Chemogenetic modulation of histaminergic neurons in the tuberomammillary nucleus alters territorial aggression and wakefulness

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Research Article

Keywords: Histaminergic neuron, tuberomammillary nucleus, locomotor activity, aggressive behaviour, sleep–wake cycle, DREADDs

Posted Date: April 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-414232/v1>

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Abstract

Designer receptor activated by designer drugs (DREADDs) techniques are widely used to modulate the activities of specific neuronal populations during behavioural tasks. However, DREADDs-induced modulation of histaminergic neurons in the tuberomammillary nucleus (HA^{TMN} neurons) has produced inconsistent effects on the sleep–wake cycle, possibly due to the use of Hdc-Cre mice driving Cre recombinase and DREADDs activity outside the targeted region. Moreover, previous DREADDs studies have not examined locomotor activity and aggressive behaviours, which are also regulated by brain histamine levels. In the present study, we investigated the effects of HA^{TMN} activation and inhibition on the locomotor activity, aggressive behaviours and sleep–wake cycle of Hdc-Cre mice with minimal non-target expression of Cre-recombinase. Chemoactivation of HA^{TMN} moderately enhanced locomotor activity in a novel open field. Activation of HA^{TMN} neurons significantly enhanced aggressive behaviour in the resident–intruder test. Wakefulness was increased and non-rapid eye movement (NREM) sleep decreased for an hour by HA^{TMN} chemoactivation. Conversely HA^{TMN} chemoinhibition decreased wakefulness and increased NREM sleep for 6 hours. These changes in wakefulness induced by HA^{TMN} modulation were related to vigilance status transition. These results indicate the influences of HA^{TMN} neurons on exploratory activity, territorial aggression, and wake maintenance.

Highlights

- DREADDs was used to modulate TMN histaminergic (HA^{TMN}) neuron activity in mice
- Excitation enhanced novel locomotor activity but reduced non-novel locomotor activity
- Activation of HA^{TMN} neurons also promoted aggression against intruders
- Modulation altered wakefulness through regulation of sleep–wake transitions
- HA^{TMN} neurons project to regions regulating locomotion, aggression and wakefulness

1. Introduction

Histaminergic neurons in the tuberomammillary nucleus (TMN) of the posterior hypothalamus (HA^{TMN} neurons) modulate a multitude of physiological processes and behaviours, and altered neuronal histamine signalling is associated with changes in the sleep–wake cycle, motor activity and aggression among other behavioural effects^{1,2}. Several technical challenges have limited research on the specific behavioural functions of HA^{TMN} neurons. Notably, histamine (HA) receptors are widely distributed throughout the central nervous system (CNS) and peripheral tissues, limiting the utility of pharmacologic modulation, while clusters of HA neurons may be difficult to stimulate specifically using implanted electrodes. Alternatively, chemogenetic and optogenetic techniques allow for the modulation of neuronal activities with regional, cellular and temporal specificity^{3,4}. However, such techniques applied to HA^{TMN} neurons have also yielded inconsistent effects on the sleep–wake cycle. In one recent study, chemogenetic inhibition decreased wakefulness and increased non-rapid eye movement (NREM) sleep

without changing REM sleep during active periods (Yu et al., 2019). Similarly, another optogenetic study reported that acute silencing of HA^{TMN} neurons during wakefulness promoted NREM sleep, but not REM sleep (Fujita et al., 2017). However, Venner et al. (2019) reported that optogenetic inhibition of HA^{TMN} neurons during wakefulness did not alter the duration of NREM sleep. Furthermore, chemogenetic activation of HA^{TMN} neurons at Zeitgeber time (ZT) 3 did not alter the sleep/wake ratio or electroencephalogram (EEG) spectra ⁵.

In addition to the sleep–wake cycle, the brain histaminergic system is involved in the regulation of locomotor activity and aggressive behaviours. Genetic deletion (knockout, KO) of histamine *N*-methyltransferase (*Hnmt*) (EC 2.1.1.8), which metabolizes histamine into inactive 1-methylhistamine, induced a sustained elevation of extracellular histamine concentration in the mouse CNS and concomitantly reduced locomotor activity during the dark periods, enhanced aggressive behaviours and disrupted the normal sleep–wake cycle without changing the total amount of wakefulness ⁶.

We speculated that these discrepancies in the behavioural effects of HA^{TMN} neuron modulation could reflect relatively minor differences in behavioural assessment methods and the emotional status of the animal. Furthermore, we suggest that the histidine decarboxylase (Hdc) (EC 4.1.1.22)-Cre mouse line may provide more cell type-specific chemogenetic or optogenetic manipulation of HA^{TMN} neuron activity ^{2,7}. Therefore, we re-examined histaminergic system functions in regulation of the sleep–wake cycle, locomotor activity and aggressive behaviours using designer receptor exclusively activated by designer drugs (DREADDs) in Hdc-Cre mice, and further examined the projection distribution of HA^{TMN} fibres by immunohistology to identify the neural circuits underlying the behavioural effects of HA^{TMN} neuron activity.

2. Results

2.1. Chemogenetic receptor expression by HA^{TMN} neurons after microinjection

Cell type-specific expression of chemogenetic receptors is crucial for establishing relationships between activity changes and behaviour. We therefore first confirmed that expression of chemogenetic receptors was exclusive to HA^{TMN} neurons in the posterior hypothalamus by scanning brain slices obtained after behavioural experiments for immunoreactivity to the vector marker mCherry. Indeed, mCherry immunoreactivity (brown) was detected only in posterior hypothalamus (TMN) in mice injected with hM3Dq (Fig. 1A) or hM4Di (Fig. 1B). Moreover, nuclear c-Fos expression (black) was markedly higher in hM3Dq-mCherry-immunoreactive cells (Fig. 1A') compared to hM4Di-mCherry-immunoreactive cells (Fig. 1B') 3.0 h after administration of clozapine N-oxide (CNO) (0.3 mg/kg i.p.), consistent with greater neuronal activity specifically in CNO responsive cells ⁸.

2.2. Activation of HA^{TMN} neurons enhanced locomotor activity in a novel environment

Previous studies have yielded inconsistent or equivocal results regarding the effects of HA^{TMN} neuron excitation or inhibition on mouse locomotor activity. Acute pharmacological inhibition of histamine decarboxylase (HDC) or deletion of the *Hdc* gene, interventions that would reduce brain histamine levels, have been reported to decrease locomotor activity in rodents^{9–13}. However, a chronic increase in histamine level also decreased locomotor activity in dark periods, while an acute moderate increase in brain histamine was found to enhance locomotor activity^{6,14,15}. Histamine receptor stimulation/inhibition outside the TMN, effects of histamine on other neuronal populations and (or) compensatory changes after knockout may obscure effects of HA^{TMN} neurons on locomotion. Therefore, we established a DREADDs system to assess the effects of direct acute activation or inhibition of HA^{TMN} neurons. Chemogenetic activation of HA^{TMN} neurons with CNO did not significantly alter locomotor activity during the first 30-min trial compared to saline (SA) (Fig. 2A–D), but there were significant differences in movement time and time in the central area between trials 1 and 2 (Fig. 2C and D). Similarly, inhibition of HA^{TMN} neurons by hM4Di-CNO did not decrease mean locomotor activity during two 30-min trials compared to controls (Fig. 2E–H) but there were significant differences in total movement distance, average speed and total movement time between trials 1 and 2 (Fig. 2E–G). In the subgroup analyses, the hM3Dq-CNO group demonstrated increased total movement distance and average speed in the first trial (Fig. S1A and B). Time-resolved analyses indicated that these indices were significantly increased in the first 5-min period (Fig. S1A and B). In the second trial, however, hM3Dq-CNO significantly decreased movement distance and average speed (Fig. S1I and J). On the other hand, subgroup analysis of hM4Di did not reveal any significant differences (Fig. S1E–H and M–P). These results indicate that HA^{TMN} neuron activation enhances locomotor activity in a novel environment but actually decreases activity in a non-novel environment, suggesting that HA signalling influences exploratory behaviour.

2.3. Chemoactivation of HA^{TMN} neurons increases territorial aggression

Like locomotor activity, histaminergic influences on aggressive behaviours have been inconsistent across studies using pharmacologic manipulations or *Hnmt* KO^{6,16–18}. Specific activation of HA^{TMN} neurons by hM3Dq-CNO significantly increased the number and duration of aggressive behaviours (Fig. 3A and B) compared to saline control in the resident–intruder test, and also tended to reduce the latency to first aggressive behaviour, although the difference did not reach statistical significance (Fig. 3C). Conversely, inhibition of HA^{TMN} neurons by hM4Di-CNO had no significant influence on aggression compared to saline control (Fig. 3A–C). These results indicate that the activation of HA^{TMN} neurons triggers and maintains territorial aggression in mice.

2.4. Modulation of HA^{TMN} neuron activity alters the sleep–wake cycle

Next, we examined whether specific activation of HA^{TMN} alters the sleep–wake cycle by administering CNO or SA to AAV-hM3Dq mice at ZT3 (light period) or ZT12 (dark period). In two-arm crossover experiments, hM3Dq-CNO did not alter wake, NREM sleep, or REM sleep times within the first 6-h during light (Fig. 4A) or dark periods (Fig. S2A). Time-resolved analyses, however, revealed that CNO injection at

ZT3 significantly increased total waking time and decreased NREM sleep time within the first 1 h (ZT3–ZT4) while REM sleep time was not changed (Fig. 4B). The latency to the first NREM sleep episode was significantly extended by hM3Dq-CNO (Fig. 4E), while the number and the mean duration of waking, NREM sleep and REM sleep episodes during the first 1 h were not changed (Fig. 4C and D, and table S1). The frequency of Wake to Wake transitions (W ∇ W) was significantly increased whereas the frequency of NREM to NREM transitions (NR ∇ NR) was significantly decreased in the hM3Dq-CNO group, indicating that activation of HA^{TMN} neurons contributes to arousal maintenance (Fig. 4F). There were no differences in EEG power spectra during wakefulness, NREM sleep and REM sleep between CNO and SA groups (Fig. S4A). In addition, CNO injection at ZT12 did not alter sleep–wake status in sub-analyses (Fig. S2 and S4B, and Table S1).

We also examined whether specific inhibition of HA^{TMN} neurons suppressed wakefulness by comparing AAV-hM4Di mouse responses to CNO and SA administered at ZT3 or ZT12. Injection of CNO decreased waking time and increased NREM sleep time during the dark periods (Fig. 5A), while REM sleep in the dark periods and all sleep and wake stages in the light periods did not differ between CNO and SA groups (Fig. 5A and S3). Analysis of sleep–wake status during the first 1 h after CNO injection revealed decreased waking duration and increased NREM sleep duration but no changes in REM sleep duration (Fig. 5B). The mean duration of NREM sleep was significantly increased by hM4Di-CNO at ZT12 (Fig. 5D and table S2), while the latencies to first NREM sleep and REM sleep episodes, mean durations of wakefulness and REM sleep and the number of individual sleep or waking episodes were not significantly changed (Fig. 5C–E). The number of W-W transitions was reduced while the number of NR-NR transitions was increased by hM4Di-CNO at ZT12 (Fig. 5F). Collectively, these findings demonstrate the HA^{TMN} neuron inhibition during the dark period impedes arousal maintenance. EEG power densities for each episode did not differ between NCO and SA groups (Fig. S4D). Further, CNO injection at ZT3 did not significantly alter sleep–wake status (Fig. S3 and S4C, Table S2). These results indicate that HA^{TMN} neuron activity during the dark period is crucial for maintaining arousal status.

2.5. HA^{TMN} neurons projects to brain regions known to regulate aggression, vigilance and the sleep–wake cycle

We then investigated the potential circuits mediating these effects of HA^{TMN} neuron chemoactivation/chemoinhibition on locomotion, aggression and wakefulness by conducting serial mCherry immunostaining to identify projection targets. Although the brains were harvested 2 months after AAV microinjection, mCherry-immunoreactive somata were found exclusively in the ventral and caudal TMN (Fig. 6A and B). In contrast, mCherry-positive histaminergic fibres were observed in multiple brain regions at 6 months post-AAV microinjection, including the bed nucleus of the stria terminalis (BNST) and substantia innominata (SI) regions of the preoptic area (Fig. 6C), wide regions of hypothalamus but particularly in the lateral hypothalamus (LH) (Fig. 6D), central amygdala (CeA) (Fig. 6E) and periaqueductal grey (PAG), especially the ventrolateral PAG (vIPAG) (Fig. 6F). These regions may mediate the effects of HA^{TMN} neuron activity on exploratory locomotion, territorial aggression and the sleep–wake cycle.

3. Discussion

In the present study, we demonstrate that the specific modulation of HA^{TMN} neuron activity by DREADDs can influence exploratory locomotion, territorial aggression and the sleep–wake cycle of mice. Although specific activation of HA^{TMN} neurons did not significantly alter overall locomotion in the two-arm crossover trial, activation moderately increased locomotion in trial 1 (first exposure) and moderately decreased locomotion in trial 2. In contrast to these modest effects on locomotion, activation of HA^{TMN} neurons markedly enhanced aggression of male mice toward an intruder male. Furthermore, HA^{TMN} neuron activation increased wakefulness for an hour in the light periods, whereas inhibition significantly decreased wakefulness during the dark periods. Transitions in sleep–wake status were also regulated by HA^{TMN} neuron activity. Finally, we show that TMN histaminergic neurons project to brain regions implicated in fear, aggression and arousal, such as the BNST, LH, CeA and vIPAG.

The HA^{TMN} neurons of rodents are distributed in five clusters, E1, E2 and E3 in the ventral area, E4 in the medial dorsal area and E5 more diffusely in the dorsal area¹⁹. At 2–3 months after injection of Hdc-hM3Dq or Hdc-hM4Di vector, mCherry-positive histaminergic neurons were localised primarily in lateral E1 and E2 (Fig. 1A and B), a distribution comparable to that of Hdc-Cre mice crossed with a Cre-dependent tdTomato reporter line⁷. In addition, however, immunostaining of brain Sect. 6 months after microinjection revealed additional mCherry-positive histaminergic neurons in E3 and E5 (Fig. 6A and B). A transgene downstream of the human synapsin gene (hSyn) promoter in the AAV vector was exclusively expressed in neurons, and AAV8 induced long-term upregulation of the transgene^{20,21}. Therefore, a wider distribution of immunoreactive histaminergic neurons in the present study may result not only from a longer time period for expression but also from use of the hdc promoter. While we assume that the observed behavioural effects are due mainly to the activities of E1 and E2 histaminergic neurons (Fig. 1), contributions of E3 and E5 neurons cannot be totally excluded.

Previous studies have clearly demonstrated that HA^{TMN} neurons project to broad regions of the brain, including BSNT, LH, CeA and vIPAG^{22,23}. Our projection study confirmed these findings and revealed a particularly dense projection to the bilateral vIPAG (Fig. 6F). Neurons of the vIPAG are involved in the regulation of locomotor activity²⁴, aggressive behaviours²⁵ and the sleep–wake cycle^{26–28}, consistent with our behavioural results. Antegrade and retrograde optogenetics experiments are warranted to elucidate the precise circuit mechanisms.

A positive association between hypothalamic histamine release and physiological locomotor activity was first reported in 1992²⁹ and thereafter evidence has accrued for important contributions of HA^{TMN} neuronal excitation to locomotor activity, mainly based on experiments in which histamine production or H₁ and H₃ receptors were inhibited^{9,12,30,31}. In these loss-of-function studies, locomotor activity was generally reduced by inhibition of histaminergic signalling in the CNS, but gain-of-function studies in which brain histamine levels were increased have yielded unexpected and inconsistent results^{6,14,15}. We found no significant difference in locomotion between CNO and control groups in the two-arm crossover

experiment (Fig. 2), even in subgroup intracohort analysis between trials (data not shown). However, we found that HA^{TMN} activation increased locomotor activity in the Hdc-hM3Dq intercohort analysis for trial 1 (Fig. S1) and decreased activity in trial 2, suggesting that HA^{TMN} neuron activation enhances locomotor activity in a novel environment^{30,32}. Increased locomotor activity by specific HA^{TMN} chemogenetic activation was also reported by Yu et al.³³ but they observed a greater and more sustained increase, possibly due to administration of 5 mg/kg CNO, which may induce nonspecific neuronal activation³⁴. On the other hand, HA^{TMN} neuron activation decreased locomotor activity in trial 2 (Fig. S1). Histaminergic activation also contributes to memory retrieval^{35–37}, so this reduced locomotor activity in trial 2 may be due to strong contextual memory for an otherwise anodyne environment, resulting in lower exploratory motivation. In contrast, HA^{TMN} suppression by hM4Di-CNO did not significantly alter locomotor activity (Fig. 2 and S1). However, chemogenetic inhibition of HA^{TMN} by 1 mg/kg CNO injection significantly decreased locomotion in dark periods³⁸. We performed open-field tests with a much lower CNO dose during the light periods, the resting phase for nocturnal animals, so further studies are needed to evaluate effects of loss-of-function on locomotor activity during the dark periods.

In contrast to the subtle effects on locomotion, HA^{TMN} neuron activation clearly enhanced territorial aggression (Fig. 3). Previous studies have suggested that elevated brain histamine levels induce aggression via H₂ receptor activation^{6,16,17}, consistent with the current results. We speculate that two mechanisms underlie increased aggression upon HA^{TMN} neuron activation, direct modulation of neural pathways mediating aggression and modulation via effects on the circadian rhythm. One potential pathway is the ventromedial hypothalamus, ventrolateral subdivision (VMHvl)–vIPAG circuit³⁹, as the VMHvl is innervated by the HA^{TMN} and induces aggression upon stimulation^{23,40}. The HA^{TMN} also innervates the vIPAG (Fig. 6), which transforms higher level neuronal signals into aggressive action-specific codes³⁹. A second possible pathway is the suprachiasmatic nucleus (SCN)–subparaventricular zone (SPZ)–VMHvl circuit. The SCN is the master circadian pacemaker in the mammalian brain, and sends dense axonal outputs to the SPZ, which negatively regulates aggression via the VMHvl⁴¹. The HA^{TMN} also projects to the SCN^{23,42} and has an inhibitory effect on SCN neuronal activity through H₁ and H₂ receptor activation^{43–45}. Additional projection mapping and chemogenetic activation studies are required to clarify the contributions of these pathways to territorial aggression induced by HA^{TMN} neuron excitation.

To our knowledge, only three previous studies have used Hdc-Cre mice to examine the functions of HA^{TMN} neurons in regulation of the sleep–wake cycle^{5,7,38}. Chemogenetic inhibition of HA^{TMN} neurons during the dark periods decreased wakefulness and increased NREM sleep duration in a different Hdc-Cre mouse line, and these responses were accompanied by increased EEG δ power and decreased EEG power at higher frequencies (8–30 Hz)³⁸. A study using the same Hdc-Cre line employed in the current study found that optogenetic inhibition of HA^{TMN} neurons by archaerhodopsin (Arch) 3.0 during wakefulness in the dark periods promoted NREM sleep and increased the number of NREM episodes but not NREM duration,

and had no effect on EEG spectra⁷. Although we found prolonged mean NREM duration without alteration in the number of episodes (Fig. 5C and D) and no differences in the EEG power spectra for wake and all sleep stages (Fig. S4), the inhibitory experiments in the present study revealed an important role for HA^{TMN} neurons in maintaining wakefulness during the dark periods, in accord with the aforementioned studies. However, inhibition of HA^{TMN} neurons with ArchT during wakefulness did not impact NREM sleep in experiments using another Hdc-Cre line⁵. These authors also induced chemogenic activation of HA^{TMN} neurons by CNO injection at ZT3 and found no alterations in the relative durations of sleep and wake stages or EEG spectra⁵. In the present study, we also found no changes in the sleep–wake cycle following HA^{TMN} neuron activation in the overall analysis (Fig. 4A–C). In sub-analysis, however, Hdc-hM3Dq mice demonstrated increased wakefulness in the first hour after CNO injection at ZT3 (Fig. 4B). Venner et al. (2019) also found that a new environment augmented the increased wakefulness and reduced NREM observed in response to HA^{TMN} neuron activation during the light periods. We suggest that the significant increase in wakefulness observed during the first hour may be influenced by the novel environment of the experimental condition.

Previous electrophysiological studies have identify some TMN neurons as ‘Waking-on’ due to predominant activation during wakefulness and complete silence during NREM and REM sleep^{46–48}. These neurons also exhibited a pronounced delay in firing during transitions from sleep to wakefulness and a long delay to an arousing stimulus, indicating important contributions to the maintenance of wakefulness and vigilance, but not to state induction⁴⁷. Our findings from transition analyses, which showed W–W promotion by HA^{TMN} activation and reduction by inhibition provide further support for the role of HA^{TMN} neurons in arousal maintenance (Fig. 4F and 5F). Steininger et al. (1999) also suggested that some histaminergic neurons in rats discharged at high rates during REM sleep, termed ‘REM-related neurons’. However, we found no relation between HA^{TMN} neuron activity and REM sleep.

As previously described, the Hdc-Cre mouse line used in the present study has high specificity with minimal Cre recombinase expression outside the TMN; however, it also exhibits only ~ 50% penetrance⁷. Thus, at least half of the Hdc-neuronal population was not modulated by the chemogenetic approach, which may explain why we did not observe the significant changes in locomotor activity and REM sleep reported in previous studies using a different Cre recombinase line with higher penetrance^{5,38}. Further, several studies suggest that HA^{TMN} neurons are functionally heterogeneous^{7,49–51} and form distinct projections to influence unique physiological functions and behaviours. Further work is required to determine the individual HA^{TMN} pathways responsible for the observed changes in locomotor activity, aggression and the sleep–wake cycle. Furthermore, we found no correlations between behavioural changes across groups (data not shown). Larger cohorts for greater statistical power and projection-specific activation methods may be required to identify such relationships.

In conclusion, the specific modulation of HA^{TMN} neuron activity altered exploratory locomotion, territorial aggression and wake maintenance in mice. These responses may be explained by projections from

HA^{TMN} neurons to the POA, CeA, LH and vIPAG.

4. Materials And Methods

4.1. Animals

All animal care and use protocols were conducted in accordance with the Standards of Humane Care and Use of Laboratory Animals of Tohoku Medical and Pharmaceutical University, Sendai, Japan. All experiments involving animals also complied with the Guidelines for the Proper Conduct of Animal Experiments of the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the ARRIVE guidelines for reporting animal research⁵². All animal experiments including the use of genetically modified mice were approved by the Tohoku Medical and Pharmaceutical University Animal Experiment Committee (registration number: 19004-cn) and Tohoku Medical and Pharmaceutical University Centre for Gene Research (registration number: 2018-19). All efforts were made to reduce the number of animals used and minimize animal suffering.

For this study, we used a mutant mouse line expressing Cre recombinase under control of the Hdc promoter [B6.FVB(Cg)-Tg(Hdc-Cre)^{IM1Gsat/Mmucd} (RRID: MMRRC_037409-UCD)] (Hdc-Cre)⁷ as previous studies reported target specificity of 94.7% (albeit with penetrance of only 48.8%)⁷. Only male heterozygous Hdc-Cre mice were used for these experiments. All mice were group-housed in a temperature-controlled (23°C ± 1°C) and humidity-controlled (55.0% ± 5.0%) animal room under a 12:12 h light:dark cycle [lights on at 8:00 (ZT0), lights off at 20:00 (ZT12)] with *ad libitum* access to standard rodent chow (CLEA Japan, Tokyo, Japan) and water.

4.2. Genotyping

Genotyping was performed in accordance with protocol MMRRC_032079-UCD. Briefly, genomic DNA was extracted from tail biopsies and analysed by polymerase chain reaction (PCR) using the KOD FX Neo enzyme (TOYOBO, Osaka, Japan). The PCR reaction protocol consisted of incubation at 94°C for 2 min, followed by 35 cycles of 98°C for 10 s, 60°C for 30 s and 68°C for 40 s under control of a T100 thermal cycler (Bio-Rad, Hercules, CA). The primer sequences were as follows: Hdc-Cre (575 bp): forward, 5'-AGC CTC CTC TGT CTG TCT GC; reverse, 5'-CCC CAG AAA TGC CAG ATT ACG TAT. WT (220 bp): forward, 5'-CTC ACC CAA ATC CAA CGA CT; reverse, 5'-GCA GTT GCC TAG CAA CAC AC.

4.3. Surgery

Adult male Hdc-Cre mice (10–14 weeks old) were anaesthetised using a mixture of 0.3 mg/kg medetomidine (Zenoaq, Fukushima, Japan), 4 mg/kg midazolam (Astellas, Tokyo, Japan) and 5 mg/kg butorphanol (Meiji Seika Pharma, Tokyo, Japan), and fixed on a digital stereotactic instrument (940-XYZ, David Kopf, Tujunga, CA). A 100-nL volume of vector solution was microinjected into each TMN (AP – 2.4 mm, RL ± 1.0 mm, DV – 5.1 mm from bregma). The vector solution contained either AAV8-hSyn-DIO-hM3Dq-mCherry (titre: 2.2×10^{13} vg/mL, 44361-AAV8; Addgene, Watertown, MA) (AAV-hM3Dq) or AAV8-DIO-hSyn-hM4Di-mCherry (titre: 2.5×10^{13} vg/mL, 4362-AAV8; Addgene) (AAV-hM4Di) to establish mouse

models for chemogenetic activation and inhibition, respectively. Three weeks after microinjection, open field and resident–intruder tests were performed as described (Sect. 2.5). After the resident–intruder test, all mice were implanted with a head mount and electrodes (E363/76//NS/SPC, P1 Technologies, Manhattan Beach, CA) for EEG and electromyogram (EMG) recordings⁵³. The EEG electrodes were inserted by threading through stainless steel screws while EMG electrodes were inserted directly into the neck extensor muscles.

4.4. Histology

After completion of behavioural studies, mice were deeply anaesthetised with a medetomidine/midazolam/butorphanol mixture and transcardially perfused with 15 mL phosphate buffered saline (PBS) followed by 50 mL of 10% formalin in PBS (Wako, Osaka, Japan). Mouse brains were harvested immediately and incubated in 10% formalin/PBS overnight at 4°C, followed by incubation in 10% formalin/PBS with 20% sucrose at 4°C for 2 days. Brains were sliced into 3 series of 40-µm coronal sections using a freezing cryostat (CM3050, LEICA, Wetzlar, Germany) and processed for immunohistochemistry.

For immunohistochemistry using diaminobenzidine (DAB) as the chromogen, sections were incubated with Fos antibody (1:10,000; SC-52; Santa Cruz Biotechnology, Dallas, TX) for two nights or ds-Red (1:10,000; Z2496N; Clontech Laboratory, Mountain View, CA) overnight at room temperature, followed by incubation in the appropriate biotin-SP-conjugated secondary antibody (1: 1,000; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Sections were incubated for 75 min in avidin-biotin-complex reagent (1:1000; Vectastain ABC kit, Vector Lab, Burlingame, CA) at room temperature, washed and incubated in a solution of 0.06% DAB (Wako) and 0.02% H₂O₂ (Wako) for 2–5 min. Then, PBS containing 0.05% CoCl₂ (Wako) and 0.01% NiSO₄ (NH₄) (Wako) was added to the DAB solution for staining (black).

Sections were mounted on Superfrost glass slides (Matsunami, Osaka, Japan), dehydrated, cleared and cover-slipped using Multi Mount 480 (Matsunami). The mounted brain sections were scanned using a BZ-X700 microscope (Keyence, Osaka, Japan) and composite images were generated by BZ-X Analyzer software (Keyence).

4.5. Behavioural experiments

All behavioural tests were performed with a two-arm crossover design by experimenters blinded to treatment history. Each microinjected mouse was examined first in the open-field test, followed by the resident–intruder test and sleep–wake cycle monitoring by EEG/EMG. Mice were randomly assigned to receive CNO or SA at the beginning of the first trial, then switched to the other drug for the second trial. Again, tests were analysed by experimenters blinded to mouse treatment history.

4.5.1 Open-field test

The open-field test was performed as previously described³³. Briefly, mice were injected with CNO (0.3 mg/kg; Sigma, St Louis, MO) or saline vehicle (SA) as a control 30 min before the trial during the light

period. Each mouse was placed into the centre of a 60 cm × 60 cm square arena and allowed to explore freely for 30 min. Total movement distance, average speed, total movement time and time spent in the central area were recorded and quantified using a video tracking system (EthoVision® XT, Ver. 13, Noldus, Wageningen, Netherlands) by an experimenter blinded to treatment history.

4.5.2 Resident–intruder test

The resident–intruder test was performed as previously described⁶. Briefly, resident mice were housed individually for 1 week before the testing day to increase territorial motivation. Then, the mouse was injected with CNO (0.3 mg/kg; Sigma) or SA 30 min before the trial during the light period. An unfamiliar male (intruder, C57BL6 WT, matched for age) was introduced into the resident cage and the two mice were allowed to interact freely for 10 min. Aggression of the resident mouse was measured by the frequencies of ‘mounting’ (attempts to mount the intruder), ‘chasing’ and ‘biting’ (bites to dorsal/ventral regions of the intruder). The latency to first aggressive behaviour, number of aggressive behaviours and total duration of aggressive behaviours were measured. Another unfamiliar male mouse was used as an intruder in the second trial. All experimental data were video-recorded and analysed by an examiner blinded to treatment history.

4.5.3. Sleep recordings and analysis

A week after EEG/EMG electrode implantation, mice were connected to recording cables and habituated for 3 days in a cylinder chamber (22 cm in diameter) placed within a soundproof electrically shielded box (SP-BOX/S, Shinfactory, Fukuoka, Japan) maintained at constant temperature ($23^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and humidity ($55.0\% \pm 5.0\%$) under a 12:12h light-dark cycle (ZT0: 8:00, ZT12: 20:00) with food and water available *ad libitum*. The mice were injected with CNO (0.3 mg/kg; Sigma) or SA at 10:50 AM (10 min before ZT3) or 7:50 PM (10 min before ZT12), followed by EEG/EMG recording for 24 h. Following an interval of at least 3 days, mice were switched to the other drug intervention group for another 24 h of EEG/EMG recording. The EEG/EMG signals were amplified (Biotex, Kyoto, Japan), digitised (AD16-16U/PIC/EV, CONTEC, Osaka, Japan) and recorded by a Vital Recorder system (Kissei Comtec, Nagano, Japan). Only the first 6 h of the 24 h period were analysed. Briefly, collected data were divided into 12-s epochs and scored manually for one of three sleep or wake states, wake, NREM sleep, or REM sleep, using SleepSign 3 (Kissei Comtec). Time spent in each state, episode frequency and episode duration were calculated in 1-h bins. Latencies to the first NREM and REM sleep episodes were measured as the period following wakefulness evoked by intraperitoneal (i.p.) injection. We also quantified sleep–wake status transitions for the first hour after CNO or SA injection. Sleep–wake status in two consecutive epochs was determined, then the frequencies of status transitions from the first epoch to the second epoch (Wake to Wake, Wake to NREM, NREM to NREM, NREM to Wake, NREM to REM, REM to REM and REM to Wake) were counted. The power spectral density of EEG signals in each stage was calculated with 0.25 Hz resolution by Fast Fourier Transform using SleepSign 3 software, then normalised for each animal by calculating the % power of each bin relative to the total power from 0 to 20 Hz.

4.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism® version 8 (GraphPad Software, La Jolla, CA). In behavioural experiments, the CNO group was compared to the SA group by two-way repeated measures ANOVA followed by Sidak's *post hoc* test or by Mann–Whitney U test as indicated in the figure legends. All data are presented as the mean $\pm$ standard error of the mean (S.E.M.) unless otherwise noted. Differences were considered significant at $P < 0.05$. All statistical analysis was performed by experiments blinded to animal treatment history.

Abbreviations

AAV, adeno-associated virus; Arch, archaerhodopsin; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; CNO, clozapine N-oxide; DAB, diaminobenzidine; DREADDs, Designer receptor activated by designer drugs; EEG, electroencephalogram; EMG, electromyogram; HA, histamine; Hdc, histidine decarboxylase; Hnmt, histamine *N*-methyltransferase; LH, lateral hypothalamus; NREM, non-rapid eye movement; REM, rapid eye movement; SCN, suprachiasmatic nucleus; SI, substantia innominata; SPZ, subparaventricular zone; TMN, tuberomammillary nucleus; VMHvl, ventromedial hypothalamus, ventrolateral subdivision; vIPAG, ventrolateral periaqueductal grey; ZT, Zeitgeber time.

Declarations

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

We thank the staff of the Animal Facility and Technical Service Division of Tohoku Medical and Pharmaceutical University for technical support.

Funding

This work was supported by a Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research B (18H02771) to NO, Grant-in-Aid for Young Scientists (17K16753) and Grant-in-Aid for Scientific Research C (20K09204) to TN and a Grant-in-Aid for Young Scientists (19K16296) to FN.

Competing interest

The authors declare no competing interests.

Author contributions

FN: Conceptualisation, Methodology, Investigation, Writing-Original Draft, Visualisation, Funding acquisition. **TN:** Conceptualisation, Methodology, Validation, Resources, Formal analysis, Writing-Original

Draft, Writing-Reviewing and Editing, Project administration, Funding acquisition. **HK:** Investigation, Formal analysis. **MT:** Investigation, Formal analysis. **TY:** Conceptualisation, Methodology, Writing-Original Draft. **KY:** Writing- Reviewing and Editing, Supervision. **NO:** Conceptualisation, Writing-Reviewing and Editing, Supervision, Project administration, Funding acquisition.

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Figures

Figure 1

Specific expression of Hdc-hM3Dq/Hdc-hM4Di in the TMN and confirmation of neuronal activation or inhibition by CNO. A, A representative hypothalamic section from an Hdc-Cre mouse injected with AAV-hM3Dq. CNO injection induced c-Fos expression (black nuclei) in mCherry-expressing HATMN neurons (brown) (A'). B, Representative section from an Hdc-Cre mouse injected with AAV-hM4Di. c-Nuclear Fos expression (black) was substantially lower in mCherry-positive neurons (brown) after CNO injection (B'). Scale bar = 200 μ m. 3V, third ventricle. Arrows: mCherry-positive/c-Fos-negative neurons, Black arrow heads: mCherry-positive/c-Fos-positive neurons, White arrow heads: mCherry-negative/c-Fos-positive neurons. These coronal brain sections were located -2.18 mm anterior-posterior from bregma.

Figure 2

Chemogenetic activation of HATMN neurons enhanced locomotor activity in a novel open field but reduced locomotor activity in a pre-explored (non-novel) open field. Locomotor parameters were compared among Hdc-hM3Dq mice (A–D, $n = 6$) and Hdc-hM4Di mice (E–H, $n = 5$) for 30 min after CNO or SA injection by two-way RM ANOVA with main factors trial number (1 or 2) and drug treatment (CNO or SA), followed by Sidak's post hoc tests. A, Total movement distance of Hdc-hM3Dq mice after CNO or SA

injection (trial: $F = 4.43$, $P = 0.062$; drug: $F = 0.087$, $P = 0.77$; trial $\times$ drug interaction: $F = 4.64$, $P = 0.057$). B, Average locomotion speed of Hdc-hM3Dq mice after CNO or SA injection (trial: $F = 4.43$, $P = 0.062$; drug: $F = 0.088$, $P = 0.77$; interaction: $F = 4.64$, $P = 0.057$). C, Total movement time of Hdc-hM3Dq mice after CNO or SA injection (trial: $F = 9.61$, $P = 0.01$; drug: $F = 0.046$, $P = 0.83$; interaction: $F = 1.88$, $P = 0.20$). D, Total time spent in the central area by Hdc-hM3Dq mice after CNO or SA injection (trial: $F = 21.58$, $P = 0.0009$; drug: $F = 0.046$, $P = 0.83$; interaction: $F = 1.78$, $P = 0.21$). E, Total movement distance of Hdc-hM4Di mice after CNO or SA injection (trial: $F = 9.44$, $P = 0.015$; drug: $F = 0.96$, $P = 0.36$; interaction: $F = 0.0062$, $P = 0.94$). F, Average speed of Hdc-hM4Di mice after CNO or SA injection (trial: $F = 9.44$, $P = 0.015$; drug: $F = 0.96$, $P = 0.36$; interaction: $F = 0.0062$, $P = 0.94$). G, Total movement time of Hdc-hM4Di mice after CNO or SA injection (trial: $F = 8.82$, $P = 0.018$; drug: $F = 0.68$, $P = 0.43$; interaction: $F = 0.0086$, $P = 0.93$). H, Total time spent in the central area by Hdc-hM4Di mice after CNO or SA injection (trial: $F = 1.8$, $P = 0.22$; drug: $F = 0.24$, $P = 0.64$; interaction: $F = 0.63$, $P = 0.45$). Data are presented as mean $\pm$ S.E.M. (horizontal bars and error bars) with superimposed individual data points (closed circles). $\dagger P < 0.05$ by two-way RM ANOVA with main factors 'trial' and 'drug'.

Figure 3

Chemogenetic activation of HATMN neurons enhanced aggression in the resident–intruder test. A, Number of aggressive behaviours in 10 min by Hdc-hM3Dq and Hdc-hM4Di mice after CNO or SA injection ($n = 7$ or 9 ; $P = 0.035$ by Mann–Whitney U test). B, Cumulative duration of aggressive behaviours within 10 min by Hdc-hM3Dq and Hdc-hM4Di mice after CNO or SA injection ($n = 7$ or 9 , $P = 0.018$ by Mann–Whitney U test). C, Latency to first aggressive behaviour by Hdc-hM3Dq and Hdc-hM4Di mice after CNO or SA injection ($n = 7$ or 9 ; no significant differences by Mann–Whitney U test). Data are presented as mean $\pm$ S.E.M. (horizontal bars and error bars) with superimposed individual data points (closed circles). $\S P < 0.05$.

Figure 4

Chemogenetic activation of HATMN neurons increased wakefulness and decreased NREM sleep during light periods. Hdc-hM3Dq mice ($n = 7$) were injected with CNO or SA at the indicated times and wake and sleep phases monitored by EEG/EMG. A, Relative mean hourly wake, NREM sleep and REM sleep times (%) after CNO or SA injection at ZT3 (wake – time: $F = 4.34$, $P = 0.0019$; drug factor: $F = 1.24$, $P = 0.29$; time $\times$ drug interaction: $F = 1.28$, $P = 0.29$; NREM – time: $F = 3.06$, $P = 0.016$; drug: $F = 1.31$, $P = 0.28$; interaction: $F = 1.46$, $P = 0.22$; REM – time: $F = 8.21$, $P < 0.0001$; drug: $F = 0.44$, $P = 0.52$; interaction: $F = 0.23$, $P = 0.95$; all by two-way RM ANOVA with main factors time and drug, followed by Sidak's post hoc test). B, Mean wake, NREM sleep and REM sleep times during ZT3–4 after CNO or SA injection at ZT3 (wake $P = 0.0070$ and NREM $P = 0.0087$ by Mann–Whitney U test). C, Number of wake or sleep phase episodes during ZT3–4 after CNO or SA injection at ZT3 (no significant differences by Mann–Whitney U test). D, Mean duration of sleep–wake status after CNO or SA injection at ZT3 (no significant differences by Mann–Whitney U test). E, Latency to the first NREM and REM sleep episode after CNO or SA injection at ZT3 (NREM $P = 0.018$ by Mann–Whitney U test). F, Frequencies of specific sleep–wake status transitions after CNO or SA injection at ZT3 (wake " wake $P = 0.0064$, wake " NREM $P = 0.30$, NREM " NREM $P = 0.018$, NREM " REM $P = 0.69$, NREM " wake $P = 0.36$, REM " REM $P = 0.30$ and REM " wake $P = 0.32$ by Mann–Whitney U test). Data are presented as mean $\pm$ S.E.M. (horizontal bars and error bars) of $n = 7$ mice with (B–F) or without (A) superimposed individual data points (close circles). † $P < 0.05$ by two-

way RM ANOVA with main factors 'time' and 'drug'; * $P < 0.05$ by post hoc Sidak's multiple comparisons test; § $P < 0.05$, §§ $P < 0.01$ by Mann–Whitney U test.

Figure 5

Chemogenetic inhibition of HATMN neurons decreased wakefulness and increased NREM sleep during dark periods. Hdc-hM3Di mice ($n = 6$) were injected with CNO or SA at the indicated time and wake and sleep phases monitored by EEG/EMG. A, Hourly mean wake, NREM sleep and REM sleep durations after CNO or SA injection at ZT12 (wake – time: $F = 5.99$, $P = 0.0002$; drug: $F = 10.02$, $P = 0.010$; time $\times$ drug interaction: $F = 2.81$, $P = 0.026$; NREM – time: $F = 5.65$, $P = 0.0003$; drug: $F = 10.39$, $P = 0.0091$; interaction: $F = 2.28$, $P = 0.061$; REM – time: $F = 3.18$, $P < 0.014$; drug: $F = 1.23$, $P = 0.29$; interaction: $F = 2.32$, $P = 0.057$; all analyses by two-way RM ANOVA with main factors time and drug, followed by Sidak's post hoc test). B, Mean wake, NREM sleep and REM sleep durations during ZT12–13 after CNO or SA injection at ZT12 (wake $P = 0.015$ and NREM $P = 0.013$ by Mann–Whitney U test) C, Number of episodes during ZT12–13 after CNO or SA injection at ZT12 (no significant differences by Mann–Whitney U test). D, Mean duration of sleep and wake stages after CNO or SA injection at ZT12 (no significant differences by Mann–Whitney U test). E, Latency to the first NREM and REM sleep episodes after CNO or SA injection at ZT12 (no significant difference by Mann–Whitney U test). F, Sleep–wake status transitions after CNO or SA injection at ZT12 (wake " wake $P = 0.041$, wake " NREM $P = 0.29$, NREM " NREM $P = 0.0022$, NREM " REM $P = 0.93$, NREM " wake $P = 0.22$, REM " REM $P = 0.51$ and REM " wake $P = 0.93$ by Mann–Whitney U

test). Data are presented as mean $\pm$ S.E.M. of $n = 6$ mice with (B–F) or without (A) superimposed individual data points (closed circles). $\dagger P < 0.05$ by two-way RM ANOVA with main factors ‘time’ and ‘drug’, $* P < 0.05$ by post hoc Sidak’s multiple comparisons test. $\S P < 0.05$ by Mann–Whitney U test.

Figure 6

Projection targets of HATMN neurons. The presence of mCherry-immunoreactive HATMN somata and fibres were examined in coronal brain sections 6 months after AAV injections. A, mCherry-immunoreactive

HATMN neuronal somata and fibres in the ventrolateral TMN. B, mCherry-immunoreactive HATMN neuronal somata and fibres in the caudal TMN. C–F, HATMN fibres in the bed nucleus of stria terminalis (BNST) (C), lateral hypothalamus (LH) (D), central amygdala (CeA) (E) and ventrolateral periaqueductal grey (vlPAG) (F). Scale bar = 100 μ m. ac, anterior commissure; Aq, aqueduct; DM, dorsomedial hypothalamic nucleus; LA, lateroanterior hypothalamic nucleus; MPO, medial preoptic nucleus; opt, optic tract; SI, substantia innominata; VMH, ventromedial hypothalamic nucleus; 3V, third ventricle. Anterior-posterior locations (mm from bregma): (A) -2.18 mm, (B) -2.70 mm, (C) 0.14 mm, (D) -0.46 mm, (E) -1.34 mm, (F) -4.24 mm.

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