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REM sleep-active MCH neurons are involved in forgetting hippocampus-dependent memories

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Abstract

The neural mechanisms underlying memory regulation during sleep are not yet fully understood. We found that rapid eye movement (REM) sleep-active melanin-concentrating hormone (MCH)-producing neurons in the hypothalamus actively contribute to forgetting. Hypothalamic MCH neurons densely innervated the dorsal hippocampus. Activation or inhibition of MCH neurons impaired or improved hippocampus-dependent memory, respectively. Activation of MCH nerve terminals *in vitro* reduced firing of hippocampal pyramidal neurons by increasing inhibitory inputs. Wake- and REM sleep-active MCH neurons were distinct populations that were randomly

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Author contributions:

A.Y., A.T. and T.S.K. designed the experiments; S.I., S.C., D.O., T.M., Y.M., R.I. and Y.O. performed experiments. H.M., K.K., M.Y., A.T. and Y.O. contributed to the analysis and interpretation; S.I., T.S.K. and A.Y. wrote the manuscript.

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distributed in the hypothalamus. REM sleep state-dependent inhibition of MCH neurons impaired hippocampus-dependent memory without affecting sleep architecture or quality. REM sleep-active MCH neurons in the hypothalamus are thus involved in active forgetting in the hippocampus.

Events experienced during wakefulness are stored as memory. Depending on their importance, these memories undergo selection during sleep resulting in either memory consolidation or forgetting (1–3). Forgetting removes overloaded and unnecessary memories through synaptic renormalization (4–6). Forgetting is an active process, rather than simply passive (7–9). However, little is known about the neural mechanisms involved in forgetting during sleep. In addition, it is unclear whether forgetting occurs during non-rapid eye movement (NREM) sleep or REM sleep.

The hypothalamus is a center for instinctive and homeostasis-related behaviors. Melanin-concentrating hormone (MCH) neurons are exclusively located in the lateral hypothalamic area (LHA) but project broadly throughout the brain (10). Intracerebroventricular injection of the MCH peptide induces feeding behavior, suggesting a role in appetite (11). However, there is also a prominent role for MCH neurons in sleep/wakefulness regulation. Activation of MCH neurons increases time in REM sleep, whereas inhibition reduces transitions into REM sleep (12–16). REM sleep is characterized by a desynchronized electroencephalogram (EEG), muscle atonia and predominant theta (6–10 Hz) rhythm in the hippocampus.

To identify sources of direct innervation to the hippocampus, retrogradely-transported beads (retrobeads) were microinjected into the hippocampus. Retrobead-positive neurons were observed in brain areas already known to project to the hippocampus and in the hypothalamus (Fig. 1A–1B and S1). Immunohistochemical studies revealed that hypothalamic MCH neurons densely projected to the hippocampus.

To visualize the nerve terminals, we generated *MCH-tTA; TetO yellow Cameleon-Nano50 (YC)* mice (Fig. S2). We observed dense MCH nerve terminals in the dorsal hippocampus where orexin nerve terminals were sparse (Fig. 1C), suggesting a possible role for MCH in memory. We therefore manipulated the activity of MCH neurons using chemogenetics. To enable activation of MCH cells, AAV9-CAG-FLEX-hM3Dq-mCherry was injected into *MCH-Cre* mice. Histochemistry and electrophysiology confirmed that MCH neurons exclusively expressed hM3Dq and were activated by clozapine-N-oxide (CNO) *in vitro* and *in vivo* (Fig. 1D, S3 and S4).

To evaluate MCH neuron effects on memory, we performed a novel object recognition (NOR) test because NOR memory is hippocampus-related (17). CNO was injected after memory acquisition (Phase I), and after a retention period, memory was tested (Phase II). Surprisingly, CNO-injected mice showed significantly impaired NOR memory (Fig. 1E). Control experiments confirmed that CNO itself had no effect. To further assess the possible role of MCH neurons in memory, we performed a contextual fear conditioning (CFC) test. Activation of MCH neurons significantly reduced the freezing rate, suggesting CFC memory impairment (Fig. 1F). Fig. S5, Table S2 and S3 present the condition setting for the NOR and CFC tests. The conditions for the NOR and CFC under manipulation of MCH neuron

activity were selected according to the results of parameter-setting experiments (Fig. S5) to more clearly observe the effects on memory.

Next, MCH neurons were inhibited by injecting AAV9-CAG-FLEX-hM4Di-mCherry into *MCH-Cre* mice (Fig. 1G–1I). *In vitro* loose cell-attached recording confirmed that CNO application almost completely inhibited activity of MCH neurons expressing hM4Di (Fig. S6). In contrast to MCH neuron activation, MCH neuron inhibition significantly improved NOR and CFC memory, suggesting a role for MCH neurons in memory impairment (Fig. 1H–1I).

To assess memory after a long retention period, MCH neurons were ablated by expressing diphtheria toxin A fragment (DTA) under control of the tet-off system (15). After doxycycline (DOX) removal from diet, almost all pro-MCH mRNA-expressing neurons were ablated (MCHN(–)) in contrast to mice fed with DOX-containing chow (MCHN(+); Fig. 1J and S7A). These two groups were independently subjected to NOR tests. MCHN(–) mice exhibited significant improvement in NOR memory for at least 48 hr (Fig. 1K). NOR memory improvement in the same mice before and after ablation were consistent with a role for MCH neurons in memory (Fig. S7B). MCHN(–) mice showed a significantly higher freezing rate, suggesting improvement in CFC memory as well (Fig. 1L). However, cued-fear memory, which is amygdala-dependent, was not improved, suggesting that MCH neurons specifically affect hippocampus-dependent memory (Fig. 1M). In the Morris water maze, the latency to reach the platform was significantly shorter in MCHN(–) mice, suggesting improvement in spatial memory (Fig. 1N and S8). Anxiety behavior was not affected by MCH neuron-ablation (Fig. S9).

To further clarify a role for MCH neurons in memory, channelrhodopsin2 (ChR2) was expressed in MCH neurons in *MCH-tTA; TetO ChR2* mice (15). Utilizing wireless photoillumination, MCH neurons were optogenetically activated (Fig. 2A). Although activation during encoding or retrieval did not affect NOR memory, activation during the retention period significantly impaired memory (Fig. 2B). Activation for different 10 min periods during the retention excluded possible differences due to the duration of illumination (Fig. S10). CFC memory was also impaired by MCH neuron activation during retention (Fig. 2C).

To reveal possible cellular mechanisms, hippocampal pyramidal CA1 neurons were patch clamped and MCH nerve terminals were optogenetically stimulated (Fig. 2D–2J). Blue light significantly decreased the firing frequency of hippocampal pyramidal neurons while the frequency and amplitude of inhibitory postsynaptic currents (IPSCs) were dramatically increased, suggesting enhancement of GABAergic inhibitory inputs.

To identify the site of action, MCH nerve terminals in the hippocampus were activated. In both the NOR and CFC tests, activation of MCH nerve terminals in the hippocampus significantly impaired memory (Fig. 2K–2M and S10).

MCH neurons are mainly active during REM sleep (18). However, those recordings have been performed under head-fixed conditions and the number of cells recorded has been relatively small. In contrast, population activity of MCH neurons has been shown during

awake exploratory behavior (19, 20). To reveal MCH neuron activity across physiological sleep/wakefulness, we utilized fiber photometry (21) with EEG and electromyographic (EMG) recording. *MCH-*tTA** mice were injected with AAV9-TetO-GCaMP6 to express the Ca^{2+} indicator, GCaMP6 (Fig. 3A and S11). We inserted fiber optics into the LHA and recorded the population activity of MCH neurons in freely-behaving mice. Although MCH neurons exhibited moderate activity in wakefulness, activity increased during REM sleep. The activity significantly increased during NREM-REM and NREM-wake transitions and decreased during REM-wake transitions (Fig. 3C–3D).

To investigate MCH neuron activity further, we used microendoscopy to measure activity at the single-cell level. *MCH-Cre* mice were injected with AAV9-CMV-FLEX-GCaMP6f. We observed Ca^{2+} transients during wakefulness and REM sleep from spatially distinct MCH neurons (Fig. 3F and Movie 1). Of 146 cells recorded from 6 mice, 34.9% showed Ca^{2+} events during wakefulness without any during REM sleep, whereas 52.8% showed Ca^{2+} events during REM sleep without any during wakefulness. In addition, 12.3% showed Ca^{2+} events during both wakefulness and REM sleep. These three subpopulations were randomly distributed throughout the LHA. We confirmed the level of Ca^{2+} activity in single cells using the Z-score integral (Fig. 3J). The presence of these subpopulations suggested the possibility of different roles for wake-active and REM-active MCH neurons in memory.

The memory improvement of *MCHN*(–) mice shown in Fig. 1J–N was abolished by sleep deprivation or absence of a retention period (Fig. 4A), suggesting that MCH neuron activity during sleep might be involved in memory regulation. To reveal functional differences for REM-active vs. wake-active MCH neurons in memory, we performed sleep/wakefulness state-dependent optogenetic inhibition. The optogenetic neural silencer, Archaelhodopsin-T (ArchT), was exclusively expressed in MCH neurons by injecting AAV9-TetO-ArchT into *MCH-*tTA** mice (Fig. 4B and S12). During the retention period, sleep/wakefulness states were automatically discriminated by analysis of EEG, EMG, and locomotor activity in real-time (Fig. 4C). This procedure enabled immediate closed loop-triggering of photoillumination during specific states (Fig. 4D). State-dependent triggering of photoillumination spanned $94.8 \pm 0.7\%$ of wakefulness, $83.6 \pm 4.0\%$ of REM sleep and $93.7 \pm 0.9\%$ of NREM sleep time, with little illumination during non-target states (Fig. 4E). The photoillumination did not induce tissue damage after the experiments (Fig. 4B). Inhibition of MCH neurons during REM sleep significantly improved memory; however, inhibition during wakefulness or NREM sleep had no such effect (Fig. 4F). These results strongly suggested that REM-active MCH neurons induced memory impairment. The EEG spectrum and time spent in a vigilance state did not differ between wake-state and REM sleep-state inhibition (Fig. 4G and S13). Because MCH neurons play a role in NREM-REM sleep transitions, we observed a reduction of REM sleep time by NREM sleep-state inhibition in ArchT-EGFP mice but not in EGFP-expressing control mice (Fig. S13 and Table S4). Photoinhibition of yoked control mice confirmed that neither photoillumination itself nor the duration of photoinhibition affected memory (Fig. 4F and Fig. S14).

We found that REM-active MCH neurons are involved in forgetting hippocampus-dependent memories. MCH plays a role in food intake and seeking behaviors (19, 22, 23), suggesting that wake-active MCH neurons might be associated with these behaviors. While MCH

neurons release a variety of neurotransmitters (24, 25), the neurotransmitters involved in memory impairment are currently unknown. Cocaine- and amphetamine-regulated transcript (CART) might be expressed in REM-active MCH neurons because only MCH neurons that colocalize with CART project to the hippocampus and only CART-containing MCH neurons are active during REM sleep (24, 26). Glutamate may be involved in hippocampal inhibition, as MCH neurons release glutamate to form feed-forward inhibition via GABA interneurons in the lateral septum (25). Interestingly, the metabotropic glutamate receptor is involved in active forgetting during sleep (6).

The role of REM sleep in memory regulation remains controversial. GABAergic neurons in the medial septum are involved in theta rhythm generation in the hippocampus and have a role in consolidating contextual memory during REM sleep (27). Conversely, several reports have supported a role for REM sleep in forgetting, whereby REM sleep selectively eliminates synapses and inhibits firing in hippocampal CA1 and cortical pyramidal neurons via GABA interneuron activity, suggesting negative memory regulation (4, 28). REM sleep deprivation studies in humans have resulted in differing conclusions (29–31). Thus, it is possible that both neural mechanisms of consolidation and forgetting occur during REM sleep, and this coexistence could complicate studies. Nevertheless, among the various aspects of memory regulation during REM sleep, we show here that MCH neurons have a role in memory impairment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Effect of MCH neurons on memory

(A and B) Retrobeads were bilaterally injected into the CA1 of the hippocampus. Retrobead-positive neurons in six brain areas (A) and the hypothalamus (B). (C) MCH and orexin nerve terminals in the hippocampus. (D-F) Activation of MCH neurons using chemogenetics. (D) Histochemical confirmation of expression and function of hM3Dq-mCherry. (E and F) Activation of MCH neurons using chemogenetics. (E) NOR test (1.5 hr retention). (F) CFC test (strong foot shock). (G-I) Inhibition of MCH neurons using chemogenetics. (G) Histochemical confirmation. (H) NOR test (3 hr retention). (I) CFC test (weak foot shock). (J-N) Experiments using MCH neuron-ablated mice. (J) Protocol for ablation and histochemical confirmation of MCH neuron-specific ablation. (K) NOR test with different retention periods. (L) CFC test. (M) Cued fear test. (N) Protocol for Morris water maze (platform in quadrant A1) and performance over 7 d. Inset: trajectories (day 7). Bar graph shows results of probe tests; insets show the trajectories. Heat maps indicate time near the

objects (**E**, **H** and **K**). Data are mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$. Abbreviations: Hyp, hypothalamus; DB/MS, diagonal band/medial septum; LS, lateral septum; Amy, amygdala; DR, dorsal raphe nuclei; MR, median raphe nuclei; LC, locus coeruleus. See Table S1 for statistical analyses.

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Fig. 2. Optogenetic activation of MCH neurons

(A) Wireless stimulation using teleopto. (B) NOR test with optogenetic activation. (C) CFC test with optogenetic activation. (D-E) Current clamp recordings of hippocampal pyramidal neurons labelled with biocytin. (F-G) Bar graphs show membrane potential (F) and firing frequency (G) of hippocampal pyramidal neurons in response to blue or green light. (H) IPSCs after photoactivation of MCH nerve terminals. (I-J) Bar graphs show IPSC events and amplitude. (K) Teleopto stimulation of MCH nerve terminals in the hippocampus. (L) NOR test with optogenetic stimulation of MCH nerve terminals. (M) CFC test with optogenetic MCH nerve terminals stimulation. Heat maps indicate time near the objects (B and L). Data are mean $\pm$ S.E.M. * P <0.05, ** P <0.01. See Table S1 for statistical analyses.

Fig. 3. Activity of MCH neurons across vigilance states

(A) GCaMP6 expression in MCH neurons and fiber photometry with EEG and EMG recordings. Image showing exclusive expression of GCaMP6 in MCH neurons. (B) MCH neuron activity across vigilance states determined by fiber photometry. (C) GCaMP6 signals aligned to state transitions. Mean (green) and individual traces (gray). Transitions between states (bottom) summarized in (D). (E) Histochemical confirmation of GCaMP6f expression in MCH neurons. (F) Ca^{2+} activity from three types of MCH neurons. (G) Number of Ca^{2+} transients across states for each MCH cell type. (H) Venn diagram summary of MCH cell types. (I) Microendoscopic image of MCH neuron distribution. (J) Color-coded activity of individual MCH cells. Dashed rectangles indicate fiber optics in (A) and GRIN lens in (E). Data are mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$. See Table S1 statistical analyses.

Fig. 4. State-dependent inhibition of MCH neurons disrupts memory

(A) MCH neuron-ablated (MCHN^{-/-}) and control mice (MCHN^{+/±}) were subjected to the NOR test with no-retention time and novelty-induced sleep deprivation. (B) Histochemical confirmation of ArchT-EGFP expression. Dashed rectangles: fiber scars. (C) Decision tree-based algorithm for real-time vigilance state determination based on EEG, EMG and locomotion. (D-E) Example and traces of state-dependent inhibition. Bar graphs indicate the cumulative percent of illumination coverage time for each vigilance state. (F) NOR test with state-dependent inhibition in ArchT-EGFP and control EGFP. (G) Effect of state-dependent inhibition on EEG spectra. Heat maps in A and F indicate time near the objects. Data are mean $\pm$ S.E.M. **P < 0.01. See Table S1 for statistical analyses.