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Hypothalamic Orexin Neurons Regulate Arousal According to Energy Balance in Mice

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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. We demonstrate that hypothalamic orexin neurons monitor indicators of energy balance and mediate adaptive augmentation of arousal in response to fasting. Activity of isolated orexin neurons is inhibited by glucose and leptin and stimulated by ghrelin. Orexin expression of normal and *ob/ob* mice correlates negatively with changes in blood glucose, leptin, and food intake. 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.

Introduction

Motivated behaviors such as food seeking are critically dependent upon arousal pathways. Periods of foraging and rest are both essential, but these mutually exclusive behaviors 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 with phases of increased wakefulness and locomotor activity that support food seeking (Borbely, 1977; Danguir and Nicolaidis, 1979; Dewasmes et al., 1989; Itoh et al., 1990; Challet et al., 1997; Williams et al., 2002). The molecular and physiological basis of this evolutionarily conserved phenomenon remains poorly understood.

Orexins, also called hypocretins, are a pair of neuropeptides 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 (de Lecea et al., 1998; Sakurai et al., 1998; Chemelli et al., 1999; Willie et al., 2001). Orexin-A (hypocretin-1) and orexin-B (hypocretin-2) are derived from a common precursor peptide, the product of the *prepro-orexin* gene (Sakurai et al., 1998, 1999). The actions of orexins are mediated by two G protein-coupled receptors termed orexin receptor type 1 and orexin receptor type 2 (Sakurai et al., 1998). 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 (Peyron et al., 1998; Date et al., 1999; Nambu et al., 1999). 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 and Stricker-Krongrad, 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., 1989). 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 (Smart et al., 2002). These observations are all consistent with the hypothesis that orexin neurons provide a link between metabolic status and motivated behavior.

The importance of orexin in promoting arousal is highlighted by the discovery that mice lacking either the *orexin* gene (*prepro-orexin* knockout mice) or orexin neurons (*orexin/ataxin-3* transgenic mice), as well as mice and dogs with null mutations in the orexin receptor type 2 gene, all have phenotypes remarkably similar to the human sleep disorder narcolepsy (Lin et al., 1999; Chemelli et al., 1999; Hara et al., 2001; Willie et al., 2003 [this issue of *Neuron*]). Narcolepsy is a neurological disease characterized by excessive sleepiness and pathologic intrusions of rapid eye movement (REM) sleep-related phenomena into wakefulness. Consistent with these findings, recent reports suggest that human narcolepsy is accompanied by a specific destruction of orexin neurons in the hypothalamus (Peyron et al., 2000; Thannickal et al., 2000). Human narcolepsy also is associated with metabolic abnormalities, including increased

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frequency of non-insulin-dependent diabetes mellitus and increased body mass index (Honda et al., 1986; Schuld et al., 2000; Nishino et al., 2001). 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).

In order to identify orexin-containing neurons for direct electrophysiological recording, we generated transgenic mice expressing GFP exclusively in these neurons. We demonstrate that isolated orexin neurons are able to monitor humoral and neural indicators of energy balance in mice. To probe the physiological relevance of this interaction, we show that the presence of orexin neurons is required for mice to respond appropriately to fasting by increasing wakefulness and locomotor activity.

Results

Specific Expression of a GFP Transgene in Orexin-Containing Neurons

Because orexin neurons of the LHA are diffusely distributed and lack distinct morphological features, it is challenging to directly examine their electrophysiological activity and regulation. To facilitate identification of orexin neurons, we generated transgenic mouse lines in which the enhanced green fluorescence protein (EGFP) is expressed under control of the human *prepro-orexin* promoter (*orexin/EGFP* transgenic mice) (Figure 1A). Under fluorescence microscopy, we observed green fluorescent neurons in LHA regions of all *orexin/EGFP* transgenic lines examined (Figures 1B and 1C) but not in the brains of wild-type littermates (data not shown). Immunostaining of brains from *orexin/EGFP* mice with anti-orexin antibody revealed that fluorescence was exclusively observed in orexin neurons: no ectopic expression of EGFP was observed throughout brains in any of the transgenic lines. Furthermore, cytoplasmic samples from eight randomly selected EGFP-positive cells were collected and subjected to single-cell RT-PCR in order to confirm the expression of *orexin* mRNA. No *orexin* mRNA was detected in non-EGFP-expressing neurons examined (Figure 1D). Up to 80% of orexin-containing neurons from two transgenic mouse lines robustly fluoresced. Fluorescence in remaining orexin neurons probably fell below the detection limit since staining with anti-EGFP antibody revealed EGFP-like immunoreactivity in >95% of orexin neurons from transgenic brains (data not shown).

Whole-Cell Patch-Clamp Recordings from Orexin Neurons

We examined the electrophysiological characteristics of orexin neurons by two methods. Primarily, we isolated orexin neurons from *orexin/EGFP* transgenic mice by dissection of LHA regions followed by enzymatic dispersal of tissues. EGFP-expressing orexin neurons were identified by fluorescence microscopy and immediately subjected to whole-cell patch-clamp recordings in physiological extracellular solution. Additionally, we performed whole-cell patch-clamp studies with hypothalamic slice preparations from *orexin/EGFP* transgenic mice.

In current-clamp mode, dispersed orexin neurons showed a resting membrane potential of -47.4 ± 0.8 mV and spontaneous firing at 15.0 ± 2.6 Hz ($n = 20$). Membrane capacitance was 6.3 ± 0.5 pF. Isolated orexin neurons exhibited shallower resting potentials and higher spontaneous firing rates as compared with those observed using hypothalamic slice preparations from *orexin/EGFP* mice (-61.4 ± 4.9 mV and 5.5 ± 3.9 Hz, $n = 24$). This may suggest that orexin neurons in slice preparations have robust innervation from inhibitory neurons within the slice. In the present study, we used only isolated neurons showing resting potentials below -45 mV for further analysis. Typical action potentials of isolated neurons (Figure 2A) were followed by a narrow shoulder and deep, brief after-hyperpolarizations in all orexin neurons examined, regardless of donor age, gender, or transgenic line (data not shown).

Extracellular Factors that Influence the Activity of Orexin Neurons

To characterize which extracellular factors directly modulate the activity of orexin neurons, we first collected data from dissociated single neurons in order to exclude confounding synaptic effects. We applied various neurotransmitters and neuromodulators in superfused solution during continuous recording (Table 1). Like most CNS neurons, all orexin neurons examined (10/10) were robustly depolarized (with associated increase in action potential frequency) by a relatively low dose of glutamate (10 μM) and hyperpolarized (with associated inhibition of action potentials) by a relatively low dose of GABA (10 μM) (Table 1). This suggests that these neurons may be regulated indirectly via excitatory and inhibitory synaptic contacts from within the LHA and/or other brain regions. In addition to these amino acid neurotransmitters, isolated orexin neurons responded to three other factors tested under our experimental conditions: glucose, leptin, and the recently identified neuropeptide ghrelin (Kojima et al., 1999).

Physiological extracellular solution utilized in this study contained 10 mM glucose. We found that changes in extracellular glucose concentration produced electrophysiological changes in 12/14 cells. Increasing glucose from 10 to 30 mM induced a striking hyperpolarization (from 45.4 ± 1.8 mV to 62.1 ± 1.1 mV, $n = 10$, $p < 0.0001$), and cessation of action potentials in 8/10 isolated orexin neurons (Table 1, Figure 2B). Conversely, decreasing glucose concentration to 0 mM induced depolarization and increased the frequency of action potentials ($174.4\% \pm 15.8\%$ compared to 10 mM glucose, $p = 0.02$) in these same neurons. Responses to smaller shifts of glucose concentrations were also observed in orexin neurons; decreasing glucose concentration from 10 to 5 mM induced a depolarization of resting potential to 41.4 ± 2.9 mV ($p = 0.01$), while increasing glucose concentration from 10 to 15 mM induced a hyperpolarization to 61.2 ± 3.9 mV in 4/4 neurons tested ($p < 0.0001$).

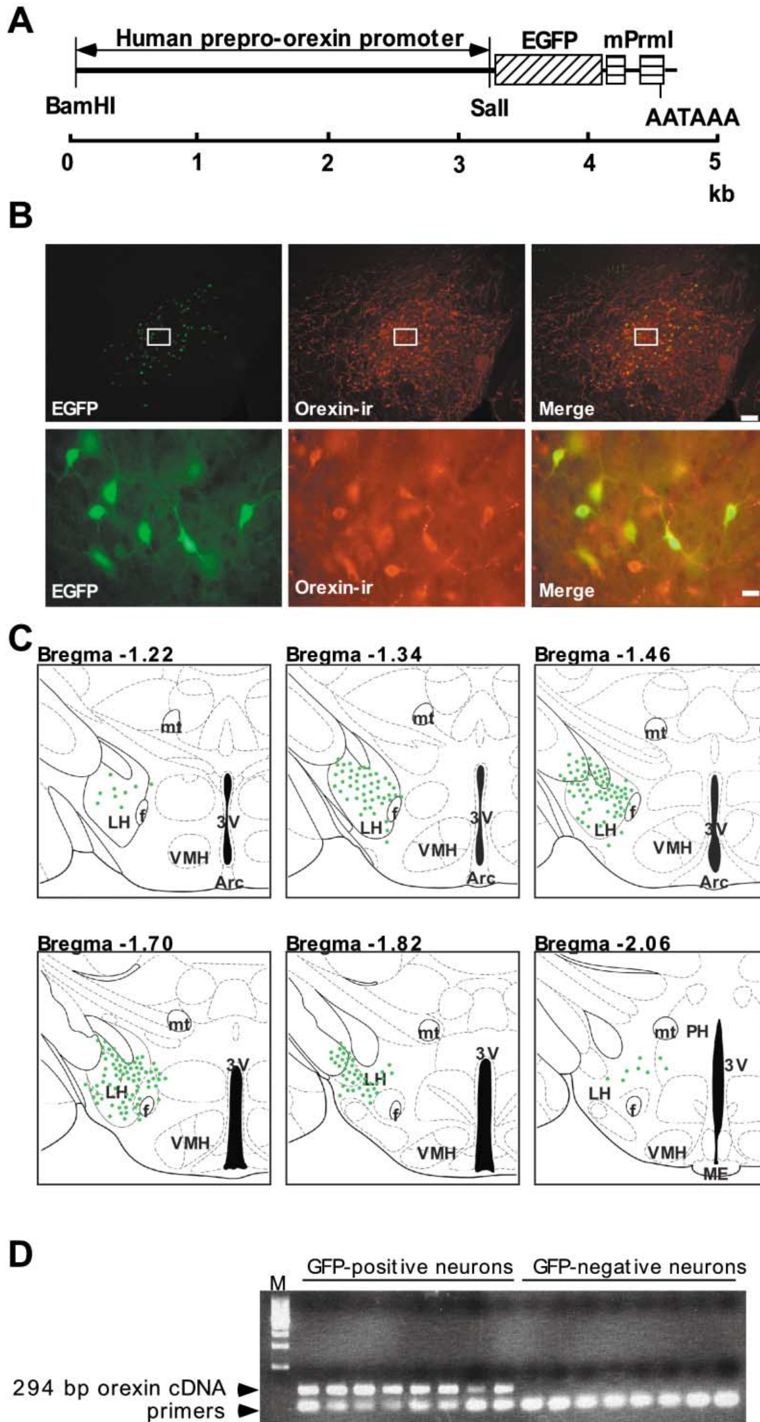

Figure 1. Transgenic Mice Expressing EGFP in Orexin Neurons

(A) Structure of the *orexin/EGFP* transgene.

(B) Specific expression of EGFP by orexin-containing neurons in the lateral hypothalamic area (LHA) of *orexin/EGFP* transgenic mice. Left, EGFP (green); middle, orexin-like immunoreactivity (red); right, merged image (scale bar equals 100 μm). Lower panels are high-power views of boxed regions from corresponding upper panels. (scale bar equals 10 μm).

(C) Schematic drawings showing distribution of EGFP-positive neurons in coronal sections through LHA.

(D) Verification of exclusive *prepro-orexin* mRNA expression in EGFP-fluorescence-positive neurons, but not fluorescence-negative neurons by RT-PCR of cytoplasmic samples from cells of the LHA. M, marker (1 kb ladder).

We also found that neuroendocrine factors influenced orexin neuron activity. The orexigenic peptide ghrelin (10 nM) activated 6/9 orexin neurons when applied in superfused solution (Table 1, Figure 2C) with depolarization and increases in action potential frequency (184.4% $\pm$ 15.5%, n $=$ 5, p $=$ 0.02). In contrast, bath application of leptin was found to robustly inhibit 7/9 orexin neurons examined, causing hyperpolarization and a decrease in firing rate (Table 1, Figure 2D). The mean resting potential was 47.6 $\pm$ 2.7 mV before and reached 62.1 $\pm$ 1.4 mV 5 min after administration of 10 nM leptin ($p < 0.0001$). Higher (30 nM) and lower (3 nM) doses of leptin also inhibited orexin neurons in a dose-dependent manner (data not shown). These doses of leptin are similar to those generally used in electrophysiological experiments (Spanswick et al., 1997). Notably, insulin exerted no direct effect on electrophysiological characteristics of these cells (n $=$ 5, data not shown).

We further examined dose-response effects of these peptidic factors to the resting membrane potentials of orexin neurons, using hypothalamic slice preparations under the presence of tetrodotoxin, which was found to

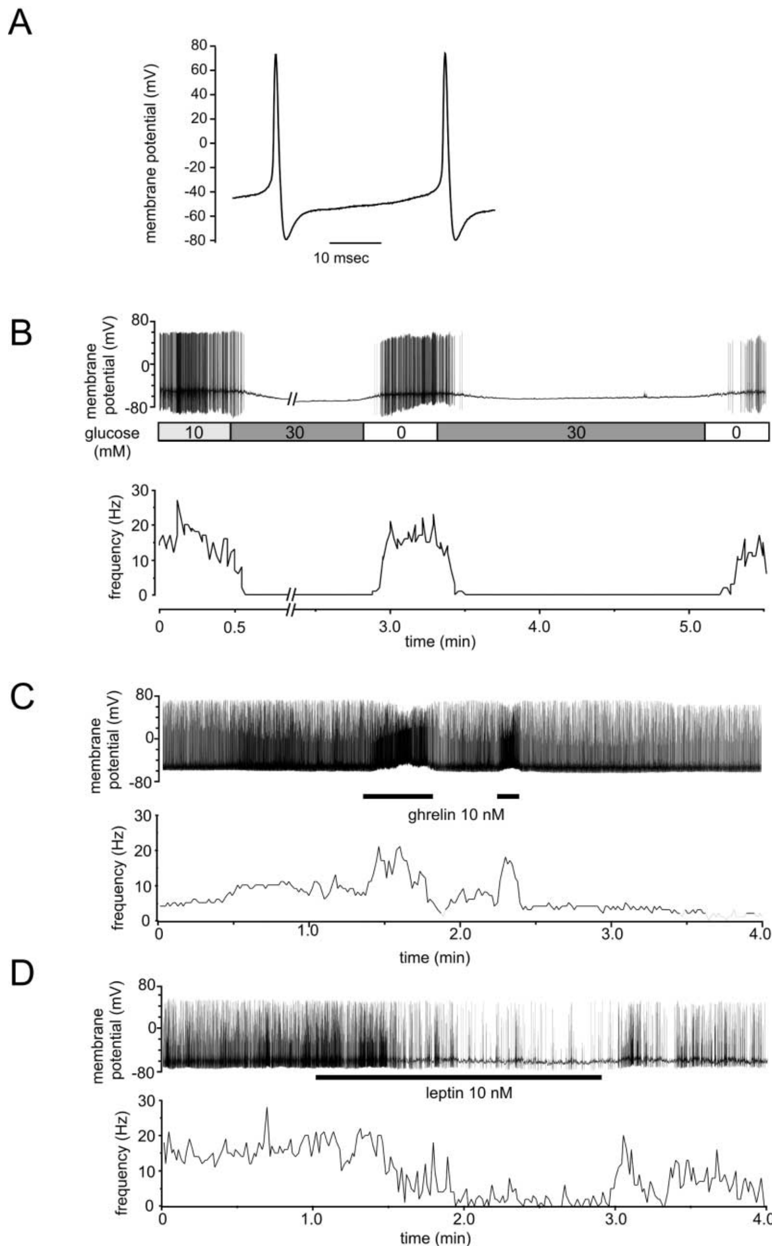

Figure 2. Modulation of Electrophysiological Activity of Isolated Orexin Neurons by Extracellular Factors

(A) A representative trace of an action potential obtained from an EGFP-expressing orexin neuron.

(B) Current-clamp recording demonstrating the effects of extracellular glucose concentration on activity of orexin neurons. Increasing glucose concentration from 10 to 30 mM induced hyperpolarization and a cessation of firing. Decreasing glucose concentration to 0 mM induced depolarization and an increase in firing rate. Upper panel, membrane potential; lower panel, action potential frequency.

(C) Current-clamp recording illustrating depolarization and increased firing rate in orexin neurons exposed to ghrelin (10 nM). Upper panel, membrane potential; lower panel, action potential frequency.

(D) Current-clamp recording illustrating hyperpolarization and reduced firing rate in an orexin neuron exposed to 10 nM leptin. Upper panel, membrane potential; lower panel, action potential frequency.

render the neurons more electrophysiologically stable in the slice. Under these conditions, ghrelin depolarized orexin neurons in a dose-dependent manner, while leptin induced dose-dependent hyperpolarization (Figure 3).

Changes in Blood Glucose and Leptin Levels Alter Orexin Expression in Mice

In apparent contrast to our present findings that orexin neurons are inhibited by leptin, hypothalamic *orexin* mRNA expression is reportedly suppressed in genetically obese *ob/ob* or *db/db* mice in which leptin signaling is absent (Yamamoto et al., 1999). However, these mice have complex metabolic abnormalities including hyperglycemia, which, as we have shown, may directly suppress orexin neurons. To clarify this issue at the whole-animal level, we treated wild-type and *ob/ob* mice with leptin. Two weeks of continuous central administration of leptin (100 ng/hr) to freely behaving, ad lib fed wild-type mice resulted in a significant reduction of hypothalamic *orexin* mRNA expression compared to vehicle-treated controls (Figure 4). Exogenous leptin treatment did not alter the normoglycemia of wild-type mice. In contrast, ad lib fed *ob/ob* mice treated with leptin, but not vehicle, demonstrated a normalization of blood glucose levels and a significant increase in *orexin* mRNA expression to levels comparable to leptin-treated wild-type mice ($p = 0.01$). These results are consistent with activation and inhibition of orexin neurons under hypoglycemic and hyperglycemic conditions, respectively. Furthermore, pair-feeding of *ob/ob* mice by matching the food intake to the lower levels of the wild-type group

Table 1. Factors Found to Affect the Electrophysiological Characteristics of Dispersed Orexin Neurons

Factors	Dose (μM)	Number of Cells Responding	Responses
Ghrelin	0.01 μM	6/9	Depolarization, ↑ firing frequency
Glutamate	10 μM	10/10	Depolarization, ↑ firing frequency
GABA	10 μM	10/10	Hyperpolarization, ↓ firing frequency
Leptin	0.01 μM	7/9	Hyperpolarization, ↓ firing frequency
Glucose	10→5 mM	4/4	Depolarization, ↑ firing frequency
Glucose	10→0 mM	8/10	Depolarization, ↑ firing frequency
Glucose	10→15 mM	4/4	Hyperpolarization, ↓ firing frequency
Glucose	10→30 mM	8/10	Hyperpolarization, ↓ firing frequency

(from 3.5 to 3.0 g/day) normalized blood glucose but increased *orexin* mRNA expression to levels even higher than that in wild-type mice ($p = 0.01$). These findings are compatible with a model in which orexin neurons integrate complex and even contradictory metabolic signals (i.e., hyperglycemia with hypoleptinemia) as previously suggested (Willie et al., 2001). Furthermore, the marked elevation of *orexin* mRNA expression in obese mice exposed to food restriction is consistent with the idea that the orexin system responds to negative energy balance.

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, we 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 wild-type 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 hr.

Hourly analysis of sleep/wake states revealed significant increases in wakefulness of food-deprived wild-type mice compared to all other experimental groups: fed wild-type mice, fed *orexin/ataxin-3* transgenic mice, and fasted *orexin/ataxin-3* mice (Figure 5). Within hours of food removal, wild-type 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, wild-type 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 following the onset of sleep (Dewasmes et al., 1989). In contrast to the fasting-induced changes in wakefulness and non-REM sleep that were observed exclusively in wild-type mice, REM sleep was significantly suppressed in both wild-type 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

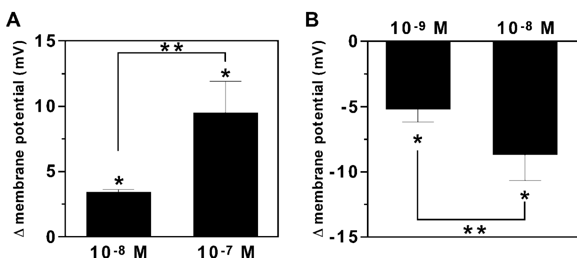

Figure 3. Effects of Ghrelin and Leptin on Membrane Potential of Orexin Neurons in Hypothalamic Slice Preparations under the Presence of Tetrodotoxin

Ghrelin (A) dose dependently depolarizes orexin neurons, while leptin (B) dose dependently hyperpolarizes them. Asterisk, significant differences ($p < 0.05$) between vehicle and agonists. Double asterisk, significant differences ($p < 0.05$) between doses.

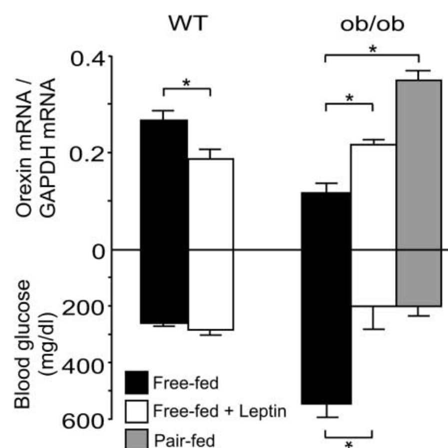

Figure 4. Effects of Central Leptin Administration or Food Restriction on Blood Glucose and Hypothalamic Expression of *prepro-orexin* mRNA in Wild-Type and *ob/ob* Mice

Leptin was continuously administrated i.c.v. at 100 ng/hr. Food restriction was done by pair feeding the mice at 3.0 g/day. Asterisk, significant difference ($p < 0.05$) by ANOVA.

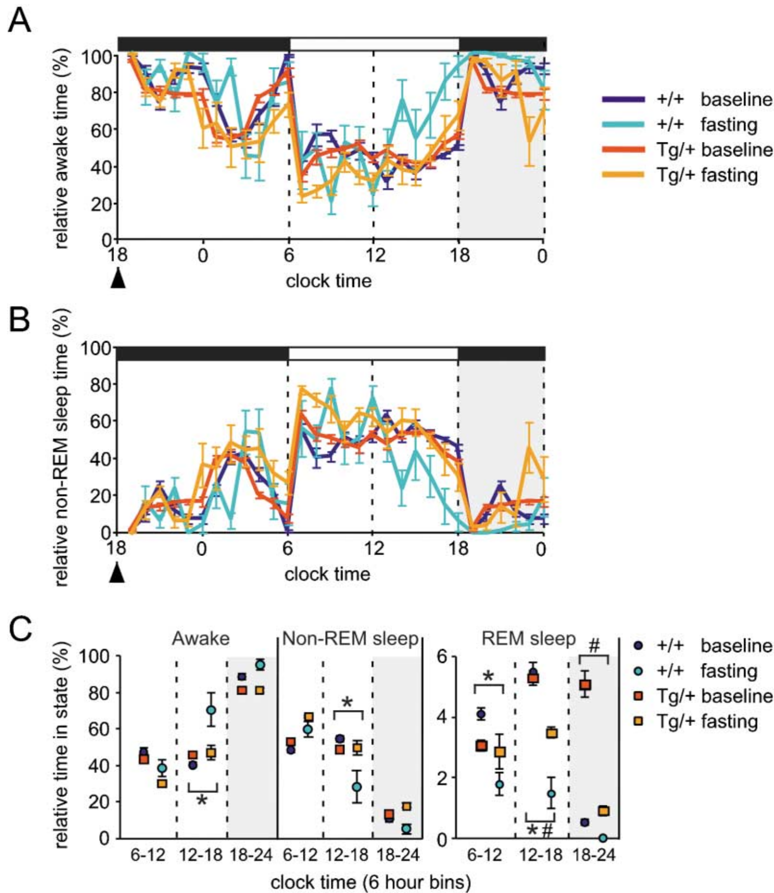

Figure 5. Impaired Fasting-Induced Arousal in Orexin Neuron-Ablated *orexin/ataxin-3* Transgenic Mice

(A and B) Time courses of amounts of wakefulness (A) and non-REM sleep (B) in hemizygous transgenic mice (red, fed; orange, fasted) and their weight-matched wild-type 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 hr 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). Asterisk, significant difference ($p < 0.01$) in vigilance states of wild-type mice upon fasting. Hatch mark, significant difference ($p < 0.01$) in vigilance states of transgenic mice upon fasting.

0–12 of fasting) in wild-type 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 wild-type 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 wild-type 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 wild-type 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 6A). 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, wild-type mice exhibited a more pronounced prolongation of the exploratory phase than *orexin/ataxin-3* mice (Figure 6A). 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

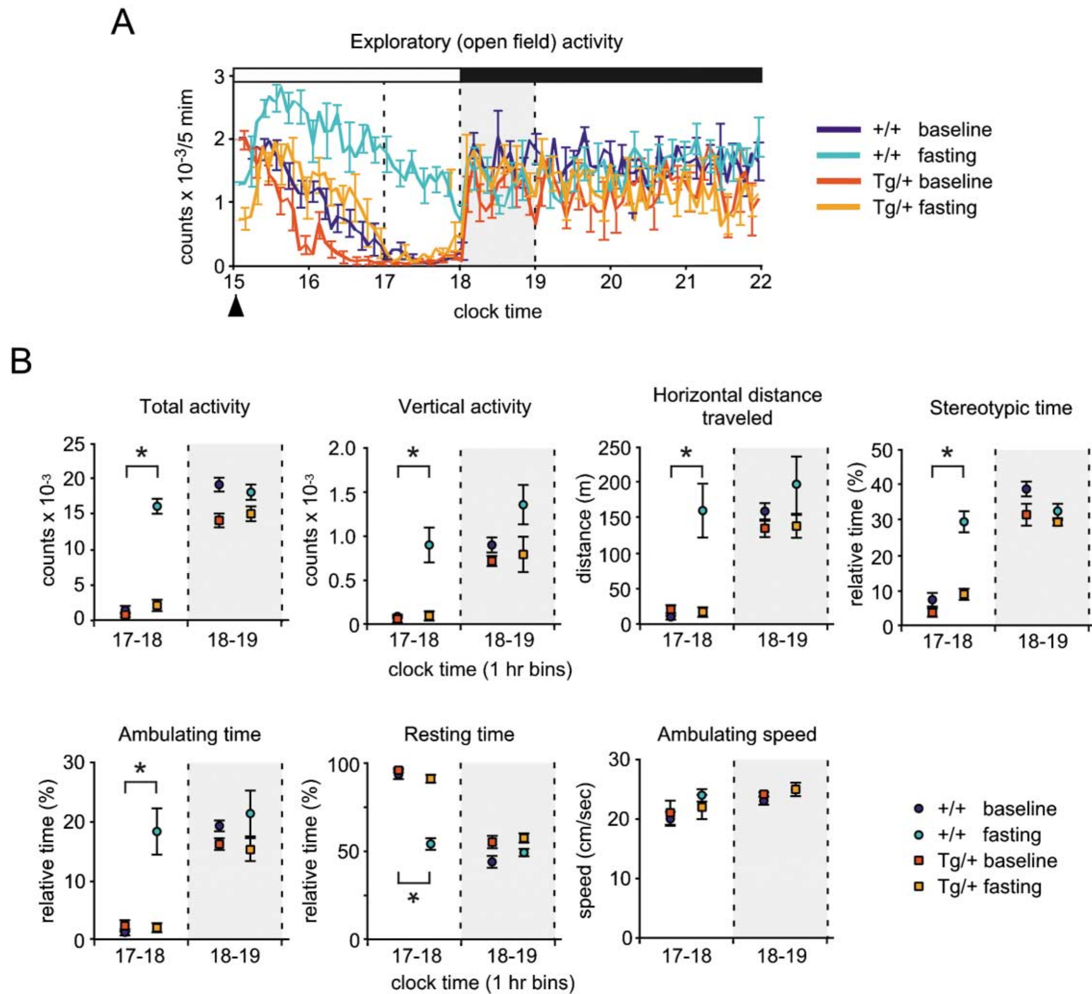

Figure 6. *Orexin/ataxin-3* Transgenic Mice Fail to Maintain Exploratory Motor Activity during Fasting

(A) 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 wild-type littermates (+/+, n = 6) correlated with the first 7 hr (baseline) and last 7 hr (fasted) of a 31 hr 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 hr bins (mean ± SEM). Distinct categories of motor activity are displayed as separate graphs. Asterisk, significant differences ($p < 0.001$) were found exclusively in wild-type mice upon fasting.

baseline activity of each group (two-way ANOVA) revealed that the total activity count (horizontal plus vertical beam breaks) was significantly increased for fasting wild-type mice ($p = 0.001$), but not for fasting *orexin/ataxin-3* mice during the light phase (Figure 6B). Additionally, fasted wild-type 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 5).

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 wild-type controls in either fed or fasted states (Figure 6B). 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 s/4 hr; fasted, 185.0 ± 45.0 s/4 hr). However, changes in body weight of transgenic (fed, 25.4 ± 0.5 g; fasted, 20.1 ± 0.4 g) and control wild-type 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 meta-

bolic rate. Overall, however, the robust behavioral changes observed in food-deprived wild-type 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

Technical Considerations

It has been difficult to identify orexin neurons in the hypothalamus without an electrophysiologically discernible marker, and to date, only one recent study has been published that examined the activity of these neurons by utilizing post hoc identification (Eggermann et al., 2003). In the present study, we generated *orexin/EGFP* transgenic mice in which green fluorescence can be used as a reliable marker for identifying orexin neurons among dispersed cells or within brain slice preparations. We used dispersed orexin neurons from these mice to examine the actions of neural and humoral factors in the absence of synaptic connections, such as those from surrounding neurons of the LHA or arcuate nucleus (discussed below). Using these techniques, we demonstrated that the majority of orexin neurons are glucose sensitive and many are directly responsive to leptin and ghrelin. We also presented findings collected using slice preparations in combination with the synaptic blocker tetrodotoxin. Although presynaptic effects such as those mediated by GABA or glutamate could not be completely excluded in these experiments, effects of leptin and ghrelin observed in isolated neurons were confirmed with the slice recording method, and the effects were dose dependent.

During the review process of this manuscript, another report was published in which orexin neurons were examined electrophysiologically (Li et al., 2002). While our study utilized dispersed, singly recorded orexin neurons as well as slice preparations from the *orexin/EGFP* transgenic mouse lines generated by our group, the report of Li et al. exclusively used whole-cell patch recording of lateral hypothalamic slices from the same mouse lines that we provided. While Li et al. did not examine the effects of glucose, leptin, or ghrelin on orexin neurons, they report similar effects of glutamate and GABA compared to those observed here, and they further demonstrated a mechanism by which orexin release may recruit the activity of surrounding orexin neurons via glutamatergic interneurons. Notably, Li et al. reported inhibitory effects of noradrenalin and serotonin on orexin neuron activity. Using the same transgenic mice, we also have described inhibitory effects of these monoamines on the activity of orexin neurons, as well as the additional finding of excitatory effects induced by acetylcholine (Yamanaka et al., 2003). Together, these studies demonstrate the utility of *orexin/EGFP* transgenic mice and provide a greater understanding of the mechanisms by which orexin neurons are regulated.

Extracellular Glucose Concentration Modulates Orexin Neurons

There are two distinct classes of hypothalamic neurons that are modulated by physiological alterations of extracellular glucose concentrations. So called glucose-responsive neurons of the medial hypothalamus are activated by high glucose levels and may function to suppress feeding behavior (Oomura and Yoshimatsu, 1984). Less well described are glucose-sensitive neurons in LHA, which are inhibited under high glucose concentrations and may participate in stimulating food intake (Oomura and Yoshimatsu, 1984).

We found that increasing glucose concentrations induced a striking hyperpolarization and cessation of action potentials in isolated orexin neurons, whereas insulin elicited no direct electrophysiological effect in these cells. Conversely, decreasing glucose concentration induced depolarization and increased the frequency of action potentials in these neurons. In support of these findings, we demonstrated hyperglycemia in the context of murine leptin deficiency and diabetes is associated with abnormally low hypothalamic orexin expression, and interventions that normalized blood glucose (leptin therapy or reduced food availability) normalized or even increased orexin expression compared to normal levels. In light of orexinergic projections to autonomic structures and the sympathomimetic effects of centrally administered orexin peptides (Date et al., 1999; Shirasaka et al., 1999), orexin neurons may play a role in the phenomenon of hypoglycemic awareness, in which low blood glucose triggers autonomic activity, peripheral epinephrine release, and behavioral defenses such as awakening from sleep. Such defenses are of particular importance to diabetic patients, who often undergo iatrogenic hypoglycemia resulting from imperfect insulin replacement. Interestingly, the responsiveness of the human autonomic system to hypoglycemia is blunted considerably during sleep in normal subjects (Jones et al., 1998; Gais et al., 2003) when orexin neurons are presumably least active (Estabrooke et al., 2001), and is even more compromised in patients with type 1 diabetes, who often suffer sleep-related hypoglycemia-associated autonomic failure (Banarer and Cryer, 2003).

Regulation of Orexin Neurons by Neuroendocrine Factors

Well-defined neurons in the arcuate nucleus have dendrites that lie outside the blood-brain barrier, and these neurons are responsive to circulating hormones and indicators of metabolic status. Such factors include leptin, which is secreted from adipose tissue in correlation with an animal's fat mass, and ghrelin, which is secreted from the stomach prior to meals and during fasting. Populations of neurons expressing pro-opiomelanocortin are activated by leptin, while those expressing neuropeptide Y (NPY) are inhibited by leptin (Elias et al., 1999) but also activated by ghrelin (Wang et al., 2002). Such neuronal populations in turn intensely innervate orexin neurons (Elias et al., 1998), providing for indirect regulation of orexin neurons by leptin and ghrelin. Indeed, ghrelin has been suggested to activate orexin neurons at least indirectly (Lawrence et al., 2002). We have presented evidence that leptin and ghrelin also directly regulate orexin neurons.

Increases in adiposity result in higher circulating leptin levels, which cross the blood-brain barrier to suppress feeding via neurons that express the signal-transducing

leptin receptor Ob-Rb (Rivest, 2002; Friedman, 2000). We found that bath application of leptin robustly inhibited most orexin neurons, causing hyperpolarization and a decrease in firing rate. Further, leptin administration depresses *orexin* expression in the hypothalamus of normal mice. Indeed, leptin injection suppresses the fasting-induced increase of *orexin* mRNA expression in rats, suggesting that upregulation of *orexin* mRNA by starvation may occur in part through reduced leptin signaling (Lopez et al., 2000). These findings are in accordance with reports that leptin receptor mRNA is expressed not only in the arcuate nucleus, but also in rat LHA (Elmquist et al., 1998), and immunoreactivity for leptin receptor and the leptin-activated transcription factor STAT3 are both present in orexin neurons (Hakansson et al., 1999).

With regard to ghrelin, weight loss by diet restriction in rats and humans promotes its release from the stomach, significantly increasing plasma concentrations of this factor (Lee et al., 2002; Cummings et al., 2002). Further, central or peripheral administration of ghrelin robustly increases food intake in rodents (Nakazato et al., 2001). These studies indicate that ghrelin is an important hormonal signal of low body mass. Ghrelin is also produced by interstitial hypothalamic neurons that project widely throughout much of the hypothalamus, including the NPY neurons of the arcuate nucleus and orexin neurons of the LHA (Cowley et al., 2003; Toshinai et al., 2003). The arcuate nucleus exhibits high expression of the receptor for ghrelin (growth hormone secretagogue receptor, GHSR). While GHSR expression has not been described in LHA to date, it is found in the anatomically and functionally related tuberomammillary hypothalamic nucleus and other arousal-promoting centers in the brainstem (Guan et al., 1997).

We have shown that ghrelin directly activates a population of isolated orexin neurons by depolarization with increases in action potential frequency. Circulating ghrelin, as well as ghrelin-containing neurons, may in part mediate activation of orexin neurons such as that occurring during food restriction. Indeed, Cai et al. (1999) have suggested that the absence of food in the gut is a key factor associated with increased *orexin* mRNA expression. Induction of food intake by ghrelin, which counteracts reduction in body weight, may be mediated in part by orexins, which also pharmacologically induce food intake (Sakurai et al., 1998). Furthermore, peripheral or central administration of ghrelin disrupts sleep patterns in rats (Tolle et al., 2002) and in mice (M.M., unpublished observations). Thus, ghrelin signaling pathways, like those of orexin, are anatomically well placed to affect both metabolic and sleep/wake homeostasis.

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 and Nicolaidis, 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 (Karklin et al., 1994; Crisp et al., 1971).

We have demonstrated significant increases of wakefulness in wild-type 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 and Nicolaidis, 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 low baselines of orexin neuron activity and wakefulness associated with this period (Estabrooke et al., 2001). 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, wild-type 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 wild-type 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 glutamate (Abra-

hamson 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 coexpressed factors listed above are not exclusively found in orexin neurons. Critically, *orexin/ataxin-3* transgenic and *prepro-orexin* knockout mice exhibit similar baseline behavioral and metabolic phenotypes when genetic background is controlled (J.T.W., J.H., and M.M., 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 antiobesity and antidiabetic effects of a recently described orally active orexin receptor antagonist (Smart et al., 2002). We suggest a model in which food deprivation triggers a drop in circulating glucose and leptin levels and an increase in ghrelin signalling. Together, these lead to increased firing of orexin neurons. 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 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

Animal Care

All experimental procedures involving animals were approved by the University of Tsukuba Animal Care and Use Committee or the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center at Dallas, and were in accordance with NIH guidelines.

Generation of *Orexin/EGFP* Transgenic Mice

The transgenic construct was made by substituting the *nLacZ* gene (SalI-BamHI fragment) of the *orexin/nlacZ* transgenic construct (Sakurai et al., 1999) with the SalI-XbaI fragment of pEGFP-N3 (Clontech, Inc., CA) containing the *EGFP* cassette. The transgene was linearized and microinjected into pronuclei of fertilized mouse eggs (F1 of C57BL/6 × DBA1) to generate transgenic founders. Founder animals were bred with C57BL/6 mice to produce stable *orexin/EGFP* lines.

Histological Analysis

Mouse brains were fixed and prepared as described previously (Nambu et al., 1999). For detection of EGFP fluorescence, cryostat sections (40 μm) were mounted on poly-L-lysine-coated slides and examined with a Provis AX70 fluorescence microscope (Olympus, Japan). For immunofluorescence staining of orexins, cryostat sections (40 μm) were postfixed with paraformaldehyde, incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 1 hr, and then incubated with rabbit anti-orexin antiserum (Nambu et al., 1999) in the same solution for 1 hr at room temperature. After washing three times in PBS, the sections were incubated with a Texas red-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, PA) for 1 hr at room temperature, and then mounted onto slides. The slides were then washed three times in PBS and examined with a fluorescence microscope.

Whole-Cell Patch-Clamp Recording

Brains from *orexin/EGFP* transgenic mice (3–4 weeks old) were isolated and cut into 1 mm thick coronal slices. The LHA region was isolated by microdissection and incubated at 30°C in 1 mg/ml proteinase K (Sigma) for 5 min, then in 1 mg/ml trypsin (Type XI, Sigma) for 25 min. The tissues were triturated by gentle pipetting. Isolated neurons were plated on poly-L-lysine-coated glass cover slips at 33°C–34°C in extracellular physiological solution consisting of NaCl (135 mM), KCl (5 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), HEPES (10 mM), and d-glucose (10 mM) (pH 7.4) until neurons are attached. Neurons displaying green fluorescence, identified by fluorescence microscopy as above, were selected for patch-clamp recordings. Current clamp recordings were carried out with an Axopatch 200B amplifier (Axon Instruments, CA) using a borosilicate pipette (3–6 MΩ after heat polishing) filled with intracellular solution consisting of K-gluconate (130 mM), KCl (10 mM), $CaCl_2$ (0.05 mM), $MgCl_2$ (0.1 mM), HEPES (10 mM), EGTA (0.5 mM), and ATP (2 mM) (pH 7.4). The pipette solution was aliquoted and stored at −80°C until required. During recordings, cells were superfused with extracellular physiological solution described above at a rate of 1.5–2.0 ml/min and kept at 33°C–34°C. During experiments in which glucose concentration was altered, osmolarity was equalized with mannitol. At the end of each recording, cytoplasm was siphoned into the electrode and directly subjected to RT-PCR to confirm the presence of *prepro-orexin* mRNA, using the following primers: 5′-CAGCCTCTGCCCGACTGCTGTC-3′ and 5′-TAAAGCGGTGGTAGTTACGGTCGGAC-3′.

For the recordings from the hypothalamic slices, the brains from orexin/EGFP mice of 3–6 weeks old were isolated in extracellular physiological solution (as above) and were cut coronally into 250 μm slices with a microtome (VTA-1000S, Leica, German). The slices, containing the lateral hypothalamic area, were transferred to an incubation chamber for at least 1 hr at room temperature (24°C–26°C). For electrophysiological recording, the slices were transferred to a recording chamber (RC-27L, Warner Instrument Corp.) at a controlled temperature of 34°C on a fluorescence microscope stage (BX51WI, Olympus, Tokyo, Japan). The slices were superfused with physiological solution, which was preheated before entering a recording chamber by an in-line heater (Warner) at 34°C, at a rate of 3 ml/min using a peristaltic pump (Dynamax, Rainin, CA). The fluorescence microscope was equipped with an infrared camera (C2741-79, Hamamatsu photonics, Hamamatsu, Japan) for infrared differential interference contrast (IR-DIC) imaging and a 3 charge coupled device (CCD) camera (IK-TU51CU, Olympus). Recording pipettes were advanced while under positive pressure toward fluoresced cells in the slice. The membrane patch was then ruptured by suction, and membrane current and potential were monitored using an Axopatch 200B patch clamp amplifier (Axon, Instruments, Foster City, CA).

Continuous Leptin Administration to Mice

Wild-type and *ob/ob* mice (both on C57BL/6J background) were anesthetized with pentobarbital (50 mg/kg, i.p.), positioned in a ste-

reotaxic frame, and implanted with a guide cannula into the third ventricle. After recovery for 7 days, an ALZET 1002 osmotic minipumps (ALZA, CA) containing either sterile water or leptin (Peprotech Inc., NJ) dissolved in sterile water was implanted subcutaneously in the interscapular region under pentobarbital anesthesia. Pump was connected to the inner cannula via a polyethylene tubing. Leptin was infused at a rate of 100 ng/hour for 2 weeks.

Prepro-Orexin mRNA Quantification

Total RNA was prepared from microdissected mouse hypothalami and subjected to Northern hybridization with ^{32}P-labeled mouse *prepro-orexin* cDNA as probe. Autoradiograms were scanned and analyzed by densitometry. Data were analyzed by two-way ANOVA.

Behavioral Analysis

Exploratory behavior of male *orexin/ataxin-3* hemizygous transgenic mice (n = 5) and their weight-matched male wild-type 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 hr light/dark cycle. Animals were introduced into the open field without food 3 hr before onset of dark, and activity was recorded until 4 hr into the dark phase (7 hr of continuous recording), after which mice were reintroduced into home cages with continued fasting. The following day, the procedure was repeated, resulting in a total fasting time of 31 hr. 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 hr 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 wild-type 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 2 weeks under constant 12 hr light/dark cycle at 25°C with free access to food and water. Continuous EEG/EMG traces were recorded over 78 hr, with ad lib feeding for the first 48 hr (baseline) and food deprivation starting at the onset of dark phase for the next 30 hr (fasting). Traces were captured and digitized in 20 s 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.

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