Motor Memory Consolidation Depends on the Post-Learning Activity of Ventral Tegmental Area Dopaminergic Neurons

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Abstract

Motor memory consolidation is ubiquitous in our daily lives, yet the underlying mechanisms remain largely elusive. It is well-established that sleep benefits memory consolidation, including motor memories. While the activity of dopaminergic neurons in the ventral tegmental area (VTA^{DA}) has been implicated in motor learning, its role in motor memory consolidation during sleep has not been clearly determined. My thesis research aims to bridge this knowledge gap. Initially, I developed a modified balance-beam task for mice to assess motor learning. I found that mice demonstrate overall improvement in motor performance across training days, with the most significant improvement occurring between the first and second days. To determine the necessity of VTA^{DA} signaling during sleep for motor memory consolidation, I chemogenetically inhibited VTA^{DA} neurons during the sleep phase following the first training day in hM4Di-expressing mice (n = 10 mice of both sexes). I found that the inhibition significantly reduced the rate of improvement in experimental mice compared to control mCherry-expressing mice (n = 9 mice). To discern whether this suppression of performance was due to impaired consolidation or stemmed from a reduced motivation to complete the task or diminished motor performance, I inhibited VTA^{DA} neurons in 'expert' mice (n = 12 mice). Notably, once motor skills were fully acquired, inhibiting VTA^{DA} neurons had no impact on motor performance. My findings strongly suggest that VTA^{DA} neurons play a crucial role in the processes of motor memory consolidation during sleep. My research is set to significantly advance the current understanding of motor memory consolidation, with the potential to inform clinical strategies aimed at improving motor function in various disabilities.

Keywords: motor memory consolidation, ventral tegmental area, dopamine, sleep, chemogenetics

Acknowledgments

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Motor Memory Consolidation Depends on the Post-Learning Activity of Ventral Tegmental Area Dopaminergic Neurons

What is Memory Consolidation?

Memory is a fascinating aspect of cognition that allows us to acquire, retain, and retrieve information. From recognizing your favorite dish from a single whiff to cramming flashcards the night before an exam, our memory is embedded in many of our thoughts and actions. Traditionally, memory-related processes have been attributed to a complex interplay between the neocortex and the hippocampus (Squire et al., 2015). However, it is now recognized that subcortical structures also participate in memory-related processes, though relatively little is known about their precise functions (Skelin et al., 2019).

Motor memory pertains to the acquisition, consolidation, and utilization of persistent and adaptable motor skills. Within the procedural memory paradigm, the consolidation stage follows motor learning and results in an increase in performance and resistance to interference from learning similar, new motor skills (Tallet et al., 2015). It should be noted that there is a difference between motor learning and motor memory consolidation. Motor learning is the active acquisition of new motor abilities, whereas motor memory consolidation is an offline process in which newly acquired motor memory is stabilized (Leech et al., 2022, Della-Maggiore, 2005). While motor memory is of paramount importance to a person's daily life, it remains an understudied subject. Unraveling the complexities of how the brain encodes and stores motor information can contribute to the development of effective strategies for motor skill acquisition, the rehabilitation of neurodegenerative disorders, and the optimization of performance in various domains (Aslan et al., 2021; Hadjiosif et al., 2023).

Motor memory consolidation depends on several brain regions, including the basal ganglia, prefrontal cortex, primary motor cortex (M1), and the ventral tegmental area (VTA) (Lee et al., 2020; Leemburg et al., 2018; Packard et al., 2002; Stefan et al., 2005). Nonetheless, the precise interconnections between these brain areas are not well understood. For the purpose of this thesis, the focus will be narrowed down to the VTA and its contribution to motor memory consolidation.

Sleep Facilitates Motor Memory Consolidation

Sleep is a complex neurophysiological and behavioral process that is divided into two states: rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep. Sleep has many beneficial properties, including the temporal compartmentalization of cellular processes, waste clearance, maturation and wiring of neural circuits, immune system restoration, and memory consolidation (Besedovsky et al., 2019; Girardeau & Lopes-Dos-Santos, 2021; Hartmann & Kempf, 2023; Xie et al., 2013).

Sleep has also been shown to play a critical role in motor memory consolidation (Smith & MacNeill, 1994). For example, sleep deprivation hampers improvement in complex motor tasks and fine motor performance in mice (Nagai et al., 2017). In addition, sleep (offline consolidation) is more effective than quiet wakefulness (online consolidation) in consolidating fine motor memory, which involves coordination between different organs (King et al., 2017, Sutapa et al., 2021). On the other hand, while gross motor memory–which involves the ability to perform single movements using large muscles–similarly improves with sleep, sleep deprivation does not appear to significantly affect gross motor performance (Christova et al., 2018; Nagai et al., 2017). Sleep regulates dopamine release in different brain regions, which can induce changes in dendritic spines and contribute to lasting neurobiological changes (Wu et al., 2024).

VTA^{DA} Involvement in Motor Memory Consolidation

Located within the midbrain, the VTA, which contains a major cluster of dopaminergic neurons as well as gamma-aminobutyric acid (GABA)ergic and glutaminergic neurons, plays a central role in reward processing, motivation, learning, and memory (Bouarab & Thompson, 2019; Cai & Tong, 2022). The VTA is a key component of the mesolimbic dopamine system and regulates behavioral processes by intricately connecting to various brain regions, including the nucleus accumbens and prefrontal cortex (Cai & Tong, 2022). Notably, dysregulation within the VTA has been implicated in various neuropsychiatric disorders, such as addiction and depression (Polter & Kauer, 2014). In addition to its affective functions, the mesolimbic dopamine system also enhances the formation of motor memory (Wood, 2021). VTA GABAergic neurons also affect motor acquisition and functions through their inhibitive properties on VTA^{DA} neurons (Bourdy et al., 2014), so regulating VTA^{DA} neurons should demonstrate the cumulative effect of both VTA^{DA} neurons and VTA GABAergic neurons.

When engaged in a motor task, individual VTA^{DA} neurons can simultaneously encode multiple variables, such as reward-predicting cues, movement kinematics, spatial position, and behavioral choices, suggesting a multiplexed encoding of reward and motor variables (Engelhard et al., 2019; Kremer et al., 2020). Moreover, VTA^{DA} neurons project to other brain areas, with the M1 being a notable projection target that also plays a crucial role in motor memory consolidation (Della-Maggiore, 2005). Through a lesion study, researchers have implicated VTA^{DA}-M1 projecting neurons in motor learning (Hosp et al., 2011). Additionally, exposure to new motor experiences results in motor learning-dependent activity changes in VTA^{DA} neurons and reorganization of circuit activity in the M1 (Ghanayim et al., 2023). However, it remains

unclear whether the activity of VTA^{DA} neurons is necessary for motor memory consolidation and whether this activity occurs during post-learning sleep or wakefulness.

VTA^{DA} Activity During Sleep

VTA^{DA} neuron activity has been shown to vary across arousal states as reported by Dahan et al. (2007) and Eban-Rothschild et al. (2016) using electrophysiological and calcium-dependent recordings, respectively. VTA^{DA} neurons exhibit the highest activity during motivated wakefulness and REM sleep, and the lowest activity during NREM sleep. Although VTA^{DA} neurons demonstrate lower activity during NREM sleep, they still show transient activation (Dahan et al., 2007; Eban-Rothschild et al., 2016). Preliminary data from our lab suggest that VTA^{DA} activity during NREM sleep is experience-dependent (Sulaman et al., n.d.). More specifically, salient experiences including motor learning, result in an increased VTA^{DA} activity during subsequent NREM sleep (Sulaman et al., n.d.). Nonetheless, whether this increased activity has a causal role in motor memory consolidation is undetermined.

My thesis study aims to uncover the function of VTA^{DA} neuron activity during sleep in motor memory consolidation. I hypothesize that the consolidation of motor memory is dependent upon the activity of VTA^{DA} neurons during sleep. To test this hypothesis, I set two specific goals: 1) to establish a motor learning task in which mice could demonstrate significant improvement over one day of learning, and 2) to perform a loss-of-function experiment to test the necessity of VTA^{DA} neurons for motor memory consolidation during sleep using chemogenetic manipulations and polysomnographic recordings.

Methods

Experimental Subjects

I used reproductively inexperienced wild-type (WT) C57BL/6J (The Jackson Laboratory, Stock #: 000664) and dopamine transporter (DAT)-IRES-Cre (The Jackson Laboratory, Stock #: 006660) mice of both sexes bred in-house. Mice were ~8 weeks old at the initiation of experiments. During experiments, mice were placed in individual Plexiglass chambers (28.6 × 39.4 cm and 19.3 cm high) mounted with video cameras (Arducam; 2MP OV2710) at constant temperatures (~21°C), humidity (~39%), and circadian cycle (12 hr light-dark cycle). Mice were given food and water ad libitum as well as nesting material. All experimental procedures were performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Michigan's Institutional Animal Care and Use Committee.

Viral Vectors

To chemogenetically inhibit VTA^{DA} neurons, I utilized the modified human M4 muscarinic (hM4Di) receptor. This receptor enables neuronal inhibition following the binding of the synthetic ligand, clozapine N-oxide (CNO) (Roth, 2016). To exclusively express hM4Di in dopaminergic neurons, I used a genetically modified mouse model expressing Cre-recombinase under the dopamine transporter promoter (DAT). I injected into the VTA region of these mice with Cre-dependent recombinant adeno-associated virus (AAVDJ-EF1a-DIO-mCherry control and AAVDJ-EF1a-DIO-hM4Di-mCherry experimental vector), packaged by the Gene Vector and Virus Core at Stanford University. As Cre-dependent recombination only occurs in neurons expressing Cre-recombinase, only DAT-positive neurons (i.e., dopaminergic neurons) are

capable of expressing the viral vectors. I employed a final viral concentration of 3.07E+12 vg/ml. Aliquots of virus were stored at -80°C before stereotaxic injection.

Surgery

All surgical procedures were performed under ketamine and xylazine anesthesia (100 and 10 mg kg^{-1}) along with lidocaine as a local anesthetic. During surgery mice were fixed in a stereotactic frame (David Kopf instruments). Body temperature was controlled using a heating pad. Carprofen (0.005 mL/kg, i.p.) was given for pain relief, after surgery and on the subsequent day.

I stereotaxically infused viral vectors (15 mice with AAVDJ-EF1a-DIO-hM4Di-mCherry and 10 mice with AAVDJ-EF1a-DIO-mCherry) to the VTA (at coordinates −3.20 mm posterior (AP) and 0.25 mm lateral (ML) of bregma, in a depth (DV) of -4.45 mm) of DAT-IRES-Cre mice using a microliter injection pump (UMP3T-1, World Precision Instruments, Ltd.). After injection, the injection needle was left in place for 15 min before being slowly retracted. Seven WT mice underwent a sham injection (the needle was lowered to the VTA but no virus was infused).

After injections, mice were implanted with two miniature screw EEG electrodes (frontal: AP: 1.50 mm, ML:1.50 mm; parietal: AP: -2.40 mm, ML: 2.80 mm) and two EMG wire electrodes previously soldered to a four-pin connector. The EMG electrodes were inserted between the neck musculature. The EEG-EMG device was affixed to the skull with dental acrylic (Revolution Formula; Kerr Corporation). I used surgical sutures to close the skin, and the mouse was kept in a warm environment until resuming normal activity. Sutures were removed within ten days post-surgery.

Polysomnographic Recordings and Analysis

EEG and EMG signals derived from the surgically implanted electrodes were amplified (Model 3500, A-M systems) and digitized at 512 Hz using sleep recording software (Vital Recorder, Kissei Comtec America). Using sleep analysis software (AccuSleep) I digitally filtered the signal (EEG: 0.3–25 Hz) and spectrally analyzed it by fast Fourier transformation.

Each EEG-EMG recording session lasted for 22 consecutive hours. I recorded data prior to the motor experiment (baseline data) and following learning. Baseline data was recorded at least one day before the motor experiment.

The polysomnographic data were annotated manually per 4-second epochs based on the EEG-EMG waveforms and EEG power spectra and corrected through cross-verification with video recordings. All scoring was conducted by one researcher and then double-checked by another to ensure consistency across recordings. Wakefulness was defined as desynchronized low-amplitude EEG and heightened tonic EMG activity with phasic bursts. NREM sleep was defined as synchronized, high-amplitude, low-frequency (0.5–4.5 Hz) EEG and substantially reduced EMG activity compared with wakefulness, with no phasic bursts. REM sleep was defined as having a pronounced theta rhythm (5–9 Hz) with no EMG activity.

Habituation and Experimental Preparation

Following recovery from surgery in standard cages for at least seven days, mice were moved to individual recording chambers for three days and finally habituated to flexible recording cables for at least 10 days. To habituate to the experimenter, mice were handled daily for five days. During this time the mice were also habituated to intraperitoneal injections (i.p.), which consisted of gentle touching of the skin at the approximate location of the injection site, followed by an i.p. injection (two days) and one i.p. injection of saline (0.1 mL) at least two days

before experimental dates (see Figure 2b). The cages of mice were changed not less than three days prior to the motor experiment to allow sufficient time for nest rebuilding.

Motor Task

Our modified balance-beam apparatus consists of two four-mm diameter metal rods placed four cm apart, extending over a total of 75 cm (see Figure 1a). The rods are elevated 23 cm off the ground to discourage mice from purposely jumping off the rods. One end of the rods is connected to a wall, while the other end leads to the mice's scent-retained old home cage, which has been kept in an airtight bag since its last use by the mouse. As a background setting, I used coroplast sheets to provide a uniform, white background for accurate mouse detection. Three cameras were used to record the experiments. Two cameras captured different halves of the modified balance-beam apparatus each, while the third camera recorded the entire apparatus. A 3D-printed stabilizer secured the rods, maintaining a consistent four cm distance between them after each trial. A measuring tape was used to ensure a consistent distance of 75 cm between the right cardboard sheet and the left cage between trials. Ethanol and dry paper towels are used to clean the apparatus between trials.

Prior to the initiation of the task, mice are placed in their old cages for three minutes. At the start of the task, mice are placed at the wall end of the rods. They complete the task by traversing from the wall end to their old home cage. If a mouse fails to traverse 60 cm or remains motionless for 10 minutes, it is removed from the apparatus, marking the trial as incomplete. Mice undergo four days of training. Those failing to complete the task within this period are excluded from further analysis.

Experiments began at least one month following surgery to ensure full recovery and ample viral expression. On each experimental day, mice acclimated to the behavioral room for

60 minutes. Experiments took place during the last hour of the dark phase (Zeitgeber Time, ZT 23). After each experiment, mice were returned to their housing room and home cage, reattached to their EEG-EMG cables, and left undisturbed until the next training or testing session. On a few non-test days, when certain mice exhibited signs of excessive stress, they were left untethered.

Drug Preparation and Injection

CNO (2.5 mg kg−1, Enzo Life Sciences, Inc.) was prepared in sterile 0.9% saline and stored in a dark compartment at room temperature until use (maximum 4 weeks in advance). CNO was administered i.p. following habituation to experimental handling and i.p. saline injection in the days prior to testing.

Chemogenetic Inhibition

To test my hypothesis**,** I conducted two separate experiments:

Experiment 1: To determine whether VTA^{DA} neuronal activity during post-learning sleep is necessary for motor memory consolidation, I chemogenetically inhibited VTA^{DA} neurons during the post-learning sleep phase (see Figure 2d). Following the first completed training day (Day 1) of the motor task, I administered either saline (as control) or CNO to hM4Di and mCherry-expressing mice twice during their sleep phase (ZT 0 and 3), ensuring inhibition in CNO-treated hM4Di mice for approximately six hours of sleep. The first injection was administered when the mouse began grooming in his nest, a pre-sleep behavior that immediately precedes sleep (Sotelo et al., 2023).

Experiment 2: To determine whether VTA^{DA} neuronal activity during sleep is necessary for motor skill performance, I chemogenetically inhibited VTA^{DA} neurons in ‘expert’ mice that had already learned the motor skill after 4-7 days of training (see Figure 3a). I administered CNO

to hM4Di and mCherry-expressing mice twice during their sleep phase (ZT 0 and 3) one day after the final training day (no task day). To ensure consistency between the two experiments, the mice in Experiment 2 also received two i.p. injections of saline (0.01 mL/kg) during the sleep phase on Day 1 of their motor task.

Motor Performance

Motor improvement on the balance-beam task is used as an indicator of motor memory consolidation. Using EthoVision software (Noldus), the mice's performance was qualitatively assessed by recording the completion time for the first 60 cm traveled in the horizontal dimension. Time was excluded when mice remained stationary or fell off the apparatus.

Histology

Histological verification of viral expression in the VTA was performed. hM4Di and mCherry-expressing mice were anesthetized in an isoflurane chamber and subsequently maintained under anesthesia with a constant supply of isoflurane. The mice were perfused transcardially with 1× PBS, followed by 4% paraformaldehyde in PBS. Brains were extracted, postfixed overnight at 4°C, and cryoprotected in 30% sucrose dissolved in 1× PBS containing 0.1% NaN3 for 48 hrs at 4°C. Brains were sectioned at 30 μm on a cryostat (Leica Microsystems), collected in PBS containing 0.1% NaN3 and stored at 4°C.

Microscopy and Image Analysis

Images were collected on a Zeiss Axio Imager.M2 microscope using ZEN software (Zeiss). Image analysis consists of determining the brain atlas location of the labeled cells (range of coordinates: e.g., AP -3.1 to -3.5) and using Adobe Photoshop to visually represent the locations of viral expression across mice. Analysis was performed by investigators blind to the viral transduction.

Statistical Analysis

I used Prism 10 (GraphPad software) for statistical analysis and visualization of data. Two-way repeated measures (RM) ANOVAs were used to compare the motor performance of mice across treatments (experimental and controls) and polysomnographic recordings of experimental mice across days. Additionally, post-hoc tests compared motor performance between the control and experimental mice. I excluded the data of five mice from analysis due to: (1) inability to build a proper nest over several days (n =1 mouse); (2) detachment of EEG-EMG implant from the skull (n =1 mouse); and (3) technical difficulties resulting in data loss (n = 3 mice).

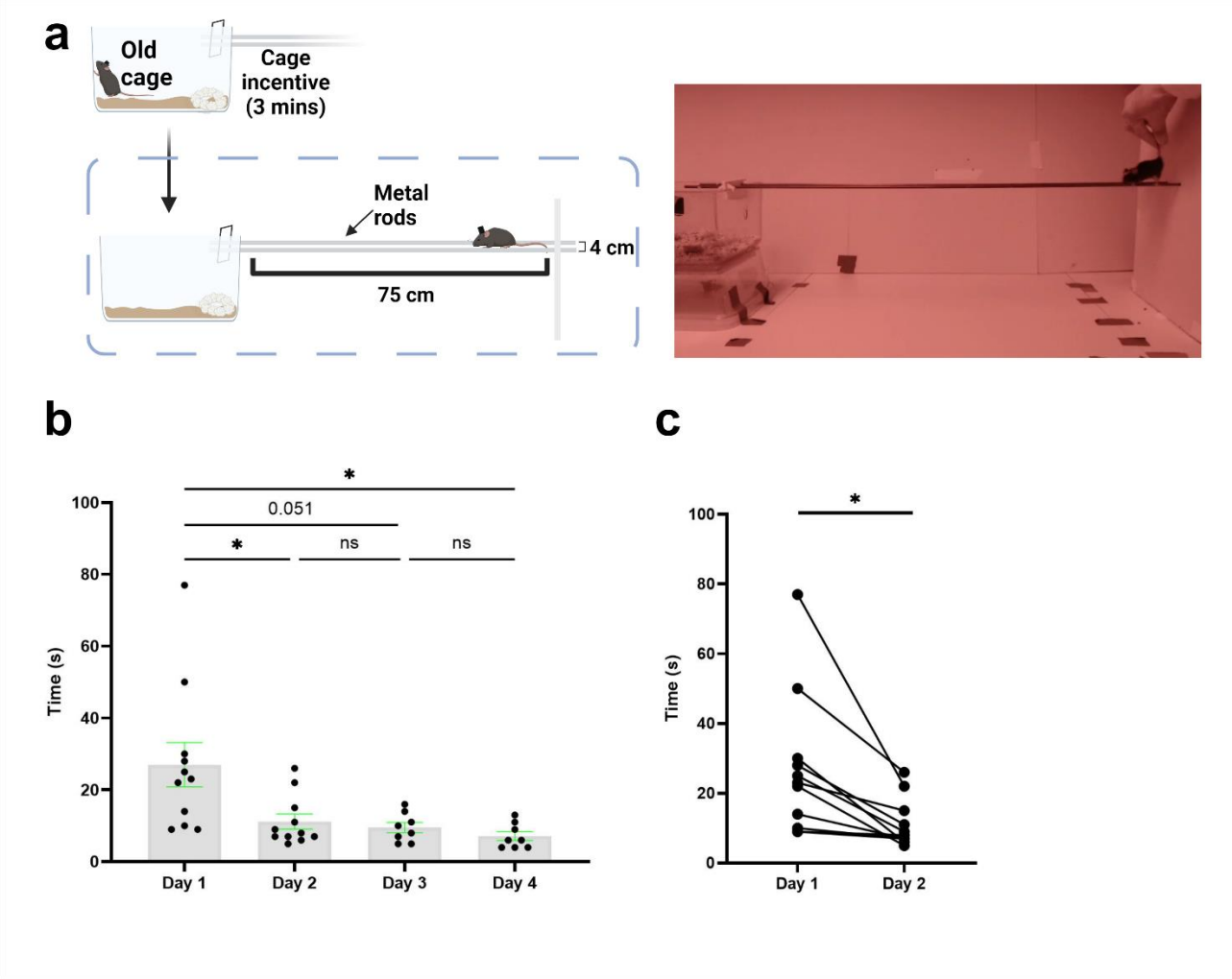
Results

Establishing a Modified Balance-Beam Motor Task

The balance-beam task evaluates motor balance and coordination in mice (Luong et al., 2011). Luong et al. (2011) employed a single, one-meter-long beam with the objective being for the mice to traverse the apparatus. Incentives for the mice included placing nesting material in a black box and gently prodding them when they paused in their movement. In my adapted balance-beam apparatus, I incorporate two shorter, circular rods as inspired by the apparatus of a different study (Carrillo et al., 2013). For incentive, I also used the old home cage for each mouse to motivate the mice to traverse the rod but refrained from prodding the mice to minimize stress, given its potential impact on sleep (Xu et al., 2023; see Figure 1a).

Figure 1

Experimental pipeline and pilot



(**a**) Schematic and picture of motor experiment procedures and picture of experiment setup. (**b**) Mice learn across a four-day training period with learning most prominent between Day 1 and Day 2. One-way RM ANOVA of completion time by pilot cohort with mean (gray shading) ± s.e.m. (green shading); start with n = 11; subjects decreased after Day 1; followed by Dunnett's post hoc test; *p < .05, **p < .01, ***p < .001. Decreases in completion time on Day 1 when compared to following days; Day 2 (*p = 0.018), Day 3 (*p = 0.033), Day 4 (**p = 0.008). Most pronounced completion time reduction in pilot cohort across consecutive days (df=10; **p =

0.003; mean dif. = 15.8; 95%CI = [5.4,26.2]) demonstrates the greatest motor improvement between Day 1 and Day 2. (**c**) Line graph demonstrates that all mice have improved between Day 1 and Day 2.

I assessed my preliminary data with pilot cohorts of mice to determine the validity of my experimental setup and to standardize my protocol. Each cohort consisted of three to six mice of both sexes. Pilot mice learned on the initial rendition of the modified balance-beam task, as the time for motor task completion decreased across the four training days. The greatest time difference for task completion occurred between Day 1 and Day 4 with a difference in mean of 19.9 ± 4.5 seconds (58%). When comparing motor performance between consecutive days, analysis revealed the most significant improvement between Day 1 and Day 2 as opposed to Day 2 and Day 3 (df = 7; p = 0.867; mean dif. = 1.7; 95%CI = [4.9,34.9]) or Day 3 and Day 4 (df = 7; p = 0.573; mean dif. = 2.4; 95%CI = [-3.5,8.3]). These results suggest that motor learning is most prominent between Day 1 and Day 2 and that substantial motor improvement occurs within four days of training on my modified balance-beam task (see Figure 1b). Additionally, all the mice in my pilot cohort have improved on the motor task between Day 1 and Day 2 again indicating that motor learning does occur between these days (see Figure 1c).

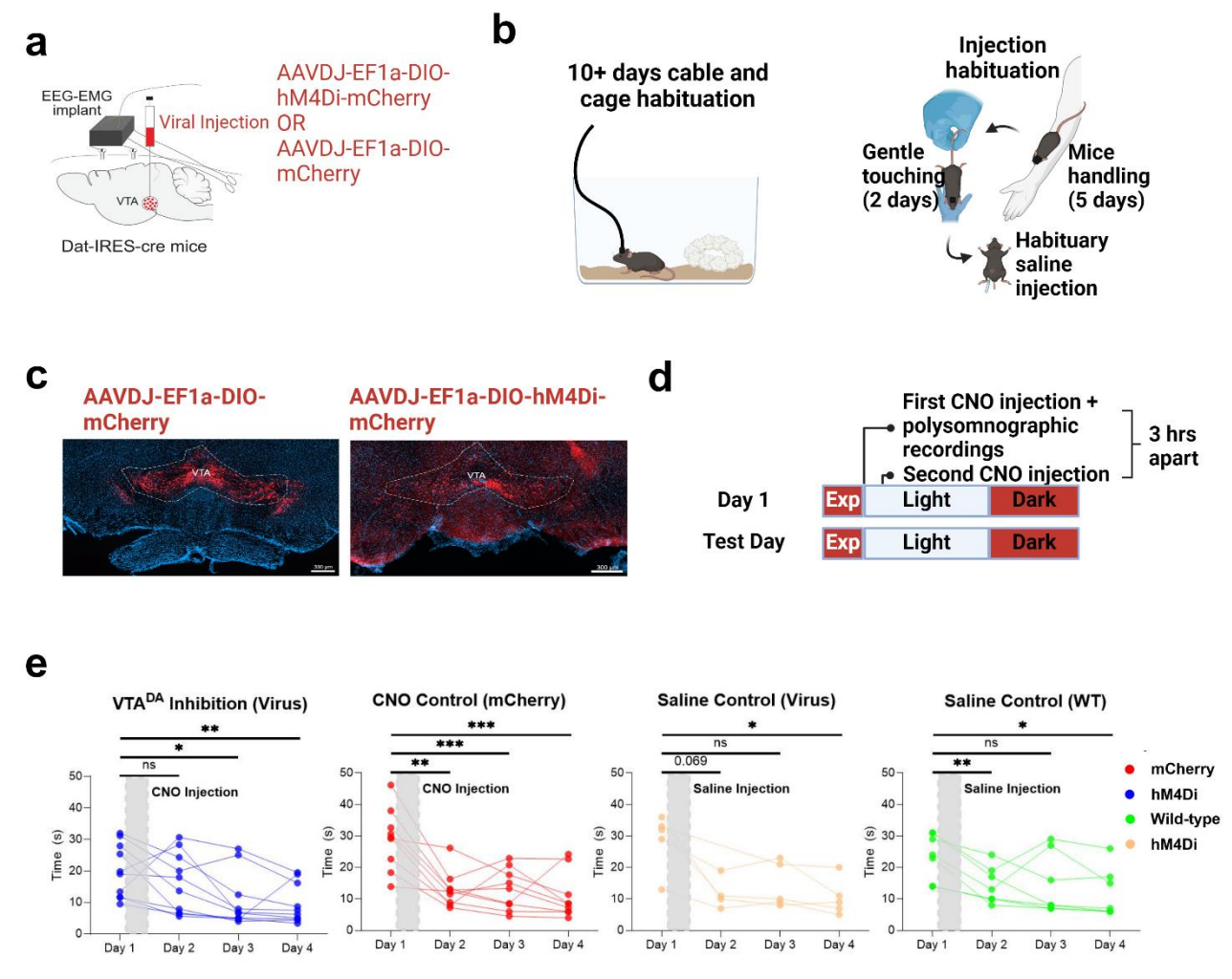
VTA^{DA} Neuronal Inhibition Suppresses Motor Memory Consolidation

To investigate the role of VTA^{DA} neurons in motor memory consolidation, I inhibited their activity during sleep after the first training day using chemogenetic tools (see Figure 2d). Histological analysis confirmed mCherry or hM4Di expression in mice (see Appendix A-F). As expected, in the mCherry-expressing and WT control mice, the time taken to complete the task on Day 2 was reduced by 16.1 ± 3.2 seconds and 9.3 ± 1.8 seconds, respectively, as compared to Day 1 (see Figure 2d). hM4Di-expressing mice administrated with saline also showed a

reduction in time to complete the task across Day 1 and Day 2, yet this reduction did not reach statistical significance ($p = 0.069$), potentially due to the low sample size of this group (n = 4 mice). In contrast, the experimental hM4Di-expressing mice showed no significant difference in task completion time between Day 1 and Day 2 following CNO treatment (see Figure 2d). Completion time reduction in mCherry-expressing control suggests that CNO alone does not inhibit motor performance. Additionally, the trend toward completion time reduction in the hM4Di-expressing control suggests that the viral vector alone does not inhibit motor performance, although further samples would be required to conclusively establish this observation. Notably, the experimental hM4Di- mice did show motor improvement on Day 3 and Day 4 by 9.7 ± 3.3 seconds and 10.7 ± 2.7 respectively, indicating that the mice are able to improve on a motor task when their VTA^{DA} neurons are not inhibited (see Figure 2e). Taken together, these results point toward the possibility that inhibiting VTA^{DA} neurons during sleep disrupts motor memory consolidation.

Figure 2

Activity in VTA^{DA} neurons during sleep is necessary for motor memory consolidation



(**a**) Schematic of viral injection and EEG implant. (**b**) Schematic of pre-experimental preparations and viral injection habituations. (**c**) Image on the left, mCherry expression in the VTA; image on the right, hM4Di expression in the VTA with co-expression of mCherry; scale bar = 300 μm. (**d**) Timeline of experiments with n = 31 mice (n = 9 mCherry; n = 7 WT; n = 15 hM4Di mice). Later days unrelated to experimental interests are left out. (**e**) Two-way RM ANOVA of completion time in mice followed by Dunnett's post-hoc tests; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Completion time reduction between Day 1 and Day 4 in respectively from

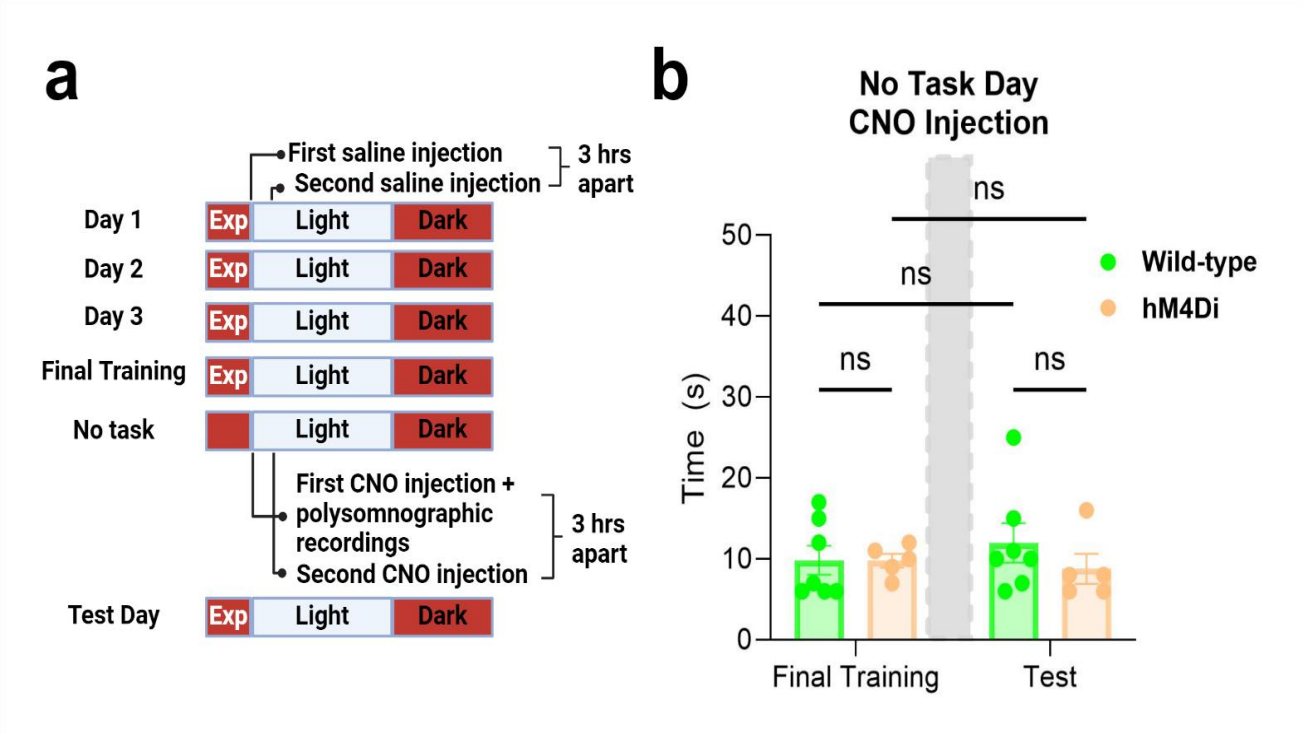
left to right: hM4Di-expressing VTA^{DA}-inhibited mice (n = 10; $^{**}p = 0.009$), mCherry-expressing CNO-control (n = 9; $^{***}p < 0.001$), hM4Di-expressing saline-control (n = 5; $^{*}p = 0.016$), and in WT saline-control (n = 7; $^{*}p = 0.010$), demonstrate overall motor improvement across the four-day training period. There was a lack of completion time reduction between Day 1 and Day 2 in hM4Di-expressing VTA^{DA}-inhibited mice (n = 10; $p = 0.523$), however, there was a completion time reduction in mCherry-expressing CNO-control mice (n = 9; $^{**}p = 0.003$) and in WT saline-control mice (n = 7; $^{**}p = 0.006$). While the completion time between Day 1 and Day 2 in hM4Di-expressing saline-control mice is also not significant, there is a strong trend toward a reduction in completion time (n = 4; $p = 0.069$).

VTA^{DA} Neuronal Inhibition Suppresses Motor Memory Consolidation

To discern whether this suppression of motor performance was due to impaired consolidation or stemmed from a reduced motivation to complete the task or diminished motor performance, I inhibited VTA^{DA} activity during sleep after mice had acquired the motor skills required for the motor task (see Figure 3a). I found that CNO-mediated inhibition of VTA^{DA} neurons during sleep in expert mice did not suppress motor performance (see Figure 3b). These findings strongly suggest that the decrease in motor performance following VTA^{DA} inhibition is directly related to the disruption in motor memory consolidation during sleep (see Figure 2e).

Figure 3

Activity in VTA^{DA} neurons during sleep is not necessary for motor performance or motivation to complete the task



(**a**) Full timeline of experiments with n = 12 mice (n = 7 WT mice; n = 5 hM4Di mice). (**b**) Two-way RM ANOVA of completion time in mice followed by Uncorrected Fisher's LSD post-hoc tests; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. No significant effect was observed between Final Training Day and Test Day in WT (n = 7) mice and hM4Di-expressing mice (n = 5) or between treatment groups demonstrating that VTA^{DA} neuron inhibition does not disrupt overall motor performance.

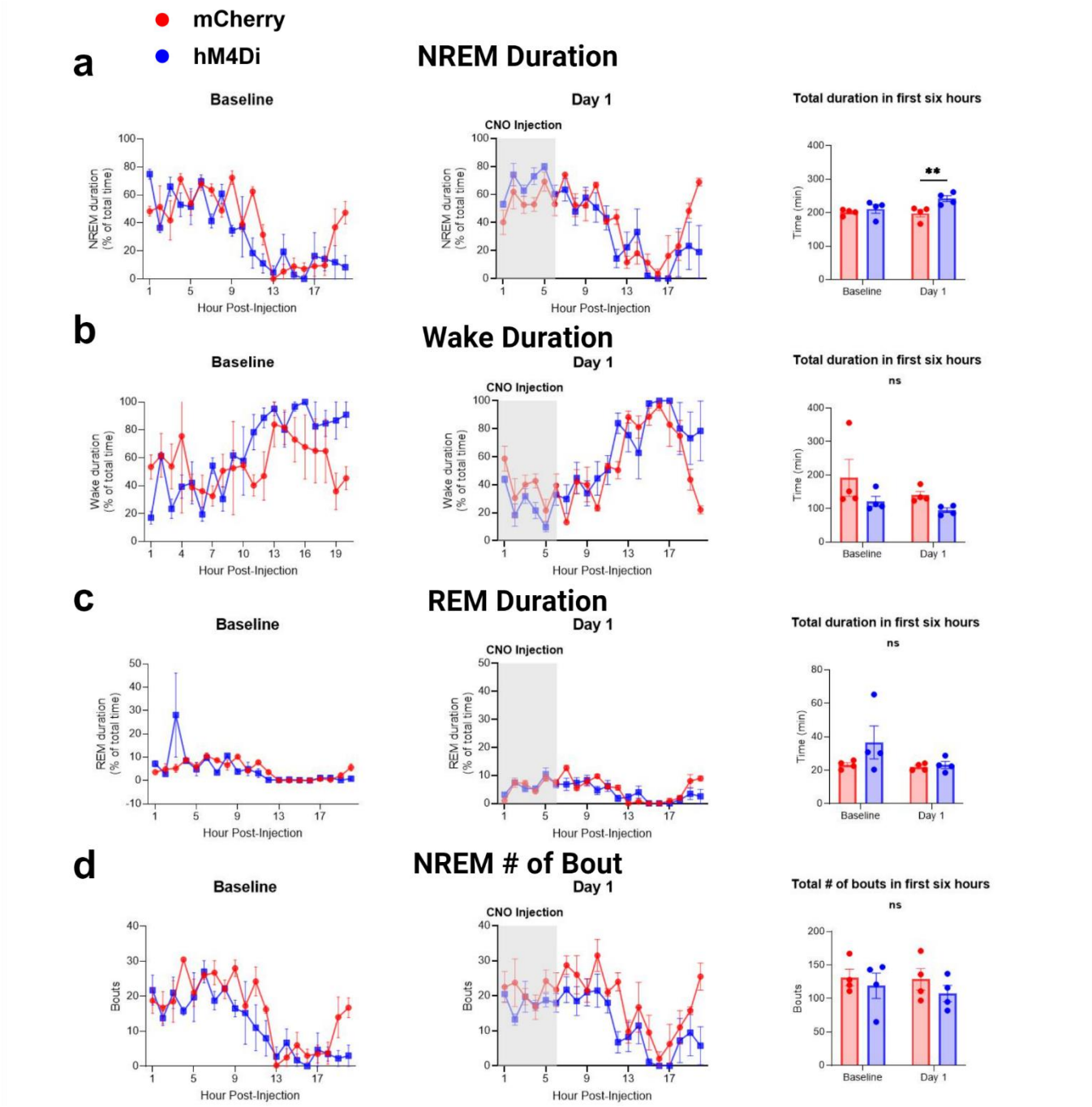
Effects of VTA^{DA} Neuronal Inhibition on Sleep

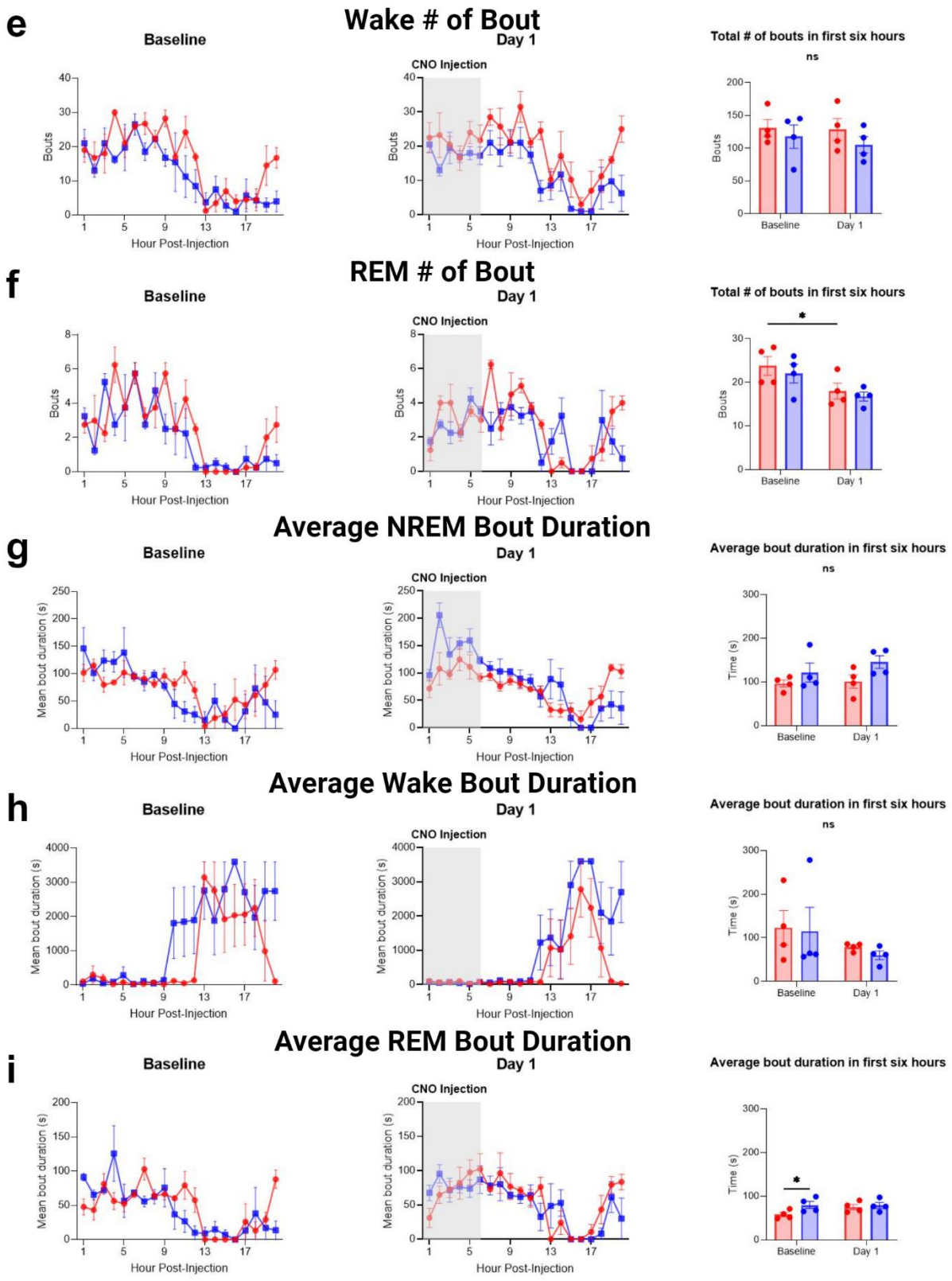
To investigate whether VTA^{DA} neuronal inhibition changes sleeping patterns, I compared the sleep/wake data of mCherry and hM4Di-expressing mice prior to and during the sleep phase

following motor learning. On Day 1, the total duration of NREM sleep increased for hM4Di mice during the first six hours of sleep (post-injection) compared to control mice, suggesting that VTA^{DA} neuronal inhibition increases the duration of NREM sleep (see Figure 4a), as previously reported (Eban-Rothschild et al., 2016). The number of REM sleep bouts decreased on Day 1 compared to baseline for mCherry-expressing mice (while a non-significant trend was observed in hM4DI mice) suggesting that a salient, motor experience increases REM sleep (see Figure 4f). The average REM bout duration between mCherry-expressing mice and hM4Di-expressing mice was significantly different on baseline suggesting variability amongst mice or that the viral vector affects average REM bout duration (see Figure 4i).

Figure 4

Sleep/wake architecture prior to and following learning in hM4Di and control mice




e
Wake # of Bout
Baseline
Day 1
CNO Injection
Bouts
Hour Post-Injection
Total # of bouts in first six hours
ns
Baseline
Day 1
f
REM # of Bout
Baseline
Day 1
CNO Injection
Bouts
Hour Post-Injection
Total # of bouts in first six hours
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Baseline
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Average NREM Bout Duration
Baseline
Day 1
CNO Injection
Mean bout duration (s)
Hour Post-Injection
Average bout duration in first six hours
ns
Time (s)
Baseline
Day 1
h
Average Wake Bout Duration
Baseline
Day 1
CNO Injection
Mean bout duration (s)
Hour Post-Injection
Average bout duration in first six hours
ns
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Day 1
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Average REM Bout Duration
Baseline
Day 1
CNO Injection
Mean bout duration (s)
Hour Post-Injection
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Baseline
Day 1

(**a-i**) Two-way RM ANOVA of polysomnographic data in mCherry-expressing control mice (n = 4) and hM4Di-expressing experimental mice (n = 4) between Baseline and Day 1 followed by Uncorrected Fisher's LSD post-hoc tests; *p < 0.05, **p < 0.01, ***p < 0.001. (**a**) After administering CNO on Day 1, hm4Di-expressing mice experienced longer NREM sleep during the first six hours of sleep (post-injection for Day 1) when compared to mCherry-expressing mice (**p = 0.008), (**b,c**) but no other difference was observed. (**f**) There is a decrease in bouts of REM sleep for mCherry-expressing mice between Baseline and Day 1 (*p = 0.048) during the first six hours of sleep, (**d,e**) but no other difference was observed. **(i**) There is a difference in baseline REM bout duration between mCherry-expressing and hM4iDi-expressing mice (*p = 0.035), (**g,h**) but no other difference was observed.

Discussion

To investigate the functions of VTA^{DA} neurons in motor memory consolidation during sleep, I have combined behavioral, chemogenetic, and polysomnographic approaches. I first established a modified motor task that allows mice to display learning-dependent motor improvement after one day of learning. I then used chemogenetic methods to specifically manipulate the activity of VTA^{DA} neurons during sleep. Lastly, I acquired polysomnographic data through EEG-EMG devices. My results indicate that motor improvement in mice is dependent on VTA^{DA} activity in the post-learning period, strongly supporting a causal role for VTA^{DA} neurons in motor memory consolidation during sleep. Furthermore, I discovered that overall motor performance, as well as the motivation to return to the home environment, is not dependent on the activity of VTA^{DA} neurons during a previous day's sleep. To the best of my knowledge, my study provides the first demonstration of the role that VTA^{DA} neurons play in motor memory consolidation during sleep.

It is widely known that information learned during wakefulness is reactivated in hippocampal-cortical circuits during sleep, leading to long-lasting memory traces (Rasch & Born, 2013). Recent research also suggests a similar mechanism in play for motor memory consolidation. During motor skill acquisition, VTA^{DA} neurons that project to the M1 are activated (Leemburg et al., 2018). Furthermore, sleep-dependent reactivation has been shown to consolidate motor memory (Fogel et al., 2017). Reactivation for motor memory occurs with spindle-oscillations, and the degree of reactivation is associated with the amount of next-day motor improvement (Ramanathan et al., 2015). This process reorganizes M1 layers 2–3 during motor learning to consolidate motor memory (Ghanayim et al., 2023). My findings suggest that reactivation in the M1 is dependent on VTA^{DA} neuron activity during sleep. While the Allen Brain Atlas does not suggest a dense innervation of the M1 by VTA^{DA} neurons in mice, other research identified functional VTA^{DA} projection in the M1 of mice (Ghanayim et al., 2023). Therefore, it is worth exploring the causal function of dopamine release in M1 neurons for reactivation mechanisms that may underlie motor memory consolidation.

The importance of sleep for motor memory consolidation is unquestionable. My results substantiate this notion by highlighting the importance of neural activity during sleep, particularly in VTA^{DA} neurons. However, the specific sleep stage that facilitates the motor memory consolidation process remains uncertain. The dual process hypothesis has suggested that different sleep stages serve to consolidate different types of memories (Ackermann & Rasch, 2014). For example, REM sleep has been suggested to facilitate the consolidation of non-declarative memories, while NREM sleep has been suggested to consolidate declarative memory (Rasch & Born, 2013). However, recent research in humans suggests that NREM sleep also helps facilitate motor memory consolation through sleep spindle activities during stage 2 of

NREM sleep (Boutin & Doyon, 2020). My own findings support this idea, as changes in NREM sleep duration due to CNO administration in hM4Di mice disrupted motor improvement. Future studies can employ an optogenetic approach by inhibiting VTA^{DA} neurons during NREM sleep to isolate to sleep stage and observe whether this intervention would lead to similar findings (Harris et al., 2022).

Strengths and Limitations

By refining my preliminary apparatus and analytical methods, my study developed an improved motor task and a standardized protocol. I have included all mice that performed in experiments throughout a two-year period and accumulated a substantial number of subjects. However, certain experiments still have a limited subject pool for specific analyses, with some analytical measurements based on as few as n = 4 subjects. Manual evaluation of the time to complete the task could introduce further variation to my data. However, I reanalyzed data for earlier cohorts to ensure greater consistency in quantifying the task completion time. In the future, I could use advanced deep learning and computer vision tools such as DeepLabCut and DAMM which are currently being employed in our lab. Additional measures of motor performance will also be beneficial, such as foot slips and/or body sliding. As mentioned previously, stress may be an unavoidable factor that could lead to behavioral variation within my cohorts. To address these challenges, I have minimized human interactions and standardized my protocols to mitigate inconsistencies in my data, but some concerns still stand.

Conclusion

By using a battery of state-of-the-art experimental techniques, I have revealed a novel function for dopaminergic neurons in motor memory consolidation during sleep. I have deepened the scientific understanding of the underlying mechanisms of motor memory consolidation and

set the stage for further innovation in rehabilitation and therapy for victims of neuromotor disorders such as Parkinson's Disease (Cristini et al., 2023). Moreover, subsequent research can help optimize motor skill acquisition in athletes or search for strategies for preserving motor-cognitive function in the elderly (Gudberg et al., 2015; Thomas et al., 2016). My research is the first step toward improving the lives of millions of individuals across diverse demographics.

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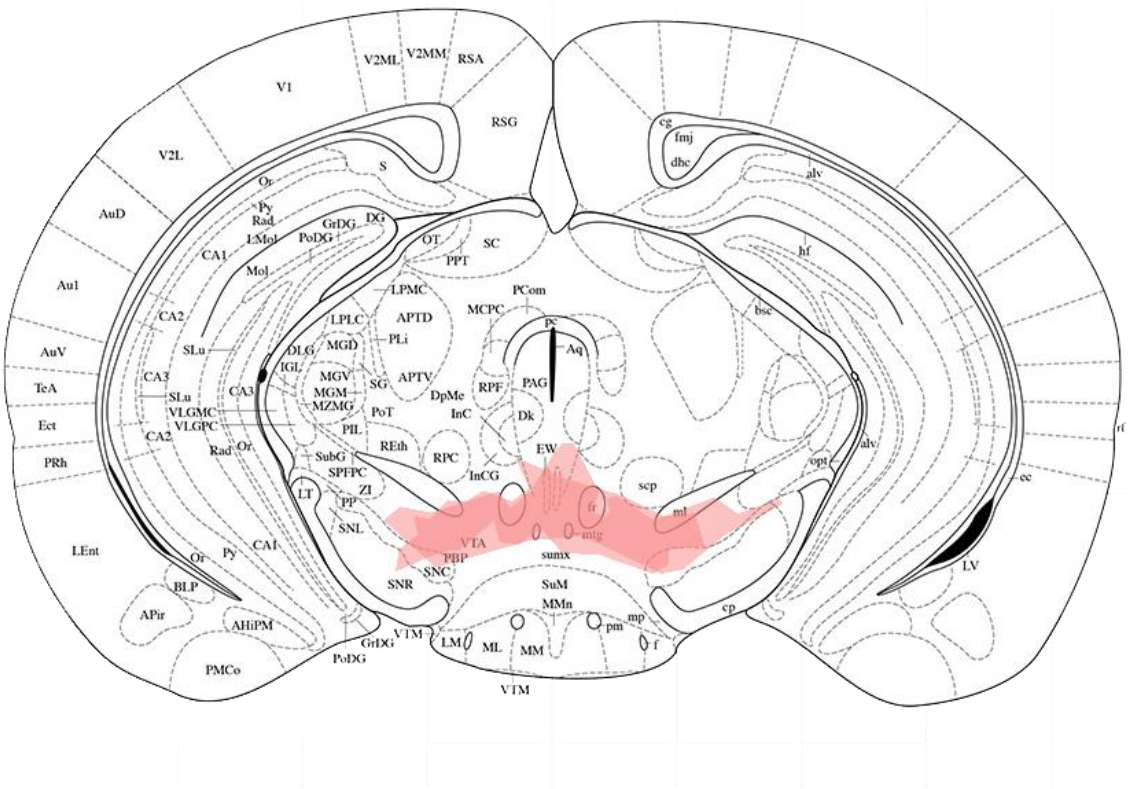
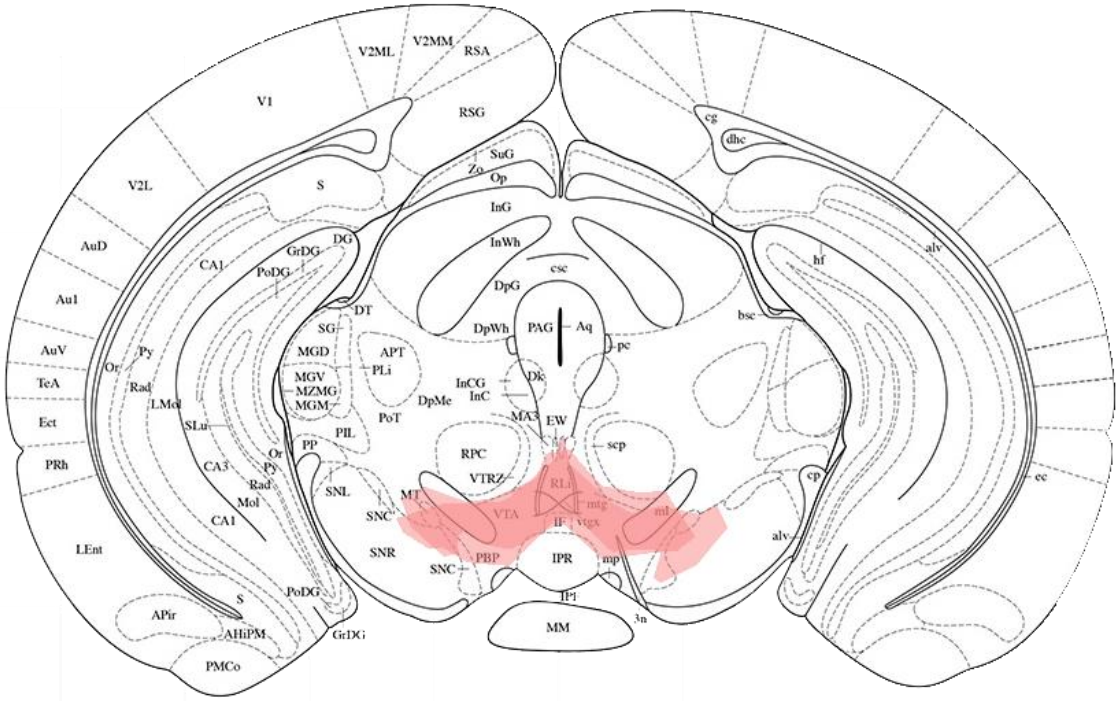
Appendix A

Example of the extent of viral expression in mCherry-expressing mice (n = 2). AP: -2.92mm, relative to bregma.



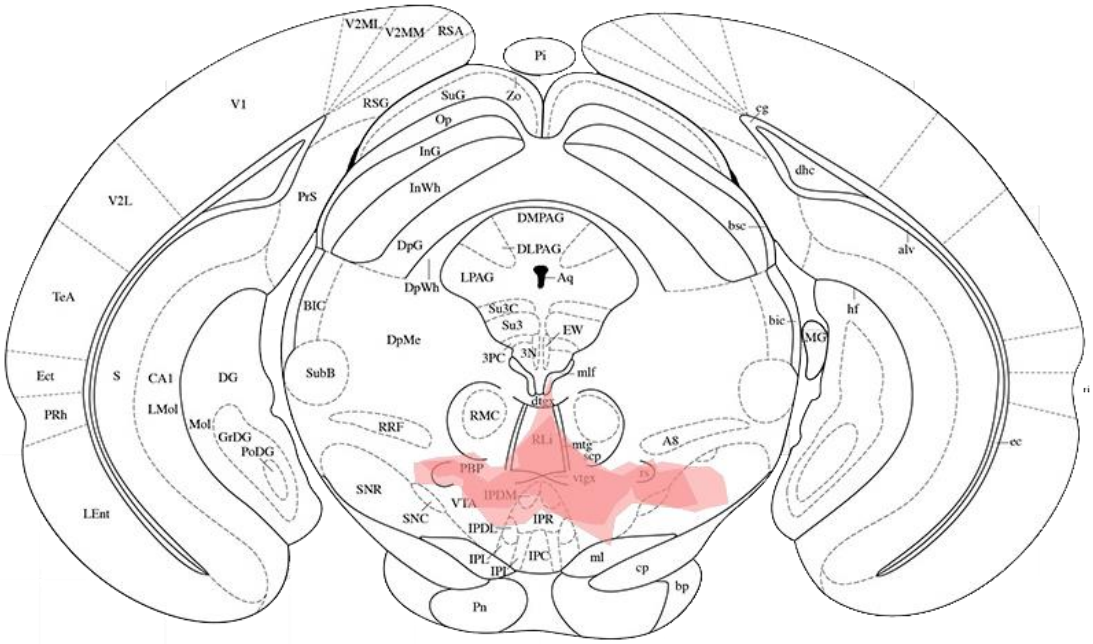
Appendix B

Example of the extent of viral expression in mCherry-expressing mice (n = 2). AP: -3.28mm, relative to bregma.



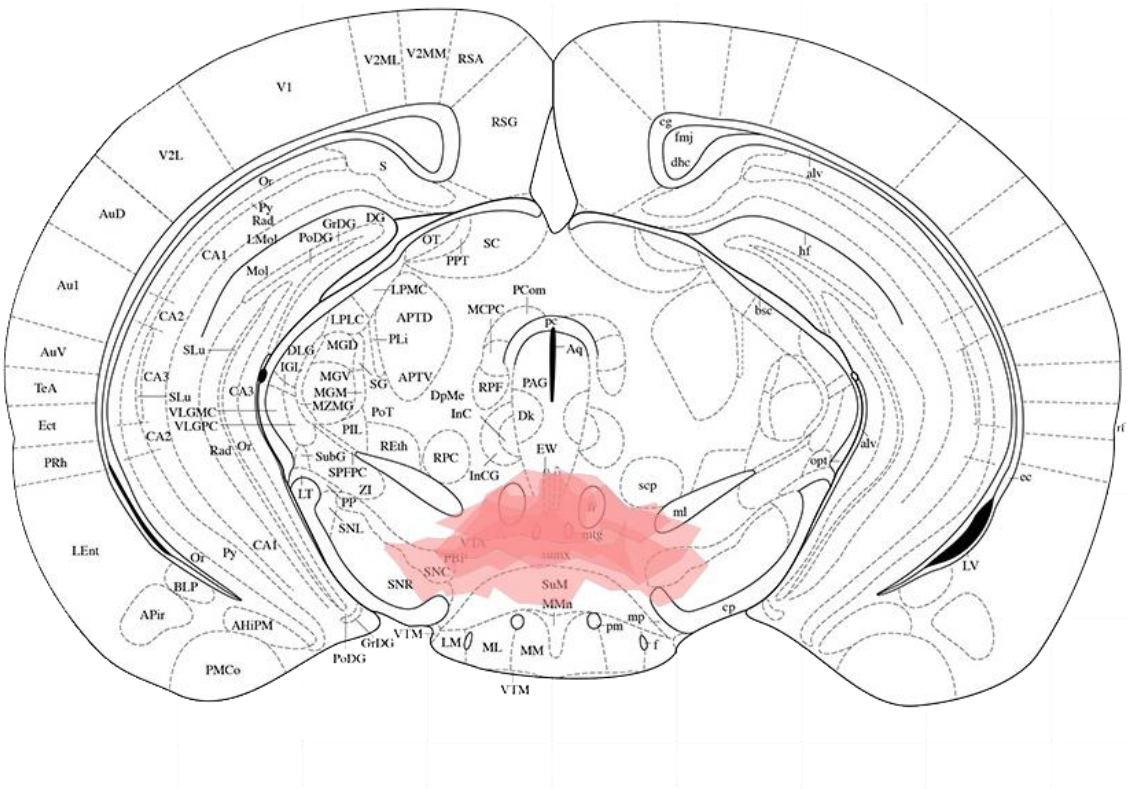
Appendix C

Example of the extent of viral expression in mCherry-expressing mice (n = 2). AP: -3.88mm, relative to bregma.



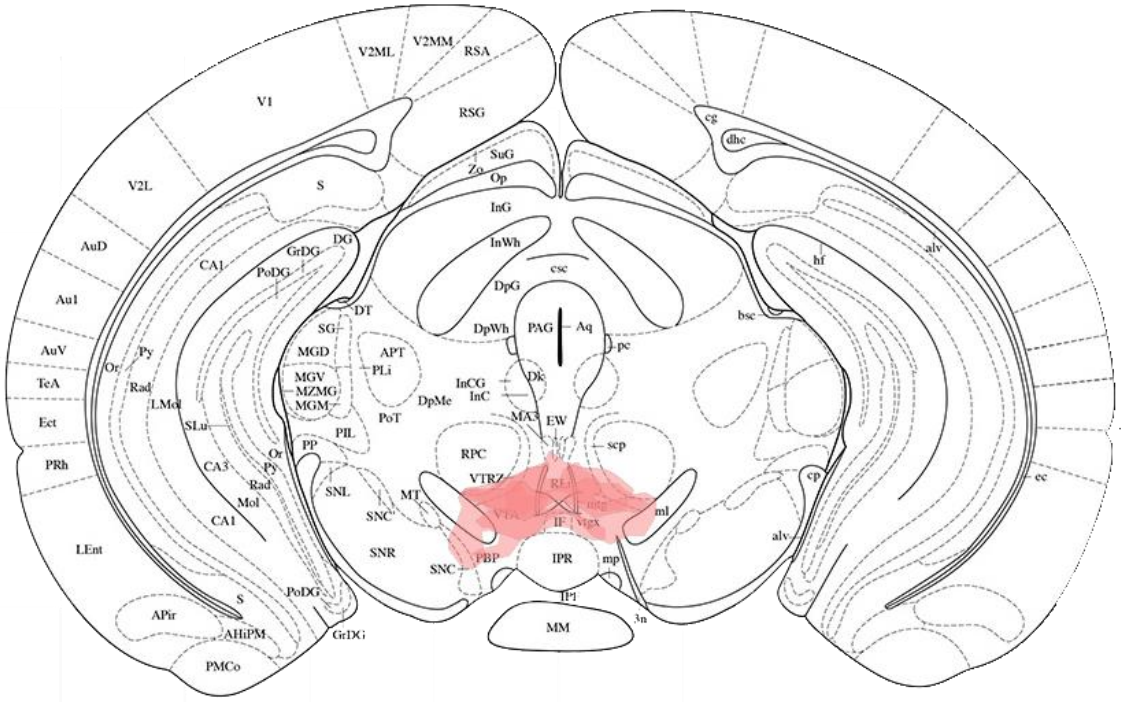
Appendix D

Example of the extent of viral expression in hM4Di-expressing mice (n = 4). AP: -2.92mm, relative to bregma.



Appendix E

Example of the extent of viral expression in hM4Di-expressing mice (n = 4). AP: -3.28mm, relative to bregma.



Appendix F

Example of the extent of viral expression in hM4Di-expressing mice (n = 4). AP: -3.88mm, relative to bregma.