradual arrests were discriminated from normal sleep onset by head "nodding" and lack of normal sleep posture. Coded videotapes of 12 *orexin*$^{-/-}$, 14 *OX2R*$^{-/-}$, and 20 wild-type littermates (males, 12-13 weeks old) were scored by each of two independent observers blinded to genotype. Identification of behavioral episodes required agreement between both observers with exclusion of ambiguous episodes (<2% of all episodes). No behavioral arrests fulfilling the above criteria were identified in wild-type mice, confirming the specificity of scoring criteria. Statistical significance was assessed by two-tailed unpaired Student's t-test.

Pharmacological Studies

Orexin$^{-/-}$ or *OX2R*$^{-/-}$ mice (n=7 per group, 4-5 months of age) were habituated to experimental conditions, including saline injections for 3-4 nights prior to the experiment. Mice were injected intraperitoneally (10 ml volume/kg body weight) with caffeine (0, 3, 10, or 30 mg/kg; Sigma), clomipramine-HCl (0 or 15 mg/kg; Sigma), or both (30 mg/kg caffeine, 15 mg/kg clomipramine) in saline just prior to onset of the dark phase. Immediately following each injection, mice were placed into cages with clear acrylic covers and filmed

individually (2 h/mouse). Each mouse received all doses of each drug (random cross-over design) over experimental sessions. Each session was separated by 2-3 days of washout during which mice were group-housed with littermates. Statistical significance was assessed by ANCOVA followed by the Tukey post-hoc test.

EEG/EMG Studies

For spontaneous sleep/wake studies, 5 *orexin*$^{-/-}$, 6 *OX2R*$^{-/-}$, and 14 wild-type control mice (males, 14-15 weeks old) were anesthetized and surgically implanted for long-term EEG/EMG monitoring as described previously (Chemelli et al., 1999). Animals were housed in a 12-h light:dark cycle at 25˚C and habituated to recording conditions for 2 weeks. Mice were recorded in matched groups for 3 consecutive days, beginning from lights-on (07:00). Food and water were replenished at 07:00 when needed, and mice were not otherwise disturbed. EEG/EMG signals were amplified using Grass Model 78 amplifiers (Grass Instruments, West Warwick, RI), filtered (EEG: 0.3-10 Hz, EMG: 30-300 Hz), digitized (sampling rate of 250 Hz), displayed on a paperless polygraph system, and archived to CD-R for off-line analysis. EEG/EMG records were visually scored according to standard criteria of rodent sleep (Radulovacki et al., 1984) and further analyzed using custom software. Statistical significance was assessed by repeated measures ANOVA followed by the Tukey post-hoc test.

For simultaneous infrared video/EEG/EMG monitoring, *orexin*$^{-/-}$ (n=5), *OX2R*$^{-/-}$ (n=5), and wild-type (n=3) mice (males, 6-8 months of age) were implanted and maintained as above. Mice were food restricted (food intake was limited to the first 4 h of the dark phase

for at least 7 days prior to experimental sessions) to promote exploratory behavior that maximizes arrest frequency (J.T.W., unpublished data). During each experiment, individual tethered mice were transferred from home cages to a modified open field apparatus with a rotating counter-balanced arm allowing free exploration of the arena. I recorded EEG/EMG traces and time-locked infrared video footage from multiple angles from the beginning of the dark phase. Additionally, samples of normal non-REM and REM sleep periods were collected from each mouse for comparison. Power spectral analysis of EEG signals was performed using custom FFT software. Comparison of spectral curves was performed by Pearson correlation test.

DISPLAY ITEMS

Table 2-1. Behaviors Preceding Abrupt Arrests in Orexin Knockout Mice

	Individuals												Group	
	A2	B1	F1	D1	S2	M2	W2	S1	C2	B2	X1	W1	Number	Percent (%)
Feeding	0	0	0	0	0	0	0	0	1	0	0	0	1	0.4
Drinking	0	0	0	0	0	0	0	0	0	0	1	0	1	0.4
Ambulating	77	26	9	10	10	10	10	1	3	2	1	1	160	68.1
Grooming	5	2	8	6	4	1	4	8	3	3	1	2	47	20.0
Burrowing	0	0	1	0	0	7	0	0	0	0	0	0	8	3.4
Climbing	0	0	1	2	1	0	0	0	1	0	0	0	5	2.1
Quiet Waking	6	0	0	1	3	0	2	0	0	1	0	0	13	5.5
Total Arrests	88	28	19	19	18	18	16	9	8	6	3	3	235	100.0

Behavioral activities in the 5 sec preceding arrests were categorized from video records by an observer that was blinded to genotype. Quiet waking includes periods of relative inactivity lacking predominant conspicuous behaviors.

Table 2-2. Behaviors Preceding Abrupt Arrests in OX2R Knockout Mice

	Individuals														Group	
	N	D	G	V	P	X	Q	B	H	Y	C	L	I	K	Number	Percent (%)
Feeding	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
Drinking	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	11.1
Ambulating	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2	22.2
Grooming	1	0	0	0	1	0	0	0	0	0	0	0	0	0	2	22.2
Burrowing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
Climbing	0	1	1	1	0	1	0	0	0	0	0	0	0	0	4	44.4
Quiet Waking	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
Total Arrests	3	2	1	1	1	1	0	0	0	0	0	0	0	0	9	100.0

Behavioral activities in the 5 sec preceding arrests were categorized from video records by an observer that was blinded to genotype. Quiet waking includes periods of relative inactivity lacking predominant conspicuous behaviors.

Table 2-3. Behaviors Preceding Gradual Arrests in Orexin Knockout Mice

	Individual												Group	
	A2	B1	F1	D1	S2	M2	W2	S1	C2	B2	X1	W1	Number	Percent (%)
Feeding	0	0	0	0	0	0	0	0	0	0	1	0	1	1.8
Drinking	0	0	0	0	0	0	0	0	0	0	1	0	1	1.8
Ambulating	0	0	2	0	0	0	1	0	3	0	4	0	10	17.9
Grooming	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
Burrowing	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
Climbing	0	0	0	0	0	0	0	0	3	0	4	0	7	12.5
Quiet Waking	0	0	12	4	3	0	0	3	7	0	7	1	37	66.1
Total Arrests	0	0	14	4	3	0	1	3	13	0	17	1	56	100.0

Behavioral activities in the 5 sec preceding arrests were categorized from video records by an observer that was blinded to genotype. Quiet waking includes periods of relative inactivity lacking predominant conspicuous behaviors.

Table 2-4. Behaviors Preceding Gradual Arrests in OX2R Knockout Mice

	Individual														Group	
	N	D	G	V	P	X	Q	B	H	Y	C	L	I	K	Number	Percent (%)
Feeding	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1.4
Drinking	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
Ambulating	2	6	4	0	1	2	1	4	0	0	1	0	0	0	21	30.0
Grooming	1	0	0	0	0	0	1	0	0	0	0	0	0	0	2	2.9
Burrowing	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1.4
Climbing	1	4	3	1	0	0	0	1	0	0	0	0	0	0	10	14.3
Quiet Waking	1	6	1	2	1	0	20	0	2	2	0	0	0	0	35	50.0
Total Arrests	5	16	8	3	2	2	23	5	2	2	1	1	0	0	70	100.0

Behavioral activities in the 5 sec preceding arrests were categorized from video records by an observer that was blinded to genotype. Quiet waking includes periods of relative inactivity lacking predominant conspicuous behaviors.

Table 2-5. EEG/EMG Correlates of Behavioral Arrests Observed during Exploration of Open Field Arena

	Gradual arrests		Abrupt arrests	
	***Orexin*$^{-/-}$**	***OX2R*$^{-/-}$**	***Orexin*$^{-/-}$**	***OX2R*$^{-/-}$**
Non-REM sleep	33/53 (62%)	40/40 (100%)	0/76 (0%)	0/10 (0%)
Non-REM sleep + rapid progression to REM sleep*	20/53 (38%)	0/40 (0%)	0/76 (0%)	0/10 (0%)
REM sleep	0/53 (0%)	0/40 (0%)	61/76 (80%)	4/10 (40%)
Ambiguous/pre-REM sleep	0/53 (0%)	0/40 (0%)	15/76 (20%)	6/10 (60%)

Behavioral arrests recorded from 5 mice of each genotype were categorized according to EEG/EMG features. No arrests were observed in wild-type controls.

*REM sleep following non-REM sleep within less than 60 seconds after onset of arrest (see text for details).

Table 2-6. Sleep/Wakefulness Parameters Recorded from *OX2R* Knockout, *Orexin* Knockout, and Wild-type Control Mice

	REM sleep			Non-REM sleep			Awake		
	OX2R-/-	Orexin-/-	Wild-type	OX2R-/-	Orexin-/-	Wild-type	OX2R-/-	Orexin-/-	Wild-type
24 hours									
Time (min)	81.11±3.78	87.67±5.92	78.84±2.89	742.2±21.6	756.3±23.5	706.5±17.1	613.9±21.8	593.3±22.7	655.0±17.4
Light (rest) phase									
Time (min)	51.72±3.75	45.98±4.47	52.62±1.59	421.2±10.9	435.7±7.29	428.6±5.76	245.8±10.7	237.0±7.31	237.2±6.14
Duration (min)	1.23±0.08	1.22±0.04	1.27±0.03	4.75±0.26	5.21±0.36	5.48±0.16	3.19±0.22	3.88±0.67	4.23±0.31
Frequency (episodes/h)	2.72±0.10# (p=0.034)	**2.21±0.04* (p=0.017)**	2.70±0.07	5.11±0.21	4.94±0.01	4.49±0.13	**3.70±0.13* (p=0.0050)**	**3.27±0.03* (p=0.044)**	2.34±0.16
REM latency (min)	8.02±0.46	**7.01±0.51* (p=0.0069)**	9.22±0.35						
Inter-REM interval (min)	17.35±0.61	19.50±1.67	17.56±0.77						
Dark (active) phase									
Time (min)	29.39±1.18# (p=0.0076)	**41.69±2.70* (p=0.0002)**	23.84±1.83	321.0±13.0	320.6±21.7	277.6±15.0	368.2±13.8	356.4±23.2	417.3±16.1
Duration (min)	1.24±0.09	1.41±0.05	1.34±0.03	**3.48±0.36* (p=0.0020)**	**3.23±0.34* (p=0.0007)**	4.74±0.12	**3.97±0.30* (p=0.015)**	**4.33±1.16* (p=0.037)**	8.43±0.95
Frequency (episodes/h)	1.64±0.12	**2.10±0.04* (p=0.012)**	1.14±0.10	**5.52±0.35* (p=0.0006)**	**5.46±0.03* (p=0.0013)**	3.25±0.18	**4.94±0.41* (p=0.0002)**	**4.67±0.04* (p=0.0002)**	2.61±0.14
REM latency (min)	**6.05±0.39* (p=0.0006)**	**5.32±0.65* (p=0.0002)**	8.51±0.29						
Inter-REM interval (min)	27.31±2.35	**21.92±2.05* (p=0.028)**	42.35±4.97						

Total time spent in each state (min), episode duration (min), episode frequency (per hour), REM latency (min), and interval between successive REM sleep episodes (min) over light and dark periods. Data are presented as means±SEM. Statistical significance was tested by repeated measures ANOVA followed by Tukey post-hoc test. *Significant differences between *OX2R*$^{-/-}$ and wild-type groups, or between *orexin*$^{-/-}$ and wild-type groups. Associated values are also shown in bold for clarity. #Significant differences between *OX2R*$^{-/-}$ and *orexin*$^{-/-}$ groups.

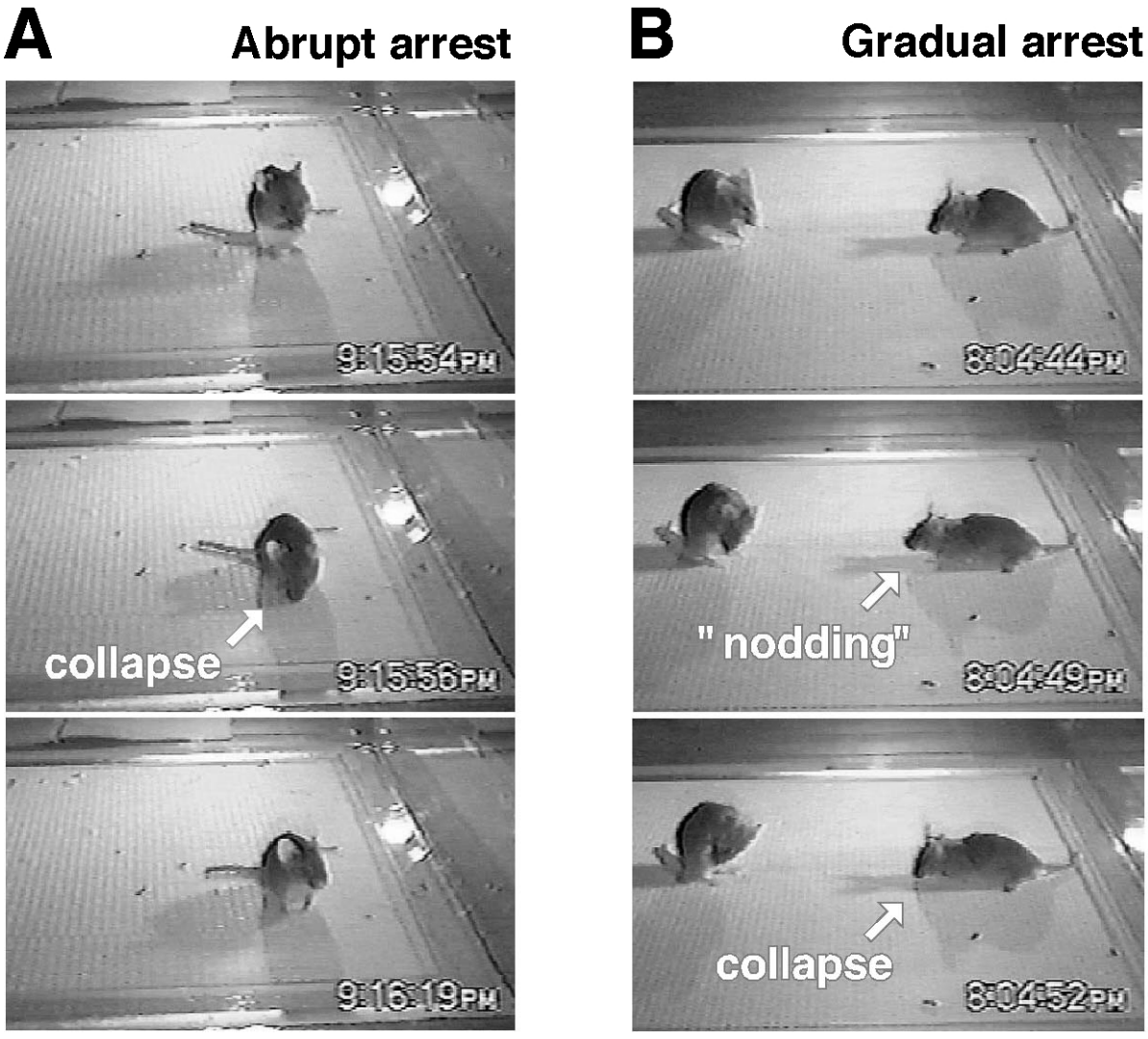
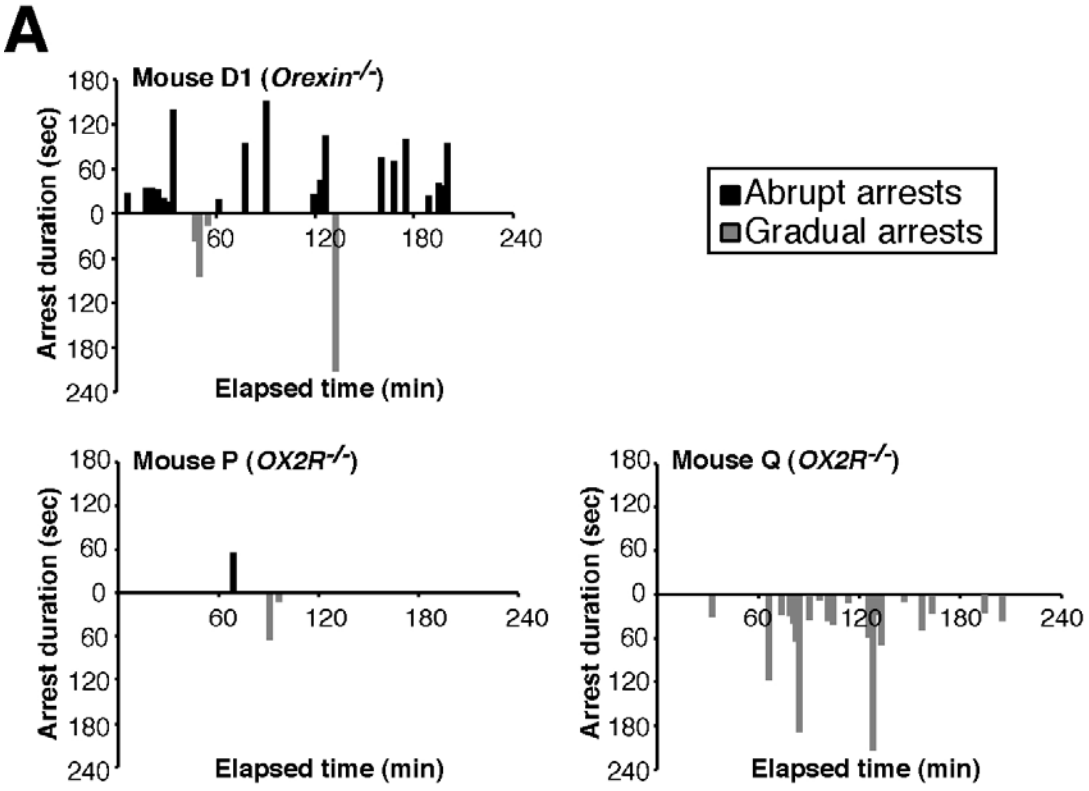

A
Abrupt arrest
9:15:54PM
collapse
9:15:56PM
9:16:19PM
B
Gradual arrest
8:04:44PM
" nodding"
8:04:49PM
collapse
8:04:52PM

Figure 2-1. Two Distinct Classes of Behavioral Arrests in Knockout Mice

(A) Time lapse infrared videophotography images portraying an abrupt arrest, rarely observed in an *OX2R*$^{-/-}$ mouse. Note collapsed posture in the second panel. View video footage of the entire arrest at http://www.neuron.org/cgi/content/full/38/5/715/DC1.

(B) Time lapse images portraying a gradual arrest in an *OX2R*$^{-/-}$ mouse. "Nodding" behavior (second panel) occurs just prior to postural collapse (third panel). View video footage of the entire arrest at http://www.neuron.org/cgi/content/full/38/5/715/DC1.


A
Mouse D1 (Orexin-/-)
Arrest duration (sec)
Elapsed time (min)
Abrupt arrests
Gradual arrests
Mouse P (OX2R-/-)
Mouse Q (OX2R-/-)

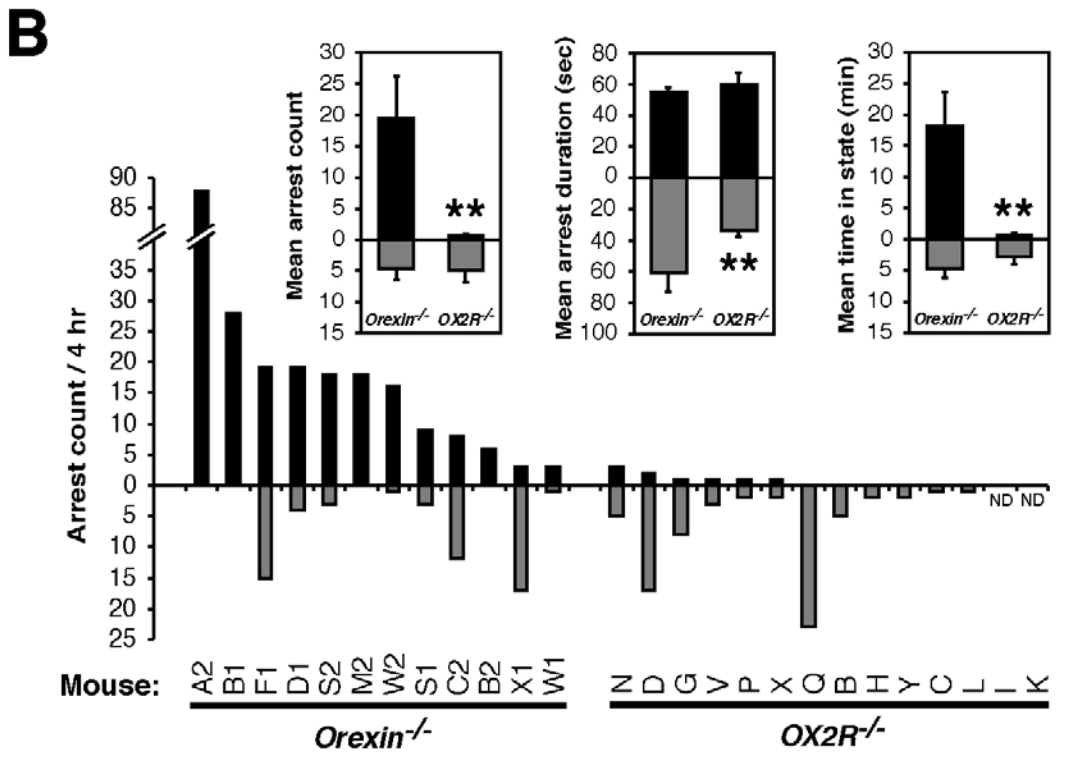

B
Arrest count / 4 hr
Mean arrest count
Mean arrest duration (sec)
Mean time in state (min)
Orexin-/-
OX2R-/-
ND ND
Mouse:
A2 B1 F1 D1 S2 M2 W2 S1 C2 B2 X1 W1
N D G V P X Q B H Y C L _ K
Orexin-/-
OX2R-/-

Figure 2-2. Comparison of Behavioral Arrests in Knockout Mice by Infrared Videophotography

(A) Behavior of typical *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ knockout mice during the first 4 h of the dark phase. Durations of individual behavioral arrests and occurrence over time from onset of dark phase are portrayed. Abrupt and gradual behavioral arrests are shown as upward solid and downward gray deflections along the Y-axis, respectively.

(B) Arrest frequencies of *orexin*$^{-/-}$ compared to *OX2R*$^{-/-}$ mice during the first 4 h of the dark phase. Bars represent arrest counts recorded for each mouse with abrupt and gradual arrests shown as upward solid and downward gray bars, respectively. Insets display frequencies, durations, and total times of arrests as means and SEM. No arrests of any kind were observed in wild-type littermate controls (not shown). ND, none detected; **, $p<0.005$ compared to *orexin*$^{-/-}$ mice. Video records were scored with the assistance of R.M. Chemelli and S. Tokita

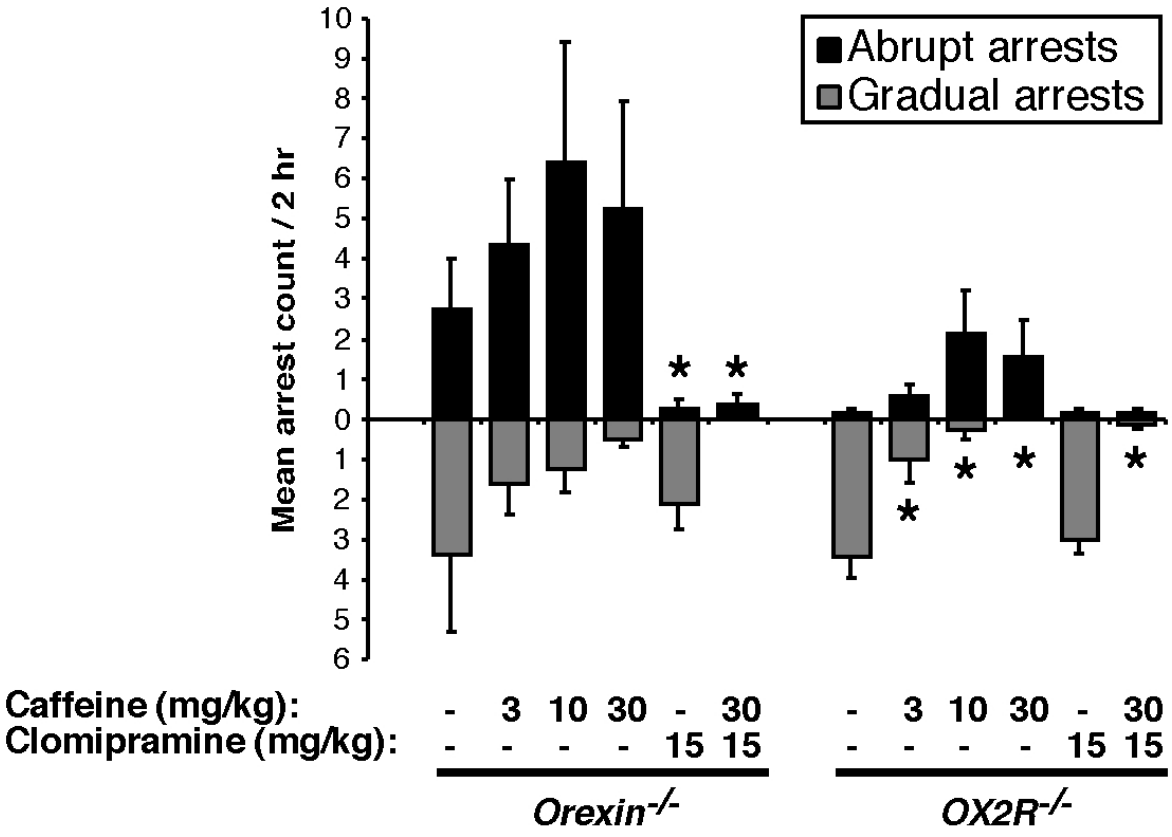


Figure 2-3. Suppression of Behavioral Arrests by Anti-Narcoleptic Drugs

Frequency of abrupt arrests (upward solid bars) and gradual arrests (downward grey bars) in knockout mice following i.p. administration of caffeine and/or clomipramine compared to vehicle administration. *, $p<0.05$ compared to vehicle.

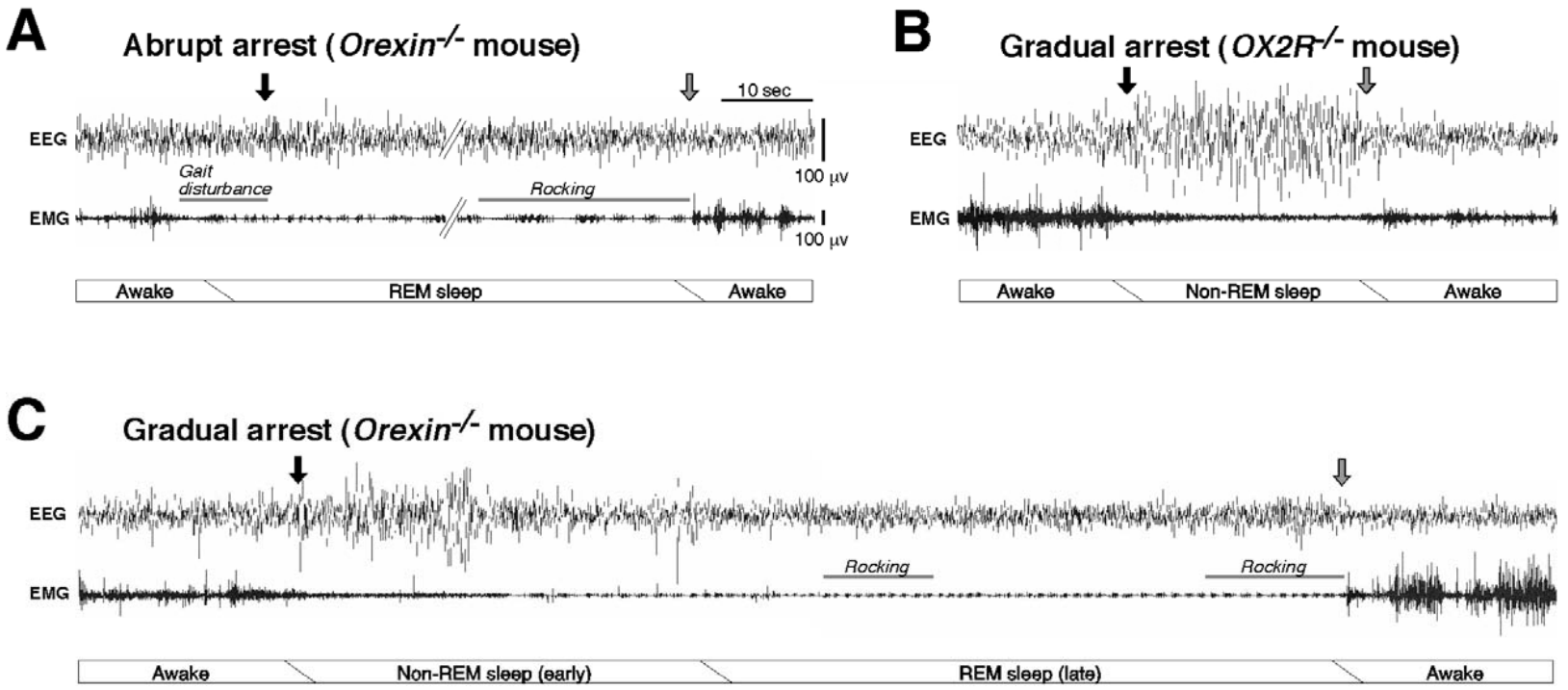

A
Abrupt arrest (Orexin-/- mouse)
10 sec
EEG
EMG
Gait disturbance
Rocking
100 μv
100 μv
Awake
REM sleep
Awake
B
Gradual arrest (OX2R-/- mouse)
EEG
EMG
Awake
Non-REM sleep
Awake
C
Gradual arrest (Orexin-/- mouse)
EEG
EMG
Rocking
Rocking
Awake
Non-REM sleep (early)
REM sleep (late)
Awake

Figure 2-4. EEG/EMG Correlates of Two Classes of Behavioral Arrests

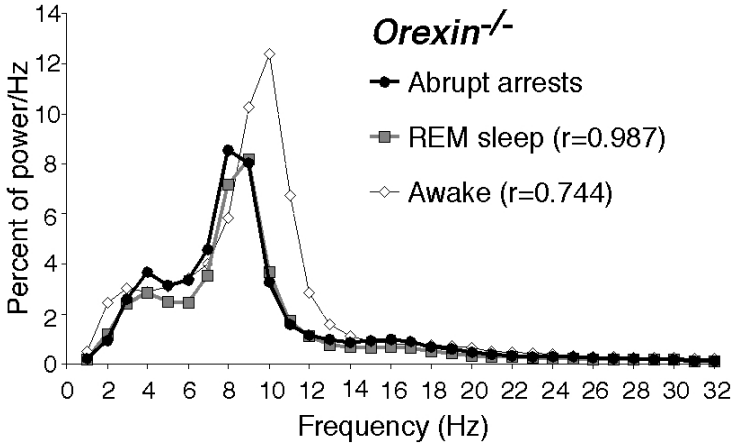
(A-C) Typical EEG/EMG traces during behavioral arrests in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice. View corresponding video footage of episodes at http://www.neuron.org/cgi/content/full/38/5/715/DC1. Solid and gray arrows demarcate onsets and terminations of arrests, respectively. Gray bars reflect timing of gait disturbances and rocking behavior associated with arrests (Chemelli et al., 1999). Time and voltage scale bars apply to all traces. Behavioral states are classified as awake, non-REM sleep, or REM sleep based on EEG/EMG features.

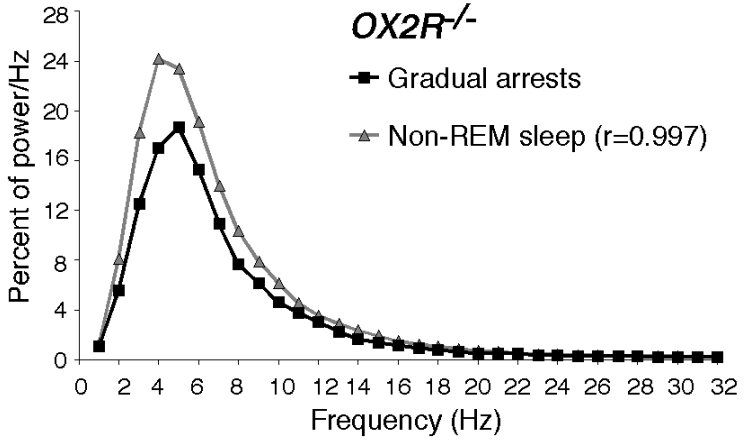
(A) Abrupt arrest in *orexin*$^{-/-}$ mouse. Excited ambulation (high amplitude nuchal EMG) accompanied by an EEG typical of normal active wakefulness (low amplitude, mixed frequency activity) gives way to rapid onset of ataxic gait, reduced neck tone, and an EEG resembling REM sleep. Postural collapse is accompanied by neck atonia and continued REM sleep EEG. Rocking behavior from limb movement occurs exclusively during periods with EEG/EMG indistinguishable from REM sleep pattern. Residual low amplitude noise remaining in the EMG during atonia consists primarily of electrocardiographic contamination.

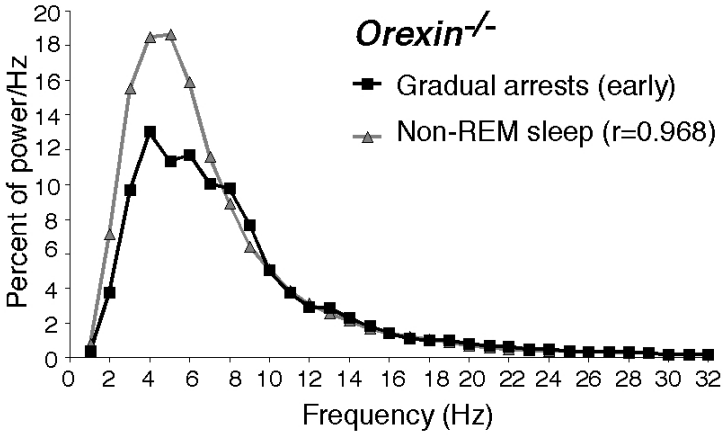
(B) Gradual arrest in *OX2R*$^{-/-}$ mouse. Feeding behavior with high amplitude EMG and a waking EEG give way to gradual onset of postural collapse with reduced but not atonic nuchal EMG and transition to an EEG indistinguishable from non-REM sleep. The mouse remains immobile until sudden recovery of waking EEG and purposeful behavior.

(C) Gradual arrest in *orexin*$^{-/-}$ mouse. Quiet wakeful behavior gives way to gradual arrest onset with transition to non-REM sleep as in (B). Note, however, the presence of a pre-REM

spindle (Chemelli et al., 1999) at arrest onset (solid arrow), rapid transition to atonia with occasional jerks, and REM sleep EEG shortly after arrest onset. Rocking behavior occurs during REM sleep EEG as in (A). This arrest ends suddenly after vigorous rocking.


Orexin-/-
Abrupt arrests
REM sleep (r=0.987)
Awake (r=0.744)
Percent of power/Hz
Frequency (Hz)


OX2R-/-
Gradual arrests
Non-REM sleep (r=0.997)
Percent of power/Hz
Frequency (Hz)


Orexin-/-
Gradual arrests (early)
Non-REM sleep (r=0.968)
Percent of power/Hz
Frequency (Hz)

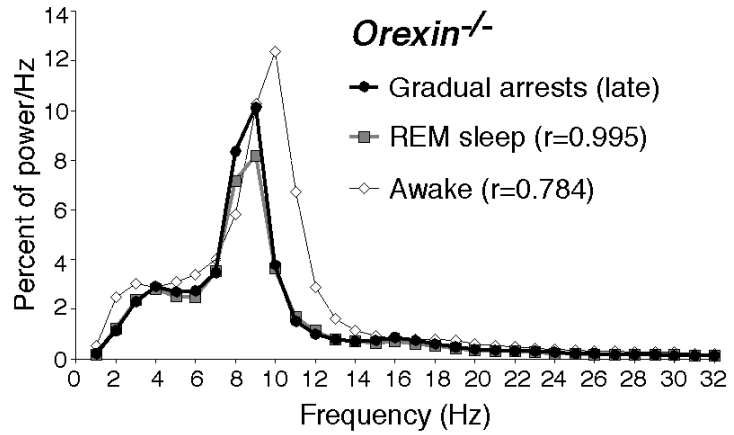
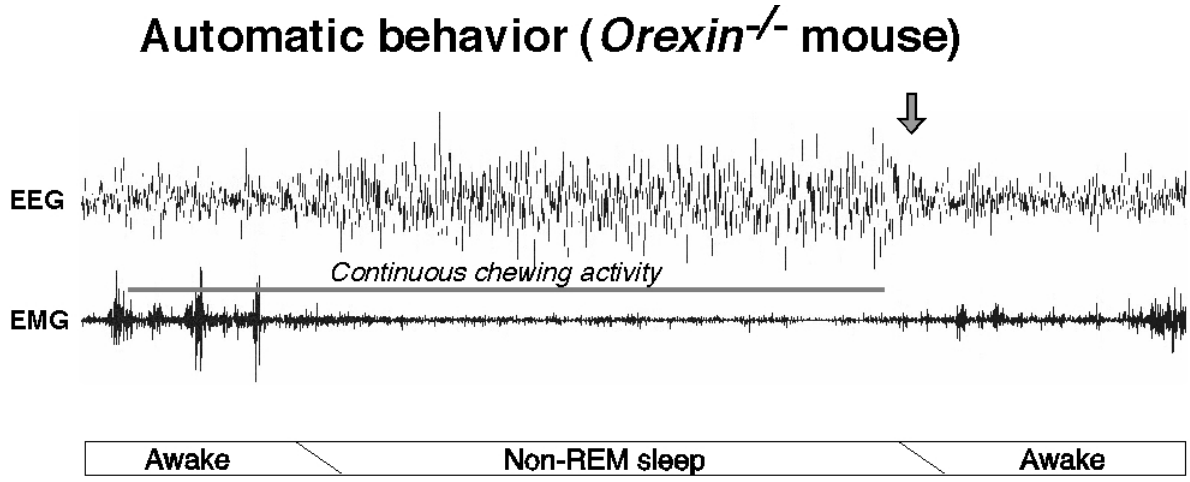

Orexin-/-
Gradual arrests (late)
REM sleep (r=0.995)
Awake (r=0.784)
Percent of power/Hz
Frequency (Hz)

Figure 2-5. Power Spectral Analysis of Behavioral Arrests

Power spectral analysis of EEG by FFT during behavioral arrests compared to normal vigilance states. Panels reflect mean spectra of designated episodes, generated for each of 5 mice per genotype, and then averaged again for each genotype. Correlation coefficients (r) demonstrate the degree to which arrest spectra resemble those of normal sleep/wake states from the same mice.


Automatic behavior (Orexin-/- mouse)
EEG
EMG
Continuous chewing activity
Awake
Non-REM sleep
Awake

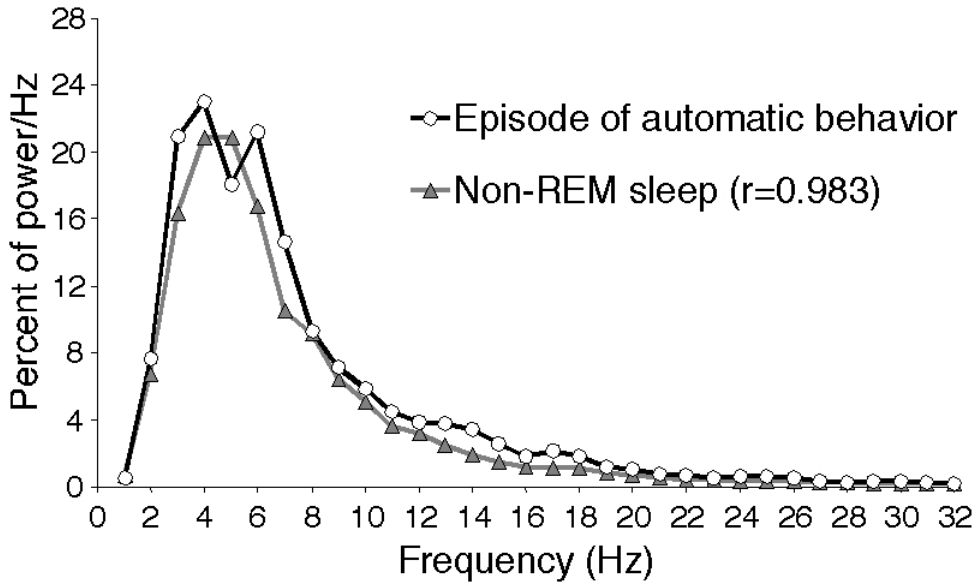
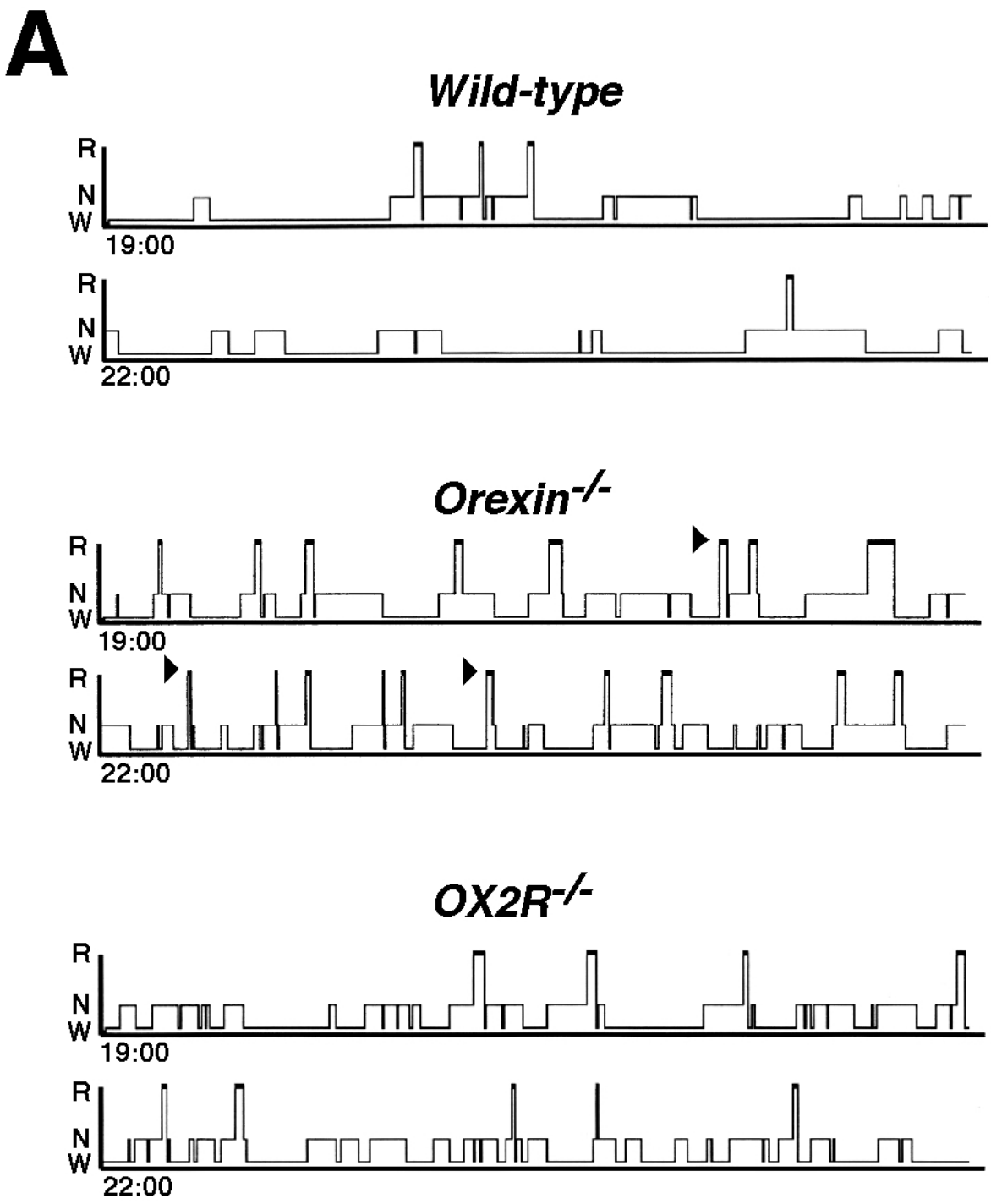

Percent of power/Hz
Frequency (Hz)
Episode of automatic behavior
Non-REM sleep (r=0.983)
0
4
8
12
16
20
24
28
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32

Figure 2-6. Automatic Behavior in Association with a Gradual Arrest in *Orexin*$^{-/-}$ Mouse

Semi-purposeful motor activity typical of wakefulness (chewing of food) continues despite onset of unambiguous non-REM sleep. This prolonged dissociated state continues unabated for 42 sec prior to the sudden termination the episode and resumption of normal wakefulness. View video footage of the entire arrest at http://www.neuron.org/cgi/content/full/38/5/715/DC1. Spectral analysis of this episode illustrates the high correlation with normal non-REM sleep in the subject. Similar brief episodes of automatic behavior episodes were observed to interrupt refeeding periods following food-deprivation in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice, but never in wild-type mice. Gray arrow demarcates termination of arrest.


A
Wild-type
R
N
W
19:00
R
N
W
22:00
Orexin-/-
R
N
W
19:00
R
N
W
22:00
OX2R-/-
R
N
W
19:00
R
N
W
22:00

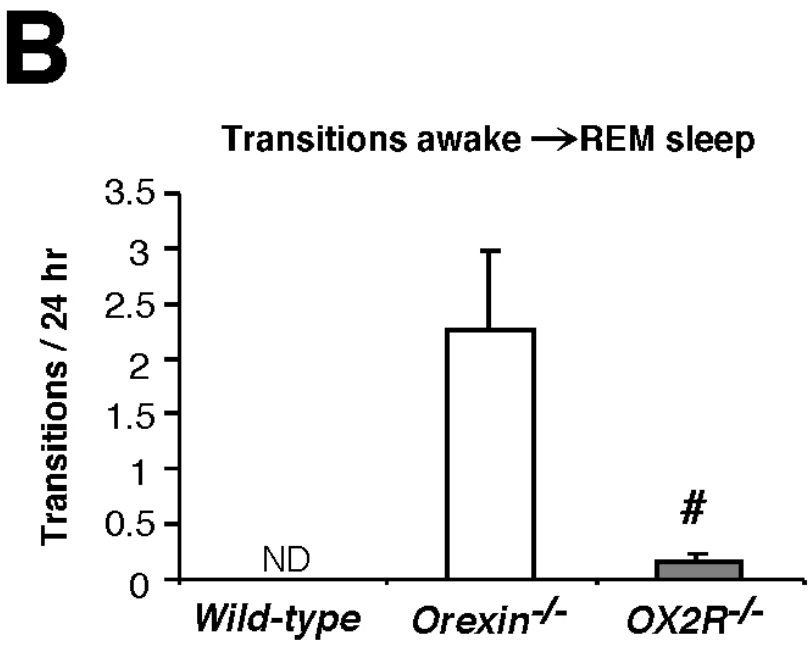
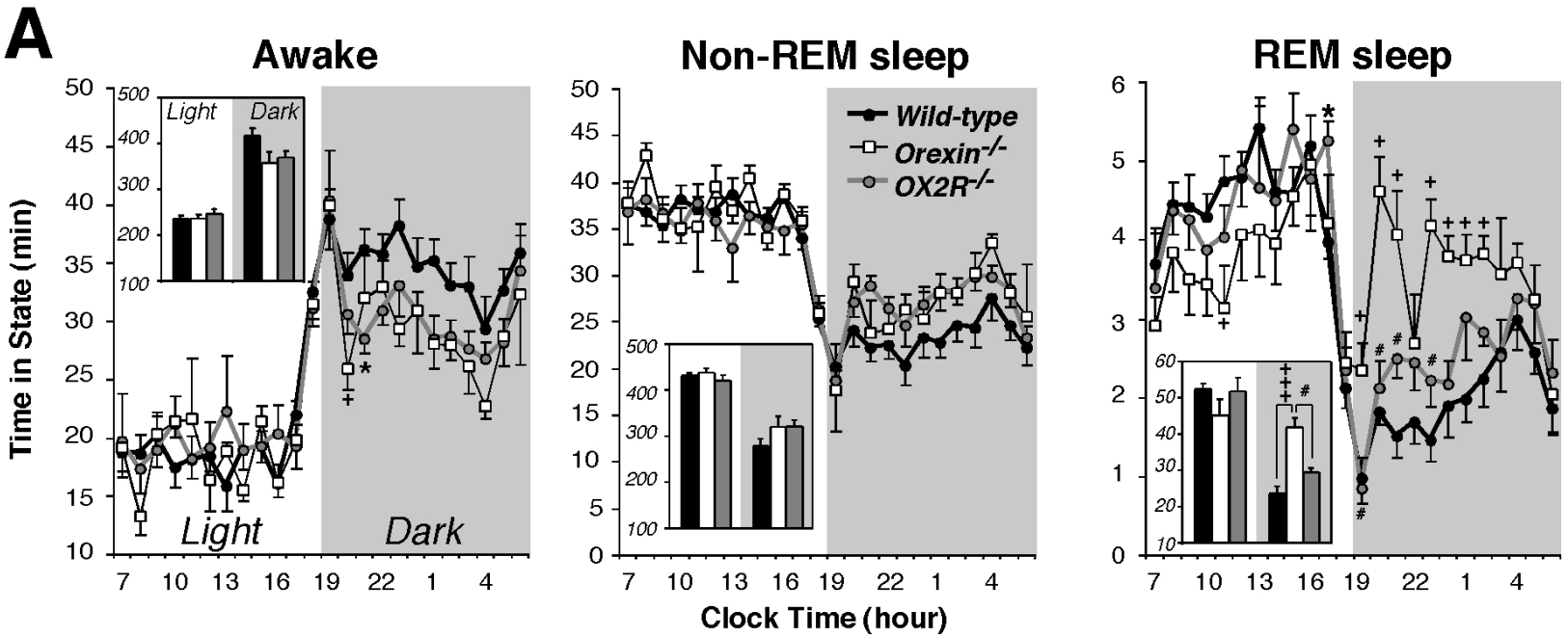


Figure 2-7. Comparison of Vigilance Patterns of Knockout Mice

(A) Representative hypnograms of wild-type, *orexin*$^{-/-}$, and *OX2R*$^{-/-}$ mice over the first 6 h of the dark phase (19:00 to 25:00) obtained by concatenating 20 s epoch EEG/EMG stage scores. The height of the horizontal line above baseline indicates the vigilance state of the mouse at the time (min) from the beginning of the dark phase. Baseline, W, represents periods of wakefulness; N, non-REM sleep; R, REM sleep. Arrowheads highlight direct transitions from wakefulness to REM sleep. *Orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice show similar fragmentation of vigilance.

(B) Frequency per day of direct transitions from wakefulness to REM sleep in each genotype. Means and SEM are shown.


A
Awake
Non-REM sleep
REM sleep
Time in State (min)
Clock Time (hour)
Light
Dark
Wild-type
Orexin-/-
OX2R-/-

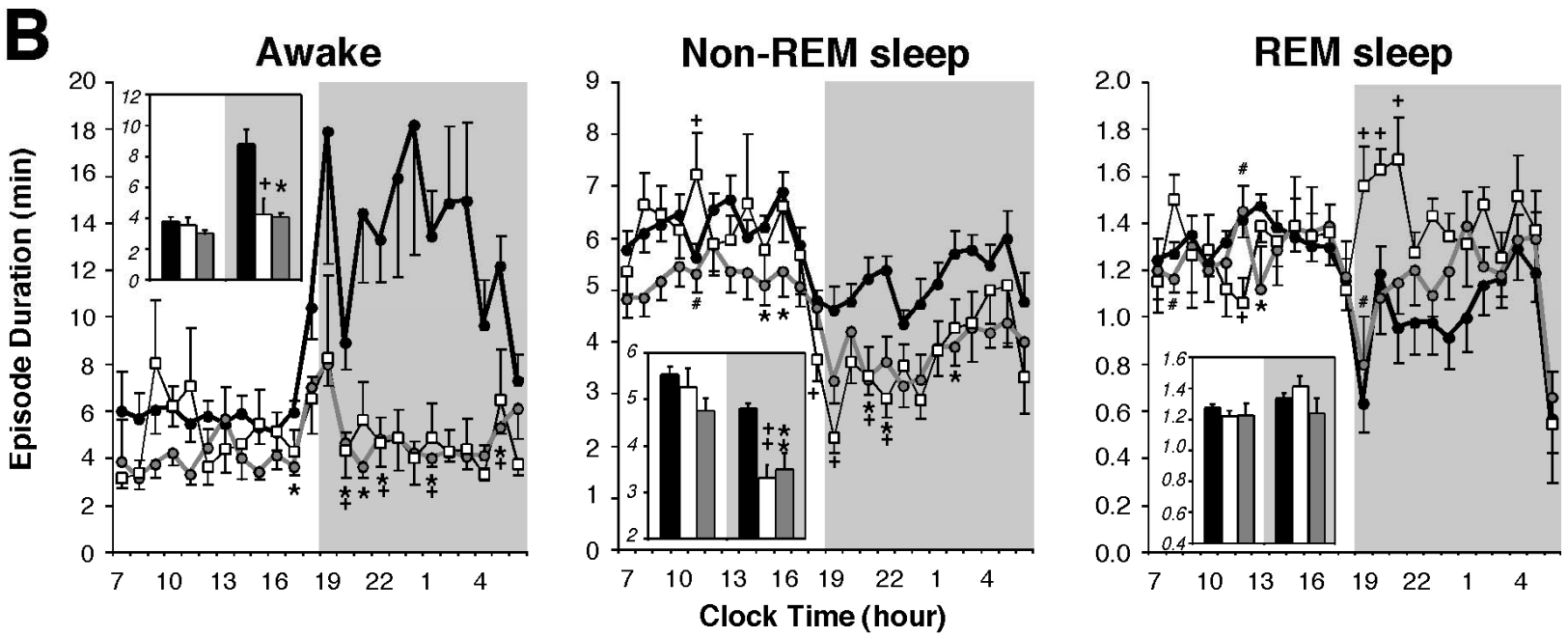

B
Awake
Non-REM sleep
REM sleep
Episode Duration (min)
Clock Time (hour)

Figure 2-8. Hourly Analysis of Vigilance States of Knockout Mice

Hourly amounts (**A**) and durations (**B**) of awake, non-REM sleep, and REM sleep states (means and SEM) plotted over the 24-h day for each group. Data collapsed over 12-h light and dark phases are displayed as graph insets. Data for the light and dark phases are displayed on white and light gray backgrounds, respectively. Wakefulness and non-REM sleep are disrupted to a similar degree in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice, especially during the dark phase. *Orexin*$^{-/-}$ mice consistently show significantly increased REM sleep times during the dark phase compared to normal mice; *OX2R*$^{-/-}$ mice do not. Significant differences between *OX2R*$^{-/-}$ and wild-type mice: *$p<0.05$, **$p<0.005$. Significant differences between *orexin*$^{-/-}$ and wild-type mice: +$p<0.05$, ++$p<0.005$, +++$p<0.0005$. Significant differences between *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice: #$p<0.05$. EEG records were collected by the author and scored with the assistance of C. M. Sinton.

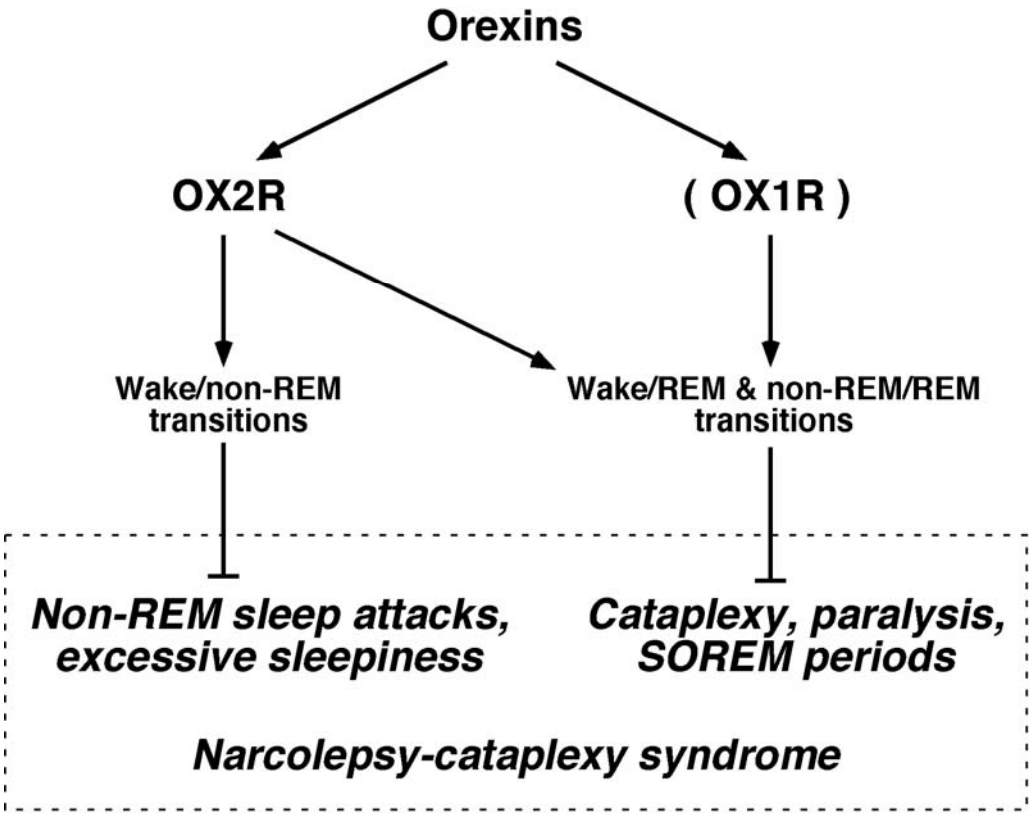


Figure 2-9. Model Depicting the Role of the Orexin Pathways in Regulating Transitions among Vigilance States

Whereas *Orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice are equally affected with attacks of non-REM sleep, *OX2R*$^{-/-}$ mice are much less severely affected with cataplexy, paralysis, and SOREM periods. Intact orexin-OX2R signaling provides proper regulation of transitions between wakefulness and non-REM sleep, thus preventing non-REM sleep attacks. In contrast, both OX2R-dependent and OX2R-independent mechanisms play critical roles in maintaining normal transitions between non-REM and REM sleep, and preventing transitions from wakefulness to REM sleep, which lead to cataplexy and other REM-associated symptoms of narcolepsy. Orexin-OX1R signaling may be implicated in such OX2R-independent REM sleep control.

CHAPTER THREE
Role of Orexin in Coordinating Energy Balance with Arousal

SUMMARY

Mammals respond to reduced food availability by becoming more wakeful and active, yet the central pathways regulating arousal and instinctual motor programs (such as food seeking) according to homeostatic need are not well understood. Our group has demonstrated that hypothalamic orexin neurons directly monitor indicators of energy balance. Here I present evidence that orexin neurons mediate adaptive augmentation of arousal in response to fasting. Transgenic mice, in which orexin neurons are ablated, fail to respond to fasting with increased wakefulness and activity. These findings indicate that orexin neurons provide a crucial link between energy balance and arousal.

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INTRODUCTION

Motivated behaviors such as food seeking are critically dependent upon arousal pathways. Periods of foraging and rest are essential but mutually exclusive behaviors that require appropriate coordination and cycling at environmentally advantageous times and in response to homeostatic needs. When faced with negative energy balance due to reduced food availability, mammals respond behaviorally with phases of increased

wakefulness and locomotor activity that support food seeking (Borbely, 1977; Challet et al., 1997; Danguir and Nicolaidis, 1979; Dewasmes et al., 1989; Itoh et al., 1990; Williams et al., 2002). The molecular and physiological basis of this highly conserved phenomenon remains poorly understood.

As discussed in **Chapter 1**, orexins are expressed by a specific population of neurons in the lateral hypothalamic area (LHA), a region of the brain implicated in feeding, arousal, and motivated behavior. Orexin-containing neurons project from the LHA to numerous brain regions, with the limbic system, hypothalamus, and monoaminergic and cholinergic nuclei of brainstem receiving particularly strong innervations. Thus, the orexinergic system is anatomically well placed to influence the arousal, motivational, metabolic, autonomic, and motor processes necessary to elicit homeostatically appropriate behaviors.

When orexins are administered centrally to rodents, they are reported to elevate sympathetic tone, plasma corticosterone levels (Hagan et al., 1999), metabolic rate (Lubkin et al.,1998), food intake (Sakurai et al., 1998), locomotor activity (Nakamura et al., 2000), and wakefulness (Hagan et al., 1999). While these effects highlight the complexity of the orexin system and the responses it regulates, most of these effects resemble those observed in fasted animals (Itoh et al., 1990; Challet et al., 1997; Dewasmes et al., 1998). Indeed, dopamine antagonists attenuate increases in locomotor activity induced by either fasting or orexin administration in rodents (Nakamura et al., 2000; Itoh et al., 1990). Importantly, pharmacological antagonism of orexin receptor type 1 is associated with reduced food intake and weight reduction in rodents (Haynes et al.,

2002; Haynes et al., 2000). These observations are all consistent with the hypothesis that orexin neurons provide a link between metabolic status and motivated behavior.

Human narcolepsy, a disorder resulting from destruction of orexin neurons, is also associated with metabolic abnormalities, including increased frequency of non insulin-dependent diabetes mellitus and increased body-mass index (Honda et al., 1986; Nishino et al., 2001; Schuld et al., 2002; Schuld et al., 2000). Complex disruptions of energy homeostasis in *prepro-orexin* knockout and *orexin/ataxin-3* transgenic mice are indicated by hypophagia, obesity, and inactivity relative to control littermates (Willie et al., 2001; Hara et al., 2001). Orexin neurons appear to be sensitive to nutritional status as prepro-orexin mRNA is upregulated in rats during fasting (Sakurai et al., 1998). Since insulin-induced hypoglycemia also increases orexin mRNA expression as well as expression by orexin neurons of c-Fos, a marker of neuronal activation, changes in circulating glucose concentration might directly or indirectly mediate some of these effects (Griffond et al., 1999; Moriguchi et al., 1999).

Our group has demonstrated that isolated orexin neurons are able to monitor humoral and neural indicators of energy balance in mice: orexin neurons are activated under conditions of low glucose, low leptin, and elevated ghrelin (Yamanaka et al., 2003a). To probe the physiological relevance of this interaction, C.T. Beuckmann and I show that the presence of orexin neurons is necessary for mice to respond appropriately to fasting, by increasing wakefulness and locomotor activity.

RESULTS

Orexin Neuron-Ablated Mice Fail to Increase Vigilance during Fasting

To examine whether orexin neurons are required for adaptive arousal responses to a metabolic stress, C.T. Beuckmann and I observed *orexin/ataxin-3* hemizygous transgenic mice, in which orexin neurons are specifically ablated (Hara et al., 2001), under food-deprived conditions. We recorded states of wakefulness, non-REM sleep, and REM sleep during food deprivation by continuously monitoring simultaneous electroencephalographic and electromyographic (EEG/EMG) traces from minimally restrained *orexin/ataxin-3* transgenic mice and weight-matched wildtype littermates. After baseline (ad lib fed) recording across dark and light phases, animals were fasted beginning with onset of the dark phase and recorded for an additional 30 hours.

Hourly analysis of sleep/wake states revealed significant increases in wakefulness of food-deprived wildtype mice compared to all other experimental groups: fed wildtype mice, fed *orexin/ataxin-3* transgenic mice, and fasted *orexin/ataxin-3* mice (**Figure 3-1**). Within hours of food removal, wildtype mice exhibited mild intermittent augmentation of arousal during the dark phase. Continued fasting resulted in a robust increase in arousal during the following light phase. However, *orexin/ataxin-3* mice exhibited no appreciable increase in vigilance during fasting. Similarly, wildtype mice, but not *orexin/ataxin-3* mice, showed significant reductions in amounts of non-REM sleep when fasted. Thus, orexin neuron-ablated animals failed to show normal fasting-induced augmentation of vigilance.

In response to fasting, normal rodents exhibit reduced amounts of REM sleep and an increased latency to REM sleep (Dewasmes et al., 1989), defined as the mean time elapsed between sleep onset and onset of subsequent REM sleep. In contrast to the fasting-induced changes in wakefulness and non-REM sleep that were observed exclusively in wildtype mice, REM sleep was significantly suppressed in both wildtype and *orexin/ataxin-3* mice under fasted conditions. Although narcoleptic mice exhibited high baseline levels of REM sleep during the dark phase compared to normal mice as previously described (Chemelli et al., 1999; Hara et al., 2001), fasting REM sleep amounts of both groups were relatively depressed in the latter light phase and the following period of darkness. Even before fasting-induced changes in amounts of wakefulness, non-REM sleep, or REM sleep were detected, we noted a significant increase in mean latency to REM sleep during the dark phase (hours 0-12 of fasting) in wildtype mice (7.9±0.5 min versus 12.2±1.8 min for fed and fasted states, respectively; $p=0.006$), but not in orexin neuron-deficient mice (2.5±0.3 min versus 4.5±0.7 min). This effect was even more pronounced during the following light phase (hours 12-24 of fasting) in wildtype mice (9.2±0.5 min versus 13.7±0.7 min; $p=0.0001$), but again not in orexin neuron-deficient mice (4.7±0.3 min versus 5.9±0.7 min). This indicates that orexin neurons are also required for inhibition of REM sleep onset under fasted conditions.

Orexin Neuron-Ablated Mice Fail to Exhibit Increased Exploration during Fasting

Changes in sleep/wake states in fasting mice may indicate changes in activity and foraging behavior given environmental conditions in which food might be found. Presented with a novel environment, mice normally exhibit an exploratory phase with

high levels of motor activity followed by habituation and resumption of normal circadian activity patterns (Valentinuzzi et al., 2000). We reasoned that an increased drive toward food seeking behavior in fasting mice would result in increased exploratory activity when fasted mice are presented with the challenge of a novel environment. We examined whether orexin neuron-ablated mice might differ from normal mice in this response.

We monitored the distance traveled, stereotypic activity, and other motor parameters of *orexin/ataxin-3* transgenic and weight-matched wildtype littermates during exposures to a novel open field arena under fed and fasted conditions. Based on the findings in our fasting EEG/EMG studies, activity under baseline fed conditions and hours 24-31 of a fast, which encompassed segments of both light and dark phases, were determined by monitoring interruption of infrared light beams in the horizontal and vertical planes of the arena. We also measured body weights before and after fasting to examine metabolic responses to fasting in each genotype.

Under baseline fed conditions during the light phase, both wildtype and *orexin/ataxin-3* mice exhibited a robust exploratory response to novelty followed by a phase of complete habituation (defined operationally as the period in which activity no longer exhibited statistical change over time) by the third hour of the experiment (**Figure 3-2A**). With onset of the dark phase when mice are generally most active, fed mice of both genotypes exhibited normal timing of circadian increases in activity. In accordance with previous observations, the quantity of locomotor activity in fed *orexin/ataxin-3* mice was mildly reduced compared to normal controls (Hara et al., 2001).

Under conditions of food deprivation, wildtype mice exhibited a more pronounced prolongation of the exploratory phase than *orexin/ataxin-3* mice (**Figure 3-**

2A). During the time period in which mice of both genotypes were habituated under fed baseline conditions, statistical analysis that took into account the differences in baseline activity of each group (two-way ANOVA) revealed that the total activity count (horizontal plus vertical beam breaks) was significantly increased for fasting wildtype mice ($p=0.001$), but not fasting *orexin/ataxin-3* mice during the light phase (**Figure 3-2A**). Additionally, fasted wildtype mice, but not fasted *orexin/ataxin-3* mice, exhibited significantly increased vertical activity (rearing and jumping), ambulating time, stereotypic time, and horizontal distance traveled, as well as significantly reduced resting time during this period. None of these changes reached statistical significance for either genotype during the following dark phase, possibly due to high baseline activity during this period. Indeed, mice normally spend 95-100% of the first hour of the dark phase in wakefulness regardless of genotype or fed/fasting condition (**Figure 3-1**).

The deficient locomotor response of the orexin neuron-ablated mice was not due to a primary motor disability, since ambulating speed (distance traveled per ambulating time) did not differ from that of wildtype controls in either fed or fasted states (**Figure 3-2B**). Nor could the observed defects in behavioral adaptation be explained by brief episodes of cataplexy-like arrests that are observed in *orexin/ataxin-3* mice (Hara et al., 2001): filming of mice during the experiment revealed that time spent in these arrests (a tiny minority of overall experimental time) did not change significantly with fasting (fed: 239.0 +/- 112.2 sec/4 hr, fasted: 185.0 +/- 45.0 sec/4 hr). However, changes in body weight of transgenic (fed 25.4 +/- 0.5 g, fasted 20.1 +/- 0.4 g) and control wildtype mice (fed 24.5 +/- 0.2 g, fasted 18.8 +/- 0.2 g) induced by fasting did differ mildly but significantly by genotype ($p=0.05$), indicating that behavioral differences in these groups

are associated with differences in fasting metabolic rate. Overall, however, the robust behavioral changes observed in food-deprived wildtype mice are consistent with an adaptive exploratory response required for increased food-seeking behaviors and contrasts with the failure of *orexin/ataxin-3* transgenic mice to respond normally.

DISCUSSION

Orexin Neurons Link Regulation of Arousal and Energy Balance

In response to reductions in food availability, animals adapt acutely with increased vigilance and disruption of normal sleeping patterns (Dewasmes et al., 1989; Borbely, 1977; Danguir et al., 1979), a response that would presumably enhance the ability to find food in nature. Humans as well, show disruption of normal sleep/wake patterns in response to energy restriction from reductive dieting or in the pathological context of anorexia nervosa (Crisp et al., 1971; Karklin et al., 1994).

This study demonstrated significant increases of wakefulness in wildtype mice during food deprivation, especially in the light phase when ad lib fed mice are typically at rest. In contrast, orexin neuron-ablated animals failed to exhibit fasting-induced arousal under the same conditions. Previous studies in fasting rats demonstrated that sleep/wake disruptions are normalized by peripheral glucose infusions (Danguir et al., 1979). Moreover, peripheral leptin administration increases non-REM sleep in fed rats, an effect that is reversed by fasting (Sinton et al., 1999). These findings are all consistent with a model in which peripheral signals reflecting negative energy balance, such as reduced

plasma glucose and leptin, induce fasting-related arousal by triggering increased activity of orexin neurons. That these differences were most striking during the latter light phase may have resulted from time-dependent effects of fasting. It is also possible that this observation relates to the low baselines of orexin neuron activity and wakefulness associated with this period. In rats, orexin peptide levels in cerebrospinal fluid (CSF) or brain dialysates peak during the dark phase and are minimal at the end of the light phase, and CSF orexin levels are exclusively increased during late light phase following a 72 hr food deprivation (Fujiki et al., 2001; Yoshida et al., 2001). Together, with our studies, these results suggest that fasting-induced changes are most robust during periods in which orexin release would normally be low.

Fasting of rodents induces complex changes in metabolism and behavior, with periods of reduced energy expenditure and metabolic rate punctuated by periods of hyperactivity consistent with food seeking (Itoh, et al., 1990; Challet et al., 1997; Williams et al., 2002). Our findings corroborate these previous studies by showing that, under conditions of food deprivation, wildtype mice exhibit an increase in both horizontal (ambulation) and vertical (rearing/jumping) locomotor activities compared to fed baselines in an assay of novelty-induced exploratory behavior. The robust fasting-induced increase of locomotor activity observed especially in the light phase is consistent with intensified foraging behaviors at the expense of normal rest and sleep. In contrast to wildtype mice, *orexin/ataxin-3* transgenic mice failed to respond normally with prolonged exploration and increased locomotor activity when food deprived. Thus, *orexin/ataxin-3* mice failed to exhibit the normal increase of both wakefulness and locomotor activity during fasting, illustrating the essential role of orexin neurons in these

behavioral adaptations. These abnormalities were also associated with evidence of subtle differences in fasting metabolic rate, as orexin-neuron deficient mice lost slightly less weight over the fasting period. Whether these effects were due primarily to differences in basal metabolic rate or secondarily from differences in energy consumption related to wakefulness and motor activity must be addressed by future studies combining indirect calorimetry with activity monitoring.

While the behavioral deficits we observed are most likely the result of absent orexin neuropeptide signaling following destruction of orexin neurons, it should be noted that these cells also produce dynorphin (Chou et al., 2001), and some may also contain galanin (Hakansson et al., 1999), angiotensin II (Hara et al. 2001), or unknown factors. Unlike orexin, however, the co-expressed factors listed above are not exclusively found in orexin neurons. Critically, *orexin/ataxin-3* transgenic and prepro-orexin knockout mice exhibit similar behavioral and metabolic phenotypes when genetic background is controlled (J. Hara and M. Mieda, unpublished observations).

Destruction of orexin neurons in human narcolepsy is accompanied not only by abnormal sleep/wake regulation but also by metabolic disruptions that have yet to be fully characterized (Honda et al., 1986; Schuld et al., 2000; Nishino et al., 2001). Our findings may also provide insight into physiological mechanisms that contribute to the anti-obesity and antidiabetic effects of a recently described, orally active orexin receptor antagonist (Haynes et al., 2002; Haynes et al., 2000). We suggest a model in which food deprivation triggers a drop in circulating glucose and leptin levels, which together lead to increased firing of orexin neurons. Ghrelin, too, may play an important role in this response. The activation of orexin neurons promotes wakefulness and locomotor activity,

stabilizing highly vigilant states required for food-seeking behaviors. Simultaneously, orexin neurons may activate other hypothalamic orexigenic mechanisms, such as those mediated by NPY (Yamanaka et al., 2000) or other factors, which may account in part for increased food intake following orexin administration.

Our present work highlights the complexity of the orexin system and behavioral and autonomic responses controlled by these peptides. Orexin neurons provide a critical link between peripheral energy balance and the CNS mechanisms that coordinate sleep/wakefulness and motivated behaviors such as food seeking, especially in the physiological state of fasting stress. It is tempting to speculate that modulation of arousal by orexin neurons is affected by more subtle circadian and ultradian changes in energy balance. For example, whether orexin neurons play a role in the well-known phenomenon of postprandial somnolence, for which the central mechanism is essentially unknown (Harnish et al., 1998), merits further investigation. Components of the orexin system have already been identified as unique targets in the development of pharmacological therapies for sleep disorders such as narcolepsy. Our study further suggests that orexin receptor agonists and antagonists might also be useful in novel treatments for obesity, eating disorders, or other autonomic/metabolic disorders.

EXPERIMENTAL PROCEDURES

Behavioral Analysis

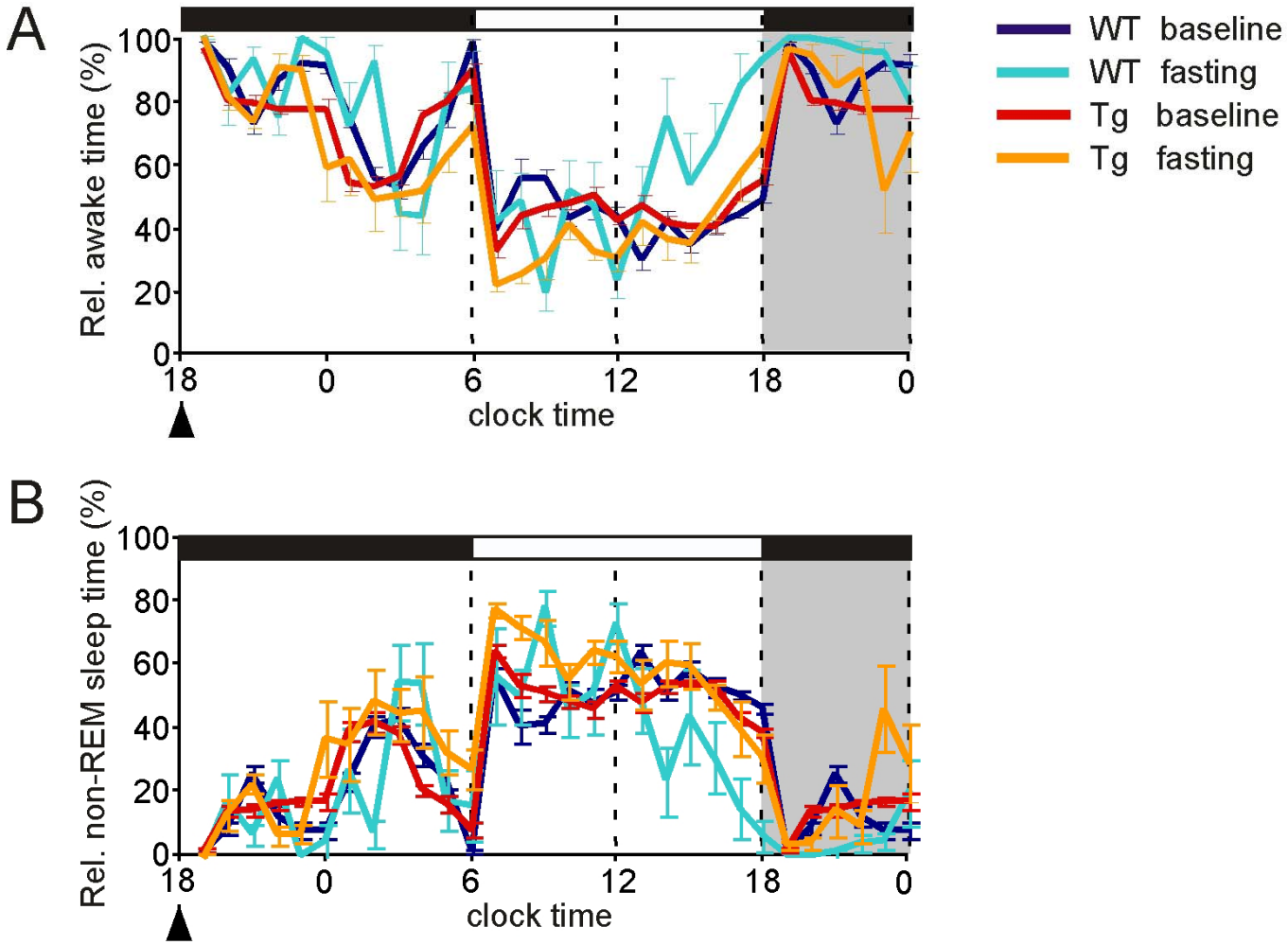
Exploratory behavior of male *orexin/ataxin-3* hemizygous transgenic mice (n=5) and their weight-matched male wildtype littermates (n=6; N4 or N5 backcross to C57BL/6J) was determined using the Opto-Varimex automated tracking system (Columbus Instruments; Columbus, OH) at 10 weeks of age under a constant 12-hour light/dark cycle. Animals were introduced into the open field without food 3 hours before onset of dark, and activity was recorded until 4 hours into the dark phase (7 hours of continuous recording), after which mice were re-introduced into home cages with continued fasting. The following day the procedure was repeated, resulting in a total fasting time of 31 hours. Following each introduction, animals exhibited exploratory activity that habituated (steadily decreased) over time. Complete habituation, defined as that period in which data (collected in 5-min bins) exhibited no statistical change over time (by ANOVA), occurred for both genotypes under fed baseline conditions by the third hour of the experiment. Samples of baseline habituated motor activity (last 1 hour of the light phase and the following first hour of the dark phase) were collapsed, quantified, and compared to the equivalent periods under fasting conditions. Data were analyzed by two-way ANOVA to detect interactions between genotype and feeding status.

Recording of Vigilance Parameters

Male *orexin/ataxin-3* hemizygous mice (n=6) and their weight-matched male wildtype littermates (n=6; N4 or N5 backcross to C57BL/6J) were implanted at 13 weeks

of age with miniature electrodes to record simultaneous EEG/EMG as described (Chemelli et al., 1999). Animals were allowed to recover and habituate for two weeks under constant 12-hour light/dark cycle at 25˚C with free access to food and water. Continuous EEG/EMG traces were recorded over 78 hours, with ad lib feeding for the first 48 hours (baseline) and food deprivation starting at the onset of dark phase for the next 30 hours (fasting). Traces were captured and digitized in 20-second epochs. Each of two investigators, blinded to genotype, scored and categorized all epochs as wakefulness, REM sleep, or non-REM sleep according to standard criteria of rodent sleep. Data are mean hourly scores from both observers, analyzed by two-way ANOVA to detect interactions between genotype and feeding status.

DISPLAY ITEMS



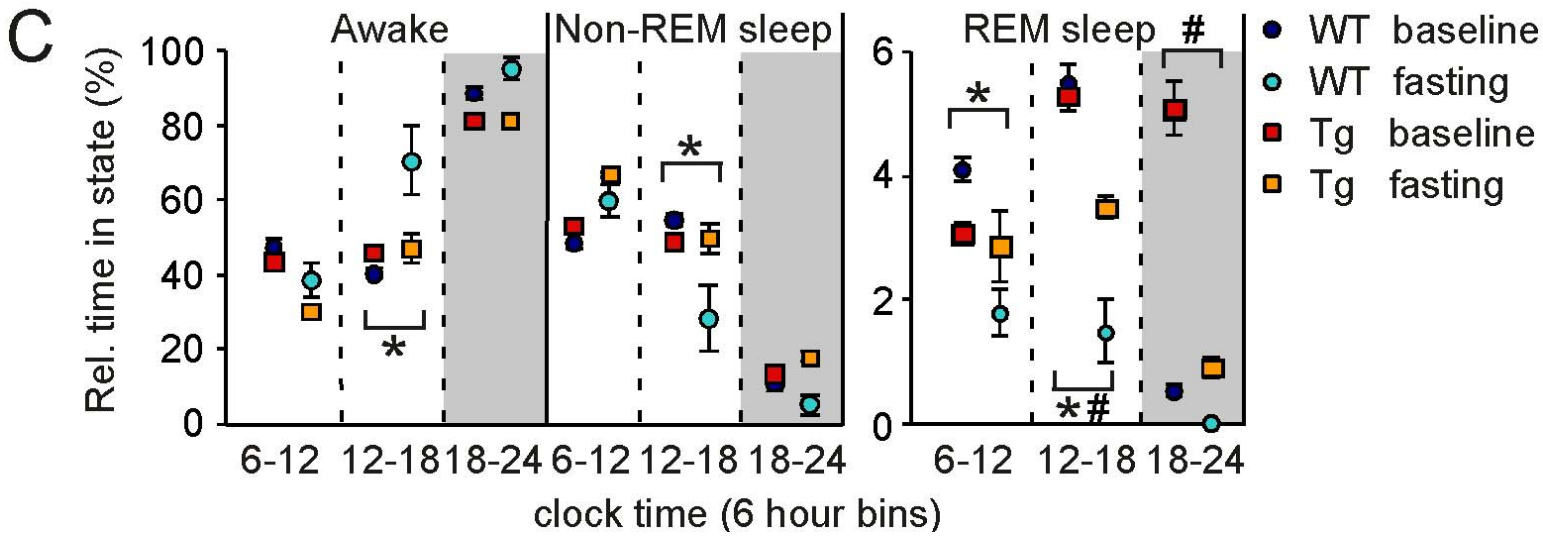
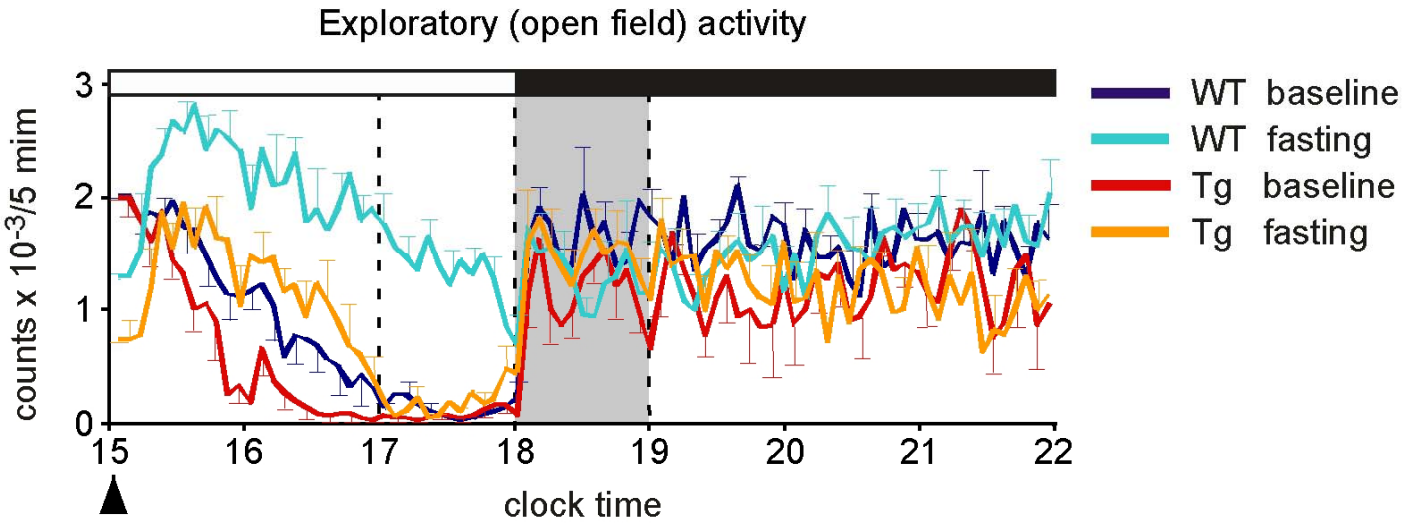

C
Awake
Non-REM sleep
REM sleep
Rel. time in state (%)
100
80
60
40
20
0
6
4
2
0
6-12
12-18
18-24
clock time (6 hour bins)
*
#
WT baseline
WT fasting
Tg baseline
Tg fasting

Figure 3-1. Impaired Fasting-Induced Arousal in *Orexin/ataxin-3* Transgenic Mice. (A) Time courses of amounts of wakefulness and (B) non-REM sleep of hemizygous transgenic mice (red, fed; orange, fasted) and their weight-matched wildtype littermates (dark blue, fed; light blue, fasted) before and during fasting (n=6 per group, mean±SEM). Solid bars, dark phase; empty bars, light phase. Arrowhead indicates the time at which food was removed from cages for the fasting portion of the study that followed baseline recordings. Dotted lines demarcate periods quantified in (C). (C) Data were collapsed into 6-hour bins over the periods indicated for comparison of time spent in wakefulness, non-REM sleep, and REM sleep (mean±SEM; colors same as A and B). *Significant difference ($p<0.01$) in vigilance states of wildtype mice upon fasting. #Significant difference ($p<0.01$) in vigilance states of transgenic mice upon fasting. EEG records were scored jointly by the author and by C.T. Beuckmann.

A


Exploratory (open field) activity
counts x 10-3/5 mim
clock time
WT baseline
WT fasting
Tg baseline
Tg fasting

B

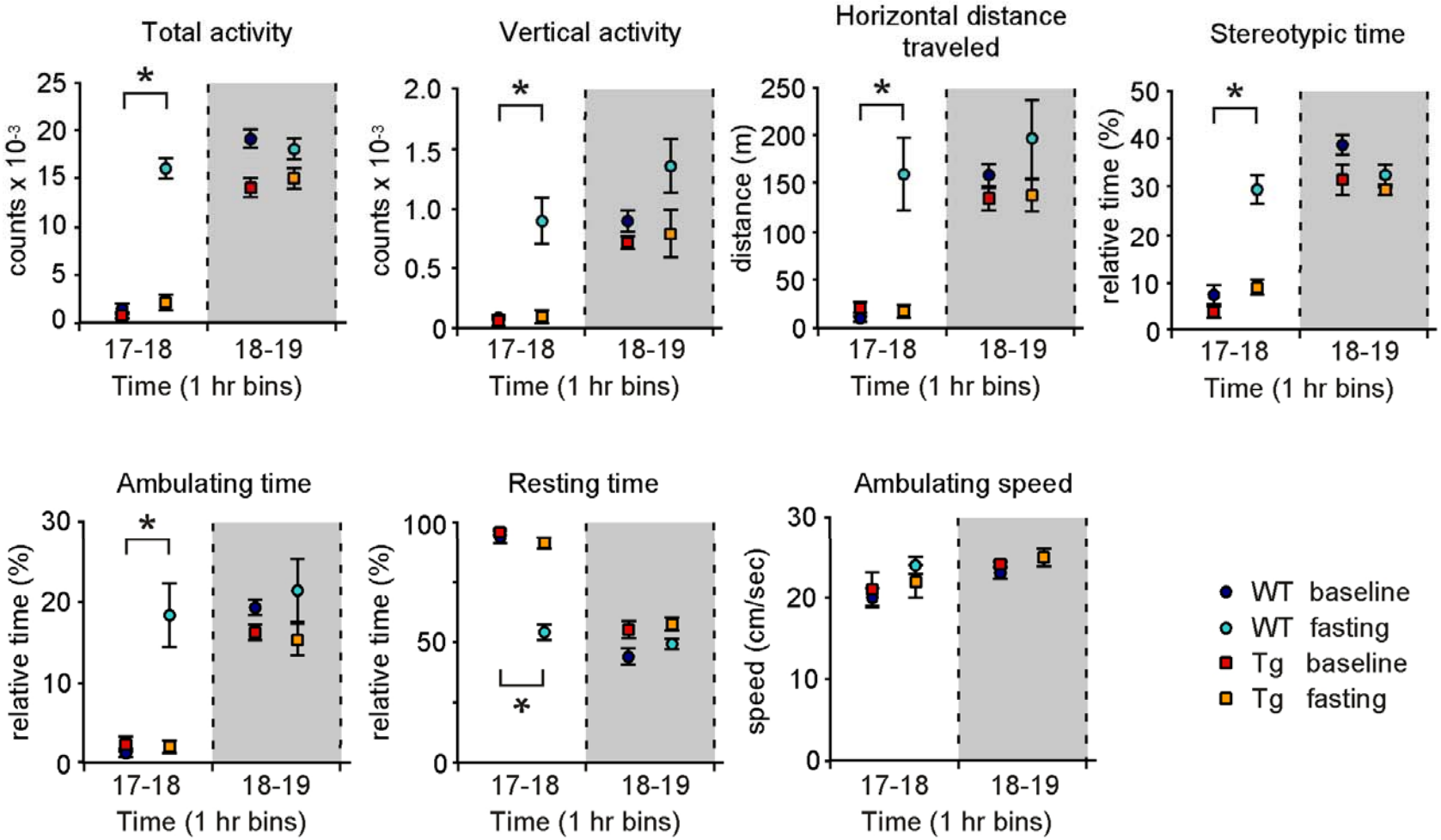

Total activity
counts x 10-3
Time (1 hr bins)
Vertical activity
counts x 10-3
Time (1 hr bins)
Horizontal distance traveled
distance (m)
Time (1 hr bins)
Stereotypic time
relative time (%)
Time (1 hr bins)
Ambulating time
relative time (%)
Time (1 hr bins)
Resting time
relative time (%)
Time (1 hr bins)
Ambulating speed
speed (cm/sec)
Time (1 hr bins)
17-18
18-19
WT baseline
WT fasting
Tg baseline
Tg fasting

Figure 3-2. *Orexin/ataxin-3* Transgenic Mice Fail to Maintain Exploratory Motor Activity During Fasting.

Time course of total motor activity (horizontal and vertical infrared beam break counts) during the light phase (empty bar) and dark phase (solid bar) following introduction to the open field. Activity-monitoring of hemizygous *orexin/ataxin-3* transgenic mice (Tg/+, n=5) and their weight-matched wildtype littermates (+/+, n=6) correlated with the first 7 hours (baseline) and last 7 hours (fasted) of a 31-hour fast. Under baseline conditions, mice of both genotypes exhibited complete habituation by the third hour (see text for details). Arrowhead indicates the time at which fed or fasting mice were introduced into the open field. Dotted lines demarcate periods quantified in (B). (B) Data following the third hour of exposure to novelty (latter light phase and early dark phase) from (A) were collapsed into 1-hour bins (mean±SEM). Distinct categories of motor activity are displayed as separate graphs. *Significant differences ($p<0.001$ by two-way ANOVA) were found exclusively in wildtypes upon fasting. The experiment was designed by the author and performed by C.T. Beuckmann.

CHAPTER FOUR
Genetic Interaction of Two Lateral Hypothalamic Peptides, Orexin and MCH

SUMMARY

Two distinct sets of neurons in the lateral hypothalamic area produce the orexin and MCH neuropeptides, respectively. While the absence of orexin, a peptide implicated also in feeding pathways, results in sleep-wakefulness abnormalities, MCH is recognized for its primary role in feeding behavior and energy homeostasis. Notably, MCH neurons also innervate areas of the brain that generate sleep/wake states. Using *orexin*$^{-/-}$, *MCH*$^{-/-}$, and double null (*orexin*$^{-/-}$;*MCH*$^{-/-}$) mice, I investigated the genetic interactions between these peptides in the regulation of energy homeostasis, and unexpectedly discovered a novel role for MCH in arousal. While the lean mass of these mutant mice is unaltered, *orexin*$^{-/-}$ and *MCH*$^{-/-}$ mice have increased and decreased adiposity and susceptibility to diet-induced obesity, respectively. The abnormalities are balanced in double null mice, which do not differ significantly from wild-type mice with respect to energy homeostasis. Differences in energy substrate utilization (calculated by indirect calorimetry) among the mutants account for these phenotypes. In terms of arousal, narcoleptic *orexin*$^{-/-}$ mice exhibit sleepiness, fragmented sleep-wake states, and cataplexy. *MCH*$^{-/-}$ mice, despite normally consolidated sleep-wake patterns, are more wakeful than wild-type mice during the active phase and exhibit increased aggression when exposed to an unfamiliar mouse. *MCH*$^{-/-}$ mice also exhibit mildly increased locomotor activity under normal fed conditions, but exhibit extreme hyperactivity relative to controls under conditions of

fasting. Double null mice exhibit an unanticipated exacerbation of the narcolepsy phenotype, with profoundly fragmented behavioral states, greater susceptibility to cataplexy, and increased aggression compared to *orexin*$^{-/-}$ controls. This study indicates a novel role for MCH in the regulation of sleep/wakefulness. I conclude with a neurobiological model of the relationship between orexin and MCH in the modulation of arousal, cataplexy, emotionality, and autonomic activity.

INTRODUCTION

The LHA, by light microscopy, consists of overlapping clusters of neurons excluded from the more densely packed nuclei of the basal and medial areas of the hypothalamus (Bernardis and Bellinger, 1993). The LHA receives rich afferentation with numerous synapses from all major regions of the hypothalamus as well as from numerous other forebrain and brainstem structures, consistent with an integrative role in hypothalamic and limbic functions. Neurons of the LHA are the largest in the hypothalamus and they project densely to discrete regions throughout the neuroaxis. Classical lesion studies of this region defined the "LHA syndrome", marked most notably by short-term anorexia, dramatic weight loss and a new permanently lower body-weight "set-point", and poorly characterized deficits in arousal (that some have suggested to be the primary deficit) (Bernardis and Bellinger, 1993; Bernardis and Bellinger, 1996; Danguir and Nicolaidis, 1980a; Devenport and Balagura, 1971). These and other studies lead to the hypothesis that the LHA controls hypothalamic "output" mechanisms of

feeding and motivated behavior, metabolic rate, arousal, and autonomic reactivity. The interpretation of these results must be tempered, however, since such lesions frequently destroy not only intrinsic neurons, but also important fibers *en passant* (e.g. fornix and medial forebrain bundle).

The neuropeptide melanin-concentrating hormone (MCH) is expressed within the LHA with some overlapping expression in the anatomically and functionally-related zona incerta and tuberomammillary nuclei. Following functional characterization in fish and amphibians, MCH was "re-discovered" to play important roles in feeding and obesity in mammals (Qu et al., 1996). Indeed, MCH knockout mice exhibit reduced appetite, reduced adiposity, and an increased metabolic rate, mirroring in several respects the LHA syndrome (Shimada et al., 1998). Based upon anatomical and pharmacolical studies, other roles of MCH in stress responses and olfaction have also been suggested (Nahon, 1994; Saito et al., 2001). With the description of orexin, a second LHA-specific neuropeptide was identified as playing a role in the regulation of feeding and metabolism. The discovery that orexin knockout mice exhibit characteristics of the sleep/wakefulness disorder narcolepsy-cataplexy enriched understanding of the physiological processes controlled by this region of the brain.

The orexin and MCH neuropeptide systems are strikingly similar with respect to anatomy. Neurons producing orexins and MCH make up distinct and non-overlapping neuronal populations within the LHA (Broberger et al 1998, Elias et al 1998), but these large neurons are morphologically similar by light microscopy. The similarity of far-reaching projection patterns of orexin and MCH neurons as well as the overlapping distributions of their receptors indicates that these systems may be functionally related

(Kilduff and de Lecea, 2001). In view of the fact that orexins may be largely neuroexcitatory (G_q-mediated) while MCH is neuroinhibitory (G_i-mediated) (Gao and van den Pol, 2001; Gao and van den Pol, 2002; Sutcliffe and de Lecea, 2000; van den Pol et al., 1998), I hypothesized that orexin and MCH may provide counter-regulatory influences on physiological functions such as sleep and metabolic rate. To test this hypothesis I have compared the metabolic and sleep/wakefulness phenotypes of wild-type, *MCH*$^{-/-}$, *orexin*$^{-/-}$ and *orexin*$^{-/-}$;*MCH*$^{-/-}$ mice on a controlled genetic background.

RESULTS

Survival of Knockout Mice

Orexin$^{-/-}$, *MCH*$^{-/-}$, and DKO mice born to heterozygous or double heterozygous parents of the predominantly C57BL/6J genetic background (N5F1 and N5F2) were born and survived until weaning at roughly the expected Mendelian frequency (data not shown). Experienced mothers of all genotypes exhibited similar fertility, producing litters of comparable size in which 5-7 offspring generally survived until weaning.

Dual –Label Immunohistochemistry

The histochemical pattern of orexin and MCH, as revealed by dual immunohistochemical labeling with both orexin and MCH antisera, is shown in a wild type mouse (**Figure 4-1A**). Despite the intermingling of neurons producing these peptides in the perifornical LHA (**Figure 4-1C**), MCH neurons are more widely

distributed laterally, dorsally, and medially. As previously described, MCH neurons are observed within regions of the zona incerta and dosal medial hypothalamus as well as the tuberal and posterior LHA. High power photomicrographs of immunolabelled neurons of the LHA suggest that MCH-positive axons may be observed in close proximity to orexin neurons (**Figure 4-1D**) and orexin-positive axons may be found in close proximity to MCH neurons (**Figure 4-1E**). Brains of *orexin*$^{-/-}$ mice were unlabeled by anti-orexin antisera (data not shown). Similarly, anti-MCH antisera did not label *MCH*$^{-/-}$ brains. Brain tissues of double null mice were unstained by dual-label immunohistochemistry (**Figure 4-1B**) compared to wild type brains (**Figure 4-1A**).

Body Composition of Knockout Mice

MCH$^{-/-}$ mice exhibit a lean phenotype relative to controls. Body composition analysis using NMR confirmed this observation: 21 week-old MCH mice exhibited reduction of adiposity to 36% of that of wild types (2.21 g versus 6.16 g, respectively; P=0.0002) (**Figure 4-2A**). In contrast, orexin mice exhibited greater adiposity to 146% (8.96 g, P=0.01) compared to wild types. Notably, the fat mass of *orexin*$^{-/-}$;*MCH*$^{-/-}$ mice (4.03 g) was intermediate between those of the single knockout groups, but differed significantly from the orexin group alone (P=0.0002). Lean mass did not vary significantly among the various genotypes (**Figure 4-2A**).

Leptin and Corticosterone Levels

In normal mammals, circulating leptin levels closely correlate with fat mass. We found that plasma leptin concentrations in knockout lines varied by genotype (**Figure 4-**

2B inset). *MCH*$^{-/-}$ mice exhibited a very low mean leptin level relative to wild types (3.67 +/- 0.92 ng/ml versus 25 +/- 3.7 ng/ml, respectively; P<0.01). *Orexin*$^{-/-}$ mice, however, exhibited only a tendency (not significant) toward increased mean leptin level (39.6 +/- 8.3 ng/ml). This value differed significantly from that of *MCH*$^{-/-}$ mice (P=0.0002). *Orexin*$^{-/-}$;*MCH*$^{-/-}$ mice had a low leptin level (10.4 +/- 2.6 ng/ml) intermediate between that of the *MCH*$^{-/-}$ and wild type groups, but not differing significantly from either group. Double null mice had a significantly lower mean leptin level than did the o*rexin*$^{-/-}$ group (P=0.0007). A scatter plot of leptin levels against fat mass (as determined from NMR analysis) for each animal suggested that leptin levels tended to be linearly related to fat mass in individuals of each genotype (**Figure 4-2B**).

Glucocorticoid levels are associated with stress and may contribute to adiposity in humans and rodents, and anatomical evidence suggests that the hypothalamic-pituitary-adrenal axis may be modulated by MCH and/or orexin (Ida et al., 2000; Nahon, 1994). We therefore measured plasma corticosterone, the predominant functional corticosteroid in rodents, as a screen for abnormalities of adrenal function in knockout mice. However, *MCH*$^{-/-}$ mice and DKO groups exhibited insignificant tendencies toward elevated mean corticosterone levels (27.8 +/- 4.7 ng/ml and 26.2 +/- 7.1 ng/ml, respectively) compared to wild type mice (18.3+/- 2.0 ng/ml). The mean corticosterone level of *orexin*$^{-/-}$ mice (19.0 +/- 2.8 ng/ml) was similar to that of wild types.

Diet-induced Obesity

Specific mouse strains (such as C57BL/6) exhibit susceptibility to diet-induced obesity (DIO). We examined the responses of each genotype to obesity-inducing (CM)

chow. Responses to DIO over time differed by genotype (P<0.0001 by ANOVA): after a two-week challenge of CM chow, *orexin*$^{-/-}$ mice gained a mean of 3.92 +/- 0.26 g while wild type mice gained 1.12 +/- 0.27 g (114% versus 104% of original body weight, respectively; P<0.001) (**Figure 4-2C**). In contrast, *MCH*$^{-/-}$ mice lost 1.28 g (95% original body weight, P<001 compared to wild types), and DKO mice were unchanged with a gain of 0.089 +/- 0.26 g (101% of original body weight) that did not differ significantly from wild type mice. At the end of one week (data not shown) and two weeks (**Figure 4-2C inset**), mean daily amounts of food consumed did not differ significantly by genotype, suggesting that weight gain by *orexin*$^{-/-}$ mice and weight loss by *MCH*$^{-/-}$ mice were not the result of changes in amounts of consumed chow over the course of the experiment. Separate long-term studies in which each mice of each genotype were fed CM chow for 20 weeks produced similar patterns of weight gain (data not shown).

Metabolic Analysis

Oxygen consumption (VO_2) was evaluated hourly in mice of each genotype. Overall, circadian patterns of VO_2 in mutants were intact compared to wild types. Total VO_2 during the day, an estimate of resting metabolic rate, tended to be slightly elevated for *MCH*$^{-/-}$ (591 +/- 17 ml/animal/day) and DKO mice (599 +/- 27 ml/animal/day) compared to wild types (555 +/- 6.2 ml/animal/day), but these effects were not significant. Day-time total VO_2 of *orexin*$^{-/-}$ mice (562 +/- 14 ml/animal/day) was similar to that of wild types. At night, when high metabolic activity reflects increased motor activity in mice, total VO2 also tended to be elevated in *MCH*$^{-/-}$ (821 +/- 24 ml/animal/night, not significant) and DKO (865 +/- 35 ml/animal/night, P=0.02)

compared to wild-types (760 +/- 9.8 ml/animal/night). *Orexin*$^{-/-}$ mice (740 +/- 18 ml/animal/night) were again similar to wild types at night. Total VO_2 over 24 h was not found to be significantly different among genotypes.

Calculation of metabolic substrate utilization over 24 h demonstrated the overall tendency of *MCH*$^{-/-}$ mice to utilize lipid at a higher rate than wild type mice (298 +/- 9.4 mg/animal/24 h versus 267 +/- 13 mg/animal/24 h, respectively), especially during the light phase when mice are typically at rest (**Figure 4-2D**). In contrast, orexin mice exhibited reduced lipid utilization during most of both dark and light phases, causing an overall reduction (212 +/- 18 mg/animal/24 h), and *MCH*$^{-/-}$ and *orexin*$^{-/-}$ mice differed from each other very significantly (P=0.0015). Notably, DKO mice, like *orexin*$^{-/-}$ mice, tended to utilize lipid poorly during the dark phase, but like *MCH*$^{-/-}$ mice, tended to have higher levels of lipid utilization during the light phase. These opposite tendencies cancelled each other such that overall lipid utilization over 24 h was effectively unchanged (272 +/- 21 mg/animal/24 h) compared to wild type mice.

Susceptibility to Cataplexy

Infrared video photography studies were carried out to analyze behavior of mice under emotive conditions during the dark phase. *MCH*$^{-/-}$ mice, like wild type mice, were never observed having behavioral arrests of any kind during this experiment or under any other circumstances. *Orexin*$^{-/-}$ mice, however, exhibited cataplectic arrests without exception during the observation period (**Figure 4-3B**). Surprisingly, *orexin*$^{-/-}$;*MCH*$^{-/-}$ mice experienced cataplectic attacks at a 5-fold greater rate than did *orexin*$^{-/-}$ littermates (18.3 +/- 1.8 attacks/h versus 4.0 +/- 1.2 attacks/h, respectively; P=0.000002). Other than

the frequency of attacks, no obvious differences in the behavioral sequence or quality of attacks were noted when these mice were compared to *orexin*$^{-/-}$ mice.

Sleep/Wakefulness Studies

To further delineate the effect of MCH deficiency on sleep/wakefulness regulation and narcolepsy, EEG/EMG studies of wild-type, *MCH*$^{-/-}$, *orexin*$^{-/-}$, and *orexin*$^{-/-}$;*MCH*$^{-/-}$ mice were carried out. Hypnograms compiled from the EEG/EMG traces of mice recorded in home cages revealed that wild type mice exhibit long uninterrupted bouts of wakefulness following the onset of the dark phase (**Figure 4-3A**), as previously described. REM sleep episodes were observed only after considerable periods of non-REM sleep, and were less abundant during the dark phase. Compared to those of wild-type mice, hypnograms from *MCH*$^{-/-}$ mice suggested increased wakefulness and reduced sleep (**Figure 4-3A**). Indeed, during the dark phase, *MCH*$^{-/-}$ mice spent significantly more time awake than wild type mice (617 +/- 13 min versus 570 +/- 5.7 min, respectively; P=0.02; **Figures 4-4A1 and 4-4A2**) and significantly less time in non-REM sleep than wild types (98 +/- 12 min versus 143 +/- 5.8, respectively; P=0.01; **Figures 4-4B1 and 4-4B2**). *MCH*$^{-/-}$ and wild type mice spent similar amounts of time in REM sleep during the dark phase (5.1 +/- 1.2 min versus 6.9 +/- 1.1 min, respectively; **Figures 4-4C1 and 4-4C2**). During the light phase, *MCH*$^{-/-}$ mice were indistinguishable from wild types with respect to amounts of wakefulness, non-REM sleep, and REM sleep.

Hypnograms of narcoleptic *orexin*$^{-/-}$ mice are remarkable for the fragmented appearance of sleep/wakefulness states and frequent bouts of REM sleep that occur with little or no preceding non-REM sleep during the active phase (**Figure 4-3A**). Such

premature transitions to REM sleep are the electrophysiological hallmark of human and animal narcolepsy and have been shown to correlate well with sleep attacks and cataplexy in mice (see **Chapter 2**). While direct transitions to REM sleep were never observed in wild type mice, they spontaneously occurred at a frequency of 4.8 +/- 2.0 transitions/dark phase at night in *orexin*$^{-/-}$ mice. *Orexin*$^{-/-}$ mice did not exhibit such transitions during the light phase. *Orexin*$^{-/-}$ mice exhibited significantly less wakefulness (477 +/- 11, P=0.0002; **Figures 4-4A1 and 4-4A2**) and more non-REM sleep (211 +/- 8.9, P=0.0007; **Figures 4-4B1 and 4-4B2**) than wild types during the dark phase; no compensatory changes were observed in the light phase. Notably, however, *orexin*$^{-/-}$ mice have profoundly elevated amounts of REM sleep during the dark phase (32.0 +/- 3.1, P=0.0002; **Figures 4-4C1 and 4-4C2**), an effect that is partially compensated by decreased REM sleep during the light (P=0.0002).

Compared to *orexin*$^{-/-}$ mice, hypnograms of double null mice exhibit a startling degree of fragmentation during the dark phase (**Figure 4-3A**). Wakeful behavior is spontaneously interrupted by brief, disorganized bouts of non-REM and REM sleep to such a degree that DKO mice appear unable to maintain wakefulness for more than a matter of minutes at a time. Consistent with cataplexy experiments, DKO mice display premature and direct transitions to REM sleep at a high rate during the dark phase (**Figure 4-3A**). Indeed, double null mice exhibited significant increases in direct transitions to REM sleep during both the light (2.4 +/- 0.8, P=0.003) and dark (25.1 +/- 7.3 transitions, P=0.006) phases compared to *orexin*$^{-/-}$ mice. DKO mice have significantly less wakefulness (497 +/- 14 min, P=0.001; **Figures 4-4A1 and 4-4A2**) than wild types, but only had an insignificant tendency toward more non-REM sleep (182 +/- 12 min;

Figures 4-4B1 and 4-4B2) than wild types during the dark phase. Compensatory changes in wakefulness and non-REM sleep were not observed during the light phase. Also like *orexin*$^{-/-}$ mice, DKO mice have profoundly elevated amounts of REM sleep relative to wild types at night (40.8 +/- 5.3 min, P=0.0002, **Figures 4-4C1 and 4-4C2**), an effect that is partially compensated by decreased REM sleep during the day (P=0.0004). Overall, DKO mice are more similar to *orexin*$^{-/-}$ mice than to *MCH*$^{-/-}$ with respect to amounts of sleep and wakefulness.

Locomotor Activity and Response to Fasting

Under conditions of negative energy balance such as fasting, rodents must adapt by balancing food-seeking locomotor activity with energy conservation. We compared the effect of food deprivation on the locomotor activities and body weights of *MCH*$^{-/-}$, *orexin*$^{-/-}$, *orexin*$^{-/-}$;*MCH*$^{-/-}$ mice, and wild type mice monitored in familiar surroundings (without the influence of novelty).

Under baseline fed conditions, *orexin*$^{-/-}$ mice exhibited locomotor activity patterns that did not differ significantly from wild types at night (8720 +/- 980 counts versus 7460 +/- 1000 counts, respectively; **Figure 4-5A,B**) or during the day (2230 +/- 180 counts versus 1940 +/- 180 counts, respectively). In contrast, *MCH*$^{-/-}$ mice were more active over the first half of the dark phase (**Figure 4-5A**), but this effect was not significant over the entire dark phase (10400 +/- 910 counts, **Figure 4-5B**). During the light phase, the activity level of fed *MCH*$^{-/-}$ mice was similar to that of wild types (2150 +/- 390 counts). While fed double null mice did not differ significantly from other genotypes at night

(9100 +/- 1500 counts), this group was more active than wild types during the day (3100 +/- 290, P=0.015).

When fasting levels of activity were compared to fed levels for each group, wild type mice did not differ significantly at night (5500 +/-690 counts) or during the following day (1460 +/- 93 counts) compared to baseline levels under our experimental conditions (**Figure 4-5A,B**). Similarly, fasting *orexin*$^{-/-}$ mice did not differ significantly (night, 6760 +/- 840 counts; day, 1080 +/- 120 counts) compared to baseline activity or to activity of fasted wild types. In contrast, *MCH*$^{-/-}$ were profoundly hyperactive when fasted during the dark (18300 +/- 1400 counts) and light phases (6800 +/- 1700 counts) compared to baselines (dark, P=0.0001; light, P=0.01) and compared to fasted wild types (dark, P=0.0002; light, P=0.0002). Hourly analysis demonstrated two separate peaks of hyperactivity in *MCH*$^{-/-}$ mice (**Figure 4-5A**): one beginning within two hours of the onset of fasting (and lasting most of the night), and the second occurring after about 19-20 hours of fasting (and lasting throughout the last third of the light phase). Double null mice were also profoundly hyperactive with the onset of fasting. This lasted throughout the night (16000 +/- 1500 counts, **Figure 4-5B**) and was increased over baseline activity (P=0.005), an effect similar in magnitude to that observed in *MCH*$^{-/-}$ mice. This activity was also significantly greater than the activity of fasted wild types during the dark phase (P=0.0002). In contrast to *MCH*$^{-/-}$ mice, double null mice displayed no second peak of hyperactivity during the light phase (**Figure 4-5A**) and did not differ significantly from fed baseline or from fasted wild types over the day (2600 +/- 240 counts, **Figure 4-5B**). The resulting difference between fasting double null and *MCH*$^{-/-}$ mice during the light-phase was significant (P=0.007).

With food deprivation, rates of weight loss reflect rates of energy utilization by basal metabolism, muscle activity, and non-shivering thermogenesis. Fasting wild type and *orexin*$^{-/-}$ mice (**Figure 4-5C**) lost body weight at similar rates (2.88 +/- 0.096 g/24 h or 10% body weight/24 h versus 2.96 +/- 0.10 g/24 h or 11% body weight/24 h, respectively). Consistent with locomotor responses, *MCH*$^{-/-}$ lost weight at an accelerated rate (4.24 +/- 0.15 g/24 h or 16% of body weight) compared to wild types (P=0.0002), as previously reported (Shimada et al., 1998). Notably, double null mice also exhibited accelerated weight loss (3.42 +/- 0.18 g/24 h or 14% of body weight) compared to wild types (P=0.02), but not to the same degree as *MCH*$^{-/-}$ mice. Indeed, *MCH*$^{-/-}$ and double null mice differed significantly in this respect (P=0.0009).

Aggression

As the lateral hypothalamus has a recognized role in emotionality, aggression, and defensive behavior, the resident-intruder paradigm was used to screen for levels of irritability/aggression in mutant mice (**Figure 4-6**). In this paradigm, increased or decreased aggression is detected as a change in the latency to attack an unfamiliar (129 SvEv) male that is introduced into the resident's home cage. Among resident wild type mice, 10/11 exhibited a mean latency to attack of 11.5 +/- 1.3 min. The remaining wild type resident mouse was attacked first by the intruder and was therefore excluded from analysis. Six of 10 *orexin*$^{-/-}$ mice tested exhibited a mean attack latency of 9.67 +/- 1.3 min (not significantly different from wild types), the remaining four *orexin*$^{-/-}$ mice were excluded as above. Nine of 10 *MCH*$^{-/-}$ mice (excluding one animal) attacked an intruder with a decreased mean latency of 4.29 +/- 0.90 min (P=0.0005 compared to wild types).

Similarly, 6 of 10 (excluding four) double null mice displayed a reduced mean attack latency of 4.02 +/- 1.2 min (P=0.001 compared to wild types). Thus, mutation of the MCH allele is associated with increased aggression/irritability while no clear effect was detected with mutation of the orexin allele in this assay.

DISCUSSION

The absence of orexins in human and animal narcolepsy has implicated these neuropeptides in the stabilization of wakefulness and maintenance of normal REM sleep regulation, but their putative role in energy homeostasis has remained controversial (de Lecea et al., 2002; Willie et al., 2001). MCH is clearly involved in energy homeostasis, but a role for this peptide in arousal has been hypothesized on the basis of neuroanatomy as well (Kilduff and de Lecea, 2001). The neuroanatomic proximity and axonal interactions between orexin and MCH neurons, the similar projections to widespread target nuclei, and the understanding that orexins and MCH are generally neuroexcitatory and neuroinhibitory, respectively, led me to the hypothesis that orexin and MCH neurons may modulate a variety of metabolic and behavioral processes in an antagonistic manner.

We have demonstrated, using a controlled genetic background, that *orexin*$^{-/-}$ mice exhibit increased adiposity and susceptibility to diet-induced obesity while *MCH*$^{-/-}$ mice have reduced levels of adiposity and decreased susceptibility to diet-induced obesity. Leptin levels in these mutants reflected differences in fat mass. Adiposity of *orexin*$^{-/-}$ mice was associated, perhaps causally, with low utilization of lipid as metabolic substrate

and decreased arousal. Likewise, elevated lipid utilization and increased arousal in *MCH*$^{-/-}$ mice may contribute to the lean phenotype of these mutants. Abnormal homeostatic tendencies of *MCH*$^{-/-}$ mice were further exemplified in the profound hyperactivity and high rate of weight loss induced by fasting in these mice. Compared to *orexin*$^{-/-}$ and *MCH*$^{-/-}$ mice, the metabolic parameters (adiposity, serum leptin levels, susceptibility to DIO, and lipid utilization) of double null mice were consistently intermediate between those of the *orexin*$^{-/-}$ and *MCH*$^{-/-}$ mice.

Likewise, we have shown that *MCH*$^{-/-}$ mice, in contrast to *orexin*$^{-/-}$ mice, tend to exhibit increased wakefulness and decreased non-REM sleep during the dark phase. Little effect upon sleep-wake patterns was observed during daylight hours in *MCH*$^{-/-}$ mice. *MCH*$^{-/-}$ mice have a mild increase in locomotor activity at night under baseline fed conditions but demonstrate a remarkable upsurge in activity under conditions of negative energy balance. These mice also demonstrate increased aggression compared to controls, furthering the likelihood that deficiency of MCH causes behavioral disinhibition.

Double null mice exhibit overall sleep-wake amounts that resemble narcoleptic *orexin*$^{-/-}$ mice and responded to negative energy balance with a psychomotor response resembling that of *MCH*$^{-/-}$ mice except for a notable attenuation during the daylight hours. Hypnograms and cataplexy scores of double null mice revealed a more severe form of murine narcolepsy-cataplexy with extreme behavioral state instability and more frequent cataplectic attacks than *orexin*$^{-/-}$ controls. As in *MCH*$^{-/-}$ mice, DKO mice are highly irritable/aggressive.

Neurobiological Relationship between Orexin and MCH in the Regulation of Behavioral, Emotional, and Autonomic States

How might the above findings be unified to explain the relationship among orexin cells, MCH cells, and the diverse inputs and outputs of this hypothalamic arousal system? The following model (**Figure 4-7**) provides just such a framework and is consistent with the best available anatomic and physiologic understanding of the illustrated components (reviewed in **Chapter 1**).

Orexin neurons are intrinsically depolarized (Eggerman, 2003). They fire actively at night in the rodent but are suppressed by direct or indirect inhibition from the suprachiasmatic nucleus (circadian oscillator) and the sleep-active ventral lateral preoptic area (a putative component of the "sleep homeostat") during daylight hours. This typically daily pattern may be disrupted under conditions of negative energy balance in which hypoglycemia, reduced circulating leptin, and increased ghrelin culminate in direct activation of orexin neurons (Yamanaka et al., 2003), regardless of phase.

In a normal animal at the onset of the dark phase, increased orexinergic activity provides excitatory input to monoaminergic and cholinergic nuclei of the ascending cortical activating system (ACAS) to promote wakefulness via thalamic relays and cortical activation. Orexinergic drive indirectly inhibits neurons of the mesopontine tegmentum that would otherwise generate REM sleep and trigger inhibition of muscle tone via brainstem glyinergic inputs onto the spinal cord. Orexin may also provide excitatory input to the limbic system to amplify the aroused state via limbic input to midbrain generators of psychomotor and autonomic activity, and altering metabolic rate. In a twist of fate, however, excessive limbic input can also have an excitatory effect upon

the brainstem reticulospinal system that inhibits muscle tone (John, 2004). Such a mechanism is thought to result in triggering of cataplexy by strong emotion in susceptible (narcoleptic) individuals.

MCH neurons are not intrinsically depolarized, but are instead conditionally activated (van den Pol, 2004). Orexin is a highly potent activator of MCH neurons via direct (orexinergic) as well as indirect (glutamatergic) inputs, so orexin and MCH neurons are likely to be coordinately activated. This might have the makings of a negative feedback loop except that orexin neurons are unresponsive to MCH (van den Pol, 2004). MCH neurons do appear, however, to provide an inhibitory sedative influence upon the very same nuclei of the ACAS and various limbic components that would be innervated by orexin. Excitatory orexinergic influences during the active phase would allow environmentally- and phase-appropriate maintenance of arousal, suppression of sleep states, and expression of state-appropriate muscle tone, while the inhibitory influence of MCH may allow for temporary reversal of such activity (i.e. brief periods of rest), such as might occur following a meal during the dark phase in rodents. Balanced orexinergic and MCHergic inputs would also result in balanced emotional, autonomic, and psychomotor pattern generation. By inhibiting limbic activity, MCH prevents overactivity of the muscle tone suppression sytem of the brainstem that would exacerbate cataplexy.

In light of this model, the typical narcolepsy-cataplexy phenotype as observed in an *orexin*$^{-/-}$ mouse is the result not only of withdrawal of orexinergic tone but also of the unbalanced sedative-hypnotic effect of MCH. The frequency of cataplexy in these mice results in good part from overactivity of the REM sleep generator. Likewise, withdrawal

of MCHergic tone would lead to increased arousal and emotionality via unbalanced orexinergic activation of the ACAS and limbic system, respectively.

Likewise in the double null animal, complete withdrawal of critical tonic modulators of the ACAS (orexin and MCH) leave this sytem susceptible to rapid transitions between states, or unstable control over "the sleep switch" (Saper et al., 2001), possibly due to the remaining influence of faster acting neurotransmitters such as GABA and glutamate. Similarly, the extremely frequent attacks of cataplexy in double null mice may result from the particular combination of an overactive REM sleep generator with an overactivated limbic system.

EXPERIMENTAL PROCEDURES

Production of Double Knockout Mice

MCH$^{+/-}$ mice (C57BL6/J:129SvEv F1, the kind gift of E. Maratos-Flier) were backcrossed to C57BL6/J for 5 generations (N5). Orexin and MCH knockout lines were intercrossed at N5 to produce double heterozygous knockout (*orexin*$^{+/-}$*;MCH*$^{+/-}$) mice that were then intercrossed to produce F1 double homozygous knockout (*orexin*$^{-/-}$*;MCH*$^{-/-}$) mice. All experiments were performed using F2 and F3 generations of this line as well as with F2 and F3 generations of the N5 MCH line. Male mice were weaned at 21-22 days of age, genotyped, and housed 3-5 mice per cage at 25 C under alternating 12-h light/dark cycle with ad lib access to water and 11% fat food, unless otherwise specified.

Genotyping

Genotypes were confirmed by PCR amplification of genomic DNA. Separate PCR amplications to detect the MCH (Shimada et al., 1998) and orexin (Willie et al., 2003) alleles were performed with primers as described previously. We verified that orexin allele PCR amplified samples from *MCH*$^{-/-}$ mice and MCH allele PCR amplified samples from *orexin*$^{-/-}$ mice.

Immunostaining

Immunostaining with anti-orexin-A and anti-MCH antisera was performed by S.C. Williams as described (Chemelli et al., 1999; Elias et al., 1998).

Body Composition

Fat mass and lean body mass were evaluated in live 21-week-old mice (n=10-18 per genotype, maintained on 11% fat chow) by NMR scanning analysis (Minispec NMR Analyzer, Bruker) as directed by the manufacturer. The device was calibrated daily using standards provided by the manufacturer. Data acquisition time for each mouse was 1 min.

Plasma Leptin and Corticosterone Measurements

At 21 weeks of age, during the early light phase, cohorts of individually-housed mice were rapidly anaesthetized using isoflurane gas and exsanguinated. Plasma was isolated and stored at -20 deg C. Plasma leptin was detected by EIA (Linco); assays were performed by Y. Xiong. Corticosterone was detected by liquid chromatography by D. Hess.

Diet-Induced Obesity

Individually-housed 18-week old mice (n=10-18 per genotype), previously maintained on standard 11% fat chow, were challenged with two weeks of exposure to highly palatable, carbohydrate-rich, 17% fat condensed milk (CM) diet (Research Diets). Food intake and body weight were monitored at weekly intervals.

Indirect Calorimetry

Metabolic parameters were measured by indirect calorimetry in 17-week-old mice (n=10-18 per genotype) using a ten-chamber open-circuit Oxymax system (Columbus Instruments, Columbus, OH). Mice were maintained at 25 C under 12-h light/dark cycle. Food and water were available ad libitum. Animals were housed individually in custom Plexiglas cages through which room air was passed at a constant rate. Exhaust air from each chamber was sampled at 1-h intervals for a period of 1 min; O_2 and CO_2 content of exhaust was determined by comparison to O_2 and CO_2 content of standardized air samples (recalibrated daily). Mice were acclimatized to cages for 5 days before beginning recordings, weighed daily, and underwent 72 hours of monitoring, beginning at lights-off (CT 1200). Fat utilization for each time point was calculated from VO_2 and VCO_2 measurements based upon the following formula: $[(VO_2)(1\text{-}RQ)] / [(1\text{-}0.71)(4.74 \text{ kcal heat/L O2})/(9.3 \text{ kcal heat/g fat oxidized})]$ where 0.71 is the RQ for 100% utilization of fat.

EEG/EMG Studies

Five mice of each genotype (littermate pairs) were prepared for long-term EEG/EMG monitoring and recorded as in previous chapters. Two days of EEG/EMG data per mouse were compiled and analyzed as in **Chapter 2**. Statistical significance was assessed using ANOVA and Tukey post-hoc tests.

Infrared Videophotography

Orexin$^{-/-}$ and *orexin*$^{-/-}$*;MCH*$^{-/-}$ littermate pairs were introduced to a novel environment and the frequency of cataplexy-like arrests in each mouse were recorded and scored by the methods described in previous chapters.

Locomotor Activity

Activity was calculated from infrared beam breaks in a standard open field apparatus (VersaMax Animal Activity Monitor, AccuScan Instruments, Inc.). Individual mice were continually monitored, following a 6-h habituation period, for 48 h beginning at the onset of the dark phase. The first 24 h constituted baseline (fed) activity. The second 24 h, beginning with removal of food at onset of the second dark phase, constituted activity under fasting conditions.

Aggression Testing

The resident-intruder paradigm was used to evaluate emotional lability/ aggression in 19-week old male mice (n=10-18 per genotype) that had been individually housed for 3 weeks. Experimental mice (residents of each genotype) were tested for

latency to attack an unfamiliar intruder (129SvEv wild type male mice of similar age and weight) after introduction to the resident's home cage. We scored latency to an aggressive encounter including either an attack, which is defined as biting of the opponent mouse, or wrestling, which is vigorous shoving and sparring when both animals (usually simultaneously) take on an upright posture. Animals were separated promptly following an aggressive encounter in order to avoid injury, or after 15 min if no such encounter was observed. Results from experiments in which the resident was attacked without provocation by the intruder were excluded. All experiments were documented by CCD video camera for later review.

DISPLAY ITEMS

Table 4-1. Sleep/Wakefulness Parameters Recorded from *MCH* Knockout Mice and Wild-Type Controls

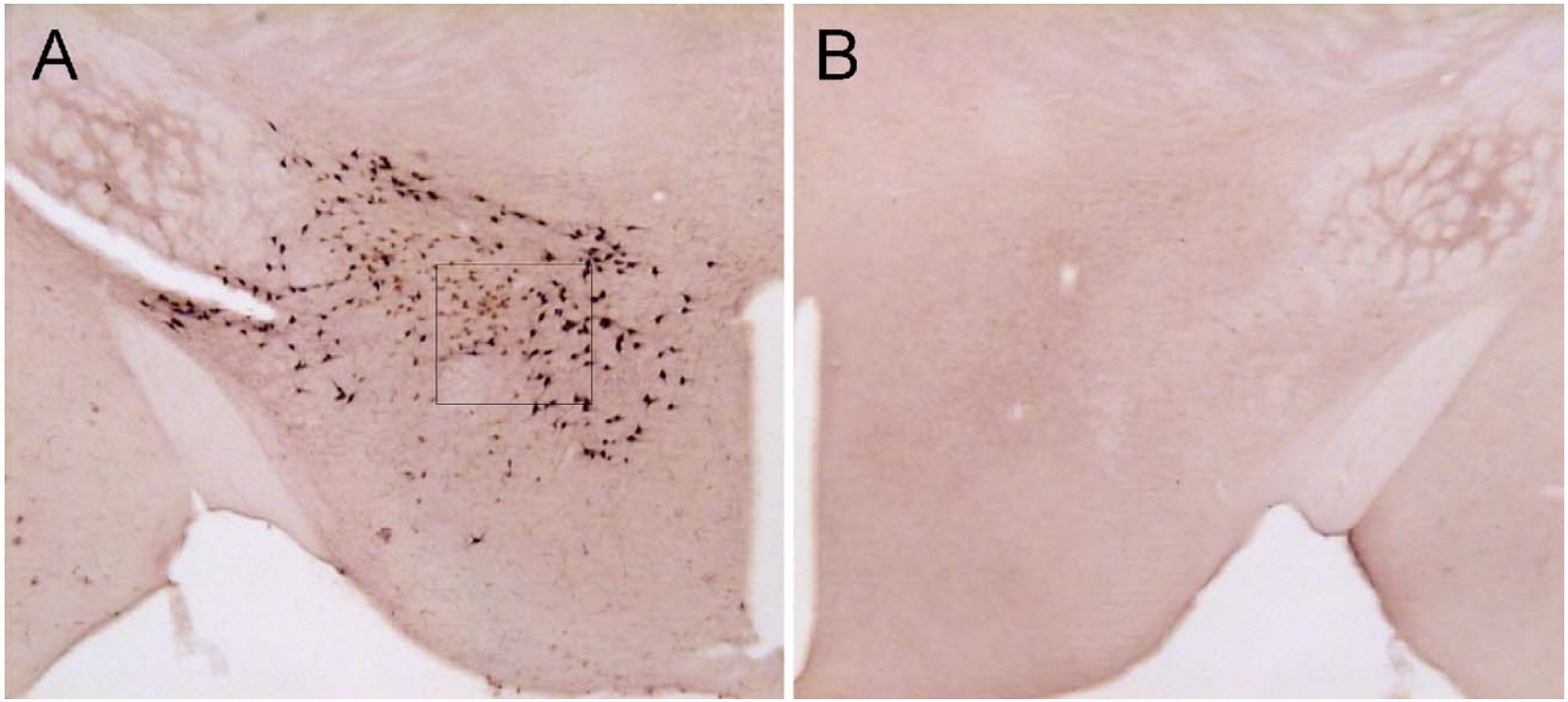
	REM				NREM				Awake			
	WT		MCH KO		WT		MCH KO		WT		MCH KO	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
24 hours												
Time (min)	53.14	2.32	47.64	2.05	559.00	12.79	486.33	12.58	827.87	11.81	906.03	12.87
Light (rest) phase												
Time (min)	44.37	2.75	42.86	1.83	417.47	11.76	393.19	13.05	258.17	10.93	283.95	13.34
Duration (sec)	68.92	4.01	61.04	2.58	290.92	32.10	268.13	13.09	313.29	64.60	446.13	72.56
Cyclicity (Min/episode)	18.91	1.71	17.27	0.74	8.30	0.70	8.24	0.51	12.60	1.97	14.56	1.68
REM latency (min)	9.46	1.19	9.19	0.61								
REM Interval (min)	23.90	3.20	23.35	1.07								
Dark (active) phase												
Time (min)	8.77	0.71	4.78	1.29	141.53	9.39	93.14	10.03*	569.70	9.37	622.08	11.24
Duration (sec)	58.43	6.50	47.92	5.87	283.34	40.45	238.73	23.78	1193.67	207.03	1320.89	171.43
Cyclicity (Min/episode)	82.10	5.84	164.97	42.82	25.38	5.30	31.94	2.54	25.63	4.43	26.69	2.41
REM latency (min)	8.73	0.71	9.58	1.08								
REM Interval (min)	54.18	5.10	119.96	35.75								

Total time spent in each state (min), episode duration (min), episode cyclicity (min/episode), REM latency (min), and interval between successive REM sleep episodes (min) over light and dark periods. Data are presented as means±SEM. *Statistical significance ($p<0.05$) was tested by repeated measures ANOVA followed by Tukey post-hoc test.

Table 4-2. Sleep/Wakefulness Parameters Recorded from MCH;orexin Double Knockout (DKO) Mice and Orexin Knockout Controls

	REM				**NREM**				**Awake**			
	Orx KO		**DKO**		**Orx KO**		**DKO**		**Orx KO**		**DKO**	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
24 hours												
Time (min)	60.17	5.49	71.10	9.08	616.03	26.89	576.73	27.33	763.80	31.23	792.07	35.23
Light (rest) phase												
Time (min)	30.83	1.93	30.77	1.89	410.30	16.54	400.40	22.50	278.87	18.36	288.83	24.07
Duration (sec)	60.86	1.20	52.04	2.68	286.22	33.29	234.27	19.85	259.55	16.93	213.04	4.43
Cyclicity (Min/episode)	24.18	1.48	20.87	2.31	8.28	0.66	6.97	0.27	11.26	1.19	9.00	0.60
REM latency (min)	7.32	0.47	6.30	0.63								
REM Interval (min)	24.24	1.48	20.38	2.41								
Dark (active) phase												
Time (min)	29.33	3.76	40.33	7.43	205.73	11.83	176.34	15.15	484.93	14.32	503.24	18.14
Duration (sec)	66.26	5.36	51.26	2.54	145.88	23.17	79.75	6.95	358.72	27.34	230.10	30.41
Cyclicity (Min/episode)	28.68	2.92	17.58	3.05	8.40	0.89	5.52	0.57	8.99	0.89	5.44	0.57
REM latency (min)	3.05	0.33	1.53	0.14								
REM Interval (min)	27.30	3.02	16.83	2.94								

Total time spent in each state (min), episode duration (min), episode cyclicity (min/episode), REM latency (min), and interval between successive REM sleep episodes (min) over light and dark periods. Data are presented as means±SEM. Statistical significance was tested by repeated measures ANOVA with no differences found in these parameters.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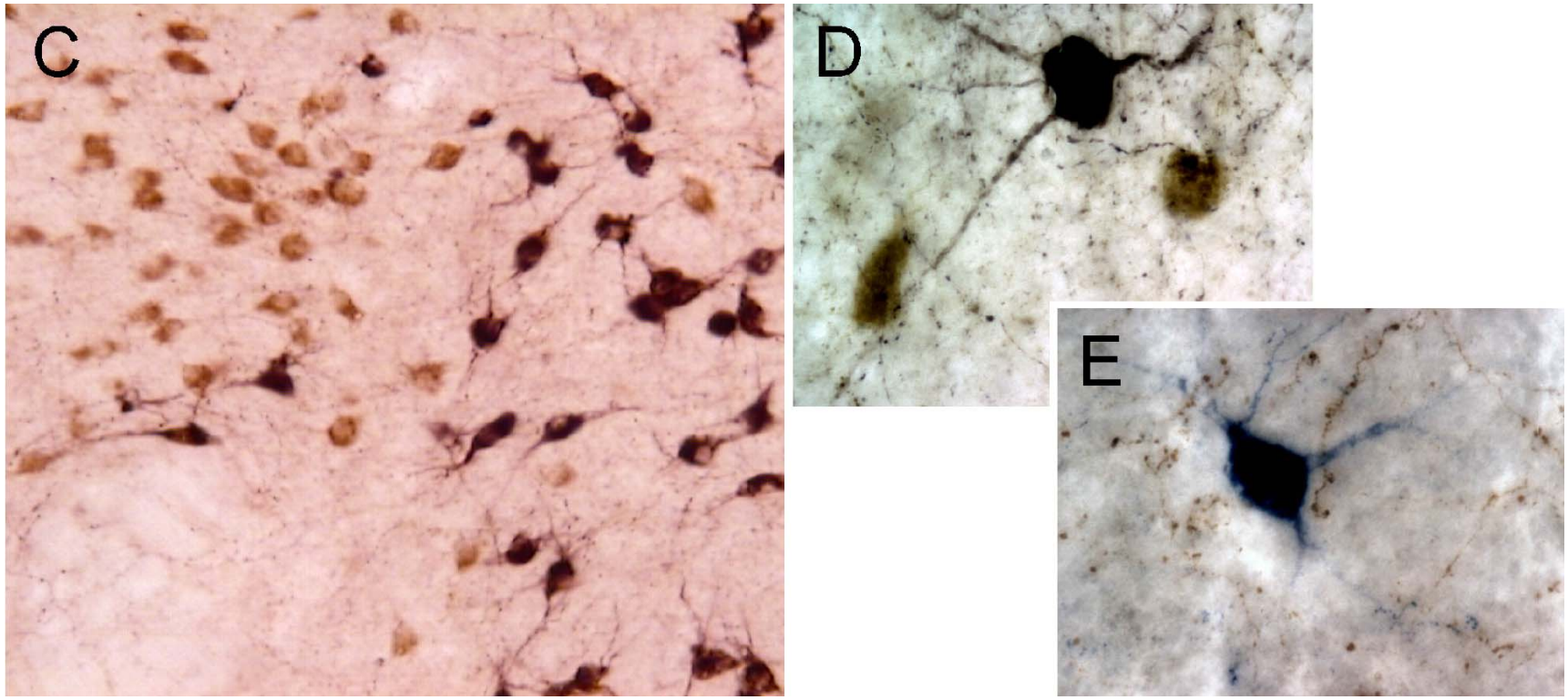
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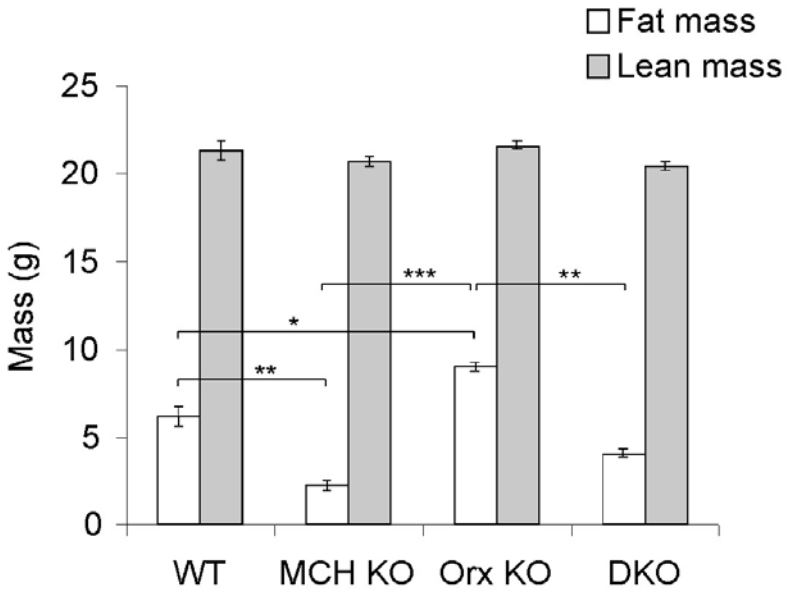
E

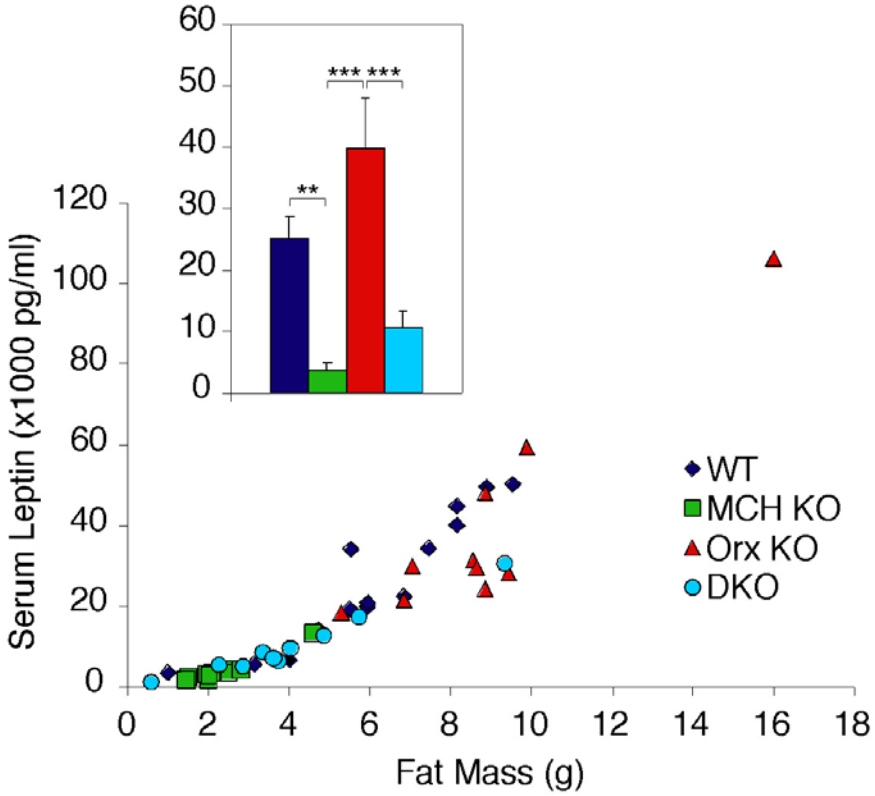
Figure 4-1. Dual –Label Immunohistochemistry.

Anti-orexin-A (brown) and anti-MCH (black) immunostaining of coronal sections of brain tissue (at the level of the median eminence) from a wild-type **(A,C, D, E)** and an *orexin*$^{-/-}$; *MCH*$^{-/-}$ mouse **(B)**. Note that neurons producing each peptide are intermingled in the perifornical region of LHA **(A,C)**, that MCH-positive axons (black) may be observed in close proximity to orexin neurons **(D)** and orexin-positive axons (brown) may be found in close proximity to MCH neurons **(E)**. No anti-orexin or anti-MCH immunoreactivity is observed in brain tissues of double null mice **(B)**. (A inset, the location of size of magnified image in C). Immunostaining performed by S.C. Williams.

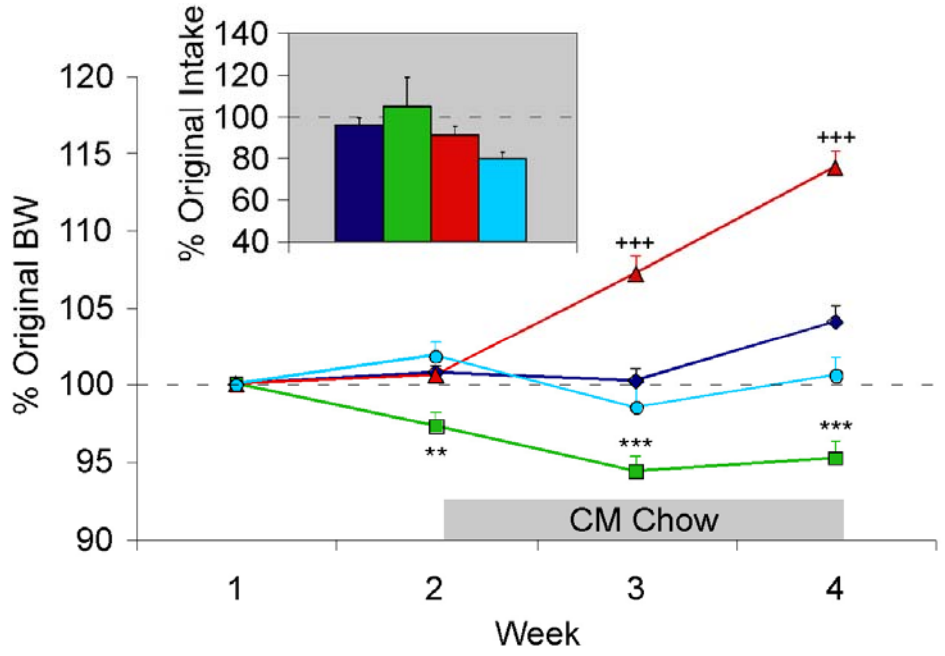
A


Fat mass
Lean mass
Mass (g)
25
20
15
10
5
0
*
**
***
**
WT
MCH KO
Orx KO
DKO

B


Serum Leptin (x1000 pg/ml)
120
100
80
60
40
20
0
60
50
40
30
20
10
0
**
*** ***
0
2
4
6
8
10
12
14
16
18
Fat Mass (g)
WT
MCH KO
Orx KO
DKO

C


% Original BW
% Original Intake
Week
CM Chow
+++
***
**

D

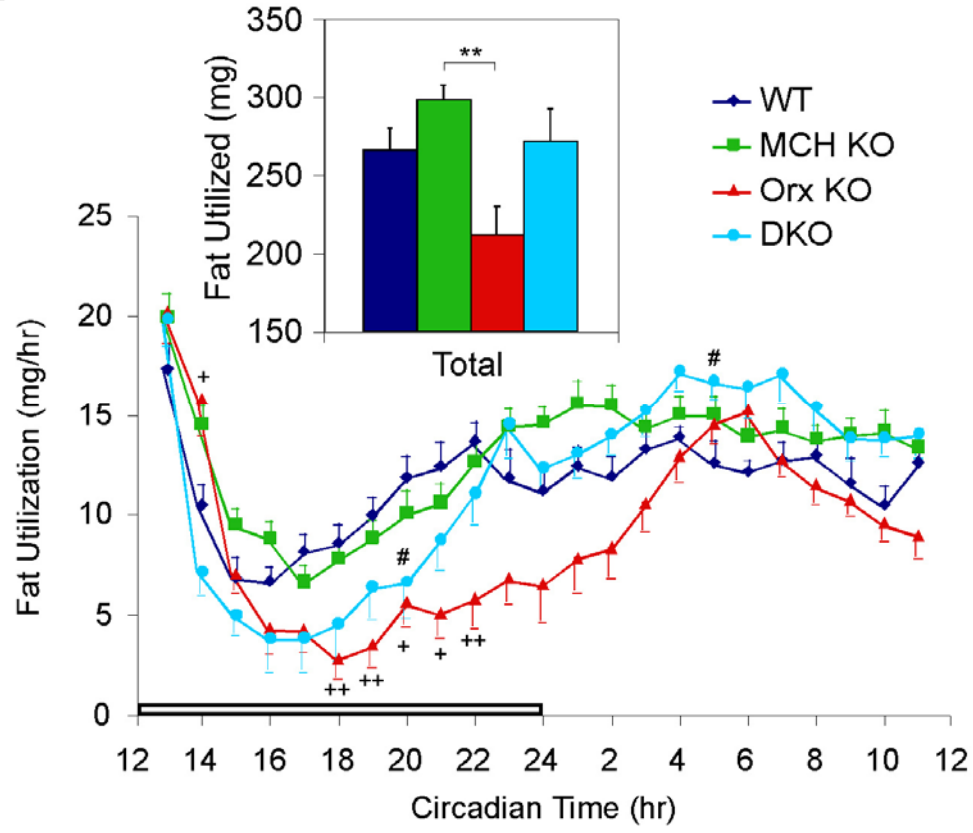

Fat Utilization (mg/hr)
Fat Utilized (mg)
Total
Circadian Time (hr)
WT
MCH KO
Orx KO
DKO

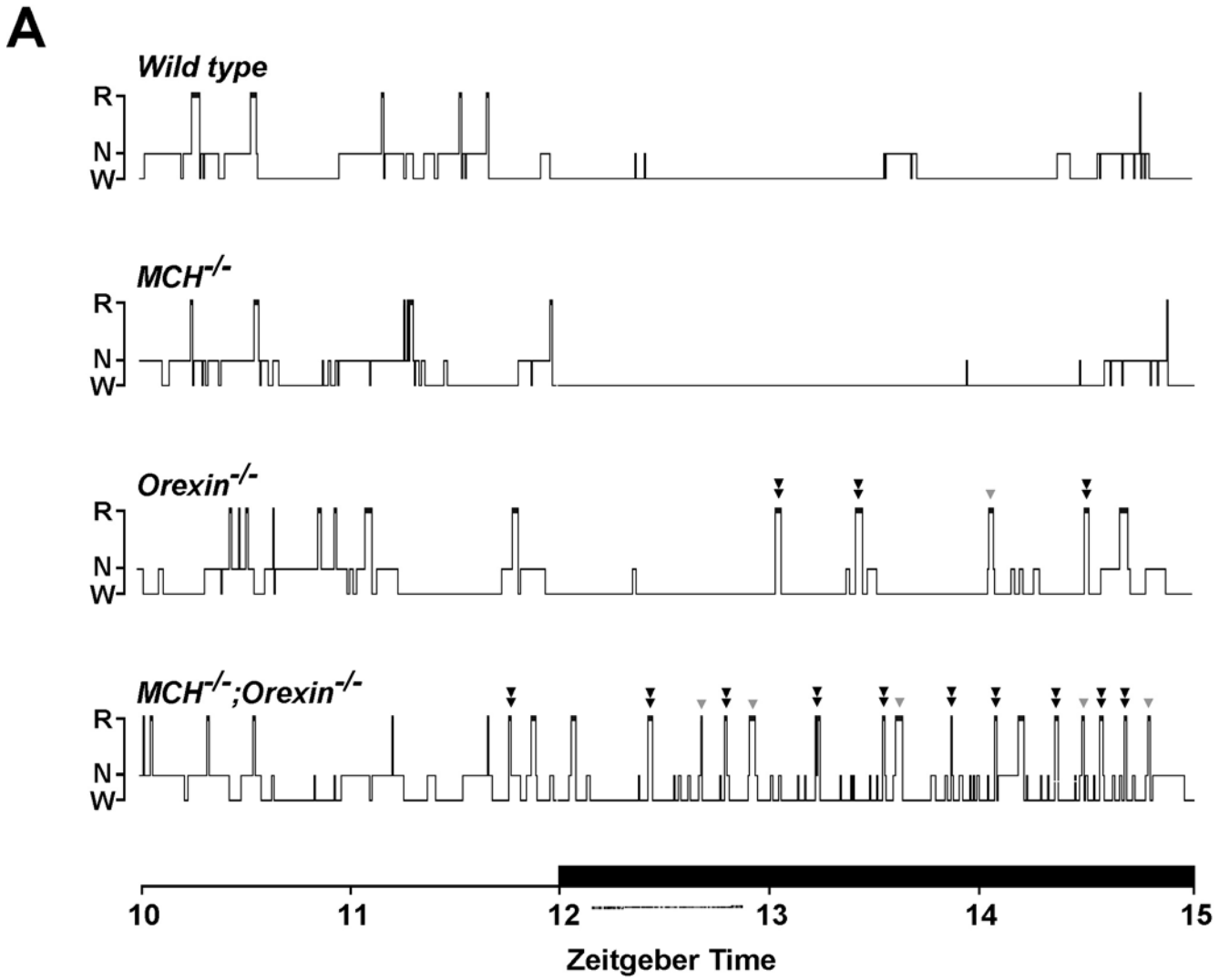
Figure 4-2. Metabolic Parameters in Knockout Mice.

(A) Body composition analysis (lean mass and fat mass) of each genotype (means and standard error) as determined by NMR scan. While the genotypes did not differ with respect to lean mass, fat mass is decreased in *MCH*$^{-/-}$ mice and elevated in *orexin*$^{-/-}$ mice. *Orexin*$^{-/-}$;*MCH*$^{-/-}$ (DKO) mice exhibited an intermediate phenotype. *p<0.05, **p<0.01, ***p<0.001 by ANOVA and Tukey post-hoc tests.

(B) Plasma leptin levels as a function of fat mass in individual subjects of each genotype. Plasma leptin levels were directly proportional to fat mass in all genotypes. Inset shows mean leptin levels by group with *MCH*$^{-/-}$ and *orexin*$^{-/-}$ mice having reduced and elevated levels of plasma leptin, respectively. DKO mice had intermediate leptin levels (**p<0.01, ***p<0.001 by ANOVA and Tukey post-hoc tests).

(C) Body mass and feeding patterns with caloric challenge. Baseline Weekly measurement of body weights (graph) and daily food intake (were were examined under baseline conditions (exclusive access to 11% fat chow) and following a two-week caloric challenge (exclusive access to highly palatable condensed milk/17% fat chow). *MCH*$^{-/-}$ and *orexin*$^{-/-}$ mice lost and gained weight, respectively while wild-type and DKO mice did not gain weight appreciably. Inset shows changes in amounts of daily food intake following caloric challenge (relative to baseline). Food intake amounts did not change significantly in any group by the end of two weeks (**p<0.01, ***p<0.001).

(D) Indirect calorimetric assessment of substrate utilization in each genotype over time. Hourly rates of fat utilization (mg fat/h/g body weight) are calculated from VO_2 and VCO_2 measurements and are displayed in the graph (+$p<0.05$ and ++$p<0.01$ for *orexin*$^{-/-}$ versus wild-type mice; #$p<0.05$ for DKO versus wild-type mice). Inset shows cumulative fat utilization over a 24-h period. Note that *MCH*$^{-/-}$ and *orexin*$^{-/-}$ mice exhibit increased and decreased fat utilization compared to each other over time (**$p<0.01$). DKO mice exhibit an intermediate level of fat utilization.


A
Wild type
R
N
W
MCH-/-
R
N
W
Orexin-/-
R
N
W
MCH-/-;Orexin-/-
R
N
W
10
11
12
13
14
15
Zeitgeber Time

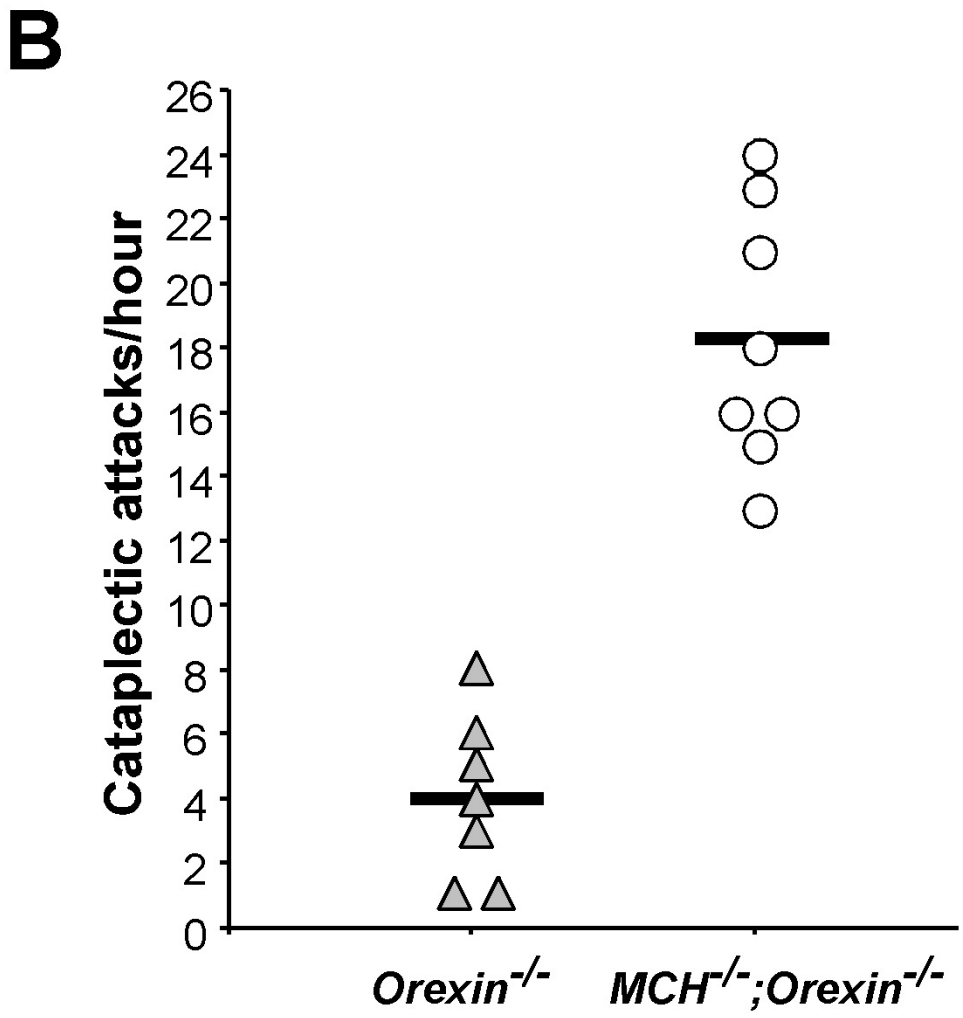
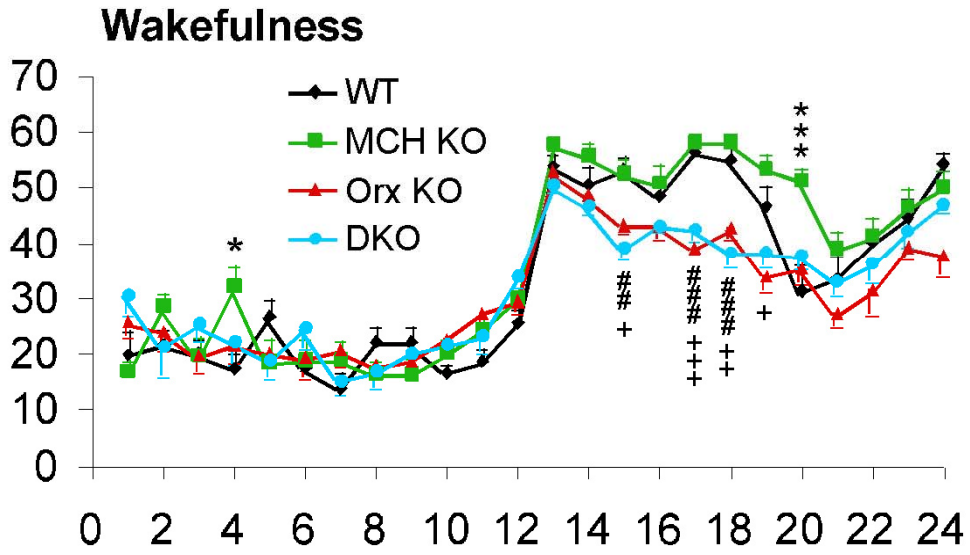

B
Cataplectic attacks/hour
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Orexin-/-
MCH-/-;Orexin-/-

Figure 4-3. Sleep/wake Hypnograms and Cataplexy Scores of Knockout Mice

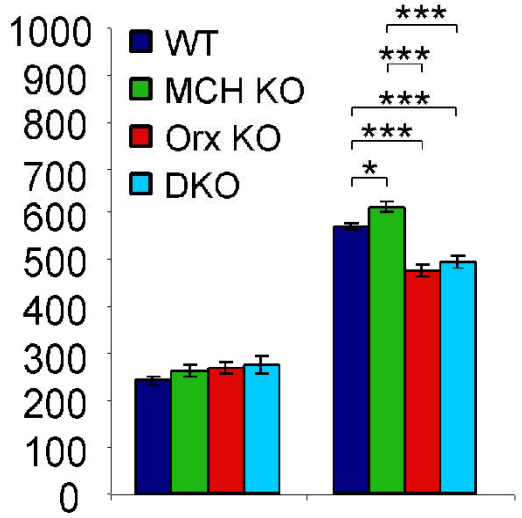
(A) Hypnograms represent concatenated 20-sec epochs of EEG/EMG activity, scored as awake (W), non-REM sleep (N), or REM sleep (R). Five hours per mouse, including transitions from light phase to dark phase (solid bar), are shown. Compared to wild type mice, *MCH*$^{-/-}$ littermates exhibited grossly normal daytime sleep periods and long consolidated periods of nocturnal wakefulness. *Orexin*$^{-/-}$ mice, however, display hallmarks of rodent narcolepsy including fragmentation of wakefulness during the dark phase and frequent premature onsets of REM sleep that occur immediately after wakefulness (double arrowheads) or after <1 min of preceding non-REM sleep (single arrowheads). Compared to *orexin*$^{-/-}$ mice, *orexin*$^{-/-}$;*MCH*$^{-/-}$ littermates have more profound fragmentation of awake, non-REM, and REM sleep states with very high frequencies of direct and premature transitions from wakefulness to REM sleep. Abnormal transitions to REM sleep were never observed in wild type or *MCH*$^{-/-}$ mice.

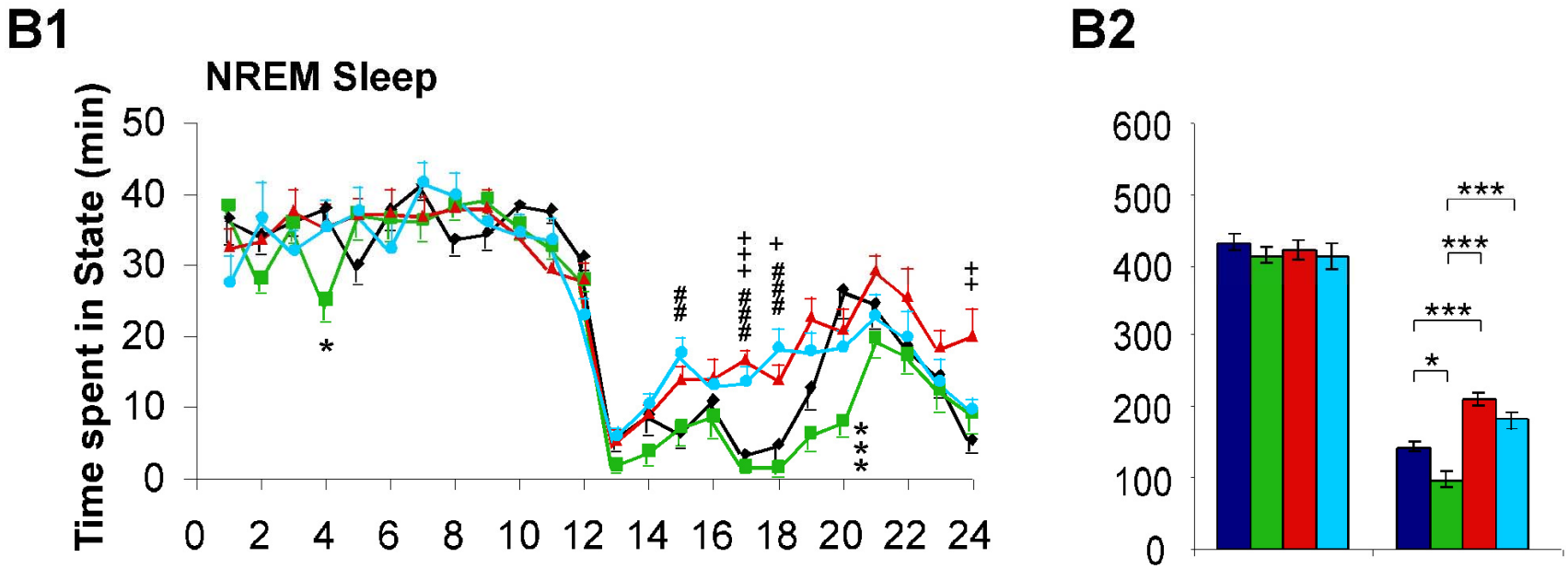
(B) Frequency of observed cataplectic attacks in individual *orexin*$^{-/-}$ and *orexin*$^{-/-}$;*MCH*$^{-/-}$ littermates. *Orexin*$^{-/-}$;*MCH*$^{-/-}$ mice experienced cataplectic attacks at a 5-fold greater rate than did *orexin*$^{-/-}$ littermates ($p=0.000002$). *MCH*$^{-/-}$ and wild type mice, do not exhibit cataplexy (data not shown).

A1

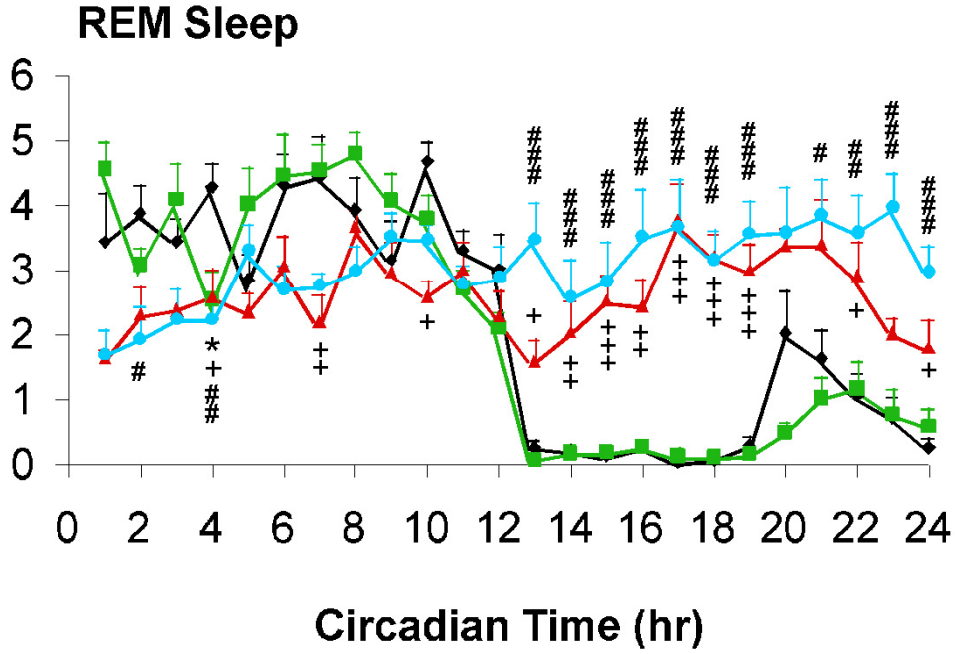

Wakefulness
WT
MCH KO
Orx KO
DKO
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24

A2


WT
MCH KO
Orx KO
DKO
1000
900
800
700
600
500
400
300
200
100
0


B1
NREM Sleep
Time spent in State (min)
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24
B2
600
500
400
300
200
100
0
***
***
***
*

C1


REM Sleep
6
5
4
3
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Circadian Time (hr)

C2

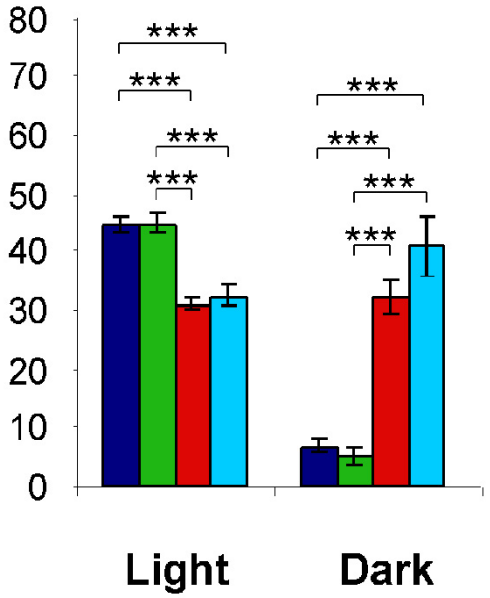

80
70
60
50
40
30
20
10
0
***
Light
Dark

Figure 4-4. Amounts of Sleep and Wakefulness in Knockout Mice.

Time spent in wakefulness, non-REM sleep, and REM sleep are demonstrated as hourly plots over 24 h **(A1, B1, C1)** and as total amounts per phase **(A2, B2, C2)** for each genotype. *MCH*$^{-/-}$ mice spent significantly more time awake and less time in non-REM sleep during the dark phase, and they exhibited normal amounts of REM sleep compared to wild-type mice. By contrast, *orexin*$^{-/-}$ controls spent less time awake and more time in non-REM sleep during the dark phase, and they had increased amounts of REM sleep during the night with a compensatory decrease in REM sleep during the day. DKO mice exhibited amounts of wakefulness, non-REM sleep, and REM sleep that closely resembled those of *orexin*$^{-/-}$ mice. Left panels: *$p<0.05$ and ***$p<0.001$ for *MCH*$^{-/-}$ compared to wild-type mice; +$p<0.05$, ++$p<0.01$, and +++$p<0.001$ for *orexin*$^{-/-}$ compared to wild-type mice; and #$p<0.05$, ##$p<0.01$, and ###$p<0.001$ for DKO compared to wild-type mice. Right panels: *$p<0.05$, **$p<0.01$, and ***$p<0.001$ for comparisons between bracketed pairs. Analysis by ANOVA and Tukey post-hoc tests.

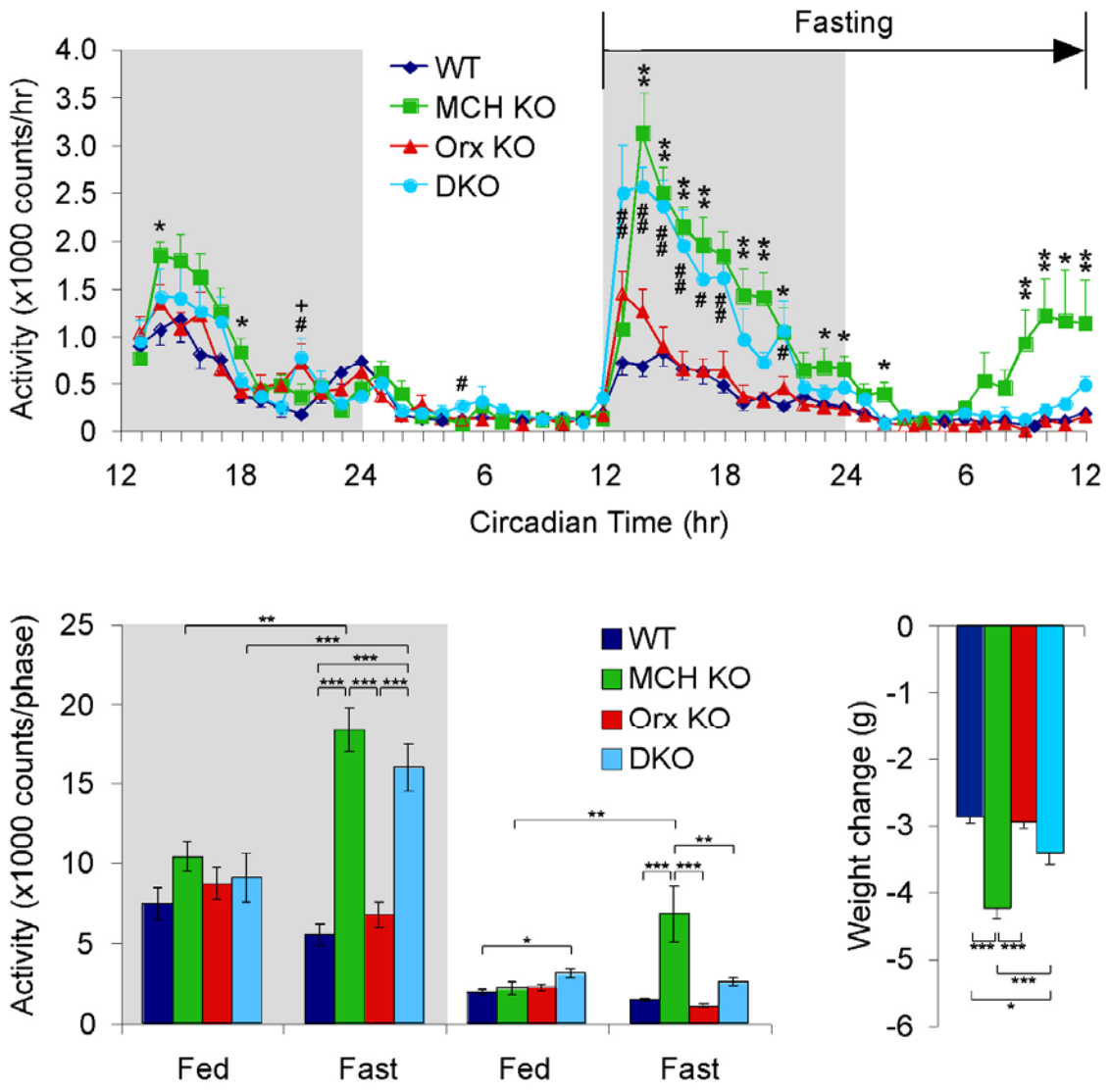


Figure 4-5. Locomotor Activity of Knockout Mice under Fed and Fasted Conditions. Top panel (A) shows locomotor activity counts in 10-min bins from the onset of the dark phase. Following 24 h of continuous baseline recording in the presence of normal chow, all food was removed and another 24 h of continuous recording took place. Gray backgrounds demarcate dark phases from light phases. Lower left panel (B) shows total activity counts collapsed over 12-h by phase and fed or fasted condition for each genotype. Note that under fed conditions, *MCH*$^{-/-}$ and DKO mice exhibit only mild

increases in activity over wild-type mice during the dark and light phases, respectively. By contrast, onset of fasting rapidly induces a significant hyperactivity in *MCH*$^{-/-}$ mice in the dark and successive light phase. DKO mice are also hyperactivity in response to fasting at night, but not during the following day. Lower right panel (C) demonstrates the consequences of altered psychomotor activity in response to fasting as *MCH*$^{-/-}$ mice lose significantly more weight over 24 h than do wild-type or *orexin*$^{-/-}$ mice. DKO mice exhibit a milder, intermediate phenotype relative to *MCH*$^{-/-}$ and wild-type groups. Top panel: *$p<0.05$ and **$p<0.01$ for *MCH*$^{-/-}$ compared to wild-type mice; +$p<0.05$ for *orexin*$^{-/-}$ compared to wild-type mice; and #$p<0.05$ and ##$p<0.01$ for DKO compared to wild-type mice. Lower panels: *$p<0.05$, **$p<0.01$, and ***$p<0.001$ for comparisons between bracketed pairs. Analysis by ANOVA and Tukey post-hoc tests.

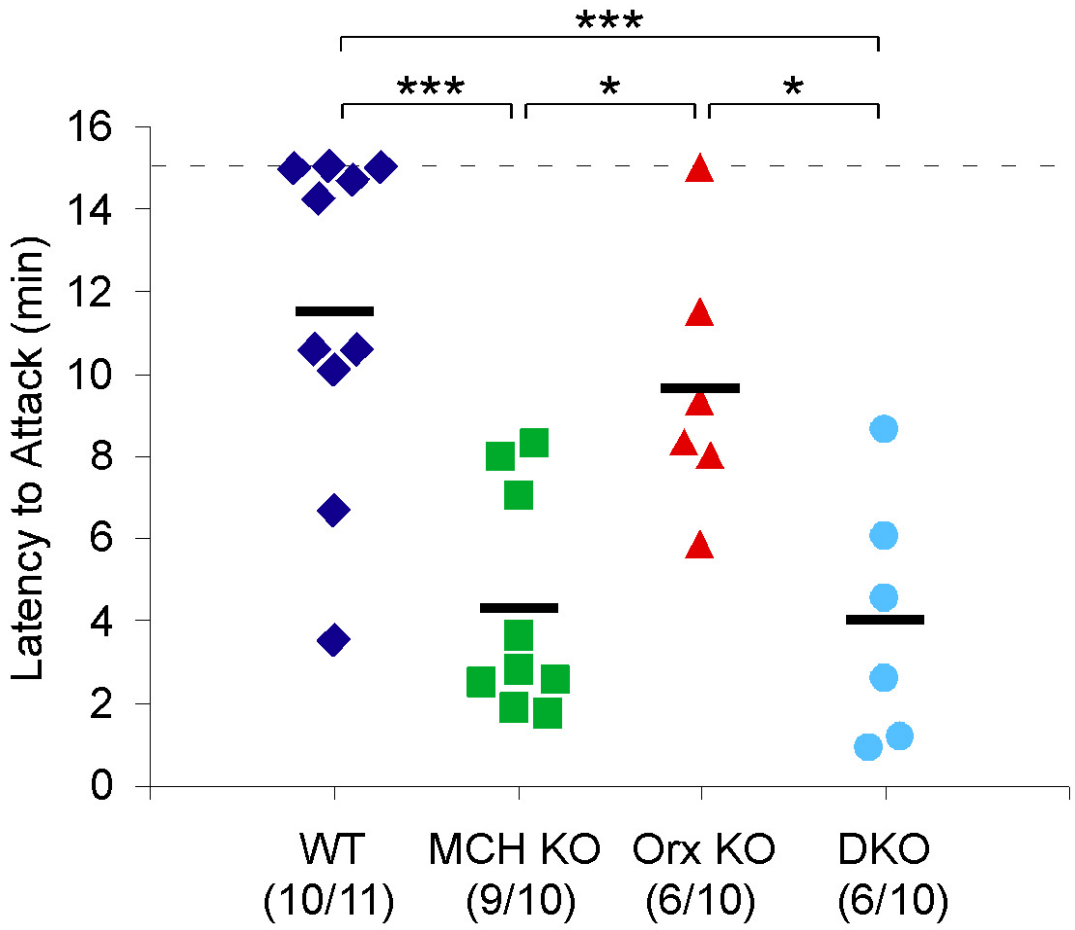


Figure 4-6. Inactivation of MCH promotes aggression.

Resident-intruder paradigm. Resident *MCH*$^{-/-}$ and *orexin*$^{-/-}$;*MCH*$^{-/-}$ males exhibited decreased latencies to attack an intruder male, while *orexin*$^{-/-}$ mice did not differ significantly from wild types. Bars represent means. *p<0.05 and ***p<0.001 for comparisons between bracketed pairs. Analysis by ANOVA and Tukey post-hoc tests.

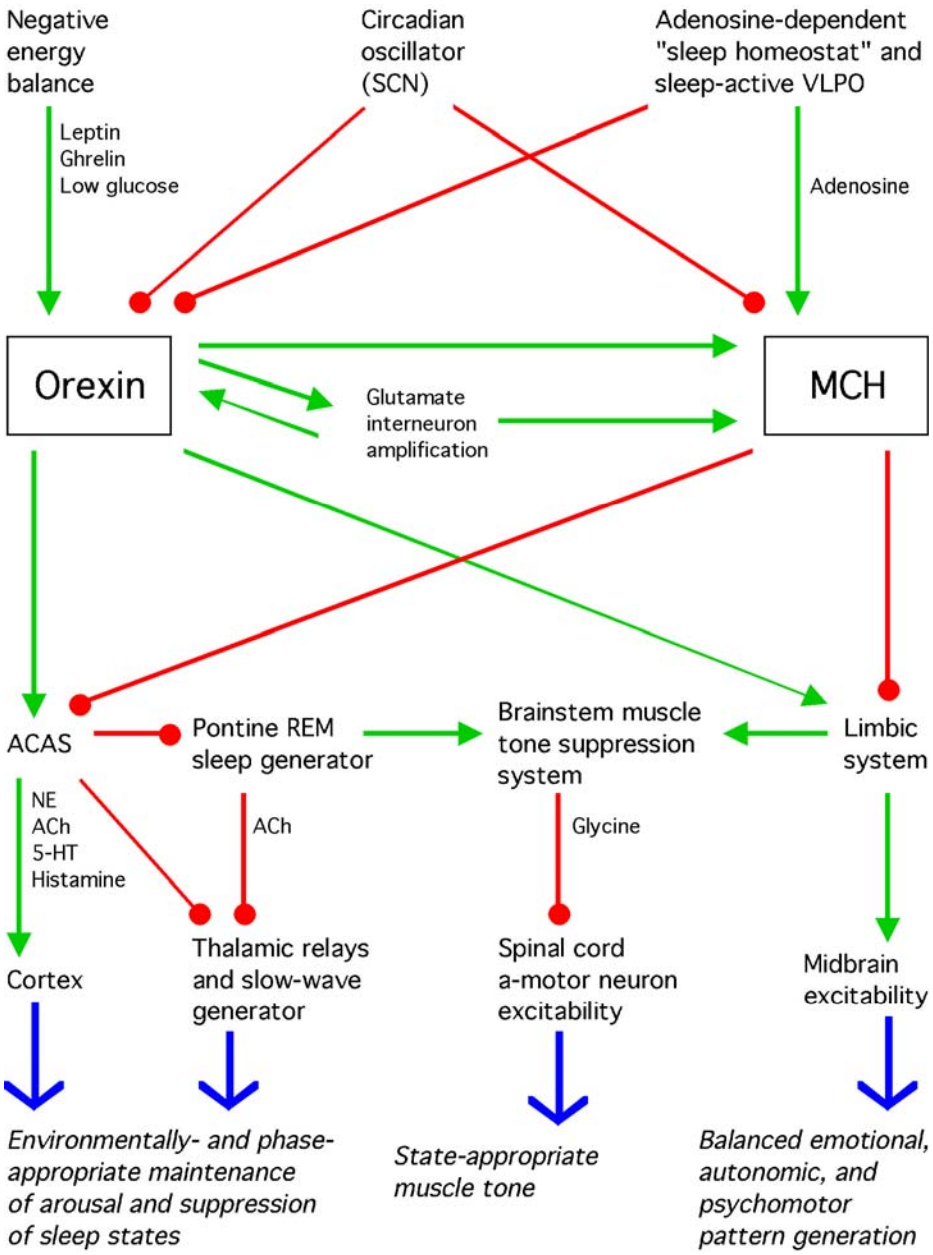


Figure 4-7. Model Depicting the Neurobiological Relationship between Orexin and MCH in the Regulation of Behavioral, Emotional, and Autonomic States.

See discussion for further details. SCN, suprachiasmatic nucleus. VLPO, ventral lateral preoptic area. ACAS, ascending cortical activating system. NE, norepinephrine. ACh, acetylcholine. 5-HT, serotonin.

CHAPTER FIVE
Effects of Unregulated Orexin Expression

SUMMARY

Orexin peptides, when administered exogenously, promote arousal and energy expenditure via receptors expressed throughout the central nervous system. Mice lacking orexins have a sleep disorder resembling narcolepsy and become obese despite reduced food intake. I characterized the phenotype of mouse strains that exhibit wide-spread overexpression of a rat prepro-orexin transgene driven by a β-actin/cytomegalovirus hybrid promoter (*CAG/orexin* transgenic mice). Despite hyperphagia, these mice exhibit reduced body weight and resistance to diet-induced obesity relative to wild-type controls. Indirect calorimetry suggests that transgenic mice utilize lipids at a higher rate than normal mice. *CAG/orexin* mice also exhibit abnormalities of sleep with fragmentation of non-REM sleep and a specific reduction in REM sleep. EEG/EMG studies reveal incomplete REM sleep atonia with abnormal myoclonic activity during this sleep stage. These results suggest that *CAG/orexin* mice have abnormalites of growth and metabolism and an inability to maintain sleep states. Orexins influence homeostatic set-points affecting both energy metabolism and sleep/wakefulnuss.

INTRODUCTION

As described in Chapter 1, administrations of orexin peptides at pharmacological doses in rodents have been reported to induce feeding (Sakurai et al., 1999), increase metabolic rate (Lubkin and Stricker-Krongrad, 1998), alter neuroendocrine homeostasis (Hagan et al., 1999), increase motor activity (Nakamura et al., 2000), and promote arousal (Hagan et al., 1999; Piper et al., 2000). A selective antagonist of OX1R reduces food intake, prevents obesity, and selectively suppresses REM sleep (Haynes et al., 2002; Haynes et al., 2000; Smith et al., 2003). Abnormalities observed in orexin-deficient animals are consistent with some of these pharmacological observations. While narcolepsy induced by loss-of-function studies of the orexin system highlight the primary importance of orexins in the regulation of sleep/wakefulness, more detailed gain-of-function studies may lead to new insights regarding interaction of the orexin system with other homeostatic processes, and such insights might have clinical implications. To examine the effects of chronically increased orexinergic tone in mice, I characterized the phenotype of lines of *CAG/orexin* transgenic mice utilized in the previous chapter.

RESULTS

Independent Lines of *CAG/orexin* Transgenic Mice Overexpress Orexin

The production of *CAG/orexin* transgenic mice by T. Sakurai is described in the previous chapter. Although multiple lines overexpressed orexin peptides in ectopic brain patterns similar to that observed in **Figure 5-1**, the absolute amounts of brain orexin expression (as determined by Northern blots) and peptide levels (as determined by orexin-A RIAs) varied by line. In this study I utilized two lines, L2 and L62, in which the whole-brain orexin contents were increased by about 50- and 100-fold, respectively, as compared with wild-type mice (data not shown). The transgene produces functional orexin peptides since its expresson in orexin neuron-ablated mice results in rescue of the narcoleptic phenotype (*orexin/ataxin-3* transgenic mice were bred to L62 for experiments in **Chapter 7**). No major phenotypic differences were observed between lines L2 and L62 in metabolic studies. Therefore, they are not differentiated in such results reported below. In contrast, a subtle gene-dosage effect was observed in parameters of vigilance between the L2 and L62 lines. These data are reported seperately for each line.

Studies of Growth, Body Adiposity, and Energy Homeostasis

To probe the effects of orexin overexpression on body weight, I recorded growth curves for male and female wild-type and transgenic littermates maintained on various diets. Transgenic animals of both sexes maintained on a relatively lean chow (6% fat by weight) exhibited a mild reduction in body weight that amounted to a non-significant 2-g difference by 4-5 weeks of age (**Figure 5-1**). When maintained on breeder chow with a

higher caloric density (11% fat by weight), male wild-type mice gained significantly more weight than wild-type mice maintained on lean chow (diet induced obesity, DIO). In contrast, transgenic male mice resisted obesity: no significant difference in body weight was observed among transgenic groups maintained on low and higher fat chows. Females exhibited a similar resistance to obesity relative to controls when transferred from an 11% fat chow to a highly palatable condensed milk (17% fat) diet (**Figure 5-2A**). Crude measures of body mass index (weight divided by the square of length) suggested that the heaviness induced in wild-type mice compared to transgenic mice related to increased obesity. To confirm these findings, female mice maintained on condensed milk chow were subjected to nuclear magnetic resonance (NMR) scanning to produce more specific estimates of lean and fat masses. NMR analysis confirmed that differences observed in body weight resulted predominantly from differences in fat mass (**Figure 5-2B**). Unexpectedly, however, orexin overexpression also resulted in a mild but significant 2-g reduction in mean lean mass as well. Thus *CAG/orexin* mice are not only leaner than wild-type littermates, but smaller as well. This size difference was verifed by measures of body length, which were reduced in transgenic mice (data not shown).

To determine whether resistence to obesity in *CAG/orexin* mice was associated with differences in energy consumption, I monitored food intake in transgenic mice and wild-type littermates. While absolute daily amounts of food intake did not differ significantly in these two groups, when food intake/day was adjusted for differences in daily body weight, *CAG/orexin* mice were found to be hyperphagic compared to controls (**Figure 5-3A**).

To examine whether *CAG/orexin* mice expend more energy than controls, M. Mieda and I utilized indirect calorimetry. We monitored the hourly rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) of both groups for several days in preliminary studies with small numbers of animals. From such data, circadian patterns of respiratory quotient (RQ) and lipid substrate utilization were also calculated over circadian phases . Wild-type mice exhibited circadian rythyms of metabolic activity. During the dark phase when mice are highly active, VO_2 and RQ were increased, and lipid utilization reached its lowest circadian level due presumably to the preferential utilization of carbohydrates as primary substrate (**Figure 5-3B**). Compared to littermate controls of similar body weight, orexin overexpressing *CAG/orexin* transgenic mice exhibited similar circadian patterns of VO_2, RQ, and substrate utilization at night. During the light (resting) phase, transgenic mice exhibited non-significant changes in VO_2, but RQ was reduced (data not shown), and rates of lipid utilization were abnormally elevated compared to wild-type mice (**Figure 5-3B**). An overall trend toward increased lipid utilization was observed over 24 h in this preliminary study.

EEG/EMG Studies

To examine the effects of orexin overexpression on spontaneous sleep/wake patterns, I monitored the EEG/EMG of mice of two indepependent lines (L2 and L62) of *CAG/orexin* transgenic mice and their wild-type littermates. Hypnograms from individual mice of each genotype during the light and dark phases illustrate the behavior of these animals (**Figure 5-4**). Compared to wild-type controls, *CAG/orexin* mice exhibited evidence of disturbed sleep, especially during the light phase. In particular, *CAG/orexin*

mice have increased fragmentation of sleep patterns during the light phase when mouse behavior is normally predominated by sleep. Increases in the frequency of wakefulness episodes and decreases in the durations of non-REM and REM sleep support this observation (**Table 5-1**). Most notably, normal patterns of REM sleep are profoundly disturbed in transgenic mice during both phases: *CAG/orexin* mice have significant suppressions of the amounts of REM sleep exhibited during the light phase and significantly reduced durations of REM sleep time accross light and dark phases. Additionally, orexin overexpressing mice exhibit reduced REM latencies and REM intervals.

Close observation of the structural features of EEG/EMG patterns reveal other abnormalities in *CAG/orexin* mice as well. The onset of REM sleep in normal wild-type mice is characterized by REM sleep atonia: a further reduction of postural muscle tone compared to that of preceding non-REM sleep. Although twitching of peripheral muscles (e.g. hands, feet, facial musculature, etc.) often occurs during REM sleep, postural muscles remain virtually quiet. In contrast, mice that overexpress orexin exhibited sporadic increases in postural muscle tone with the onset of REM sleep. A striking increase in the frequency and amplitude of phasic twitches, recorded from nuchal muscles, were also observed ($p=0.05$) (**Figure 5-5A, -5B**). Abnormalities of atonia in REM sleep have also been reported in association with narcolepsy in humans. For comparison, narcoleptic *orexin/ataxin-3* transgenic mice of a similar genetic background were also examined in this experiment. Notably, *orexin/ataxin-3* mice also exhibited a mild increase in phasic muscle activity during REM sleep, although it was of an intermediate frequency relative to wild-type and *CAG/orexin* transgenic mice and was not

found to be statistically significant (**Figure 5-5B**). Notably, *orexin/ataxin-3* mice showed normal frequencies of tonic muscle activity during REM sleep.

DISCUSSION

Orexin Overexpression Disrupts Energy Homeostasis

Chronic orexin overexpression resulted in multiple abnormalities of energy homeostasis in mice. As expected from pharmacological studies of orexin peptides and an OX1R antagonist (Haynes et al., 2002; Haynes et al., 2000; Sakurai et al., 1998b), increased orexin tone caused hyperphagia. Despite this, *CAG/orexin* transgenic mice exhibited a mild reduction in lean body mass and length suggesting subtle alterations in growth and somatotrophic homeostasis. Indeed one study reported suppressions of circulating growth hormone and prolactin following central administrations of orexin-A in rats (Hagan et al., 1999).

Additionally, transgenic mice were resistant to diet-induced obesity when fed palatable energy-dense diets. Both males and females exhibited this resistance. Evidence of decreased RQ and increased lipid utilization that was limited to the light phase further suggested an abnormality of metabolism in transgenic mice that provides a possible mechanism for resistance to obesity. Notably, preliminary experiments measuring locomotor activity of *CAG/orexin* transgenic mice maintained on normal (6% fat) chow suggested no significant differences in overall activity (data not shown). However, it will be necessary to repeat these experiments in mice fed high fat diets to examine whether

compensatory changes in locomotor activity contribute to resistance to diet-induced obesity.

Our findings of abnormal metabolism in orexin overexpressing mice may be consistent with one report suggesting that orexin administrations induce an increase in energy expenditure and basal metabolic rate. In contrast, a selective OX1R antagonist not only to reduce feeding, but also exerts anti-obesity and antidiabetic effects that might have resulted in part from increases in metabolic rate. Whether individual orexin receptors exert distinct or even opposing metabolic effects merits further investigation. Our results support the notion that components of the orexin system may provide therapeutic targets for disorders of energy homeostasis such as obesity and diabetes.

Orexin Overexpression Disrupts the Patterns and Structure of Sleep

CAG/orexin transgenic mice exhibit complex alterations of sleep/wakefulness states. Primarily, orexin overexpression induced an inability to maintain sleep states as evidenced by increased frequency of wakefulness and non-REM sleep episodes across light and dark phases and a selective decrease in the duration of non-REM sleep episodes during the light phase. These findings contrast with similar studies of mice suffering orexin-deficient narcolepsy in which animals are primarily unable to maintain wakefulness states during the dark phase (**Chapters 2, 4** and **Appendix 1**). Strong disruption of normal sleep states were also evident: while narcoleptic mice display frequent and abnormal intrusions of REM sleep into wakefulness, *CAG/orexin* transgenic mice displayed a significant suppression of the amounts of REM sleep during the light

phase, and reduced REM sleep durations across phases. These findings are consistent with a role of orexin in inhibiting the expression of REM sleep.

That orexin imbalance also disrupted mechanisms of REM sleep atonia may have particular clinical relevance for disorders of abnormal muscle activity and myoclonus during sleep, such as Restless Legs Syndrome (RLS) or REM Sleep Behavior Disorder (RBD) (Schenck and Mahowald, 2002). Indeed, one recent study indicated that early-onset familial forms of RLS are associated with higher levels of orexin-A in the CSF (Allen et al., 2002). RBD, on the other hand, is often associated with human narcolepsy. Indeed, in this study I identified increases in phasic muscular activity during REM sleep in both orexin overexpressing mice as well as orexin neuron ablated-controls. A potentially complex role of orexins in such processes is indicated by orexin microinjection studies in cats that suggest that REM sleep-related muscle atonia can be facilitated or inhibited depending on the site of injection in brainstem areas that have previously been implicated in the control of REM sleep atonia (Mileykovskiy et al., 2002).

Sleep and Energy Homeostasis: Chicken or the Egg?

The association of metabolic and sleep abnormalities in this study is intriguing. While ablation of orexin neurons is associated not only with narcolepsy but also obesity despite hypophagia and locomotor inactivity under some conditions (Hara et al., 2001), orexin overexpression induced inability to maintain sleep and subtle deficits of somatic growth despite hyperphagia and increased metabolism of lipids. It is possible that orexins exert disparate effects upon homeostatic processes that are not intrinsically related.

Alternatively, disruptions of sleep may result in secondary effects upon energy homeostasis or *vice versa*. Pulsatility of growth hormone secretion is strongly correlated with patterns of non-REM sleep, and deprivation of non-REM sleep reduces circulating growth hormone. It is therefore possible that sleep disruptions in *CAG/orexin* mice cause growth deficits. Increased arousal during the light phase might also contribute to subtle alterations in metabolism that contribute to resistance to obesity. Clearly further studies will be needed to delineate the multiple effects of increased orexin signaling in *CAG/orexin* transgenic mice.

EXPERIMENTAL PROCEDURES

CAG/orexin Transgenic Mice

The production and maintenance of orexin overexpressing mice is described in the previous chapter. All experiments utilized male and female mice from crosses in which hemizygous transgenic mice were bred to wild-type C57BL6/J mice. Offspring (DBA1 backcrossed to C57BL6/J, N4-N5) were weaned at 21 days and genotyped by PCR.

Metabolic Experiments

Growth curves were generated from weekly weight starting at 21 days of age. Animals were maintained on normal 6% fat chow, 11% fat breeder chow, or condensed milk (17% fat chow) (Research Diets). BMI estimates were obtained using nosal-anal

length measurements. Fat and lean mass measurements were obtained by NMR scanning (Minispec NMR Analyzer, Bruker) carried out on live mice at 38-weeks of age in the laboratory of C. Li at UTSW. Food intake of individually-housed mice was recorded for 5 days and averaged. VO_2 and VCO_2 measurements by indirect calorimetry were recorded hourly for three days from individual singly-housed mice using an Oxymax gas analyzer (Columbus Instruments). Fat utilization was calculated based upon the following formula: $[(VO_2)(1\text{-}RQ)] / [(1\text{-}0.71)(4.74 \text{ kcal heat/L O2})/(9.3 \text{ kcal heat/g fat oxidized})]$ where 0.71 is the RQ for 100% utilization of fat. Statistical analysis was performed by Student t-test or ANOVA as appropriate.

Sleep Studies

EEG/EMG monitoring and analysis was performed as described in previous chapters. For quantifying abnormal motor activity during REM sleep, all REM sleep events during a 24-h period in each of 3 animals per genotype were scored. Phasic muscle activity during REM sleep was defined as a deflection of the EMG of greater than 50% above baseline. A tonic activity event was scored when a series of deflections lasted for longer than 0.3 sec. Statistical comparisons were performed by ANOVA.

DISPLAY ITEMS

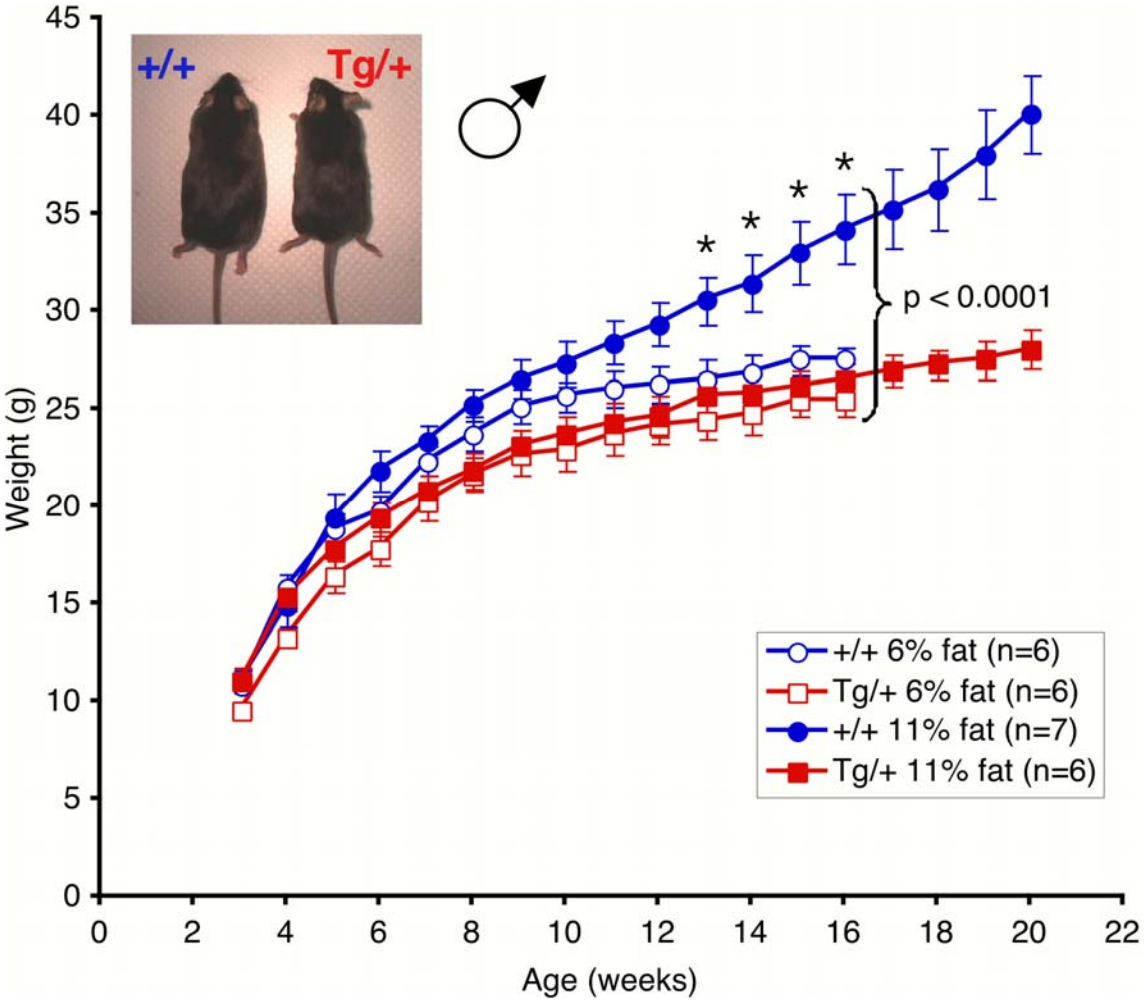
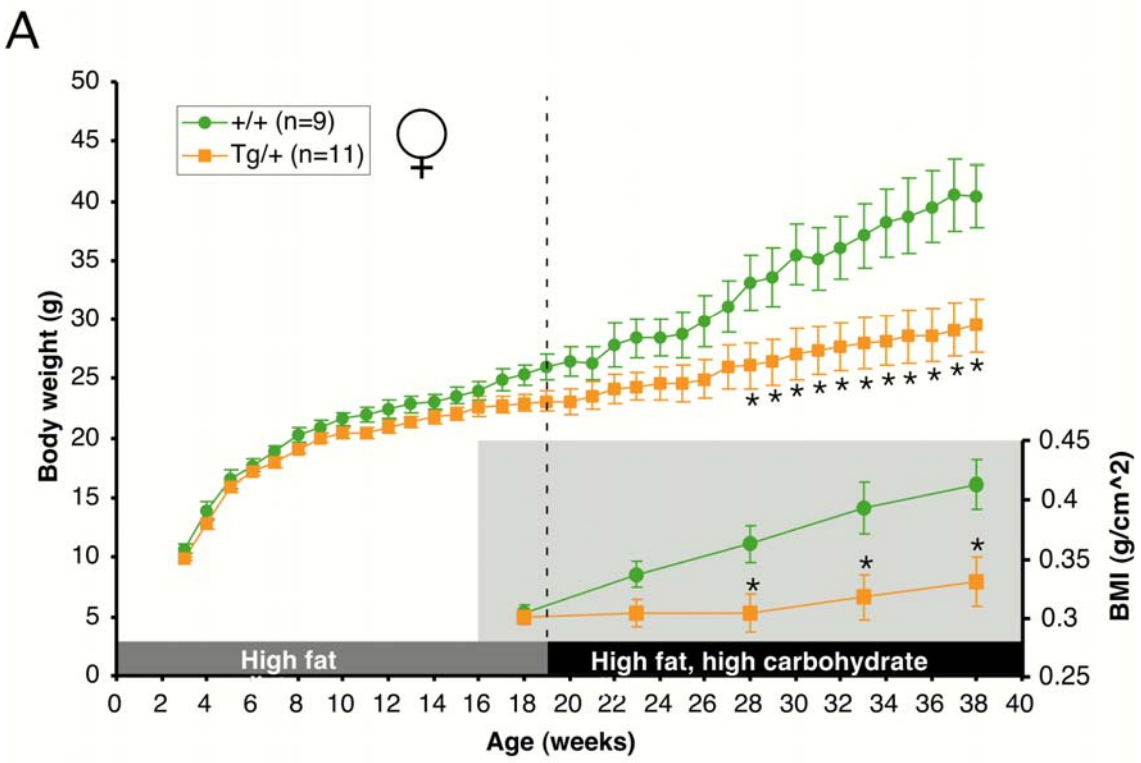


Figure 5-1. Growth Curves of Male *CAG/orexin* Transgenic Mice.

Growth curves on 6% chow reveal similar rates of growth in wild-type and transgenic mice, although transgenic mice reveal a non-significant tendency to weigh about 2 g less than controls after 4-5 weeks of age. In contrast, when mice of both genotypes are fed 11% fat chow, wild-type mice gain significantly more weight (diet-induced obesity) than wild-type mice fed 6% chow and also gained significantly more weight than transgenic mice fed an 11% chow. Thus, orexin overexpressing mice are resistant to diet-induced obesity. The image inset illustrates the size difference between typical littermates at 18 weeks of age. The p-value above represents statistical significant differences among genotypes until week 16 by two-way ANOVA (genotype by time). The Tukey post-hoc test shows differences are between the wild-type group fed 11% fat (*$p<0.05$ at each week shown) and all other groups.


A
+/+ (n=9)
Tg/+ (n=11)
Body weight (g)
BMI (g/cm^2)
High fat
High fat, high carbohydrate
Age (weeks)

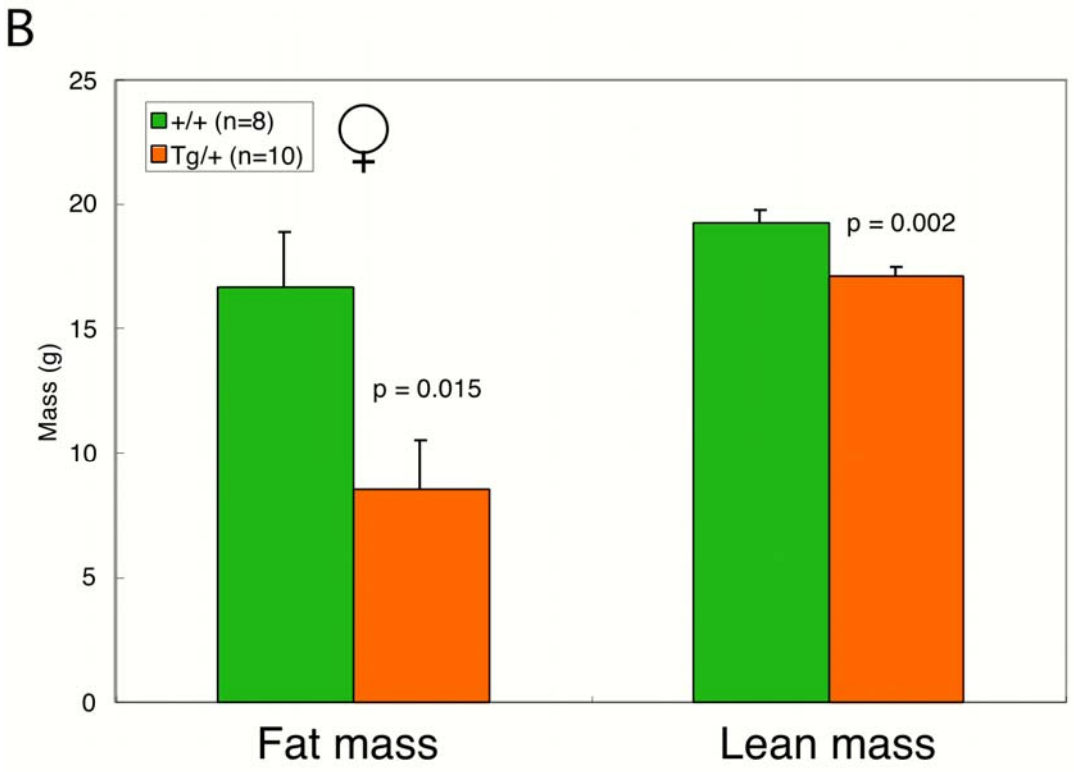

B
+/+ (n=8)
Tg/+ (n=10)
Mass (g)
p = 0.015
p = 0.002
Fat mass
Lean mass

Figure 5-2. Growth Curves and Body Composition Analysis of Female *CAG/orexin* Trangenic Mice.

(A) Female wild-type and transgenic mice exhibit less of a difference than do male mice when maintained on 11% fat chow. When mice are shifted to a highly palatable condensed milk chow (17% fat) after 21 weeks of age, however, wild-type mice exhibit diet induced obesity relative to transgenic mice which are relatively resistant. Body mass index (weight/length/length) is also shown after 18 weeks of age. *$p<0.05$.

(B) Differences in body composition following diet-induced obesity using NMR scanning confirmed that weigh differences resulted from reductions in both fat as well as lean masses.

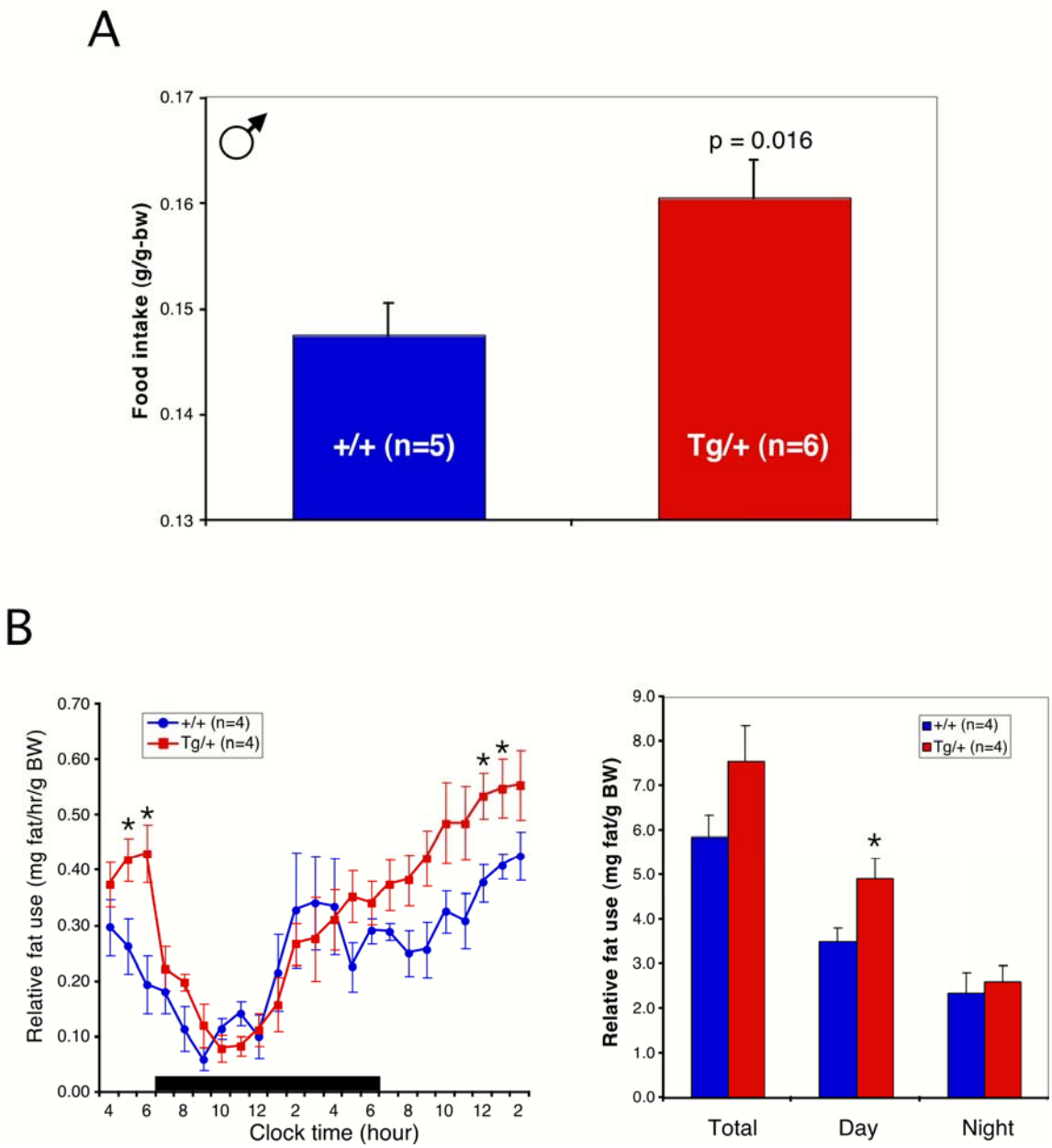
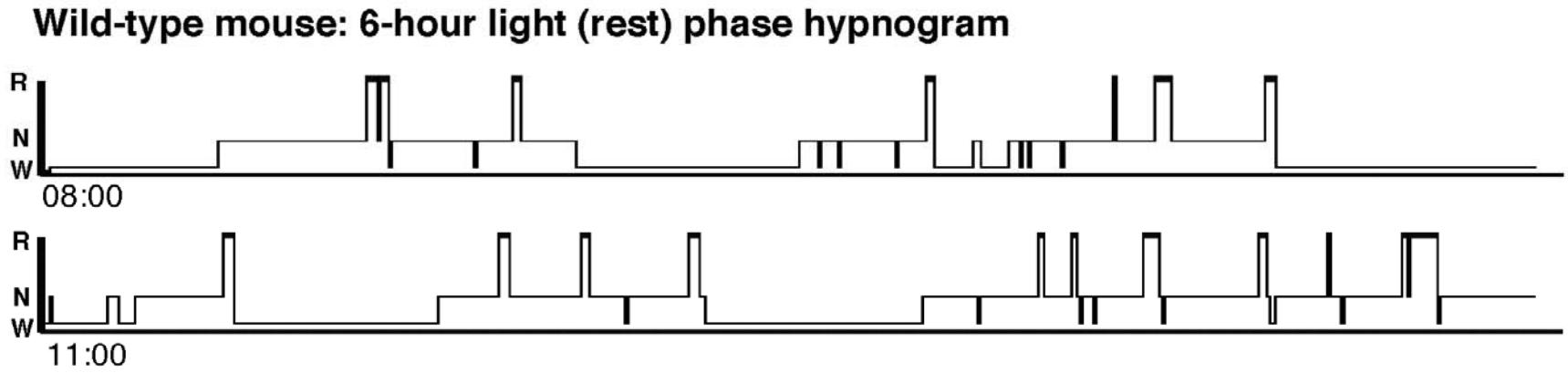

A
0.17
0.16
0.15
0.14
0.13
Food intake (g/g-bw)
♂
p = 0.016
+/+ (n=5)
Tg/+ (n=6)
B
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Relative fat use (mg fat/hr/g BW)
+/+ (n=4)
Tg/+ (n=4)
* *
* *
4 6 8 10 12 2 4 6 8 10 12 2
Clock time (hour)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Relative fat use (mg fat/g BW)
+/+ (n=4)
Tg/+ (n=4)
*
Total
Day
Night

Figure 5-3. Food Intake and Fat Utilization in CAG/orexin Transgenic Mice.

(A) Transgenic mice are hyperphagic relative to controls. Daily food intake, relative to body weight, of singly housed wild-type and transgenic mice, averaged over 5 days.

(B) Transgenic mice exhibit increased utilization of fat as a substrate compared to controls during the rest phase by indirect calorimetry. Hourly rates of fat utilization (mg fat/h/g body weight) are calculated from VO_2 and VCO_2 measurements and are displayed in the left panel. The right panel displays calculated amounts of fat utilized (mg fat/g) during light phase, dark phase, and overall. Indirect calorimetry experiment was assisted by M. Mieda.


Wild-type mouse: 6-hour light (rest) phase hypnogram
R
N
W
08:00
R
N
W
11:00

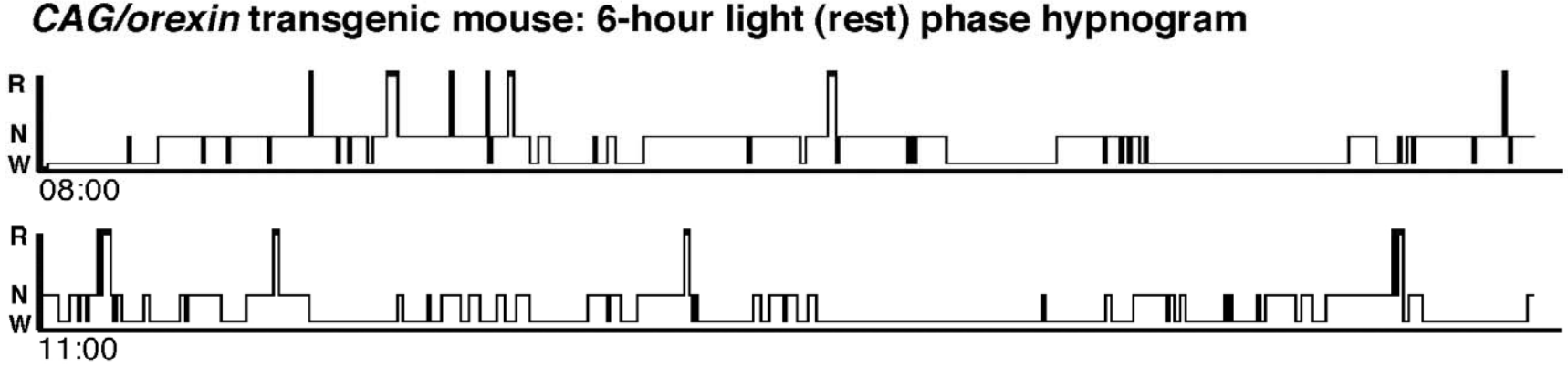
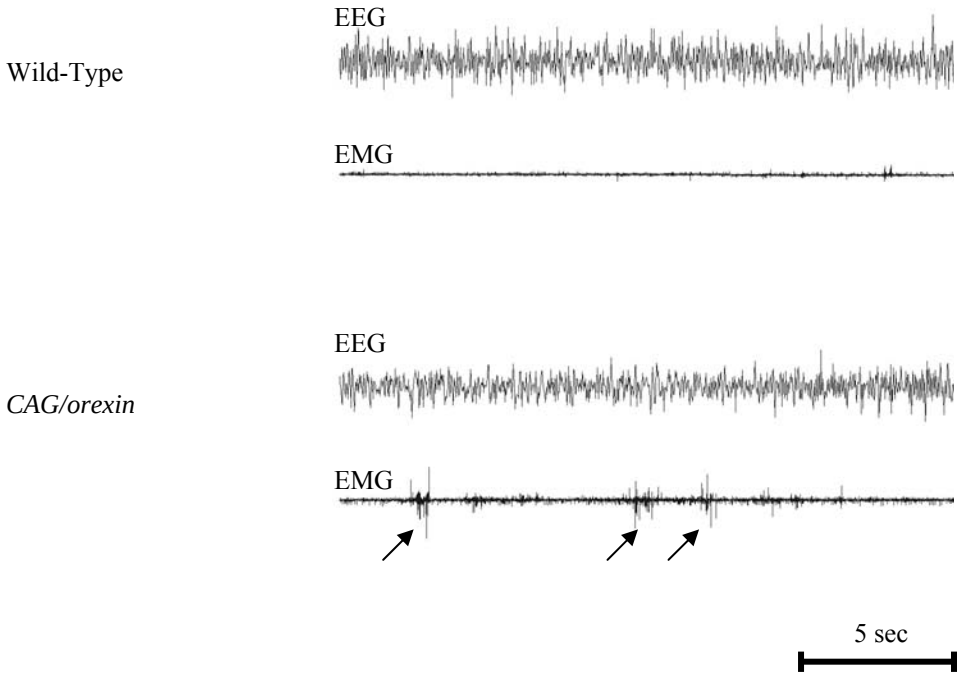

CAG/orexin transgenic mouse: 6-hour light (rest) phase hypnogram
R
N
W
08:00
R
N
W
11:00

Figure 5-4. Illustrative Hypnograms from Wild-type and *CAG/orexin* Transgenic Mice.

Sleep/wake patterns (W, wakefulness; N, non-REM sleep; and R, REM sleep) generation from concatenating EEG/EMG stage scores over the first 6 h of the light phase are shown. Note the increased fragmentation of non-REM sleep and reduced amounts and durations of REM sleep in transgenic mouse. *CAG/orexin* mouse shown is from line L2.

A


Wild-Type
EEG
EMG
CAG/orexin
EEG
EMG
5 sec

B

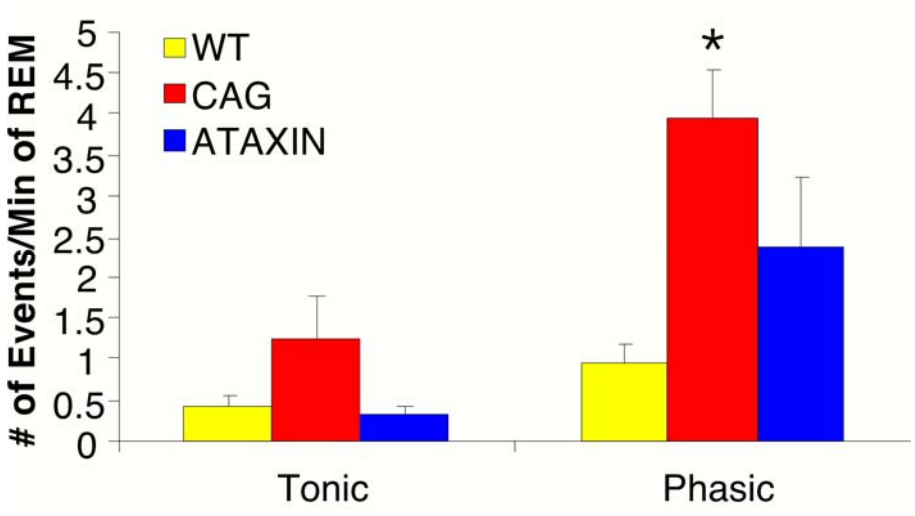

# of Events/Min of REM
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
CAG
ATAXIN
*
Tonic
Phasic

Figure 5-5. Abnormalities of REM sleep Atonia in *CAG/orexin* Transgenic Mice.

(A) Illustrative EEG/EMG traces reflect REM sleep in wild-type and transgenic mice. Note increased postural tone and increased phasic activity (arrows) evident in the EMG of the transgenic mouse.

(B) Frequencies of tonic and phasic activities in wild-type, *CAG/orexin*, and *Orexin/ataxin-3* transgenic mice during REM sleep. Note high frequencies of abnormal tone and phasic activity in *CAG/orexin* mice but a milder elevation of phasic activity in *orexin/ataxin-3* transgenic mice as well. Visual scoring was performed by H. Kirane (rotation student supervised by the author).

CHAPTER SIX
Treatment of Narcolepsy - Modafinil

SUMMARY

Modafinil, an atypical wakefulness-promoting agent with an unknown mechanism of action, is used to treat hypersomnolence in narcolepsy patients. Fos protein immunohistochemistry has previously demonstrated that orexin neurons are activated after modafinil administration, and it has been hypothesized that the wakefulness-promoting properties of modafinil might therefore be mediated by the neuropeptide. Here I tested this hypothesis by immunohistochemical, electroencephalographic, and behavioral methods using modafinil at doses of 0, 10, 30 and 100 mg/kg i.p in *orexin*$^{-/-}$ mice and their wild-type littermates. I found that modafinil produced similar patterns of neuronal activation, as indicated by Fos immunohistochemistry, in both genotypes. Furthermore, modafinil more effectively increased wakefulness time in *orexin*$^{-/-}$ mice than in the wild-type mice. This may reflect compensatory up-regulation in components of central arousal following the prolonged absence of orexin in the null mice. In contrast, the compound did not suppress direct transitions from wakefulness to REM sleep, a marker of narcolepsy in mice. Spectral analysis of the electroencephalogram in awake *orexin*$^{-/-}$ mice under baseline conditions revealed reduced power in the theta band frequencies (8-9 Hz), an index of alertness and attentive vigilance in the rodent. Modafinil administration only partly compensated for this vigilance deficit in the orexin

null mice. I conclude that the presence of orexin is not required for the wakefulness-promoting action of modafinil.

INTRODUCTION

Modafinil (a diphenylmethyl-sulphinyl-2 acetamide derivative) is a wake-promoting substance used for the treatment of hypersomnolence, in particular that associated with narcolepsy (Bastuji and Jouvet, 1986; 1988; US Modafinil in Narcolepsy Multicenter Study Group, 1998; 2000). It effectively enhances wakefulness in all species tested to date, including drosophila, mice, rats, cats, dogs, monkeys and humans (Lagarde and Milhaud, 1990; Touret et al., 1995; Lin et al., 1992; Shelton et al., 1995; Hermant et al., 1991; Hendricks et al., 2003; Simon et al., 1996; Panckeri et al., 1996). Modafinil is pharmacologically distinct from classical wakefulness-inducing compounds such as the amphetamines, methylphenidate and pemoline (Simon et al, 1995; Mignot et al., 1994), and, in comparison with these agents, the compound is well tolerated and has a relatively low potential for abuse (Janinski and Kovacevic-Ristanovic, 2000; Malcolm et al., 2002). Despite exhaustive research, the exact mechanism for the wakefulness-promoting action of modafinil remains unknown (Saper and Scammell, 2004).

Significant research has focused on the dopaminergic actions of the compound. Modafinil has been shown to increase extracellular dopamine (DA) levels, as measured by microdialysis, in rat nucleus accumbens (Ferraro et al., 1996; 1997) and prefrontal cortex (de Saint Hilaire et al., 2001), as well as in the caudate of narcoleptic dogs (Wisor et al., 2001). The most intriguing evidence for a dopaminergic role in modafinil-induced

wakefulness, however, comes from a study in DA transporter (DAT) knockout mice (Wisor et al., 2001). These mice showed no wakefulness-enhancing response to 300 mg/kg modafinil, a dose much higher than that normally required for this effect in mice. Although DAT has not been shown to be a direct molecular target of modafinil, these results nevertheless underline the role that the DAT must play in modafinil-induced wakefulness.

Another experimental approach, Fos immunohistochemistry, has been used to determine those brain regions that are activated after modafinil administration (Lin et al., 1996; Engber et al., 1997; Chemelli et al., 1999; Scammell et al., 2000). Modafinil-induced activation was noted in regions such as the tuberomammillary nucleus (TMN) and the orexin (also called hypocretin) cell group in the perifornical region of the hypothalamus (Scammell et al., 2000) that have been implicated in the promotion of wakefulness. Indeed, the demonstration that modafinil induced Fos in orexin cells, and the putative involvement of these cells in the regulation of wakefulness, together suggest that orexin might be necessary for the wake-promoting effects of the compound. I therefore measured the effectiveness of modafinil at inducing wakefulness in *orexin*$^{-/-}$ mice. I also examined whether the absence of orexin affected the pattern of Fos activation induced by modafinil administration in these mice.

RESULTS

Immunohistochemistry

The pattern of Fos immunoreactivity was examined in brains of wild-type and *orexin*$^{-/-}$ mice treated with vehicle or modafinil (10 or 100 mg/kg i.p.) (**Fig. 6-1**) in collaboration with T. Scammell. In both genotypes treated with vehicle, the pattern of Fos expression was similar to that previously reported for the spontaneously awake rat (Scammell et al., 2000). Scattered Fos-immunoreactive (Fos-IR) nuclei were present in most cortical regions with slightly higher levels in cingulate and pyriform cortex. The lateral septum contained moderate numbers of Fos-IR neurons, but labeling was very rare in the nucleus accumbens and striatum. The medial and ventral parts of the bed nucleus of the stria terminalis contained mild to moderate labeling, but Fos-IR nuclei were uncommon in the laterodorsal subdivision. The medial and lateral parts of the preoptic area contained scattered cells, and the ventrolateral preoptic area contained at most occasional labeled nuclei. All parts of the basal forebrain contained a few Fos-IR neurons. A small number of labeled nuclei were often present around the dorsolateral border of the suprachiasmatic nucleus, extending into the subparaventricular zone, and tapering off into the anterior hypothalamic area. One wild-type and one *orexin*$^{-/-}$ mouse had moderate labeling in the paraventricular nucleus of the hypothalamus, but this nucleus typically contained only mild labeling. The perifornical region and lateral hypothalamus contained moderate numbers of Fos-IR nuclei, with no substantial differences between the genotypes. All mice had mild-moderate labeling in the posterior

hypothalamus. Moderate numbers of nuclei were labeled in the tuberomammillary nucleus. Occasional labeling was also present in the central nucleus and other parts of the amygdala. In the brainstem, the periaqueductal grey contained occasional Fos-IR neurons, but cells were rare in the raphe nuclei. Similar to previous findings in the rat (Scammell et al., 2000) the retro-ventral tegmental region was never labeled in vehicle-treated mice. A few cells were present in the pedunculopontine and laterodorsal tegmental regions, lateral parts of the parabrachial nucleus, and lateral subdivision of the nucleus of the solitary tract. Mice treated with 10 mg/kg modafinil had a pattern of Fos expression very similar to that seen in vehicle-treated mice.

In contrast, wild-type and *orexin*$^{-/-}$ mice treated with 100 mg/kg modafinil had substantial increases in Fos expression in many brain regions with no difference apparent between the genotypes (**Fig. 6-1**). After the 100 mg/kg dose of the compound, mice had many Fos-IR neurons in frontal, parietal, and cingulate cortex, with greater than normal labeling in the pyriform cortex. Fos-IR nuclei were abundant throughout the striatum and were moderately common in the accumbens core and shell. The islands of Calleja contained intense labeling, but labeling was light in the olfactory tubercle. Many nuclei were labeled in the laterodorsal subdivision of the bed nucleus of the stria terminalis and the lateral subdivision of the central nucleus of the amygdala. The number of Fos-IR neurons in the preoptic area (including the ventrolateral preoptic area) was similar to that seen with vehicle. No increase in labeling was apparent in the suprachiasmatic nucleus, subparaventricular zone, or anterior hypothalamic area. The number of Fos-IR nuclei was moderately increased in the parvocellular regions of the paraventricular nucleus. The perifornical region, lateral hypothalamus (especially caudally, adjacent to the

cerebral peduncle), posterior hypothalamus, and tuberomammillary nucleus also contained more Fos-labeled neurons. The substantia nigra and ventral tegmental area contained only a few lightly labeled nuclei, except in one brain with a moderate number of Fos-IR nuclei in both regions. Within the brainstem, the dorsal raphe often contained moderate labeling, but Fos immunoreactivity was rare in the locus coeruleus. All mice that received 100 mg/kg of modafinil had moderate labeling in the interfascicular nucleus and retro-ventral tegmental area.

Vigilance states

I recorded EEG/EMG signals for 24 hr after vehicle or modafinil administration at the onset of the dark phase (19:00). Vigilance state parameters, analyzed over the 12 hr dark and subsequent light periods following all doses are presented in detail in **Appendix D**. Notably, amounts of sleep and wakefulness recorded after vehicle administrations differed only minimally from those previous reported for *orexin*$^{-/-}$ and wild-type mice under baseline conditions (Chemelli et al., 1999; Willie et al., 2003) (see also **Chapter 2**).

The hourly distribution of vigilance states under baseline conditions (i.e., after vehicle administration) were examined (**Table 6-1**). Despite elevated amounts of REM sleep, which is a characteristic of sleep in *orexin*$^{-/-}$ mice during the active phase, the times spent in wakefulness or non-REM sleep (data not shown) did not differ between the genotypes during the 12 hr dark period after vehicle administration. Next, the hourly distribution of the effect of modafinil on vigilance states in the 2 genotypes relative to baseline was determined (**Fig. 6-2**). Compared to control levels, administration of 100

mg/kg of modafinil to the *orexin*$^{-/-}$ mice increased wakefulness time during each of the first 8 hr of recording ($P < 0.05$). In contrast, when wild-type mice were administered the same modafinil dose, significant increases in wakefulness over baseline were observed only over the first 5 hr after drug administration ($P < 0.05$). Indeed, during hour 8 of recording, the effect of 100 mg/kg modafinil on wakefulness in the orexin-null mice was significantly greater than the effect of the compound in the wild-type mice ($t [18] = 2.14$, $P < 0.05$). The differential sensitivity of the genotypes to the wakefulness-inducing properties of modafinil was also apparent at lower doses: during the first hour of recording, modafinil increased wakefulness time in the *orexin*$^{-/-}$ mice at both 10 and 30 mg/kg, but in the wild-type mice only at 30 mg/kg ($P < 0.05$).

To examine the dose-response effect of modafinil on wakefulness in more detail, the total cumulative increase in wakefulness induced over the corresponding vehicle data was determined for each mouse and for each dose of modafinil. Means (± SEM) for the 3 doses were plotted by genotype (**Fig. 6-3**) as a Lineweaver-Burk double-reciprocal plot to ensure linearity. Adopting the method of Kleinbaum et al. (1988), the dose response regression lines were not parallel ($F [1, 56] = 4.1$, $P < 0.05$), indicating that modafinil was more effective in inducing wakefulness in the *orexin*$^{-/-}$ mice.

Significant reductions in hourly REM sleep times from levels recorded after vehicle administration were also noted after modafinil was administered at 100 mg/kg (**Fig. 6-2**). In the *orexin*$^{-/-}$ mice these differences were significant during each of the first 5 hr, and in the wild-type mice during hours 2 through 5, following compound administration ($P < 0.05$). Also, during the first 5 hr, the reduction in REM sleep time caused by modafinil administration at 100 mg/kg was greater in the orexin null mice (3.0

± 0.5 min; mean ± SEM) than in the wild-type mice (2.0 ± 0.4 min) (**Fig. 6-2**). This difference between the genotypes in REM sleep time reached significance during the third hour after compound administration ($t [18] = 2.63$, $P < 0.02$). However, these differences must be considered in light of the baseline excess of REM sleep in *orexin*$^{-/-}$ mice compared with wild-type mice during the early dark phase (**Table 6-1**). No significant differences in REM sleep were induced by lower doses of modafinil in either genotype.

Next, the patterns of sleep debt and recovery of each genotype after modafinil administration were examined. Modafinil at 100 mg/kg resulted in accumulated non-REM sleep debts of 125 min during 8 hr and 100 min during 5 hr in *orexin*$^{-/-}$ and wild-type mice respectively (**Fig. 6-4 A,B**). During the first 5 hr, the rates at which non-REM sleep debt were accumulated in *orexin*$^{-/-}$ and wild-type mice were indistinguishable (19 and 20 min/hr, respectively, $P > 0.05$ by regression analysis). Including the additional 3 hr of compound effect for the analysis in the *orexin*$^{-/-}$ mice resulted in an overall accumulation of non-REM sleep debt of 16.5 min/hr. Notably, and despite the greater accumulated sleep debt in the null mice, the recovery slope for this genotype was significantly less than for the wild-type mice during the light period (0.9 and 2.1 min/hr respectively, $P < 0.05$). Even by the end of 24 hr after treatment with 100 mg/kg modafinil, 110 min (88 %) of the non-REM sleep debt remained unrecovered in the *orexin*$^{-/-}$ mice (**Fig. 6-4A**). Correspondingly, 90 min (90 %) of this deficit was unrecovered in the wild-type mice by the end of the recording (**Fig. 6-4B**). After lower doses of modafinil, the recovery of the non-REM sleep debts was also very limited in each genotype: orexin-null mice exhibited a 16 min deficit in non-REM sleep after 10

mg/kg and a 32 min deficit after 30 mg/kg at the end of recording, and wild-type mice exhibited corresponding deficits of 12 min and 26 min.

In contrast, the patterns of REM sleep debt and recovery differed somewhat from those observed for non-REM sleep. *Orexin*$^{-/-}$ mice accumulated more REM sleep debt than wild-type mice (16 min during 7 hr versus 13 min during 8 hr, respectively; **Fig. 6-4 C,D**), and this debt was accumulated more rapidly in *orexin*$^{-/-}$ than in wild-type mice (2.4 and 1.8 min/hr, respectively, $P < 0.05$). In contrast, the rate of recovery of the REM sleep debt during the light period was the same in both genotypes (0.4 min/hr, $P > 0.05$). At the end of the 24 hr recording period after 100 mg/kg modafinil, 8 min (50 %) of the REM sleep deficit remained unrecovered in the *orexin*$^{-/-}$ mice. Likewise, 8 min (62 %) of the REM sleep deficit in the wild-type mice were unrecovered after 24 hr. Notably, REM sleep deficits were completely recovered at the end of the 24 hr period in both genotypes after the 10 and 30 mg/kg doses of modafinil.

EEG power spectra during wakefulness

As modafinil had a differential effect on the duration of induced wakefulness in the 2 genotypes, spectral analysis was used to evaluate the effect of compound administration on the frequency spectrum of the wakefulness EEG. For each mouse, the normalized mean spectrum obtained after vehicle was subtracted from that obtained after each dose of compound. These data, by mouse, were then averaged across genotype (**Fig.6-5A**). Significant EEG spectral differences caused by modafinil administration to the wild-type mice in the 8-9 Hz bins (increased spectral power versus control) and in the 11-15 and 17-23 Hz bins (decreased spectral power versus control) ($P<0.05$, two-tailed

paired t-test) were noted. Importantly, and despite a similar behavioral expression of wakefulness, the effect of 100 mg/kg modafinil on the mean EEG spectrum in the *orexin*$^{-/-}$ mice occurred over a smaller range of frequencies: a significant increase in power was noted in the 9-10 Hz bins and a significant decrease in the 12-13 and 21-22 Hz bins ($P<0.05$, two-tailed paired t-test). These effects of modafinil on EEG spectral power were responsive to dose. After the 10 and 30 mg/kg doses of the compound, the wakefulness EEG power spectra were statistically indistinguishable from the vehicle condition.

In view of these genotypic differences in the effect of modafinil on wakefulness EEG spectral power, the difference in spectral power between the genotypes after vehicle administration and 100 mg/kg modafinil was plotted (**Fig. 6-5B**). Under control conditions, *orexin*$^{-/-}$ mice showed significantly less power than wild-type mice at 8 Hz and significantly more power at 13 Hz ($P<0.05$). After 100 mg/kg modafinil, these differences in EEG spectral power between the genotypes were accentuated: a significant decrease at 8 Hz and a significant increase over the range 12-14 Hz when *orexin*$^{-/-}$ mice were compared with wild-type controls ($P<0.05$).

Abnormal REM sleep transitions

From the EEG/EMG records of the *orexin*$^{-/-}$ mice during the first 4 hr of recording, I determined the number of abnormal REM sleep transitions, either entered directly from wakefulness or following less than 60 sec of non-REM sleep (i.e., episodes showing rapid progression to REM sleep) (**Table 6-2**). I also confirmed the overt incidence of these behavioral arrests from the videotape monitoring during the first 4 hr

of recording. Modafinil treatment did not affect the number of direct transitions to REM sleep (χ^2 [3] = 3.7, P = 0.3, Ordinal logistic Wald test) but tended to reduce the number of episodes showing rapid progression to REM sleep (χ^2 [3] = 7.2, P = 0.06).

DISCUSSION

Using Fos expression as a marker of direct and indirect neural activation after modafinil treatment in wild-type and orexin null mice, activation patterns similar to those in the rat (Scammell et al., 2000) were observed. In particular, the islands of Calleja were intensely labeled and many nuclei were labeled in the laterodorsal subdivision of the bed nucleus and the lateral central nucleus of the amygdala. As noted by Scammell et al. (2000), these are regions that have been shown to be activated after treatment with DA agonists or drugs that promote dopaminergic transmission.

No differences were evident in the pattern of c-Fos immunoreactivity between the genotypes either at baseline or after modafinil treatment. Orexin null mice were also responsive to the wakefulness-promoting effects of modafinil: the orexin system is therefore neither a direct nor indirect target through which modafinil must act to increase wakefulness or to activate brain regions associated with the expression of wakefulness. Hence, non-selective activation of orexin neurons by modafinil as previously reported (Chemelli et al., 1999; Scammell et al., 2000) is a consequence of the wakefulness induced by the compound rather than a reflection of its primary locus of action. These findings thus highlight a technical limitation of Fos immunohistochemistry, which does

not distinguish between the primary and secondary neuronal activation patterns that result from an experimental intervention. Hence other putative neuroanatomical substrates for the action of modafinil, as demonstrated by Fos immunohistochemistry (Lin et al 1996; Engber et al., 1997; Scammell et al., 2000), might also be state-related rather than compound-induced.

Unexpectedly, however, orexin null mice were not only responsive to the wakefulness-prolonging effect of modafinil, but they were actually more sensitive to this effect. Despite similar baseline amounts of wakefulness, *orexin*$^{-/-}$ mice responded with greater increases in wakefulness time than wild-type mice. In the following chapter, the stimulating effects of intra-cerebroventricular orexin-A in *orexin/ataxin-3* transgenic mice is explored. As with modafinil in the present study, orexin-A more effectively increased wakefulness in the orexin-deficient mice compared to wild-type controls (Mieda et al., 2004). Together, these results may suggest that the absence of orexin signaling over prolonged periods can induce compensatory up-regulation in components of central arousal. Indeed, narcoleptic dogs show an abnormal balance between adrenergic and cholinergic systems and in the level of histamine (Nishino and Mignot, 1997; Nishino et al., 2001). Orexin innervates these and other brainstem and basal forebrain loci of systems that play a role in central arousal (Peyron et al, 1998; Chemelli et al., 1999). Several components of the central activating system may therefore undergo compensatory changes in signaling after prolonged absence of orexin.

Such compensatory mechanisms, however, do not completely substitute for orexin during wakefulness. When the distribution of spectral power in the awake EEG was determined to examine changes in arousal and vigilance, differences were observed,

most importantly in the theta band, between *orexin*$^{-/-}$ and wild-type mice after vehicle administration. An increase in EEG spectral power in the theta (i.e., 8-9 Hz) band has long been viewed as indicative of more active, movement-related wakefulness in rodents (Vanderwolf, 1967; Bland, 1985). More recently, EEG theta power has been described as a characteristic of cortical activation and may represent the engagement of attentional processes (Buzsáki, 1989; Huerta and Lisman, 1993; Sutherland and McNaughton, 2000). EEG oscillation in the theta range is derived primarily from the hippocampus in rodents. It is driven through the septo-hippocampal system and is dependent on ascending GABAergic and cholinergic innervation and the pontine reticular activating system (Lee et al., 1994; Vinogradova, 1995; Vertes and Kocsis, 1997). An important link to basal forebrain activation has also been noted recently (Manns et al., 2002). These findings indicate that awake EEG theta power can be influenced by multiple systems. Since orexin excites basal forebrain cholinergic neurons (Eggermann et al., 2001; Espana et al., 2001; Thakkar et al., 2001) and also activates the GABAergic septo-hippocampal pathway (Wu et al., 2002), I would expect orexin to promote the theta rhythm. That power in the EEG theta band is less in orexin null mice under baseline conditions is thus consistent with these data.

Any effect of the lower doses of modafinil on awake EEG spectral power distribution was not detectable in this study. At a dose of 100 mg/kg, however, the compound increased power in the 8-9 Hz band and decreased power between 12 and 15 Hz. These effects of modafinil in the wild-type mice are comparable to those reported previously in the OF1 strain of mice after administration of 200 mg/kg modafinil, though they did not reach significance under the conditions of that study (Kopp et al., 2002).

Also Béracochéa et al (2002) reported that modafinil at 64 mg/g, but not at 32 mg/kg, improved spatial reversal learning of C57Bl/6 mice in a T-maze: spatial memory in the T-maze is correlated with hippocampal theta power (Markowska et al., 1995). The action of modafinil in the *orexin*$^{-/-}$ mice was similar to that found in the wild-type mice, but less pronounced. Thus, despite the presence of apparent compensatory changes in the components of central activation after the prolonged absence of orexin signaling, modafinil accentuates the difference between the genotypes in awake EEG theta power. Either modafinil administration or the presence of orexin shifts the awake EEG spectral distribution towards an activated pattern and these effects are additive.

The EEG spectral data demonstrate, therefore, that modafinil increases alertness and vigilance partly through orexinergic innervation because, after compound administration, *orexin*$^{-/-}$ mice demonstrate less vigilant wakefulness than the wild-type mice, as indicated by reduced theta rhythm generation. Despite this, *orexin*$^{-/-}$ mice remain awake longer. The activation of orexin-containing neurons by modafinil in the wild-type mice, demonstrated by Fos immunohistochemistry (Chemelli et al., 1999; Scammell et al., 2000) may help to maintain active wakefulness, as reflected functionally in theta band power. When orexin is absent, this level of vigilance is compromised. Thus the putative up-regulated compensatory response in terms of the effect of modafinil on wakefulness time in the *orexin*$^{-/-}$ mice apparently does not extend to the level of vigilance. These data may be interpreted to suggest that modafinil is more effective in increasing the amount of wakefulness but less effective in increasing qualitative degrees of vigilance in *orexin*$^{-/-}$ mice.

Modafinil suppressed REM sleep in both wild-type and orexin null mice, but this effect was significant only at the highest dose. Although at this dose modafinil appeared to be more effective in the orexin null mice, this result is difficult to interpret because wild-type mice exhibit less REM sleep than the *orexin*$^{-/-}$ mice during the first part of the dark period. Also the REM sleep deficit at the end of the 24 hr recording period was equivalent, suggesting that the effect of modafinil administration with respect to REM sleep may be comparable in the two genotypes.

Direct transitions to REM sleep were not altered by modafinil. This result confirms the minimal effect of modafinil on murine cataplexy, as previously observed in humans and dogs (Billiard et al., 1994; Shelton et al., 1995). However, modafinil has been reported to be an effective treatment for sleep attacks in narcolepsy (Grozinger et al., 1998) and I noted here that the compound tended to reduce the frequency of premature REM sleep transitions. These have been associated with sleep attacks in narcoleptic mice (as shown in **Chapter 2**). In contrast, orexin-A, when administered intra-cerebroventricularly to *orexin/ataxin-3* transgenic mice, suppresses both cataplexy episodes and sleep attacks (see **Chapter 7**). The inability of modafinil to reduce direct transitions to REM sleep is evidence that the compound is unlikely to act post-synaptically on targets of orexinergic neurons that modulate these transitions. In fact, this finding may have general applicability since modafinil, up to a concentration of 10 μM, is unable to inhibit orexin-A activation at the orexin-1 receptor (D. Bozyczko-Coyne, personal communication), or displace orexin-B from this receptor (Wieland et al., 2002).

As previously reported after modafinil, and in contrast to amphetamine (Touret et al., 1995; Edgar and Seidel, 1997; Lin et al., 2000), no evidence of rebound hypersomnia was found in this study. I have also shown that the wakefulness-promoting action of modafinil does not require orexin, a putative component of central activation. By inference, and in line with the lack of effect of modafinil on noradrenergic cell discharge rates (Akaoka et al., 1991), the compound similarly may not directly enhance other substrates of central activation. Amphetamine, however, acts primarily through such activation. Considering both this difference in mechanism of action and in rebound hypersomnolence, it may be concluded that, whereas amphetamine appears to increase alertness until the drive for sleep is irresistible, modafinil may directly inhibit the increased drive for sleepiness that normally follows prolonged wakefulness.

EXPERIMENTAL PROCEDURES

Animals

F3 and F4 homozygote orexin$^{-/-}$ mice and wild-type littermates, on a C57Bl/6-J-129/SvEv background, were derived as previously described (see **Chapter 2**).

Modafinil

Modafinil (Cephalon, Inc., West Chester, PA) was freshly prepared before each experiment. The compound was suspended in vehicle (sterile saline with 0.25% carboxy methylcellulose; Sigma, St Louis, MO). Less than 60 sec before each injection, modafinil was re-suspended by vortexing and sonication.

Immunohistochemistry

Wild-type and *orexin*$^{-/-}$ mice (N = 2 - 6/group) were injected with vehicle or modafinil (10 or 100 mg/kg, i.p.) at lights-off (19:00). Two hr later, mice were deeply anesthetized by overdose of 5% chloral hydrate in saline (0.8 ml, i.p.) and transcardially perfused with 12-14 ml of saline (4 ml/min) followed by 15 ml of phosphate-buffered 10% formalin (5 ml/min). Brains were removed, post-fixed for 3 hr in formalin, and then allowed to equilibrate in 20% sucrose in 0.1 M phosphate buffered saline (PBS) with 0.02% sodium azide. Brains were then sectioned (30 μm, 1:3 series) on a freezing microtome and stored in PBS-azide at 4°C. One series was immunostained for Fos in the laboratory of T. Scammell as previously described (Scammell et al., 2000). In brief, sections were incubated for 48 hr in rabbit anti-Fos antiserum (Ab-5, 1:25,000 dilution, Oncogene Research Products), reacted with biotinylated donkey anti-rabbit secondary antiserum (1:1,000, Jackson ImmunoResearch, West Grove, PA) for 1 hr, and incubated in avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA). A black reaction product was produced in cell nuclei using diaminobenzidine with $NiS0_4$ and $CoCl_2$. The pattern of Fos immunoreactivity was examined throughout the entire brain.

Animal surgery

For chronic electroencephalogram/electromyogram (EEG/EMG) monitoring, 12-14 week-old matched pairs of *orexin*$^{-/-}$ and wild-type male mice (N = 10 /genotype) were anaesthetized with sodium pentobarbital (60 mg/kg i.p.; Nembutal, Abbott Laboratories) and chronically implanted with electrodes as in previous chapters. Mice were also implanted with indwelling intraperitoneal catheters to allow administration of modafinil

or vehicle without handling of the mice, so minimizing disturbance during the experimental procedure. Custom catheters were fabricated from PE50 polyethylene tubing with PE90 cuffs, so that the total dead space was less than 0.1 ml. The sites for insertion of the catheters were shaved and prepared with Betadine solution (Baxter Healthcare Corp., Deerfield, IL) and 70% ethanol. Using a large-bore blunted 18 g needle, a subcutaneous tunnel between the dermis and underlying musculature was made, starting at the nape of the neck, following down the dorsum of the back, and around to the lower right abdomen. A 1 cm midline incision exposed the abdominal wall. A puncture hole was then created in the abdominal musculature with a 22 g needle, and the catheter was threaded down the subcutaneous tunnel and its cuff placed through the puncture. The catheter was fastened to the muscle wall by a small figure-eight stitch of 6-0 silk, and secured above the neck to the EEG/EMG implant through a stainless steel loop screw. The exposed extent of the catheter was attached to the EEG/EMG tether with surgical tape. A resistance-free injection of saline through the exposed end of the catheter, positioned 10 cm above the head, insured patency of the cannula. The cannula was then sealed with an airtight stainless steel stylet, and the surgical sites were closed with 6-0 silk suture and treated with Betadine ointment (Baxter Healthcare Corp., Deerfield, IL).

Behavioral studies

All mice were housed singly and allowed to recover and habituate to tethers, catheters, and the experimental procedures for 14-15 days prior to EEG/EMG recording. Catheters were swabbed externally with 70% ethanol pads and flushed internally with

sterile saline every 48 hr to maintain patency. Food and water were made available ad libitum throughout the study and mice were housed under a 12:12 Light:Dark cycle (lights on at 07:00) and a constant temperature of 24 ± 1 °C.

Just before the onset of the dark phase at 19:00, mice were administered modafinil (0, 10, 30, or 100 mg/kg; 10 ml volume/kg body weight; 0.12-0.17 ml absolute volume) through the indwelling cannulae. Each injection was immediately followed by an injection of the same volume of sterile saline. Simultaneous EEG/EMG/video recording was initiated at the onset of the dark phase and continued for 24 hr. Each mouse received all doses of modafinil or vehicle in a randomized crossover design over the experimental sessions. Each session was separated by 72 hr to ensure washout of the compound. After completion of experiments, animals were sacrificed by sodium pentobarbital overdose (Nembutal, 100 mg/kg i.p.) and cervical dislocation. Cannulae were examined post mortem to confirm correct placement and checked for continued patency during the study.

Data analysis

EEG/EMG signals were collected, stored, and analyzed in 20 sec epochs as in previous chapters. by an experimenter blinded as to condition and genotype. Hourly vigilance state data as well as 12-hour summary results were analyzed by condition and by genotype using two-way or one-way repeated measures ANOVA followed by Dunnett's post-hoc test (JMP 4.0, SAS Institute, Cary, NC).

Determination of the efficacy of the wake-promoting action of modafinil was based on the method previously described by Edgar and Seidel (1997). For each mouse

and for each dose of modafinil, the total cumulative increase in wakefulness induced during the period after compound treatment was derived by difference from the corresponding vehicle data. This assured equality of treatment across doses when the duration of the initial compound effects can vary. Means (± SEM) for the 3 doses were then plotted by genotype as a Lineweaver-Burk double-reciprocal plot to ensure linearity.

The fronto-occipital EEG frequency spectrum was analyzed by power spectral analysis (i.e., fast Fourier transform, FFT) in 1 Hz bins from 1 to 32 Hz. Only the spectral power of concatenated 20 sec epochs during the initial wakefulness period was analyzed. After the 100 mg/kg dose of modafinil, all mice were awake throughout the first 60 min of recording. This period was therefore used for the analysis. For the vehicle and the 10 and 30 mg/kg doses of modafinil, however, the first 20 min of recording were analyzed. Few mice remained consistently awake after this time under these conditions, thus precluding the inclusion of a greater number of epochs to reduce variance in these groups. Any epochs with movement artifacts were deleted before the spectral data were averaged. Thus between 8.3 and 13.2 % of epochs in the wild-type and between 8.7 and 16.5 % of epochs in the *orexin*$^{-/-}$ mice, depending on the dose and mouse, were excluded from analysis because of artifact. The resulting data were normalized by determining power in each bin as a proportion of the total power, and the mean power spectrum for each condition and each genotype was then determined. Power differences among the bins were statistically analyzed by two-tailed paired t-test following an overall ANOVA.

Abnormal REM sleep transitions

Direct transitions from wakefulness into REM sleep in *orexin*$^{-/-}$ mice appear to fulfill the criteria for cataplexy in this species (**Chapter 2**). Likewise, episodes of rapid progression to REM sleep (i.e., a transition from wakefulness to REM sleep after less than 60 sec of intervening non-REM sleep) follow a pattern associated with a behavioral state resembling sleep attacks in the human. I examined here the effect of modafinil treatment on the incidence of these abnormal REM sleep transitions. Their incidence was determined from the EEG/EMG records during a 4 hr period from the beginning of the dark phase. Infrared video tape recording (Sony TRV-CCD66) was also used to monitor the behavior of the mice during this period and confirm the events detected from the EEG/EMG records. As in **Chapter 2**, no episodes fulfilling these criteria were observed in the wild-type mice.

DISPLAY ITEMS

Table 6-1. Hourly distribution of vigilance states after vehicle treatment during the 12 hr dark period

	Wakefulness		**REM Sleep**	
	Wild-type	**Orexin -/-**	**Wild-type**	**Orexin -/-**
19:00 – 20:00	49.9 ± 1.8	48.2 ± 2.6	0.3 ± 0.2	2.1 ± 0.6 *
20:00 – 21:00	34.2 ± 4.0	35.7 ± 3.6	2.4 ± 0.5	4.3 ± 0.8 *
21:00 – 22:00	32.3 ± 2.1	31.9 ± 2.9	2.3 ± 0.5	4.7 ± 0.5 *
22:00 – 23:00	32.4 ± 3.2	36.1 ± 4.3	3.0 ± 0.6	3.3 ± 0.6
23:00 – 24:00	33.5 ± 3.4	35.5 ± 3.3	2.7 ± 0.6	3.6 ± 0.5
24:00 – 01:00	33.8 ± 3.2	35.3 ± 3.4	3.1 ± 0.6	3.2 ± 0.6
01:00 – 02:00	35.7 ± 3.5	31.0 ± 3.5	2.0 ± 0.5	4.5 ± 0.6 *
02:00 – 03:00	31.0 ± 2.9	29.8 ± 2.5	3.1 ± 0.6	3.6 ± 0.6
03:00 – 04:00	31.2 ± 3.5	35.1 ± 3.1	2.6 ± 0.5	3.5 ± 0.6
04:00 – 05:00	28.4 ± 3.0	28.1 ± 3.7	3.2 ± 0.5	3.5 ± 0.6
05:00 – 06:00	32.7 ± 3.4	34.1 ± 2.9	2.9 ± 0.5	3.8 ± 0.4
06:00 – 07:00	37.4 ± 2.9	42.8 ± 3.6	1.7 ± 0.4	2.5 ± 0.5

Time in minutes (mean ± SEM) spent in wakefulness and REM sleep during each hour of the dark period in the control condition for both genotypes. Significant differences ($P < 0.05$) between orexin-/- and wild-type mice are denoted by an asterisk. Note that there are no significant differences in the hourly durations of wakefulne

Table 6-2. Abnormal REM sleep transitions characterized from EEG/EMG records.

	Modafinil treatment			
	0 mg/kg	**10 mg/kg**	**30 mg/kg**	**100 mg/kg**
Rapid progression to REM sleep	12	11	8	1
Direct transition to REM sleep	10	8	11	16

Total number of abnormal REM sleep transitions in 10 orexin-/- mice during the first 4 hr after modafinil or vehicle administration. There were no similar episodes recorded in the wild-type mice during this period. Rapid progression to REM sleep was defined as an episode in which wakefulness was followed by an abnormally brief period (not exceeding 60 sec) of non-REM sleep prior to the onset of REM sleep. Direct transition to REM sleep was defined as REM sleep that directly followed wakefulness. Modafinil did not affect the number of direct transitions to REM sleep ($P = 0.3$) but tended to decrease the number of rapid progressions to REM sleep ($P = 0.06$).

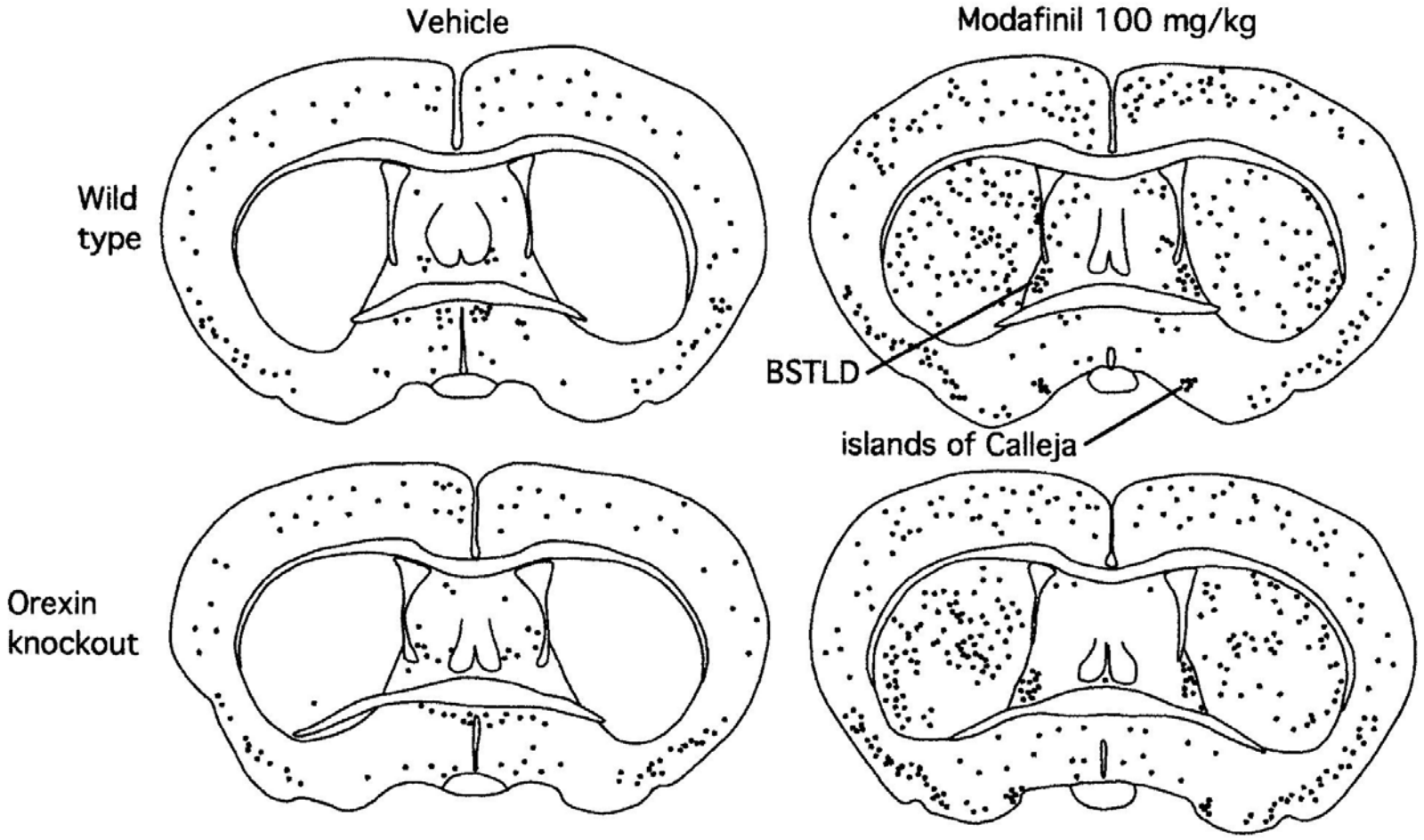

Vehicle
Modafinil 100 mg/kg
Wild type
BSTLD
islands of Calleja
Orexin knockout

Figure 6-1. Effect of modafinil on Fos-IR patterns.

A series of line drawings illustrating the distribution of Fos-IR neurons in a typical mouse of each strain 2 hr after administration of vehicle or modafinil (100 mg/kg i.p.) at the onset of the dark period. Wild-type and *orexin*$^{-/-}$ mice treated with vehicle showed occasional Fos-IR neurons in the cortex, lateral septum, and ventral parts of the bed nucleus of the stria terminalis (BSTLD), with a few scattered cells in the preoptic area. Mice of either genotype treated with 100 mg/kg modafinil had many more Fos-IR neurons throughout much of the cortex and marked increases in Fos expression in the striatum, laterodorsal subnucleus of the BSTLD, and islands of Calleja. Drawings generated by T. Scammell from experiments performed by the author in collaboration.

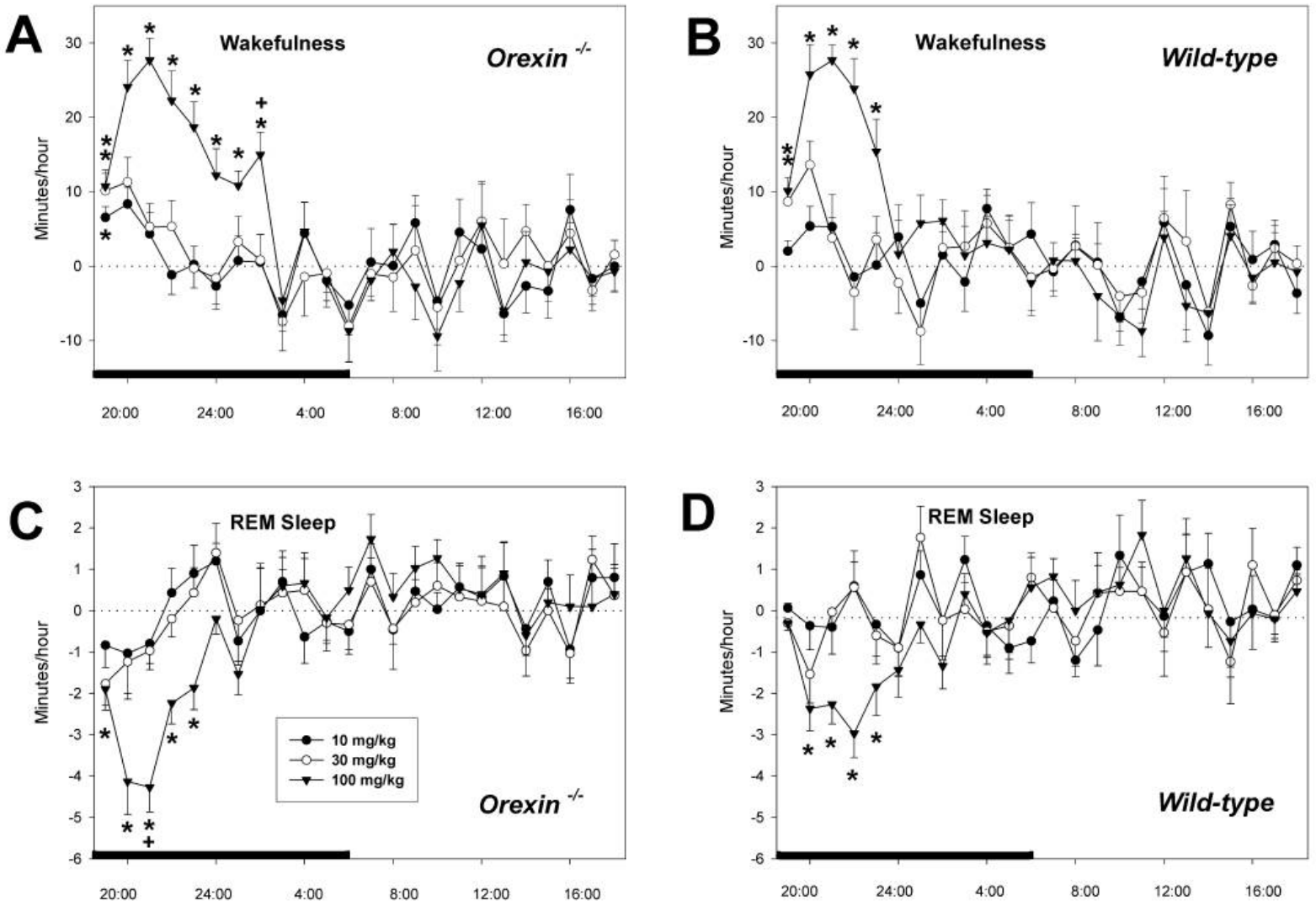

A
Wakefulness
Orexin -/-
Minutes/hour
30
20
10
0
-10
20:00
24:00
4:00
8:00
12:00
16:00
B
Wakefulness
Wild-type
Minutes/hour
C
REM Sleep
Minutes/hour
3
2
1
0
-1
-2
-3
-4
-5
-6
10 mg/kg
30 mg/kg
100 mg/kg
Orexin -/-
D
REM Sleep
Wild-type

Figure 6-2. Effect of modafinil upon hourly wakefulness and REM sleep.

Hourly differences in wakefulness and REM sleep (mean ± SEM) between times recorded after vehicle and modafinil treatments (10, 30 and 100 mg/kg i.p.) for *orexin*$^{-/-}$ (A and C) and wild-type mice (B and D). Significant differences ($P < 0.05$) from vehicle are designated by an asterisk (*) and between the genotypes by a cross (+). Note that the increase in wakefulness resulting from 100 mg/kg i.p. modafinil has a similar maximum in both genotypes but continues for 3 hr longer in the null mice. In contrast, the effect on REM sleep was greater in the *orexin*$^{-/-}$ mice but continued for the same time in both genotypes. During the first hour of recording, wakefulness was significantly increased in the *orexin*$^{-/-}$ mice after both the 10 and 30 mg/kg doses of modafinil, but in the wild-type mice this effect was noted only after the 30 mg/kg dose .

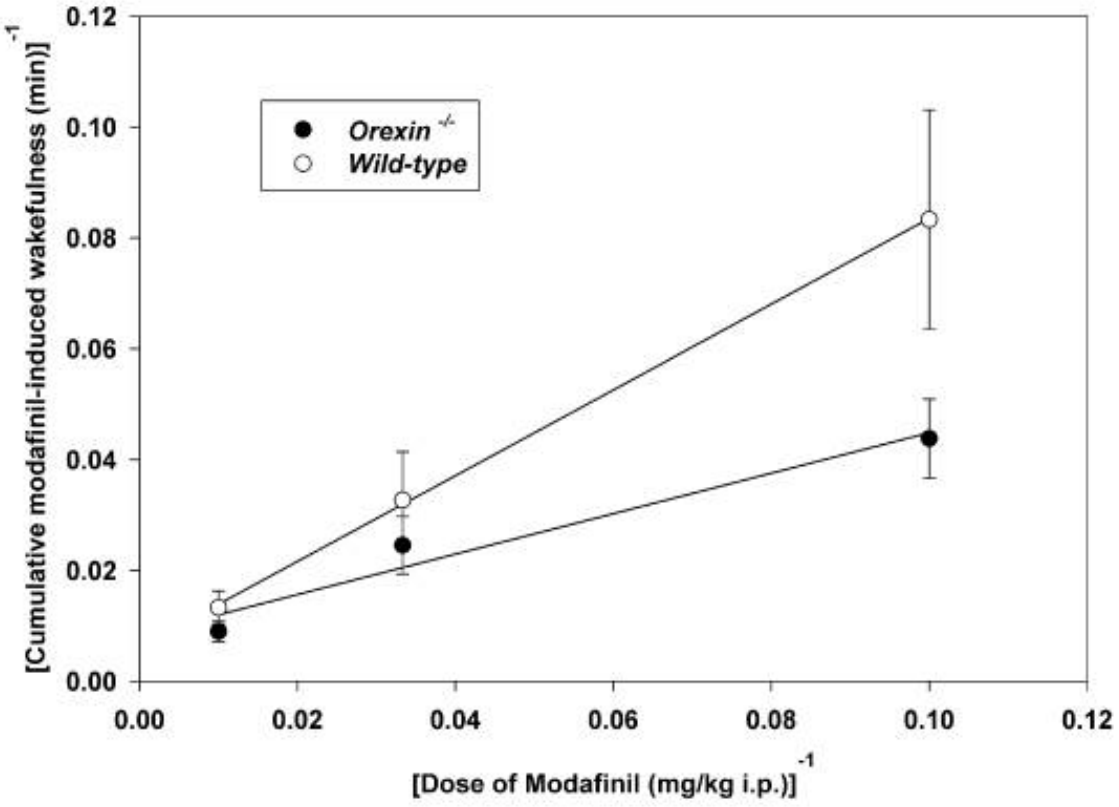


Figure 6-3. Dose response of the effect of modafinil on maximal cumulative wakefulness in each genotype.

For each mouse and each dose of modafinil, the hourly differences between wakefulness times recorded after modafinil and after vehicle were summed for the period through to the maximal increase in wakefulness induced by compound treatment. The means of these results were plotted as a double-reciprocal plot for each genotype. The slopes of the regression lines, a measure of the efficacy of the compound in inducing wakefulness, were different ($P < 0.05$), indicating that modafinil was more effective in inducing wakefulness in the *orexin*$^{-/-}$ mice

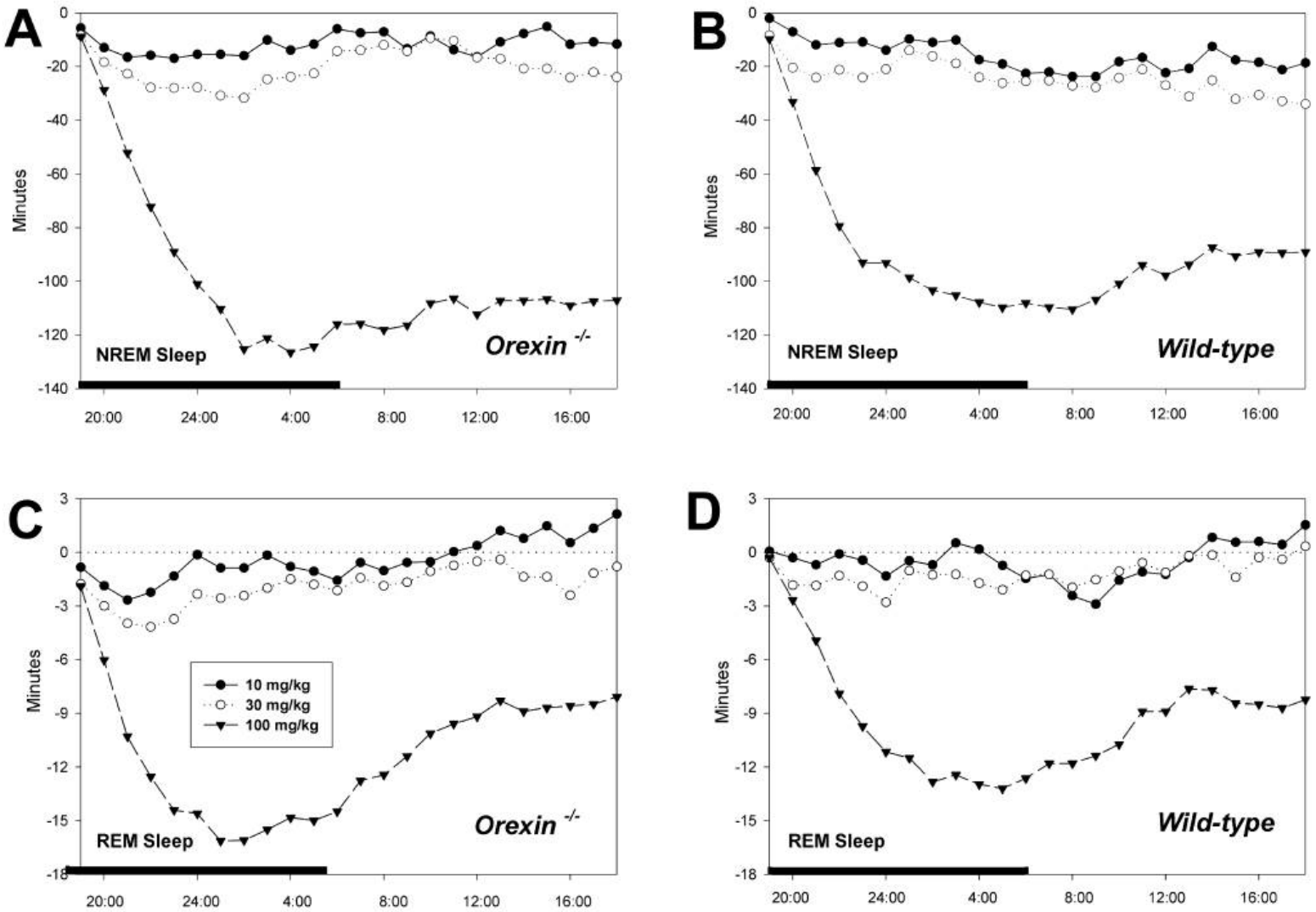

A
B
C
D
Minutes
NREM Sleep
REM Sleep
Orexin -/-
Wild-type
10 mg/kg
30 mg/kg
100 mg/kg
20:00
24:00
4:00
8:00
12:00
16:00

Figure 6-4. Cumulative loss and partial recovery of non-REM and REM sleep times after modafinil administration.

The cumulative difference from vehicle is plotted on an hourly basis over 24 hr. Recovery of the non-REM sleep debt after modafinil administration (A and B) was limited. This effect was similar at all doses of the compound tested and for both genotypes; but note the difference between genotypes in the time course of the effect of modafinil. I noted partial recovery of REM sleep times (C and D) at the 100 mg/kg dose of modafinil and complete recovery at the lower doses. This effect was similar in both genotypes. See text for details.

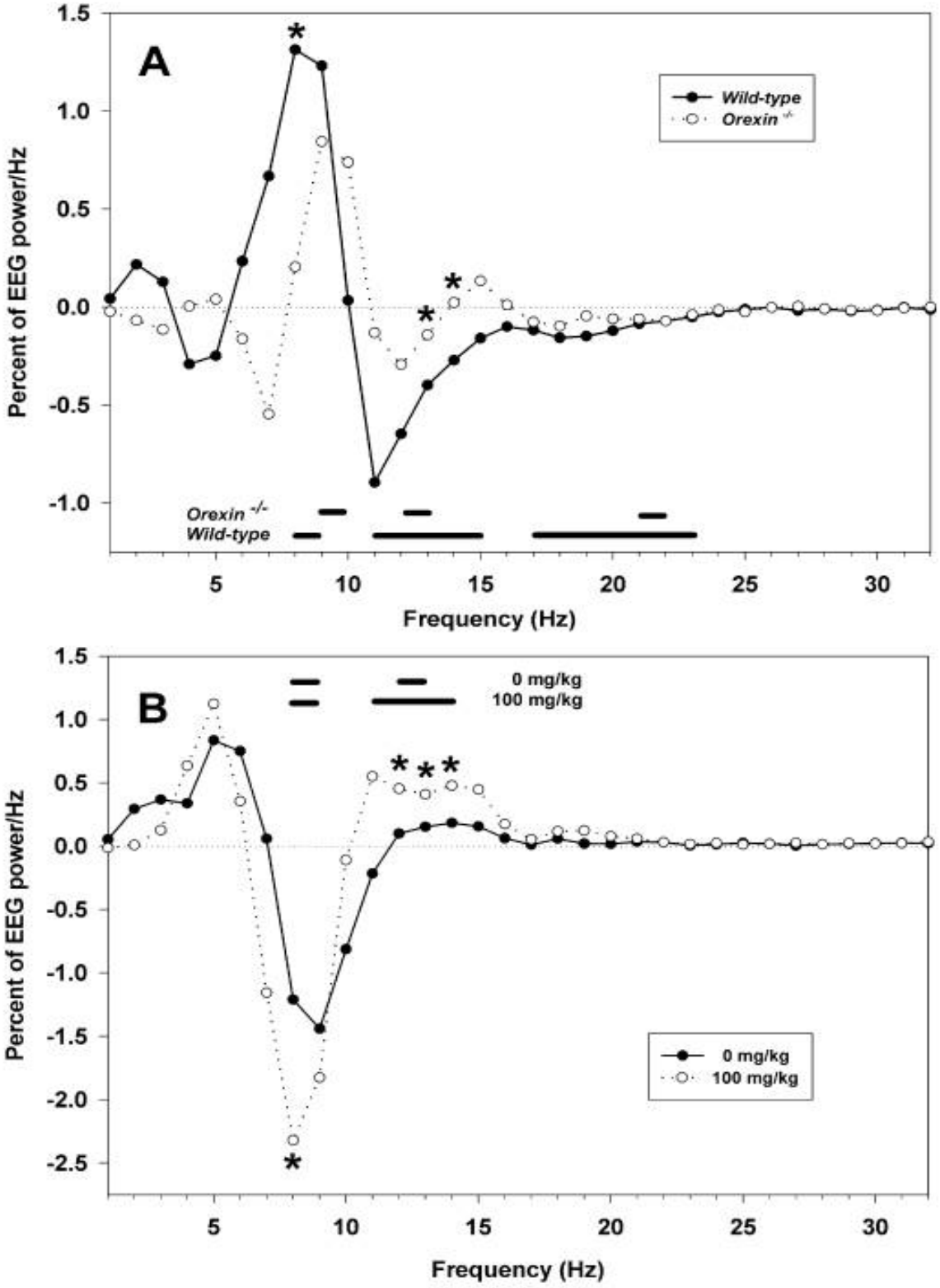

A
Wild-type
Orexin -/-
Orexin -/-
Wild-type
Percent of EEG power/Hz
Frequency (Hz)
B
0 mg/kg
100 mg/kg
0 mg/kg
100 mg/kg
Percent of EEG power/Hz
Frequency (Hz)

Figure 6-5. Mean differences in relative EEG spectral power recorded during wakefulness episodes.

The FFT analysis was in 1 Hz bins from 1 to 32 Hz. The horizontal lines designate those bins in which each curve on the plot is significantly different ($P < 0.05$) from zero. Significant differences between the curves on each plot are designated by asterisks at the corresponding frequency bins. **(A)** Differences between vehicle and 100 mg/kg i.p. modafinil are plotted for each genotype. The effect of modafinil on awake EEG spectral power, including that in the theta frequency range (8 – 9 Hz), was significantly less in the *orexin*$^{-/-}$ mice than in the wild-type mice. **(B)** Differences between *orexin*$^{-/-}$ mice and wild-type mice are plotted for the vehicle and 100 mg/kg i.p. modafinil conditions. Note that the reduced power in the theta frequency range (8 – 9 Hz) seen in the *orexin*$^{-/-}$ mice after vehicle, is increased after modafinil treatment.

CHAPTER SEVEN
Treatment of Narcolepsy – Genetic and Pharmacological Rescue by Orexin

SUMMARY

In this study, rescue of the narcolepsy-cataplexy phenotype of orexin neuron-ablated mice is demonstrate by genetic and pharmacological means. Ectopic overexpression of a *prepro-orexin* transgene in the brain completely prevented cataplectic arrests and other abnormalities of REM sleep in the absence of endogenous orexin neurons. Intracerebroventricular administration of orexin-A acutely suppressed cataplectic behavioral arrests and increased wakefulness for 3 hours. These results indicate that orexin neuron-ablated mice retain the ability to respond to orexin neuropeptides and that orexin receptor agonists would be of potential value for treating human narcolepsy.

INTRODUCTION

Human narcolepsy-cataplexy is characterized by excessive daytime sleepiness, premature transitions to REM sleep ("sleep-onset REM periods"), and cataplexy. Excessive sleepiness is treated using amphetamines, modafinil, or gamma-hydroxybutyrate (sodium oxybate), while cataplexy is treated with tricyclic antidepressants such as clomipramine (Mignot et al., 2002b; Scammell, 2003). This therapeutic regimen is problematic due to

limited effectiveness, undesirable side effects (such as insomnia or symptom rebounds), and the potential for abuse.

Narcolepsy-cataplexy in humans and animal models results from failure of signaling mediated by orexin (hypocretin) neuropeptides (Chemelli et al., 1999; Hara et al., 2001; Lin et al., 1999; Nishino et al., 2000b; Peyron et al., 2000; Thannickal et al., 2000). Because of a strong association with certain human-leukocyte-antigen alleles, it has been speculated that narcolepsy-cataplexy may result from selective autoimmune degeneration of orexin neurons (van den Pol, 2000). These findings open up the possibility that replacement therapies based on administration of orexin receptor agonists might prove beneficial (Mignot et al., 2002b), a possibility not yet convincingly investigated in humans or animal models of narcolepsy. Intracerebroventricular (i.c.v.) injections of orexin peptides, however, when administered acutely in normal mice and rats, have been shown to increase wakefulness and suppress both non-REM and REM sleep (Huang et al., 2001; Piper et al., 2000).

Previously, our group generated mice in which orexin neurons are specifically and post-natally ablated due to expression of a neurotoxic gene fragment (*ataxin-3*) from the *prepro-orexin* promoter (*orexin/ataxin-3* transgenic mice) (Hara et al., 2001). These mice exhibited a phenotype analagous to human narcolepsy, including cataplexy-like behavioral arrests, premature onset of REM periods, fragmented wake and non-REM sleep episodes, and increased body weight. The *orexin/ataxin-3* mice exhibited sleep abnormalities strikingly similar to that reported for *orexin*$^{-/-}$ mice despite their difference in genetic backgrounds in these original studies. The striking similarity of these two mouse models further demonstrated the importance of orexins in the regulation of the sleep/wake state. These

observations also suggest that, although orexin-containing neurons have some co-localized neuromodulators (such as dynorphin) and possibly glutamate, orexin is the factor that is primarily important for the regulation of the sleep/wake state. Nevertheless, *orexin/ataxin-3* mice, like humans, become narcoleptic following a post-natal neurodegenerative process. They are therefore a biochemically and behaviorally accurate model of human narcolepsy.

In this chapter, it is examined whether symptoms of murine narcolepsy-cataplexy can be reversed either by ectopic production of orexin peptides from a *prepro-orexin* transgene (in *CAG/orexin* transgenic mice) or by pharmacological administrations of synthetic orexin-A. These interventions were evaluated by behavioral and electrophysiological criteria using orexin neuron-ablated *orexin/ataxin-3* transgenic mice, an accepted model of human narcolepsy-cataplexy.

RESULTS

Genetic Rescue of Narcolepsy in Orexin Neuron-Ablated Mice.

In order to examine whether the narcolepsy-cataplexy phenotype of orexin neuron-ablated mice could be rescued by ectopic production of orexin peptides, T. Sakurai produced transgenic mice that overexpress a *prepro-orexin* transgene under the control of a *β-actin/cytomegalovirus* hybrid promoter (*CAG/orexin* transgenic mice). Several stable transgenic lines overexpressed the orexin as determined by northern blots, radioimmunoassays, and anti-orexin-A immunohistochemistry. In this study, I utilized one

line in which whole-brain orexin-A and -B peptide levels were increased by nearly 30- and 80-fold, respectively. Mice of this line were crossed to *orexin/ataxin-3* transgenic mice to produce offspring that carry both transgenes (double hemizygous mice). Littermate pairs carrying either transgene (*CAG/orexin* or *orexin/ataxin-3* transgenic mice), both transgenes (*orexin/ataxin-3;CAG/orexin* double transgenic mice), or neither transgene (wildtype mice), as determined by genotyping with PCR, were selected for further experiments.

Anti-orexin-A immunohistochemistry of the brains of 15-week-old wild type mice produced a pattern of staining in which orexin-producing cells were clustered in the perifornical LH (**Figure 7-1*a* and *b***), as described (Peyron et al., 1998; Sakurai et al., 1998b). In contrast, no orexin staining was found in any part of the brain of *orexin/ataxin-3* transgenic littermates (**Figure 7-1*c* and d**), consistent with destruction of endogenous orexinergic neurons by this age as previously reported (Hara et al., 2001). Littermates carrying the *CAG/orexin* transgene alone exhibited a widespread, diffuse orexin-immunoreactive staining throughout most of the brain, with highest levels observed in the hypothalamus, amygdala, hippocampus, and brain stem (**Figure 7-1e and f**). Notably, the native orexinergic neurons in *CAG/orexin* transgenic mice were still visible above background in sections containing the LH due to the extremely high levels of orexin immunoreactivity in endogenous neurons relative to those expressing the transgene ectopically. *Orexin/ataxin-3*;*CAG/orexin* double transgenic mice exhibited a similar pattern of widespread orexin signal throughout the hypothalamus and remaining brain. Notably absent, however, were endogenous orexinergic neurons (**Figure 7-1g and h**).

I tested mice of all four genotypes for the presence of cataplectic arrests during the dark (active) phase using infrared videophotography. I adapted a behavioral paradigm in which narcoleptic mice are exposed to a novel environment that tends to increase the frequency of such arrests (see **Appendix B**), a similar method to that utilized in the previous chapters. As in wild type mice, mice carrying the *CAG/orexin* transgene alone exhibited no behavioral arrests of any kind (data not shown). The frequencies of arrests in *orexin/ataxin-3* mice and *orexin/ataxin-3*;*CAG/orexin* double transgenic littermates were then compared (**Figure 7-2**). All *orexin/ataxin-3* mice exhibited cataplexy-like arrests within the 3-h observation period as described (Hara et al., 2001). In contrast, no arrests were observed in any of the *orexin/ataxin-3*;*CAG/orexin* double transgenic littermates under these same conditions. Thus, ectopic transgenic expression of orexin peptides prevents cataplectic arrests in mice in which endogenous orexin neurons have been ablated.

I then examined sleep/wake patterns over the entire 24-h day in each group of mice, by carrying out EEG/EMG recordings. As expected, the dark-phase sleep/wake patterns of *orexin/ataxin-3* mice, summarized in hypnograms (**Figure 7-3**), exhibited severe fragmentation of wakefulness and sleep with numerous brief non-REM sleep episodes and frequent transitions to REM sleep with little or no latency from preceding wakefulness. Such transitions were observed without exception in each mouse carrying the *orexin/ataxin-3* transgene alone. In contrast, *orexin/ataxin-3*;*CAG/orexin* double transgenic littermates exhibited longer, more consolidated bouts of wakefulness and normalized amounts of REM sleep during the dark phase. Most importantly, no direct or premature wake-REM sleep transitions were ever detected in any double transgenic mice. Hypnograms of wildtype mice

from the same line were typical of C57Bl/6J strain and illustrate normal sleep/wake patterns during this period. Notably, compared to wildtype and narcoleptic mice, hypnograms of *orexin/ataxin-3*;*CAG/orexin* double transgenic mice exhibited increased levels of fragmentation of sleep specifically during the light (resting) phase, resulting possibly from chronic, abnormally high orexin tone due to the *CAG/orexin* transgene.

Sleep/wake states in these mice are also illustrated by hourly plots of amounts of time spent in wakefulness, non-REM sleep, and REM sleep over 24 h (**Figure 7-4**). Consistent with a previous description8, *orexin/ataxin-3* mice showed selective increases in amounts of REM sleep during the dark phase compared to wild type controls. Importantly, mice carrying both transgenes exhibited a normalization of REM sleep amounts during the dark phase with an additional tendency toward reduced amounts of REM sleep during the light phase as well. Overall, ectopic expression of the *CAG/orexin* transgene prevented the development of symptoms of narcolepsy-cataplexy despite the post-natal ablation of endogenous orexin neurons, supporting a specific role for orexin peptides in preventing narcolepsy-cataplexy.

Pharmacological Rescue of Narcolepsy in Orexin Neuron-Ablated Mice.

The rescue of the narcoleptic phenotype in orexin neuron-ablated mice by ectopic prepro-orexin expression led M. Mieda and I to examine whether acute exogenous administrations of orexin might also rescue this phenotype. In pharmacological studies we administered orexin-A, a non-selective agonist for OX1R and OX2R (Sakurai et al., 1998b). The dose and route of administration chosen (3 nmol/mouse i.c.v.) is comparable to that used

previously to examine the effects of orexin-A on behavior and metabolism in rodents (Hagan et al., 1999; Lubkin and Stricker-Krongrad, 1998; Piper et al., 2000; Stricker-Krongrad et al., 2002), and is effective in dose-response studies of sleep/wakefulness in wild type mice, but produces no observable effects in knockout mice lacking both OX1R and OX2R (M. Mieda, unpublished observations).

We examined whether i.c.v. orexin-A administration suppresses cataplectic arrests in *orexin/ataxin-3* transgenic mice. Using a randomized crossover design, we administered vehicle and orexin-A to all mice during separate experimental sessions. When narcoleptic mice were treated with vehicle alone, they exhibited variable frequencies of arrests as well as variable cumulative times spent in arrests during the first 3 hours following injections (**Figure 7-5**). In contrast, when the same mice were administered orexin-A, the frequency of arrests and overall time spent in cataplexy were significantly reduced in each mouse during the same 3 hour time period, despite increases in observable wakeful activity. Thus, i.c.v. administration of orexin-A is sufficient to acutely suppress behavioral arrests in *orexin/ataxin-3* mice.

To quantify the effects of orexin-A administration on sleep/wake status, we recorded EEG/EMG in *orexin/ataxin-3* mice as well as wild type controls. As narcoleptic humans and animals exhibit sleepiness and cataplexy during their respective active phases, it is likely that orexin-based therapies would be administered to humans during the active phase. Nevertheless, some pharmacological effects of exogenous orexins have been reported to vary with time of day (zeitgeber-dependent effects) (Espana et al., 2002; Haynes et al., 1999). We therefore examined the effects of orexin-A administrations in mice both at the

onset of the dark (active) phase as well as during the light (resting) phase, again using a randomized crossover design.

Patterns of wakefulness, non-REM sleep, and REM sleep revealed a robust arousal effect of orexin-A in transgenic narcoleptic as well as wild type mice. I.c.v. administration of 3 nmol orexin-A strikingly increased wakefulness and suppressed both non-REM and REM sleep, regardless of genotype and time of administration (**Figure 7-6a**). Interestingly, equivalent doses of orexin-A, administered during either the nocturnal or diurnal phases, produced arousal with greater effectiveness in narcoleptic mice compared to normal controls. While both wild type and narcoleptic mice exhibited similar amounts of wakefulness and non-REM sleep in a 3-h period following vehicle administrations, orexin-A induced significantly greater amounts of wakefulness in *orexin/ataxin-3* transgenic mice than it did in wild type mice. These increases in wakefulness were essentially mirrored by significantly greater suppressions of non-REM sleep in narcoleptic animals.

As expected, *orexin/ataxin-3* mice exhibited significantly higher amounts of REM sleep than did wild type controls during the dark phase under baseline (vehicle-administered) conditions. Critically, orexin-A effectively suppressed this elevation of REM sleep in narcoleptic mice. Again, orexin-A was more effective at suppressing REM sleep in narcoleptic mice than in wild type controls, regardless of the time of administration (**Figure 7-6a**).

Unlike effects reported with amphetamine administrations in humans (Valerde et al., 1976), rats (Edgar and Seidel, 1997), and mice (Kitahama and Valatx, 1979), no immediate rebounds of sleep were observed following dark-phase or light-phase orexin-A

administrations (**Figure 7-6b**). Only narcoleptic animals maintained a statistically significant increase of cumulative wakefulness (**Figure 7-6b**) and a decrease of cumulative sleep (data not shown), even after 24 h following a nocturnal administration of orexin-A. This difference compared to wild type controls likely resulted in part from the greater effectiveness of orexin-A in narcoleptic mice. In contrast, when orexin-A was administered diurnally, mice of both genotypes recovered sleep losses by the 24-h mark, but this recovery was gradual in nature. Overall, these results point to the feasibility of treating symptoms of narcolepsy-cataplexy using pharmacological agonists of orexin receptors.

DISCUSSION

Narcolepsy-cataplexy is a disorder of the organization of the sleep-wake cycle resulting, in the vast majority of patients, from absence of orexin peptides. Replacement therapy using orexin receptor agonists may provide treatment directed toward this fundamental pathophysiology. By two distinct methods of replacing orexin peptides, M. Mieda and I have demonstrated the reversal of symptoms of narcolepsy-cataplexy in a rodent model of the disorder in which native orexin neurons have degenerated.

Chronic over-production of orexin peptides from an ectopically expressed transgene prevented the development of the narcolepsy-cataplexy syndrome following genetic ablation of endogenous orexin neurons. Similarly, i.c.v. bolus administrations of the non-selective orexin receptor agonist orexin-A acutely increased wakefulness, suppressed sleep, and

inhibited cataplectic attacks in narcoleptic mice. Together, these findings provide strong evidence of the specific causal relationship between absence of orexin peptides in the brain and the development of the narcolepsy-cataplexy syndrome. From genetic studies alone, the formal possibility remains that the prepro-orexin transgene rescues the phenotype of *orexin/ataxin-3* mice by precluding any period of orexin deficiency following the degenerative loss of native orexin neurons in young *orexin/ataxin-3* mice. However, our pharmacological rescue experiments suggest that even after substantial periods of complete orexin deficiency, the neural mechanisms required for orexin-mediated arousal and suppression of cataplexy (orexin receptors, intracellular signaling, post-synaptic neural networks, and other downstream neurotransmitter pathways) remain anatomically and functionally intact.

Our study suggested that acutely administered orexin-A is of greater effectiveness in *orexin/ataxin-3* transgenic mice than in wild type controls. This improved effectiveness may result from increased sensitivity of orexin-responsive pathways. Indeed, complex alterations in downstream neurotransmitter systems leading to cholinergic/monoaminergic imbalance are a well-described feature of animal narcolepsy (Nishino et al., 2001a; Nishino and Mignot, 1997). While such changes in the brains of narcoleptics could theoretically result from compensatory increases in expression of excitatory orexin receptors, we have observed normal whole-brain expression levels of OX1R and OX2R mRNAs in *prepro-orexin* knockout mice (Willie et al., 2003), a phenotypic equivalent of the *orexin/ataxin-3* mice used in this study.

We achieved definitive suppressions of behavioral cataplexy utilizing both genetic and pharmacological replacements of orexins, and these changes are unlikely to have resulted indirectly from increased arousal alone. The behavioral paradigm we utilized (exposure to novelty), induces high baseline levels of active, exploratory wakefulness among all genotypes (see **Appendix B**). Indeed, the elicitation of murine cataplectic attacks by novelty is itself probably related to this increased level of activity. Furthermore, other agents that promote wakefulness, such as amphetamines, modafinil, and caffeine, either have no effect on the frequency of human or mouse cataplexy or are mildly exacerbatory (Nishino and Mignot, 1997; Scammell, 2003; Willie et al., 2003). Orexin peptides are therefore quite distinct from these agents in that they promote wakefulness while specifically suppressing cataplexy.

Another potential advantage of orexin agonist-based therapies follows from our results: arousal induced by a bolus administration of 3 nmol orexin-A resulted in no sharp rebounds of sleep. When administered in coordination with the active phase, the orexin-induced gains in cumulative wakefulness lasted as long as 24 h after injection in narcoleptic mice. The absence of such rebounds, a potentially confounding factor in therapies for excessive daytime sleepiness based on classical psychostimulants (Valerde et al., 1976), suggests that orexin-based therapies may more safely maintain wakefulness. Interestingly, recent studies suggest that orexins may promote attention and memory processes (Jaeger et al., 2002; Wu et al., 2002); orexin-based therapies may therefore also improve cognitive function in coordination with increased arousal.

One other report, utilizing OX2R-deficient narcoleptic Dobermans, has suggested that large peripheral doses of orexin-A may reverse sleep/wake fragmentation and cataplexy in some dogs (John et al., 2000). If true, such findings suggest that increased signaling mediated by OX1R may play a role in reducing cataplexy and normalizing sleep patterns. Unfortunately, other investigators have consistently failed to verify the findings presented in that study even at higher doses (S. Nishino and E. Mignot, personal communication). Considering that narcolepsy in Dobermans results from a defect in OX2R (Lin et al., 1999), lack of response to exogenous orexin would be a plausible finding in these dogs.

Our demonstration of a genetic rescue of narcolepsy-cataplexy may also provide implications for future human therapies, which might involve orexin gene therapy utilizing viral vectors, or transplantation of orexin neurons or stem cell precursors. Interestingly, we noted that fragmentation of sleep during the light (rest) phase was associated with chronic unregulated orexin overexpression, a phenotype described in greater detail in the following chapter. As we have demonstrated pharmacologically, orexin-A has the potential to alter sleep/wake cycles when administered during either active or resting phases. Therefore, we predict that potential therapies based on orexin receptor agonists should rely on titration and timing of doses to reinforce rather than counteract normal circadian rhythms. Our observation that administrations of orexin-A promote wakefulness at the expense of non-REM and REM sleep in both wild type and narcoleptic-cataplectic animals suggests that such agonists might also be useful in the treatment of other conditions of excessive daytime sleepiness in humans, including narcolepsy without cataplexy, idiopathic hypersomnia, and

sleep deprivation of various causes. The effects of chronic orexin administrations in animals and the effects of orexin receptor agonists in humans clearly merit further investigation.

EXPERIMENTAL PROCEDURES

Production of Transgenic Mice

The *CAG/orexin* transgene was constructed by T. Sakurai, so that the rat prepro-orexin gene is expressed under the control of the β-actin/cytomegalovirus (CAG) hybrid promoter, which drives wide-spread expression (Niwa et al., 1991). The transgene was injected into fertilized ova of DBA1 mice. Full details of the characterization of these mice are described in the following chapter. *CAG/orexin* hemizygous transgenic mice (line L62 backcrossed N4-N5 with C57BL/6J mice) were bred to *orexin/ataxin-3* hemizygous transgenic mice (backcrossed N5 to C57BL/6J) to generate double hemizygous mice.

Cataplexy Testing

Screening of 8 *orexin/ataxin-3* hemizygous transgenic mice and 13 *orexin/ataxin-3*; *CAG/orexin* double hemizygous transgenic mice (generally littermates, 12-20 weeks old, C57BL/6J N4 and N5 prior to filial crosses) was carried out as described in previous chapters during the dark phase using infrared video recording, except that chambers contained additional novelties (plastic test tube racks and cotton nesting pads) to increase natural

activity of the mice. Scoring criteria used by genotype-blinded observers were the same as those in previous chapters.

Following the cannulation of *orexin/ataxin-3* transgenic animals for orexin administrations (see below), it was noted that cataplexy was not reliably elicited in some individuals, possibly due to stress associated with indwelling cannulas, restraint, or injection procedures. Only animals consistently showing cataplectic attacks following vehicle administrations (5/16) were subjected to further analysis.

Surgical Cannulation and Orexin Administration

All procedures were approved by the appropriate institutional animal care and use committees, and were carried out in strict accordance with NIH guidelines. *Orexin/ataxin-3* hemizygous transgenic mice (16-19 week-old, male, C57BL/6J:129SvEv F1 genetic background) were anesthetized with sodium pentobarbital (50-60 mg/kg i.p.) and implanted with indwelling stainless steel guide cannulas into the left lateral ventricle (stereotaxic coordinates: 0.3 mm posterior to bregma, 0.9 mm lateral from midline, 2.4 mm depth from skull surface). Mice were treated postoperatively with penicillin G benzathine/penicillin G procaine suspension (100,000 U/kg s.c.). All animals were recovered for at least 10 days prior to experiments; mice utilized for further study regained presurgical body weights and exhibited no obvious signs of infection.

For cataplexy testing, doses of synthetic orexin-A (3 nmol/mouse i.c.v., generous gift of Glaxo SmithKline Pharmaceuticals) or vehicle (sterile artificial cerebrospinal fluid) were administered using a randomized crossover design at an interval of 4-7 days. All doses

were delivered through cannulas to gently restrained mice using gas-tight syringes (Hamilton Company, Reno, Nevada) with a blunt needle. An injection volume of 1 µl was delivered over a 30-sec period to each mouse, with the needle remaining in position for an additional 15-sec to ensure dispersal of the peptide. Mice were injected just prior to onset of the dark phase with cataplexy testing following immediately.

For i.c.v./EEG/EMG experiments (see below), mice were injected either at the onset of the dark phase or, alternatively, at 3 h into the light phase. Mice were then returned to home cages for immediate recording of EEG/EMG.

EEG/EMG Recordings

For genetic studies, 6 *orexin/ataxin-3* transgenic, 5 *CAG/orexin*;*orexin/ataxin-3* double transgenic littermates, and 10 wild type control mice (all genotypes 14-15 week-old males, C57BL/6J N4 and N5 prior to filial crosses) were anesthetized and surgically implanted for long-term EEG/EMG monitoring as described in previous chapters. For i.c.v./EEG/EMG experiments, 4 *orexin/ataxin-3* transgenic and 3 wildtype mice (16-19 week-old males, C57BL/6J:129SvEv F1) were simultaneously implanted with a modified EEG/EMG implant (with only two EEG electrodes implanted over one hemisphere) in addition to a guide cannulas (placed as above).

DISPLAY ITEMS

Table 7-1. Sleep/Wakefulness Parameters Recorded from *Orexin/ataxin-3* transgenic, *CAG/orexin;orexin/ataxin-3* double transgenic, and Wild-type Control Mice

	REM						NREM						Awake					
	WT		Ataxin		CAG;Ata		WT		Ataxin		CAG;Ata		WT		Ataxin		CAG;Ata	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
24 hours																		
Time (min)	55.54	2.44	63.39	2.94	41.30	2.90	568.59	28.61	566.83	21.80	546.93	28.08	813.20	30.00	807.11	22.12	849.07	29.15
Light (rest) phase																		
Time (min)	47.46	2.28	35.58	2.26	31.53	2.03	400.52	12.70	389.72	10.34	394.87	10.64	270.53	13.65	293.36	10.10	292.23	10.02
Duration (sec)	72.45	1.83	74.84	6.24	51.74	1.61	277.55	17.60	261.98	15.78	237.46	19.34	333.98	61.52	274.16	22.52	257.15	17.51
Frequency (episodes/h)	2.56	0.10	1.85	0.13	2.17	0.18	4.82	0.24	4.74	0.27	5.52	0.38	2.76	0.36	3.45	0.19	3.92	0.34
REM latency (min)	9.05	0.64	7.45	0.70	6.79	0.58												
REM Interval (min)	23.02	0.99	25.81	1.72	22.51	0.96												
Dark (active) phase																		
Time (min)	7.95	1.08	27.81	2.36	9.77	1.92	168.07	20.11	177.11	13.47	152.07	18.30	542.64	20.12	513.75	14.23	556.83	19.92
Duration (sec)	58.58	2.68	80.70	2.91	46.04	3.86	262.47	29.36	157.52	10.89	170.37	21.47	851.29	133.79	450.82	55.28	543.59	51.32
Frequency (episodes/h)	0.51	0.08	1.51	0.12	0.65	0.14	2.34	0.36	3.78	0.26	3.45	0.27	2.25	0.39	3.99	0.24	3.60	0.27
REM latency (min)	8.33	0.63	3.91	0.41	5.05	0.55												
REM Interval (min)	75.99	12.46	33.07	2.62	51.59	6.61												

Total time spent in each state (min), episode duration (min), episode frequency (per hour), REM latency (min), and interval between successive REM sleep episodes (min) over light and dark periods. Data are presented as means±SEM. Statistical significance was tested by repeated measures ANOVA followed by Tukey post-hoc test.

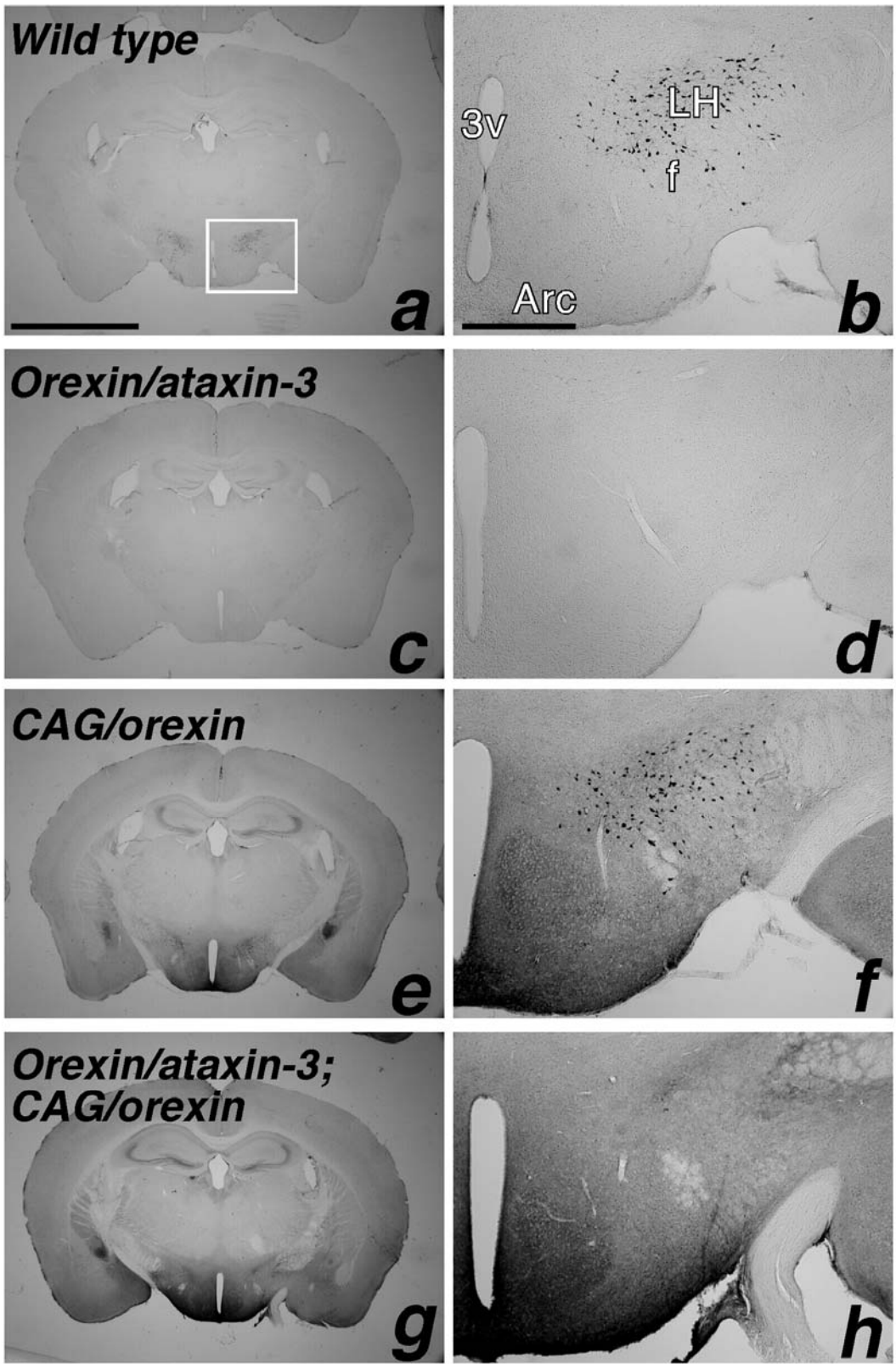

Wild type
a
3v
LH
f
Arc
b
Orexin/ataxin-3
c
d
CAG/orexin
e
f
Orexin/ataxin-3;
CAG/orexin
g
h

Figure 7-1. Immunohistochemical Analysis of Brains from Transgenic Mice.

Anti-orexin-A immunostaining of coronal sections of brain tissue from wild type, *orexin/ataxin-3* hemizygous transgenic, *CAG/orexin* hemizygous transgenic, and double hemizygous transgenic mice were performed as described31. Note the punctate staining of native orexin-A-immunoreactive neurons clustered in the perifornical lateral hypothalamus of wild type mice (a, b), and absence of orexin-A in the brains of *orexin/ataxin-3* transgenic mice in which native orexinergic neurons have degenerated (c, d). *CAG/orexin* transgenic mice have widespread, diffuse, ectopic production of orexin-A in addition to that observed in native neurons (e, f). In contrast, *orexin/ataxin-3*;*CAG/orexin* double transgenic mice exhibit only the ectopic pattern of orexin-A; native neurons are absent (g, h). The inset frame in panel (a) illustrates the location and size of magnified panels on the right. LH, lateral hypothalamus; f, fornix; Arc, arcuate nucleus of the hypothalamus; 3V, third ventricle. (a, c, e, g) Bar, 3 mm; (b, d, f, h) bar, 0.5 mm. Histology performed by J. Hara.

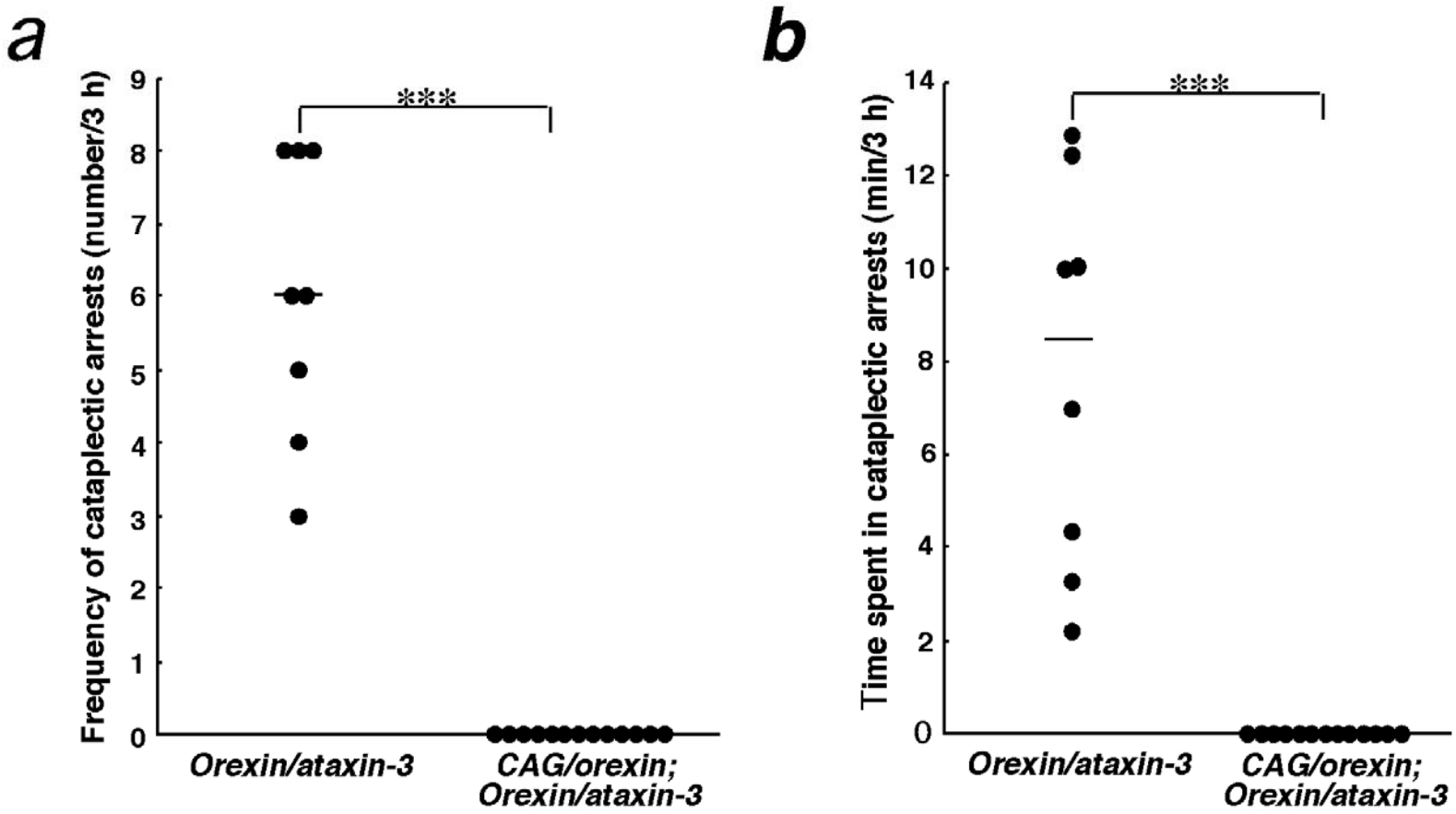

a
Frequency of cataplectic arrests (number/3 h)
9
8
7
6
5
4
3
2
1
0
***
Orexin/ataxin-3
CAG/orexin;
Orexin/ataxin-3
b
Time spent in cataplectic arrests (min/3 h)
14
12
10
8
6
4
2
0
***
Orexin/ataxin-3
CAG/orexin;
Orexin/ataxin-3

Figure 7-2. *CAG/orexin* Transgene Prevents Cataplectic Behavioral Arrests in Orexin Neuron-Ablated Mice.

Cataplectic arrests are absent in *orexin/ataxin-3*;*CAG/orexin* double hemizygous transgenic mice compared to *orexin/ataxin-3* hemizygous transgenic littermates. Number of cataplectic arrests observed (a) and total time spent in cataplexy (b) in each mouse are shown for 3-h sessions. Bars represent medians. ***P<0.0001 by Mann-Whitney Test. Experiment was carried out by the author. Videos were scored by both the author and M. Mieda.

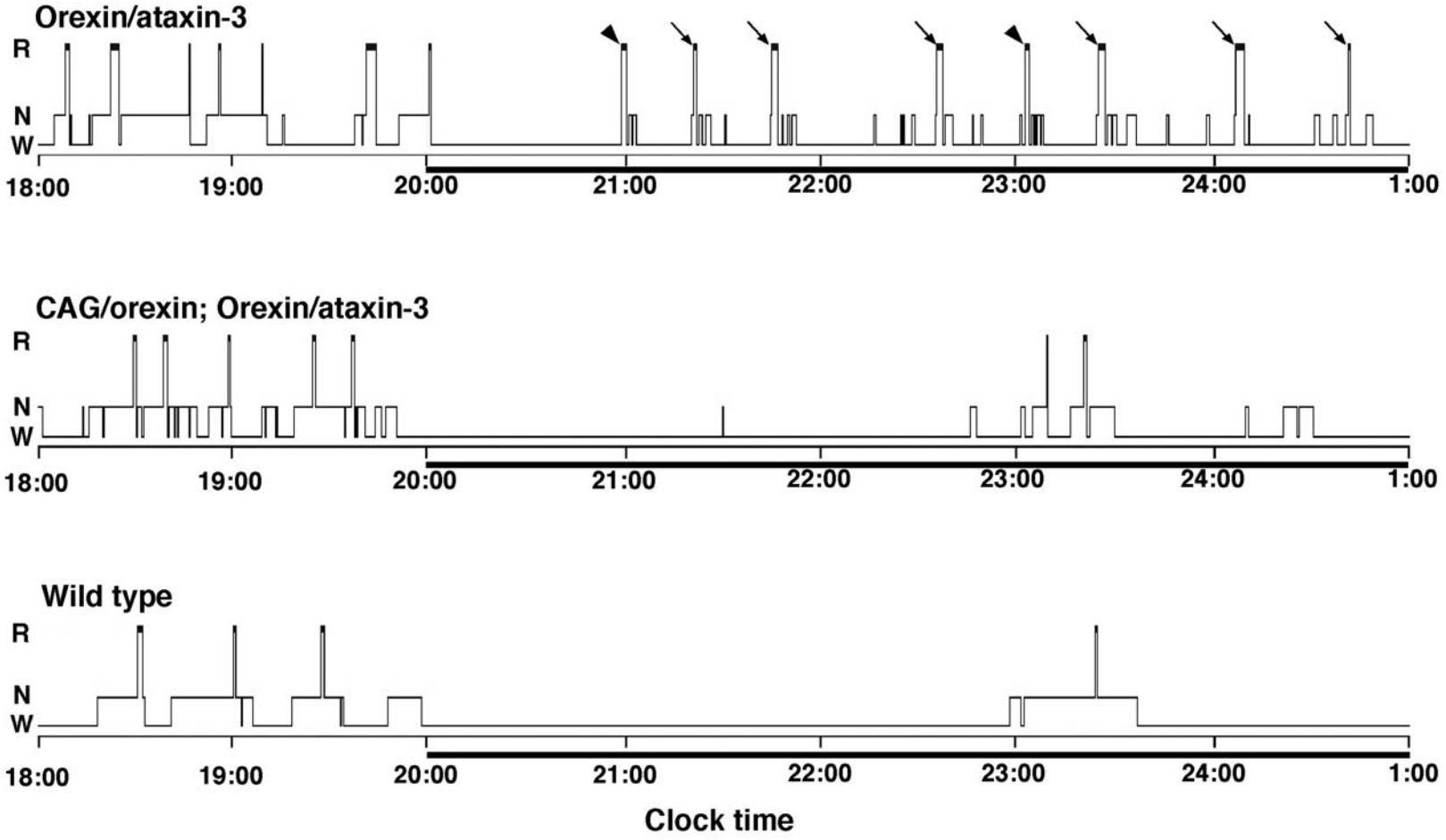

Orexin/ataxin-3
R
N
W
18:00
19:00
20:00
21:00
22:00
23:00
24:00
1:00
CAG/orexin; Orexin/ataxin-3
R
N
W
18:00
19:00
20:00
21:00
22:00
23:00
24:00
1:00
Wild type
R
N
W
18:00
19:00
20:00
21:00
22:00
23:00
24:00
1:00
Clock time

Figure 7-3. Sleep-Wake Cycles of Typical Transgenic and Wild-type Mice.

Hypnograms represent concatenated 20 sec epochs of EEG/EMG activity, scored as either awake (W), non-REM sleep (N), or REM sleep (R). Seven hours per mouse, including transitions from light phase to dark phase (solid bar) are shown. The *orexin/ataxin-3* transgenic mouse exhibits fragmentation of wakefulness during the dark phase and frequent premature onsets of REM sleep that occur immediately after wakefulness (arrowheads) or after less than 1 min of preceding non-REM sleep (arrows). In contrast, the *orexin/ataxin-3*;*CAG/orexin* double transgenic mouse has more consolidated wakefulness during the dark phase. As in the wild type mouse, no direct or premature transitions from wakefulness to REM sleep were ever observed in the double transgenic mouse.

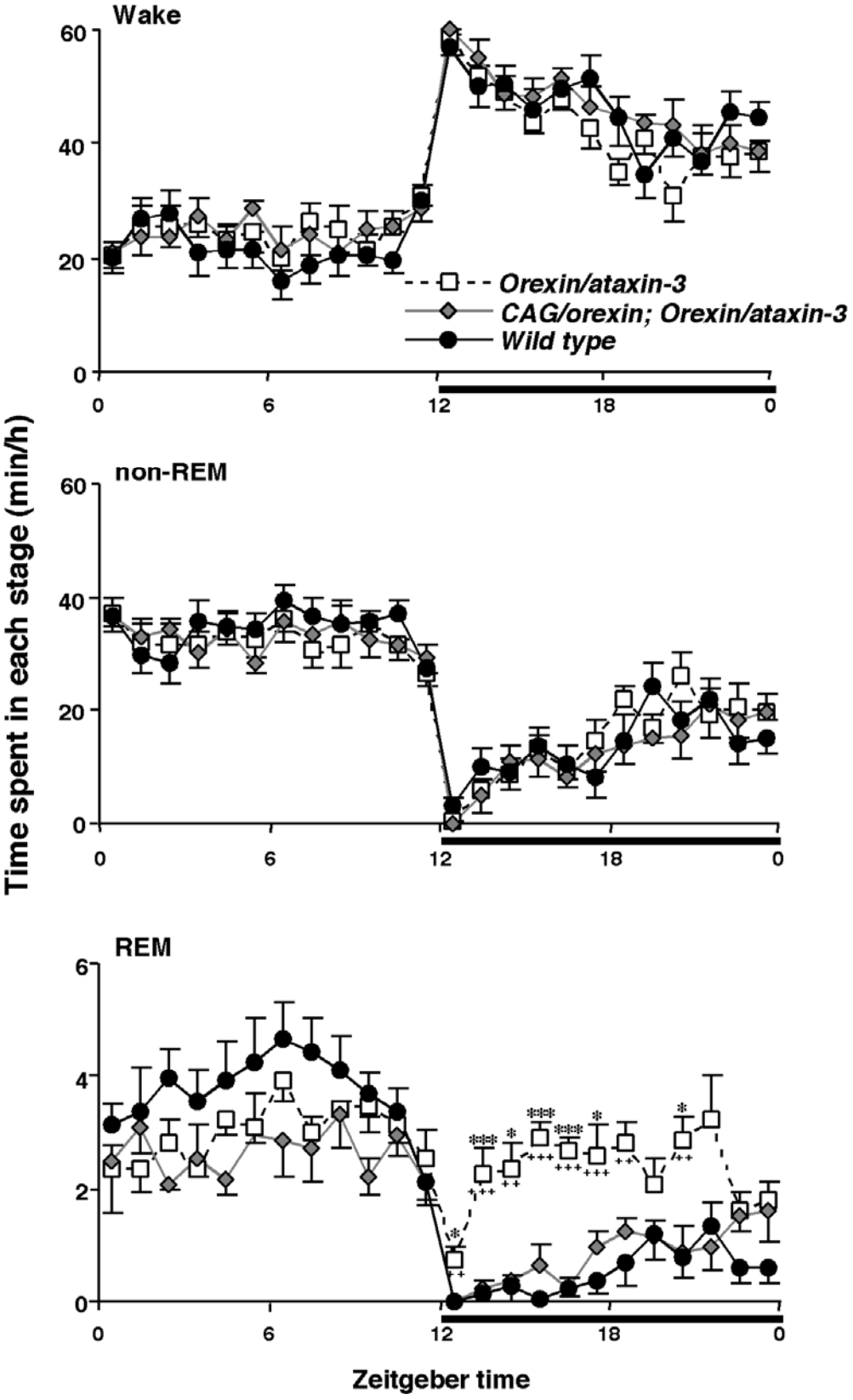

Wake
non-REM
REM
Orexin/ataxin-3
CAG/orexin; Orexin/ataxin-3
Wild type
Time spent in each stage (min/h)
Zeitgeber time
0
20
40
60
0
2
4
6
0
6
12
18
0

Figure 7-4. Hourly Plots of Sleep/wake States in Transgenic and Wild-type Mice.

Narcoleptic *orexin/ataxin-3* transgenic mice as well as *orexin/ataxin-3;CAG/orexin* double transgenic mice exhibit hourly amounts of wakefulness and non-REM sleep that are similar to that of wild type mice. In contrast, *orexin/ataxin-3* transgenic mice exhibit significantly increased amounts of REM sleep during the dark phase (solid horizontal bars). Double transgenic mice exhibit a specific rescue of this abnormality compared to wild type mice. *P<0.05, **P<0.005, ***P<0.0005 compared to wild type mice, and +P<0.05, ++P<0.005, +++P<0.0005 compared to *orexin/ataxin-3;CAG/orexin* double transgenic mice by ANOVA and Tukey post-hoc tests. Values are means +- S.E. (n = 10 for wild type mice, n = 6 for *orexin/ataxin-3* mice, n = 5 for *orexin/ataxin-3;CAG/orexin* double transgenic mice).

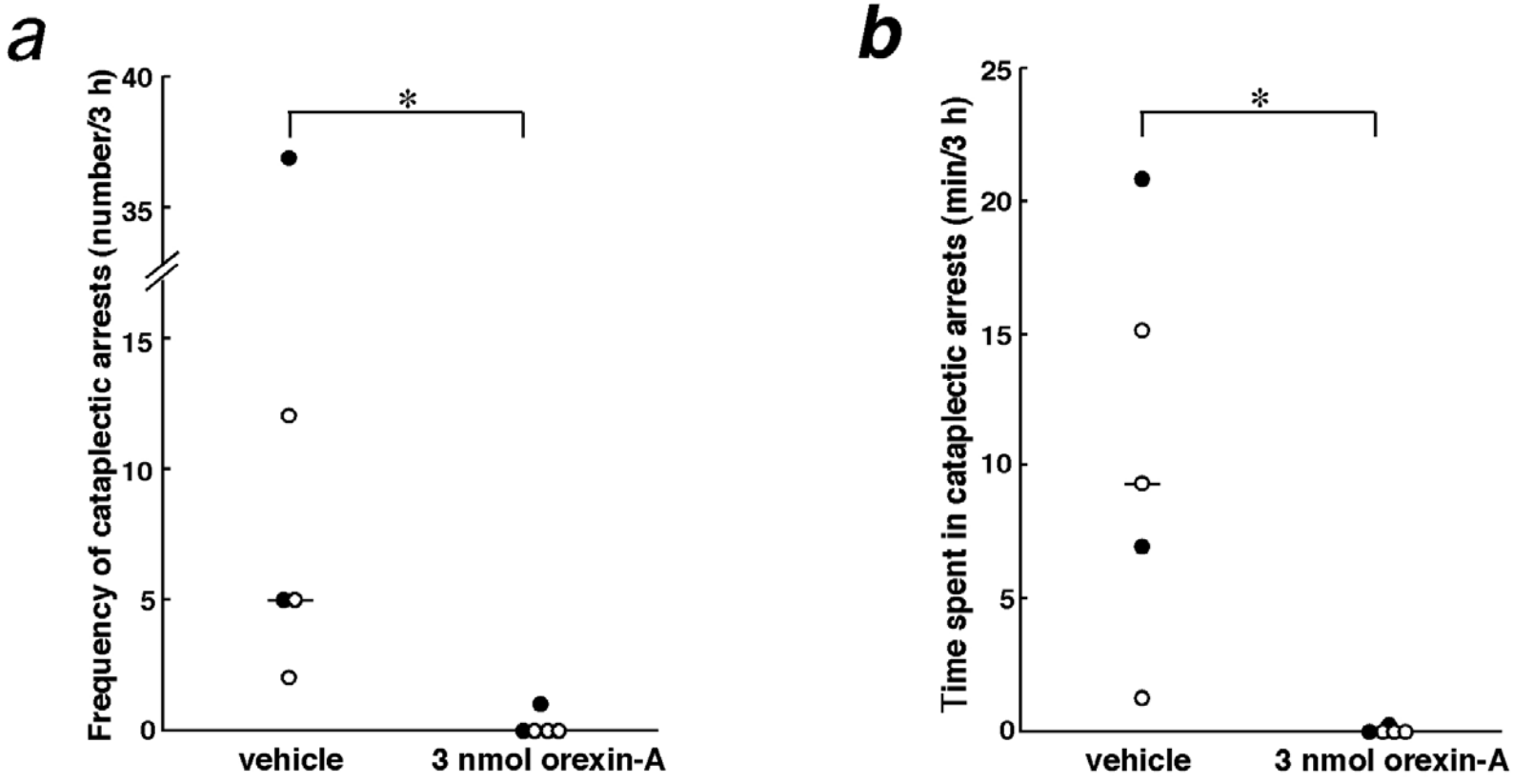
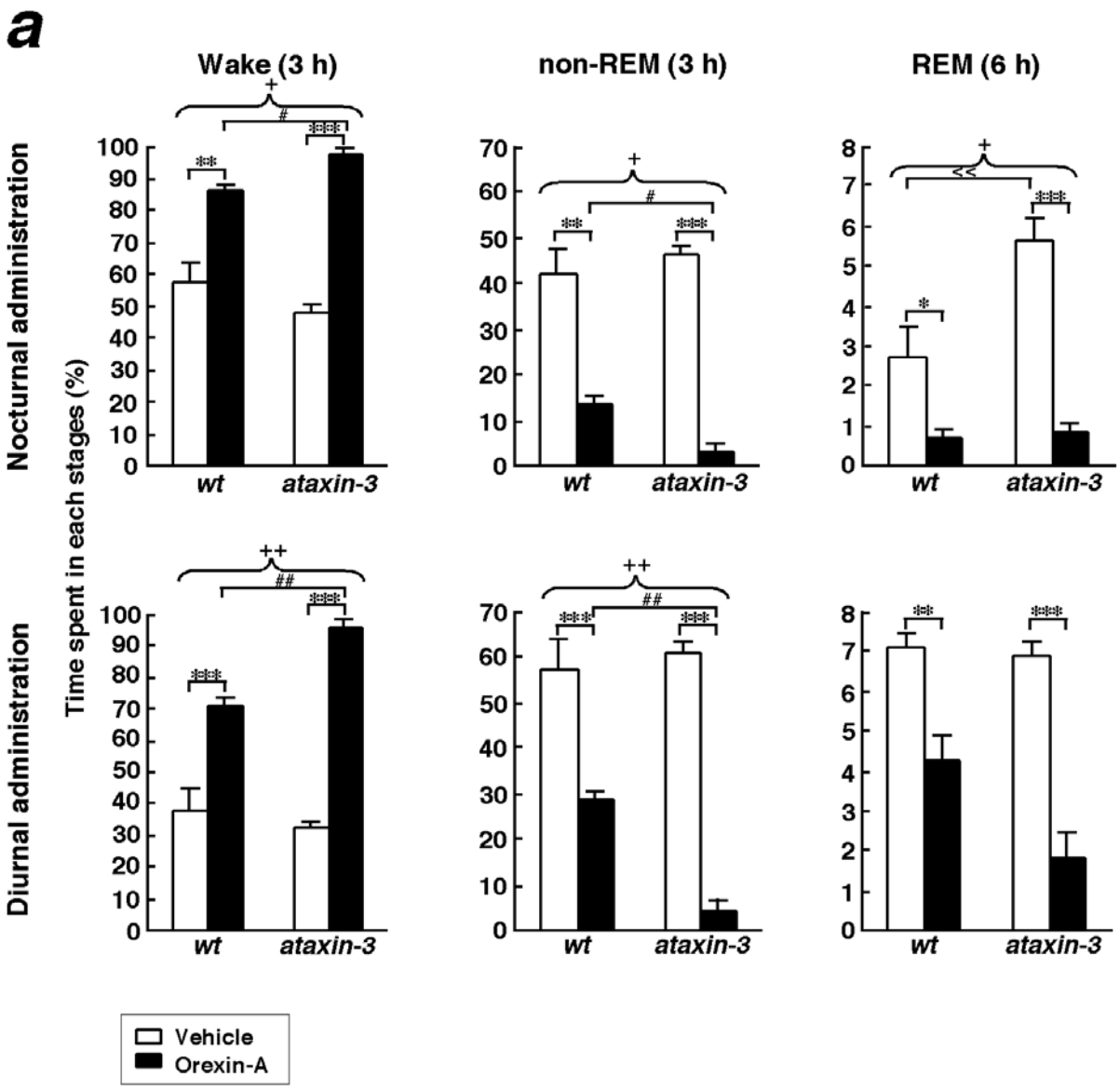

a
Frequency of cataplectic arrests (number/3 h)
40
35
15
10
5
0
*
vehicle
3 nmol orexin-A
b
Time spent in cataplectic arrests (min/3 h)
25
20
15
10
5
0
*
vehicle
3 nmol orexin-A

Figure 7-5. Suppression of Cataplectic Arrests by I.c.v. Orexin-A Administration. Vehicle (artificial cerebrospinal fluid) and orexin-A (3 nmol/mouse) were administered by bolus injections into the lateral ventricles of 5 narcoleptic *orexin/ataxin-3* transgenic mice prior to onset of the dark phase in a randomized crossover design, over two consecutive experimental sessions. The order in which animals were dosed is indicated by symbols: filled circles, orexin-A first; open circles, vehicle first. Number of cataplectic arrests observed (a) and total time spent in cataplexy (b) in each mouse are shown for 3-h sessions. Bars represent medians. *$P<0.05$ by Wilcoxon signed-ranks test. Experiment was designed by the author and carried out by M. Mieda. Video records were scored by the author and M. Mieda.


a
Wake (3 h)
non-REM (3 h)
REM (6 h)
Nocturnal administration
Diurnal administration
Time spent in each stages (%)
wt
ataxin-3
Vehicle
Orexin-A

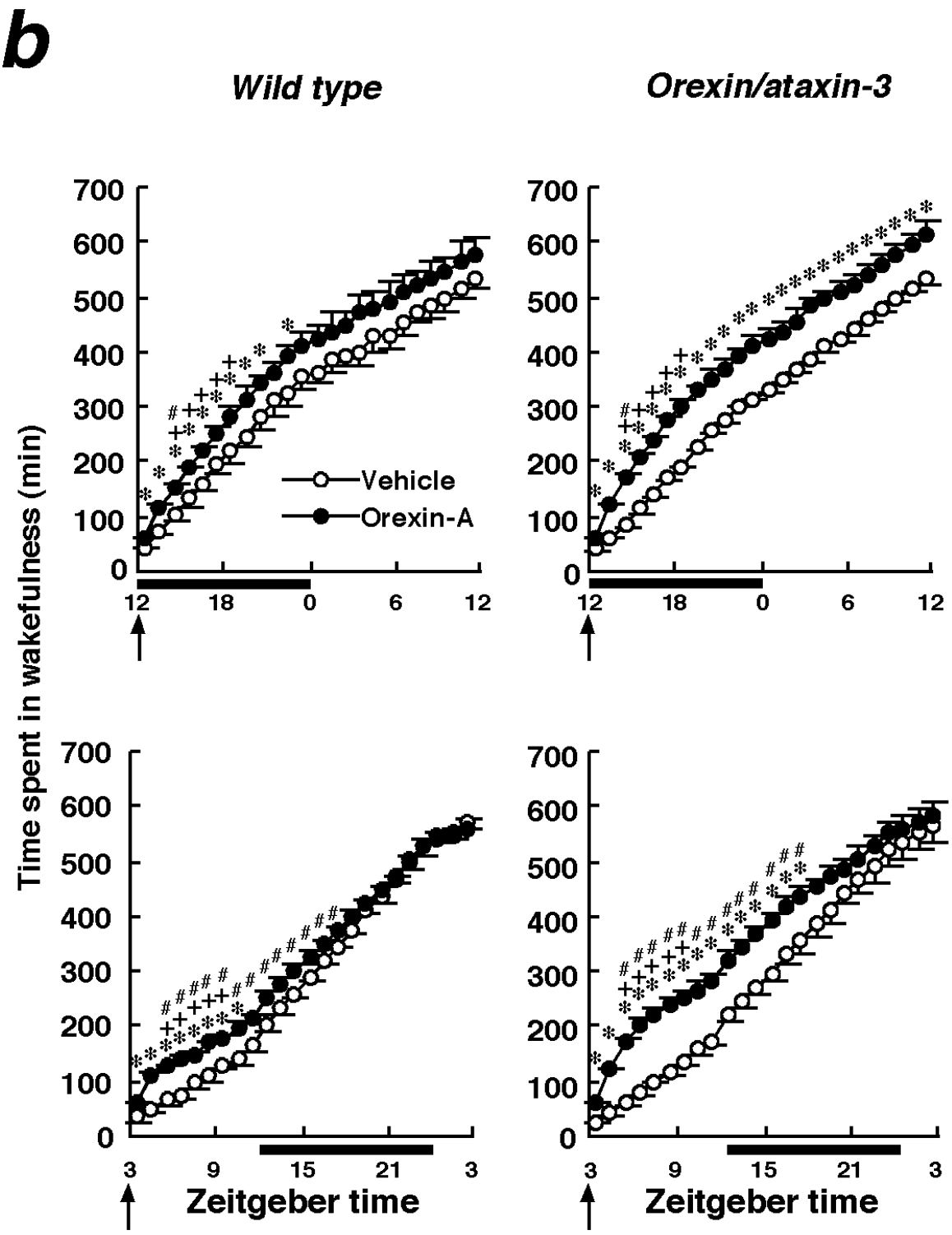

b
Wild type
Orexin/ataxin-3
Time spent in wakefulness (min)
Vehicle
Orexin-A
Zeitgeber time
Zeitgeber time

Figure 7-6. Orexin-A Administrations Increase Wakefulness and Suppress Sleep in Narcoleptic Mice.

(a) Amounts of time spent in each stage within 3 h (wakefulness, NREM) or 6 h (REM) following vehicle (open bar) and orexin-A (filled bar) administrations at either the beginning of the dark phase (nocturnal administration) or during the light phase started (diurnal administration) in wild type (wt) or *orexin/ataxin-3* mice (ataxin-3). *, P<0.05; **, P<0.01; ***, P<0.001 vehicle vs. orexin-A in each genotype; #, P<0.05; ##, P<0.01 wild type mice administered orexin-A vs. *orexin/ataxin-3* mice administered orexin-A; <<, P<0.01 wild type mice administered vehicle vs. *orexin/ataxin-3* mice administered vehicle; +, P<0.05; ++, P<0.01 interaction of dose and genotype; by two-way ANOVA. (b) Cumulative amounts of time spent in wakefulness following nocturnal and diurnal administrations of vehicle and orexin-A administrations in wild type or *orexin/ataxin-3* mice. Times of administration are indicated by arrows. The dark phase is indicated by solid horizontal bars. *, P<0.05 vehicle vs. orexin-A in each genotype; #, P<0.05 wild type mice administered orexin-A vs. *orexin/ataxin-3* mice administered orexin-A; +, P<0.05 interaction of dose and genotype, by two-way ANOVA. Values are means +- S.E. (n = 3 for wild type, n = 4 for *orexin/ataxin-3* mice) for both (a) and (b). Experiment was carried out by M. Mieda.

CHAPTER EIGHT
Conclusion

I have demonstrated that the narcolepsy-cataplexy symptoms are induced by loss of signaling through OX2R-dependent and OX2R-independent pathways. I predict that agonists of OX1R and OX2R, once developed, will provide selective therapeutic benefit in the treatment of specific symptoms of narcolepsy-cataplexy and other disorders of sleep and wakefulness. Orexin neurons provide a critical link between peripheral energy balance and the CNS mechanisms that coordinate sleep-wakefulness and motivated behaviors such as food-seeking, especially in the physiological state of fasting stress. The interaction between the *orexin* and *MCH* gene products reveals complementary functions with respect to arousal and metabolic regulation. Orexin overexpression studies demonstrate a role for orexin signaling in energy substrate utilization and resistance to obesity. Modafinil does not inhibit mouse cataplexy and does not require intact orexin signaling for its wake-promoting effects, suggesting that its mechanism of action is unlikely to be related to orexin signaling. In contrast, the genetic and pharmacologic rescue of narcolepsy-cataplexy in mice in which endogenous orexin neurons have been ablated have important therapeutic implications for the treatment of this debilitating neurologic disorder.

APPENDIX A
Narcolepsy in Orexin Knockout Mice

SUMMARY

Neurons containing the neuropeptide orexin (hypocretin) are located exclusively in the lateral hypothalamus and send axons to numerous regions throughout the central nervous system, including the major nuclei implicated in sleep regulation. By behavioral and electroencephalographic criteria, orexin knockout mice exhibit a phenotype strikingly similar to human narcolepsy patients, as well as *canarc-1* (OX2R-mutant) dogs, the only previously described monogenic model of narcolepsy. Based upon these findings it was first demonstrated that orexin regulates sleep/wakefulness states, and that orexin knockout mice are a model of human narcolepsy, a disorder characterized primarily by REM sleep dysregulation.

INTRODUCTION

Here, data are presented in which a null mutation induced by targeted disruption of the mouse orexin gene results in an autosomal recessive phenotype with characteristics remarkably similar to narcolepsy. Initial studies on these knockout mice focused on the putative involvement of orexins in energy homeostasis. While studying the 24-hour open

field activity of these mice as an index of energy expenditure, R.M. Chemelli and I found that the orexin knockout mice showed periods of reduced activity during the dark phase when mice are normally most active. We then used infrared videotaping to investigate possible reasons for this abnormal behavior. Close study of these videotapes unexpectedly revealed brief periods of behavioral arrest that occurred exclusively in homozygous knockout mice. Twenty-four-hour EEG/EMG recording, assisted by C. M. Sinton, were undertaken to determine if these episodes might be seizure-related. Instead we demonstrated a complete absence of seizures and revealed an unanticipated disruption of REM sleep regulation. Orexin neurons project directly onto major nuclei implicated in the processes of cortical activation known to play a critical role in modulating sleep-wakefulness (Chemelli et al., 1999). These observations firmly identify orexins as neuropeptides with an important function in sleep regulation. In conjunction with the report that the OX2R gene is mutated in the narcoleptic dogs from Mignot's group (Lin et al., 1999), our findings suggest that orexin–OX2R interaction is essential for proper regulation of REM sleep.

RESULTS

Production of Prepro-orexin Knockout Mice

The targeting of the mouse prepro-orexin gene by replacement of exon 1 (Sakurai et al., 1999) in-frame with nuclear lac-Z and neo cassettes by homologous recombination in embryonic stem cells is as reported (Chemelli et al., 1999). Heterozygous F1 mice were

phenotypically normal, and progeny of F1 heterozygote crosses survived until weaning in a ratio consistent with Mendelian inheritance (homozygosity did not cause significant lethality in utero or up to the time of weaning). Homozygous mice were anatomically and histologically normal except that orexin mRNA and orexin peptides were absent from the brain. Serum electrolytes and glucose levels were also within normal ranges.

Behavioral Characterization of Narcoleptic Attacks

Intensive studies of the behavior of orexin knockout mice using conventional methods failed to reveal overt behavioral abnormalities during the day time, when mouse behavior is normally quiet. To assess behavior at night, when mice are normally most active, infrared videophotography was performed. The videotapes revealed frequent periods of obvious behavioral arrest in orexin-null mice during the dark phase. An infrared video study of progeny derived from independent ES cell clones confirmed that this behavior occurred with 100% penetrance in orexin-null mice. These episodes of behavioral arrest are described as “narcoleptic episodes” for the rest of this chapter because at the time these studies were performed, we could not readily distinguish episodes with preserved consciousness (cataplexy) from those without consciousness (see Discussion). My later experiments address this issue and are described in the following chapter.

In order to identify and characterize these narcoleptic episodes objectively, R.M. Chemelli and I developed strict criteria to differentiate them from quiet behavioral states with decreased overt activity, as well as from normal transitions into sleep. Pilot video studies of knockout mice and wild-type littermates demonstrated that this behavior could be

specifically recognized by the abrupt cessation of purposeful motor activity associated with a sudden, sustained change in posture that was maintained throughout the episode (**Appendix A Figure 2B**), ending abruptly with complete resumption of purposeful motor activity. Essentially, the episodes looked as if a behavioral switch had been turned "off" and then "on". As independent observers, we used these criteria to identify narcoleptic episodes in all of the behavioral video studies reported. Episodes judged ambiguous in any aspect, by either observer, were excluded from the analysis.

We studied the characteristics of the narcoleptic episodes in 14-15 week old male mice in a standard open-field apparatus with an infrared video camera mounted overhead. Nine homozygote, 5 wild-type and 4 heterozygote F2 littermates were filmed for the first 4 h of the dark phase, after an initial 3 h acclimation period during the light cycle. Videotapes were coded and then given to two blinded observers independently for scoring. Two mice were filmed in two separate open-fields at once. They were assigned randomly to filming so that all combinations of genotypes were present (control-control, control-knockout, knockout-knockout, etc.). No narcoleptic episodes were identified on videotapes of the wild-type or heterozygote mice. Individual knockout mice had from 8 to 27 episodes (group average 17.1 ± 2.1 episodes) during the 4 h filming period (**Appendix A Figure 1A**). The average duration of episodes for individuals varied from a low of 48.8 ± 8.8 sec to a high of 81.7 ± 18.7 sec with an overall group average of 65.6 sec. The duration of single narcoleptic episodes varied widely among and within individual mice with the shortest ending after only 6 sec (mouse E) and the longest lasting 214 sec (mouse I). The time course and duration of each narcoleptic episode for mouse F is plotted in **Appendix A Figure 1B** as a representative

example. A parallel video study of female homozygotes and controls revealed similar episode frequency and duration (data not shown).

The predominant behavior for the 5 sec immediately preceding and the 10 sec immediately after each episode were categorized as feeding, drinking, ambulating, grooming, burrowing, climbing, or other (Group means presented in **Appendix A Figure 1C**). Interestingly, while burrowing and climbing were often observed before an episode they never occurred afterward. Conversely, while feeding and drinking rarely preceded an attack they frequently occurred afterward. Individual mice appeared to have characteristic triggering behaviors with mouse A exhibiting ambulatory behavior in 22 of 27 episodes, mouse F burrowing before 8 of 17 episodes and mouse G grooming in 6 of 8 episodes. Clearly observed gait ataxia lasting 1-3 sec immediately preceded 26.5 ± 8.7% of all observed episodes with a range of 0%-77.8% in individual homozygotes. Grossly observable motor activity causing side-to-side rocking, without change in overall posture, frequently occurred several seconds after the start of the attack. This lasted anywhere from 2-10 sec and was observed during 75.5 ± 7.0% of all episodes with a range of 46.2%-100% of all attacks in individual knockouts. Often these bouts of motor activity intensified and they appeared to abruptly terminate some narcoleptic attacks.

I performed close-up video studies of individual knockout mice filmed from various angles to further explore the nature of the postural changes and motor activity noted during the narcoleptic attacks. The postural changes characteristically involved sudden collapse of the head and neck with simultaneous buckling of the limb, medially and/or laterally from the body, causing the ventral surface to fall to the cage floor at angles of 45˚-perpendicular to the

sagittal plane. Occasionally the mouse fell completely onto its side. Gross motor activity during attacks always resulted from episodic limb twitching that rocked the mouse about its central axis. Bulbar motor activity was often noted in the close-up videos during attacks, with ear twitching, eye blinking, eye movements and chewing movements sometimes observed.

Developmental Aspects of Narcoleptic Attacks

We studied the onset and development of narcoleptic episodes in two sets of male homozygote littermates from F2 homozygote crosses. Both cohorts were housed in groups of 4-5 per cage. Mice from one set were placed into individual cages for videotaping. The other set was videotaped in the group cage, allowing social interaction. Videotaping was conducted at weekly intervals from 3-6 weeks of age. **Appendix A Figure 2A** shows the numbers of episodes recorded in the first 4 h of the dark phase for five individually filmed knockout mice, designated M-Q. The right panel of **Appendix A Figure 2A** compares the total number of episodes in the individually videotaped mice versus the total number in the group filmed mice. The grouped mice exhibited more episodes; 55 vs. 8 at 4 weeks of age and 64 vs. 26 at 6 weeks of age (4 mice in-group vs. 5 mice in individual). Interestingly, while the entire group-filmed cohort had attacks at 4 weeks of age, this did not occur until 6 weeks in the individually filmed mice. In the group-filmed mice, chasing, tail-biting, and social grooming were observed immediately prior to narcoleptic attacks on some occasions. Narcoleptic attacks in the group-filmed setting also appeared to be cut short by stimulation from littermates. Average episode duration was 45.1 ± 4.1 sec vs. 56.9 ± 11.7 sec ($p = 0.026$ by Welch t-test) at 4 weeks and 44.4 ± 4.1 sec vs. 89.8 ± 11.7 sec ($p < 0.0001$) at 6 weeks in the

group vs. individually filmed littermates, respectively. Categorization of narcoleptic posture at 6 weeks revealed that, in 37/90 (41.1%) of episodes, the young mice completely fell over to their side either in group or individually filmed settings (**Appendix A Figure 2B**). In another 37/90 (41.1%) of episodes, the mice collapsed onto their ventral surface without gross motor activity (**Appendix A Figure 2C**); and only in 16/90 (17.7%) of episodes they exhibited adult-like "rocking" motor activity.

EEG/EMG Studies

Bodily collapse associated with episodic rocking motor activity initially suggested the possibility of a seizure disorder in the orexin knockout mice. To evaluate this hypothesis, our collaborator C. M. Sinton chronically implanted EEG and EMG electrodes in these mice and recorded EEG/EMG continuously while the mice were allowed ad lib movement in their acclimated cages. After analyzing more than 300 h of EEG recordings from 6 knockout mice, he was unable to find any evidence of seizure activity. Rather, these EEG recordings revealed that REM sleep was affected in the knockout mice (**Appendix A Figure 3A,B**). While I delve further into abnormalities of sleep-wakefulness transitions in orexin null mice using EEG/EMG in **Chapter 2**, the basic findings from these original experiments were that (i) sleep/wake periods of orexin knockout mice were highly fragmented, especially during the dark phase, relative to indistinguishable heterozygote and wild-type controls, and (ii) orexin knockout mice but not controls exhibited direct transitions from wakefulness to REM sleep, a highly abnormal phenomenon in mammals (Chemelli et al., 1999).

Despite unexpected difficulties due to the infrequency of behavioral arrests in implanted tethered animals relative to our previous experiments described above, I successfully correlated a small number of infrared videotaped narcoleptic attacks in the first 4 h of the dark phase against concurrent EEG/EMG records. These periods corresponded to one of two distinct EEG/EMG patterns: they occurred either during direct transitions from waking to REM sleep (similar to **Appendix A Figure 3C**), or high amplitude spindle oscillations in the EEG, superimposed on a slow-wave or non-REM sleep background. These spindles are characteristic of the normal pre-REM phase in the mouse (Glin et al., 1991). In contrast to wild-type mice, however, in the knockout mice these spindle oscillations occurred immediately after a waking period. An example of this oscillatory EEG activity is displayed in **Appendix A Figure 3E**. These oscillations can be distinguished from spindles that are recorded during sleep onset because pre-REM spindles are of higher amplitude, longer duration and frequently show a pointed aspect (Gottesmann, 1996). The existence of pre-REM spindles at sleep onset is a further indication of abnormalities in the processes of REM sleep initiation and control in the orexin knockout mouse (for more detailed descriptions and discussion of this phenomenon see **Chapter 2**). After further development of behavioral paradigms that reliably elicit behavioral arrests (**Appendix B**), I address the correlates of behavioral arrests quantitatively and in further detail in **Chapter 2**.

DISCUSSION

These studies, using behavioral and electrophysiological approaches, comprise the original observation that mice lacking orexin neuropeptides exhibit a syndrome of sleep dysregulation remarkably similar to human and canine narcolepsy. Homozygous knockout mice experience episodes of narcoleptic attacks, and are hypersomnolent during their active dark phase. Their waking periods are fragmented and they have direct transitions from wakefulness to REM sleep. The narcoleptic phenotype, combined with documented orexin projections to the major sleep-wakefulness regulating nuclei of the brain (Chemelli et al., 1999), strongly suggest an important role for orexins in sleep regulation. This work lays the groundwork for further development of the orexin and orexin receptor null mice as models of narcolpsy and exploration of the role of orexin receptor signaling pathways in regulation of sleep-wakefulness transitions and the narcolepsy-cataplexy syndrome (see **Chapter 2**)

EXPERIMENTAL PROCEDURES

Experimental Subjects

The generation and basic characterization of orexin knockout mouse lines is described (Chemelli et al., 1999). Animals were genotyped by PCR, which had been verified by Southern blotting. All experiments using wild-type and heterozygote controls were performed on F2 C57Bl/6J-129/SvEv mixed background littermates from F1 heterozygote

crosses. Experiments performed exclusively on homozygotes used F3 mixed background littermates from F2 homozygote crosses. All mice were provided food and water ad lib, maintained on a 12 h light:dark cycle at all times and were housed under conditions that controlled for temperature and humidity. All mouse procedures used in this study were approved by the Institutional Review Board for Animal Research of the University of Texas Southwestern Medical Center at Dallas.

Infrared Videotaping and Scoring of Narcoleptic Episodes

An 8-mm CCD videocamera with infrared and digital time recording capabilities (Sony TRV-CCD66) was used for documentation and scoring of dark cycle behavior. Characterization of dark phase behavior in 14 to 15 week old mice was performed in a standard open field apparatus (Opto-Varimex-3, Columbus Instruments, Columbus, OH) within a plexiglass arena covered with bedding material and modified for ad lib food and water delivery. Group housed mice were brought to the behavior room 4 to 5 h prior to the their usual dark phase, acclimated to individual arenas for 3 h and then remained there for the 12-h dark phase. Investigations into the developmental aspects of mouse narcoleptic behavior were conducted in knockout littermates videotaped weekly from 3 to 6 weeks of age. These mice were videotaped group housed or individually housed in shoebox cages modified for side-delivery of ad lib food and water. The first 4 h of the dark phase was videotaped from overhead for all behavioral paradigms described. Close-up videotaping of mouse behavior was performed in home cages photographed from various acute angles. Still images and

illustrative video segments were captured and edited with a video capture board (Dazzle Multimedia).

Open field narcoleptic episodes were scored with observers blinded to genotype for 9 homozygote, 5 wild-type and 4 heterozygote F2 littermates from coded videotapes. Narcoleptic episodes for all experimental paradigms were strictly defined by the following features: 1) Abrupt transition from obvious purposeful motor activity associated with; 2) A sustained change in posture maintained throughout the episode and; 3) An abrupt end to the episode with resumption of obvious purposeful motor activity (Essentially as if a switch had been turned "off" and then "on"). This strict scoring methodology likely reduced the sensitivity of episode detection, especially during quiet awake behavior, but enhanced specificity to enable unambiguous identification of narcoleptic episodes. The exact time recorded on the video for the start and end of each episode was recorded along with the following additional observations; The predominant activity for the 5 sec preceding and the 10 sec following an episode were categorized as feeding, drinking, ambulating, grooming, burrowing, climbing or other; Obvious gait ataxia preceding and non-purposeful motor activity (twitching) during the episode were recorded as present or absent. Developmental cohorts were scored similarly with the exception that observers were not blinded since all mice were homozygote littermates. All videotapes were scored by 2 independent observers with disputes settled by quorum.

EEG/EMG Recording

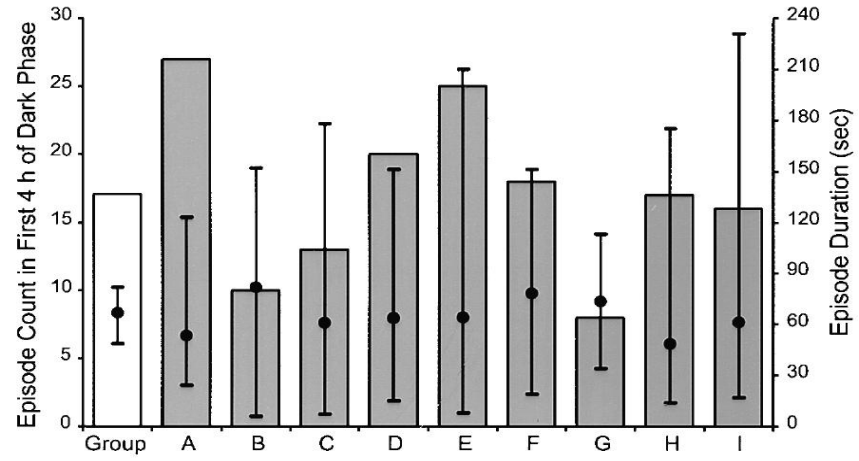
Male mice (n = 12, 14-15 weeks old, 30-35 g at the time of surgery) were prepared for chronic monitoring of EEG/EMG signals using a recently developed lightweight implant and cabling procedure. Full details of this technique will be published elsewhere. Briefly, the EEG/EMG implant was based on a 6-pin double in-line microcomputer connector, modified to form four EEG electrodes, each 1.3 mm by 0.3 mm (h x w) positioned 4.6 mm by 2.9 mm (l x w) apart with two EMG electrodes soldered to the center pins. Mice were anesthetized with sodium pentobarbital (Nembutal, 50-60 mg/kg i.p.) and standard sterile surgical and stereotaxic procedures were employed for implant placement. The cranium was exposed and four burr holes were drilled, anterior and posterior to bregma, bilaterally (AP 1.1, ML ±1.45 and AP -3.5, ML ±1.45) according to the atlas of Franklin and Paxinos (1997). The implant was then inserted into these holes, cemented to the skull with dental acrylic, and the EMG electrodes secured to the nuchal musculature. The design of this EEG/EMG implant allowed precise insertion of electrodes in a reproducible manner, targeting bilateral stereotaxic coordinates over the frontal and occipital cortices at a consistent depth, just touching the dura, while minimizing surgical trauma.

Immediately after recovery from anesthesia the mouse was housed individually and the head-mounted connector was coupled via a 15 cm light weight cable to a slip ring commutator which was suspended from a counter-balanced arm mounted to a standard shoebox cage (19 cm x 30 cm, Allentown Caging, Allentown, NJ). This allowed full freedom of movement and the cage was modified to provide side-delivery of food and water that were available ad lib throughout the experiment. All mice recovered from surgery and were

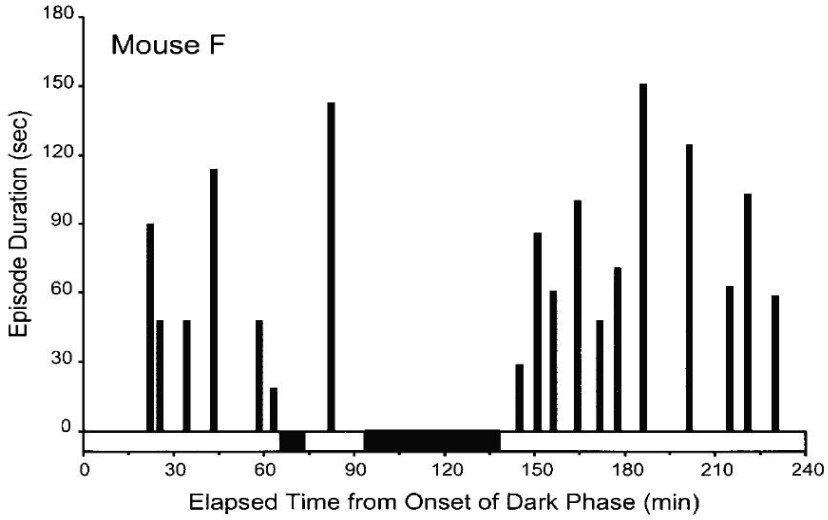
habituated to these conditions for a minimum of 14 days before recording began. Each mouse was then recorded for three consecutive 24-h periods, beginning at lights-on at 07:00. Food and water were replenished at 07:00 and mice were not otherwise disturbed in any way except the minimal perturbation caused by initiation of infrared video recording at 19:00. Four mice were recorded concurrently in matched littermate pairs of homozygotes and wild-type controls. EEG/EMG signals were amplified using a Grass Model 78 (Grass Instruments, West Warwick, RI) and filtered (EEG:0.3-100 Hz, EMG:30-300 Hz) before being digitized at a sampling rate of 250 Hz, displayed on a paperless polygraph system, and archived to CD-R for off-line sleep staging and analysis. EEG/EMG records were visually scored into 20 second epochs of Awake, rapid eye movement (REM) and non-REM sleep according to standard criteria of rodent sleep (Radulovacki et al., 1984). In particular, non-REM sleep was scored from the onset of drowsiness, characterized by a reduction of muscle tone and increasing EEG amplitude. Differences between vigilance state data for homozygous and wild-type mice were analyzed by repeated measures ANOVA (JMP statistical software v. 3.2, SAS Institute, Cary NC) and grouped means calculated across the three recording periods for display.

DISPLAY ITEMS

A



B



C

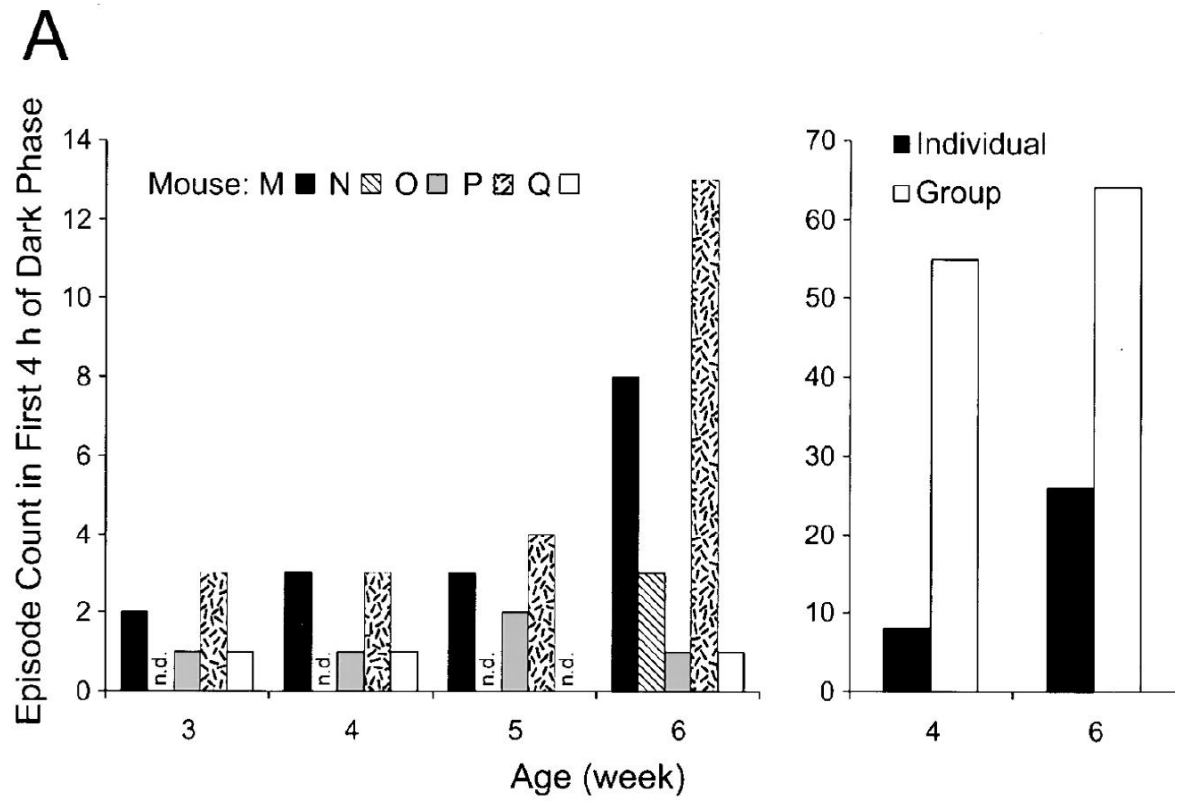
Behavior	Before (%)		After (%)	
Feeding	5	(3.2)	44	(28.6)
Drinking	2	(1.3)	24	(15.6)
Ambulating	63	(40.9)	21	(13.6)
Grooming	36	(23.4)	31	(20.1)
Burrowing	17	(11.0)	0	(0.0)
Climbing	11	(7.1)	0	(0.0)
Other	20	(13.0)	34	(22.1)

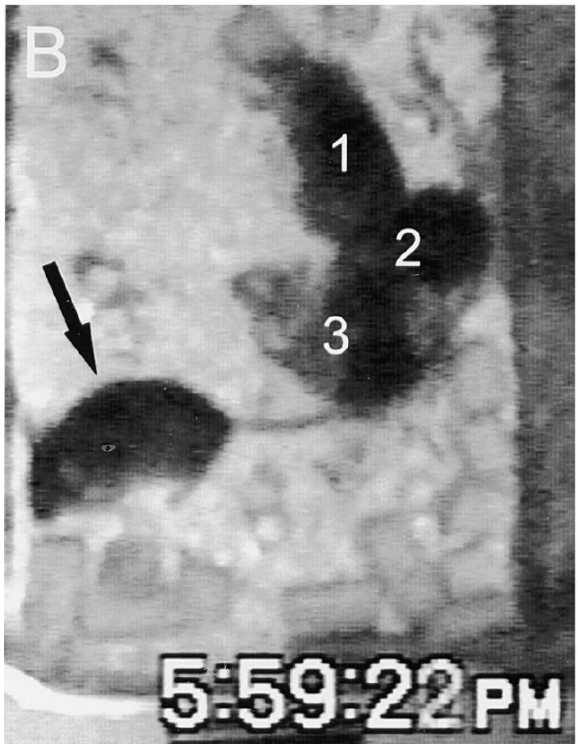
Appendix A Figure 1. Infrared Video Characterization of Narcoleptic Episodes

(A) Episode number and duration. Columns represent total number of episodes recorded in the first 4 h after onset of dark phase. Filled circles represent the mean duration of all recorded episodes. T-bars indicate the minimum and maximum duration observed. Data for individual knockout mice are designated A to I. Group is the average count and duration for all mice (A to I), with the T-bars indicative of minimum and maximum individual averages. No narcoleptic episodes were observed for any of the wild-type (5) or heterozygote (4) control mice by blinded observers. The experiment was designed by R.M. Chemelli and carried out by the author. Videos were scored with the assistance of R.M.Chemelli.

(B) Typical behavior during the first 4 h of the dark phase in knockout mouse. Duration of individual narcoleptic episodes and the time they occurred from dark-phase onset are plotted as vertical lines for mouse F. Periods designated as awake and sleeping were judged by gross behavioral observation and are indicated by the white and black bars above the timeline, respectively.

(C) Behavior of mice before and after each narcoleptic episodes. The predominant behavior observed for the 5 seconds preceding each episode was categorized as feeding, drinking, ambulating, grooming, burrowing, climbing or other (Before). Most behaviors classified as "other" were combinations of behavioral categories where a predominant behavior was not manifest. The predominant behavior for the 10 seconds after each episode was categorized similarly (After). Number of observations and percentage of total observations are reported for all knockouts.


A
Episode Count in First 4 h of Dark Phase
Mouse: M N O P Q
n.d.
Age (week)
Individual
Group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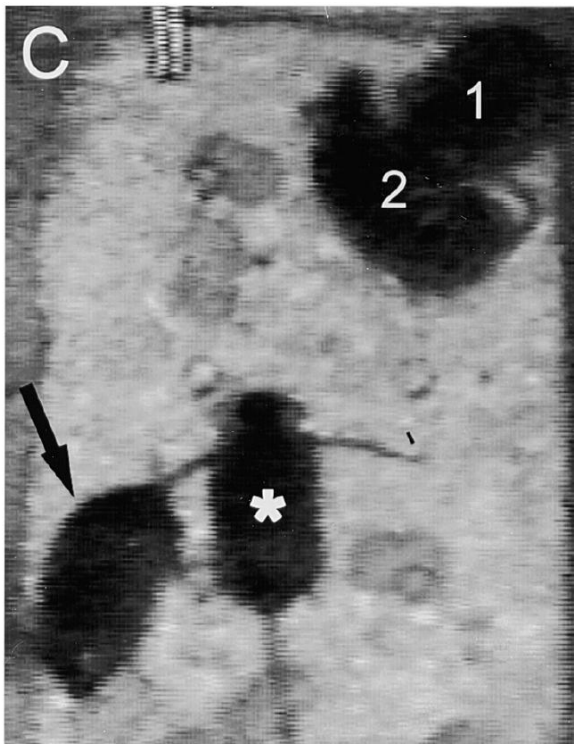
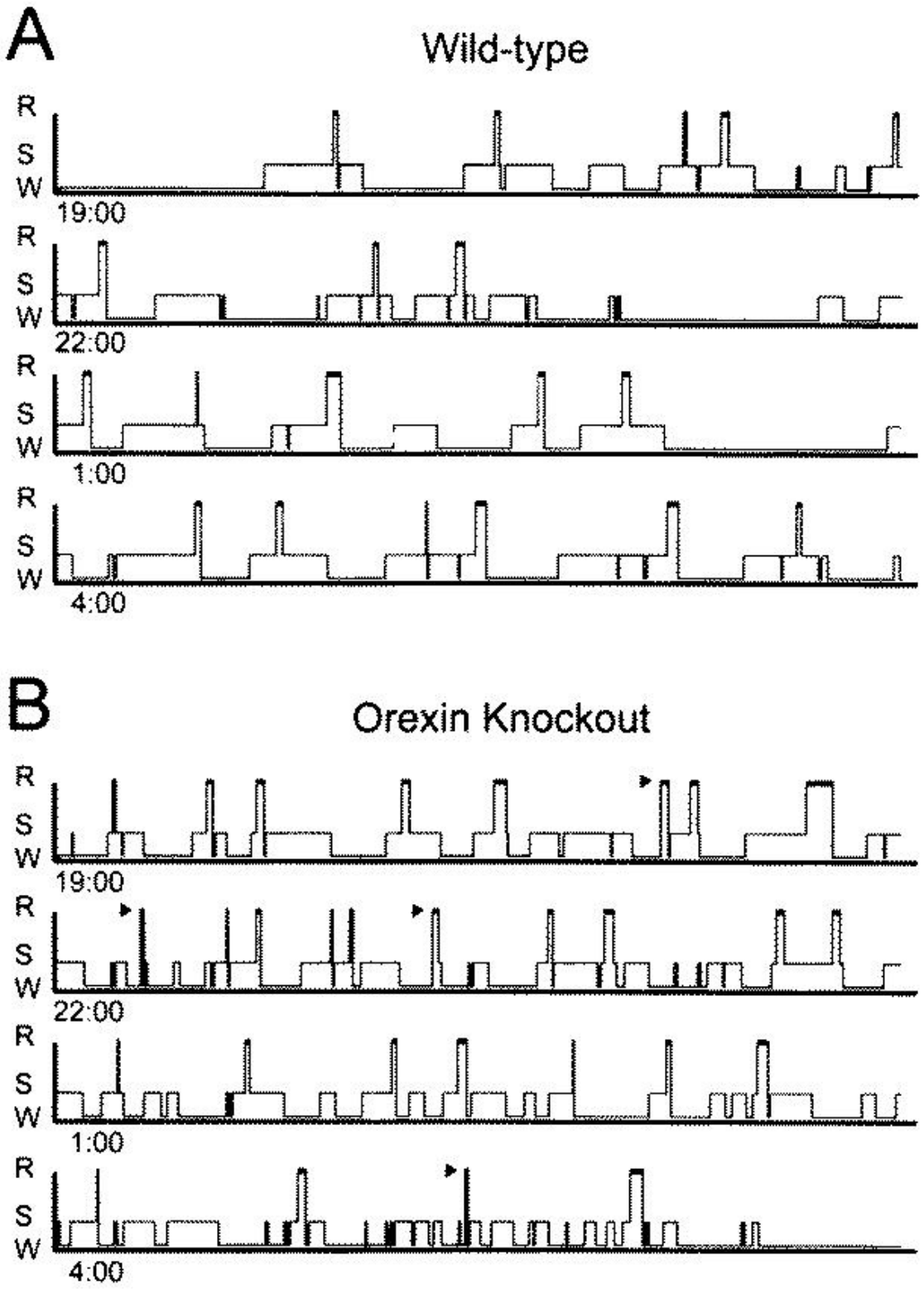

C
1
2
*

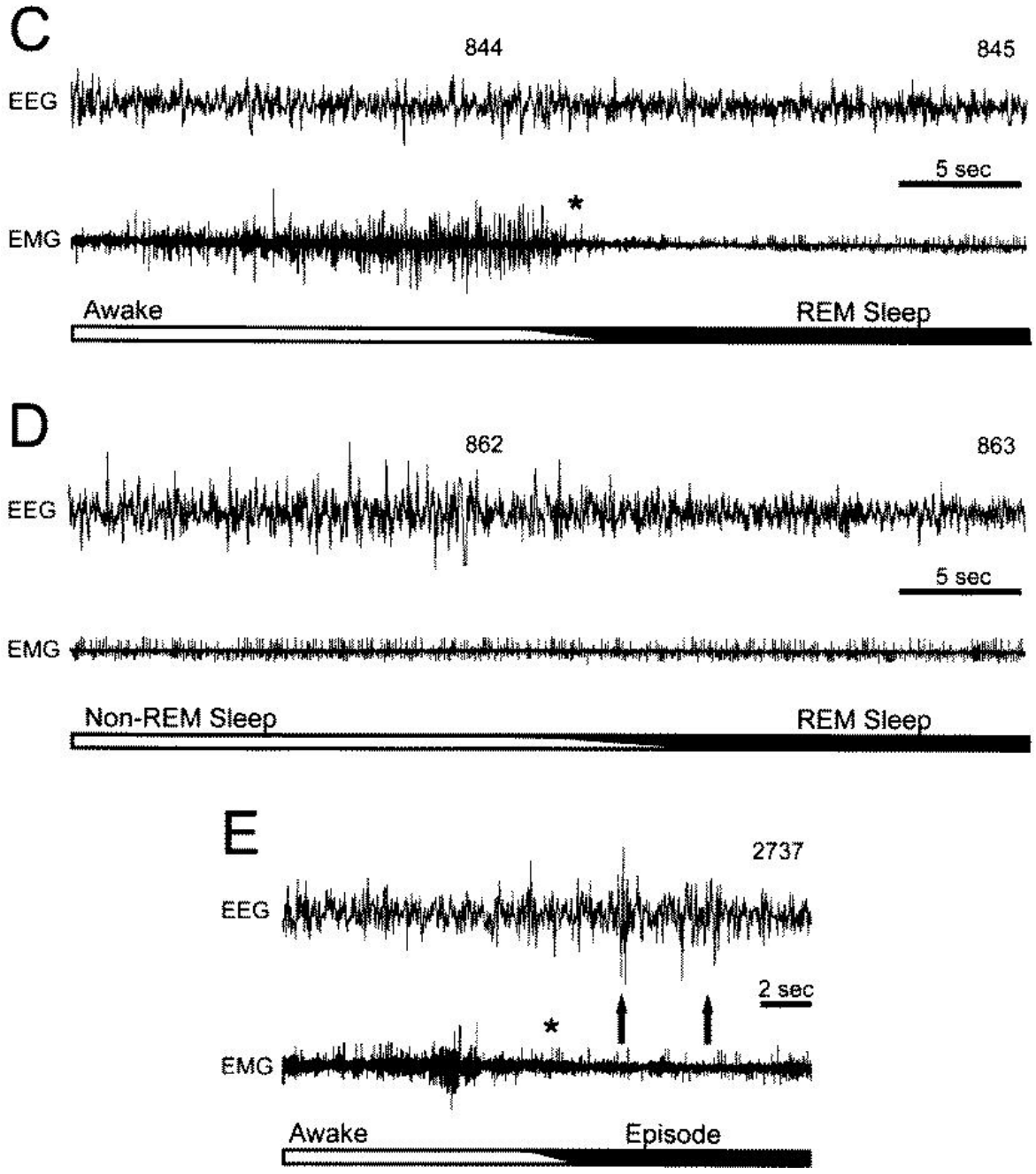
Appendix A Figure 2. Development of Narcoleptic Episodes.

(A) Episode count in first 4 h dark phase for individually-filmed and group filmed F3 homozygote littermates. First graph reports episode counts for individual orexin knockout mice (M to Q). Note that mouse N did not record an episode until 6 weeks of age, and mouse Q did not have one at week 5. n.d., none detected. The right panel contrasts total episodes of individually-filmed vs. group-filmed littermates at 4 and 6 weeks of age. The experiment was designed by R.M. Chemelli and carried out by the author.

(B) Digitally-captured infrared video image of group-filmed knockout mice at 4 weeks of age. Note that one mouse (arrow) has completely fallen onto his side. The film shows the fuzziness (motion artifact) associated with body movement in normally acting littermates designated 1 to 3. Dark phase onset at 17:30 (see video of complete episode at http://www.cell.com/cgi/content/full/98/4/437/DC1).

(C) Digitally-captured infrared video image of group filmed knockout mice at 6 weeks of age. Note that one mouse has fallen completely onto his side (arrow) while the another is collapsed onto his ventral surface (star). Littermates designated 1 and 2 are quietly sleeping in their usual corner of the cage. Dark phase onset at 17:30, episodes recorded at 20:26 (see video of complete episode at http://www.cell.com/cgi/content/full/98/4/437/DC1).


A
Wild-type
R
S
W
19:00
22:00
1:00
4:00
B
Orexin Knockout
19:00
22:00
1:00
4:00


C
844
845
EEG
5 sec
EMG
*
Awake
REM Sleep
D
862
863
EEG
5 sec
EMG
Non-REM Sleep
REM Sleep
E
2737
EEG
2 sec
*
EMG
Awake
Episode

Appendix A Figure 3. EEG/EMG Characterization of Orexin Knockout Mice

(C-D) Initiation of a sleep-onset REM episode (C) compared with a normal transition from non-REM to REM sleep (D) in an orexin knockout mouse. These examples are extracted from a continuous record, epochs 844/845 occurring at 22:08, and epochs 862/863 six min later at 22:14. Each epoch represents 20 sec of recording. Epoch 844 is characterized by rhythmic grooming activity with high amplitude EMG and low amplitude, mixed frequency EEG, typical of an awake period. But at the beginning of epoch 845 this is rapidly replaced, over a few seconds, by muscle atonia, leaving only the heart beat registering on the EMG leads combined with low amplitude EEG dominated by theta frequencies, characteristic of REM sleep in this species. The star marks the onset of the observable concurrent behavioral collapse. In contrast, epoch 862 is a typical non-REM sleep period with minimal EMG activity and high amplitude, low frequency EEG that is replaced during the first half of epoch 863 by low amplitude EEG dominated by theta, marking a normal transition to REM sleep. Wild-type mice demonstrated only normal non-REM sleep to REM sleep transitions, indistinguishable from tracing (D).

(E) EEG/EMG recording during a typical narcoleptic episode identified from concurrent infrared video photography as sudden immobility associated with muscular twitches immediately following a period of normal activity. The EEG shows that the start of this episode corresponds to two high amplitude spindling epochs, marked with arrows, associated with phasic EMG activity as muscle tone declines at the onset of attack. The star marks the onset of observable immobility. These spindles in the EEG are normally observed only

during the transition phase immediately prior to REM sleep. EEG traces were collected by C. M. Sinton.

APPENDIX B
Behavioral Paradigms that Elicit Behavioral Arrests in Narcoleptic Mice

INTRODUCTION

Pharmacological studies of narcoleptic dogs have been useful in understanding the neurochemical mechanisms of cataplexy, which in dogs is triggered by emotional stimuli such as presentation of appetitive food (food elicited cataplexy test) (Nishino and Mignot, 1997). To study the neurochemistry of severe narcolepsy in *orexin* knockout mice, I undertook the development of a behavioral bioassay for mouse narcoleptic attacks. Exposure of *orexin* knockout mice to novel environments elicits narcoleptic attacks, yet inconsistent episode frequency over several experimental sessions is observed. To achieve consistent performance, experimental paradigms based on the food elicited cataplexy test and restricted feeding schedules (food shift) were tested. Using the food shift paradigm, preliminary pharmacological data was obtained.

EXPERIMENTAL PROCEDURES

Scoring criteria of narcoleptic attacks in mice using nighttime infrared video photography is as described in chapter 2 (**Appendix B Figure 1**). Since no effects of age or sex on the frequency of narcoleptic attacks have been observed in adult *orexin* knockout mice

(unpublished observations), adult mice of varying ages (3-10 months) and both sexes were used. All mice in this study were homozygous for deletion of the *orexin* gene.

Behavioral responses of *orexin* knockout mice to a novel environment were recorded at the beginning of the dark cycle by removing four age-matched male, group-housed, handling-acclimated littermates from their wire-top-covered home cages and introducing them individually to novel, larger cages with clear acrylic tops. Mice were filmed in novel cages for the first 90 minutes of the dark phase on each of four consecutive nights.

In the food shift paradigm, five age-matched female, group-housed, handling-acclimated *orexin* knockout mice were weighed and introduced individually to plexiglas-covered cages stocked with normal chow for the first three hours of the dark phase every day for 22 consecutive days. After the first night, food was removed from home cages, and was thereafter available only when mice were placed in the plexiglas-covered cages for the first three hours of every dark phase. After the weights of all mice stabilized, they were filmed again on nights 14, 15, 17, 18 and 22.

For the food elicited cataplexy test, 3 age-matched female *orexin* knockout mice were maintained on the food shift paradigm. Over seven consecutive days, they were removed from their home cages and trained to dig up and consume appetitive food (pieces of sugary cereal buried in bedding material) for ten minutes before the dark cycle.

The effects on narcoleptic attack frequency of the muscarinic cholinergic antagonist atropine (0.5 mg/Kg, i.p.) and the a2 adrenergic antagonist yohimbine (0.5 mg/Kg, i.p.) were examined in four age-matched knockout littermates (3 females and 1 male) maintained on the

food shift. In this case, mice were also acclimated to nightly i.p. injections of vehicle (saline) for at least two weeks before drug administration.

RESULTS

Narcoleptic attack frequency of *orexin* knockout mice exposed to a novel environment at the beginning of the dark phase over consecutive nights displays significant habituation (**Appendix B Figure 2A**). Total attack time is more variable, but displays a trend toward habituation as well (**Appendix B Figure 2B**). The marked decrease in attack frequency is accompanied by an increase in normal resting behavior over consecutive nights (data not shown).

When *orexin* knockout mice are maintained on the food shift paradigm, attack frequency (**Appendix B Figure 3**) and total attack time (data not shown) are maintained at relatively stable levels. Further, normal rest appears to be limited during this period (data not shown). By contrast, knockout mice trained in the food elicited cataplexy test revealed no attacks over the entire training period despite improved performance at finding and consuming the cereal. By day 7, all three knockout mice consumed all available cereal within the allotted 10-minute periods, and no attacks were ever recorded during these training sessions (data not shown).

Atropine significantly reduced narcoleptic attack frequency compared to vehicle controls in *orexin* knockout mice using the food shift paradigm (**Appendix B Figure 4**).

Yohimbine, on the other hand, significantly increased attack frequency in knockout mice (**Appendix B Figure 5**).

CONCLUSIONS

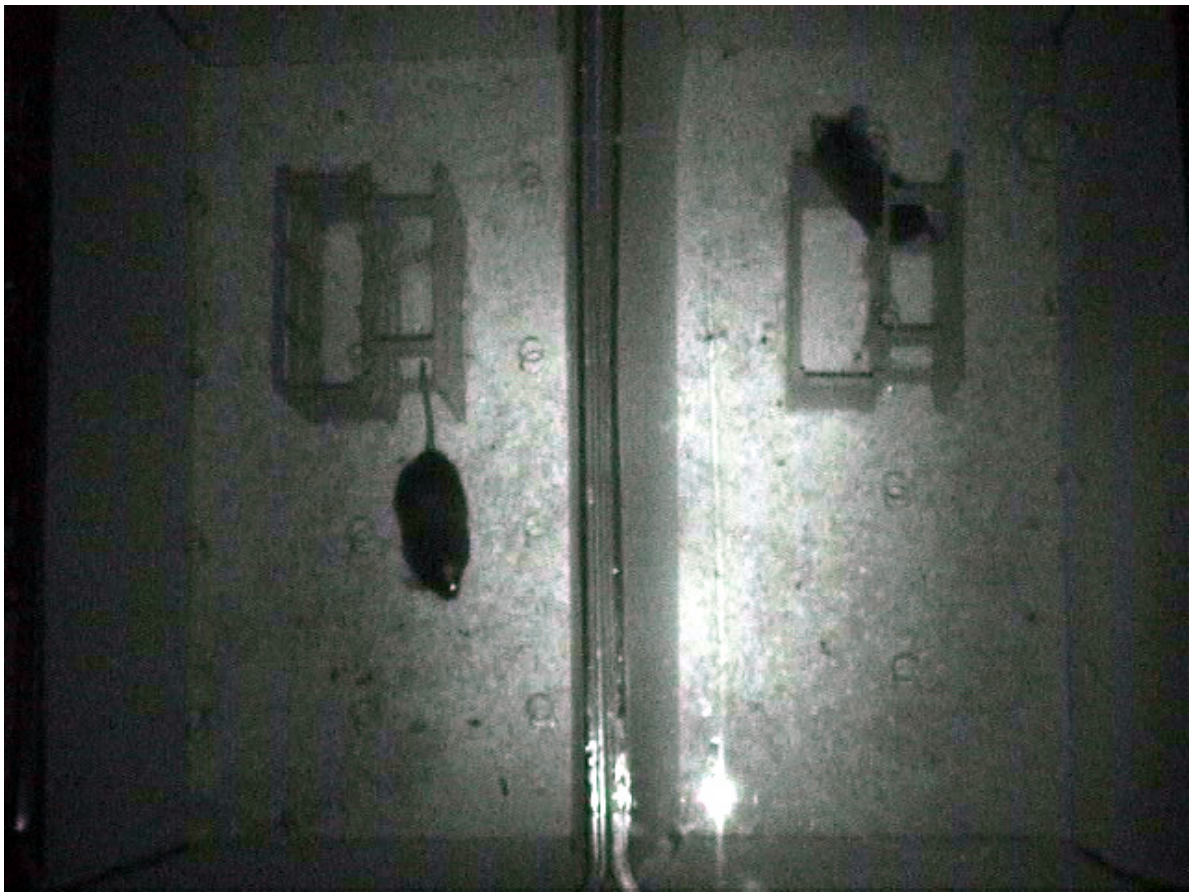
Behavioral studies of *orexin* knockout mice suggest that exposure to novelty elicits narcoleptic attacks, but attack frequency shows marked habituation with time. The instability of behavior elicited by novelty makes this paradigm inappropriate for use in pharmacological studies in which individual animals are used as their own controls. In contrast, the food shift paradigm, in which animals are maintained on a restricted feeding schedule that promotes extended periods of arousal, elicits narcoleptic attacks with stable frequency over consecutive experimental sessions, allowing pharmacological studies. A food elicited cataplexy test similar to that used in narcoleptic dogs, however, does not elicit attacks in mice.

The novelty and food shift paradigms promote accumulation of sleep debt in *orexin* knockout mice, which may be a cause of increased narcoleptic attacks. I can not rule out the possibility that emotional triggers play a role in eliciting attacks with these paradigms, but the failure of the food elicited cataplexy test to elicit attacks suggests that manipulating emotional triggers of cataplexy in mice may be more difficult than in dogs. As both cataplexy (interrupting an awake EEG) and sudden sleep-onset REM periods contribute to the narcoleptic attacks observed in *orexin* knockout mice (Chemelli et al., 1999), future EEG/EMG studies combined with the food shift will better differentiate these. The food shift

paradigm should allow greater understanding of triggers of narcoleptic attacks in mice and is a behavioral bioassay useful in pharmacological studies of *orexin* knockout mice.

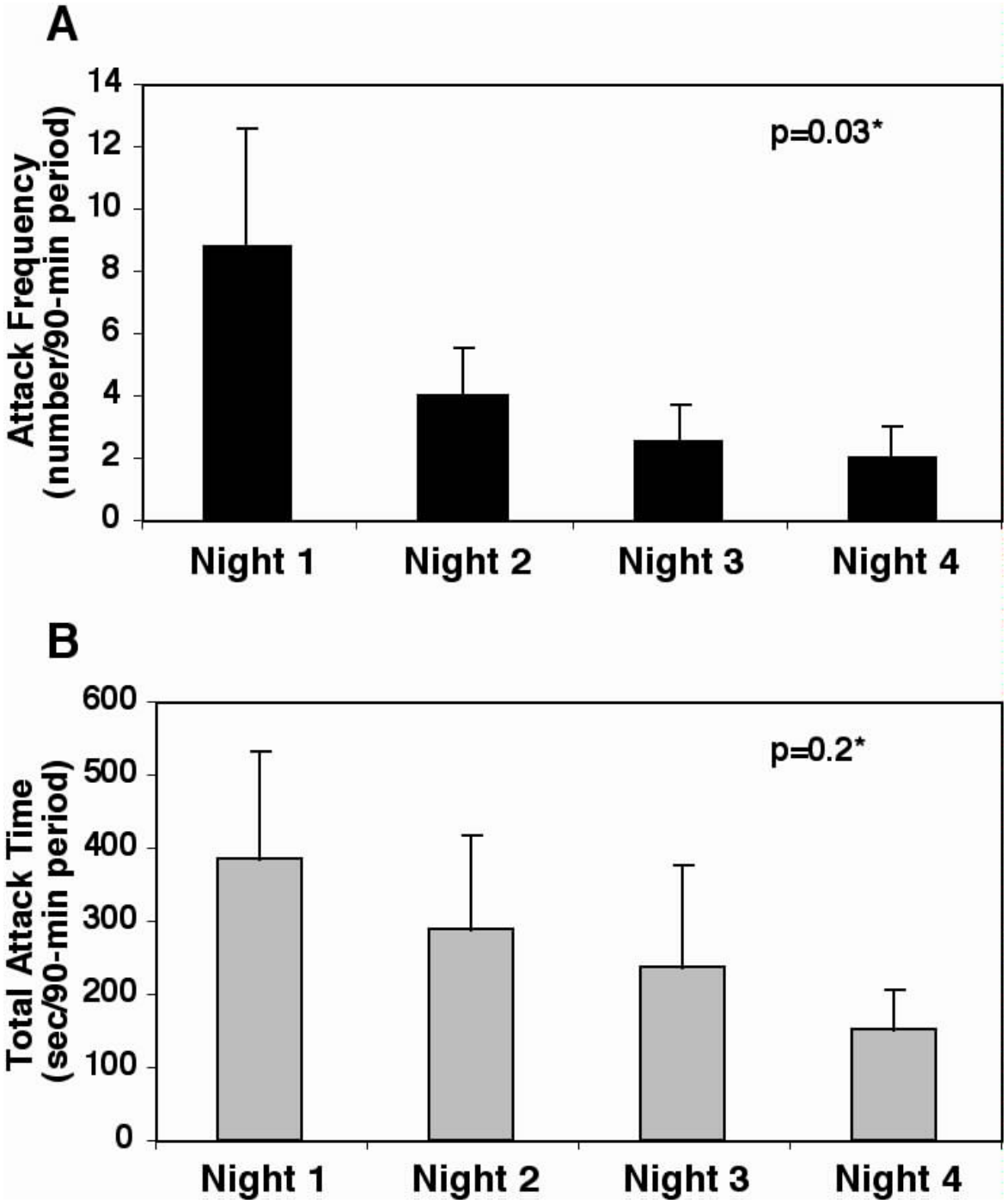
Our finding that the muscarinic cholinergic antagonist atropine significantly reduces narcoleptic attacks is consistent with atropine's effects in humans and dogs, and reflects the importance of increased cholinergic activity in the triggering of REM-related phenomena. Our finding of increased attacks with administration of the a2 adrenergic antagonist yohimbine contrasts with its reported reduction of cataplexy in dogs (Nishino and Mignot, 1997). Simple species differences are unlikely to explain this discrepancy since yohimbine is an effective stimulant in both dogs and rodents. While the different bioassays used may contribute to different results, it is notable that yohimbine is not effective in treating narcolepsy in humans (Nishino and Mignot, 1997). Our preliminary findings may reflect the importance of the *orexin* knockout mouse as an alternative animal model of human narcolepsy.

DISPLAY ITEMS

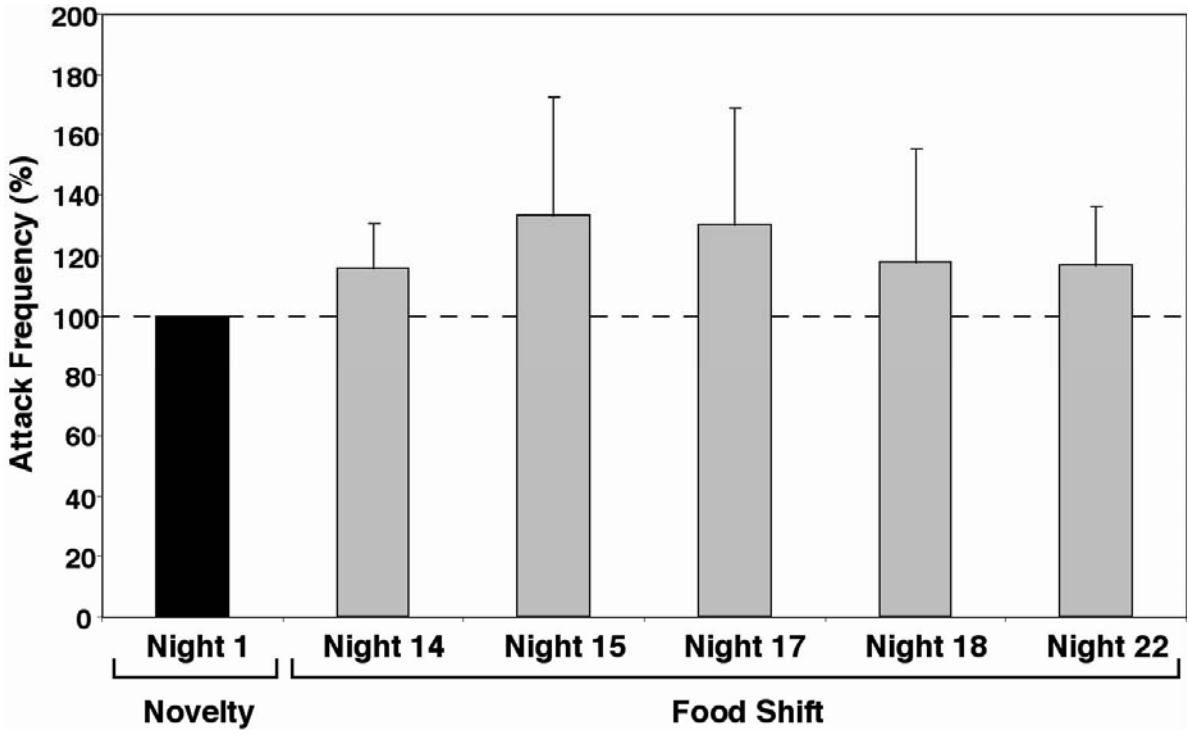


Appendix B Figure 1. Nighttime infrared video photography of orexin-/- mice in a novel environment.

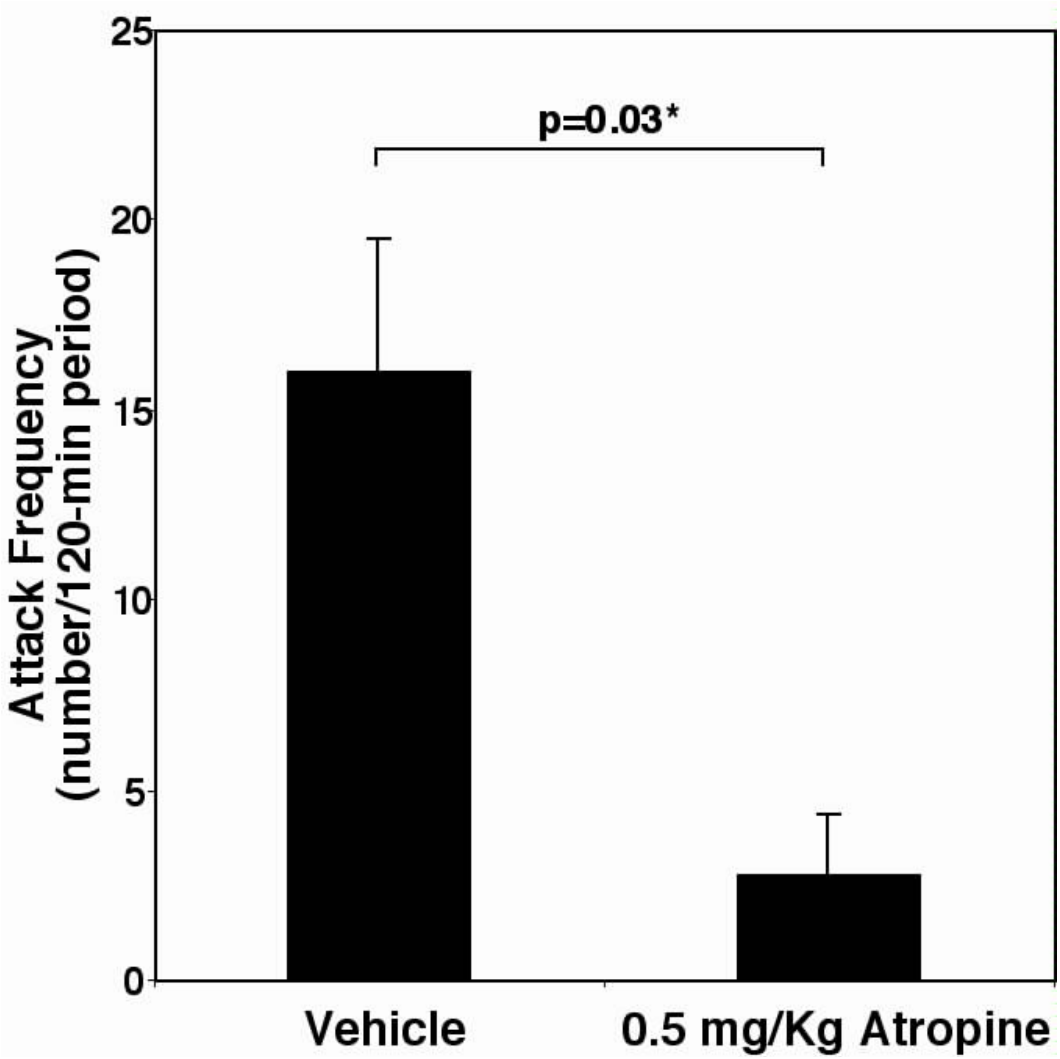
Mouse on right is exhibiting a cataplectic attack.



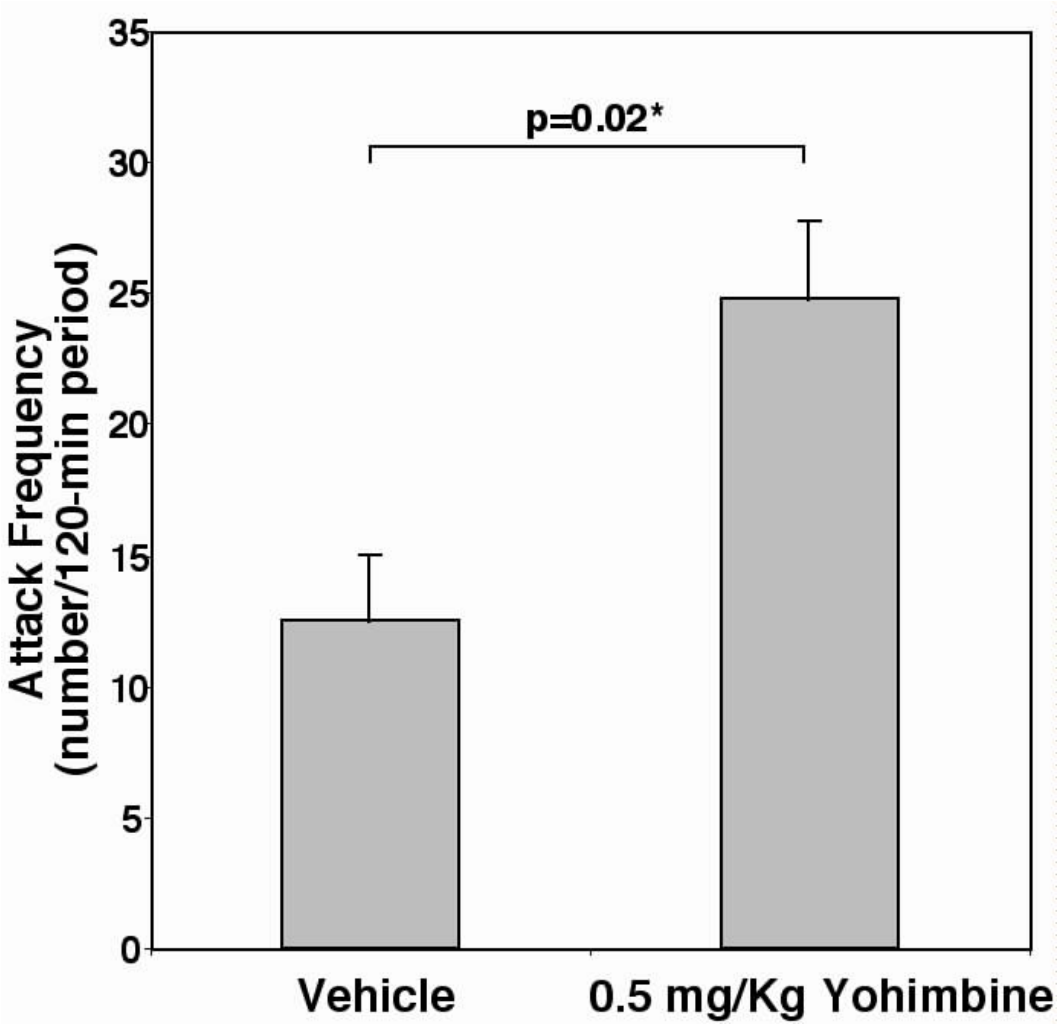
Appendix B Figure 2. Effect of Habituation on Cataplectic Attacks



Appendix B Figure 3. Food Shift Paradigm Stabilizes Attack Frequency



Appendix B Figure 4. Atropine Reduces Cataplectic Attacks



Appendix B Figure 5. Yohimbine Increases Cataplectic Attacks

APPENDIX C
Hypophagia in Orexin Knockout Mice

INTRODUCTION

To test the hypothesis that orexin plays a role in hypothalamic feeding mechanisms, food intake was recorded for male group-housed orexin heterozygous and homozygous knockout mice, as well as wild-type mice, that had been matched for weight and age.

RESULTS AND CONCLUSIONS

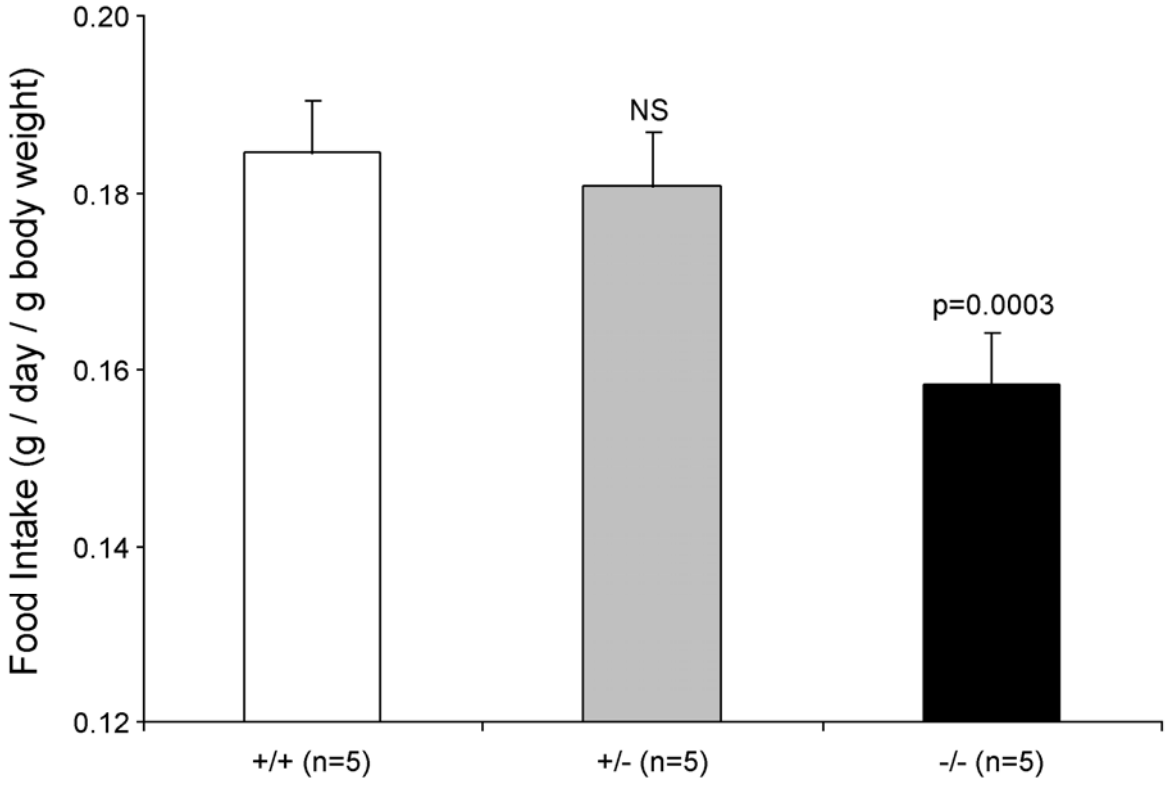
As expected, orexin knockout mice were significantly hypophagic relative to heterozygous mice and wild-type controls (**Appendix C Figure**). Notably, growth curves for orexin heterozygous and homozygous knockout mice of this mixed genetic background were relatively normal when mice were fed a lean chow (6% fat by weight), although some individual mice exhibited a tendency toward late-onset obesity (R.M. Chemelli, data not shown). On a highly palatable condensed milk (17% fat by weight) diet, orexin knockout mice exhibited a greater tendency toward diet-induced obesity than did normal controls (R.M. Chemelli, data not shown).

Thus, it was observed that mice that lack orexin neuropeptides are hypophagic despite maintainence of normal or increased weights. These findings are consistent with (i) the notion that orexin plays a role in the regulation of feeding and metabolic rate, and (ii) the observation that a greater susceptibility to obesity may be an intrinsic feature of narcolepsy in humans. The role(s) of orexin signaling in feeding, metabolism, and behavioral responses to fasting are further explored in **Chapters 3, 4, and 5**.

EXPERIMENTAL PROCEDURES

Food intake and body weights of 14 to 15 week old group-housed male F2 mice (5 per genotype) were collected daily for 7 days and averaged. Statistical comparison was performed by ANOVA.

DISPLAY ITEM



Appendix C Figure. Food Intake of Heterozygous and Homozygous Orexin Knockout Mice.

Orexin knockout mice are hypophagic relative to controls. Data represent 7-day mean food intake adjusted for body weight. Age- and weight-matched group-housed male C57Bl/6J-129/SvEv F2 mice were fed freely throughout the study. Despite hypophagia, these mice maintain normal growth curves, suggesting a reduced metabolic rate. The experiment was designed by R.M. Chemelli and carried out by the author.

APPENDIX D
Behavioral Vigilance State Analysis by 12 h Light and Dark Periods for Wild-Type and Orexin Null Mice Following Various Doses of Modafinil

INTRODUCTION

The following is an expansion of data analysis from chapter 6. It provides further corroboration of results from the above chapter.

RESULTS AND CONCLUSIONS

During the 12 hr dark period after vehicle administration, total REM sleep time was longer in orexin null mice (43 min versus 29 min in the *orexin*$^{-/-}$ and wild-type mice respectively; $F [1, 18] = 9.29$, $P < 0.01$), mean REM sleep episode duration was longer (83 sec versus 67 sec; $F [1, 18] = 5.12$, $P < 0.05$), vigilance states were more fragmented (159 sec mean non-REM sleep episode duration versus 234 sec; $F [1, 18] = 18.1$, $P < 0.001$), and the mean latency to REM sleep was shorter (3.8 min versus 7.0 min; $F[1, 18] = 41.5$, $P < 0.0001$) (Appendix, Table 2). Comparing the results obtained here and those recorded when the mice were left undisturbed (Chemelli et al., 1999; Willie et al., 2003), showed that the *orexin*$^{-/-}$ mice were awake longer during the 12 hr dark period here (423 min versus 350 min). Thus the previously reported increased non-REM sleep time during

the dark period in the *orexin*$^{-/-}$ mice was no longer apparent (F [1, 18] = 0.9, P = 0.3). In addition, the mean inter-REM sleep interval, a measure of sleep cyclicity in this species, was shorter in the wild-type mice (28.4 ±3.0 min) than that previously recorded under undisturbed conditions (42.1 ± 7.8 min). Consequently, the mean inter-REM sleep intervals of the wild-type and *orexin*$^{-/-}$ mice were not significantly different in the present study (F [1, 18] = 0.7, P = 0.4). During the 12 hr light period, as previously reported in undisturbed mice (Chemelli et al., 1999; Willie et al., 2003), the only difference between the strains was in the mean latency to REM sleep (6.6 min versus 8.5 min in the *orexin*$^{-/-}$ and wild-type mice, respectively; F [1, 18] = 7.80, P = 0.01).

From the overall analysis, modafinil increased wakefulness time during the 12 hr dark period (F [3, 54] = 57.1, P < 0.0001), but subsequently, during the 12 hr light period, no effect on wakefulness was noted (F [3, 54] = 2.6, P = 0.06). Correspondingly, non-REM sleep time was decreased after modafinil during the initial 12 hr dark period (F [3, 54] = 57.9, P < 0.0001), but recovery of non-REM sleep during the subsequent 12 hr light period was insignificant in both genotypes (P > 0.1). REM sleep time was also reduced by modafinil during the 12 hr dark period (F [3, 54] = 19.7, P < 0.0001); a subsequent tendency to recover REM sleep during the 12 hr light period was noted, but only in the *orexin*$^{-/-}$ mice after 100 mg/kg modafinil (F [3, 27] = 2.9, P = 0.05). During the 12 hr dark period after modafinil, both the mean interval between successive REM sleep episodes (F [3, 54] = 31.0, P < 0.0001), and the mean wakefulness episode duration (F [3, 54] = 18.6, P < 0.0001), were increased. Both parameters reflect a more consolidated vigilance state pattern.

One interaction between the effect of modafinil and the genotypes was observed. This interaction, in mean inter-REM sleep interval during the 12 hr dark period ($F [3, 54] = 3.8$, $P = 0.01$), resulted from a greater increase in inter-REM sleep interval in the wild-type mice in comparison with the *orexin*$^{-/-}$ mice. With this exception, the lack of significant interaction between modafinil and genotype in the overall analysis demonstrated that the compound had a very similar effect on vigilance state parameters in both genotypes.

EXPERIMENTAL PROCEDURES

Detailed in **Chapter 6.**

DISPLAY ITEM

Appendix D Table. Vigilance state parameters recorded from *orexin*$^{-/-}$ mice and wild-type mice after modafinil (0, 10, 30 and 100 mg/kg i.p.).

		+/+				-/-			
		Vehicle	**10 mg/kg**	**30mg/kg**	**100mg/kg**	**Vehicle**	**10 mg/kg**	**30 mg/kg**	**100 mg/kg**
24 h									
REM	Total time (min)	72.2 ± 5.4	73.9 ± 6.1	72.5 ± 4.5	64.1 ± 4.0	84.6 ± 5.1	86.9 ± 4.6	83.8 ± 3.8	76.6 ± 3.0
	Episode duration (sec)	64.6 ± 3.0	66.2 ± 3.1	67.5 ± 3.6	65.4 ± 2.7	74.8 ± 3.9	75.0 ± 3.3	78.2 ± 5.0	74.9 ± 3.9
	REM latency (min)	7.7 ± 0.2	8.2 ± 0.3	7.8 ± 0.4	7.9 ± 0.6	5.0 ± 0.3	5.2 ± 0.3	5.6 ± 0.4	5.4 ± 0.4
	Inter-REM interval (min)	21.2 ± 1.9	21.4 ± 1.3	21.7 ± 1.6	24.4 ± 1.9	20.7 ± 1.9	20.0 ± 1.4	21.3 ± 1.6	22.4 ± 1.5
NREM	Total time (min)	696.7 ± 19.5	677.8 ± 20.7	662.6 ± 21.7	607.6 ± 21.2	669.3 ± 35.4	657.5 ± 24.3	645.2 ± 24.1	562.5 ± 18.1
	Episode duration (sec)	259.1 ± 12.8	252.7 ± 10.7	258.3 ± 12.9	273.2 ± 13.3	218.8 ± 12.6	207.3 ± 12.1	220.4 ± 11.3	234.3 ± 15.3
Awake	Total time (min)	668.4 ± 22.1	685.7 ± 23.4	702.2 ± 24.1	765.7 ± 19.7	683.4 ± 38.8	693.0 ± 28.3	708.3 ± 26.4	798.2 ± 20.4
	Episode duration (sec)	325.2 ± 33.5	323.2 ± 27.4	345.1 ± 32.5	450.2 ± 46.4	262.4 ± 27.9	260.0 ± 30.7	280.5 ± 23.6	381.7 ± 33.5
Dark period									
REM	Total time (min)	**29.0 ± 3.1**	***27.6 ± 3.6***	***27.7 ± 2.3***	***16.5 ± 1.7***	**42.6 ± 4.2**	***41.1 ± 3.8***	***40.5 ± 3.0***	***28.2 ± 2.7***
	Episode duration (sec)	**66.8 ± 3.5**	68.4 ± 3.6	67.4 ± 4.6	65.5 ± 3.8	**82.8 ± 4.8**	81.4 ± 4.7	88.5 ± 6.9	79.5 ± 6.9
	REM latency (min)	**7.0 ± 0.3**	7.2 ± 0.5	6.9 ± 0.4	6.7 ± 0.5	**3.8 ± 0.2**	3.6 ± 0.2	4.0 ± 0.3	2.9 ± 0.3
	Inter-REM interval (min)	***28.4 ± 3.0***	***31.4 ± 3.7***	***30.0 ± 3.4***	***48.7 ± 4.4***	***24.2 ± 3.3***	***24.0 ± 2.4***	***25.8 ± 2.6***	***34.0 ± 4.1***

NREM	Total time (min)	***277.9 ± 16.5***	***255.1 ± 17.8***	***252.4 ± 14.6***	***169.9 ± 16.7***	***253.1 ± 25.8***	***247.1 ± 19.5***	***238.8 ± 19.3***	***137.3 ± 12.2***
	Episode duration (sec)	**233.7 ± 13.6**	222.8 ± 8.2	224.3 ± 12.3	239.2 ± 16.5	**158.5 ± 12.3**	148.4 ± 10.4	161.7 ± 12.2	150.5 ± 12.7
Awake	Total time (min)	***411.7 ± 18.9***	***435.9 ± 20.3***	***438.6 ± 15.8***	***532.3 ± 17.8***	***423.0 ± 28.7***	***430.4 ± 23.1***	***439.4 ± 21.2***	***553.2 ± 14.4***
	Episode duration (sec)	***449.9 ± 68.2***	***483.2 ± 73.1***	***478.1 ± 55.7***	***991.5 ± 207.3***	***313.4 ± 45.6***	***303.7 ± 47.6***	***335.7 ± 44.4***	***678.8 ± 103.5***
Light period									
REM	Total time (min)	43.2 ± 3.1	46.2 ± 2.6	44.8 ± 3.2	47.6 ± 4.0	***42.0 ± 1.5***	***45.8 ± 1.7***	***43.3 ± 2.0***	***48.4 ± 1.5***
	Episode duration (sec)	63.8 ± 3.5	65.9 ± 4.0	68.0 ± 4.0	65.3 ± 3.5	68.3 ± 3.5	70.2 ± 2.9	70.4 ± 4.0	72.9 ± 2.7
	REM latency (min)	**8.5 ± 0.3**	8.9 ± 0.4	8.6 ± 0.5	8.5 ± 0.7	**6.6 ± 0.4**	6.8 ± 0.4	7.3 ± 0.5	7.1 ± 0.5
	Inter-REM interval (min)	17.5 ± 1.9	16.5 ± 1.1	17.2 ± 1.4	16.4 ± 1.5	18.5 ± 1.3	17.4 ± 1.1	18.5 ± 1.4	16.9 ± 1.9
NREM	Total time (min)	418.8 ± 7.3	422.7 ± 8.8	410.2 ± 12.5	437.7 ± 7.5	416.2 ± 11.4	410.3 ± 9.2	406.4 ± 11.1	425.3 ± 8.4
	Episode duration (sec)	283.2 ± 15.4	276.9 ± 14.9	287.4 ± 17.2	294.1 ± 14.7	282.9 ± 17.0	269.8 ± 15.6	280.8 ± 11.0	288.5 ± 19.7
Awake	Total time (min)	***256.7 ± 8.1***	***249.8 ± 7.9***	***263.6 ± 14.5***	***233.4 ± 7.2***	***260.4 ± 11.7***	***262.5 ± 9.0***	***268.9 ± 12.2***	***245.0 ± 8.8***
	Episode duration (sec)	232.7 ± 23.3	212.4 ± 12.9	241.9 ± 26.6	205.3 ± 14.5	217.8 ± 20.4	214.8 ± 17.8	230.2 ± 13.2	198.7 ± 17.9

Total time spent in each state (min, mean ± SEM), episode duration (sec, mean ± SEM), mean latency to REM sleep and mean interval between successive REM sleep episodes (min, mean ± SEM) are expressed for the 12 hr light and dark periods and the total 24 hr recording period. Bold data entries correspond to significant differences between wild-type and orexin-/- mice after vehicle, and bold italic data entries correspond to significant effects of modafinil.

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VITAE

Jon Timothy Willie was born in Minneapolis, Minnesota, on April 3, 1973, the son of Glen Robert Willie, M.D. and Patricia Sue Willie, R.N. After completing his work at Shawnee Mission Northwest High School, Shawnee, Kansas in 1991, he entered The University of Texas at Austin. During the summer of 1993 he attended the Georgetown University in Georgetown, Virginia. He received the degree of Bachelor of Arts in biochemistry and graduated with honors from The University of Texas at Austin in December, 1995. He carried out research first as a student and then later as a research technician in the laboratory of Thomas Kodadek, Ph.D. at The University of Texas at Austin between 1994 and 1996. In the summer of 1996, Jon married Abigail Marie Bright of Pittsburgh, Pennsylvania and entered the Medical Scientist Training Program of The University of Texas Southwestern Medical Center at Dallas. He has performed his research studies to fulfill the requirements for a doctorate in philosophy under the mentorship of Masashi Yanagisawa, M.D., Ph.D. His first child, Samuel Barrett Willie, was born in July 2004. He will graduate having earned his doctorate of medicine and doctorate of philosophy in June, 2005. Beginning in July, 2005, he will enter residency training in neurological surgery at Washington University Barnes-Jewish Hospital in St. Louis, MO.

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