HHS Public Access

Author manuscript

Neuroscience. Author manuscript; available in PMC 2017 November 12.

Published in final edited form as:

Neuroscience. 2016 November 12; 336: 102–113. doi:10.1016/j.neuroscience.2016.08.046.

Melanin-concentrating hormone neurons specifically promote rapid eye movement sleep in mice

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Abstract

Currently available evidence indicates that neurons containing melanin-concentrating hormone (MCH) in the lateral hypothalamus are critical modulators of sleep-wakefulness, but their precise role in this function is not clear. Studies employing optogenetic stimulation of MCH neurons have yielded inconsistent results, presumably due to differences in the optogenetic stimulation protocols, which do not approximate normal patterns of cell firing. In order to resolve this discrepancy, we 1) selectively activated the MCH neurons using a chemogenetic approach (Cre-dependent hM3Dq expression) and 2) selectively destroyed MCH neurons using a genetically targeted diphtheria toxin deletion method, and studied the changes in sleep-wake in mice. Our results indicate that selective activation of MCH neurons causes specific increases in rapid eye movement (REM) sleep without altering wake or non-REM (NREM) sleep. On the other hand, selective deletions of MCH neurons altered the diurnal rhythm of wake and REM sleep without altering their total amounts. These results indicate that activation of MCH neurons primarily drives REM sleep and their presence may be necessary for normal expression of diurnal variation of REM sleep and wake.

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Conflict of Interest:

The authors declare no competing financial interests.

Keywords

Lateral hypothalamus; sleep-wake; paradoxical sleep; feeding; chemogenetics; conditional deletion

Introduction

Neurons containing melanin-concentrating hormone (MCH), localized in the lateral hypothalamus (LH), have long been implicated in sleep-wake regulation (Verret et al., 2003, Hanriot et al., 2007, Willie et al., 2008, Peyron et al., 2009, Peyron et al., 2011). For example, mice with deletions of the *MCH* gene or MCH neurons have been reported to display an increase in wakefulness with corresponding decrease in non-rapid eye movement (NREM) sleep, suggesting that MCH neurons may promote NREM sleep (Willie et al., 2008, Tsunematsu et al., 2014). Other evidence indicates that the MCH neurons may be specifically linked to regulation of rapid eye movement (REM) sleep. For example, more than 60% of MCH neurons express cFos during REM hypersomnia following selective REM deprivation (Verret et al., 2003). MCH, when injected intracerebroventricularly, produces a robust (~200%) increase in REM sleep (Verret et al., 2003) while infusion of an MCH receptor antagonist causes a significant reduction in REM sleep in rats (Ahnaou et al., 2008). MCH neurons heavily innervate the dorsolateral brainstem regions implicated in REM generation including the sublaterodorsal nucleus (SLD) and ventrolateral periaqueductal gray matter (vlPAG), (Hanriot et al., 2007, Peyron et al., 2009, Sapin et al., 2010, Clement et al., 2012) and local injection of MCH into the nucleus pontis oralis (in the subcoeruleus region which is considered to be an SLD-equivalent in cats) produces a significant increase (~70%) in REM sleep and reduces the latency to REM sleep (Torterolo et al., 2009). Juxtacellular recording studies show that MCH neurons are maximally active during REM sleep, silent during wake, and occasionally active in NREM sleep (Hassani et al., 2009), suggesting that they promote REM sleep.

On the other hand, recent studies using optogenetic activation of MCH neurons have found contradictory results. Brief periods of stimulation of MCH neurons during NREM and REM sleep increased NREM-to-REM transitions and REM sleep bout durations respectively whereas stimulation during wake had no effect on either wake-sleep transitions or bout durations (Jego et al., 2013). Long-term stimulation of MCH neurons (1 minute out of 5, for 6 or 24 hours), however, was reported to cause increases in both NREM and REM sleep (Konadhode et al., 2013). A third study using intermediate duration (3 hr) continuous stimulation of MCH neurons found an increase in REM sleep with significant reduction in NREM sleep (Tsunematsu et al., 2014). However, in the same study, genetically driven deletion of MCH neurons caused reduced NREM sleep (Tsunematsu et al., 2014).

One reason for these varying results may be that optogenetic stimulation produces artificial, monotonous firing that may not approximate endogenous firing patterns. MCH neurons contain both GABA and glutamate, as well as several additional peptides, so the mix of neurotransmitters released at a given terminal site may be different with different firing patterns (Arrigoni and Saper, 2014). The effects of MCH and other peptides that work

through G-protein coupled receptors may also have prolonged effects on membrane potential that are difficult to predict. Thus, the long-term effects of MCH neurons on their targets *in vivo* may be different from what is measured by brief optogenetic activation of MCH neurons either *in vivo* or *in vitro* (Arrigoni and Saper, 2014). Hence, we used a chemogenetic approach, expressing genetically targeted hM3Dq mutated muscarinic acetylcholine receptors to excite MCH neurons by using the ligand clozapine-N-oxide (CNO). We also used genetically targeted diphtheria toxin (DT) to selectively ablate MCH neurons and re-examine the changes in sleep-wake.

Finally, sleep and wake occur in the context of a variety of important behaviors such as feeding and locomotor activity (LMA), and physiological functions such as regulation of body weight and temperature (Tb). Because MCH neurons have also been implicated in these functions (Qu et al., 1996, Shimada et al., 1998, Astrand et al., 2004, Whiddon and Palmiter, 2013), we studied concomitant changes in feeding, body weight, LMA and Tb during MCH neuron stimulation and deletion.

Experimental Procedures

Animals

Two transgenic mouse lines (MCH-Cre and MCH-Cre/+; iDTR, mice) were used in this study.

MCH-Cre transgenic mice were generated as previously described (Kong et al., 2010). These mice specifically express Cre recombinase (Cre) under the MCH promoter and the eutopic expression of Cre in MCH neurons has been verified (Kong et al., 2010).

MCH-Cre/+;iDTR mice were generated by crossing the MCH-Cre mice with another transgenic mouse line, inducible diphtheria toxin receptor (iDTR) mice (Stock no. 007900; Jackson's laboratories, USA). The resultant offspring express DTR specifically in the MCH neurons (MCH-Cre/+;iDTR) and thus allow specific deletion of MCH neurons upon intraperitoneal (IP) administration of diphtheria toxin, DT.

Animal care—All mice were housed in individual cages and maintained under 12:12 light-dark cycle (lights on at 0700; 150 lux). Ambient temperature in the animal room was kept at 22 ± 1°C. 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.

Viral vector—In order to selectively activate the MCH neurons, we injected Cre-dependent adeno-associated viral vectors (AAV) containing the stimulatory hM3Dq receptor (AAV-hSyn-DIO-hM3D(Gq)-mCherry; AAV serotype 8; University of North Carolina Vector Core, USA) into the MCH-Cre mice. The construction of this vector has been described in previous publications (Krashes et al., 2011). The ligand for the hM3Dq receptors is clozapine-N-oxide (CNO), which is administered intraperitoneally.

Experiment 1: Selective stimulation of MCH neurons

Surgery and recordings—Adult male MCH-Cre mice (n=11) were anesthetized (100 mg/kg ketamine + 10 mg/kg xylazine; IP) and stereotaxically microinjected with AAV-hSyn-DIO-hM3D(Gq)-mCherry; AAV serotype 8 (AAV-hM3Dq; 520 nl per side) into the lateral hypothalamic MCH field (anteroposterior: −1.7 mm from bregma, ventral: 4.8 mm from duramater, lateral ±1.0 mm) bilaterally. These injections were performed using glass pipettes with a 10 to 20 μm diameter tip and a pressure-injection system (Scammell et al., 1998). The mice were then implanted with miniature telemetry transmitters (TLM2-F20EET; Data Science International, USA) for recording electroencephalogram (EEG), electromyogram (EMG), body temperature (Tb) and locomotor activity (LMA) (Vetrivelan et al., 2009). Baseline recording of sleep-wake (EEG and EMG), Tb and LMA from all the mice were performed for a period of 24-hrs at 2–3 weeks after surgery using Dataquest ART 3.1 software (Data Sciences International, USA) (Vetrivelan et al., 2009). Then the mice were IP injected with the vehicle (saline) or CNO (at a dose of 0.3 mg/kg body weight; Sigma, USA) and post-injection recordings were continued for 24 h on each occasion. Each animal received 4 IP injections - 2 saline and 2 CNO injections; one each at 10 AM and 7 PM. Injections were performed in a randomized crossover fashion and there was at least one week between two CNO injections in the same animal.

Histology—Three days after the final recordings, the mice were injected with CNO at 10 AM and euthanized under anesthesia by perfusion with 10% formalin 3-hrs after these injections. The brains were then removed and cut into 3 series of 40 μm sections. One series of sections were immunolabelled for cFos (as a marker of neuronal activity) and DsRed (to label hM3Dq-mCherry expressing neurons) as described previously (Anaclet et al., 2014). The following primary antibodies were used – rabbit (Rb) anti-cFos (Oncogene Sciences; cat. no: 4188; 1:30000 dilution) and Rb-Anti-DsRed (Clontech, USA; cat. no: 632496; 1:10000 dilution). All doubly-labeled neurons were counted bilaterally. A second series was labelled for MCH by immunofluorescence (Rb Anti-MCH, generous gift from Dr. Maratos-Flier, BIDMC; 1:10000 dilution) as described earlier (Oishi et al., 2013) and hM3Dq-expressing neurons were visualized using native fluorescence of mCherry on the same sections. As controls, there were very few cFos immunoreactive neurons in the MCH region in normal sleeping animals; there was no staining of neurons with the anti-DSRed antibody in areas of the brain that had not received injections of vectors expressing this protein; and there was nearly complete correspondence of MCH-Cre neurons with those that labeled with the MCH antibody (see Results). All double-labelled [mCherry+ and MCH-immunoreactive (MCH-ir)] as well as mCherry+ neurons were counted in order to quantify the specificity of the injected AAV-hM3Dq. A third series was immunolabeled for cFos and MCH to identify the MCH neurons activated by CNO (Oishi et al., 2013). All double-labelled and MCH-ir neurons were counted to estimate the percentage of MCH neurons activated by CNO. All cell counts were corrected using Abercrombie's formula (Guillery, 2002).

Data Analysis—EEG/EMG recordings were divided into 12-second epochs and scored manually into wake, NREM or REM sleep using SleepSign software (Kessei Comtec, Japan) using the criteria described in our previous publications (Lu et al., 2000, Lu et al., 2002). Sleep-wake data from the first 8 h after the saline/CNO injections were divided into two 4-h

bins and percentages of wake, NREM and REM sleep in each bin were calculated. This is because our pilot data analysis indicated that sleep-wake changes induced by CNO injections lasted for ~ 4 h in most cases. In addition, bout number and average bout duration of individual sleep wake states were also calculated for these two 4-h bins. Similarly, mean Tb and total LMA during these periods were calculated. Finally, REM latency was calculated as the time taken to the first REM episode from the time of injection. Data from Post-CNO injections were then compared with post-saline injection data using ANOVA followed by paired t test.

Activation of MCH neurons by CNO *in vitro*—Under anesthesia, male MCH-Cre mice (n=3; 4 week old) were injected with AAV-hM3Dq into the LH as explained in the previous section. 4 weeks after these injections, these mice were sacrificed and LH slices (250 μm) were prepared for electrophysiological recordings as described earlier (Ferrari et al., 2016). Whole cell current clamp recordings were then performed on the mCherry+ neurons (n=8) using a Multiclamp 700B amplifier (Molecular Devices, Foster City, CA, USA), a Digidata 1322A interface and Clampex 9.0 software (Molecular Devices) as detailed in our previous publications (Ferrari et al., 2016). After achieving stable whole cell recordings from MCH neurons for 15 minutes, artificial cerebrospinal fluid (ACSF) solution containing 500 nM CNO was perfused through the chamber and recordings continued for 5 min before the CNO was washed out by ACSF. Data from 3 min just prior to bath application of CNO was considered baseline, the response to CNO was measured during the last 1 min of CNO application. The resting membrane potentials before and during CNO were compared using paired t-test.

Experiment 2: Selective deletion of MCH neurons

Surgery and recordings—Under anesthesia (100 mg/kg ketamine + 10 mg/kg xylazine; IP), the MCH-Cre/+;iDTR mice (n=9) and their wildtype (WT) littermates (n=6) were implanted with telemetry transmitters (TLM2-F20EET; Data Science International, USA) for recording EEG, EMG, Tb and LMA (Vetrivelan et al., 2009). After a 10-day postoperative recovery period, the mice were transferred to the recording room and habituated to the conditions for at least 2 days. Baseline (Pre-DT) values of food intake were measured for a week and average daily intake was calculated. Then, telemetric recording of EEG, EMG, Tb and LMA were conducted for 72 h (~20–22 days post-surgery) using Dataquest ART 3.1 software (Data Sciences International, USA) as explained earlier (Vetrivelan et al., 2009). Following this baseline data collection, the mice were injected with a total of 100 μg/kg of DT (Sigma, USA) IP on 2 alternate days (50 μg/kg per day). Mice were then maintained for 3 weeks and post-DT data was collected during this period. Sleep-wake, Tb and LMA recordings were performed during week 3 post-DT. In addition, post-DT food intake and body weight were monitored once a week. Body weight on the day of the first DT injection (prior to the injections) was used as baseline (pre-DT) data.

Histology—One day after completion of sleep-wake recordings, the mice were deeply anesthetized (chloral hydrate 500 mg/kg-body weight) and transcardially perfused with 10% formalin. The brains were cut into 3 series of 40 μm sections on a freezing microtome. One series of sections were double-labeled for MCH (to assess the MCH neuronal loss) and

orexin (to assess any nonspecific cell loss due to DT) as described earlier (Chee et al., 2013, Oishi et al., 2013). The following primary antibodies were used – Rb Anti-MCH (1:30000; generous gift from Dr. Maratos-Flier, BIDMC) and Rb Anti-orexin A (1:10000; Cat. No: SC-8070; Santa Cruz Biotechnology, USA). The number of MCH-immunoreactive (MCH-IR) and orexin-immunoreactive (Orexin-IR) neurons were counted bilaterally on 2 sections (one every 120 μm) at the peak of the MCH group and cell counts were corrected using Abercrombie's formula (Guillery, 2002).

Data Analysis—Sleep-wake scoring was similar to Experiment 1. The amount of time spent in each sleep-wake stage during 12-h light and 12-h dark periods and during the 24-h period in each animal were calculated. In addition, circadian indices (CI) of the sleep-wake stages were calculated for the post-DT data using the following formula: C.I. = $(\text{mean}_{\text{dark}} - \text{mean}_{\text{light}})/\text{mean}_{24\text{hr}}$, normalized to 100% for pre-DT data from the same mice. Similarly, total LMA counts and mean Tb during these periods and their circadian indices were also calculated. Post-DT data from transgenic and WT mice was compared with their respective pre-DT data using ANOVA followed by post-hoc bonferroni correction (Food intake, body weight data from week 1, 2 and 3 post-DT) or paired t test (for LMA, Tb and sleep-wake data from week 3 Post-DT).

Results

Experiment 1: Selective stimulation of MCH neurons

Because measuring acute changes in food intake following saline/CNO injections requires experimenter intervention that can wake the animals up, only sleep-wake, Tb and LMA were studied after selective stimulation of MCH neurons.

Histology and *in vitro* electrophysiological recording—As expected, injections of AAV-hM3Dq-mCherry into the LH of MCH-Cre mice caused expression specifically in MCH neurons as evidenced by virtually all of the mCherry-expressing neurons being double-labeled for MCH (93.11±1.71%; n = 11; Fig. 1A). Whole cell, current clamp recordings from these mCherry-expressing MCH neurons *in vitro* indicated that bath application of CNO depolarized them (+5.97 ± 1.00 mV; resting membrane potentials: −50.98±5.23 mV pre-CNO vs −45.00 ±5.03 mV post-CNO; n = 8, $t_7 = -5.97$; $p < 0.001$) and increased their firing rate (Fig. 1B), and the depolarization induced by CNO was reversed following washout. In addition, mCherry-IR neurons also expressed cFos following IP CNO injections *in vivo* (Fig. 1C), thus indicating that CNO specifically activated the MCH neurons, which typically are found to express cFos (Fig 1 D) only after REM hypersomnia (Verret et al., 2003). AAV-hM3Dq injections into the MCH-Cre mice transfected 21.6 – 61% of MCH neurons bilaterally depending upon the volume and site of injections and hence activation of variable percentage of MCH neurons (24.3 – 56.2%of all MCH neurons expressed cFos) was achieved in our cohort of 11 mice.

Activation of MCH neurons specifically increases REM sleep—Selective activation of MCH neurons by CNO injections into the MCH-hM3Dq mice (n=11) resulted in, on average, a 70% increase in REM sleep during the next four hours in the light period

(23.41 min ± 1.15 post-CNO vs. 13.71±1.08 min post-saline; n = 11; t_{10} = − 8.767; P<0.001) and a doubling of REM sleep during the next four hours in the dark period (9.52±2.69 min vs.4.64±1.81 min; n = 9; t_8 = −9.019; P<0.001; Fig 1 E, F) when compared with saline injections in the same mice. This increase in REM sleep was mostly at the expense of wake, although the loss of wake did not reach statistical significance [wake - 84.27±3.93 min in four hours post-CNO vs. 92.27±4.78 min post-saline; (t_{10} = 1.662; P= 0.13); NREM sleep −132.31±3.81 min post-CNO vs. 134.00±4.32 min post-saline (t_{10}= 0.415; P= 0.69) during the light-period; wake - 147.71±11.65 min post-CNO vs. 163.10± 18.94 post-saline (t_8 = 2.024; P=0.08) and NREM - 82.75±10.21 min post-CNO vs. 72.29±17.22 min post-saline (t_8 = −1.326; P = 022) during the dark period]. The increase in REM sleep following CNO injections was due to an increase in the number but not in the duration of REM bouts indicating the importance of MCH neurons in REM generation and NREM-REM transitions rather than the maintenance of REM sleep (Table 1). Consistent with that observation, the latency to first REM sleep was decreased after the CNO when compared to saline injections in the same mice (43.98±3.89 min post-CNO vs. 67.64±3.77 min post-saline during the light period, t_{10} = 4.40; P<0.001; 52.7 ±16.37 min post-CNO vs. 149.23±25.48 min post-saline during the dark period, t_8 = 4.85; P<0.001). REM amounts during the second 4-h bin did not differ significantly between saline and CNO conditions indicating that there was no ‘rebound’ reduction in REM sleep following REM hypersomnia (20.91±1.19 min post-CNO vs. 18.54±0.98 min post-saline during light period; t_{10} = −2.16; P = 0.06 and 9.65±0.53 min post-CNO vs. 8.45 ± 0.91 min post-saline during dark period; t_8= −1.41; P = 0.20; Fig. 1 E, F).

Finally, total LMA or mean Tb did not change significantly after IP CNO injections either during the light or dark period in the AAV-hM3Dq injected MCH-Cre mice when compared to IP saline injections in the same mice (Table 2).

Experiment 2: Selective deletion of MCH neurons

Histology—As expected, IP injections of DT into the MCH-Cre/+;iDTR mice (n=9) resulted in nearly complete and selective destruction of MCH neurons. MCH-IR neurons were almost completely absent in these mice following DT injection (Fig 2 B, B′), with the one or two MCH-IR neurons that remained intact in some cases confirming the adequacy of the MCH immunostaining. On the other hand, DT injections into the WT littermates (n=8) had no effect and MCH neurons remained intact in these mice (Fig. 2 A, A′; 0.44 ± 0.24 MCH-IR neurons in MCH-Cre/+;iDTR mice vs. 395.75 ± 24.30 in WT mice; unpaired t test, t_{15} = 17.32; P<0.001) ruling out any non-specific effects of DT. Finally, the number of orexin-IR neurons, which are adjacent to and intermingle with MCH neurons, was not significantly different between the WT and MCH-Cre/+;iDTR mice (380.8 ± 21 vs. 342.13 ±28.94 in WT mice; unpaired t test, t_{15} = 1.16; P = 0.26; Fig. 2 A′, B′) following DT injections, which further confirms the selectivity of these lesions.

MCH deletions affect diurnal variation of sleep-wake—IP injections of DT into the MCH-Cre/+;iDTR mice (n=9) that resulted in complete deletion of MCH neurons did not produce any major changes in daily amounts of sleep-wake when compared to the pre-DT data from the same mice or post-DT recordings from WT mice (Fig 2 C). However, the

diurnal distributions of wake and REM sleep were significantly altered in these mice post-DT. There was a significant increase in the REM amounts during the light period (10.72±0.85% vs 7.02±0.63% during pre- DT; $t_8 = -4.79$; P = 0.001) and a small decrease during the dark period that did not reach statistical significance (2.77±0.41% vs. 3.72±0.48% during pre-DT, $t_8 = 1.54$; P = 0.16) resulting in increased diurnal variation of REM sleep (Fig 2 D, E). Consistently, the circadian index of REM sleep was significantly (84% more than pre-DT) increased in the MCH-Cre/+;iDTR mice following DT injections ($t_8 = 3.381$; P = 0.01; Fig 2 F). Similarly, the circadian index of wake was significantly increased at week 3 post-DT (39% more than pre-DT; $t_8 = -2.454$; P = 0.04; Figure 2 F) although total wake during the light or dark periods did not significantly differ from pre-DT values (Fig 2 D, E). Finally, the circadian index of NREM post-DT was also higher than pre-DT values, but this did not reach statistical significance ($t_8 = 1.33$; P = 0.22; Figure 2 F).

Analysis of sleep-wake architecture indicated a significant increase in number of REM bouts (69 ±8.74 vs. 44±5.85; $t_8 = -2.750$; P = 0.03) and a decrease in mean bout duration of wake (86.44±7.62 seconds vs. 115.71±9.91 seconds; $t_8 = 2.912$; P = 0.02) during the light period at week 3 post-DT when compared to pre-DT values (Table 3). No other significant changes were observed in bout number or duration of sleep-wake states (Table 3).

Mice with MCH neuronal loss are hypophagic and lean—Food intake in the MCH-Cre/+;iDTR mice was significantly reduced following DT injections. On average, daily food intake in these mice was reduced by 20% during week 1 and 35% during week 2 post-DT (3.22 ± 0.12 g and 2.69 ± 0.36 g during week 1 and 2 post-DT Vs.4.28±0.21 g pre-DT; Fig. 3A; $t_8 = 8.19$ and 7.57 respectively; P<0.001 in both cases). Although there was a trend for recovery by week 3, food intake was still significantly (~20%) lower than the pre-DT values (3.44 ± 0.17 g vs 4.28±0.21 g pre-DT; $t_8 = 7.30$; P<0.001; Fig. 3A). A similar pattern was observed in body weight. These mice weighed significantly less (3.3±0.5 g, 5.30±1.87 g and 3.95±0.55 g lower than the pre-DT values; $t_8 = 8.65$, 8.07 and 8.65 respectively; P<0.01 for all comparisons) during the post-DT week 1, 2 and 3 respectively (Fig. 3B).

Mice with MCH neuronal loss are hyperactive—MCH-Cre/+;iDTR mice displayed a significant increase (91%) in the daily amounts of LMA after the DT injections when compared to their pre-DT levels. While the LMA counts increased about 30% during the light period (350.66±50.0 post-DT vs. 270.44±40.71 pre-DT; $t_8 = -3.18$; P = 0.01), it more than doubled (1457.78±172.38 post-DT vs.684.28±73.59 pre-DT; $t_8 = -7.16$; P<0.001) during the dark period (Fig. 3C). Similarly, although the mean Tb did not change significantly during the light period (36.05±0.06°C vs. 35.91±0.04°C during pre-DT, $t_8 = -2.21$; P = 0.06), it increased by 0.5°C during the dark period (37.46±0.16°C vs. 36.99±0.03°C during pre-DT, $t_8 = -7.65$; P<0.001; Fig. 3D) indicating an altered diurnal pattern of Tb and LMA following the MCH neuronal loss. Consistent with those observations, circadian indices of LMA and Tb increased significantly by 37.3% ($t_8 = -4.71$; P = 0.002) and 28.9% ($t_8 = -6.72$; P<0.001) respectively during week 3 post-DT.

Discussion

We investigated the role of MCH neurons in sleep-wake either by selectively activating or ablating them using conditional genetic approaches in mice. Acute stimulation of MCH neurons specifically increased REM sleep whereas their ablation did not cause any changes in amount of sleep-wake or bout frequency or duration. However, ablation of MCH neurons increased the diurnal variation of wake and REM sleep as well as LMA and Tb, while decreasing food intake.

MCH neurons and sleep-wake

Selective increases in REM sleep observed following chemogenetic activation of MCH neurons in this study suggests a role for MCH neurons that is specific to REM sleep regulation, and does not support a role in NREM regulation as suggested by previous optogenetic studies (Konadhode et al., 2013, Tsunematsu et al., 2014). Konadhode et al., (2013) found an increase in NREM and REM sleep following optogenetic activation of MCH neurons at 10 Hz for one minute out of five during the dark phase but found only an increase in NREM sleep when the stimulation was continued for 24 hrs. Tsunematsu et al., (2014) found a significant decrease in NREM sleep with continuous 10Hz stimulation for three hours during the light phase, however when they deleted MCH neurons using a method similar to our study, they also observed a decrease in NREM sleep. While it is possible that the differences in methods of optical stimulation and genetic targeting could have contributed to this discrepancy, optogenetics itself is associated with various pitfalls. For example, blue laser light causes significant heating (~6°C) and profound fMRI responses in the naive brain even without optogenetic activation (Christie et al., 2013). Thus, blue laser stimulation may cause non-specific activation of other neurons in the same region and stimulation for longer periods may cause tissue damage. In addition, if there was leakage of light from the fiber optic cable or its connectors, the increase in NREM sleep and EEG delta power following the photostimulation of MCH neurons (Konadhode et al., 2013) could have resulted from direct effects of light pulses applied during the dark (photostimulation in sham-injection controls were not studied). Even millisecond light pulses during the dark period in nocturnal animals can increase NREM sleep and EEG delta power for long durations even after withdrawal of light (Morin and Studholme, 2009, Studholme et al., 2013). Finally, light penetration may be insufficient to activate all transfected MCH neurons, as they are spread over a large area (~ 1.5 mm mediolatareally, ~1 mm rostrocaudally; ~1.2 mm dorsoventrally). In contrast, the chemogenetic method used in our study does not suffer from these drawbacks and offers excellent specificity and control over neuronal activity. Chemogenetic activation raises the membrane potential of cells by a few mV, making them more excitable, but firing patterns are still driven by incoming postsynaptic potentials. Thus, our findings that chemogenetic activation of MCH neurons specifically increases REM sleep amount, REM bout numbers, and decreases REM latency, yet has no effect on NREM sleep, argues that MCH neurons primarily regulate REM sleep. Chronic deletion of MCH neurons however did not alter the REM amount (but altered its diurnal pattern; discussed below), indicating that the MCH neurons may not be critical for determining baseline levels of REM sleep and that there are other neuronal populations that can regulate the amount of REM sleep in the absence of the MCH neurons. On the other hand, MCH neurons may be

particularly important in homeostatic increases in REM sleep, such as following REM sleep deprivation. Thus 'rebound' reduction in REM sleep may not be possible if the REM increase is caused by manipulation of MCH neurons.

It has also been difficult to establish the role of MCH in sleep regulation using pharmacological approaches. Verret et al. (2003) showed that i.c.v injection of MCH caused a tripling in REM sleep but a smaller, 1.5-fold increase in NREM sleep. However, given that activation of MCH cells only doubled REM sleep in our experiments, they may have used a pharmacological dosage that exceeds the amount that is seen with the firing of MCH neurons. When Lagos and colleagues (2012) injected MCH into the basal forebrain, they reported a reduction in wake as well as an increase in REM sleep and latency to first REM bout, but when the same group injected MCH into the ventrolateral preoptic nucleus, they found that it increased NREM but not REM sleep (Benedetto et al., 2013). These studies point out the possibility that MCH may have different effects at different targets in the brain, but in our chemogenetic stimulation experiments, all of them receive MCH at the same time. Other groups have used MCHR1 antagonists to study MCH effects on sleep. Ahnaou et al. (2008) found that subcutaneous administration of MCHR1 antagonists increased wake, at the expense of both REM and NREM sleep, but Adamantidis and colleagues (2008) reported that MCHR1 null animals had increased sleep. While MCHR1 is believed to be the sole MCH receptor in rodents, it is possible that there are other receptors that are not yet known, or that MCHR1 may have additional ligands. In fact, the distribution of MCHR1 in the brain (Francke et al., 2005) is quite different from the distribution of MCH itself (Bittencourt et al., 1992, Bittencourt, 2011), and MCH neurons are known to contain other neurotransmitters, such as glutamate (Chee et al., 2015). In summary, the chemogenetic activation of MCH neurons is more likely to mimic the in vivo neuronal firing pattern and reflect the physiological role of MCH neurons than is the exogenous application of MCH peptide or MCHR1 antagonists. Our results from chemogenetic activation of MCH neurons suggest a specific REM modulatory role for MCH neurons.

Neural circuitry underlying MCH control of REM sleep

MCH neurons contain the vesicular glutamate transporter 2, (Chee et al., 2015) and glutamic acid decarboxylase-67 (Sapin et al., 2010, Jego et al., 2013), but not the vesicular GABA transporter (Chee et al., 2015). Consistent with these observations, MCH terminals in the lateral septum release glutamate, but not GABA (Chee et al., 2015). Conversely, optogenetic stimulation of MCH terminals in the tuberomammillary nucleus (TMN) was reported to cause GABAergic inhibitory post-synaptic currents (Jego et al., 2013). In addition, MCH neurons contain inhibitory neuropeptides such as CART and nesfatin-1 (Broberger, 1999, Elias et al., 2001, Brailoiu et al., 2007). Thus, MCH neurons may potentially elicit excitatory or inhibitory responses depending on the target neurons and what receptors they bear.

MCH neurons may increase REM sleep either by directly acting on the REM sleep regulatory system, thereby enhancing the initiation and/or maintenance of REM sleep, or by inhibiting wake-promoting structures, thereby delaying termination of REM bouts (which usually culminate in wake in rodents). Our results support primarily the former possibility, as activation of MCH neurons reduced REM latency and increased bout frequency, but did

not increase bout duration. The REM sleep regulatory system includes neurons in the sublaterodorsal nucleus (SLD) and adjacent precoeruleus (PC), and possibly the laterodorsal (LDT) and pedunculopontine (PPT) tegmental nuclei, which generate REM sleep by projecting to the medulla and spinal cord (causing eye movements and REM atonia) and to the forebrain (producing the characteristic faster EEG and enhanced theta rhythm) (Datta and Siwek, 1997, Datta et al., 2001, Lu et al., 2006, Luppi et al., 2006, Fuller et al., 2007, Vetrivelan et al., 2011, Luppi et al., 2012, Boucetta et al., 2014, Van Dort et al., 2015). The SLD and PC are held in check by inhibitory inputs from the ventrolateral periaqueductal gray matter (vlPAG) and adjacent lateral pontine tegmentum (LPT, also called the deep mesencephalic nucleus) (Lu et al., 2006, Vetrivelan et al., 2011, Luppi et al., 2012). Both the REM-On (SLD/PC/LDT/PPT) and the REM-Off (vlPAG/LPT) structures receive intense MCH input (Torterolo et al., 2009, Clement et al., 2012, Torterolo et al., 2013), as does the ventrolateral medullary (vM) area (Bittencourt et al., 1992) where Weber and colleagues have recently identified GABAergic neurons that generate REM sleep by inhibiting the vlPAG/LPT (Weber et al., 2015). Thus, MCH neurons are likely to regulate REM sleep by inhibiting the vlPAG/LPT and activating the SLD/PC/LDT/PPT/vM, although the physiology of these connections remains to be investigated. In contrast, MCH infusions into the SLD decreased REM sleep in rats (Monti et al., 2016), but MCH neurons also contain glutamate (Chee et al., 2015) that can stimulate the REM generating neurons in the SLD and promote REM sleep.

MCH neurons also innervate important components of the arousal system, including the locus coeruleus (LC), dorsal raphe (DR), and TMN (Bittencourt et al., 1992, Jego et al., 2013, Yoon and Lee, 2013). These neurons fire fastest during wake and are nearly silent during REM sleep (Saper et al., 2010). Augmenting these monoaminergic neurons (e.g., by administering reuptake inhibitors) prevents entry into REM sleep, thus their silence plays a permissive role in REM regulation. However, lesions of these structures do not affect REM sleep amounts (Lu et al., 2006). Jego and colleagues (2013) reported that optogenetic activation of MCH terminals in the TMN increased REM bout duration but not REM latency, whereas stimulation of MCH cell bodies did decrease REM latency. Thus, the increased entry into REM sleep in our chemogenetic experiments was probably not due to inhibition of TMN neurons, although those inputs may have prevented the TMN from prematurely terminating the excessive number of REM bouts. On the other hand, local infusions of MCH into the DR and LC increased REM sleep by increasing the number of REM bouts (Lagos et al., 2009, Monti et al., 2015) consistent with a modulatory role for these monoaminergic nuclei in REM sleep regulation.

Diurnal variation of sleep-wake

Mice with MCH neuronal deletions displayed increased diurnal variation of wake, REM sleep, LMA and Tb suggesting that MCH neurons may play a role in circadian regulation of these functions. Tsunematsu and colleagues (2014) used a different construct (a Tet off system) to control DTA expression in MCH neurons, which was not as efficient at eliminating MCH neurons (about 3% of neurons remained in their experiments compared to less than 1% in ours). They reported a reduction in NREM, but not REM sleep time in these animals. Their animals were also a different substrain from ours, which may account for the

difference. On the other hand, orexin neurons are known to be important for circadian rhythms of REM sleep (Kantor et al., 2009), and MCH and orexin neurons are mutually inhibitory *in vitro* (Guan et al., 2002) and have opposite firing patterns *in vivo* (Hassani et al., 2009). Thus the balance between orexin and MCH signaling may contribute to the circadian regulation of sleep-wake, LMA and Tb. This influence is likely to be mediated via inputs from the suprachiasmatic nucleus (SCN), relayed through the subparaventricular zone and dorsomedial hypothalamus (Saper et al., 2005, Vujovic et al., 2015).

Energy metabolism

Our findings of hypophagia, hyperactivity, and weight loss following deletion of MCH neurons are consistent with similar observations in mice congenitally lacking MCH signaling (Qu et al., 1996, Shimada et al., 1998, Alon and Friedman, 2006). A previous study using similar lesion methods reported no change in feeding (Whiddon and Palmiter, 2013), but compared transgenic mice with WT controls (whereas we compared animals with their own baseline data) and used a different strain (C57BL6 vs. mixed background in our study). Increased LMA has been reported in all the above-mentioned studies, although acute activation of MCH neurons in our study did not alter LMA levels. The increase in LMA with MCH deletion, which of course occurs only during wake, would imply that firing of MCH neurons during wakefulness might increase eating and reduce energy expenditure, consistent with a state of starvation.

A recent paper has examined MCH neuronal activity using calcium sensing dyes in freely moving mice and shows increased activation during novelty exploration (Gonzalez et al., 2016). Activation of MCH neurons during wakefulness may not cause REM phenomena in intact animals, as the activity in other circuits, such as the orexin neurons that reinforce firing of monoaminergic neurons that locks the brain out of REM sleep during wakefulness, would prevent wake-REM transitions. On the other hand in animals or patients with narcolepsy, who lack orexin neurons (that oppose wake-REM transitions), activation of MCH neurons during wakefulness would potentially drive entry into REM states such as cataplexy or hypnagogic hallucinations. Thus the activation of the MCH neurons during rewarding conditions, such as exploration of novel objects, positive emotions, or availability of highly palatable foods, could be a proximate cause of cataplexy.

Acknowledgments

Funding: This work was supported by National Institutes of Health Grants R21-NS074205 and R01-NS088482 (to RV), K01-DK094943, R01-DK108797 and Charles Hood Foundation Grant (to DK), R01-NS091126 (to EA), R01-DK096010, R01-DK089044, R01-DK071051, R01-DK075632, R37-DK053477, BNORC Transgenic Core P30-DK046200 and BADERC Transgenic Core P30-DK057521 (to BBL), R01-NS061841 and R01-NS062727 (to JL) and P01-AG09975, P01-HL095491, and R01-NS085477 (to CBS).

We thank Quan Ha, Minh Ha, Sofia Iqbal and Bushra Anis for excellent technical assistance, Celia Gagliardi for proofreading the manuscript and Dr. Nina Vujovic for helpful discussions. We also thank Drs. Eleftheria Maratos-Flier and Mellisa Chee (Department of Medicine, Beth Israel Deaconess Medical Center, Boston) for providing the rabbit anti-MCH antibody.

Abbreviations

AAV adeno-associated viral vectors

ACSF	artificial cerebrospinal fluid
CI	circadian index
CNO	clozapine-N-oxide
Cre	Cre recombinase
DT	diphtheria toxin
EEG	electroencephalogram
EMG	electromyogram
iDTR	inducible diphtheria toxin receptor
IP	intraperitoneal
ir	immunoreactive
REM	rapid eye movement
LDT	laterodorsal tegmentum
LH	lateral hypothalamus
LMA	locomotor activity
LPT	lateral pontine tegmentum
MCH	melanin-concentrating hormone
NREM	non-rapid eye movement sleep
PC	precoeruleus
SCN	suprachiasmatic nucleus
PPT	pedunculopontine tegmentum
SLD	sublaterodorsal nucleus
TMN	tuberomammillary nucleus
Tb	body temperature
vlPAG	ventrolateral periaqueductal gray matter
vM	ventrolateral medulla
WT	wildtype

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Highlights

- Chemogenetic activation of MCH neurons specifically increased REM sleep in mice
- Selective deletion of MCH neurons increased diurnal variation of REM sleep
- MCH neuronal loss produced hypophagia, hyperactivity and weight loss in mice

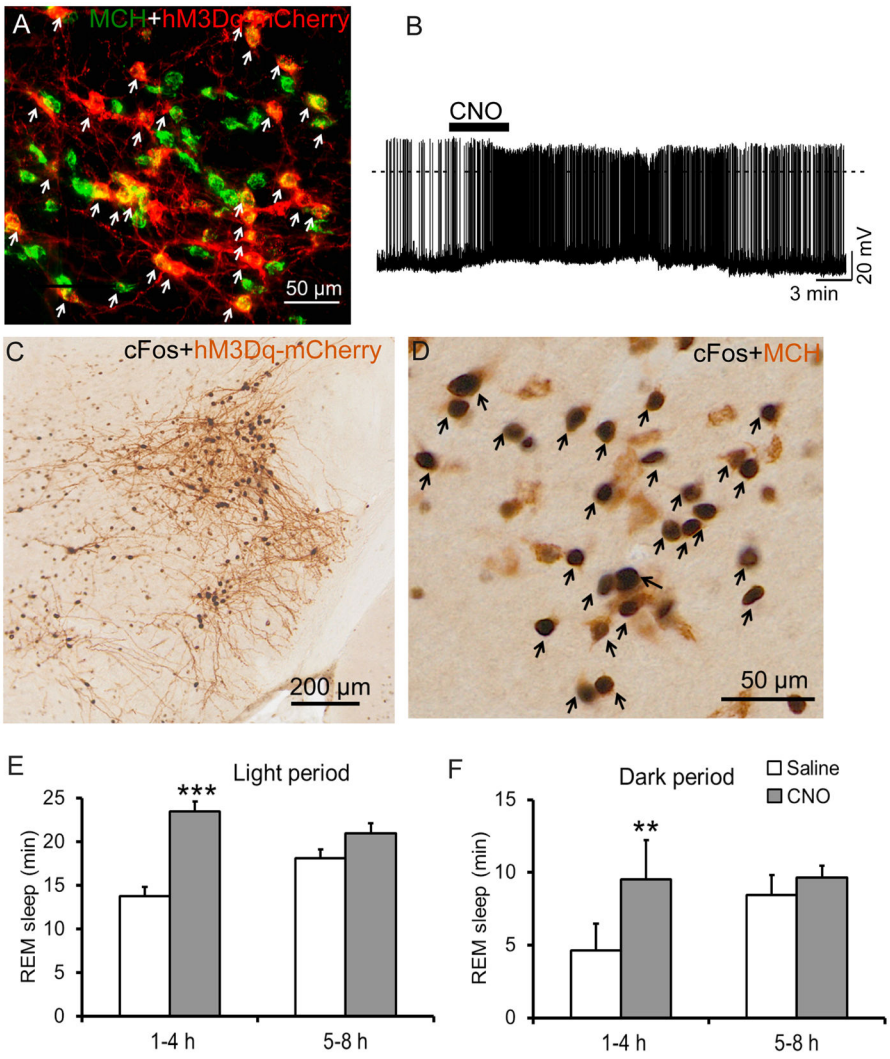

Figure 1. Specific expression of AAV-hM3Dq-mCherry in MCH neurons in MCH-Cre mice and their activation by CNO

A. Photomicrograph of a brain section labeled for mCherry (red cells) and MCH (green cells) by immunofluorescence from a mouse injected with AAV-hM3Dq into the lateral hypothalamus indicating DREADD expression in the MCH neurons (white arrows indicate doubly labeled neurons). The percentage of MCH neurons transfected ranged from 21–60%. B- Representative whole cell, current clamp recording from a MCH neuron (identified by mCherry fluorescence) indicating bath application of CNO (500 nM) induced depolarization and increased the firing rate of these neurons. Intraperitoneal (IP) injections of CNO induced cFos expression (black nuclei) in DREADD-expressing neurons (brown cytoplasm) in the lateral hypothalamus (C). Double labelling of sections for MCH (brown) and cFos (black) indicated cFos expression in MCH neurons after IP CNO (black arrows in D). Brains were obtained for cFos analysis 180 minutes after IP CNO (0.3 mg/kg of body weight). Activation of MCH neurons by IP CNO caused specific increases in REM sleep in mice. REM sleep amounts (in minutes) during the first two 4 hr periods following IP saline or CNO injections

either during the light (E; n=11) or dark period (F; n=9) in MCH-Cre mice injected with AAV-hM3Dq into the LH. Data are mean ± SEM. **P<0.01; ***P<0.001; paired t test.

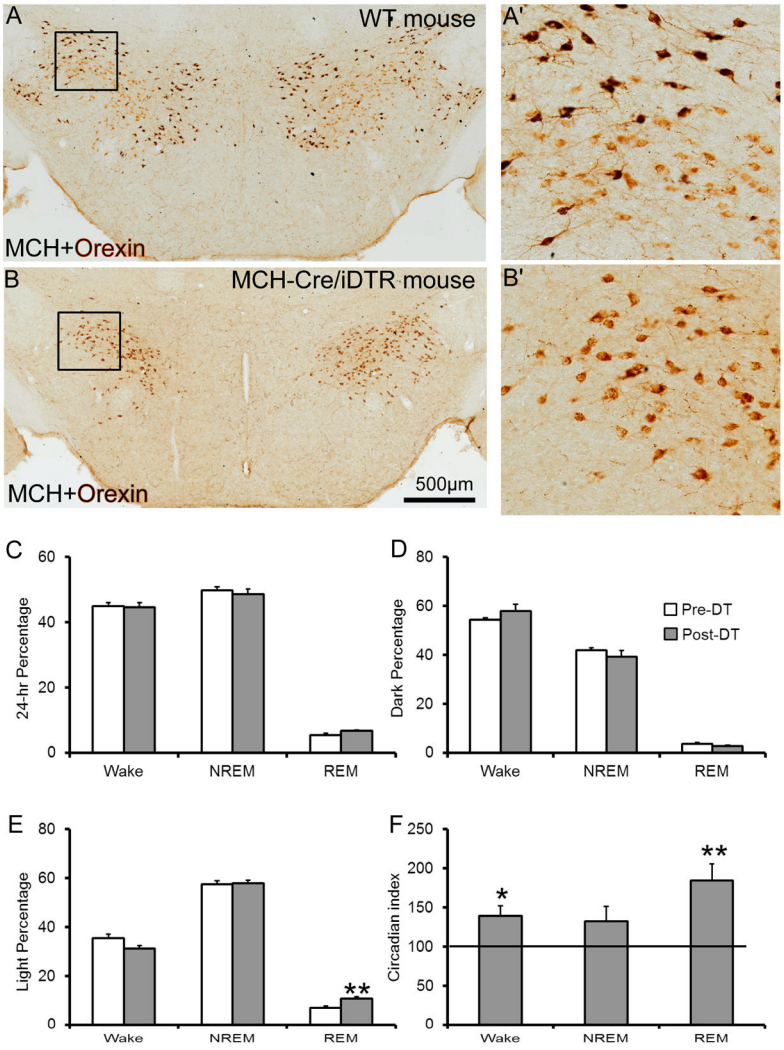

Figure 2. Changes in sleep-wake following specific deletion of MCH neurons

Photomicrographs from brain sections double-labelled for MCH (black) and orexin (Brown) from a WT mouse (A, A′) and a MCH-Cre/+;iDTR mouse (B, B′). Injections of diphtheria toxin resulted in complete elimination of MCH neurons only in the MCH-Cre/+; iDTR mouse (B, B′) but not in WT mice (A, A′). Orexin neurons remained intact in both groups of mice ruling out any non-specific cell loss (A, B). Percentages of Wake, non-REM and REM sleep during dark (D) light (E) or the entire 24-hr period (C) in MCH-Cre/+;iDTR mice (n=9) before (white bars) and at 3 weeks after (grey bars) the diphtheria toxin injections. Circadian index (amount of behavior in light phase minus dark phase, over total amount of behavior) of sleep-wake stages expressed as percentage of control mice are shown in F.

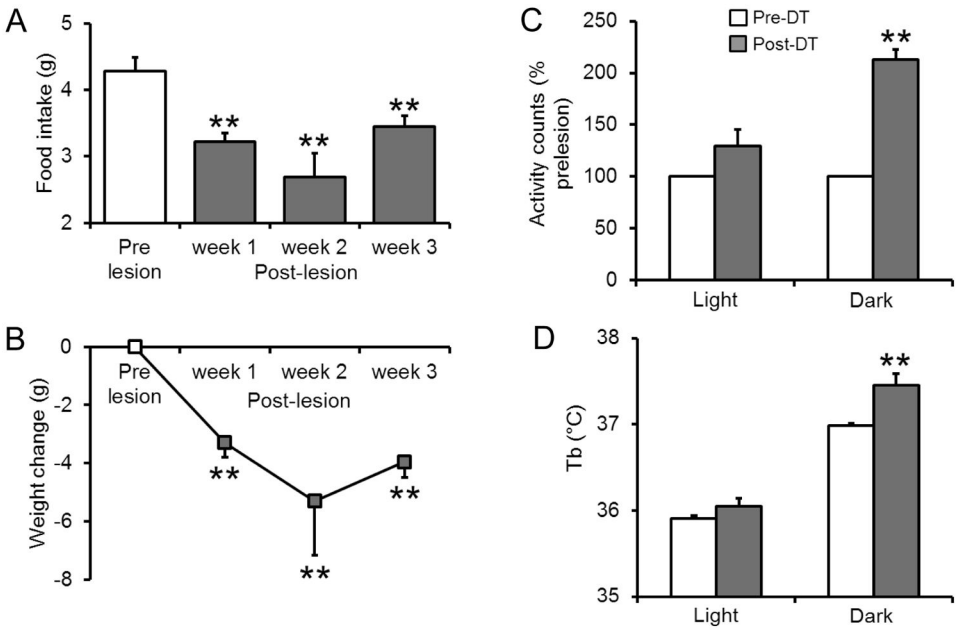

Figure 3. Metabolic changes following MCH neuronal deletions

Food intake and body weight gain in MCH-Cre/+;iDTR mice (n=9) before (white bar in A and white square in B) and 1, 2 and 3 weeks after diphtheria toxin (DT) injections (grey bars in A and grey squares in B), showing dramatic reduction following deletion of MCH neurons. Total locomotor activity (LMA; C) and mean body temperature (Tb; D) during the light and dark phases in MCH-Cre/+;iDTR mice before (white bars) and 3 weeks (grey bars) after DT injections show the increase in both LMA and Tb during the dark phase after deletion of MCH neurons.

Table 1

Changes in sleep architecture following MCH neuronal activation

Bout number and duration of individual sleep-wake states following intraperitoneal administration of saline or CNO in MCH-Cre mice injected with AAV-hM3Dq into the LH. Data from the first 8 h following saline or CNO injections was divided into two 4 hr bins and analyzed

Stage		Number of bouts		Mean bout duration (seconds)	
		Post-Saline	Post-CNO	Post-Saline	Post-CNO
Injections at light period					
Wake	**1–4 hr**	40.27±3.39	41.0±3.61	85.82±9.52	76.55±6.91
	4–8 hr	42.91±2.27	45±3.29	111.00±7.02	100.73±9.98
NREM	**1–4 hr**	41.55±3.44	44.09±3.63	195.64±12.14	187.73±12.97
	4–8 hr	44.0±2.0	47.09±3.32	188.27±10.30	188.09±13.15
REM	**1–4 hr**	10.73±1.27	18.27±1.1***	72.82±3.99	77.64±3.59
	4–8 hr	15.00±0.36	15.73±1.04	73.55±3.92	81.82±6.0
Injections at dark period					
Wake	**1–4 hr**	36±4.81	30±4.81	311.8±121.97	299.6±93.85
	4–8 hr	37.2±8.15	38.2±8.26	237.2±42.16	181±29.20
NREM	**1–4 hr**	36.4±10.35	29.2±4.4	136.8±25.72	169±17.58
	4–8 hr	37.4±8.26	34.4±7.99	178.2±34.86	192.6±34.37
REM	**1–4 hr**	4.4±1.75	8.8±1.93**	66±11.33	64.2±11.03
	4–8 hr	7.2±1.39	8±0.89	75.4±13.87	75.2±10.11

Data are mean ± SEM.

** P<0.01;

*** P<0.001; paired t test.

Table 2

Changes in locomotor activity and body temperature

Locomotor activity and body temperature following intraperitoneal administration of saline or CNO in MCH-Cre mice injected with AAV-hM3Dq into the LH. Data from the first 8 h following saline or CNO injections was divided into two 4 hr bins total LMA and mean Tb for was calculated for these periods. Data are mean ± SEM

		Injections at light period		Injections at dark period	
		Post-Saline	**Post-CNO**	**Post-Saline**	**Post-CNO**
Total LMA (counts)	**1–4 hr**	172.97 ± 40.30	172.46±31.60	162.24±30.15	206.32±25.49
	5–8 hr	115.09±21.88	114.31±18.97	342.92±22.81	385.39±67.76
Mean Tb (°C)	**1–4 hr**	36.00±0.22	35.94±0.10	37.43±0.26	37.26±0.21
	5–8 hr	35.62±0.13	35.65±0.08	36.99±0.10	37.06±0.11

Table 3

Changes in sleep architecture following MCH neuronal deletions

Bout number and duration of individual sleep-wake states in MCH-Cre/+;iDTR mice (n=9) before (Pre-DT) and at 3 weeks after (Post-DT) diphtheria toxin injections

Stage		Number of Bouts		Mean bout duration (seconds)	
		Pre-DT (n=7)	**Post-DT (n=9)**	**Pre-DT (n=7)**	**Post-DT (n=9)**
Wake	**Light**	126.71±10.79	153.22±15.79	115.71±9.91	86.44±7.62 *
	Dark	125.57±12.21	148.33±16.49	180.00±16.00	175.44±21.07
	24-hr	252.86±21.46	302.33±28.96	149.43±11.88	130.89±10.35
NREM	**Light**	134.00±10.98	160.89±15.11	191.86±17.25	166.67±16.36
	Dark	128.57±12.29	149.67±16.21	146.43±11.87	125.56±17.31
	24-hr	262.86±22.09	310.78±28.44	169.00±13.83	145.33±15.56
REM	**Light**	44.00±5.85	69.00±8.74 *	71.86±6.21	69.78±3.87
	Dark	25.71±5.60	19.44±2.64	67.57±7.01	61.56±4.35
	24-hr	69.71±10.81	88.44±7.71	69.14±3.97	67.33±3.21

Data are mean ± SEM.

* P<0.05; paired t test.