Pharmacogenetic dissection of neural mechanisms underlying the regulation of sleep-wakefulness using DREADDs

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Pharmacogenetic dissection of neural mechanisms underlying the regulation of sleep/wakefulness using DREADDs

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Abstract

Sleep and wakefulness are controlled by a complex network of neurons harboring diverse neurochemical characteristics, including glutamatergic, GABAergic, monoaminergic, cholinergic, and peptidergic neurons. To understand the precise role of each type of neuron in this circuit, it is useful to artificially manipulate the activity of a particular type of neuron to see its effect on behavior. The DREADD system has made such a strategy possible. Here, we review our recent work using DREADD to pharmacogenetically dissect neural mechanisms regulating sleep/wakefulness, describe the protocol we used, and discuss the technical aspects of our studies.

Key words: Sleep/wakefulness, Orexin, Narcolepsy, Neuropeptide, Preoptic area, Hypothalamus, Transgenic mice

1. Introduction

Designer receptors exclusively activated by designer drugs (DREADD) are artificially generated mutants of human muscarinic receptors that lose the ability to bind natural ligands, acetylcholine, while they gain the ability to be activated by the otherwise pharmacologically inert ligand clozapine-N-oxide (CNO) with nanomolar potency (1, 2). The stimulatory DREADD, designated "hM3Dq," couples with the Gq pathway to depolarize neurons, while the inhibitory DREADD, "hM4Di," couples with the Gi/o pathway to hyperpolarize neurons. Therefore, in combination with genetic techniques to target expression of exogenous genes, DREADDs function as powerful tools of neuroscience research to artificially manipulate the activity of a certain population of neurons to reveal its physiological and pathological roles.

Compared to optogenetics (3), which utilizes photoactivatable cation channels, ion pumps, or GPCRs, which manipulate neural activities, the time resolution of DREADD technology is limited. On the other hand, it is less invasive and its effects last for longer periods, which may be suitable for studying events occurring on a time scale of minutes to hours, such as sleep/wakefulness cycles. In addition, since CNO is a stable substance that can cross the blood brain barrier, it can be readily administered intraperitoneally (i.p.) or orally while only minimally interfering with the behavioral state in freely-moving animals.

We have been studying the neural mechanisms underlying the regulation of sleep/wakefulness since our discovery of the neuropeptide orexin (4, 5)(further explained later). Proper wakefulness and vigilance are essential for us to behave in an appropriate manner, while sleep is vital for the normal function and homeostasis of our brains, as well as for efficient memory formation. Thus, understanding the regulation of

the sleep/wakefulness cycle is of tremendous importance. Since the development of DREADD technology by Dr. Bryan Roth's group at the University of North Carolina (1, 2), we have utilized DREADD technology to dissect neural circuits regulating the vigilance states. We so far have demonstrated (i) that changes in the activity of orexin neurons can alter the behavioral state of animals (6), (ii) that activation of dorsal raphe (DR) serotonergic neurons and locus coeruleous (LC) noradrenergic neurons suppress cataplexy and fragmentation of wakefulness, respectively, in narcoleptic mice (7), and (iii) that stimulation of GABAergic neurons in the preoptic area (POA) leads to an increase in the amount of non-rapid eye movement (NREM) sleep (8). These findings will be described below in more detail.

2. Pharmacogenetic Modulation of Orexin Neurons Alters Sleep/Wakefulness States in Mice (6)

The neuropeptides orexin A and orexin B, also called hypocretin 1 and hypocretin 2, are produced by cleavage of a single precursor polypeptide, prepro-orexin, in neurons located in the lateral hypothalamic area (LHA) (5, 9). These neuropeptides act on G protein-coupled receptors, termed orexin receptors type 1 and type 2 (OX1R and OX2R). Intracerebroventricular administration of orexin in animals increases the amount of wakefulness, accompanied by decreases in the amount of both NREM and REM sleep (10-12). Neurons expressing orexins (orexin neurons) send projections throughout the brain and spinal cord, with particularly dense innervations to nuclei containing monoaminergic and cholinergic neurons in the hypothalamus and brainstem, which are implicated in the promotion of wakefulness and constitute the ascending reticular activating system (4, 5, 9, 13-15). *In vitro* slice electrophysiology studies have

further shown that orexin increases the firing rates of monoaminergic neurons in the LC (16, 17), DR (18, 19), and tuberomammillary nucleus (TMN) (20-22), as well as the cholinergic neurons in the basal forebrain and laterodorsal tegmental nucleus (LDT) (23). The critical roles of orexins and orexin neurons in the consolidation of wakefulness are further highlighted by the fact that the disruption of orexin signaling results in narcolepsy in mice, rats, dogs, and humans (4, 24-29). Narcolepsy is a sleep disorder characterized by difficulties in maintaining prolonged episodes of wakefulness (30) (described in detail later).

Consistent with wake-promoting roles of orexin neurons, their activity increases during wakefulness and decreases during sleep (31-33). However, it remains unclear whether changes in the activity of orexin neurons result in changes of sleep/wakefulness states. Since orexin neurons receive input from various brain regions, including those involved in sleep/wakefulness regulation (34, 35), changes in their activity may occur secondarily to changes in sleep/wakefulness states. Alternatively, the alterations in activity of orexin neurons may actively affect the vigilance states of animals.

To address this question, we utilized DREADD technology to artificially manipulate the activity of orexin neurons to see its consequence on sleep/wakefulness (6). To target the expression of DREADDs specifically in orexin neurons, we injected recombinant adeno-associated virus (AAV) vectors, utilizing the flip-excision (FLEX) switch (36) that restores the open reading frame in a Cre-dependent manner, into the LHA of *Orexin-Cre* transgenic mice, in which Cre-recombinase is expressed exclusively in orexin neurons (37). The successful targeting of the expression of DREADDs in orexin neurons was confirmed by double-immunostaining, demonstrating that hM3Dq and hM4Di were expressed in 78.3% and 73.3% of orexin neurons, respectively, without

any ectopic expression in non-orexin neurons.

Stimulation by i.p CNO administration of excitatory hM3Dq expressed in orexin neurons significantly increased Fos expression, a marker of neuronal activity, while stimulation of inhibitory hM4Di in orexin neurons significantly decreased Fos expression in these neurons during light and dark periods, respectively. This demonstrates that the DREADD system can be used to manipulate the activity of orexin neurons. Electroencephalogram/electromyogram (EEG/EMG) recordings demonstrated that excitation of orexin neurons significantly increased the amount of time spent in wakefulness and decreased both NREM and REM sleep times, while inhibition of orexin neurons decreased wakefulness time and increased NREM sleep time (Fig. 1). These findings clearly show that changes in the activity of orexin neurons can alter the behavioral state of animals and also validate this novel approach for manipulating neuronal activity in awake, freely moving animals.

Artificial manipulation of the activity of orexin neurons was initially achieved by optogenetics: the photostimulation of orexin neurons expressing channelrhodopsin 2 increased the probability of transition to wakefulness from either NREM or REM sleep (38). However, chronic photo-stimulation (15 ms pulses at 20 Hz for 10 s every minute for 1 h) of orexin neurons did not increase total wake time, although the number of NREM sleep-to-wake transitions was increased (39). This fact suggests that DREADD-mediated pharmacogenetic stimulation has an advantage, evoking a greater potent and chronic stimulation of orexin neurons than ChR2-mediated photo-stimulation does, although the time resolution was less for the former than for the latter.

3. Pharmacogenetic activation of DR serotonergic and LC noradrenergic neurons differentially ameliorate narcolepsy in a mouse model (7)

As mentioned previously, the degenerative loss of orexin neurons in humans is associated with narcolepsy, a debilitating neurological disorder that provides a unique perspective on the mechanisms of sleep/wakefulness control (26-28). The narcolepsy syndrome consists of excessive daytime sleepiness that often results in sleep attacks (sudden onset of NREM sleep), cataplexy (sudden bilateral skeletal muscle weakening triggered by emotions without impairment of consciousness), hypnagogic hallucinations, and sleep paralysis. These symptoms can be divided into two independent pathological phenomena (30). One is the inability to maintain a consolidated waking period, characterized by abrupt transitions from wakefulness to NREM sleep (i.e., dysregulation of NREM sleep onset). This phenomenon manifests clinically as excessive daytime sleepiness or sleep attacks. The other key phenomenon is the pathological intrusion of REM sleep or REM atonia into wakefulness or at sleep onset (i.e., dysregulation of REM sleep onset; normal sleep is characterized by an orderly progression from wakefulness to NREM sleep and then to REM sleep) (30). It is during these periods that patients may experience cataplexy, hypnagogic hallucinations, and sleep paralysis.

Similarly, mice deficient in orexin signaling, such as mice lacking the *prepro-orexin* gene (*Orexin*$^{-/-}$ mice), orexin neurons (*orexin/ataxin-3* mice), or orexin receptors (*OX1R*$^{-/-}$*;OX2R*$^{-/-}$ mice), display a phenotype strikingly similar to human narcolepsy: markedly decreased duration of wakefulness episodes during the dark phase (i.e., inability to maintain a long waking period, or sleepiness) and abrupt behavioral arrests with muscle atonia (i.e., potentially cataplexy) that manifest as direct transitions from wakefulness to REM sleep in EEG/EMG recordings (4, 29, 40).

However, the precise neural mechanisms downstream of orexin neurons, which play important roles in consolidating wakefulness and inhibiting inappropriate transitions from wakefulness to NREM or REM sleep, have remained largely unknown. In vitro studies have shown that orexin activates wake-active monoaminergic and cholinergic neurons in the hypothalamus and brainstem (16-23). However, neurons activated by the pharmacological application of exogenous orexin are not necessarily essential for the endogenous mechanisms by which orexin neurons regulate sleep/wakefulness in a physiological condition. In order to identify neurons that are directly activated by endogenous orexins and mediate their wake-stabilizing effect in a particular natural context, we searched for monoaminergic and cholinergic nuclei in which the focal rescue of orexin receptor expressions in *OX1R*$^{-/-}$*; OX2R*$^{-/-}$ mice ameliorates their narcoleptic phenotype. As a result, we found that the targeted restoration of orexin receptor expression in the DR and in the LC of *OX1R*$^{-/-}$*; OX2R*$^{-/-}$ mice differentially inhibited their cataplexy-like episodes and pathological fragmentation of wakefulness (i.e., sleepiness), respectively (7). The suppression of cataplexy-like episodes was correlated with the number of serotonergic neurons restored with orexin receptor expression in the DR, while the consolidation of fragmented wakefulness was correlated with the number of noradrenergic neurons restored in the LC.

To further confirm that our conclusion originated from the receptor-restoration experiments, we used the DREADD system to examine whether the artificial activation of DR serotonergic and LC noradrenergic neurons using orexin neuron-ablated narcoleptic mice (*orexin/ataxin-3* mice)(7). In these mice, orexin neurons degenerate postnatally due to the transgenic expression of a truncated Machado-Joseph disease gene product, mimicking the pathophysiological condition of human narcolepsy (29).

For this purpose, we generated AAV vectors encoding hM3Dq under the control of either a serotonergic neuron-selective promoter (*Pet1* promoter) (41) or a noradrenergic neurons-selective promoter (*PRSx8* promoter) (42). Although the specificity of these neuron type-selective promoters was not perfect, causing some leaked expression, most of the neurons expressing hM3Dq were cells of interest. This suggests that these promoters were acceptable for demonstrating serotonergic or noradrenergic neuron-specific effects of neuronal activation mediated by hM3Dq when careful control experiments were combined. As described in the previous section, we could ideally target the hM3Dq expression in DR serotonergic and LC noradrenergic neurons by microinjecting AAV vectors utilizing the FLEX switch in the DR and LC of mice expressing Cre specifically in serotonergic neurons and noradrenergic neurons, respectively. However, for the purpose of this study, we needed *orexin/ataxin-3* mice that had a Cre-expressing allele, which were laborious to obtain for our experiments. Thus, we considered our approach using neuron type-selective promoters in recombinant AAV vectors to be practical.

When AAV-Pet1/hM3Dq was microinjected into the DR, *hM3Dq* mRNA expression was detected in 78.4 ± 1.5% of serotonergic neurons in the DR, and 83.1 ± 1.0% of *hM3Dq*(+) cells were serotonergic (7). No *hM3Dq* mRNA expression was detected in the area surrounding the DR. I.p. administration of CNO into *orexin/ataxin-3* mice injected with AAV-Pet1/hM3Dq in the DR immediately before the start of the dark period substantially reduced the frequency of and time spent in cataplexy-like episodes during the subsequent 6 h in the dark period (Fig. 2A). In contrast, fragmentation of wakefulness was not improved by CNO administration (Fig. 2B).

Similarly, when AAV-PRSx8/hM3Dq was microinjected into the LC, *hM3Dq*

mRNA expression was detected in 68.1 ± 3.9% of noradrenergic neurons in the LC, and 81.1 ± 1.5% of *hM3Dq*(+) cells were noradrenergic (7). In these mice, the duration of wakefulness episodes during the subsequent 6 h of the dark period was significantly increased in the CNO-treated condition as compared to the saline-injected control condition (Fig. 2C). The total time spent in wakefulness during the same period was not significantly increased when CNO was administered. In contrast, the administration of CNO did not significantly affect the frequency of cataplexy-like episodes in these mice (Fig. 2D). Importantly, the wake-stabilizing effect of CNO was not observed in *orexin/ataxin-3* mice with off-target hM3Dq expression, which have similar numbers of hM3Dq-expressing cells in the regions surrounding the LC but have almost no such cells in the LC. In addition, the wake-stabilizing effect of CNO was strongly correlated with the number of *hM3Dq*(+) noradrenergic neurons in the LC noradrenergic neurons, but not with the number of *hM3Dq*(+) non-noradrenergic neurons in any of surrounding areas.

These data suggest that the pharmacogenetic activation of DR serotonergic and LC noradrenergic neurons in narcoleptic mice is sufficient to significantly suppress cataplexy-like episodes and consolidate wakefulness, respectively (7). In addition, our success in improving narcoleptic symptoms by DREADD may lead to a novel type of gene therapy: the delivery of an artificial receptor into neurons using a viral vector—together with the subsequent administration of an artificial ligand for the receptor with the appropriate timing, in which the effectiveness, specificity, and safety of that artificial receptor-ligand system have been well established—can be used to ameliorate symptoms of interest.

4. Pharmacogenetic stimulation of POA GABAergic neurons increases NREM sleep in mice (8)

The POA of the hypothalamus is thought to play an important role in the initiation and maintenance of sleep. Electrical or chemical stimulation of the lateral POA in animals promotes EEG slow-wave activity and sleep onset (43-46). Consistently, lesions in the POA have been shown to result in profound and persistent sleep loss (47, 48). The extracellular recording studies in vivo have identified sleep-active neurons in a region extending from the medial through the lateral POA (49, 50). In addition, neurons in the rat ventrolateral preoptic area (VLPO) and median preoptic nucleus (MPO) have been reported to drastically increase Fos expression following consolidated sleep (51, 52).

The POA sends GABAergic inhibitory projections to wake-active monoaminergic regions, including the LC, DR, and the TMN (52-55). The VLPO receives projections from wake-active monoaminergic neurons, such as TMN histaminergic, DR serotonergic, and LC noradrenergic neurons, and electrophysiological studies have demonstrated that VLPO neurons are inhibited by noradrenaline and serotonin (56). These facts suggest mutually inhibitory interactions between VLPO and the monoaminergic arousal systems (57).

Thus, we aimed to specifically activate GABAergic neurons in the POA using excitatory DREADD hM3Dq and examine its effects on sleep/wakefulness and on the activity of wake-promoting orexin neurons (8). For this purpose, we injected an AAV vector containing the inverse hM3Dq cDNA in combination with the FLEX switch into the POA of Gad67-Cre mice, in which GABAergic neurons specifically express Cre recombinase. Specific stimulations of hM3Dq-expressing GABAergic neurons in the POA by i.p. CNO administrations significantly increased the amount of NREM sleep,

regardless of whether CNO was administered during a light or dark phase (Fig. 3). We further confirmed direct connectivity between POA GABAergic neurons and orexin neurons, in addition to their widespread projections to wakefulness-related areas in the hypothalamus and brainstem, using channelrhodopsin 2 (ChR2) as an anterograde tracer. Optogenetic stimulation of the nerve terminals of POA GABAergic neurons on orexin neurons rapidly inhibited the activity of orexin neurons and evoked fast IPSC in slice preparations, and pharmacogenetic stimulation of POA GABAergic neurons in vivo mediated by hM3Dq significantly reduced Fos expression in orexin neurons. These observations suggest that activation of POA GABAergic neurons is sufficient to increase NREM sleep, which might be at least partly through the inhibition of orexin neurons (8).

5. Technical considerations on the strategy of DREADD expression

As in the previous sections, we used recombinant AAV vectors to express DREADDs in the neurons of interest in the brain. Even with ubiquitous, powerful promoters, such as EF1α and CAG promoters, stereotaxic microinjections of AAV vectors can target the expression of transgenes in the areas of interest. Practically, however, it is difficult to perfectly restrict expression in only the small areas of interest in mice, often resulting in additional expression in the adjacent areas. Additionally, multiple types of neurons are usually intermixed within a particular brain area. Thus, Cre-dependent AAV expression vectors, utilizing the FLEX switch (36), are an excellent strategy for targeting DREADD expression, specifically in a neurochemically-defined neuronal population located in a particular brain area of interest when suitable transgenic mice expressing Cre recombinase are available. Note that specificity of Cre

expression is required only in the brain area of interest and its surrounding regions. Undesired Cre expression in the brain regions located apart from the area of interest (> ~1mm) is of no matter, since stereotaxic injection of AAV vectors can avoid the infection and expression in such areas. On the other, the expression of DREADDs should be confirmed in every mouse that receive AAV injections because the location and spread of AAV infection inevitably varies among individual injected mice.

Alternatively, DREADDs can, of course, be expressed by generating transgenic mice with cell-specific promoters. It is worth noting that two useful transgenic mouse lines have been developed. One expresses hM3Dq specifically in tTA (tetracycline-controlled transactivator)-expressing cells (1), while the other expresses hM4Di in Cre and/or Flp recombinase-dependent manner (58). We also generated transgenic mice in which hM3Dq or hM4Di fused with the loxP-flanked transcriptional blocker are knocked-in in the Rosa26 allele so that DREAD receptors are expressed in a Cre-dependent manner. By using these transgenic mice in combination with other transgenic mice expressing tTA, Cre or Flp specifically in the neuronal population of interest, we can target the DREADD expression in the cells of interest with minimal variation of expression among individual mice. However, in these cases, systemic administration of CNO would result in the activation or inhibition of not only the cells of interest but also of other neurons expressing Cre or Flp in the regions of no interest. Thus, the outcomes of DREADD activation should be carefully interpreted. This problem may be resolved by focal administration of CNO in the brain area of interest (59, 60).

6. Protocols

6.1. AAV production and purification

First of all, it should be noted that multiple AAV vectors of DREADDs, which are purified and have high titer, are currently available from the Gene Therapy Center of the University of North Carolina at Chapel Hill (http://genetherapy.unc.edu/services.htm).

Viruses are produced using a triple-transfection, helper-free method in which the recombinant expression plasmid, containing the AAV-2 (serotype 2) inverted terminal repeat (ITR) sequences and an expression cassette of gene of interest, is co-transfected into the HEK293 cells with a helper plasmid carrying adenovirus-derived genes and a plasmid carrying AAV replication (rep) and capsid (cap) genes.

6.2. AAV serotypes

There are many AAV serotypes available, each incorporating a different viral capsid protein and each mediating different transduction characteristics within the brain and peripheral tissues (61). The AAV2 serotype has been most studied and utilized as a recombinant vector. However, when the expression cassette flanked by AAV2 ITRs is cross-packaged into capsid proteins of other serotypes, the hybrid AAV vectors have been reported to infect neurons more efficiently as compared to AAV2 vectors. On the other hand, AAV2 can be relatively easily purified using a heparin-agarose column (62): purification of viral particles of other serotypes needs ultracentrifugation over either a CsCl or iodixanol gradient, which is cumbersome and expensive. Thus, we decided to utilize AAV2 vectors containing a mutant form of AAV2 capsid protein to increase the efficiency of transgene expression (kindly provided by Dr. Arun Srivastava of the University of Florida) (63), which retains the ability to bind to heparin for purification, and this was used for the three studies described above. We will describe the protocol of

viral production and purification in detail later.

Recently, it was discovered that microinjection of unpurified AAV viral solutions in the brain may not cause significant pathological damage to the tissue (64). Therefore, we are currently using unpurified AAV vectors with capsid proteins of rh10 serotype (65), which seem to infect neurons more efficiently than mutant AAV2.

6.3. Plasmids

We initially constructed ITR-containing plasmids with DREADD cDNAs in combination with the FLEX switch by replacing ChR2::EYFP genes of pAAV-double floxed-hChR2(H134R)-EYFP-WPRE-pA (66), provided by Dr. Karl Deisseroth of Stanford University (http://www.stanford.edu/group/dlab/optogenetics/), with HA-hM3Dq or HA-hM4Di cDNAs derived from the plasmids pcDNA5/FRT-HA-hM3Dq and pcDNA5/FRT-HA-hM4Di (2), provided by Dr. Bryan Roth of University of North Carolina. Currently, mCherry-fusioned DREADDs have been developed and are available from Dr. Roth's laboratory, which is easier than using HA-tagged DREADDs to detect protein expression. Therefore, we are currently using Cre-dependent AAV vectors expressing mCherry-fusioned DREADDs.

For producing AAV-2 vectors used in the studies described previously, we used pACG-2-Y730F (63), which contains a mutant form of AAV2 capsid protein, as the rep/cap plasmid. To produce AAV-rh10 vectors, we replaced pACG-2-Y730F with pAAV2/rh10, which contains AAV2 rep gene and AAVrh10 cap gene (available from the Vector Core of the University of Pennsylvania, http://www.med.upenn.edu/gtp/vectorcore/index.shtml). For the helper plasmid carrying adenoviral genes, we used pHelper (Strategene), but a similar helper plasmid

pAd-DeltaF6 is also available from the Vector Core of the University of Pennsylvania.

6.4. HEK293 cells

We used 293A cells (Invitrogen), which are a subclone of the HEK293 cell line and were originally established for the production of recombinant adenoviral vectors. They grow efficiently and adhere to the culture dishes better. The commonly-used HEK293 cell line should work for AAV production, and another subclone AAV-293, which had been screened for AAV production, is available from Stratagene. We have no information about which 293 cells are the best for the production of recombinant AAV vectors. Note that the following protocol is on the 293A cells.

6.5. Packaging

1. Plate ~4.5 x 10^6 cells per 100-mm tissue culture dish in 10 ml of DMEM medium 24 ~ 28 hours prior to transfection. We usually prepare 10 dishes for producing one AAV-2 vector with a purification step (the following protocol describes the amount of reagents for 10 dishes). When producing AAV-rh10 vectors without purification, we downscale to 4 culture dishes per vector.
2. Inspect the host cells that were split one day before; they should be approximately 90 - 100% confluent.
3. Add 100 μg of each of the three plasmid DNA solutions: pHelper, pACG-2-Y730F, and the ITR-containing expression plasmid) to 10 ml of 0.3 M $CaCl_2$ in a 50-ml conical tube and mix gently.
4. Pipet 10 ml of 2 × HBS (NaCl 8.182g, Na_2HPO_4 0.106g, HEPES 5.958g/500mL, pH7.10, filter-sterilized; adjust pH to 7.06 ~ 7.07 because filtration slightly

increases pH) into a second 50-ml conical tube. Add the 10-ml DNA/$CaCl_2$ mixture (step 3) dropwise with gentle rocking of the conical tube. Mix gently by inversion or by repeated pipetting.

5. Leave the DNA/$CaCl_2$/HBS suspension for 20 minutes at room temperature.
6. Apply the DNA/$CaCl_2$/HBS suspension to the dish of cells (2 ml/dish) in a dropwise fashion, swirling gently to distribute the DNA suspension evenly in the medium.
7. Return the tissue culture dishes to the 37°C CO_2 incubator overnight.
8. At the end of the incubation period, remove the medium from the dishes and replace it with 10 ml/dish of fresh DMEM growth medium.
9. Return the dishes to the 37°C incubator for an additional 2 days.
10. At the end of incubation, the density of cells is much higher than at transfection, with each cell appearing compressed. Most cells remain attached to the dishes.
11. Scrape and transfer the transfected cells plus DMEM growth medium to two 50-ml conical tubes.
12. Collect cells by centrifugation at ~150 g for 10 minutes at room temperature.
13. Discard the supernatant and resuspend the cells in binding buffer (10ml of 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0). The cells can be stored frozen in the -80°C deep freezer for a long period before purification.

For production of AAV-rh10 vectors, wash cells once with PBS, pellet the cells, and resuspend in 400 μl (for 4 dishes) of PBS containing 1mM of $MgCl_2$. The following steps (step 14~) are essentially same, except that we use benzonase nuclease instead of DNaseI and RNaseA, pellet cellular debris by microcentrifuge at the maximal speed, and omit the purification step (step 19~).

6.6. Purification

14. Subject the cell suspension to two rounds of freeze/thaw by alternating the tubes between the -80°C deep freezer and the 37°C water bath.
15. Pellet cellular debris by centrifugation at ~2,000 × g for 15 minutes.
16. Transfer the supernatant (~10 ml) to a fresh tube and add 40 µl of DNaseI (10 mg/ml) and 80 µl of RNaseA (5 mg/ml).
17. Incubate for 30 minutes in the 37°C water bath.
18. Centrifuge the viral solution at ~2,000 × g for 15 minutes and transfer the supernatant to a fresh tube.
19. Add 500 µl (1/20 volume) of 10% Sodium deoxycholate to the viral solution, and then incubate it for 30 minutes in the 37°C water bath.
20. Centrifuge the viral solution at ~2,000 × g for 15 minutes and recover the supernatant.
21. Pass the viral solution through a 5 µm-syringe filter, then through a 0.4 µm-syringe filter.
22. (In parallel to the treatment of the viral solution, prepare heparin-agarose resin) Transfer 1.5ml of Heparin-agarose suspension (Sigma) into a 15-ml conical tube with 5 ml of binding buffer.
23. Centrifuge the Heparin-agarose suspension for a minute, discard the supernatant, and wash the Heparin-agarose with another 5 ml of biding buffer.
24. Centrifuge the Heparin-agarose suspension for a minute and discard the supernatant. Add the viral solution prepared in Step 21 to the Heparin-agarose.
25. Suspend the viral solution/Heparin-agarose by rocking at 4°C.

26. Transfer the viral solution/Heparin-agarose into a bio-spin column (Bio-Rad) and allow the column to empty by gravity flow.
27. Wash the Heparin-agarose resin three times with 2 ml of binding buffer.
28. Add 1 ml of elution buffer (0.5 M NaCl, 50 mM Tris, pH 8.0) to the resin and collect the eluate in a fresh tube.
29. Cap the tip of column, add another 1ml of elution buffer, and leave the column for 5 minutes.
30. Remove the cap at the tip of column and collect the eluate in the tube.
31. Add another 1ml of elution buffer to the column and collect the eluate (total ~3ml of eluate).

6.7. Dialysis and Concentration

32. Transfer the eluate containing AAV particles into a Slide-A-Lyzer Dialysis Cassette (Pierce, Slide-A-Lyzer Gamma Irradiated Dialysis Cassete Extra-strength, 0.5-3ml, 10,000 MWCO) and dyalize it against 1L of PBS (KH_2PO_4 0.144g, NaCl 9.0g, $Na_2HPO_4.12H_2O$ 1.062g, $MgCl_2.6H_2O$ 0.10g, $CaCl_2.2H_2O$ 0.133g/1L, pH7.4, filter sterilized; adjust pH to 7.36 ~ 7.37 because filtration slightly increases pH) overnight at 4°C. Change the dialysate once.
33. Concentrate the dialyzed viral solution by placing the Dialysis Cassette into the Slide-A-Lyzer Concentration Solution (Pierce) inside the resealable plastic bag and rock the bag for 60 ~ 90 minutes at 4°C.
34. Recover the concentrated viral solution from the cassette, make aliquots, freeze rapidly, and store at -80°C. Typically, we can obtain 400~800 μl of concentrated viral solution.

35. Measure the titer of the viral stocks by real-time PCR. Prior to PCR, 2 µl of viral solution is pre-treated in 100 µl of 50 mM NaOH for 30 minutes at 95°C to liberate AAV genome DNA from viral particles, followed by neutralization by adding 8.4 µl of 1M Tris-HCl pH8.0. Further dilute the DNA solution 20-fold and use 2.5µl of diluted solution per PCR reaction in 10µl. Use serial dilution of ITR-containing plasmid as the reference to write standard curves for calculating viral titers. We amplify the WPRE sequence, which is contained in most of the recombinant AAV vectors we generated, by Roche LightCycler 480 using two primers 5'-actgtgtttgctgacgcaac-3' and 5'-agcgaaagtcccggaaag -3' in combination with an universal probe #77. When using other types of real-time PCR reagents and cyclers, primer sequences may need to be optimized. Typically, we obtain final viral stocks with the titer of $2 \times 10^{12} \sim 2 \times 10^{13}$ genome copies/ml.

6.8. Surgery of mice

Male mice (10 to 20 weeks old) were anesthetized with sodium pentobarbital (0.5 mg/kg, i.p.) and positioned in a stereotaxic frame (David Kopf Instruments). For microinjection of viral solutions, we used the Hamilton syringe (10 µl, 701RN) in combination with a 33-gage needle (1 inch long).

The coordinates for injection sites depend on the location and size of the area of interest. The following are the coordinates we used, with reference to the bregma; anteroposterior, AP ("-" means posterior to the bregma); medilateral, ML ("±" means bilateral injections); dorsoventral, DV ("-" means ventral to the skull surface):

LHA (targeting orexin neurons)(6): AP, -1.4 mm; ML, ±0.9 mm; DV, -5.5 mm; and AP, -1.8 mm; ML, ±0.9 mm; DV, -5.7 mm. Since orexin neurons are scattered in the LHA

and perifornical area, we microinjected viruses in 4 sites. LC: AP, -5.4 mm; ML, ± 0.9 mm; DV, -3.7 mm. DR: AP, -4.4 mm; ML, ± 0 mm; DV, -3.0 and -3.5 mm. TMN: AP, -2.5 mm; ML, ± 1.0 mm; DV, -5.6 mm. PPT: AP, -4.5 mm; ML, ± 1.0 mm; DV, -3.7 mm. PB: AP, –5.4 mm; ML, ± 1.0 mm bilaterally; DV, -3.5 mm (7). POA: AP, +0.3 mm; ML, ± 0.65 mm; DV, -5.72 mm (8). Note that the coordinates of injection may need to be optimized for each experimenter and mouse age and strain.

Small holes were drilled into the skull at the position corresponding to the injection sites, and the needle was inserted slowly and progressively to minimize tissue damage until the tip of needle reaches to the injection site. Then, 0.2 ~ 1 μl of viral solution was delivered to each site over a 10-min period using KDS310 Nano Pump Syringe Pumps (KD Scientific). After 5 min of rest, the needles were slowly removed. Injection using 33-gage stainless needles caused minimal tissue damage in most cases. However, when relatively small nuclei are targeted, the thickness of the needle and the volume of viral solution injected may cause broader infection than expected. Microinjection of smaller volume using fine glass micropipettes would be more suitable for finer expression, although targeting small volume to very restricted areas might make the targeting technically more difficult. From this perspective, the Cre-dependent expression system utilizing the FLEX switch is a useful strategy to target the expression into the cells of interest in spite of the broader infection of AAV vectors in the surrounding cells/areas.

Following virus administration, electrodes for EEG and EMG recording were implanted in the skull of each mouse (4, 6-8). The EEG/EMG implant was based on a six-pin double inline microcomputer connector, modified to form four EEG electrodes and two EMG wire electrodes soldered to the pins. Four holes were drilled on the skull, and the four arms of the EEG electrode were placed anterior and posterior to the bregma

(AP, +1.2 mm; ML, ±1.2 mm and AP, -1.2 mm; ML, ±1.2 mm). Stainless steel wires for EMG recording were bilaterally sutured to the neck muscles of each mouse, and each electrode was glued solidly to the skull.

Silicon tubes (Shinetsu Polymer, CP-N, outside diameter: 1mm, inside diameter: 0.5mm) were implanted for remote CNO injection (6, 8). The tube was implanted subcutaneously from the abdominal to the neck. The tip of a 30 cm-long silicon tube was inserted 1 cm into the peritoneal cavity and sutured to the abdominal wall. The other end of the silicon tube was placed outside the body through an incision in the neck, and all incisions were sutured. Implanting silicon tubes minimizes the disturbance due to the CNO administrations. Alternatively, the CNO can be administered i.p. by conventional injections using syringes and needles with proper acclimations to the injection before the experiments, reducing the invasiveness of surgery (7).

All animals were then housed individually for a recovery period of at least 7 days. Generally, transgene expression by AAV vectors plateaued about 2 weeks after injection.

6.9. Sleep recordings

After the recovery period, animals were moved to a recording cage placed in an electrically shielded and sound-attenuated room. A cable for signal output was connected to the implanted electrode and animals were allowed to move freely. Signals were amplified through an amplifier (AB-611J, Nihon Koden, Tokyo) and digitally recorded on a PC using EEG/EMG recording software (Vital recorder, Kissei Comtec). Animals were allowed at least seven days to adapt to the recording conditions prior to any EEG/EMG recording session. Following the adaptation period, each animal was

injected with both CNO and saline on separate experimental days with at least a 2-day interval. The order of injection (i.e. either CNO first or saline first) should be randomized. EEG/EMG data were evaluated and staged for 24 hours on the day of CNO or saline administration. Data acquired on the day of saline administration were used as controls (6-8).

6.10. Histological confirmation of DREADD expression

After EEG/EMG recordings, efficiency and specificity of DREADD expression should be determined histologically. Mouse brains were fixed by transcardial perfusion with 4% paraformaldehyde and serial coronal brain sections were prepared by standard procedures. Immunostaining with a mouse anti-HA antibody (Covance, 1:1000) detects HA-tagged DREADDs expressed at relatively high level (1, 6, 8). However, the sensitivity of detection is not good enough to detect low-level expression. Thus, as an alternative method, DREADD mRNA can be detected by nonradioisotope *in situ* hybridization using digoxigenin-labeled antisense riboprobes for DREADDs (7). Using mCherry-fusioned DREADDs makes it much easier to check their expression, by the fluorescence of mCherry or immunostaining with rabbit anti-DsRed antibody (Clontech 632496, 1:2000).

7. Conclusion

Overall, there are clear benefits to using DREADD technology in the analysis of neural circuits underlying sleep/wakefulness regulation. We successfully manipulated the activity of neurons involved in sleep/wake regulation and detected the effects of manipulation. The same strategy can be applicable to other types of neurons, which may

lead to the understanding of the whole picture of how our vigilance states are controlled, and further to a novel therapeutical approach to multiple sleep disorders, such as insomnia and narcolepsy.

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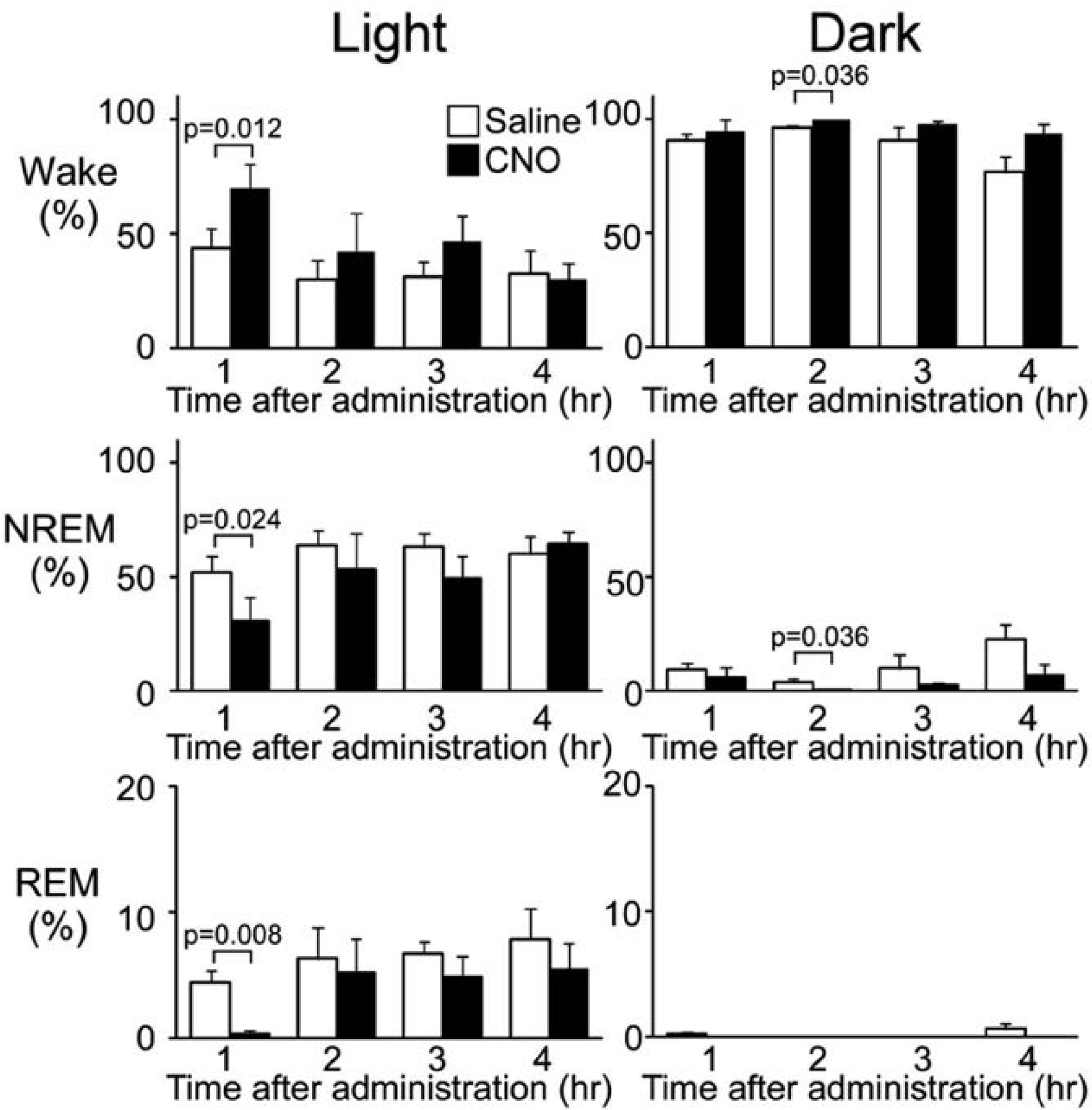
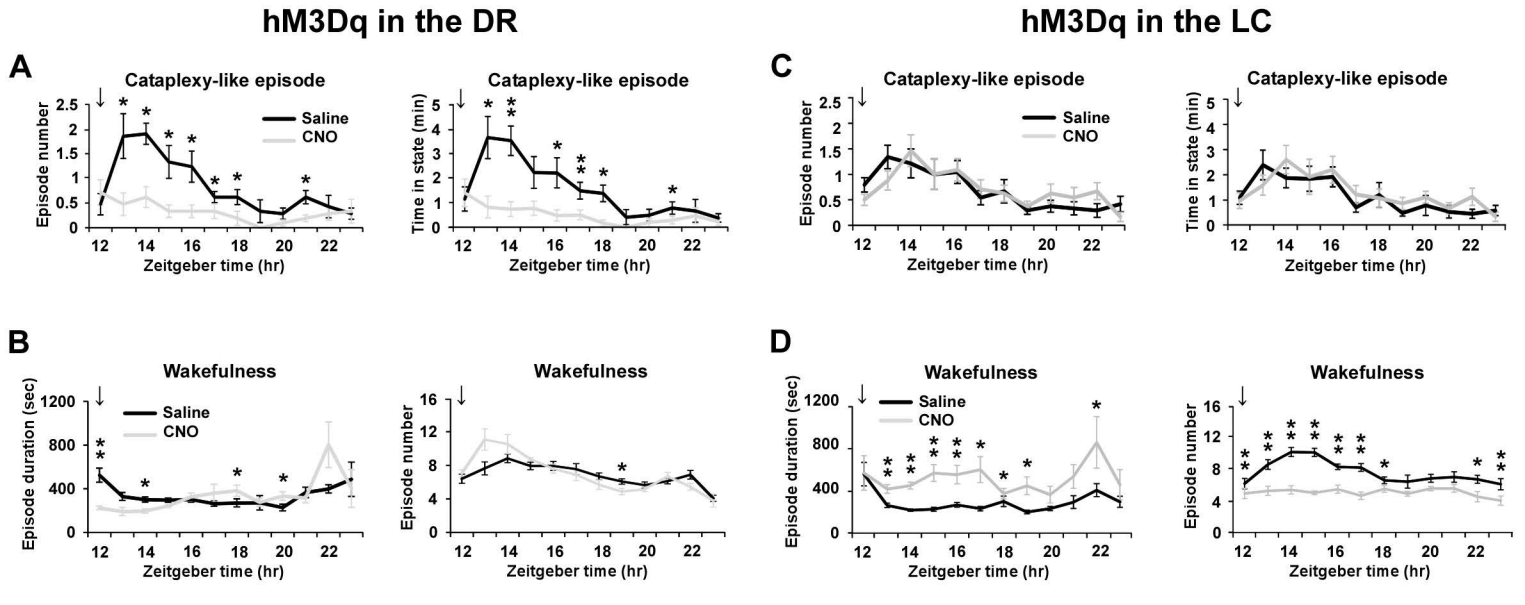
Legends to figures

Fig. 1. Effect of stimulation of orexinergic tone by hM3Dq on vigilance states of mice during light and dark periods (left and right panels, respectively)(6). Hourly analysis of sleep/wake states in transgenic and wild-type mice, both injected with rAAV-DIO-HAhM3Dq after administration of CNO at ZT4 or ZT12. Amounts of wakefulness (Wake, upper panels), NREM sleep (middle panels), and REM sleep (lower panels) are shown.

Fig. 2. Pharmacogenetic activation of DR serotonergic and LC noradrenergic neurons suppresses cataplexy-like episodes and consolidates wakefulness, respectively (7). *Orexin/ataxin-3* mice with DR serotonergic neuron–selective (A and B) or LC noradrenergic neuron–selective (C and D) expression of hM3Dq were injected with saline or CNO. Hourly plots of number and total time of cataplexy-like episodes (A and C) or hourly plots of wakefulness duration and number (B and D) within 12 hours after administration at ZT 12 (arrows). $*P < 0.05$, $\#P < 0.01$, 2-tailed Student's paired *t* test. Values are mean ± SEM.

Fig. 3. Specific pharmacogenetic stimulation of GABAergic neurons in the POA increased NREM sleep amount (8). Total time of wakefulness (WAKE), NREM sleep, and REM sleep for 3 h after CNO (or saline) administration at ZT12 (upper panels) and at ZT4 (lower panels).


Light
Dark
Saline
CNO
Wake (%)
NREM (%)
REM (%)
p=0.012
p=0.036
p=0.024
p=0.036
p=0.008
100
50
0
20
10
1
2
3
4
Time after administration (hr)


hM3Dq in the DR
hM3Dq in the LC
A
Cataplexy-like episode
Episode number
Saline
CNO
Zeitgeber time (hr)
Cataplexy-like episode
Time in state (min)
Zeitgeber time (hr)
C
Cataplexy-like episode
Episode number
Saline
CNO
Zeitgeber time (hr)
Cataplexy-like episode
Time in state (min)
Zeitgeber time (hr)
B
Wakefulness
Episode duration (sec)
Saline
CNO
Zeitgeber time (hr)
Wakefulness
Episode number
Zeitgeber time (hr)
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Wakefulness
Episode duration (sec)
Saline
CNO
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Wakefulness
Episode number
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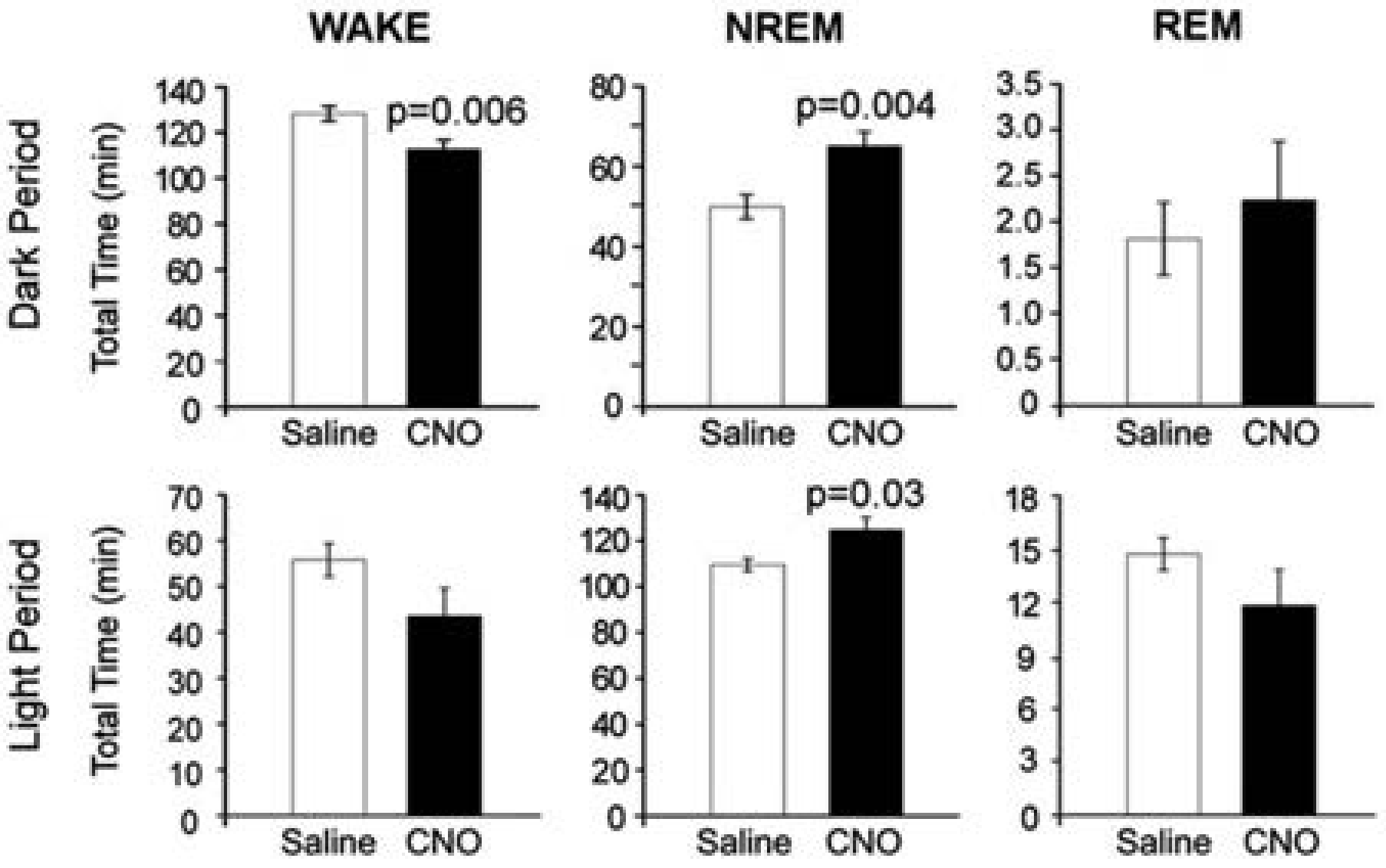
WAKE
NREM
REM
Dark Period
Light Period
Total Time (min)
p=0.006
p=0.004
p=0.03
Saline
CNO
0
20
40
60
80
100
120
140
10
30
50
70
0.5
1.0
1.5
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18