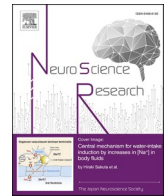




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Regulation of REM sleep in mice: The role of dopamine and serotonin function in the basolateral amygdala[☆]

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ABSTRACT

Animals have a sleep cycle that involves the repetitive occurrence of non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. In a previous study, we discovered that a transient increase in dopamine (DA) levels in the basolateral amygdala (BLA) during NREM sleep terminates NREM sleep and initiates REM sleep by acting on *Drd2*-positive neurons (Hasegawa et al., 2022). In this study, we identified the neurons activated by the transient increase of DA in the BLA and found that chemogenetic excitation of these neurons increased REM sleep. Additionally, we demonstrated that acute inhibition of serotonin (5HT) in the BLA elicited a transient increase in DA in the BLA, which triggered REM sleep.

1. Introduction

Sleep is composed of two main stages: NREM sleep and REM sleep. During REM sleep, the brain is more active compared to NREM sleep. Neuroimaging and intracranial recording studies in humans have indicated that the amygdala becomes activated during REM sleep (Corsi-Cabrera et al., 2016; Maquet, 1996). Narcoleptic patients exhibit increased amygdala activity during cataplexy, which is typically considered a pathological intrusion of REM sleep into wakefulness (Meletti et al., 2015).

In a previous study, we discovered that dopamine (DA) signaling in the basolateral amygdala (BLA) during NREM sleep triggers the transition to REM sleep by affecting dopamine D2 receptor (*Drd2*)-expressing neurons. Likewise, during wakefulness, this signaling triggers cataplexy (Hasegawa et al., 2022). Following manipulations aimed at inducing REM sleep, there was a rise in Fos expression in the cells surrounding *Drd2*-positive cells in the BLA, but not within the *Drd2*-positive cells themselves. To identify the activated neurons subsequent to the activation of DA fibers in the BLA, we utilized activity-dependent genetic labeling techniques, Targeted Recombination in Active Populations 2 (TRAP2) (Allen et al., 2017) and observed that the labeled neurons projected into the mesopontine-junction regions, which have been

implicated in REM sleep regulation (Hasegawa et al., 2022).

In this study, we investigated the impact of reactivating these stimulated neurons within the BLA on the sleep/wakefulness states of mice. Additionally, we explored the influence of serotonergic modulation on the role of DA function in the BLA. This inquiry stemmed from our prior research, which demonstrated the essential role of 5HT neurons in the dorsal raphe (DR) in suppressing cataplexy—an occurrence resembling REM sleep states that manifest during wakefulness (Hasegawa et al., 2014, 2017).

2. Materials and methods

2.1. Animals

All animal experiments were performed at the International Institute of Integrative Sleep Medicine (IIS), University of Tsukuba, according to its guidelines for animal experiments. They were approved by the animal experiment committee of each institute and thus were in accordance with NIH guidelines. Mice were given food and water ad libitum and maintained at T_A (Ambient Temperature) of 23 °C, relative humidity of 50%, with a 12-hr light/12-hr dark cycle. *Fos2A-iCreER* (TRAP2) (*Fosm2.1(icre/ERT2) Luo/J*, ID #030323, Jaxson Laboratory (Allen

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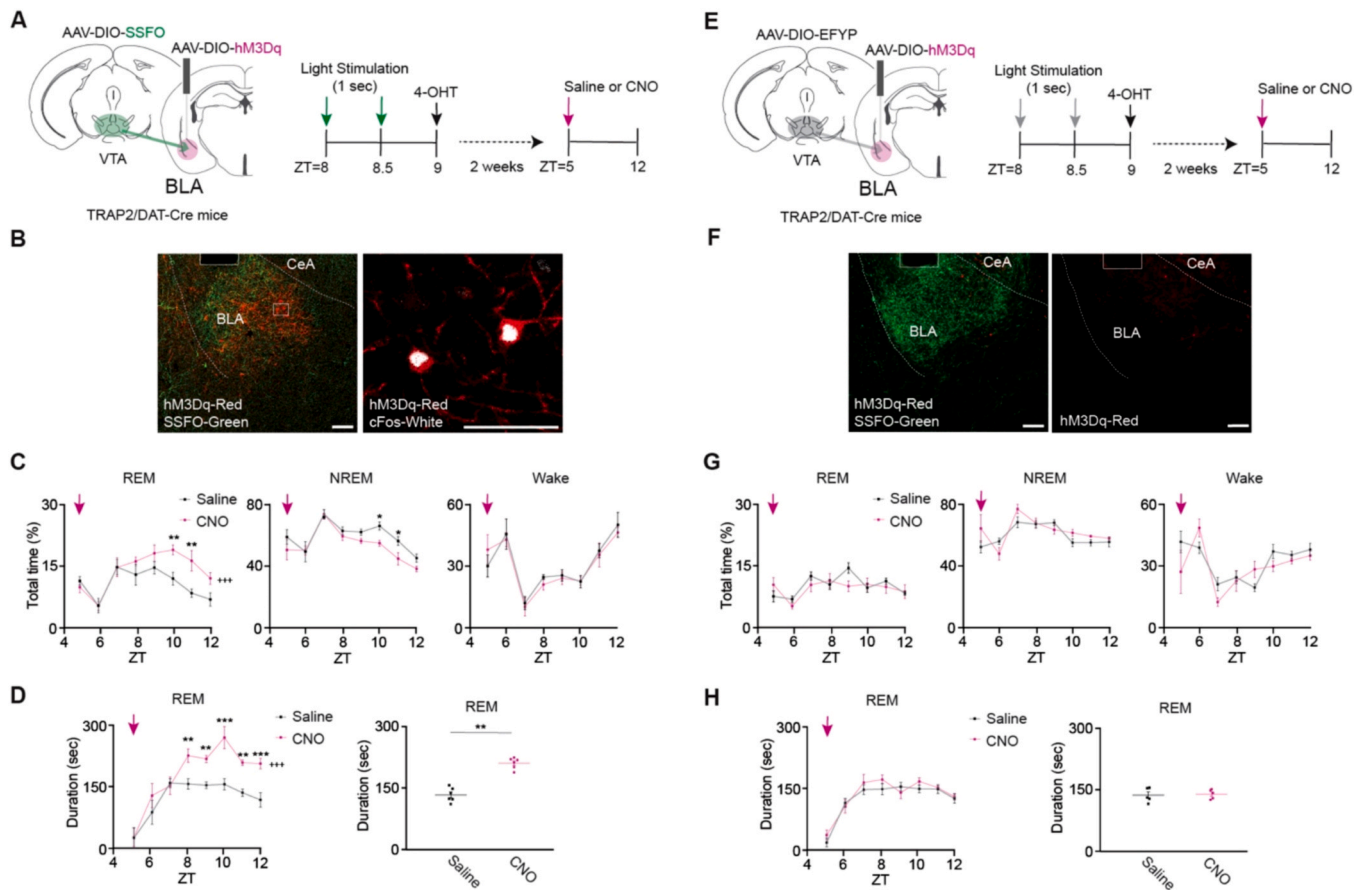


Fig. 1. Re-activation of BLA neurons activated by transient DA increases REM sleep. (A) and (E) The schematic drawings of AAV injection and optic fiber implantation in VTA and BLA. Right, experimental design. ZT, zeitgeber time. (B) and (F) DA fibers expressing hM3Dq+SSFO (left) and hM3Dq with cFos (right), perfusion was performed 90 min after administration of CNO) -positive neurons in the amygdala. Placements of optic fibers are shown. Scale bar= 100 μ m. (C) and (G) The hourly amount of each state. (D) and (H) The hourly amount and total duration of REM sleep after administration of saline or CNO in ZT 5–12. TRAP2; DAT-Cre mice expressing SSFO or EYFP in VTA and hM3Dq mCherry in BLA ($n = 6$, $n = 5$). Arrow shows administration of saline or CNO in ZT 5 (relative to Saline, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, paired t -test; $+++P < 0.001$, two-way repeated measures ANOVA).

et al., 2017.), *DAT-ires-Cre* mice (B6. SJL-Slc6a3tm1.1(cre)Bkmm/J; ID# 006660, Jaxson Laboratory (Bäckman et al., 2006) and *ePet1-Cre* (B6. Cg-Tg(Fev-cre)1Esd/J; ID# 012712, Jaxson Laboratory (Scott et al., 2005) were used. Mice were maintained under a strict 12-hour light/12-hour dark cycle in a temperature- and humidity-controlled room and fed ad libitum. *TRAP2; DAT-Cre* mice were generated by mating *DAT-ires-Cre* and *Fos2A-iCreER* mice.

2.2. Viruses

AAVs were produced using a triple-transfection, helper-free method as described previously (Hasegawa et al., 2014). The final purified viruses were stored at -80°C . Titers of recombinant AAV vectors were determined by quantitative PCR: *AAV10-EF1 α -DIO-SSFO-EYFP*, 1.35×10^{12} ; *AAV10-EF1 α -DIO-hM3Dq-mCherry*, 1.15×10^{11} ; *AAV10-EF1 α -DIO-vLWO-EGFP*, 2.17×10^{12} genome copies/ML.

2.3. Surgery

For injection of AAV vectors, male *TRAP2; DAT-Cre*, and *ePet1-Cre* (12–16 weeks old) mice were anesthetized with isoflurane (Pfizer) and placed in a stereotaxic frame (David Kopf Instruments). We controlled the rate of injection at 0.07 μ l per min using a Hamilton needle syringe (SYRINGE, NEUROS, 1701; needle 6PK, NEUROS, 33GA). The needle was kept in place for 5 min after injection.

For optogenetic and chemogenetic manipulation, we injected *AAV10-*

EF1 α -DIO-SSFO-EYFP into the ventral tegmental area (VTA) (anterior-posterior (AP), -3.30 mm; medial-lateral (ML), ± 0.3 mm; dorsal-ventral (DV), -4.50 and 4.20 mm from bregma; 0.50 μ l in each site) and *AAV10-EF1 α -DIO-hM3Dq-mCherry* into the BLA (AP, -1.80 mm; ML, ± 3.20 mm; DV, -4.50 mm from bregma; 0.50 μ l in each site), respectively. Optical fibers were then implanted bilaterally in the BLA (AP, -1.80 mm; ML, ± 3.20 mm; DV, -4.20 mm). For fiber-photometry and optogenetic manipulation, we injected *AAV10-hsyn-DA2m* unilaterally into the BLA (AP, -1.80 mm; ML, -3.20 mm; DV, -4.50 mm; 0.50 μ l) and *AAV10-EF1 α -DIO-vLWO-EGFP* into the DR (AP, -4.40 mm; ML, ± 0.00 mm; DV, -3.50 and 3.00 mm from bregma; 0.50 μ l). Optical fibers were then implanted unilaterally in the BLA (AP, -1.80 mm; ML, -3.20 mm; DV, -4.20 mm) and DR (AP, -4.40 mm; ML, ± 0.00 mm; DV, -3.00 mm). For optogenetic manipulation of 5HT neurons, we injected *AAV10-EF1 α -DIO-vLWO-EGFP* into the DR (AP, -4.40 mm; ML, ± 0.00 mm; DV, -3.50 and 3.00 mm from bregma; 0.50 μ l). Optical fibers were then implanted bilaterally in the BLA (AP, -1.80 mm; ML, ± 3.20 mm; DV, -4.20 mm) or VTA (AP, -3.30 mm; ML, -0.30 , $+1.0$ mm; DV, -3.00 mm).

For sleep recording, EEG/EMG electrodes were implanted after virus injection and optical fiber implantation. After a recovery period of at least 2 weeks in individual cages, mice were used for experiments.

2.4. Activity-dependent genetic labeling (TRAP2)

AAV10-EF1 α -DIO-SSFO-EYFP or *AAV10-EF1 α -DIO-EYFP* and *AAV10-*

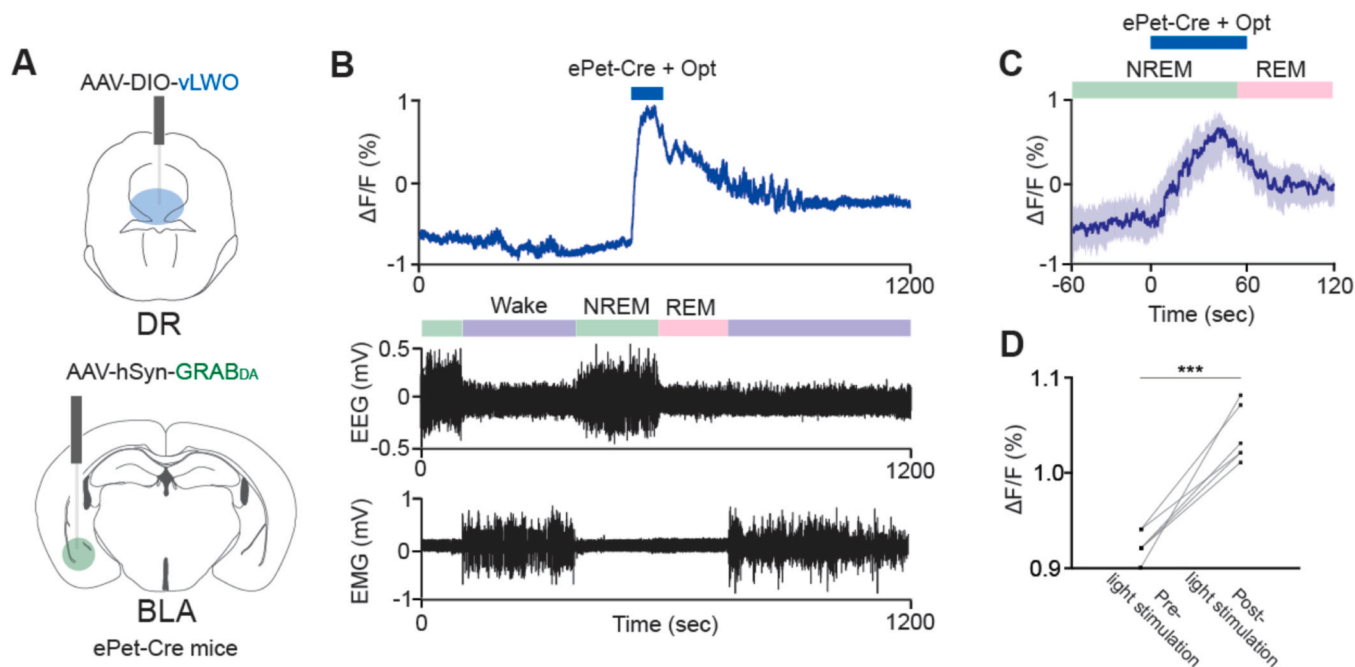


Fig. 2. Inhibition of DR-5HT neurons cause a transient increase in DA level in BLA and NREM-to-REM sleep transition. (A) Placement of optical fiber and GRAB_{DA} and vLWO expression in BLA and DR. (B) Top, the representative trace of DA level in BLA in *ePet1-Cre* mouse before and after optogenetic excitation. Blue bars show the time of light stimulation in the DR. Middle and Bottom, the waveform of EEG and EMG. Purple, green, and pink bars show wakefulness, NREM sleep, and REM sleep, respectively. (C) Temporal change in DA level of light stimulation (Average \pm SEM). (D) Time is relative to the transition of states. *ePet-Cre +Opt*; 3 mice, each recorded two times; $n = 6$ DA level in BLA before (Pre-light stimulation, -60 to 0 s) and after (Post-light stimulation, 0–60 s) light stimulation in DR-5HT neurons (relative to Pre-light stimulation, *** $P < 0.001$, paired t-test).

EF1 α -DIO-hm3Dq-mCherry were injected in the VTA and BLA, respectively in *TRAP2; DAT-Cre* mice. Optic fibers were implanted in the BLA. Two weeks after the virus injection, optogenetic excitation (1-s) was applied two times at ZT8 and ZT8.5. 30 min after the last optogenetic excitation, 4-hydroxy tamoxifen (4-OHT, #H9712, LKT Laboratories, 33 mg kg⁻¹) was injected intraperitoneally. Two weeks later, mice were subjected to CNO injection under sleep recording. After the experiment, mice were fixed and subjected to histological examination.

2.5. Biological signal recordings and sleep recordings

The protocols for biological signal and sleep recordings were described previously (Hasegawa et al., 2022). *AAV10-hsyn-DA2m* and *AAV10-EF1 α -DIO-vLWO-EGFP* were injected and a 200- μ m core, 0.50 NA optical fiber (Lymyth) was implanted unilaterally into the BLA and DR in *ePet1-Cre* mice. The mice were allowed to recover for at least 2 weeks before the experiments. On the day of the experiment, the mice were first recorded in their home cage while being fed regular chow for 1.5 h, starting at zeitgeber time (ZT) around 6–8. The time-resolved power spectrum of the EEG trace was recorded from the cortical surface, and EMG integral values were used to define states of wakefulness, NREM sleep, and REM sleep. All analyses were calculated using MATLAB.

2.6. Optogenetics

Optogenetic experiments were performed as described previously (Hasegawa et al., 2017). To activate DA neurons, *AAV10-EF1 α -DIO-SSFO-EYFP* or *AAV10-EF1 α -DIO-EYFP* was injected bilaterally into the VTA of *TRAP2; DAT-Cre* mice. Then 200- μ m core, 0.50 NA optical fibers (Lymyth) were bilaterally implanted in the BLA. Mice were allowed to recover for at least 2 weeks prior to experiments. On the test day, DA axon terminals were activated by laser (1-s, 462 nm, 8–10 mW, Shanghai Laser). To examine Fos expression, animals were killed 90 min after laser application.

To inhibit 5HT neurons, *AAV10-EF1 α -DIO-vLWO-EGFP* was injected in the DR in *ePet1-Cre* mice. Then 200- μ m core, 0.50 NA optical fibers (Lymyth) were unilaterally or bilaterally implanted in the DR, BLA, or VTA. 5HT-DR soma or their axon terminals in the DR, BLA, or VTA were inhibited by laser (1 Hz for 1 min, 462 nm, 5–8 mW, Shanghai Laser). The first laser stimulation was applied during stable NREM sleep. Then light stimulation was applied every 30 min during ZT5 to ZT7. To examine Fos expression in the DR, BLA, or VTA, animals were killed 90 min after light application.

2.7. Sleep recordings

These protocols of sleep recording were described previously (Hasegawa et al., 2014). EEG/EMG recordings were performed for 12 consecutive hours in the light phase. EEG/EMG data were analyzed as previously described and EEG/EMG signals were scored in wakefulness, NREM sleep, and REM sleep. All analyses were calculated by Sleep Sign (KISSEI COMTEC), followed by manual corrections through visual inspections for accuracy.

2.8. Immunohistochemistry

Double immunostaining was performed as described previously (Hasegawa et al., 2014). Serial coronal brain sections (30 μ m thick) were collected in 4 series, one of which was further immunostained. The primary antibodies used in this study were: rat anti-GFP (1:1000, 04404–84, Nacalai Tesque); goat anti-mCherry (1:1000, AB0040–200, Sicgen); rabbit anti-cFos (1:3000, ABE457, Millipore). The secondary antibodies were: Alexa Fluor 488 donkey anti-rat, 594 donkey anti-goat, and 647 donkey anti-rabbit (1:1000; Thermo Fisher). Sections were washed with PBS and coverslipped with FluorSave Reagent (Millipore). Brain sections were observed using a TCS SP8 laser confocal microscope (Leica).

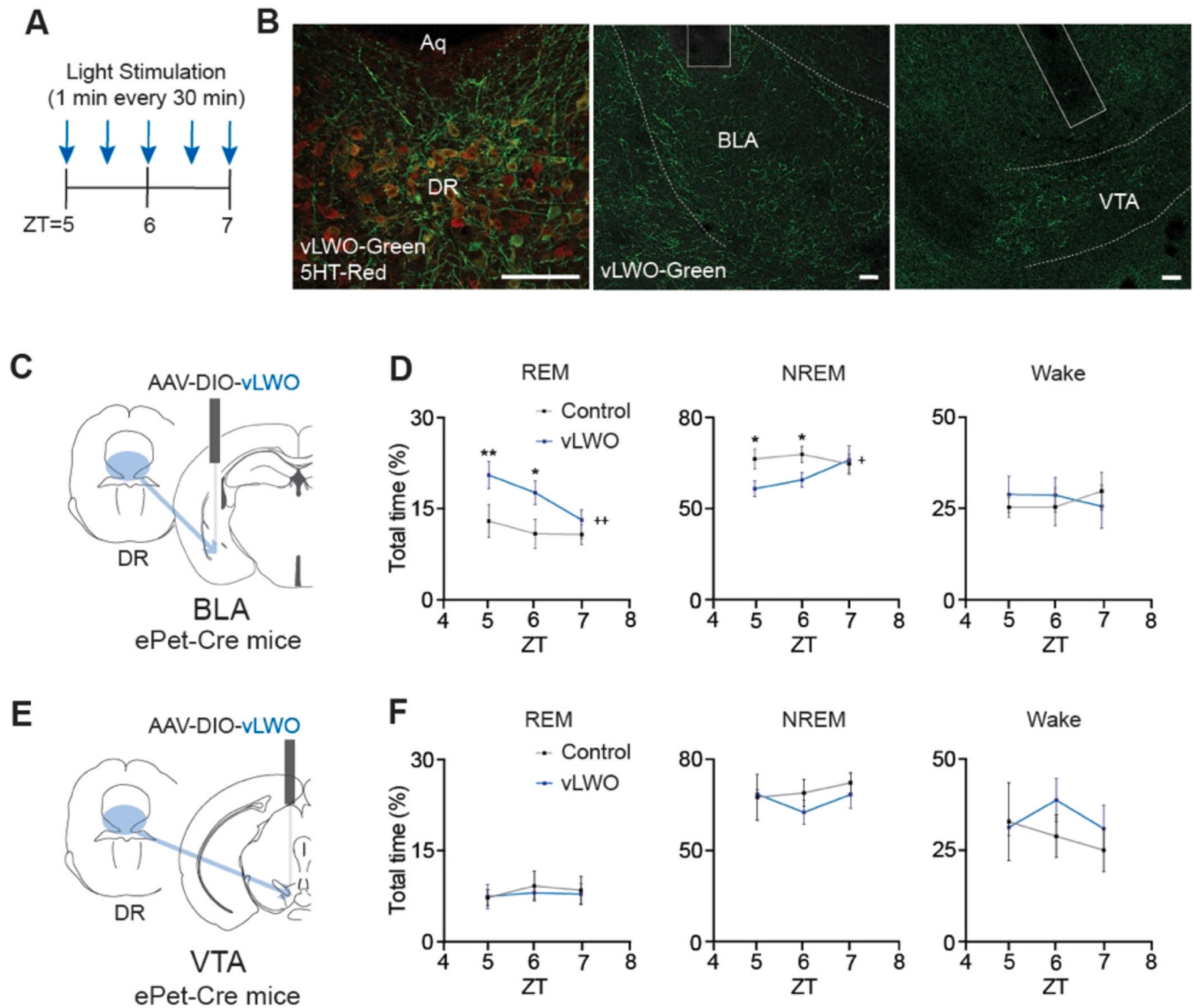


Fig. 3. Optogenetic inhibition of 5HT DR fibers in BLA during NREM sleep increases REM sleep. (A) Experimental design. ZT, zeitgeber time. (B) Coronal brain section stained with anti-GFP (green) and anti-5HT (red) antibodies. The white line marks the position of optical fibers. Scale bar, 100 μ m. (C) and (E) Schematic drawings of fiber placement and AAV injection in *ePet1-Cre* mice. (D) and (F) Total amount of REM sleep (REM), NREM sleep (NREM), and wakefulness (Wake) in ZT5–7. Control is mice connected to optical fiber without light stimulation (relative to control, * $P < 0.05$ and ** $P < 0.01$, paired t-test; + $P < 0.05$ and ++ $P < 0.01$, two-way repeated measures ANOVA).

2.9. Statistical analysis

All results are expressed as mean \pm SEM. Differences between individuals were analyzed by Paired t-test and two-way repeated measures ANOVA.

3. Result

To label the activated neurons in the amygdala following optogenetic activation of DA fibers in the BLA, we generated *TRAP2:DAT-Cre* mice by crossing *DAT-ires-Cre* and *Fos2A-iCreER* mice (DeNardo et al., 2019). We expressed the stabilized step function opsin (SSFO), a bistable excitatory channelrhodopsin variant or EYFP (Yizhar et al., 2011), in the VTA in *TRAP2:DAT-Cre* mice using a Cre-dependent AAV vector (Fig. 1A-B and E-F). We also injected a Cre-dependent AAV vector carrying hM3Dq in the BLA. Then, we bilaterally implanted optic fibers in the BLA for optogenetic stimulation by applying a light pulse (1-s width), as we previously discovered that exciting DA fibers in the BLA during NREM

sleep caused a transition to REM sleep (Hasegawa et al., 2022). 30 min after optogenetic stimulation, mice were intraperitoneally administered 4-OHT to activate CreER and express hM3Dq. We examined the effect of intraperitoneal injection of CNO on sleep/wakefulness states and observed a robust increase in REM sleep time and duration accompanied by a decreased NREM sleep time (Fig. 1C-D and Table S1).

To clarify the cell populations responsible for regulating REM sleep, namely the cFos-expressing cells activated following the stimulation of dopaminergic terminals in the basolateral amygdala (BLA), we utilized *TRAP2:DAT-Cre* mice as the control. These mice lacked SSFO in the ventral tegmental area (VTA) but were equipped with EYFP in the VTA (Fig. 1E-F). We conducted an investigation into the effects of intraperitoneal administration of CNO on sleep/wakefulness states and observed no noticeable effects (Fig. 1G-H and Table S1).

Next, we aimed to investigate the involvement of 5HT in the mechanism that triggers transitions from NREM sleep to REM sleep when DA peaks occur in the BLA. This is based on our previous discovery that 5HT neurons in the dorsal raphe (DR) play a crucial role in inhibiting

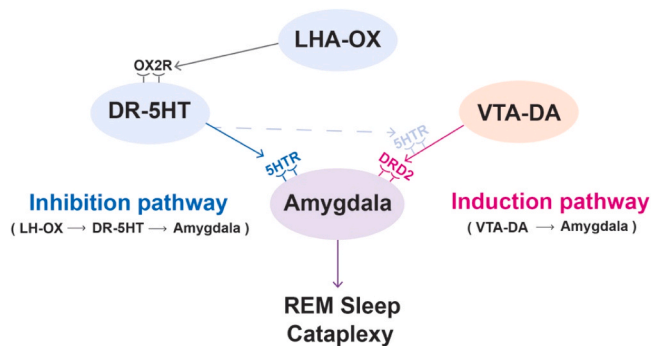


Fig. 4. The neural pathways of REM sleep gating involving the amygdala. Schema summarizing neuronal pathway involving the amygdala in the regulation of REM sleep/cataplexy. In the transition of NREM sleep to REM sleep, DA levels in the BLA transiently increase and inhibit DRD2-neurons to disinhibit amygdala neurons that send innervations to the brainstem of regulatory REM sleep. In narcolepsy patients, cataplexy attacks are triggered when DA level in the BLA is increased by positive emotions because the inhibition of DA release during wakefulness is weak in the loss of the orexin system. Orexin activates 5HT neurons in the DR to increase 5HT levels in the amygdala for the suppression of cataplexy. In healthy persons, 5HT levels were maintained high due to the activation of the orexin system during wakefulness. Orexin neurons in the lateral hypothalamus (LHA-ORX), dopamine neurons in the ventral tegmental area (VTA-DA), serotonin neurons in the dorsal raphe (DR-5HT), orexin 2 receptors (OX2R), dopamine 2 receptors (DRD2) and serotonin receptor (5HTR).

cataplexy (Hasegawa et al., 2014, 2017), which is a pathological intrusion of REM sleep-like state during wakefulness. Additionally, we observed that 5HT levels in the BLA began to decrease just before the transition from NREM sleep to REM sleep (Hasegawa et al., 2022). These findings led us to hypothesize that the decrease in 5HT levels in the BLA triggers DA peaks in the BLA, initiating REM sleep. To test this hypothesis, we examined whether a transient decrease in 5HT affected DA levels in the BLA. We expressed low wave-length opsin (vLWO), which couples to the Gi subclass of G proteins, and transiently induces hyperpolarization by light (Massek et al., 2014), in the 5HT neurons in the DR using a Cre-dependent AAV vector injected in *ePet1-Cre* mice. Simultaneously, we expressed a fluorescent G-protein-coupled receptor-activation-based sensor for DA (GRAB_{DA}) (Sun et al., 2018) in the BLA by injecting an AAV vector carrying GRAB_{DA} and implanted optic fibers in the BLA to monitor fluorescence to examine the DA levels in the BLA (Fig. 2A). After applying the laser for 1 min during NREM sleep, the mice exhibited a transient increase in DA levels in the BLA that closely resembled the pattern observed during the transition from NREM sleep to REM sleep (Fig. 2B). As a result, the mice transitioned to REM sleep. Light stimulation during NREM sleep in DR-5HT neurons increased DA levels in BLA and transitions to REM sleep (Fig. 2C-D).

To investigate further, we implanted optic fibers bilaterally in the BLA of *ePet1-Cre* mice expressing vLWO in the 5HT neurons in the DR and optogenetically inhibited 5HT fibers in the BLA while monitoring sleep/wakefulness states (Fig. 3A-C). We applied a light pulse every 30 min, starting from ZT5 to ZT7, in the BLA. We observed that this manipulation resulted in an increase in the amount of REM sleep and a decrease in NREM sleep (Fig. 3D and Table S2). Notably, inhibiting 5HT fibers from the DR in the VTA did not have any effect on REM sleep (Fig. 3E-F and Table S2).

4. Discussion

The amygdala is commonly referred to as the emotional center of the brain and is known to be involved in various behaviors such as motivation, defense, and fear. While the amygdala is activated during REM sleep, the regulatory mechanism and function of the amygdala during REM sleep remained unclear. Our recent study suggested that a transient

increase in DA levels in the BLA during NREM sleep terminates NREM sleep and initiates REM sleep (Hasegawa et al., 2022). Furthermore, *Drd2*-positive neurons in the BLA play a crucial role in this process by disinhibiting BLA neurons. Inhibitory manipulation of these neurons in the BLA caused long-lasting hyperpolarization, leading to an increase in Fos-positive neurons in the BLA and central nucleus of the amygdala. This ultimately results in an increase in REM sleep.

In our present study, we examined the function of activated neurons following the transient increase in DA levels in the BLA. We utilized the TRAP2 method to capture these activated neurons. Our findings demonstrate that the re-activation of these neurons, after optogenetically inducing a DA peak in the BLA, resulted in an increase in REM sleep (Fig. 1). This strongly supports our hypothesis that transient DA peaks in the BLA during REM sleep activate amygdala neurons through inhibition of *Drd2*-positive inhibitory neurons, leading to the induction of REM sleep.

Our previous study revealed that the activated neurons in the amygdala send projections to regions in the brainstem that regulate REM sleep (Hasegawa et al., 2020). Re-activation of these cells increased the amount of REM sleep increased by 6 h after the administration of CNO (Fig. 1). It is possible that CNO requires time to activate the amygdala cells responsible for initiating REM sleep. This activation then signals the brainstem regions that control REM sleep, ultimately adjusting the various states of REM sleep within the brain. This process could potentially be time-consuming, and its duration might be influenced by the condition of the brain (Sakurai and Hasegawa, 2023). These factors could potentially explain the reason behind the extended period required for CNO administration to elevate REM sleep.

Cataplexy, which is one of the symptoms of narcolepsy, is a pathological intrusion of REM sleep into wakefulness. In our earlier study, we discovered that a dopamine (DA) peak occurs before cataplexy in narcoleptic mice. Furthermore, we demonstrated that cataplexy can be triggered by replicating DA dynamics in the BLA while the mice are awake, using optogenetic or chemogenomic techniques. This implies that cataplexy attacks are initiated through the same mechanism that regulates REM sleep by means of DA in the BLA. Additionally, we have previously demonstrated that the activity of 5HT neurons in the DR, which are activated by orexin, is involved in suppressing cataplexy (Hasegawa et al., 2014, 2017). This suggested a possibility that 5HT inhibits the release of DA in the BLA through 5HT-mediated inhibition of DA axonal terminals. We examined whether the DA signal involved in the transition from NREM sleep to REM sleep was influenced by 5HT. Our findings showed that the transient inhibition of DR^{5HT} neurons by optogenetics manipulation resulted in a transient increase in DA levels in the BLA (Fig. 2) accompanied by an increase in the amount of REM sleep (Fig. 3D). We need to consider the potential impact of nonspecific factors, such as a local temperature increase resulting from optical stimulation, on sleep states. However, our previous study (Hasegawa et al., 2022) might actually contradict this possibility. In that study, we introduced the expression of EYFP in the VTA of *DAT-Cre* mice using a Cre-dependent AAV vector. Afterward, we administered optogenetic stimulation bilaterally in the BLA via a 5-minute light pulse. We observed that both the group exposed to light stimulation and the group without light stimulation showed minimal effects on REM sleep.

This present work further deepens our understanding of how the amygdala plays a role in regulating REM sleep, and how DA and 5HT are involved in this process. Our results suggest a possibility that a decrease in 5HT signals during NREM sleep is one of the mechanism that generates transient DA peak in the BLA triggering REM sleep (Fig. 4). As the activity of 5HT neurons is strongly influenced by orexin, a potent REM-inhibitory factor, this study also highlights the importance of the orexin-DR^{5HT} pathway in the REM regulation.

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Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.neures.2023.09.003](https://doi.org/10.1016/j.neures.2023.09.003).

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