Orexin receptor 2 expression in the posterior hypothalamus rescues sleepiness in narcoleptic mice

Takatoshi Mochizuki[a], Elda Arrigoni[a], Jacob N. Marcus[b,c], Erika L. Clark[a], Mihoko Yamamoto[a], Michael Honer[d], Edilio Borroni[d], Bradford B. Lowell[b], Joel K. Elmquist[b,e], and Thomas E. Scammell[a,1]

[a]Department of Neurology and [b]Division of Endocrinology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; [c]Merck Research Laboratories, West Point, PA 19486; [d]F. Hoffmann-La Roche, CH-4070 Basel, Switzerland; and [e]Departments of Internal Medicine and Pharmacology, Division of Hypothalamic Research, University of Texas Southwestern Medical Center, Dallas, TX 75390

Narcolepsy is caused by a loss of orexin/hypocretin signaling, resulting in chronic sleepiness, fragmented non-rapid eye movement sleep, and cataplexy. To identify the neuronal circuits underlying narcolepsy, we produced a mouse model in which a loxP-flanked gene cassette disrupts production of the orexin receptor type 2 (OX2R; also known as HCRTR2), but normal OX2R expression can be restored by Cre recombinase. Mice lacking OX2R signaling had poor maintenance of wakefulness indicative of sleepiness and fragmented sleep and lacked any electrophysiological response to orexin-A in the wake-promoting neurons of the tuberomammillary nucleus. These defects were completely recovered by crossing them with mice that express Cre in the female germline, thus globally deleting the transcription-disrupter cassette. Then, by using an adeno-associated viral vector coding for Cre recombinase, we found that focal restoration of OX2R in neurons of the tuberomammillary nucleus and adjacent parts of the posterior hypothalamus completely rescued the sleepiness of these mice, but their fragmented sleep was unimproved. These observations demonstrate that the tuberomammillary region plays an essential role in the wake-promoting effects of orexins, but orexins must stabilize sleep through other targets.

histamine | Cre-loxP | G protein-coupled receptors | arousal

Narcolepsy is caused by a selective loss of the hypothalamic neurons producing the orexin (i.e., hypocretin) neuropeptides and is one of the most common causes of chronic sleepiness (1). In humans and mice, loss of orexin signaling results in unstable sleep/wake states, with poor maintenance of wakefulness, fragmented sleep, and intrusions into wakefulness of elements of rapid eye movement (REM) sleep, including brief episodes of paralysis known as cataplexy. The orexin neuropeptides strongly excite many brain regions that regulate sleep/wake behavior, yet the key pathways through which orexins stabilize wakefulness and sleep remain unknown.

Orexins act through two receptors, OX1R and OX2R (also known as HCRTR1 and HCRTR2), and the OX2R seems to play a critical role in the maintenance of wakefulness. Mice constitutively lacking OX2R are unable to maintain long bouts of wakefulness and can fall asleep rapidly (2). In addition, an OX2R antagonist strongly promotes sleep, whereas an OX1R antagonist has no effect (3).

Although it is clear that OX2R signaling is necessary for the normal maintenance of wakefulness, the anatomic sites through which this occurs remain unknown. OX2R is expressed in many wake-promoting brain regions, including the histaminergic neurons of the tuberomammillary nucleus (TMN), other monoaminergic regions, cholinergic systems, and forebrain regions, including the thalamus and cortex (4). Several researchers have hypothesized that the TMN is a key site because orexin-A excites the TMN neurons and infusion of orexin-A near this region promotes wakefulness (5–7). However, this perspective is controversial as optogenetic activation of the orexin neurons promotes arousal in mice lacking histamine (8), and mice lacking

both OX1R and histamine H1 receptors have normal sleep/wake behavior (9).

The main goal of the present study was to determine the key pathways through which OX2R signaling promotes wakefulness. We hypothesized that orexins stabilize wake through the TMN and adjacent regions. In addition, we predicted that if narcoleptic mice could produce longer periods of wakefulness, they would develop stronger homeostatic sleep drive and their fragmented sleep would become more consolidated. To define these pathways, we produced mice with a loxP-flanked transcription-disrupter (TD) gene cassette that prevents expression of functional OX2R, but OX2R signaling in these mice can be reactivated by Cre recombinase. The advantage of this reactivation approach is that OX2R expression can occur only in neurons that would normally express the receptor because the OX2R gene is driven by the native promoter. We then induced local expression of OX2R by microinjecting an adeno-associated viral vector (AAV) coding for Cre recombinase (AAV-Cre) to determine whether focal rescue of orexin signaling in and around the TMN improves the sleepiness and fragmented sleep of narcolepsy.

Results

Generation of OX2R Transcription-Disrupted Mice. We inserted a loxP-flanked TD cassette into intron 1, 137 bp upstream of exon 2 (Fig. 1). RT-PCR with cDNA prepared from the hypothalamus demonstrated that OX2R TD mice lacked normal *Hcrtr2* (*Ox2r*) mRNA, but instead produced mutant mRNA containing a 115- or 187-bp fragment of the *engrailed 2* (*En2*) splice acceptor of the TD cassette between exons 1 and 2. These abnormal inserts should cause frame shifts and prevent expression of functional OX2R receptors.

Electrophysiological Characterization of Disrupted OX2R. To test the function of the disrupted OX2R directly, we performed patch-clamp recordings of TMN neurons. The basic electrophysiological properties of TMN neurons were identical in WT and OX2R TD mice, including spontaneous activity, a relatively broad action potential as a result of activation of Ca^{2+} conductances, a robust outward transient rectification by "A"-type K^+ current, and a depolarizing sag during hyperpolarizing pulses produced by activation of a hyperpolarization-activated current (Fig. 2 and Table S1). All neurons from WT mice responded to orexin-A (300 nM) with a marked increase in their firing rate associated with membrane depolarization. These characteristics are all very
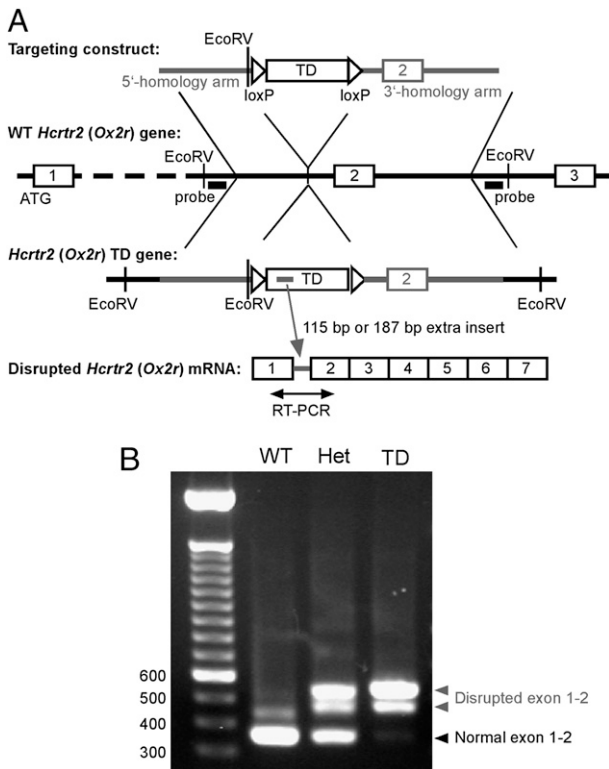
NEUROSCIENCE

Fig. 1. Generation of OX2R TD mice. (*A*) The loxP-flanked TD cassette was inserted into intron 1 of the *Hcrtr2* (*Ox2r*) gene encoding OX2R. The resulting *Hcrtr2* mRNA contains parts of the TD cassette (115 or 187 bp) between exons 1 and 2, causing frame shifts and preventing expression of functional OX2R. Cre-mediated removal of the TD cassette results in normal *Hcrtr2* mRNA. (*B*) RT-PCR of the exon 1–2 boundary region shows the longer *Hcrtr2* mRNA in homozygous and heterozygous OX2R TD mice.

similar to those previously reported in rat TMN neurons (6, 10–13). In contrast, TMN neurons from OX2R TD mice failed to respond to orexin-A, demonstrating a lack of functional OX2R.

To restore OX2R function throughout the animal, we crossed OX2R TD mice with Zp3-Cre mice, which express Cre recombinase in the female germline. The offspring lacked the entire TD cassette, and TMN neurons from these mice had completely normal responses to orexin-A (Fig. 2 and Table S1). These findings demonstrate that the disruption of OX2R expression is reversible, and Cre restores functional OX2R signaling. These results also confirm that TMN neurons respond to orexin-A only through the OX2R.

Poor Maintenance of Wakefulness and Fragmented Sleep in OX2R TD Mice. Wake and non-REM (NREM) sleep bouts in OX2R TD mice were significantly shorter and occurred more frequently than in normal mice, especially during the dark period (Fig. 3). In WT mice, most wakefulness during the dark period occurred in bouts longer than 32 min, but OX2R TD mice had much less wakefulness in these long bouts and much more in bouts of intermediate length (4–16 min) instead. Despite this fragmented behavior, OX2R TD mice had normal hourly amounts of wake and NREM and REM sleep (Fig. S1). Cataplexy occurs frequently in orexin-KO mice (14, 15) but was very rare in OX2R TD mice. This pattern of short wake bouts and fragmented sleep is very similar to that described in constitutive OX2R-KO mice (2).
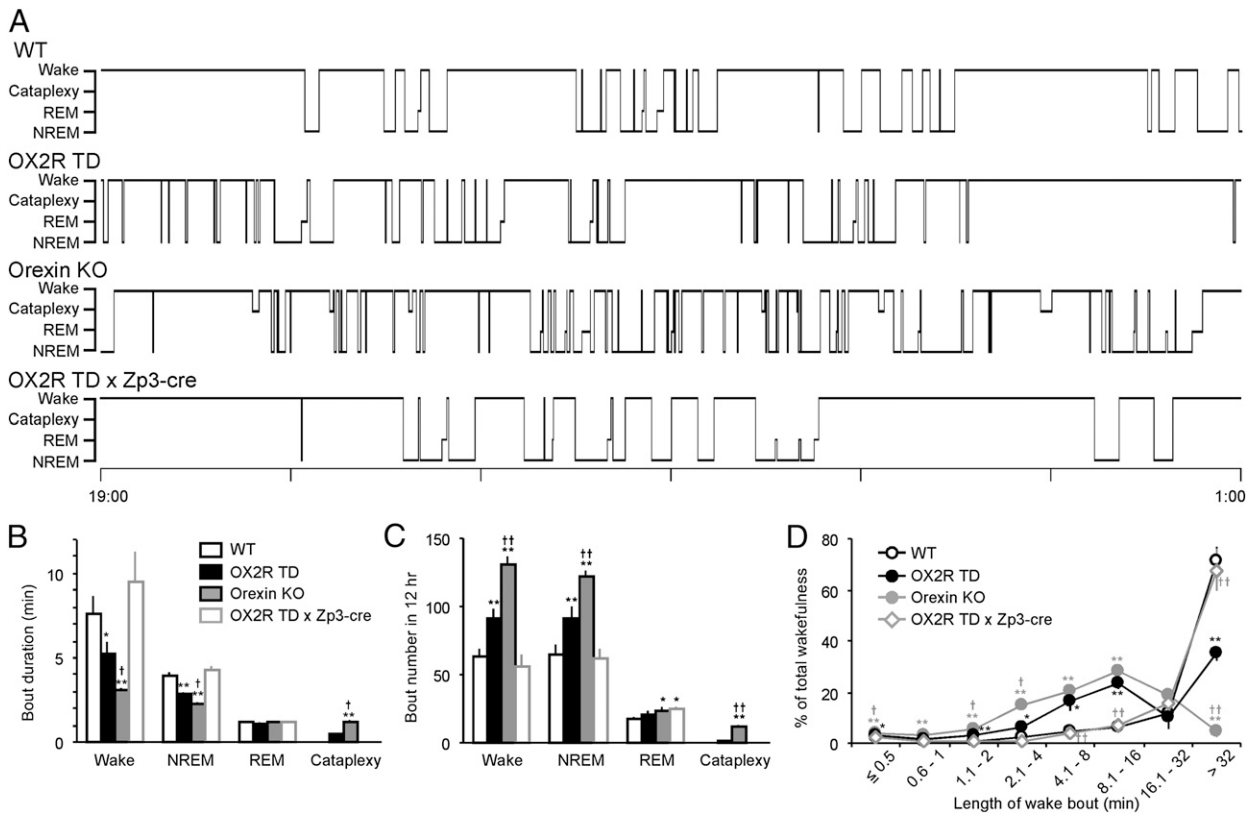
OX2R TD mice have frequent transitions from wake into NREM sleep. This might be caused by increased homeostatic sleep drive, but OX2R TD mice had normal EEG power spectra during NREM and REM sleep during baseline recordings (Fig.
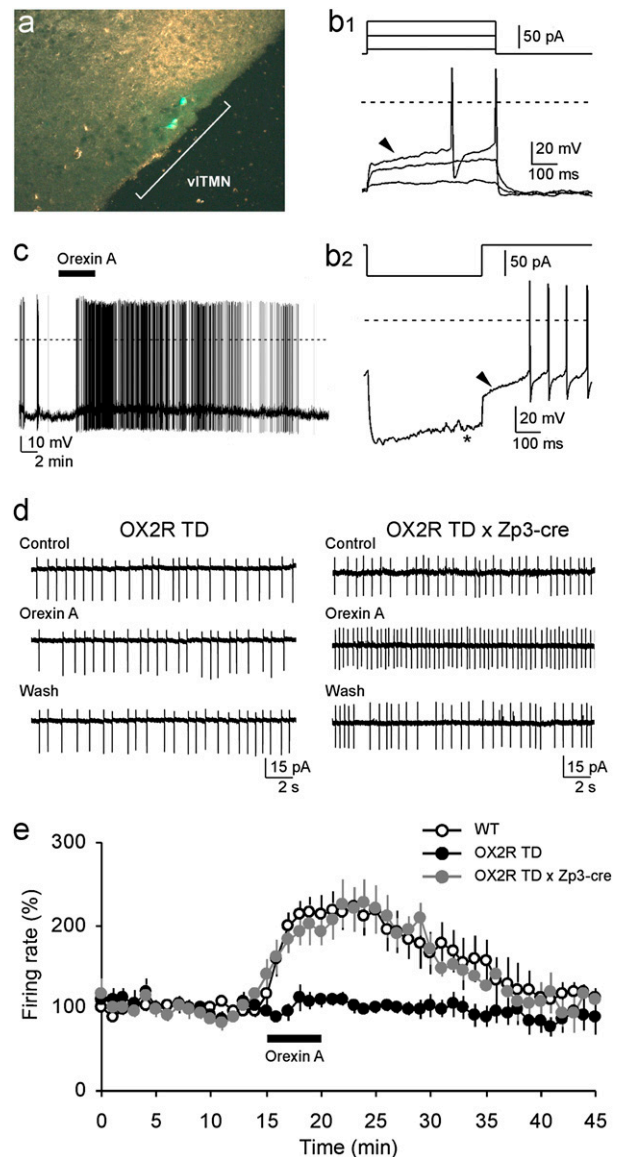
Fig. 2. OX2R receptors are necessary for the excitation of TMN neurons by orexin-A. (*A*) Three recorded neurons labeled with Lucifer yellow in the ventrolateral subnucleus of the TMN (vlTMN). (*B*, *1–2*) Responses of TMN neurons to depolarizing and hyperpolarizing pulses. TMN neurons display both "A"-type K⁺ current (arrowheads) and an hyperpolarization-activated current (star). (*C*) Application of orexin-A (300 nM) depolarizes and increases the firing rate of a TMN neuron from WT mice. (*D*) Orexin-A has no effect on TMN neurons from OX2R TD mice, but it excites TMN neurons from OX2R TD × Zp3-Cre mice, showing that OX2R function is restored. (*E*) The firing rate of WT TMN neurons ($n = 9$) more than doubles in response to orexin-A and remains increased for 15 min. TMN neurons from OX2R TD mice ($n = 8$) show no response to orexin-A, whereas TMN neurons from OX2R TD × Zp3-Cre ($n = 7$) respond just like WT neurons.

S2). To test homeostatic sleep pressure further, we deprived OX2R TD mice of sleep for the first 4 h of the light period. Their responses to sleep deprivation were normal, with longer NREM bouts and increased EEG delta power, very similar to WT mice (Fig. S2). OX2R TD mice also had normal daily rhythms of locomotor activity and core body temperature (Fig. S3).

To determine whether global deletion of the TD cassette could rescue sleep/wake behavior, we analyzed sleep recordings in OX2R TD × Zp3-Cre mice. These mice had completely normal sleep/wake behavior, with long wake bouts during the

Fig. 3. OX2R TD mice have fragmented wakefulness and sleep. (*A*) Representative hypnograms from the first 6 h of the dark period show that WT mice have long periods of wake and consolidated periods of sleep, whereas OX2R TD mice have frequent transitions between wake and sleep. Orexin-KO mice have even more fragmented behavior than OX2R TD mice. Cataplexy occurs frequently in orexin KO mice but is very rare in OX2R TD mice. OX2R TD × Zp3-Cre mice, with global restoration of OX2R, have normal sleep/wake architecture. (*B* and *C*) OX2R TD mice ($n = 8$) have shorter and more frequent wake and NREM sleep bouts than WT mice ($n = 8$) in the dark period, and orexin-KO mice ($n = 11$) have even shorter bouts. OX2R TD × Zp3-Cre mice ($n = 7$) have completely normal sleep/wake bouts. (*D*) Time-weighted frequency histograms showing the proportion of wake bouts of each length to the total amount of wakefulness in the dark period indicate that 70% of wake in WT mice occurs in bouts longer than 32 min, but in OX2R TD and orexin-KO mice, most wake occurs in bouts of intermediate length. The production of long wake bouts is completely rescued in OX2R TD × Zp3-Cre mice (*$P < 0.05$ and **$P < 0.01$ vs. WT mice; †$P < 0.05$ and ††$P < 0.01$ vs. OX2R TD mice).

dark period and consolidated sleep (Fig. 3). This clearly indicates that Cre recombinase can fully restore OX2R signaling.

Focal Rescue of OX2R Signaling in TMN Region Improves Fragmented Wakefulness. We then bilaterally microinjected 50 nL AAV-Cre into the TMN region to determine whether local restoration of OX2R signaling could normalize sleep/wake behavior. These microinjections induced expression of Cre in neurons of the TMN and adjacent parts of the posterior hypothalamus, including the lateral mammillary nucleus, the supramammillary nucleus (SuM), and the most caudal parts of the lateral hypothalamus (Fig. 4*A*). In some mice, we mapped the expression of OX2R by using the highly selective OX2R antagonist N-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonyl)-amino]-N-pyridin-3-ylmethyl-acetamide (EMPA) (16). In WT mice, [³H]EMPA labeled neurons in a pattern very similar to that reported for *Hcrtr2* (*Ox2r*) mRNA (4), but in OX2R TD mice injected with AAV-Cre, [³H]EMPA labeling was limited to just the TMN and adjacent SuM (Fig. 4*B*).

These AAV-Cre microinjections markedly improved the maintenance of wakefulness, with most wakefulness during the dark period occurring in bouts lasting more than 32 min, just as in WT mice (Fig. 4*D*). Control OX2R TD mice injected with an AAV coding for GFP (AAV-GFP) showed no improvement. The rescue with AAV-Cre also appeared to be specific to the TMN/SuM region, as more dorsal injections of AAV-Cre into the posterior thalamus failed to improve wakefulness.

Surprisingly, even though microinjections of AAV-Cre in the TMN/SuM normalized the maintenance of wakefulness, these mice continued to have fragmented sleep, with NREM bouts that were clearly shorter than normal (Fig. S4). These results suggest that OX2R signaling in the TMN/SuM maintains wakefulness, but consolidation of sleep must be regulated by other regions.

Discussion

These studies show that OX2R signaling in the TMN region is sufficient for the normal maintenance of wakefulness. We found that orexin-A excites TMN neurons through the OX2R, and focal restoration of orexin signaling in the TMN/SuM of OX2R TD mice produced wake bouts just as long as those seen in WT mice.

Most likely, this marked improvement in sleepiness is mediated by OX2R excitation of histaminergic neurons in the TMN. TMN neurons appear to play an essential role in promoting wakefulness as inactivation of the TMN region of cats with muscimol potently increases sleep (17), and mice lacking histamine have less wakefulness at the beginning of the dark period (18, 19). Orexins excite TMN neurons (6, 7), and microdialysis of orexin-A near the TMN increases wakefulness and induces release of histamine in the cortex (5). In addition, the orexin-induced increase in wake is absent in mice lacking histamine H1 receptors (5) and is blocked by an H1 receptor antagonist (7). Of clinical relevance, hypocretin-deficient individuals with narcolepsy have a 44% reduction

NEUROSCIENCE
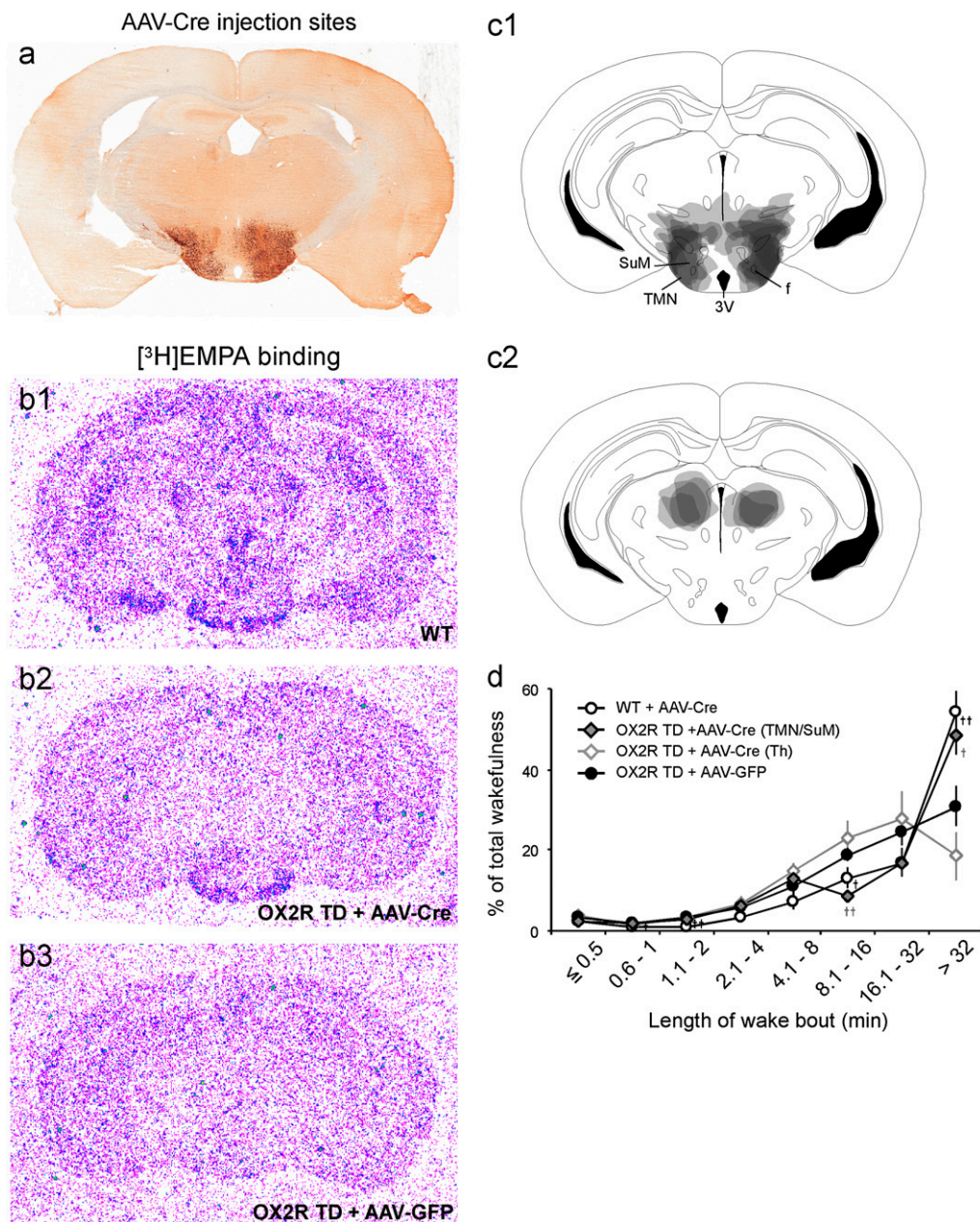
Fig. 4. Microinjection of AAV-Cre into the TMN and adjacent parts of the posterior hypothalamus of OX2R TD mice consolidates wakefulness. (*A*) Cre-immunoreactive neurons are confined bilaterally to the TMN and adjacent area, including the SuM. (*B*) Total binding signals of [³H]EMPA in a WT mouse (*1*) show the localization of OX2R in the TMN and SuM as well as in parts of the thalamus, hippocampus, and cerebral cortex, but in an OX2 TD mouse injected with AAV-Cre (*2*), binding is limited to the TMN/SuM area. An OX2R TD mouse injected with AAV-GFP (*3*) shows no [³H]EMPA binding. (*C*) Superimposed mapping images of Cre-immunoreactive neurons in the TMN/SuM group (*1*; *n* = 8) and in the posterior thalamus (*2*; *n* = 4) as an anatomical control group. (*D*) Time-weighted frequency histograms of wake bout lengths in the dark period. Almost half of all wake occurs in bouts longer than 32 min in OX2R TD mice with AAV-Cre injection into the TMN/SuM, just as in WT mice (*n* = 7). Injections of AAV-Cre into the thalamus (Th) result in no improvement ([†]$P < 0.05$ and [††]$P < 0.01$ vs. control OX2R TD mice injected with AAV-GFP; *n* = 8).

in cerebrospinal fluid (CSF) histamine concentrations (20). These observations, combined with our studies on the focal expression of OX2R, strongly suggest that orexins can promote wake by activating neurons of the TMN.

The present observations do not rule out the possibility that orexins may also promote wake through pathways outside the TMN/SuM. For example, orexins can increase wake when injected near the basal forebrain, locus coeruleus, and laterodorsal tegmental nucleus (21–23). These or other sites may also be capable of mediating the normal wake-promoting effects of orexins, as

optogenetic stimulation of the orexin neurons promotes arousal, even in mice lacking histamine (8). However, these previous studies may not reflect normal orexin signaling because they used high concentrations of orexin peptides, and intense optogenetic activation of the orexin neurons that may be supraphysiologic. In contrast, the present study used global and focal restoration of normal OX2R signaling, so orexin peptide levels should be close to normal.

A major strength of the reactivation approach is that expression of OX2R is eutopic because it is driven by its native pro-

Mochizuki et al.

www.manaraa.com

moter. Thus, focal expression of Cre recombinase can restore normal OX2R signaling in the TMN or other specific nuclei. However, it is challenging to limit microinjections of AAV-Cre to small nuclei, and several of our injections extended into the SuM, an area that contains OX2R-expressing neurons (4). The role of these cells in the control of wakefulness has not yet been tested directly, but they could promote arousal through their projections to the cortex and basal forebrain (24, 25). In future studies, it will be important to determine selectively the role of the TMN by crossing OX2R TD mice with mice that express Cre only in histaminergic neurons.

These results provide an improved understanding of how orexins promote wakefulness, but the sites through which orexins stabilize sleep remain unknown. Global restoration of OX2R signaling in OX2R TD × Zp3-Cre mice normalized sleep, but surprisingly, OX2R TD mice rescued with AAV-Cre in the TMN/SuM continued to have fragmented sleep despite a complete improvement in their maintenance of wakefulness. This dissociation suggests that sleep fragmentation is not a consequence of shorter wake bouts and low homeostatic sleep drive, but instead, orexins must stabilize sleep through other mechanisms. How orexins influence sleep is one of the more challenging questions in orexin neurobiology, as, during sleep, the orexin neurons are generally inactive and extracellular levels of orexin-A are low (26–28). It may be that orexins produce lasting changes in neuronal activity that persist into sleep, or perhaps low synaptic levels of orexins during sleep still excite OX2R-expressing neurons.

Compared with orexin-KO mice, OX2R TD mice had a less severe narcolepsy phenotype. OX2R TD mice had fragmented wakefulness and sleep, with bout lengths intermediate between that of orexin KO and WT mice, and their behavior was very similar to that previously described in constitutive OX2R-KO mice (2). Unlike orexin-KO mice, OX2R TD mice had no reduction in locomotor activity and only rare cataplexy. These results clearly indicate that a lack of OX2R signaling can produce moderate to severe sleepiness, but a complete loss of orexin signaling is necessary for the full narcolepsy phenotype in mice. Several orexin antagonists are under development for the treatment of insomnia, and selective OX2 antagonists may be an excellent approach for the induction of sleepiness without concern of cataplexy (3).

Sleepiness is the symptom that most disrupts the lives of people with narcolepsy, and these experiments highlight the importance of orexin signaling in the TMN/SuM. It will be essential to determine just which neurons in this region promote wakefulness because stimulation of these target cells may provide a highly effective therapy for improving the sleepiness of narcolepsy and other sleep disorders.

Materials and Methods

All experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the institutional animal care and use committees of Beth Israel Deaconess Medical Center and Harvard Medical School. All efforts were made to minimize the number of animals used and their suffering.

Generation of OX2R Transcription-Disrupted Mice. We generated the targeting construct by using ET cloning and related techniques in EL250 bacterial cells (29, 30). Previously, we produced the loxP-flanked TD cassette that includes an engrailed 2 (En2) splice acceptor, SV40 enhancer, Neo, and a Myc-associated zinc finger protein-binding site (MAZ) (31, 32). To produce OX2R TD mice, we used the 4C8 clone from the RPCI-22 mouse bacterial artificial chromosome library (Invitrogen), which carries at least 25 kb upstream of exon1 and 10 kb downstream of exon 7 of the Hcrtr2 (Ox2r) gene. We inserted the TD cassette into intron 1, 137 bp upstream of exon 2 (Fig. 1). The targeting construct, consisting of a 4.0-kb 5′-homology arm, the 2.9-kb floxed TD cassette (with an extra EcoRV site next to the first loxP site), and a 4.0-kb 3′-homology arm, was electroporated into ES cells. Clones with

correct recombination were identified by Southern blot analysis by using EcoRV digestion of ES cell genomic DNA. The selected ES cell clones were expanded and injected into C57 BL/6 blastocysts. The chimeric animals with successful germline transmission were identified by mating with C57 BL/6J mice and genotyping their F1 offspring for the floxed TD Ox2r allele by using tail DNA and PCR primer pairs of 5′-CTGCCTCCCAAGGCTAAGAT-3′ (common) and 5′-GGGACTGTCCAAAGAACCAA-3′ for the WT allele or 5′-ACCTGGTTGTCATGGAGGAG-3′ for the TD allele. For the behavioral studies, these F1 heterozygotes of mixed genetic background were backcrossed with C57 BL/6J mice for five generations.

Surgery for Sleep Recordings and Microinjection of AAV. Male, 11-wk-old OX2R TD mice ($n = 28$) and WT littermates ($n = 15$) were anesthetized with ketamine/xylazine (100 and 10 mg/kg i.p.) and implanted with EEG and electromyography (EMG) electrodes as described previously (15). EEG signals were recorded by using two ipsilateral stainless steel screws [anteroposterior (AP), 1.0 mm; lateral, 1.5 mm from bregma and lambda, respectively]. EMG signals were acquired by a pair of multistranded stainless steel wires inserted into the neck extensor muscles. A telemetric temperature and locomotor activity transmitter (TA10TA-F20; Data Sciences International) was placed in the peritoneal cavity.

To delete the loxP-flanked TD cassette and focally restore OX2R expression, OX2R TD mice were then injected with recombinant AAV-Cre (serotype 10) (33). With a glass micropipette and air pressure injector system, eight OX2R TD mice were injected with 50 nL (1.2×10^{12} particles/mL) of AAV-Cre into the TMN region bilaterally (AP, −2.54 mm; 1.0 mm lateral; ventral, 4.9 mm from bregma). As controls, seven WT mice were also injected with AAV-Cre, and another eight OX2R TD mice were injected with AAV-GFP (serotype 2). As anatomical controls, four OX2R TD mice were injected with AAV-Cre into the posterior thalamus region bilaterally (AP, −2.54 mm, 1.0 mm lateral; ventral, 2.8 mm from bregma).

Crossing OX2R TD Mice with Zp3-Cre Mice. To rescue OX2R signaling globally, OX2R TD mice were crossed with Zp3-Cre mice (Jackson Laboratory), which express Cre recombinase in the female germline. The deletion of the TD cassette and one loxP site was confirmed in the offspring by genotyping tail DNA with the use of: (i) the primer set for the WT allele, which results in a product that is 34 bp longer than normal and (ii) the primer set for the TD allele to confirm lack of the TD cassette.

Recording and Analysis of Sleep. Nine days after surgery, mice were transferred to individual recording cages and acclimated to the recording cables for another 5 d under a 12:12 h light:dark cycle (lights on at 7:00 AM) and at a constant temperature of approximately 23 °C. The EEG/EMG signals were acquired by using model 12 amplifiers (Grass) and digitized at 128 Hz by using Sleep Sign software (Kissei Comtec). The EEG/EMG signals were digitally filtered (EEG, 0.3–30 Hz; EMG, 2–50 Hz) and semiautomatically scored in 10-s epochs as wake, NREM sleep, or REM sleep. This preliminary scoring was visually inspected and corrected when appropriate. Cataplexy was scored in line with previously published criteria and simultaneously captured video data (34).

To examine the ability of mice to maintain behavioral states, we analyzed the distribution of behavioral states as a function of bout length (e.g., Figs. 3D and 4D). We separated bouts into eight bins of increasing duration. We then produced time-weighted frequency histograms by time-weighting the amount of state in each bin and normalizing by the total amount of the state.

After the baseline 24-h recordings, we deprived mice of sleep by gentle handling for 4 h starting at light onset. Sleep/wake behavior was then recorded for the following 20 h.

Immunohistochemistry. To map the AAV-Cre and AAV-GFP injection sites, we immunostained brain sections as described previously (35). In brief, 30-μm coronal sections were incubated overnight with rabbit anti-Cre (1:10,000; Novagen) or anti-GFP (1:10,000; Molecular Probes) antiserum diluted in PBS with 0.25% Triton X-100 and 0.02% sodium azide. Next, the sections were incubated for 1 h in biotinylated anti-rabbit secondary antiserum (Jackson ImmunoResearch) at 1:500. Tissue was then reacted with avidin–biotin complex (1:500; Vectastain ABC Elite kit; Vector Laboratories), and immunoreactive cells were visualized by reaction with 3,3′-diaminobenzidine and 3% hydrogen peroxide (Vector Laboratories).

Receptor Autoradiography. To map the distribution/reactivation of OX2R, we conducted receptor autoradiography by using [^{3}H]-labeled EMPA, a highly selective OX2R antagonist (16). We microinjected OX2R TD mice with AAV-

Mochizuki et al.

NEUROSCIENCE

www.manaraa.com

Cre ($n = 3$) or AAV-GFP ($n = 2$) and used WT mice ($n = 2$) as normal controls. Briefly, 10-μm coronal sections were incubated for 60 min with 5 nM [^{3}H] EMPA in assay buffer containing (in mM): CaCl$_2$ 1, MgCl$_2$ 5, and Hepes 25, pH 7.4. The sections were exposed to tritium-sensitive imaging plates (BAS-TR2025; Fuji Film) for 5 d, then scanned by BAS-5000 (Fuji Film) and analyzed with an MCID M2 image analysis system (Imaging Research).

Patch-Clamp Recordings. Coronal slices (230 μm) of 2- to 3-wk-old mice (WT, $n = 9$; OX2R TD, $n = 8$; OX2R TD × Zp3-Cre, $n = 7$) were cut with a vibrating microtome in ice-cold artificial CSF containing (in mM): NaCl 124, KCl 2, KH$_2$PO$_4$ 3, MgCl$_2$ 1.3, CaCl$_2$ 2.5, NaHCO$_3$ 26, and glucose 10, pH 7.4. Slices were recorded submerged and perfused with oxygenated artificial CSF (2 mL/min) maintained at 32 °C. Patch-clamp recordings were performed in cell-attached mode and whole-cell mode by using a Multiclamp 700A amplifier (Molecular Devices) (36). Signals were digitized by using a Digidata 1322A interface and acquired using Clampex 9.0 software (Molecular Devices). Patch electrodes were filled with (in mM): K-gluconate 120, KCl 10, MgCl$_2$ 3, Hepes 10, K-ATP 2, and Na-GTP 0.2, pH 7.2. Lucifer yellow CH ammonium salt (0.1%) was added to the pipette solution. Cell-attached recordings were performed in voltage clamp mode. Signals were low-pass filtered at 800 Hz and digitized at 10 kHz. Orexin-A (300 nM; Tocris) was bath-applied to the recording chamber. After the recordings in cell-attached

mode, we switched to whole-cell configuration for the electrophysiological characterization of the neurons and to label them with Lucifer yellow. Whole-cell recordings were performed in current-clamp mode. Signals were low-pass filtered at 20 kHz and digitized at 40 kHz. Series resistance was monitored at regular intervals with current pulses (−5 to −10 nA; 50–100 ms). Data were analyzed by using Clampfit 9.0 (Molecular Devices) and IGOR Pro-4.0 (WaveMetrics) software.

Statistical Analysis. All results are expressed as means ± SEM. Changes in behavioral state were compared across groups by using one-way factorial ANOVA with a post-hoc Fisher protected least significant difference test. Time-weighted frequency histograms were compared between WT mice and other groups by using two-way, repeated-measures ANOVA with a post-hoc, two-tailed Student t test. Electrophysiologic responses were compared by using one-way ANOVA.

1. Dauvilliers Y, Arnulf I, Mignot E (2007) Narcolepsy with cataplexy. *Lancet* 369:499–511.
2. Willie JT, et al. (2003) Distinct narcolepsy syndromes in orexin receptor-2 and Orexin null mice: Molecular genetic dissection of Non-REM and REM sleep regulatory processes. *Neuron* 38:715–730.
3. Dugovic C, et al. (2009) Blockade of orexin-1 receptors attenuates orexin-2 receptor antagonism-induced sleep promotion in the rat. *J Pharmacol Exp Ther* 330:142–151.
4. Marcus JN, et al. (2001) Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 435:6–25.
5. Huang ZL, et al. (2001) Arousal effect of orexin A depends on activation of the histaminergic system. *Proc Natl Acad Sci USA* 98:9965–9970.
6. Eriksson KS, Sergeeva O, Brown RE, Haas HL (2001) Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. *J Neurosci* 21:9273–9279.
7. Yamanaka A, et al. (2002) Orexins activate histaminergic neurons via the orexin 2 receptor. *Biochem Biophys Res Commun* 290:1237–1245.
8. Carter ME, Adamantidis A, Ohtsu H, Deisseroth K, de Lecea L (2009) Sleep homeostasis modulates hypocretin-mediated sleep-to-wake transitions. *J Neurosci* 29:10939–10949.
9. Hondo M, et al. (2010) Histamine-1 receptor is not required as a downstream effector of orexin-2 receptor in maintenance of basal sleep/wake states. *Acta Physiol (Oxf)* 198:287–294.
10. Haas HL, Reiner PB (1988) Membrane properties of histaminergic tuberomammillary neurones of the rat hypothalamus in vitro. *J Physiol* 399:633–646.
11. Greene RW, Haas HL, Reiner PB (1990) Two transient outward currents in histamine neurones of the rat hypothalamus in vitro. *J Physiol* 420:149–163.
12. Stevens DR, Eriksson KS, Brown RE, Haas HL (2001) The mechanism of spontaneous firing in histamine neurons. *Behav Brain Res* 124:105–112.
13. Bayer L, et al. (2001) Orexins (hypocretins) directly excite tuberomammillary neurons. *Eur J Neurosci* 14:1571–1575.
14. Chemelli RM, et al. (1999) Narcolepsy in orexin knockout mice: Molecular genetics of sleep regulation. *Cell* 98:437–451.
15. Mochizuki T, et al. (2004) Behavioral state instability in orexin knock-out mice. *J Neurosci* 24:6291–6300.
16. Malherbe P, et al. (2009) Biochemical and behavioural characterization of EMPA, a novel high-affinity, selective antagonist for the OX(2) receptor. *Br J Pharmacol* 156:1326–1341.
17. Lin JS, Sakai K, Vanni-Mercier G, Jouvet M (1989) A critical role of the posterior hypothalamus in the mechanisms of wakefulness determined by microinjection of muscimol in freely moving cats. *Brain Res* 479:225–240.
18. Parmentier R, et al. (2002) Anatomical, physiological, and pharmacological characteristics of histidine decarboxylase knock-out mice: Evidence for the role of brain histamine in behavioral and sleep-wake control. *J Neurosci* 22:7695–7711.
19. Anaclet C, et al. (2009) Orexin/hypocretin and histamine: Distinct roles in the control of wakefulness demonstrated using knock-out mouse models. *J Neurosci* 29:14423–14438.
20. Nishino S, et al. (2009) Decreased CSF histamine in narcolepsy with and without low CSF hypocretin-1 in comparison to healthy controls. *Sleep* 32:175–180.
21. Bourgin P, et al. (2000) Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. *J Neurosci* 20:7760–7765.
22. España RA, Baldo BA, Kelley AE, Berridge CW (2001) Wake-promoting and sleep-suppressing actions of hypocretin (orexin): basal forebrain sites of action. *Neuroscience* 106:699–715.
23. Xi MC, Morales FR, Chase MH (2001) Effects on sleep and wakefulness of the injection of hypocretin-1 (orexin-A) into the laterodorsal tegmental nucleus of the cat. *Brain Res* 901:259–264.
24. Saper CB (1985) Organization of cerebral cortical afferent systems in the rat. II. Hypothalamocortical projections. *J Comp Neurol* 237:21–46.
25. Vertes RP (1992) PHA-L analysis of projections from the supramammillary nucleus in the rat. *J Comp Neurol* 326:595–622.
26. Yoshida Y, et al. (2001) Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light-dark cycle and sleep-wake activities. *Eur J Neurosci* 14:1075–1081.
27. Lee MG, Hassani OK, Jones BE (2005) Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. *J Neurosci* 25:6716–6720.
28. Mileykovskiy BY, Kiyashchenko LI, Siegel JM (2005) Behavioral correlates of activity in identified hypocretin/orexin neurons. *Neuron* 46:787–798.
29. Copeland NG, Jenkins NA, Court DL (2001) Recombineering: A powerful new tool for mouse functional genomics. *Nat Rev Genet* 2:769–779.
30. Liu P, Jenkins NA, Copeland NG (2003) A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* 13:476–484.
31. Balthasar N, et al. (2005) Divergence of melanocortin pathways in the control of food intake and energy expenditure. *Cell* 123:493–505.
32. Zigman JM, et al. (2005) Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J Clin Invest* 115:3564–3572.
33. Lazarus M, et al. (2007) EP3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses. *Nat Neurosci* 10:1131–1133.
34. Scammell TE, Willie JT, Guilleminault C, Siegel JM; International Working Group on Rodent Models of Narcolepsy (2009) A consensus definition of cataplexy in mouse models of narcolepsy. *Sleep* 32:111–116.
35. Yoshida K, McCormack S, España RA, Crocker A, Scammell TE (2006) Afferents to the orexin neurons of the rat brain. *J Comp Neurol* 494:845–861.
36. Scammell TE, et al. (2003) Focal deletion of the adenosine A1 receptor in adult mice using an adeno-associated viral vector. *J Neurosci* 23:5762–5770.

www.manaraa.com