

Genetic Ablation of Orexin Neurons in Mice Results in Narcolepsy, Hypophagia, and Obesity

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Summary

Orexins (hypocretins) are a pair of neuropeptides implicated in energy homeostasis and arousal. Recent reports suggest that loss of orexin-containing neurons occurs in human patients with narcolepsy. We generated transgenic mice in which orexin-containing neurons are ablated by orexinergic-specific expression of a truncated Machado-Joseph disease gene product (ataxin-3) with an expanded polyglutamine stretch. These mice showed a phenotype strikingly similar to human narcolepsy, including behavioral arrests, premature entry into rapid eye movement (REM) sleep, poorly consolidated sleep patterns, and a late-onset obesity, despite eating less than nontransgenic littermates. These results provide evidence that orexin-containing neurons play important roles in regulating vigilance states and energy homeostasis. Orexin/ataxin-3 mice provide a valuable model for studying the pathophysiology and treatment of narcolepsy.

Introduction

Orexins are pairs of neuropeptides that are expressed in the lateral hypothalamic area (LHA), a region of the brain classically implicated in feeding behavior (Sakurai et al., 1998). Orexin-A is a 33 amino acid residue polypeptide with an N-terminal pyroglutamyl residue and C-terminal amidation. It has two intrachain disulfide bridges, while orexin-B is a 28 amino acid linear polypeptide with C-terminal amidation. The actions of orexins are mediated via two G protein-coupled receptors named the orexin-1 (OX1R) and orexin-2 receptor (OX2R) (Sakurai et al., 1998). de Lecea et al. (1998) independently identified an mRNA encoding the same peptides by a

differential cloning approach and named the putative encoded peptides hypocretins. Orexin-A (hypocretin-1) and orexin-B (hypocretin-2) are expressed together as a single precursor peptide undergoing subsequent proteolytic cleavage (Sakurai et al., 1998). These neuropeptides, when injected intracerebroventricularly, influence feeding behavior, metabolic rate and arousal (Sakurai et al., 1998; Lubkin and Striker-Krongrad, 1998; Hagan et al., 1999; Willie et al., 2001).

Recently, several reports implicate a dysfunction of the orexin system in the human sleep disorder narcolepsy. Narcolepsy is the only neurological disorder characterized by a primary disorganization of sleep and wakefulness. Patients with narcolepsy suffer from excessive daytime sleepiness, cataplexy (a sudden weakening of posture muscle tone usually triggered by emotion), and an alteration in the amount of and entry into REM sleep (Mignot, 1998). Nocturnal sleep is also frequently disturbed by insomnia, sleep paralysis, and vivid dreaming. The debilitating lifelong disorder affects ~1 in 2000 individuals. Familial transmission of narcolepsy is rare; however, in these families the relative risk for first-degree relatives is 10 to 40 times higher than that seen in the general population. One of the predisposing factors is a specific class II HLA haplotype on human chromosome 6, with HLA DQB1*0602 and DQA1*0102 alleles being found in more than 85% of all narcoleptic patients (Kadotani et al., 1998). The genetics of human narcolepsy, however, have remained unclear.

Positional cloning has identified mutations in the *OX2R* (*hcrtr-2*) gene as the cause of narcolepsy in a canine model (Lin et al., 1999), and mice with a targeted deletion of the *prepro-orexin* gene display a phenotype strikingly similar to human narcolepsy (Chemelli et al., 1999). In contrast to monogenic murine and canine narcolepsy models, human narcolepsy is rarely familial, and may result from undefined environmental factors acting on a susceptible genetic background (Mignot, 1998). Recently, it was demonstrated that orexin-A was undetectable in the cerebrospinal fluid (CSF) of the majority of patients with narcolepsy, indicating that abnormal orexin neurotransmission also exists in human narcolepsy (Nishino et al., 2000). Since these patients do not carry mutations in the *prepro-orexin* or *orexin receptor* genes, decreased orexin levels in these patients may result from dysfunction or loss of orexin-expressing neurons. More recently, postmortem studies in six narcoleptic subjects indicated undetectable orexin peptides in projection sites such as the cortex and pons and an 80%–100% reduction in the number of orexin-containing neurons in the hypothalamus as determined by in situ hybridization (Peyron et al., 2000). Thannickal et al. (2000) independently reported a global loss of orexin-containing neurons and residual gliosis in four brains examined in the perifornical area of brains of narcoleptic patients by immunohistochemistry. These results strongly suggest a selective loss of orexin-containing cells in brains of narcoleptic patients. Thus, mice in which orexin-containing neurons are specifically ablated would make a useful model of human narcolepsy. Furthermore,

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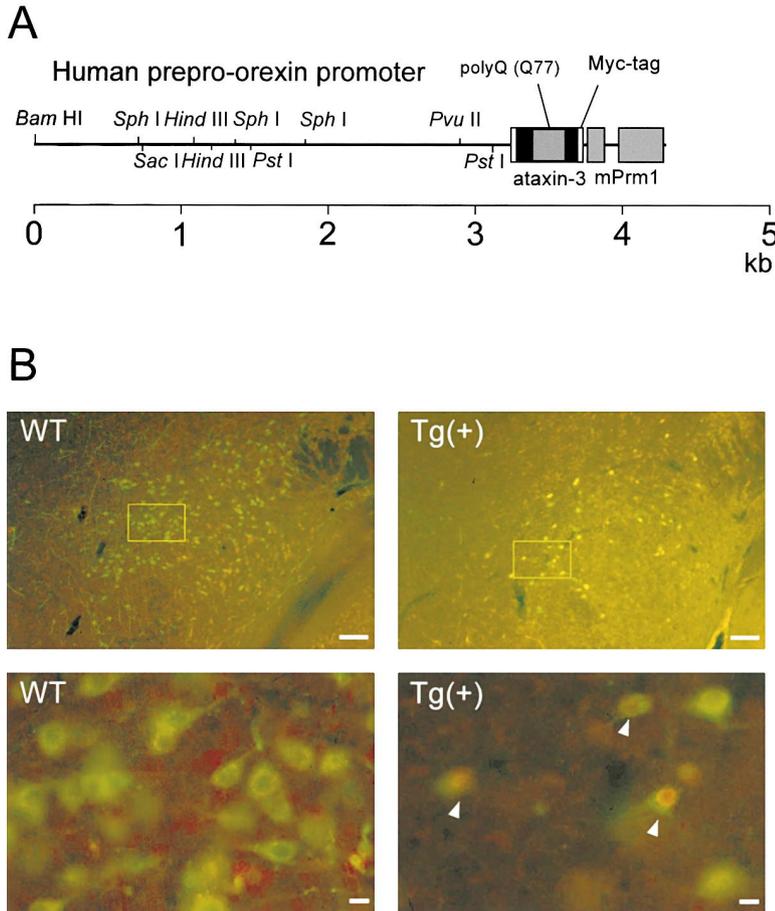


Figure 1. Expression of Truncated Ataxin-3 with Polyglutamine Repeat Stretch in Orexin-Containing Neurons in Transgenic Mice

(A) Orexin/ataxin-3 transgene. The genomic DNA fragment containing the 3149-bp 5'-flanking region and the 122-bp 5'-noncoding region of exon 1 of human *prepro-orexin* gene was ligated to a cDNA fragment, which encodes from amino acid 286 to the C terminus of elongated *ataxin-3* isolated from a MJD patient. The 5' terminus of this cDNA fragment has the HA epitope and the 3' terminus has the myc-epitope. The 3' end of *ataxin-3* cDNA was followed by a murine protamine 1 genomic fragment (mPrm1) that provides an intron and a polyadenylation signal. Southern blot analysis of tail DNA from the F1 generation revealed that copy numbers ranged from 2 to 4 among lines.

(B) Double immunofluorescence photomicrographs show matched coronal brain sections (bregma -2.1 mm) of the lateral hypothalamic area (LHA) of a 2-week-old orexin/ataxin-3 transgenic mouse (right panels) and transgene-negative littermate (left panels). Orexins are represented by green fluorescence (fluorescein isothiocyanate) and myc epitope-immunoreactivity is shown by red fluorescence (Cy-3). Lower panels show high power views of the yellow-boxed areas in the upper panels. Arrowheads indicate the existence of nuclear aggregates stained by anti-myc antibody in orexin-containing neurons. Bar = 100 μ m for the upper panels, and 10 μ m for the lower panels.

the phenotype of such mice might be different from that of *prepro-orexin* knockout mice because orexin neurons of the LHA express several other neuropeptides including galanin (Hakansson et al., 1999), dynorphins (T. Chou and T. Scammell, personal communication), and angiotensin II (T. N., unpublished data).

We have previously reported transgenic mouse lines in which the 3.2 kb fragment of the 5'-upstream region of the human *prepro-orexin* gene is sufficient to express the *Escherichia coli beta-galactosidase (lacZ)* gene eutropically in orexin containing neurons (Sakurai et al., 1999). In the present study, we used the same human *prepro-orexin* gene fragment as the promoter to specifically express a toxic transgene only in orexin-containing neurons to selectively remove orexin-containing neurons in transgenic mice. This transgene, the N-terminal truncated cDNA for human ataxin-3, which was isolated from a patient with Machado-Joseph disease, includes abnormally expanded polyglutamine repeats, and has been shown to induce apoptosis in many cell types when expressed exogenously (Yoshizawa et al., 2000). In these mice, hypothalamic orexin-containing neurons gradually decrease in number, and are almost completely absent by 15 weeks of age. We examined the phenotype of these mice, focusing especially on abnormalities of sleep/wake states and energy homeostasis.

Results

Specific Expression of Polyglutamine Repeat in Orexin-Containing Neurons

We constructed a transgene with the 3.2 kb fragment of the 5'-upstream region of the human *prepro-orexin* gene as a promoter, which was ligated to the N terminally truncated cDNA for human ataxin-3 with 77 polyglutamine repeats. Additionally, we myc-tagged the C terminus of the transgene for histological examination (Figure 1A). The transgene was injected into pronuclei of fertilized mouse eggs to generate founder animals, which were bred to produce four orexin/ataxin-3 transgenic lines.

Brains from 2-week-old F1 orexin/ataxin-3 mice were examined by double-immunofluorescence histochemistry using anti-myc monoclonal antibody and anti-orexin antiserum to assess transgene expression. We observed a common expression pattern of specific myc-like immunoreactivity in the hypothalamic orexin-containing neurons in all lines (Figure 1B). These neurons contained anti-myc positive nuclear aggregates (Figure 1B, right panels). No ectopic expression of myc-like immunoreactivity was observed. These results suggest that this transgene directs expression of cDNA encoding the N terminally truncated human ataxin-3 with expanded polyglutamine repeats specifically in orexin neurons.

Ablation of Orexin-Containing Neurons in the Hypothalamus of Orexin/Ataxin-3 Mice

Brains of orexin/ataxin-3 mice showed a temporal progression of apparent loss of orexin-containing neurons as determined by anti-orexin immunostaining. At postnatal day 5 we were not able to detect any differences in the number of orexin-containing neurons in orexin/ataxin-3 mice and their wild-type littermates (data not shown). However, we found that $56\% \pm 12\%$ of orexin-containing neurons were lost by 2 weeks of age ($n = 4$) (Figure 1B). Thereafter, $75\% \pm 5\%$ and $90\% \pm 8\%$ of orexin-containing neurons were eliminated by 4 and 8 weeks of age, respectively ($n = 4$) (Figure 2A). At 12 weeks of age, over 99% of orexin-containing neurons were already lost (not shown), and at 15 weeks of age, nearly no orexin-containing neurons could be found in the hypothalamic regions of all lines. These observations suggest that ablation of orexin-containing neurons occurred postnatally, and most drastically within 2 weeks after birth.

Immunostaining of progeny derived from all independent transgenic founders confirmed that the ablation of orexin-containing neurons occurred with full penetrance.

In situ hybridization of coronal sections of the mice also revealed that *prepro-orexin* mRNA expression in the LHA was markedly reduced in the transgenic mice (Figure 2B)

Although orexin-containing cell bodies are exclusively localized to the LHA, these neurons innervate regions throughout the entire brain, including monosynaptic projections to several regions of the cerebral cortex, the limbic system, specific areas of the thalamus, and the brain stem (Peyron et al., 1998; Date et al., 1999; Nambu et al., 1999). We examined some of these projections in orexin/ataxin-3 mice using anti-orexin antiserum, and found that the number of orexin-immunoreactive fibers was markedly decreased in the brains of the transgenic mice. Notably, orexin-immunoreactive fibers in the locus coeruleus, a region of the brain normally receiving dense orexin neuronal projections, were markedly reduced in transgenic mice (Figure 2C).

Gross anatomical and histological studies, using hematoxyline-eosin and Nissl staining, failed to detect any structural abnormality in the brains of the transgenic mice at 12 weeks of age (not shown). Melanin-concentrating hormone (MCH)-containing neurons, a group of lateral hypothalamic neurons that are distinct from orexin-containing neurons (Elias et al., 1998), were immunostained in transgenic and wild-type control mice using anti-MCH antiserum. The MCH-containing neurons were unaffected by the presence of the transgene as the number of MCH-positive cells was the same as that in the transgene-negative controls (Figure 3A). Northern blot analysis revealed that the levels of *MCH* mRNA in orexin/ataxin-3 were comparable with wild-type mice when normalized by *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) mRNA levels, while *prepro-orexin* mRNA levels in the hypothalamus of orexin/ataxin-3 mice were markedly reduced at 4 weeks of age (Figure 3B). After 8 weeks of age, we could not detect *prepro-orexin* mRNA in the hypothalamus of orexin/ataxin-3 mice of any lines by Northern analysis (not shown). Immunostaining for other hypothalamic peptides using anti-neu-

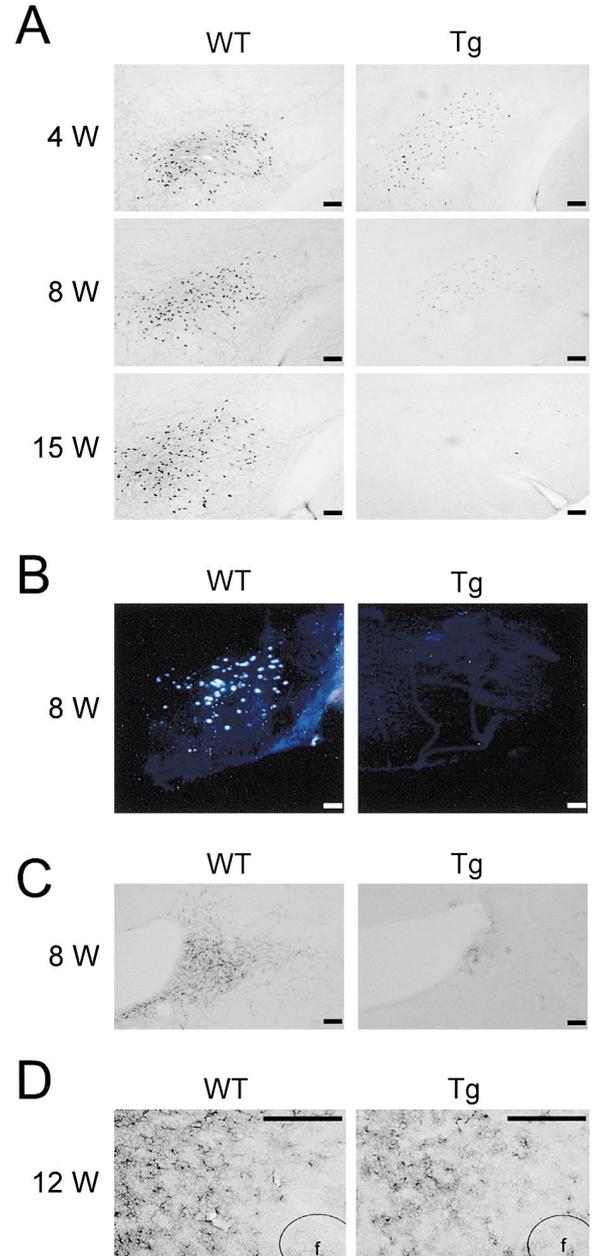


Figure 2. Ablation of Orexin Neurons in Orexin/Ataxin-3 Transgenic Mice

(A) Matched brain sections (bregma -2.1 mm) from 4-, 8-, and 15-week-old orexin/ataxin-3 transgenic mice (right panels) and their transgene negative littermates (left panels) were stained with anti-orexin antiserum.

(B) In situ hybridization of brain of orexin/ataxin-3 mouse (right panel) and their transgene negative control (left panel). Dark-field images of matched coronal brain sections of the LHA hybridized with 32 P-labeled antisense riboprobe for *prepro-orexin* mRNA. The signal was barely detectable in transgenic mice.

(C) Orexin-immunoreactive fibers in the locus coeruleus of an 8-week-old orexin/ataxin-3 mouse (right panel) and its transgene negative littermate (left panel) are shown. Note that orexin-immunoreactive fibers are vastly removed in orexin/ataxin-3 mouse.

(D) Immunostaining of glial fibrillary acidic protein (GFAP)-like immunoreactivity in the LHA of a 12-week-old orexin/ataxin-3 mouse (right panel) and its transgene negative littermate (left panel) are shown. There is no obvious difference between the two genotypes. f, fornix. Bar = $100 \mu\text{m}$

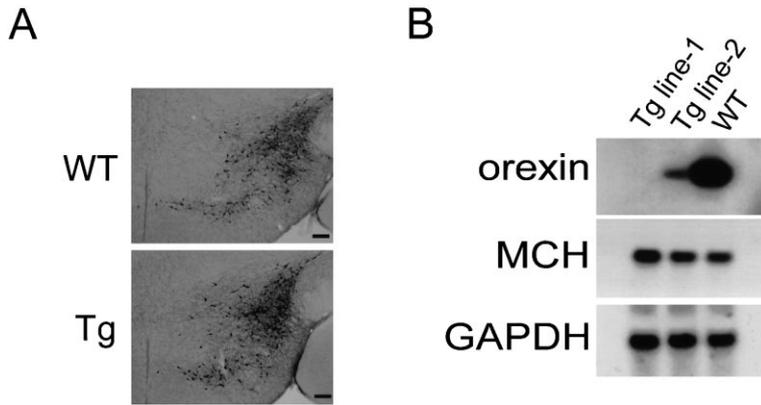


Figure 3. Normal Expression of MCH in Orexin/Ataxin-3 Mice

(A) Matched brain sections (bregma -2.1 mm) from an 8-week-old orexin/ataxin-3 transgenic mouse (lower panel) and a transgene-negative littermate (upper panel) stained with anti-MCH antiserum. We obtained similar results using the other transgenic lines. Bar = $100 \mu\text{m}$.

(B) Northern blot analysis for expression of *prepro-orexin* mRNA of hypothalami from 4-week-old orexin/ataxin mice of two independent orexin/ataxin-3 lines and transgene-negative mice (WT). The same blot was rehybridized with *MCH* and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) probes.

ropeptide Y or anti-melanocyte-stimulating hormone antibodies failed to detect any abnormality in the brains of the transgenic mice (not shown). We also stained brain sections of 12-week-old orexin/ataxin-3 mice with anti-glial fibrillary acidic protein (GFAP) antiserum, but we did not observe any evidence of gliosis in the hypothalamus of orexin/ataxin-3 mice (Figure 2D).

Taken together, these observations suggest that orexin-containing neurons are both postnatally and specifically removed in orexin/ataxin-3 transgenic mice.

Behavioral Arrests in Orexin/Ataxin-3 Mice in the Dark Period

Since mice are most active at night, we observed nocturnal behavior of orexin/ataxin-3 mice and wild-type littermate mice by infrared video photography. Frequent periods of obvious behavioral arrest were observed in orexin/ataxin-3 mice, similar to the "narcoleptic episodes" in *prepro-orexin* knockout mice (Chemelli et al., 1999). As previously reported, such episodes were recognized in orexin/ataxin-3 mice by an abrupt cessation of purposeful motor activity associated with a sustained change in posture that was maintained throughout the episode, ending abruptly with complete resumption of purposeful motor activity (Chemelli et al., 1999). The postural changes are characterized by sudden collapse of the head and neck with simultaneous buckling of the limb. An infrared video study of progeny derived from three independent transgenic lines confirmed that this behavior occurred with 100% penetrance in orexin/ataxin-3 mice ($n = 6$ for each line). 12-week-old transgenic mice had from 10 to 30 episodes during the 4 hr filming period (group average 20 ± 6.6 episodes) (Figure 4A). The duration of episodes varied from a few seconds to 150 s. Overall, the average duration of episodes was 41.4 s. As with *prepro-orexin* knockout mice, attacks often occurred during grooming or excited ambulation (Figure 4B). Similar to *prepro-orexin* knockout mice, feeding and drinking were rarely observed before an episode, but were frequently observed afterward. These behavioral arrests typically began at about 6 weeks of age. We obtained similar results from transgenic mice of the two other lines (not shown). We observed similar episode frequency and duration in both genders. No

narcoleptic episodes were identified in transgene-negative (wild-type) littermates.

Sleep State Characterization of Orexin/Ataxin-3 Mice

Sleep state patterns of orexin/ataxin-3 mice and wild-type littermate mice were revealed by simultaneous electroencephalographic/electromyographic (EEG/EMG) recording as previously reported (Chemelli et al., 1999). Typical hypnograms, graphic representations of sleep-waking states over time, for an orexin/ataxin-3 mouse and a wild-type littermate for the 12 hr dark period (active phase) are shown in Figure 5A. The hypnogram for the transgenic mouse is characterized by the appearance of direct transitions from wakefulness to REM sleep, and more fragmented waking and non-REM sleep episodes (Figure 5A). A typical example of an EEG/EMG recording showing the direct transition from the awake state to REM sleep is depicted in Figure 5B. The mean \pm SEM frequency of direct transition to REM observed in orexin/ataxin-3 mice for 12 hr light and dark period was 0.25 ± 0.16 and 3.17 ± 1.45 , respectively ($n = 4$ for 3 days). No direct REM transition was ever observed in wild-type littermates.

Sleep state patterns of orexin/ataxin-3 mice were very similar to that of *prepro-orexin* knockout mice (Chemelli et al., 1999), despite some differences in the genetic background. During the dark period, both REM sleep time and REM episode duration were longer in the transgenic mice ($n = 4$) compared to wild-type littermates ($n = 3$) (Table 1). These changes occurred together with a decrease in the interval between successive REM sleep periods, indicating increased pressure for entry into REM sleep in these mice. There was also a significant decrease in awake episode duration, reflecting an increased fragmentation of the sleep-wake cycle and an inability to maintain consolidated wakefulness during the dark period in orexin/ataxin-3 mice (Figure 5A, Table 1). REM latency was also significantly shorter in orexin/ataxin-3 mice compared with wild-type littermates during the dark period.

During the light period time spent in REM sleep and the episode duration of the awake state was significantly shorter in orexin/ataxin-3 mice as compared with their

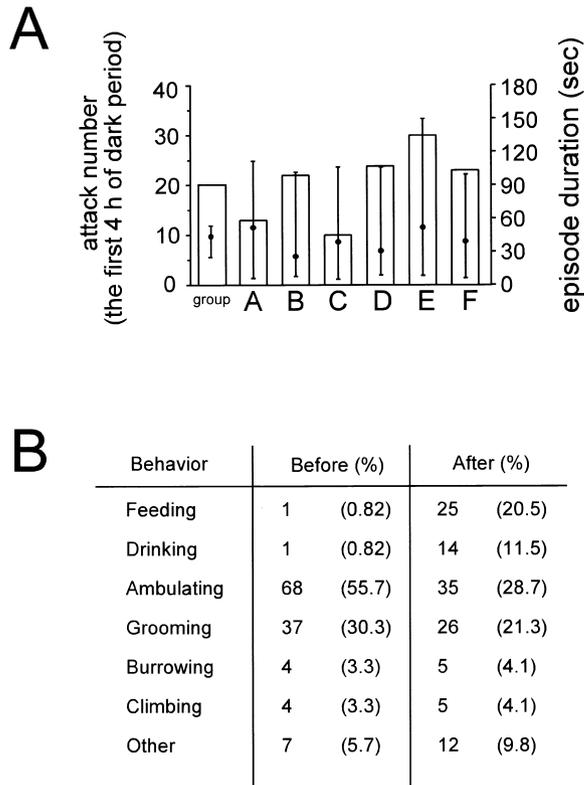


Figure 4. Characterization of Behavioral Arrests in Orexin/Ataxin-3 Mice

(A) Behavioral arrest (narcoleptic episode) number and duration. Columns represent total number of episodes recorded in the first 4 hr after onset of dark period. Filled circles represent mean duration of all recorded episodes. T-bars indicate the minimum and maximum episode durations observed. Data for individual male transgenic mice are designated A-F. No narcoleptic cataplexy-like episodes were observed in any of the wild-type mice ($n = 6$). Group is the average count and duration for all mice (A-F), with the T bars indicative of minimum and maximum individual averages.

(B) Behavior of orexin/ataxin-3 mice before and after each narcoleptic episode. The predominant behavior observed for the 5 s preceding each episode and the predominant behavior for the 10 s after each episode were categorized as previously reported (Chemelli et al., 1999). Number of observations and percentage of total observations are reported.

wild-type counterparts (Table 1). Direct transitions from waking to REM were also occasionally observed in the light period.

Orexin Neuron-Ablated Mice Exhibit Late-Onset Obesity despite Being Hypophagic

We found that orexin/ataxin-3 mice showed late-onset obesity (Figures 6A and 6B). At 10 to 12 weeks of age, the male transgenic mice started to gain excess weight (Figure 6B). Female transgenic mice displayed a similar pattern of obesity (not shown). Obesity occurred despite an almost 30% reduction in food intake during the dark period at 8 to 10 weeks of age, suggesting the importance of orexin-containing neurons in the regulation of feeding behavior and energy homeostasis (Figure 6C). Since orexin/ataxin-3 mice became obese despite eating less, we speculated the energy expenditure of

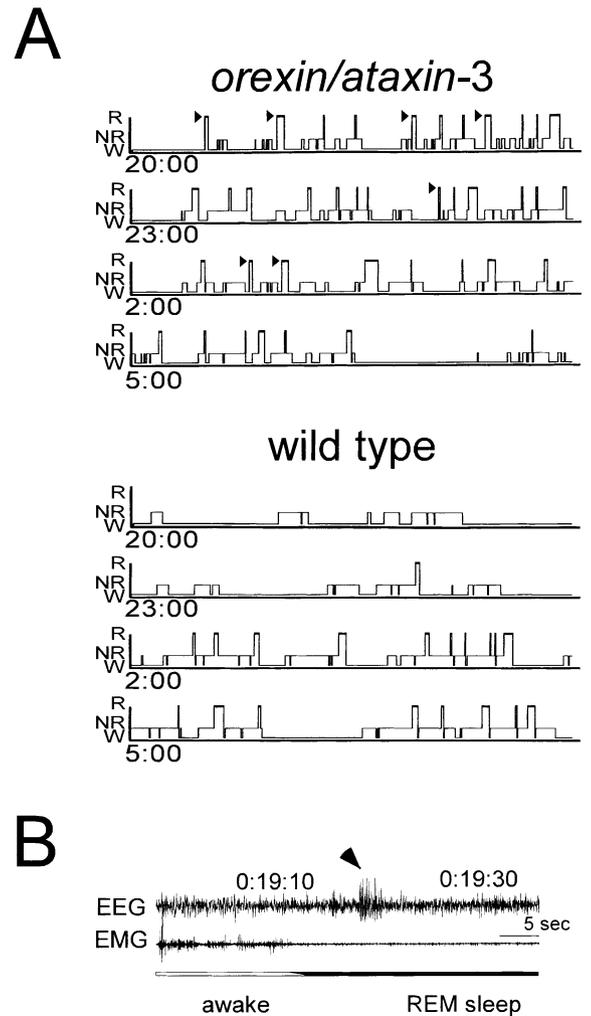


Figure 5. Sleep State Abnormalities Seen in Orexin/Ataxin-3 Mice

(A) Representative 12 hr dark period (20:00 to 08:00) hypnogram for an orexin/ataxin-3 mouse (upper panel) and its transgene-negative littermate (lower panel). The height of the horizontal line above baseline indicates the vigilance state of the mouse at the time. W, wakefulness; NR, non-REM sleep; R, REM sleep. Note episodes of direct transition from awake state to REM sleep (marked by arrowheads), greater non-REM sleep episode fragmentation, and reduced wakefulness periods in the hypnogram of the orexin/ataxin-3 mouse. Hypnograms were obtained by simultaneous EEG/EMG recording as described previously (Chemelli et al., 1999).

(B) EEG/EMG simultaneous recording showing a typical example of a direct transition from awake state to REM sleep, extracted from a continuous record. Awake state, which is characterized by a high-amplitude EMG and a low amplitude, mixed frequency EEG, is directly followed by a REM sleep episode, which is characterized by muscle atonia, leaving only the heart beat registering on the EMG, combined with a low-amplitude EEG dominated by theta-frequencies. The arrowhead indicates a sleep spindle, which is sometimes seen shortly before or at the entry into REM sleep periods in rodents.

these mice must be decreased. As lower energy expenditure may result from differences in motor activity, we measured spontaneous motor activity, using an infrared ambulation monitoring system (Masuo et al. 1997). Wild-type mice showed a higher level of spontaneous activity than orexin/ataxin-3 mice for most of the dark period, while no obvious difference between genotypes was

Table 1. Vigilance State Parameters Recorded from Orexin/Ataxin-3 Hemizygous Transgenic (Tg) and Wild-Type Littermate Control (WT) Mice

	REM Sleep		Non-REM Sleep		Awake	
	Tg	WT	Tg	WT	Tg	WT
24 hr						
Total time (min)	88.2 ± 3.5	91.6 ± 2.3	731.4 ± 7.9	692.1 ± 15.3	617.7 ± 6.6	653.7 ± 15.7
Episode duration (s)	80.4 ± 2.1*	70.3 ± 2.9	265.8 ± 10.7	298.3 ± 17.1	252.0 ± 11.8*	383.5 ± 14.4
REM latency (min)	6.4 ± 0.3*	9.4 ± 0.5				
Inter-REM interval (min)	20.8 ± 1.2	17.1 ± 0.7				
Light Period						
Total time (min)	41.8 ± 1.8*	64.4 ± 2.3	443.6 ± 5.1	428.6 ± 4.6	233.3 ± 5.1	225.7 ± 5.6
Episode duration (s)	75.7 ± 2.8	72.8 ± 3.5	363.8 ± 15.8	309.6 ± 16.0	265.0 ± 10.7*	310.1 ± 13.4
REM latency (min)	9.0 ± 0.4	9.7 ± 0.4				
Inter-REM interval (min)	22.0 ± 1.2*	16.5 ± 0.8				
Dark Period						
Total time (min)	46.4 ± 2.0*	27.1 ± 3.3	287.9 ± 9.6	263.6 ± 14.4	384.4 ± 9.1	428.0 ± 17.1
Episode duration (s)	85.7 ± 2.8*	67.1 ± 5.6	188.7 ± 8.8	282.7 ± 21.5	247.0 ± 17.6*	474.7 ± 32.9
REM latency (min)	4.5 ± 0.4*	9.2 ± 0.6				
Inter-REM interval (min)	19.8 ± 1.0	19.2 ± 1.1				

Total time spent in each state (minutes, mean ± SEM), episode duration (seconds ± SEM), REM latency, and interval between successive REM sleep episodes (minutes, mean ± SEM) over 24 hr and itemized separately for light and dark periods.

Significant differences ($p < 0.05$; repeated measurements ANOVA) between Tg and WT mice are indicated with an asterisk.

observed in the light period, consistent with a lower energy expenditure in transgenic mice over the recorded 24 hr period (Figure 6D).

Discussion

Technical Considerations

An understanding of the functions of the brain, which depends on neuron-neuron interactions, will progress with the development of methods that permit the selective elimination of neuronal types with particular chemical identities. When ablation of a subpopulation of neurons leads to physiological and behavioral changes, it is possible to determine the role of a specific type of neurons in brain function. General methods of genetic cell ablation are based on expression of a toxic gene product under the control of a cell-specific enhancer/promoter (Palmiter et al., 1987). The diphtheria toxin gene product is widely used for this purpose in nonneuronal cell lineage. An alternative approach is conditional obliteration of target cells with herpes simplex virus 1 thymidine kinase (Heyman et al., 1989). However, these techniques have not been used to eliminate postmitotic cells, including neurons, because these toxins may not be adequately effective in neurons.

We used a C-terminal fragment of ataxin-3 gene product, which was isolated from a Machado-Joseph disease patient to ablate orexin-containing neurons. This cDNA product has been shown to induce apoptosis in many cell types when expressed exogenously in vitro (Yoshizawa et al. 2000). We showed that expression of this cDNA effectively removes orexin-containing neurons in vivo in transgenic mice. This method should be broadly applicable in genetic ablation of a defined population of neurons, and provides an approach to elucidate the physiological and behavioral functions of specific neuronal types in the mammalian central nervous system. It could also be used to create experimental models of human brain disorders that are caused by degeneration of certain neuronal groups.

Postnatal Loss of Orexin-Containing Neurons in Mice Results in Narcolepsy

The first clue suggesting the possible involvement of the orexin system in narcolepsy came from animal models. The positional cloning approach in the canine narcolepsy model led to the identification of mutations in the *OX2R* (*hcrtr2*) gene (Lin et al., 1999). Independently, *prepro-orexin* knockout mice were reported to exhibit episodes of behavioral arrest similar to cataplexy and have fragmented sleep/wake pattern during the dark period (Chemelli et al., 1999). In humans, however, most cases of narcolepsy are not monogenic. Although a mutation screening of *prepro-orexin OX1R* and *OX2R* genes was carried out on 74 patients with a family history of narcolepsy, only one mutation has been identified so far, in a highly atypical patient with symptomatic onset at 6 months of age (Peyron et al., 2000). Interestingly, the early presentation of this particular case was similar to genetically induced narcolepsy in animals and therefore different from most cases of human narcolepsy, which usually start during adolescence (Bassetti and Aldrich, 1996). Recent reports indicated that narcoleptic human brains have undetectable *prepro-orexin* mRNA and markedly reduced immunocytochemical staining of orexin peptides in the perifornical area (Peyron et al., 2000; Thannickal et al., 2000), supporting an earlier report showing undetectable CSF orexin-A levels in most narcolepsy patients (Nishino et al., 2000). These results suggest either a loss of orexin-containing neurons or a lack of orexin production in these neurons if still present. Thannickal et al. (2000) reported that global loss of orexin-immunoreactive neurons was accompanied by residual gliosis in the perifornical area. With the well-established HLA association of narcolepsy (Kadotani et al., 1998), these observations suggest an autoimmune mediation of human narcolepsy with highly selective destruction of orexin-containing cells in the disorder. This mechanism would be consistent with the hypothesis that narcolepsy is caused by environmental factors that act on a susceptible genetic background (Mignot, 1998). Since orexin-containing neurons in the LHA ex-

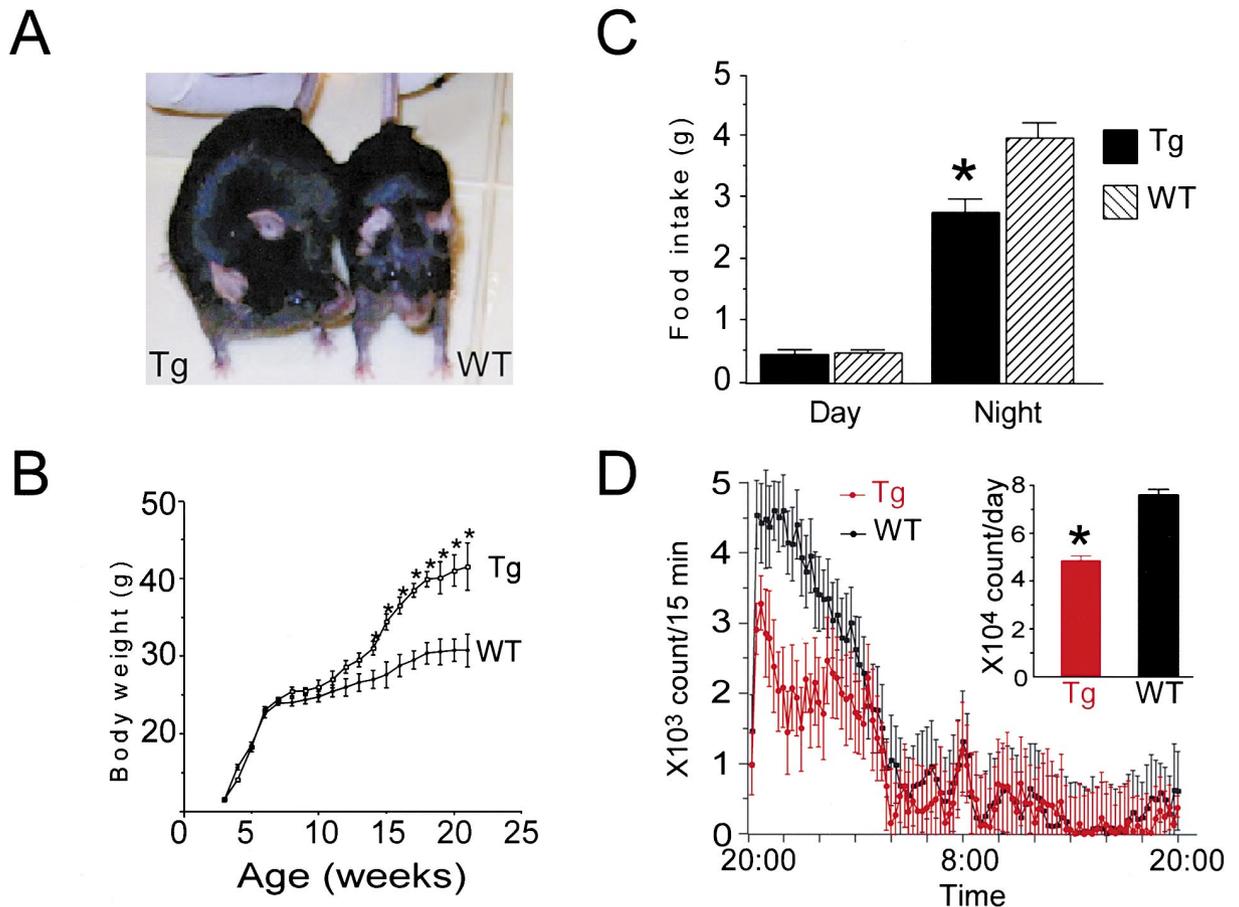


Figure 6. Abnormality in Energy Homeostasis Observed in Orexin/Ataxin-3 Transgenic Mice

(A) Photograph showing an 18-week-old orexin/ataxin-3 mouse (left) and its nontransgenic littermate (right). Note obvious obesity in the orexin/ataxin-3 mouse.

(B) Growth curves of wild-type ($n = 8$) and orexin/ataxin-3 mice ($n = 8$). *From 12 to 15 weeks, $p < 0.05$; from 16 weeks onward, $p < 0.01$.

(C) Food consumption in wild-type and orexin/ataxin-3 mice at 8 to 10 weeks of age, $p < 0.002$ ($n = 8$).

(D) Spontaneous motor activity of 10- to 12-week-old orexin/ataxin-3 mice (Tg) and their transgene-negative littermates (WT) measured by infrared monitoring system (Supermex system; Muromachi Kikai). Measured activity during 15 min intervals are plotted. Data are indicated as mean \pm S.E. ($n = 24$). Inset shows counted activity during 24 hr in orexin/ataxin-3 mice (Tg) and transgene-negative littermates (WT). $p < 0.001$ ($n = 24$). Statistical analyses were performed with ANOVA (two-way layout with repetition).

press other neuropeptides (Hakansson, et al., 1999), loss of these neurons might produce a phenotype distinct from that of *prepro-orexin* deficiency. Moreover, postnatal loss of orexins might result in a different phenotype compared with the *prepro-orexin* knockout mice that do not produce orexins at any stage of their lives.

In the present study, we generated mice in which orexin-containing neurons are genetically ablated to examine the phenotype caused by postnatal loss of orexin-containing neurons. These mice exhibited a phenotype strikingly similar 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 of *prepro-orexin* knockout mice despite their different genetic backgrounds (Orexin/ataxin-3 mice in this study are 75% C57BL/6 and 25% DBA1, while *prepro-orexin* knockout mice reported previously are 50% C57BL/6 and 50% 129SvEv). The

similarity of these two mouse models further demonstrates the importance of orexins in the regulation of the sleep/wake state. Our observations also suggest, that although orexin-containing neurons produce other neuromodulators, it is orexin that is important for the regulation of the sleep/wake state by these neurons.

The behavioral arrests seen in orexin/ataxin-3 mice typically began about 6 weeks of age. We never observed these episodes earlier than 6 weeks of age in any lines of orexin/ataxin-3 mice. On the other hand, attacks were observed in some *prepro-orexin* knockout mice earlier than 3 weeks of age (Chemelli, et al., 1999). We observed one fourth of orexin-containing neurons still exist in the hypothalamus of orexin/ataxin-3 mice at 4 weeks of age (Figure 2A). Residual orexin-containing neurons in orexin/ataxin-3 mice might function to prevent the emergence of the behavioral arrests. Thus, postnatal loss of orexin-containing neurons is more comparable to the typical narcolepsy syndrome in humans, which usually starts at adolescence.

Other than the timing of phenotypical emergence, we did not observe significant differences in the characteristics of behavioral arrests seen in orexin/ataxin-3 mice and *prepro-orexin* knockout mice. In particular, average episode frequency in the first 4 hr of the dark period is 20 ± 6.6 episodes; the duration of episodes varied from a few seconds to 150 s (Figure 4A). These are similar findings to those reported in *prepro-orexin* knockout mice (Chemelli et al., 1999). Although the overall average duration of episodes was 42.4 s, shorter than that reported in *prepro-orexin* knockout mice (65.6 s) (Chemelli et al., 1999), this discrepancy likely stems from small sample sizes and high variability of individual episode durations among and between the orexin/ataxin-3 and *prepro-orexin* knockout mouse cohorts studied. As with *prepro-orexin* null mice, attacks often occurred during grooming or excited ambulation, implicating the animal's emotional state in the onset of episodes (Figure 4B).

Sleep state patterns of orexin/ataxin-3 mice revealed by simultaneous EEG/EMG recording were very similar to that of *prepro-orexin* knockout mice (Chemelli et al., 1999), despite some differences in the genetic background. However, we observed the duration of awake state during the light period was significantly shorter in orexin/ataxin-3 mice as compared with wild-type littermates (Table 1), a difference that was not observed in *prepro-orexin* knockout mice (Chemelli et al., 1999). Although these differences might be due to small sample sizes and divergent genetic backgrounds of the transgenic and knockout mice studied, the finding of sleep abnormalities during the daytime in orexin/ataxin-3 mice is more consistent with human narcolepsy, in which sleep disruptions also occur during the rest phase (night).

Role of Orexin-Containing Neurons in Maintenance of the Sleep/Wakefulness State

Orexin-containing neurons have been shown to heavily innervate those brainstem and hypothalamic regions implicated in regulation of sleep/wake state. Such regions include the pedunculopontine and laterodorsal tegmental nuclei, locus coeruleus, dorsal raphe nucleus, ventral tegmental nucleus, and tuberomammillary nucleus (Chemelli et al., 1999; Nakamura et al., 2000). These neurons diffusely innervate cerebral cortices and thalamocortical neurons to maintain arousal (Saper, 1987).

Indeed, orexins activate adrenergic cells from the locus coeruleus (Hagan et al., 1999) and dopaminergic cells from the ventral tegmental area in vitro (Nakamura et al., 2000). Additionally, orexin neurons and monoaminergic neurons directly innervate the preoptic area of the hypothalamus, the location of the ventrolateral preoptic nucleus (VLPO). The VLPO is thought to modulate the onset of sleep via sleep-promoting neurons that can be inhibited in vitro by noradrenaline, acetylcholine and serotonin (Gallopini et al., 2000). The orexin system might interact directly with VLPO neurons, or indirectly via monoamines and acetylcholine.

Maintenance of arousal by orexin-containing neurons may thus be achieved by coordinated regulation of the cholinergic systems in the pedunculopontine nucleus and laterodorsal tegmental nucleus, and the monoaminergic systems in the tuberomammillary nucleus, dorsal

raphe nucleus, ventral tegmental area, and locus coeruleus, as well as direct or indirect inhibition of the VLPO. Consistent with this, pharmacological studies of canine narcolepsy have suggested that narcolepsy is associated with hyperactivity of cholinergic neurotransmission and decreased monoaminergic tone, which are consistent with narcoleptic symptoms including daytime sleepiness and REM abnormalities (Mignot et al., 1993). Our present observations that orexin neuron-ablated mice lose projections to sleep/wake nuclei and exhibit behavioral arrests resembling cataplexy, a tendency toward premature entry into REM sleep, and poorly consolidated sleep patterns, unequivocally indicate the essential nature of orexin-containing neurons in the maintenance of the waking state and a proper architecture of sleep/wake cycles.

Obesity in Orexin/Ataxin-3 Mice

A significant body of evidence has been accumulated for a role of orexins in the regulation of energy metabolism and autonomic function (reviewed in Willie et al., 2001). Besides increasing feeding behavior in a dose-dependent manner (Sakurai et al., 1998), central injection of orexin-A increases oxygen consumption in mice indicating an increased metabolic rate (Lubkin and Stricker-Krongrad, 1998). Orexins also increase mean arterial blood pressure and heart rate in rats when administered into the lateral ventricle or ventrolateral medulla, suggesting positive influences on sympathetic outflow (Samson et al., 1999; Chen et al., 2000; Shirasaka et al., 1999). These effects generally result in increased energy consumption.

Metabolic abnormalities in food intake and/or energy expenditure may exist in human narcolepsy. This is evidenced by increased frequencies of obesity and noninsulin dependent (Type II) diabetes in narcoleptic patients compared with a control group (Schuld et al., 2000; Honda et al., 1986). Orexin/ataxin-3 mice showed late-onset obesity (Figures 6A and 6B), consistent with human cases. However, food consumption of orexin/ataxin-3 mice was decreased by almost 30% at 8 to 10 weeks of age (Figure 6C), before obesity became apparent. Thus, reduced food intake is unlikely to be a simple compensation for increased weight gain. Rather, reduced feeding and obesity may together reflect an underlying reduction in energy expenditure due to decreased motor activity, a lower basal metabolic rate, or both. Indeed, we observed a decreased spontaneous motor activity in the dark (active) period in orexin/ataxin-3 mice (Figure 6D). Decreased basal energy expenditure may result directly from elimination of orexin-containing neurons, which normally promote energy expenditure.

The metabolic abnormality in orexin/ataxin-3 mice seems more severe than that of *prepro-orexin* knockout mice reported by Chemelli et al. (1999). *Prepro-orexin* knockout mice reportedly display normal growth rather than obesity, although they are also hypophagic (Chemelli et al., 1999; Willie et al., 2001). The observed metabolic differences between orexin/ataxin-3 mice and *prepro-orexin* knockout mice may stem from different genetic backgrounds and environmental effects. Indeed, metabolic phenotypes are known to be very sensitive to genetic background and environmental factors.

Loss of neuropeptides or modulatory factors expressed by orexin-containing neurons may also contribute to phenotypic differences in these models. In particular, dynorphins, which influence feeding behavior (Walker et al., 1980). Another intriguing possibility is that phenotypic differences result in part from fetal and/or early postnatal development in the presence or absence of orexin signaling in orexin/ataxin-3 mice and knockout mice, respectively. Additional metabolic and activity studies of these two narcoleptic mouse models, under identical environmental and genetic conditions, may clarify these issues.

Conclusion

Recent observations suggest that narcolepsy is a neurodegenerative disorder that specifically affects orexin-containing neurons (Peyron et al., 2000; Thannickal et al., 2000). As orexin-containing neurons normally express additional neuropeptides, we probed whether mice with selective postnatal degeneration of orexinergic neurons differ phenotypically from *prepro-orexin* gene knockout mice, in which development occurs in the absence of orexin signaling, and residual neuropeptide functions of these neurons may be intact. As is likely the case in human narcolepsy, orexin/ataxin-3 mice are born with orexins but lose orexin-containing neurons later in life. Accordingly, the onset of narcoleptic attacks in orexin/ataxin-3 mice is later than in *prepro-orexin* knockout mice. We also observed late-onset obesity in orexin/ataxin-3 mice.

Our findings confirm the importance of orexinergic neurons in the regulation of sleep/wake states and further suggest a role in metabolic regulation. Orexin/ataxin-3 mice may represent the most accurate pathophysiological model of narcolepsy available; this model should be valuable in the study of the symptoms, genetics, and therapy of this debilitating human sleep disorder. Our findings confirm the importance of orexinergic neurons in the regulation of sleep/wake states and further suggest a role in energy homeostasis.

Experimental Procedures

Animal Use

All experimental procedures involving animals were approved by the University of Tsukuba Animal Care and Use Committee and 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 Transgenic Mouse Lines

Truncated ataxin-3 cDNA with 77 repeats of polyglutamine was donated by Dr. Yoshizawa. This cDNA fragment encodes from amino acid 286 to the C terminus of elongated ataxin-3 from a Machado-Joseph disease patient (Yoshizawa et al., 2000), and was subcloned between *HindIII* and *KpnI* sites of pcDNA3.1 myc-His (Invitrogen) (pMJD-Q77 (Δ N286)). The transgene, termed *orexin/ataxin-3*, was constructed by swapping the *HindIII-Pml* fragment of pMJD-Q77(Δ N286) for the *nLacZ* gene (*Sall-BamHI*) fragment of the *orexin-nlacZ* transgene (Sakurai et al., 1999). As a consequence, the 3' end of this DNA fragment was followed by murine *protamine-1* (*mPrr1*) gene fragment (from +95 relative to the transcription start site to +625), which includes part of exon 1 and all of intron 1 and exon 2 including the polyadenylation signal and site. The transgene was linearized and microinjected into fertilized mouse eggs (BDF1 strain, which is the F1 of C57BL/6 and DBA1) to generate transgenic founder lines. Founder animals were mated with C57BL/6 mice to

generate N1 mice, which we used for characterization. The presence and copy numbers of the transgene in the offspring were identified by tail blot and PCR analysis using tail DNA. Four transgenic lines were established.

Histological Analysis

Mouse brains were fixed and prepared as described previously (Nambu et al., 1999). For double immunofluorescence analysis, cryostat sections (40 μ m) were cut and post-fixed 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) and mouse anti-myc monoclonal antibody (9E10; Santa Cruz) in the same solution for 1 hr at room temperature. After washing three times in PBS, the sections were incubated with FITC-conjugated goat anti-rabbit IgG and Cy-3-conjugated donkey anti-mouse IgG antibodies (Cappel) for 1 hr at room temperature. The slides were then washed three times in PBS and examined under a fluorescence microscope. For the other immunostainings, cryostat sections (40 μ m) were stained using the avidin-biotin-peroxidase method as described previously (Nambu et al., 1999). For determining numbers of orexin-containing neurons or MCH-containing neurons, we counted orexin- or MCH-immunoreactive neurons in five coronal sections throughout the hypothalamic region containing these neuronal populations. For in situ hybridization histochemistry, a 0.29 kb segment of mouse cDNA encoding Gln33-Val130 of *prepro-orexin* was generated by PCR and subcloned into pBluescript II SK (+) vector. Sense and anti-sense riboprobes were generated with T7 and T3 RNA polymerases, respectively in the presence of 33 P-CTP (Amersham). In situ hybridization of the adult mouse brain sections was performed as described previously (Chemelli et al., 1999).

Infrared Videotaping and Scoring of Narcoleptic Episodes

A digital CCD video camera with infrared capability (Sony DCR-PC3) was used for documentation and scoring of dark cycle behavior of male mice at 10 to 12 weeks of age. Food and water were available ad libitum. The first 4 hr of the dark phase were videotaped from overhead for all behavioral paradigms described. Two independent observers blinded for genotype scored open field narcoleptic episodes. Narcoleptic episodes for all experimental paradigms were strictly defined by the criteria described previously (Chemelli et al., 1999) (i.e., [a] an abrupt transition from obvious purposeful motor activity, [b] a sustained change in posture maintained throughout the episode, and [c] an abrupt end to the episode with resumption of obvious purposeful motor activity).

Spontaneous Motor Activity

Spontaneous motor activity of male mice (10 to 12 weeks of age) was continuously measured individually in a home cage with a Supermex system (Muromachi Kikai, Japan). In this system, a sensor detects the radiated body heat of an animal (Masuo et al., 1997). We measured activity counted by this system during each 15 min for four consecutive 24 hr periods. Food and water were replenished at 08:00, and mice were not otherwise disturbed in any way. Six mice were recorded concurrently in matched littermate pairs of the transgenic and wild-type controls.

EEG/EMG Recording

Male mice (14 weeks old) were anesthetized and chronically implanted for continuous monitoring of EEG/EMG as described previously (Chemelli et al., 1999). Animals were housed in a 12 hr light/dark cycle and allowed to habituate to recording conditions for 2 weeks. Each mouse was then recorded for three consecutive 24 hr periods, beginning at lights-on at 08:00. Food and water were replenished at 8:00 a.m., and the mice were not otherwise disturbed in any way. Four male transgenic mice and three matched wild-type littermates were recorded concurrently. 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, and displayed on a polygraph system. EEG/EMG records were visually scored into 20 s epochs of wakefulness, REM, and non-REM sleep according to standard criteria of rodent sleep (Radulovacki et al., 1984).

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