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Melanin-concentrating hormone neurons contribute to dysregulation of rapid eye movement sleep in narcolepsy

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Abstract

The lateral hypothalamus contains neurons producing orexins that promote wakefulness and suppress REM sleep as well as neurons producing melanin-concentrating hormone (MCH) that likely promote REM sleep. Narcolepsy with cataplexy is caused by selective loss of the orexin neurons, and the MCH neurons appear unaffected. As the orexin and MCH systems exert opposing effects on REM sleep, we hypothesized that imbalance in this REM sleep-regulating system due to activity in the MCH neurons may contribute to the striking REM sleep dysfunction characteristic of narcolepsy. To test this hypothesis, we chemogenetically activated the MCH neurons and pharmacologically blocked MCH signaling in a murine model of narcolepsy and studied the effects on sleep-wake behavior and cataplexy. To chemoactivate MCH neurons, we injected an adeno-associated viral vector containing the hM3Dq stimulatory DREADD into the lateral hypothalamus of orexin null mice that also express Cre recombinase in the MCH neurons (MCH-Cre::OX-KO mice) and into control MCH-Cre mice with normal orexin expression. In both lines of mice, activation of MCH neurons by clozapine-N-oxide (CNO) increased rapid eye movement (REM) sleep without altering other states. In mice lacking orexins, activation of the MCH neurons also increased abnormal intrusions of REM sleep manifest as cataplexy and short latency transitions into REM sleep (SLREM). Conversely, a MCH receptor 1 antagonist, SNAP 94847, almost completely eliminated SLREM and cataplexy in OX-KO mice. These findings affirm that MCH neurons promote REM sleep under normal circumstances, and their activity in mice lacking orexins likely triggers abnormal intrusions of REM sleep into non-REM sleep and wake, resulting in the SLREM and cataplexy characteristic of narcolepsy.

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Keywords

Paradoxical sleep; orexin neurons; narcolepsy; chemogenetics; muscle tone; REM sleep intrusions; cataplexy

Introduction

Narcolepsy with cataplexy, a chronic, debilitating sleep disorder, is characterized by excessive daytime sleepiness plus poor regulation of rapid eye movement (REM) sleep manifest as REM sleep occurring very soon after sleep onset, and cataplexy, sudden loss of bilateral muscle tone provoked by strong emotions (Burgess and Scammell, 2012; Dauvilliers et al., 2014; Pintwala and Peever, 2017; Sakurai, 2013; Scammell, 2003; Scammell, 2015). In both animals and humans, narcolepsy with cataplexy is caused by impaired orexin signaling. For example, mice lacking the orexin neurons, orexin neuropeptides or orexin receptors exhibit signs of sleepiness plus cataplexy and sleep-wake fragmentation (Chemelli et al., 1999; Hara et al., 2001; Mochizuki et al., 2004; Willie et al., 2003). Similarly, people with narcolepsy have a huge loss of orexin neurons and very low levels of orexin-A in their cerebrospinal fluid (Heier et al., 2007; Lopez et al., 2017; Peyron et al., 2000; Ripley et al., 2001a; Ripley et al., 2001b; Thannickal et al., 2000). Thus, loss of orexin signaling is the fundamental cause of narcolepsy with cataplexy.

The orexin neurons are located in the lateral hypothalamus (LH), intermingled with a separate population of neurons expressing melanin concentrating hormone (MCH) (Broberger, 1999; Elias et al., 2001; Elias et al., 1998). The MCH and orexin neurons innervate each other, and *in vitro* and *in vivo* studies indicate that they may be mutually inhibitory (Apergis-Schoute et al., 2015; Guan et al., 2002; Rao et al., 2008). The firing pattern of MCH neurons is also reciprocal to that of the orexin neurons, discharging maximally during REM sleep when complete muscle atonia occurs (Hassani et al., 2009), whereas the orexin neurons are silent during REM sleep but fire maximally during active wakefulness when muscle tone is high. Consistent with these observations, selective activation of the MCH neurons increases REM sleep (Jego et al., 2013; Tsunematsu et al., 2014; Vetrivelan et al., 2016) whereas activation of the orexin neurons increases wake (Adamantidis et al., 2007). Collectively, these observations suggest that the MCH and orexin neurons play opposing roles, especially in the regulation of REM sleep.

The causal role of the orexin neurons in narcolepsy with cataplexy is well established, but the role of the MCH neurons remains unclear. In people with narcolepsy, the number of MCH neurons is normal (Crocker et al., 2005; Peyron et al., 2000; Thannickal et al., 2000), but it is unknown whether activity of the MCH neurons contributes to the symptoms of narcolepsy. We hypothesized that in the absence of orexins, the effects of MCH on REM sleep may be unbalanced, potentially contributing to aspects of abnormal REM sleep observed in narcolepsy such as cataplexy and rapid transitions into REM sleep. To address this issue, we chemogenetically activated the MCH neurons (Experiment 1) and pharmacologically blocked MCH signaling (Experiment 2) in a mouse model of narcolepsy (orexin null mice) and studied changes in sleep-wake behavior and cataplexy.

Experimental methods:

Animals: Orexin knockout (OX-KO) mice have a null mutation in the prepro-orexin gene and are well validated to lack orexins (Chemelli et al., 1999; Clark et al., 2009; Mochizuki et al., 2004) (Also see Fig. 1D). We have previously described eutopic Cre-mediated recombination in the MCH-Cre mice (Kong et al., 2010; Vetrivelan et al., 2016). We produced MCH-Cre::OX-KO mice by crossing MCH-Cre mice with OX-KO mice. The resultant offspring heterozygous for both Cre and the prepro-orexin mutation were crossed with homozygous OX-KO mice. We used the F2 offspring heterozygous for Cre and homozygous for the null prepro-orexin gene (MCH-Cre::OX-KO) for the experiments. All mice used in this study were on a mixed background.

Adult male and female mice were singly housed with *ad libitum* access to food and water under standard vivarium conditions - 12 h:12 h light-dark (LD) cycle with lights on at 0700; 150 lux and an ambient temperature of 22 ± 1°C. At the time of surgery, mice were 12–18 weeks old and weighed 19–26 g. Care of the animals met National Institutes of Health standards, as set forth in the Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the BIDMC Institutional Animal Care and Use Committee.

Surgery and recordings:

Experiment 1: Activation of MCH neurons in mouse-model of narcolepsy—To investigate the role of MCH neurons in sleep-wake behavior and cataplexy, we first chemogenetically activated the MCH neurons in orexin null mice (MCH-Cre::OX-KO mice) as well as in control mice (MCH-Cre mice) with intact orexin signaling. MCH-Cre mice (n=8; 7 males) and MCH-Cre::OX-KO mice (n = 6; 3 males) were anesthetized (100 mg/kg ketamine + 10 mg/kg xylazine; intraperitoneal injection) and bilaterally microinjected with AAV8-hSyn-DIO-hM3D(Gq)-mCherry (AAV-hM3Dq; University of North Carolina Vector Core, USA; 260 nl per injection) into the LH (anteroposterior: −1.7 mm from bregma, ventral: 4.8 mm from dura, lateral ±0.5 and ±1.1 mm) (Vetrivelan et al., 2016). Another set of MCH-Cre mice (n=5 males) were injected with AA8-hSyn-DIO-mCherry (AAV-mCherry; sham-control) into the LH and these mice served as negative controls. All mice were then implanted with electrodes for recording electroencephalogram (EEG) and electromyogram (EMG) (Anaclet et al., 2012; Mochizuki et al., 2004). After a post-surgical recovery period of 3 weeks, mice were transferred to light- controlled, sound-attenuated recording chambers and habituated to cables and recording conditions for at least 3 days. Intraperitoneal (i.p.) injections of saline (vehicle) or clozapine-N-oxide (CNO; Sigma, USA; 0.3 mg/kg) were performed ~10 mins before dark onset (6:50 PM), and EEG and EMG with time-locked video was recorded in all mice for the next 12 h. We chose to use a 0.3 mg/kg dose of CNO as this dose has no effects on sleep-wake behavior and cataplexy in mice lacking DREADD receptors (Kroeger et al., 2017; Mahoney et al., 2017; Vetrivelan et al., 2016). The order of injections was counterbalanced; half of the mice in each group received saline and other half received CNO as first injections. EEG/EMG signals were amplified (AM systems, USA), digitized and recorded using Vital recorder (Sleepsign software, Kissei Comtec, Japan).

Histology: After the recordings, all mice were injected with CNO (0.3 mg/kg; i.p) at 10 AM and transcardially perfused 3 h later with 30 ml saline followed by 50 ml of 10% formalin under deep anesthesia with chloral hydrate (700 mg/kg; i.p.). The brains were harvested, post-fixed and cut into 3 series of 40 pm sections. To test whether hM3Dq was expressed solely in MCH neurons, one series of sections was labelled for MCH and mCherry using immunofluorescent staining (Vetrivelan et al., 2016). A second series was immunolabeled for cFos and mCherry to detect the activation of hM3Dq-mCherry expressing neurons by CNO (Chen et al., 2017; Vetrivelan et al., 2016). A third series was labelled for orexin and mCherry to verify the loss of orexin in MCH-Cre::OX-KO mice and to verify the absence of hM3Dq expression in orexinergic neurons using DAB-immunohistochemistry (Oishi et al., 2013; Vetrivelan et al., 2016). We used the following primary antibodies: Rabbit anti-MCH, (generous gift from Dr. Maratos-Flier, BIDMC; 1:10,000 dilution), rabbit anti-DsRed (for labeling mCherry, Clontech, USA; cat.no: 632496; 1:10,000 dilution), rat anti-DsRed (for fluorescent labeling of mCherry; 1:10,000) rabbit anti-cFos (Oncogene Sciences; cat. no: 4188; 1:30,000 dilution) and rabbit anti-orexin A (1:5,000; Cat. No: SC-8070; Santa Cruz Biotechnology, USA). The specificity of the antibodies was confirmed by the absence of staining in negative controls.

Data Analysis: EEG/EMG recordings were manually scored (after semi-automatic scoring by the software) in 12-second epochs into 5 vigilance stages - wake, NREM or REM sleep, short-latency REM sleep (SLREM) and cataplexy using SleepSign software (Kissei Comtec, Japan). Wake stage was identified by high muscle tone with movement-related activity and low-amplitude high frequency (desynchronized) EEG. NREM sleep was identified by EMG activity lower than wake with high- amplitude, low frequency (synchronized) EEG with high delta activity (0.5 – 4 Hz). REM sleep was identified by very low EMG activity (muscle atonia) and desynchronized EEG with regular, high amplitude theta activity (4–9 Hz). Cataplexy episodes were scored based on previously established consensus criteria (Scammell et al., 2009) including visual confirmation using the time-locked video recordings. People with narcolepsy and narcoleptic dogs often enter REM sleep within 15 minutes of sleep onset (Andlauer et al., 2013; Nishino et al., 2000; Reiter et al., 2015). We defined SLREM episodes in mice as typical REM sleep preceded by ≤12 s NREM sleep. In contrast to cataplexy which is typically preceded by active wake, SLREM episodes were always preceded by typical sleep behavior with the mouse in its nest in a typical sleeping posture. Scorers were blind to the genotype of the mice and conditions. Scored sleep-wake data was divided into 3-h bins and the percentages of individual sleep- wake stages and the number and mean duration of their bouts in each 3-h bin were calculated. We also calculated the latency to enter the first bout of NREM and REM sleep after injection of saline or CNO. Sleep-wake data from MCH-Cre and MCH-Cre::OX-KO mice were compared using two way ANOVA followed by post-hoc comparisons or non-parametric Mann-Whitney U test. All statistics were performed using GraphPad Prism version 7.0 (GraphPad, USA). Although we analyzed sleep-wake recordings for the 12-h after saline/CNO injection, we present just the first 6 h of data as CNO induced changes in sleep- wake were observed only during the first 3 h, and we examined the second 3-h bin for any ‘rebound’ effects.

Experiment 2: Blockade of MCH signaling in a mouse model of narcolepsy— To examine whether blockade of MCH signaling can attenuate narcolepsy symptoms, we treated OX-KO mice with a MCH receptor 1 antagonist (MCHR1 is the only MCH receptor found in rodents) and studied sleep-wake behavior and cataplexy. As positive emotional stimuli are the primary triggers for cataplexy in humans and animals (Burgess et al., 2013; Clark et al., 2009; Espana et al., 2007; Overeem et al., 1999; Overeem et al., 2004), we then provided the OX-KO mice with access to chocolate and tested the effect of MCHR1 antagonist on cataplexy.

Adult OX-KO mice (n=7; 3 males) were implanted with EEG and EMG electrodes. Three weeks after the surgery, the mice were transferred to recording room and habituated to conditions similar to Experiment 1. All mice were then injected (10 min before dark onset; 6:50 PM) with an MCHR1 antagonist, SNAP 94847 (Sigma Aldrich, USA; 30 mg/kg, i.p.) or vehicle at baseline condition when the mice had access to only regular chow (without chocolate). After three to four days, all mice were presented with chocolate (Hershey's Kisses, Hershey, USA) at 05:50 PM, and vehicle and SNAP 94847 (30 mg/kg) were injected again at 06:50 PM. Sleep-wake with time-locked video was recorded for 6 h post-injection on each occasion. SNAP 94847 was dissolved in 20% (2-hydroxypropyl)-P-cyclodextrin, and the dose (30 mg/kg) was selected based on the previous publications indicating >90% receptor occupancy in the brain at this dose (David et al., 2007; Nair et al., 2009; Smith et al., 2009). Upon completion of all recordings, the mice were sacrificed, brains harvested and histologically processed for orexin-immunolabeling as described in Experiment 1.

Sleep-wake data after vehicle and SNAP 94847 in baseline and chocolate conditions were scored and analyzed as in Experiment 1. As the half-life of SNAP 94847 is 5.2 hours (David et al., 2007) and our pilot data analysis also indicated that the drug effects on cataplexy lasted 5–6 h, we plotted the data for the entire 6 h period after vehicle and SNAP 94847 injections. Percentage of time spent in wake, NREM or REM sleep, short-latency REM sleep (SLREM) and cataplexy and the number and mean bout durations across conditions during the 6 h after saline and SNAP 94847 were compared using two-way ANOVA followed by Mann-Whitney tests.

Results

Histology

AAV-hM3Dq injections into the LH of MCH-Cre or MCH-Cre::OX-KO mice resulted in selective expression of mCherry (indicating hM3Dq expression) in MCH neurons. Almost all the Cherry+ neurons were also labelled for MCH (92.8±3.5%; n=5 mice; Fig. 1A), and none of them were labelled for orexin (Fig 1 C) indicating that hM3Dq expression was restricted to the MCH neurons. These mCherry+ MCH neurons expressed cFos following i.p. CNO injections (Fig. 1B). As the MCH neurons do not express cFos under baseline sleep-wake conditions (Verret et al., 2003), robust cFos expression observed in these neurons demonstrates their activation by i.p. CNO. Finally, absence of orexin- immunoreactivity in the LH of MCH-Cre::OX-KO mice (Experiment 1) and the OX-KO mice (Experiment 2) confirmed the loss of orexin-A in these mice and demonstrated the specificity of the orexin antiserum (Fig.1D).

Experiment 1: Sleep-wake behavior after chemoactivation of MCH neurons— Chemoactivation of MCH neurons in MCH-Cre mice and in MCH-Cre:: OX-KO mice increased REM sleep without altering other vigilance states. In MCH-Cre mice, CNO (0.3 mg/kg i.p.; 6:50 PM) roughly doubled the amount of REM sleep in the first 3 h compared to saline in the same mice (7.6±1.5 min with CNO vs 3.7±1.0 with saline; two-way ANOVA followed by Sidak's multiple comparison test, p=0.033; Fig. 2 A-C). CNO injections in MCH-Cre::OX-KO mice almost tripled the amount of REM sleep (9.9±1.3 min with CNO vs. 3.4±1.3 min with saline; two-way ANOVA followed by Sidak's multiple comparison test, p=0.0096; Fig. 2B) during the same time-period, suggesting that MCH activation more potently increases REM sleep in the absence of orexins. The increase in REM sleep was mainly due to more REM sleep bouts (6.00 ±1.3 with CNO vs. 2.88 ± 0.9 with saline in MCH-Cre mice; 7.7 ±1.2 with CNO vs 3.2 ±1.2 with saline in MCH-Cre::OX-KO mice; Mann-Whitney U test; P=0.034 and 0.039 respectively). REM sleep latency (time to enter REM sleep after injection) was much shorter with CNO (49.8 ± 12.3 min with CNO vs. 130.7 ± 22.0 min with saline in MCH-Cre mice; 28.3 ± 10.9 min with CNO vs 59.2± 10.4 with saline in MCH-Cre::OX-KO mice; Mann-Whitney U test; P=0.0047 and 0.026 respectively; Fig. 2D). Compared to MCH-Cre mice, the MCH-Cre::OX-KO mice entered REM sleep faster even after saline injections, and chemoactivation of the MCH neurons reduced it further, suggesting a high propensity to REM sleep during the dark period in MCH-Cre::OX-KO mice which can be further enhanced by MCH neuron activation. Chemoactivation did not alter the duration of REM sleep bouts in either group of mice (Table 1), suggesting that the MCH neurons trigger transitions into REM sleep but do not sustain REM sleep.

In the 4–6 h period after injections, CNO did not significantly alter REM sleep in either group of mice (Fig. 2B; Table 1), indicating absence of any subsequent reduction in REM sleep (i.e. after the initial increase in the first 3 h).

Abnormal REM sleep phenomena after chemoactivation of MCH neurons

Narcolepsy is characterized by signs of REM sleep dysregulation including cataplexy and rapid transitions into REM sleep. People with narcolepsy and narcoleptic dogs (Andlauer et al., 2013; Nishino et al., 2000; Pizza et al., 2015; Reiter et al., 2015) often enter REM sleep within 15 minutes of sleep onset. Therefore, we analyzed SLREMs which are defined as REM sleep bouts after ≤12 sec of NREM sleep; SLREMs are distinct from regular REM sleep bouts that occur after considerable (>12 sec) NREM sleep or cataplexy which is a REM sleep-like state that occurs in the midst of wake.

In MCH-Cre mice with intact orexin signaling, CNO injections did not induce cataplexy or SLREMs. In contrast, MCH-Cre::OX-KO had moderate amounts of cataplexy and SLREMs after saline, and CNO increased both states. Time spent in cataplexy more than doubled (4.5±1.0 min with CNO vs. 1.9 ±0.5 min with saline; Mann-Whitney U test, p=0.039), and SLREMs increased seven-fold (3.7±1.1 min with CNO vs. 0.5±0.3 min with saline; Mann-Whitney U test, p=0.0087; Fig. 3) with CNO compared to saline. The increase in time spent in cataplexy was probably driven by more frequent and longer cataplexy bouts, though these

increases were not statistically significant. The increase in time spent in SLREMs was due to a 7-fold increase in SLREMs episodes (Table 2) but not their mean duration.

CNO did not alter sleep-wake behavior in MCH-Cre mice that received the negative control vector, AAV-mCherry into the LH. Percentages of wake, NREM sleep and REM sleep as well as bout numbers and bout durations of these states after CNO did not differ from those after saline (Supplementary Table 1). None of these mice displayed cataplexy or SLREM after CNO. We did not perform similar AAV-mCherry injections in MCH-Cre::OX-KO mice because these are MCH-Cre mice on OX-KO background, and we have recently shown that CNO does not alter sleep-wake behavior or cataplexy in OX-KO mice lacking DREADD receptors (Mahoney et al., 2017). Taken together, these data demonstrate that CNO at the 0.3 mg/kg dose used in this study does not produce any non-specific effects on sleep-wake behavior or cataplexy. Thus, the increases in REM sleep, SLREM and cataplexy observed after CNO in MCH-Cre and MCH-Cre::OX-KO mice injected with AAV-hM3Dq into the LH were mediated by CNO activation of hM3Dq receptors in MCH neurons.

Experiment 2: Sleep-wake behavior and abnormal REM sleep phenomena after an MCH antagonist—The MCHR1 antagonist, SNAP 94847 (30 mg/kg, i.p) did not substantially change the amount of sleep-wake states during the first 6 h after injections in OX-KO mice (Fig. 4), suggesting that the MCH signaling may not regulate spontaneous sleep-wake behavior, including REM sleep. In contrast, SNAP 94847 dramatically reduced baseline amounts of cataplexy and SLREMs in OX-KO mice. In the 6 hours after vehicle, the OX-KO mice displayed moderate amounts of cataplexy (3.4 ± 0.4 min) and SLREMs (1.4 ± 0.7 min), but SNAP 94847 reduced cataplexy by 80% (Fig. 5A; P = 0.008; Mann-Whitney U test) and SLREMs by 75% (Fig. 5B; P=0.12; Mann-Whitney U test).

We then analyzed the effects of SNAP 94847 on chocolate-induced cataplexy. Access to chocolate almost tripled the number of cataplexy bouts and total amounts of cataplexy (Fig. 5 A,C) during the first 6 h after vehicle injections, confirming that rewarding stimuli increase cataplexy in OX-KO mice. SNAP 94847 reduced the amounts of chocolate-induced cataplexy by 88% (Fig. 5A; P= 0.002; Mann- Whitney U test) and SLREM by 85% (Fig. 5B; P=0.02; Mann-Whitney U test) by producing fewer and shorter bouts (Fig. 5C,D; Table 3). OX-KO mice ate similar amounts of chocolate after vehicle (1.6±0.08 g) and SNAP 94847 (1.76 ± 0.16 g; P>0.05). These data demonstrate that the MCH signaling may promote abnormal intrusions of REM sleep phenomena in other vigilance states, resulting in SLREMs and cataplexy.

Discussion:

To examine whether MCH contributes to the abnormal physiology of narcolepsy, we studied chemoactivation of the MCH neurons in mice with and without orexin signaling. In both lines of mice, chemoactivation of the MCH neurons substantially increased REM sleep, and in mice lacking orexins, chemoactivation also increased cataplexy and SLREMs. In addition, a MCHR1-antagonist dramatically reduced SLREMs and cataplexy in mice lacking orexins at baseline as well after a positive emotional stimulus. These findings indicate that MCH

neurons can promote REM sleep under physiological conditions, and their activity may promote rapid transitions into REM sleep and cataplexy in an animal model of narcolepsy.

MCH neurons do not alter NREM sleep

Researchers have studied the effects of MCH on sleep for over 15 years, but the effects of MCH are still debated. We previously found that chemoactivation of MCH neurons increases REM sleep (Vetrivelan et al., 2016), but optogenetic activation of MCH neurons, on the other hand, yielded inconsistent results (Konadhode et al., 2016; Konadhode et al., 2013; Tsunematsu et al., 2014). For example, photostimulation of MCH neurons increased both NREM and REM sleep in mice and rats (Blanco-Centurion et al., 2016; Konadhode et al., 2013), whereas a third study found less NREM sleep with both optogenetic stimulation and inhibition of MCH neurons (Tsunematsu et al., 2014). In the current study, we found no effects on NREM sleep with chemoactivation of MCH neurons in both MCH- Cre and MCH-Cre::OX-KO mice. Lack of NREM sleep alterations in the latter mouse model is especially interesting as OX-KO mice have poor wake maintenance with frequent transitions into NREM sleep. As the orexin neurons heavily innervate major wake-promoting centers in hypothalamus and brainstem but not to sleep-promoting preoptic neurons and these neurons are maximally active during wake, it was proposed that orexin neurons may act to consolidate wake and thereby stabilize sleep-wake states. However, even in these mice with sleep-wake instability with low thresholds to transition between behavioral states, MCH neuron activation failed to increase NREM sleep or transitions into NREM sleep, thereby arguing against a role for the MCH neurons in promoting NREM sleep.

MCH neurons specifically promote REM sleep

Though chemoactivation of MCH neurons had no effect on NREM sleep, it substantially increased REM sleep in MCH-Cre mice and even more so in MCH-Cre::OX-KO mice. These results support our hypothesis that MCH neurons mainly promote REM sleep but not NREM sleep. OX-KO mice have normal amounts of REM sleep (Kantor et al., 2009; Mochizuki et al., 2004), but their average REM sleep latencies are shorter under constant dark conditions (Kantor et al., 2009). Moreover, mice lacking orexin neurons displayed shorter latency to REM sleep and higher REM sleep amounts especially during the dark period (Kantor et al., 2009). Compared to MCH-Cre mice, we found that MCH-Cre: :OX-KO mice entered REM sleep more quickly after saline injection, and with chemoactivation of MCH neurons, REM sleep latency was even shorter and the amount of REM sleep was increased. This increase in REM sleep after CNO in MCH-Cre::OX-KO mice was larger than that observed in MCH-Cre mice. These results indicate that MCH neurons can promote REM sleep through mechanisms independent of suppressing orexin signaling.

The specific mechanisms through which MCH promotes REM sleep remain unknown. Most likely, the MCH neurons inhibit the REM sleep-suppressing brain regions, including the ventrolateral periaqueductal gray (vlPAG) and the adjoining lateral pontine tegmentum (LPT). GABAergic neurons in the vlPAG/LPT have been shown to ‘gate’ REM sleep by inhibiting glutamatergic neurons in the sublaterodorsal nucleus (SLD) that generate REM sleep (Lu et al., 2006; Luppi et al., 2012; Vetrivelan et al., 2011). MCH neurons heavily innervate the vlPAG/LPT, and MCH receptors are densely expressed in this region (Chee et

al., 2015; Clement et al., 2012). Importantly, REM sleep suppression induced by pharmacological inhibition of LH was accompanied by with an increase in cFos in the vlPAG/LPT neurons projecting to the SLD (Clement et al., 2012). The orexin neurons also innervate the vlPAG/LPT and likely excite these REM-suppressing neurons (Adamantidis and de Lecea, 2008; Oishi et al., 2013). Thus, we propose that REM sleep is regulated by the balance between excitatory orexin and inhibitory MCH signaling in the vlPAG/LPT and similar REM sleep-regulatory brain regions.

MCH neurons contribute to narcolepsy symptoms

In addition to increasing REM sleep, activation of MCH neurons increased time in SLREM episodes and cataplexy in MCH-Cre::OX-KO mice, suggesting MCH neurons may also trigger pathological intrusions of REM sleep into other vigilance states. While the SLREMs increased 7 fold, time spent in cataplexy increased two-fold. We defined SLREMs as REM sleep with <12 s of preceding NREM sleep, and we believe these are akin to the rapid transitions into REM sleep that are common in people with narcolepsy. In fact, transitions into REM sleep within 15 min of sleep onset at night may be a reliable indicator for the diagnosis of narcolepsy with cataplexy in adults and children with narcolepsy with cataplexy (Andlauer et al., 2013; Reiter et al., 2015). We find that OX-KO mice enter REM sleep rapidly, and these events are more frequent with MCH neuron activation whereas blocking MCHR1 receptors lead to near complete attenuation of these events. Thus, it appears that in the absence of orexin peptides, signaling by the MCH neurons can promote rapid transitions into REM sleep and cataplexy.

Despite much evidence indicating a close interrelationship with orexin neurons, the role of MCH neurons in cataplexy and SLREMs has never been explored in animal models. Cataplexy is usually triggered by strong positive emotions, and rewarding or emotional stimuli may activate the MCH neurons. Chocolate strongly increases cataplexy in OX-KO mice, and chocolate roughly doubles the number of MCH neurons expressing cFos and this increase correlates with the number of cataplexy bouts (Oishi et al., 2013). A recent study using calcium imaging shows that MCH neurons are active during exploration of novel objects (Gonzalez et al., 2016). MCH neurons are also implicated in feeding behavior, and MCH levels increased significantly in the amygdala prior to, during and after eating behavior (Blouin et al., 2013). In addition, intracerebroventricular injection of MCH increases both food and sucrose intake in rats (Baird et al., 2008; Fukushima et al., 2014; Sakamaki et al., 2005). Considered together, these results suggest that the MCH neurons may be active not only during REM sleep but also with rewarding stimuli, including feeding and exposure to novel objects. Our present results show that MCH neuron activation increases cataplexy in mice lacking orexins and a MCH antagonist substantially reduces cataplexy at baseline as well as with chocolate, thereby demonstrate that MCH neurons are causally involved in pathophysiology of cataplexy. However, positive emotional or rewarding stimuli that trigger cataplexy is generally associated with an increase in wake because the animals should stay awake to secure the reward. But, activation of MCH neurons do not increase wake but only increased REM sleep (Jego et al., 2013; Tsunematsu et al., 2014; Vetrivelan et al., 2016). Thus, we speculate that the MCH neurons may not promote wake associated with reward-expecting situations but may counteract the motor

activation associated with these situations. MCH neurons fire most when EMG tone is low in a pattern opposite to that of the orexin neurons (Hassani et al., 2009) and mice lacking MCH neurons display hyperactivity which is further enhanced with psychostimulants (Vetrivelan et al., 2016; Whiddon and Palmiter, 2013). Thus, MCH neurons may reduce the hyperactivity associated with reward-expecting conditions in intact mice, and may promote muscle atonia in the absence of the opposing actions of orexin.

In addition to narcolepsy, REM dysregulation is commonly observed in patients with major depressive disorder (Adrien, 2002; Palagini et al., 2013). Interestingly, studies on animal models indicated that MCH signaling might contribute to pathophysiology of depression. For example, increased expression of prepro-MCH mRNA is observed in animal models of depression (Garcia-Fuster et al., 2012) and MCH infusions into specific brain regions, such as dorsal raphe and nucleus accumbens, induce depressive behavior in rodents (Georgescu et al., 2005; Lagos et al., 2011) indicating MCH signaling is pro-depressant. Consistent with this, MCH antagonists exhibit anti-depressant properties (Shimazaki et al., 2006). Finally, increase in REM sleep and decreased REM latency, which are hallmark features of major depressive disorder (Adrien, 2002; Palagini et al., 2013), were also observed after MCH neuron activation. Taken together, these findings indicate that MCH neurons may be a link between affective disorders and REM sleep.

Limitations of the study

We used both male and female mice for the experiments, but as numbers in each sex were small, we could not examine sex differences. However, each mouse served as its own control (vehicle injections were performed in all mice), thereby enabling us to test the effects of MCH neuron activation or MCH antagonism. Close inspection of data from individual mice also indicated that CNO injections increased REM sleep and/or cataplexy in all mice and SNAP 94847 decreased cataplexy in all mice compared to vehicle controls indicating that MCH neurons increase REM sleep and cataplexy in both male and female mice. Another limitation is that we did not test the effects of the MCHR1 antagonist in intact animals. However, this has been investigated previously (Able et al., 2009) and this study also found no significant alterations in sleep-wake in intact rats similar to our results in OX-KO mice. As the present study was designed to primarily evaluate the role of MCH neurons in narcolepsy, we injected MCHR1 antagonist, SNAP 94847 only in mouse model of narcolepsy and our results convincingly shows that this drug strongly attenuates narcolepsy symptoms.

Conclusions

Our results demonstrate that activation of MCH neurons selectively promotes REM sleep in mice with intact orexin signaling and increases abnormal REM sleep events such as rapid transitions into REM sleep and cataplexy in mice lacking orexins. Conversely, MCH antagonism strongly reduces both cataplexy and abnormal REM sleep transitions. These results, for the first time, suggest a causal role for MCH neurons in narcolepsy. Future studies in animal models of narcolepsy will be necessary to probe the specific neural

pathways and mechanisms through which MCH neurons induce cataplexy and other abnormal manifestations of REM sleep.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAV	adeno-associated viral vector
CNO	clozapine N-oxide
EEG	electroencephalogram
EMG	electromyography
LH	lateral hypothalamus
LPT	lateral pontine tegmentum
MCH	melanin concentrating hormone
NREM	non-rapid eye movement sleep
OX	orexin
REM	rapid eye movement sleep
SLD	sublateraodorsal tegmentum
SLREM	short latency REM sleep
vlPAG	ventrolateral periaqueductal gray

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Highlights

- Chemoactivation of MCH neurons selectively increases REM sleep in mice.
- MCH neuron activation, even in mice lacking orexin, does not alter non-REM sleep.
- MCH neuron activation increases short-latency REMs and cataplexy in mice lacking orexin.
- Pharmacological blockade of MCH signaling reduces short-latency REMs and cataplexy in lacking orexin.

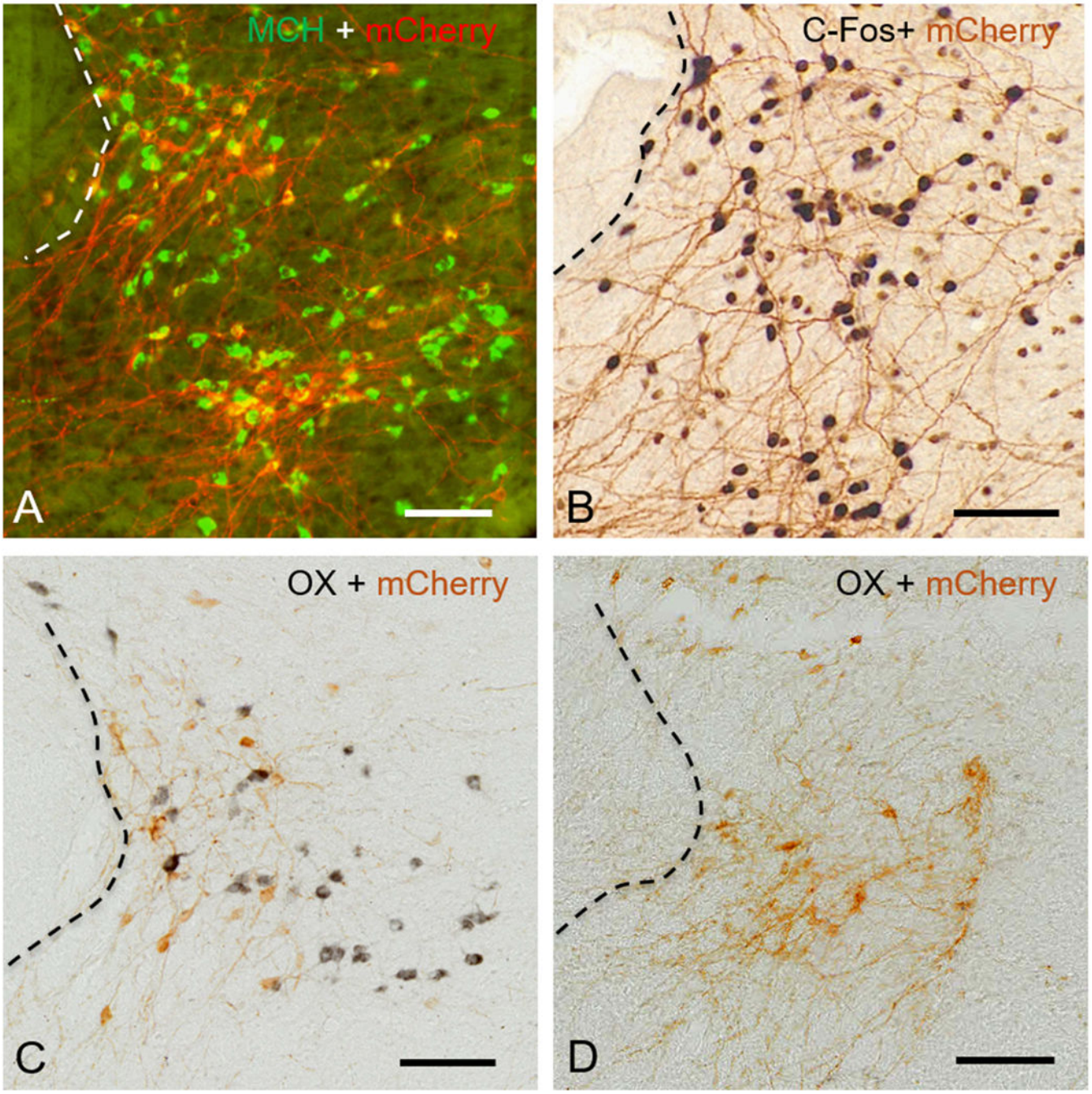

Figure 1: Chemoactivation of MCH neurons in MCH-Cre and MCH-Cre::OX-KO mice.

(A) Representative section at the level of the lateral hypothalamus (LH), immunolabeled for the hM3 marker, mCherry (red) and MCH (green) from a MCH-Cre mouse that received AAV-hM3Dq injections. Virtually all the mCherry+ neurons were also labeled for MCH, indicating that hM3 expression was specific to the MCH neurons. (B) Intraperitoneal administration of CNO resulted in cFos expression (black) in hM3Dq-mCherry neurons (brown cells) in the LH. Double labeling of sections from MCH-Cre (C) and MCH-Cre::OX-KO mice (D) for mCherry (brown) and orexin (black) showed that hM3Dq was not

expressed in orexin neurons. Also note the lack of orexin staining in MCH-Cre::OX-KO mice (D). Scale bar, 100 μm.

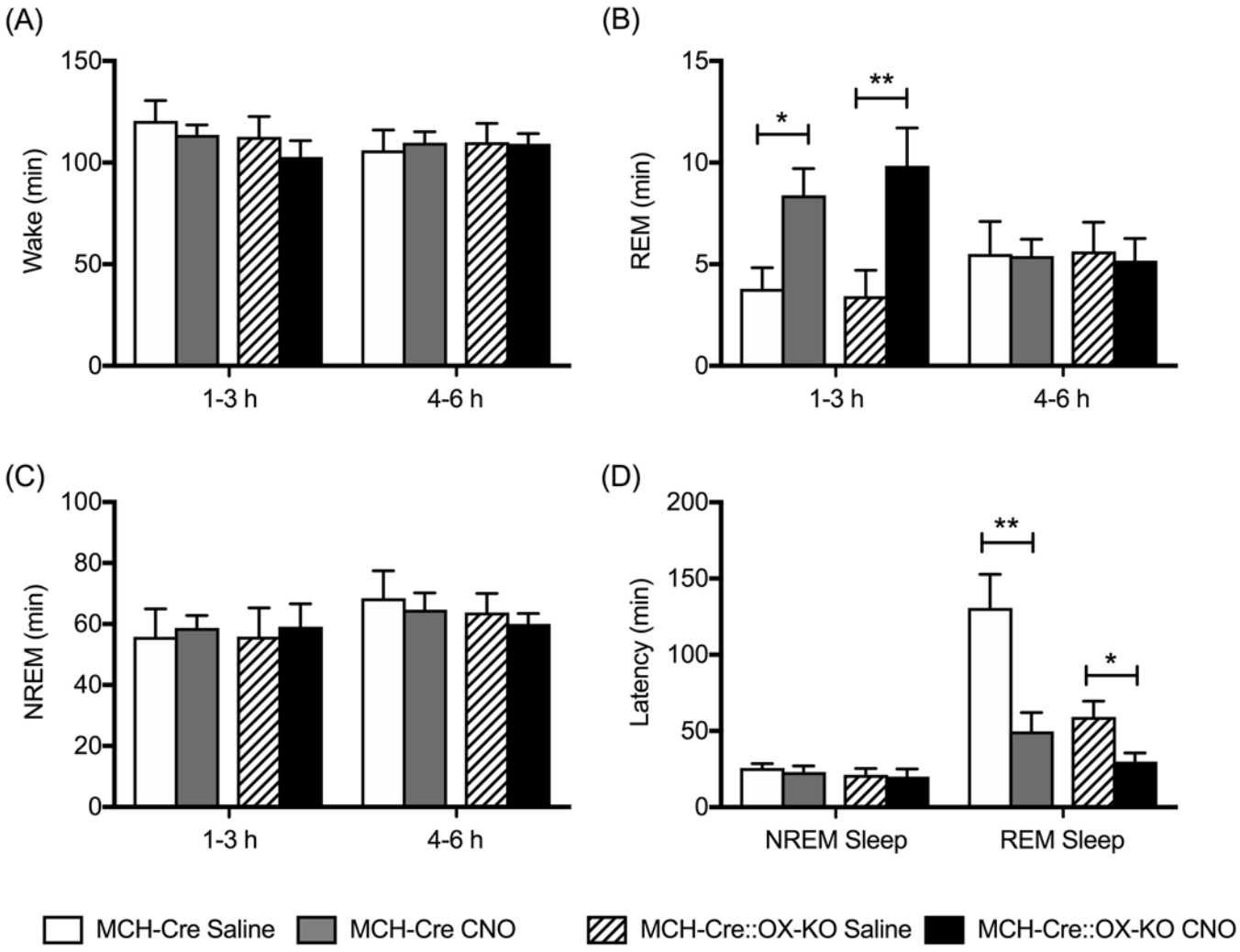

Figure 2: Chemoactivation of MCH neurons increased REM sleep in both MCH-Cre and MCH-Cre: :OX-KO mice

Amounts of wake (A), rapid eye movement (REM) sleep (B) and non-REM (NREM) sleep (C) after intraperitoneal administration (6:50 PM; 10 min before dark onset) of vehicle (saline) or CNO (0.3 mg/kg) in MCH-Cre and MCH-Cre::OX-KO mice injected with AAV-hM3Dq into the LH. First 6-h data after saline/CNO administration was summarized as two 3 h bins; Two-way ANOVA between gene and treatment (Treatment $F(1, 24)=16.24$, $p=0.0005$; Gene $F(1, 24)=0.16$, $p=0.69$; Interaction $F(1, 24)=0.45$, $p=0.51$) followed by Sidak's Multiple Comparison test; *P<0.05, **P<0.01. (D) Latency to first NREM and REM episodes after saline and CNO injections. *P<0.05; **P<0.01; non-parametric Mann-Whitney U test. Chemoactivation of MCH neurons substantially increased REM sleep during the first 3 h after CNO injections but had no effect of wake or NREM sleep. All data are mean ± SEM.

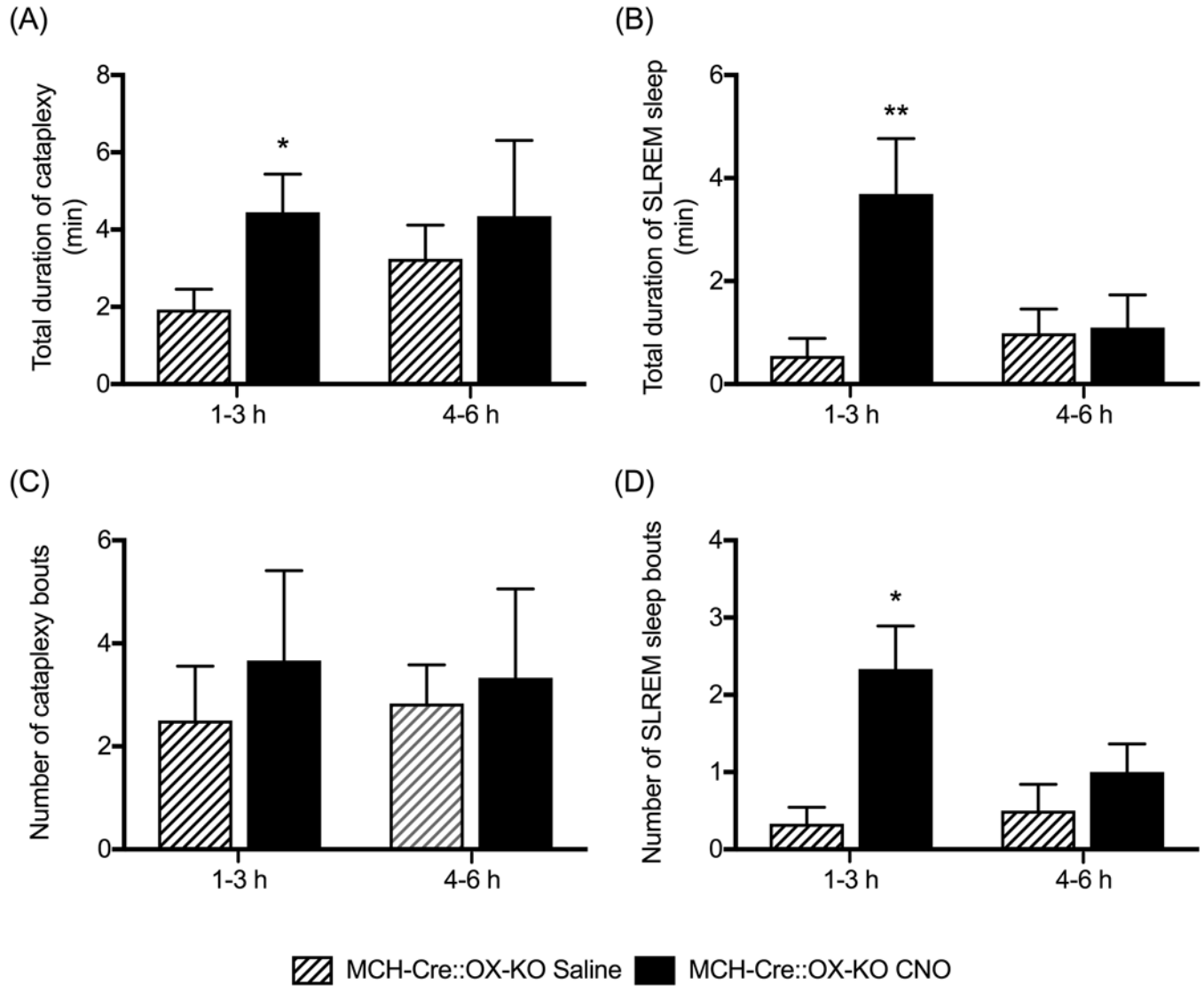

Figure 3. Chemoactivation of MCH neurons increased the occurrence of abnormal REM intrusions in MCH-Cre::OX-KO mice.

Total amounts (total duration in minutes) and number of cataplexy (A, C) and short latency-REM sleep bouts (B, D) after intraperitoneal administration (6:50 PM; 10 min before dark onset) of vehicle (saline) or CNO (0.3 mg/kg) in MCH-Cre::OX-KO mice injected with AAV-hM3Dq into the LH. First 6-h data after saline/CNO administration was summarized as two 3 h bins. Chemoactivation of MCH neurons increased the amounts of both cataplexy and SLREM in the absence of orexin. All data are mean ± SEM. *P<0.05; **P<0.01; non-parametric Mann-Whitney U test.

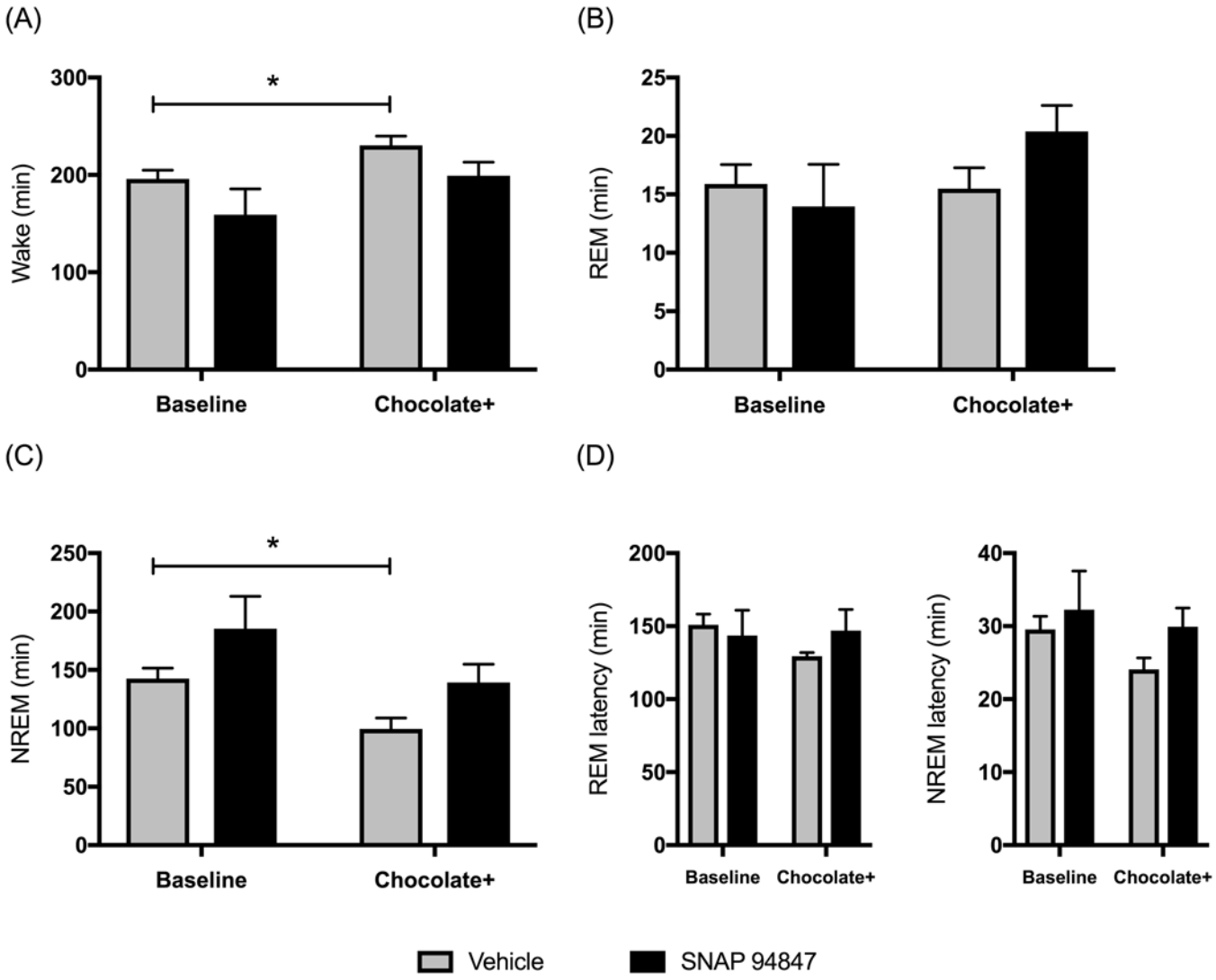

Figure 4. MCHR1 antagonist did not alter sleep-wake in OX-KO mice.

Amounts of wake (A), rapid eye movement (REM) sleep (B) and non-REM (NREM) sleep (C) during the 6 h after intraperitoneal administration (6:50 PM; 10 min before dark onset) of vehicle or an MCHR1- antagonist, SNAP 94847 (30 mg/kg) in OX-KO mice when they had access to only regular chow diet (Baseline) or chocolate in addition (Chocolate+). Two-way repeated measures ANOVA (for wake - condition $F(1,5)=9.88$, $p=0.02$; Treatment $F(1,5)=2.30$, $p=0.19$; for REM sleep - condition $F(1,5)=1.43$, $p=0.28$; Treatment $F(1,5)=0.23$; $P=0.6$; for NREM sleep - condition $F(1,5)=15.08$, $p=0.01$; Treatment $F(1,5)=2.95$, $p=0.15$) followed by Mann-Whitney U tests; *$P<0.05$. (D) Latency to first REM and NREM episodes after vehicle and SNAP 94847 injections in both conditions. Pharmacological blockade of MCHR1 did not significantly alter sleep-wake in OX-KO mice. While the access to chocolate increased the amounts of wake and decreased NREM sleep, simultaneous MCHR1 antagonism did not significant alter the wake or NREM. All data are mean ± SEM.

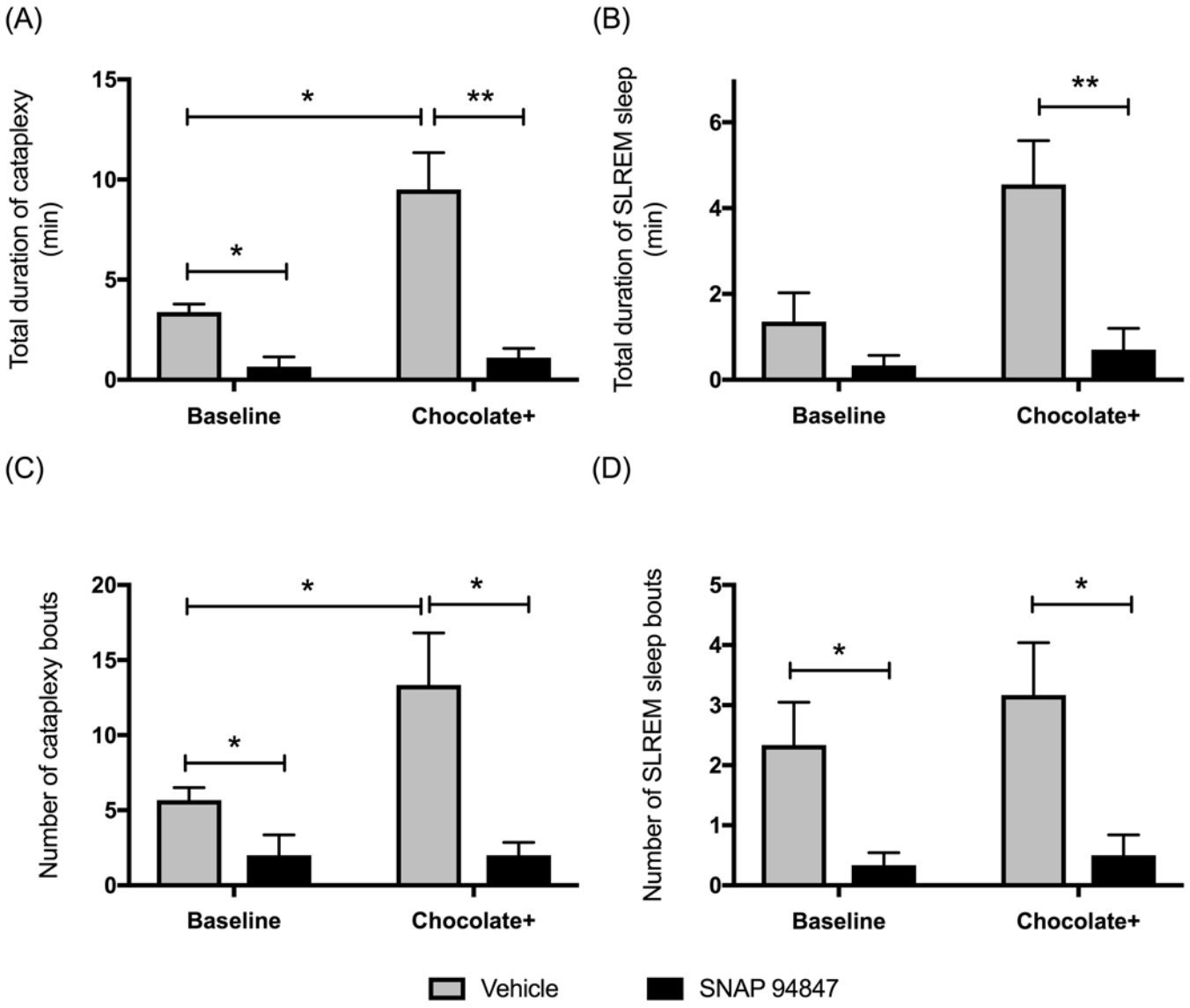

Figure 5. MCHR1 antagonist substantially reduces the abnormal REM events in OX-KO mice.

Total amounts (total duration in minutes) and number of cataplexy (A, C) and short latency-REM sleep bouts (B, D) during 6-h after intraperitoneal administration (6:50 PM; 10 min before dark onset) of vehicle or SNAP 94847 (30 mg/kg) in OX-KO mice when they had access to only regular chow diet (Baseline) or chocolate in addition (Chocolate^{+}). Two-way repeated measures ANOVA (for total duration of cataplexy - condition $F(1,5)=8.45$, $p=0.03$; Treatment $F(1,5)=26.67$, $p=0.003$; for total duration of SLREM - condition $F(1,5)=7.62$, $p=0.04$; Treatment $F(1,5)=13.76$; $P=0.01$; for number of cataplexy bouts - condition $F(1, 5)=3.37$, $p=0.12$; Treatment $F(1,5)=16.15$, $p=0.01$; For number of SLREM bouts - condition $F(1,5)=1.36$, $p=0.30$; Treatment $F(1,5)=22.27$, $p=0.005$) followed by Mann-Whitney U tests; *$P<0.05$, **$P<0.01$. Pharmacological blockade of MCHR1 substantially reduced cataplexy and SLREM amounts under both conditions. All data are mean ± SEM.

Table 1:

Changes in sleep-wake architecture after chemoactivation of MCH neurons in MCH-Cre and MCH-Cre::OX-KO mice.

Bout number and duration of sleep-wake states after saline or CNO (i.p.; 0.3 mg/kg) in MCH-Cre mice and MCH-Cre::OX-KO mice that received AAV-hM3Dq injections into the LH. Data from the first 6 h after injections was divided into two 3 h bins and presented. Chemoactivation of MCH neurons significantly increased the number of REM sleep bouts in both sets of mice.

		Number of bouts		Mean bout duration (s)	
		Post-saline	Post-CNO	Post-saline	Post-CNO
MCH-Cre					
Wake	1–3 h	25.13 ±5.49	23.75 ±3.85	382.50 ± 174.07	264.38 ± 44.88
	4–6 h	25.00 ±6.12	26.50 ±4.47	314.63 ± 94.25	240.63 ± 65.16
REM	1–3 h	2.88 ±0.90	6.00 ± 1.25*	91.17 ± 12.98	79.88 ± 10.51
	4–6 h	4.75 ± 1.05	5.25 ±0.73	60.63 ± 11.64	64.50 ±8.71
NREM	1–3 h	25.50 ± 5.43	24.75 ±3.50	143.50 ± 23.39	152.63 ± 18.50
	4–6 h	25.25 ±6.24	26.63 ±4.25	175.38 ± 22.83	162.00 ± 23.13
MCH-Cre::OX KO					
Wake	1–3 h	35.33 ±4.94	40.67 ±4.27	192.00 ± 63.33	127.83 ± 67.69
	4–6 h	34.33 ±5.81	43.33 ±4.78	254.67 ± 102.03	145.67 ± 109.96
REM	1–3 h	3.17 ± 1.17	7.67 ± 1.21 *	66.00 ± 12.68	74.33 ± 14.11
	4–6 h	4.17 ± 1.35	5.67 ± 1.34	75.60 ± 6.64	66.50 ± 7.34
NREM	1–3 h	33.00 ± 5.86	36.50 ± 5.33	111.33 ±8.28	97.33 ±7.32
	4–6 h	31.00 ±5.92	40.17 ±5.08	117.33 ± 11.52	91.67 ±8.73

All data are mean ± SEM.

* P<0.05; Mann-Whitney U test.

Table 2:

Changes in abnormal REM events after chemoactivation of MCH neurons in MCH-Cre::OX-KO mice

Bout number and duration of abnormal REM events after saline or CNO (i.p.; 0.3 mg/kg) in Cre::OX-KO mice that received AAV-hM3Dq injections into the LH. Data from the first 6 injections was divided into two 3 h bins and presented. Chemoactivation of MCH neurons in MCH-Cre::OX-KO mice significantly increased the number of SLREM sleep bouts. On the other hand, data from MCH-Cre mice is not presented as these mice neither after saline nor after CNO injections displayed SLREM or cataplexy.

		Number of bouts		Mean bout duration (s)	
		Post-saline	Post-CNO	Post-saline	Post-CNO
Cataplexy	1-3h	2.50 ±1.06	3.67 ±1.74	33.83 ± 12.10	58.17 ± 13.15
	4-6 h	2.83 ±0.75	3.33 ±1.73	46.17 ±9.74	23.50 ±6.87
SLREMs	1-3 h	0.33 ±0.21	2.33 ±0.56 **	26.00 ± 17.09	100.50 ± 18.20
	4-6 h	0.50 ±0.34	1.00 ±0.37	24.00 ± 15.49	57.00 ± 15.34

All data are mean ± SEM.

** P<0.01; Mann-Whitney U test.

Table 3:

Changes in architecture of sleep-wake cycle and abnormal REM events after blockade of MCHR1 in OX-KO mice

Bout number and duration of sleep-wake states and abnormal REM events during the 6 h after vehicle or SNAP 94847 (i.p.; 30 mg/kg) in OX-KO mice. Pharmacological blockade of MCH signaling significantly decreased both the number and duration of SLREM and cataplexy bouts, but did not alter architecture of other sleep-wake stages.

		Number of bouts		Mean bout duration (s)	
		Post-vehicle	Post-SNAP 94847	Post-vehicle	Post-SNAP 94847
Baseline					
Wake	1–6 h	104.67 ± 10.61	116.17 ± 9.22	114.17 ± 10.61	90.42 ± 23.39
REM	1–6 h	18.67 ±1.38	16.17 ±3.89	55.33 ±4.65	41.75 ±8.38
NREM	1–6 h	97.00 ± 10.98	113.33 ± 10.03	96.58 ± 10.66	101.58 ± 14.84
Cataplexy	1–6 h	5.67 ± 0.84	2.00 ± 1.37 *	31.25 ±3.76	14.67 ±9.03
SLREM	1–6 h	2.33 ±0.71	0.33 ±0.21 *	47.25 ± 16.27	10.00 ±7.04 *
Chocolate+					
Wake	1–6 h	109.17 ± 8.92	140.33 ± 14.91	142.25 ± 14.40	89.91 ± 13.78
REM	1–6 h	18.00 ± 1.97	23.50 ±2.69	50.58 ± 3.25	50.33 ± 7.54
NREM	1–6 h	95.50 ± 7.06	136.83 ± 15.37	58.00 ± 3.89	65.50 ±3.78
Cataplexy	1–6 h	13.33 ±3.47	2.00 ±0.86 *	42.08 ± 5.60	13.17 ±4.83 **
SLREM	1–6 h	3.16 ±0.87	0.50 ±0.34 *	67.42 ± 15.45	13.50 ±8.62 *

All data are mean ± SEM.

* P<0.05

** P<0.01, Mann-Whitney U test.