UNIVERSITY OF CALIFORNIA SAN DIEGO

# The functional role of thalamocortical projections in motor learning

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Yun Celeste Yang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

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Chapter 1 is co-authored with Dr. Assaf Ramot, Felix H. Taschbach, and Prof. Takaki Komiyama. The original dataset for monosynaptic retrograde rabies virus tracing is from Muñoz-Castañeda et al. *Nature* (2021).

Chapter 2 is co-authored with Dr. Assaf Ramot and Prof. Takaki Komiyama.

Chapter 3 is co-authored with Dr. Assaf Ramot, Xinyi C. Wang, and Prof. Takaki Komiyama.

ABSTRACT OF THE THESIS

The functional role of thalamocortical projections in motor learning

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Professor Takaki Komiyama, Chair

Motor learning is an essential aspect of an animal's everyday behaviors. As the environment around us evolves, animals constantly learn and adapt to new motor skills in order to survive. The primary motor cortex (M1) is required for motor learning. To learn and generate voluntary movements, M1 receives long-range inputs that carry movement-related information; this information is then processed by local M1 neural network and output to downstream circuits.

However, the functional roles of these long-range inputs to M1 remain unclear. Here, we investigate motor learning as mice learn a sound-cued forelimb lever-press task for two weeks. Upon inactivation of the motor thalamus with chemogenetic tools, mice's ability to execute the learned lever-press movement become significantly impaired. Our results suggest the thalamocortical inputs as the main driver underlying movement execution at the expert stage of motor learning.

# Chapter 1 Introduction

## Emergence of reproducible movement pattern during motor learning

Motor learning is generally defined as the animal's ability to learn and execute movement in a reliable manner. Over the course of learning, an individual acquires, refines, and eventually retains a movement pattern that would allow them to achieve the desired behavioral outcome faster and at a higher success rate. To study motor learning, a sound-cued lever-press task was employed, where in each trial, an effective lever press during cue leads to a water reward. Water-restricted mice are trained to perform this task for 14 consecutive days (sessions) with 100 trials per session. To quantify the behavioral outcome of motor learning, number of rewards, the time between cue to movement, as well as the time between cue to reward are compared between beginners (session 1 and 2) and experts (session 13 and 14). As a result, mice show a significant increase in the number of rewarded trials, as well as a significant decrease in the time it takes for movement initiation and receiving rewards. In addition, the rewarded lever-pressing movement becomes increasingly similar, as shown by the significant increase in both within session and across sessions movement correlation.

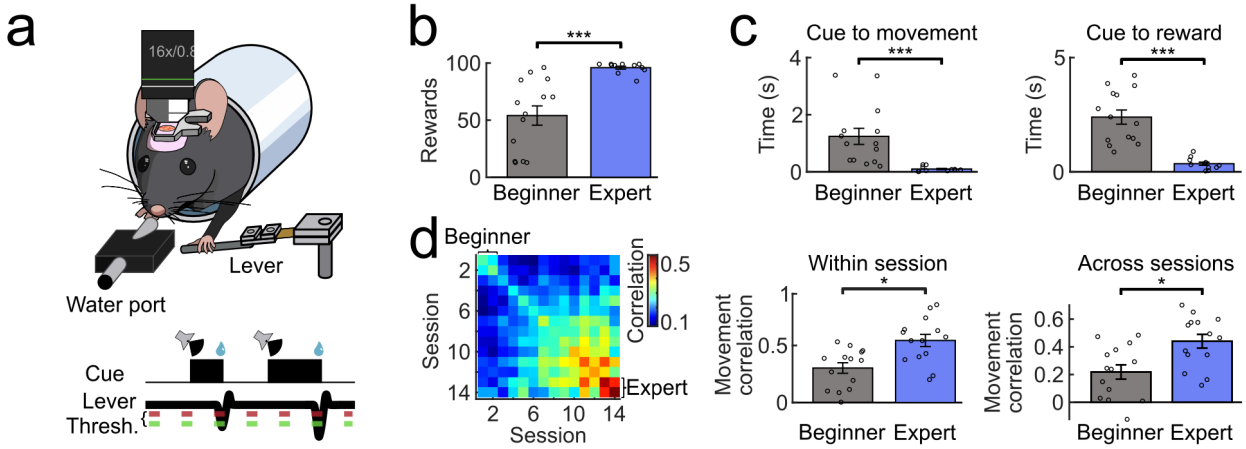


Figure 1.1: Motor learning includes increased success rate, increased movement speed and similarity with repeated practice. a. Schematic of the motor learning task; b. Significant increase in the number of rewards between beginners (session 1 and 2) and experts (session 13 and 14), circles correspond to individual sessions; c. Significant decrease in cue to movement and cue to reward between beginners and experts; d. Significant increase in both within session and across sessions rewarded movement correlation over the course of learning (n = 7 mice).

**The primary motor cortex (M1) is required for motor learning**

Past research has revealed the primary motor cortex (M1) as the central locus of change that underlies motor learning[1]. First of all, longitudinal two-photon calcium imaging of the same population of M1 Layer 2/3 pyramidal neurons reveals the formation of a reproducible activity pattern as learning occurs[1]. Secondly, M1 Layer 2/3 pyramidal neurons undergo substantial changes in their synapses over the course of learning[1-4]. Last but not least, pharmacological inactivation of M1 with muscimol, a GABA receptor agonist, impaires motor learning, as demonstrated by the significant decrease in the number of rewards that mice received in the lever-press task[1]. Optogenetic inactivation of M1 with channelrhodopsin (ChR2) in parvalbumin-expressing inhibitory neurons by blue light shows a similar effect[1]. Therefore, M1 is required for learning the lever-press task.

**Motor cortex circuit model for generating voluntary movement**

In our current model for learning and generating voluntary movements, M1 receives long-range inputs that carry movement-related information[5]. The interaction between these long-range inputs and M1 Layer 2/3 excitatory neurons, together with M1 Layer 2/3 recurrent connectivity, drive the formation of patterned neuronal activity and synaptic plasticity during learning[1, 5]. This ensemble activity in M1 Layer 2/3 then drives the deeper layer neurons in M1 and outputs to downstream circuits that are responsible for generating and executing skilled movements[5, 6]. However, it remains unclear which long-range inputs are the main drivers for the skill-specific M1 Layer 2/3 ensemble activity, and what functional roles these long-range inputs hold in dexterous movement.

**Identifying monosynaptic long-range inputs to M1 Layer 2/3**

To identify the monosynaptic long-range inputs to M1 Layer 2/3 excitatory neurons, we analyzed data from a published dataset by Muñoz-Castañeda, R. et al. (2021). In this dataset, projections from the whole brain areas to the mouse primary motor cortex, and more specifically, the upper limb area (MOp-ul), were mapped with retrograde rabies tracing techniques[7]. By injecting a mono-trans-synaptic rabies virus into MOp-ul in different Cre lines, the fraction of total inputs from each brain area, both ipsilateral and contralateral sides, were quantified[7]. We analyzed data from two specific Cre lines, Cux2 and Sepw1, which revealed the primary sensory cortex (S1), thalamus, and contralateral M1 (cM1) as the three major monosynaptic input areas into M1.

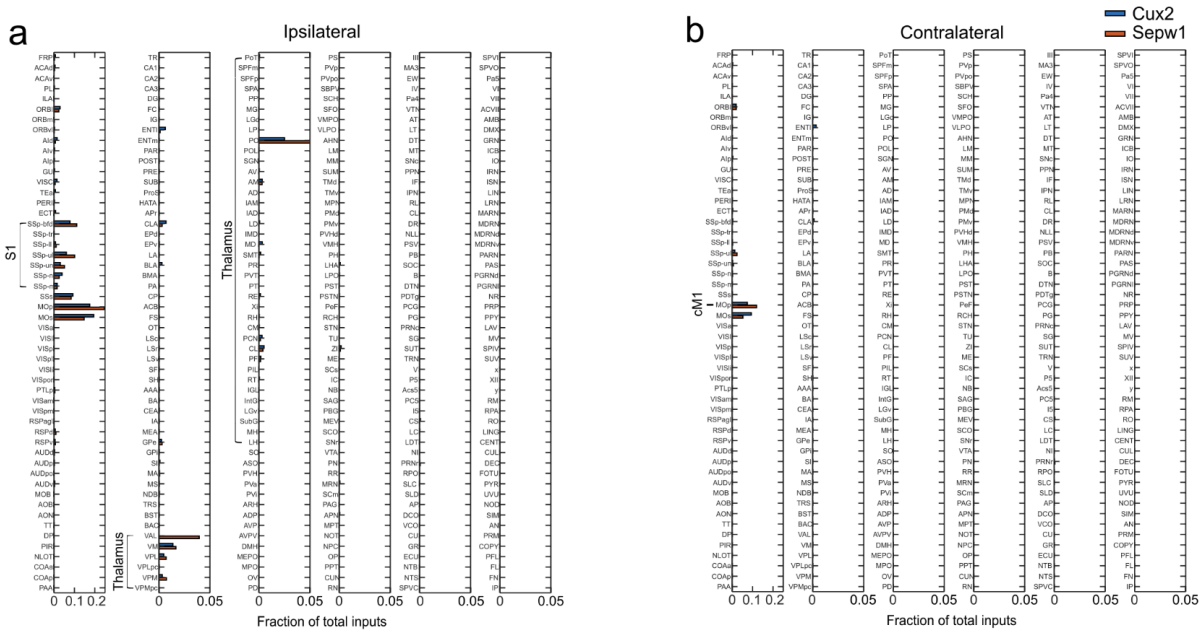


Figure 1.2: S1, Thalamus, and cM1 account for major monosynaptic inputs to M1 L2/3 excitatory neurons. a. Fraction of total inputs from ipsilateral brain regions; b. Fraction of total inputs for contralateral brain regions. Cux2 and Sepw1 Cre lines were used to limit Layer 2/3 excitatory neurons as starter cells. Original dataset is from Muñoz-Castañeda et al. *Nature* (2021).

**Rewarded movement is preferentially encoded by thalamic inputs to M1 at the expert stage of learning**

To investigate the functional roles of these inputs to M1, we expressed Axon-GCaMP6s in the motor thalamus, S1, or cM1 in a Cre dependent manner, and performed longitudinal two photon imaging of axonal boutons innervating M1 (same FOV in beginners and experts), as mice learn the lever-press task. Next, the activity of either thalamic, S1, or cM1 inputs was used to decode the timing of rewarded movements using linear SVM classifiers. The result revealed that thalamic inputs in experts have the highest decoding accuracy for rewarded movement, implying that upon learning, thalamic inputs begin to carry substantial rewarded movement related

information. Therefore, in this thesis project, we seek to understand the functional role of these thalamic inputs to M1 during the expert stage of motor learning.

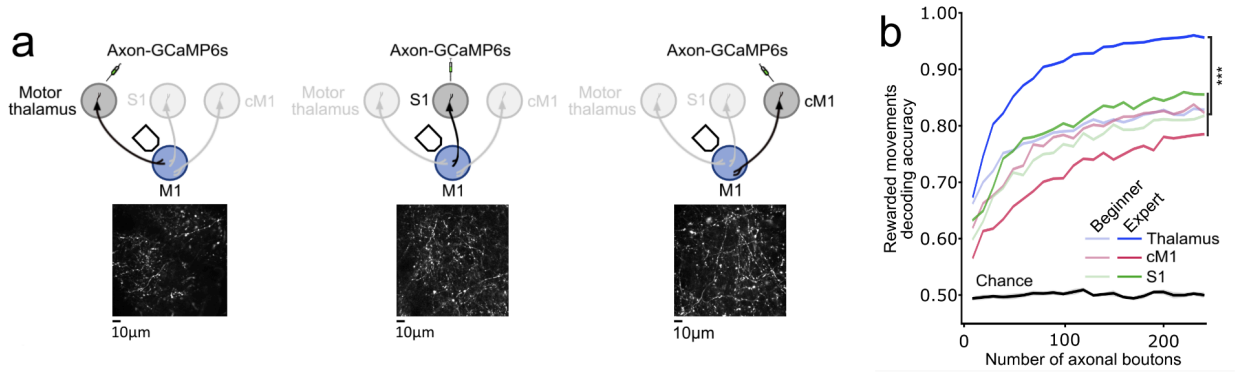


Figure 1.3: Rewarded movement is preferentially encoded by thalamic inputs to M1 at the expert stage of learning. a. Top, schematic of experiment setup; Bottom, example of two-photon images of axonal boutons innervating M1 expressing GCaMP6s; b. Thalamic inputs in experts have the highest decoding accuracy for rewarded movement compared to beginners, as well as inputs from S1 and cM1.

Chapter 1 is co-authored with Dr. Assaf Ramot, Felix H. Taschbach, and Prof. Takaki Komiyama. The original dataset for monosynaptic retrograde rabies virus tracing is from Muñoz-Castañeda et al. *Nature* (2021).

# Chapter 2 Materials & Methods

## Animals

All animal procedures were performed in accordance with guidelines and protocols approved by the UCSD Institutional Animal Care and Use Committee and the National Institutes of Health (NIH). Vglut2-Cre (Vglut2-ires-cre, JAX stock #016963) mice were group-housed in disposable cages with standard bedding in a temperature-controlled room (~21 °C) with a reversed light cycle (10.00–22.00: dark). All experiments were performed during the dark cycle. Males and females were randomly used for experiments.

## Surgeries and virus injections

Adult mice (7 weeks or older) were anesthetized with 1 – 2% isoflurane. Virus solutions of AAV5-hSyn-DIO-hM4Di-mCherry (Addgene viral prep # 44362) (diluted 1:5 for soma inactivation and 1:2 for axonal inactivation) or AAV1-FLEX-tdTomato (Addgene viral prep # 51503) (control group) were injected into the following coordinates of Vglut2-Cre mice: 1 mm posterior, 1 mm lateral to bregma at a depth of ~3.7 mm from the pia (~0.4μL). For axonal inactivation mice, M1 was marked at the following coordinates: 0.3 mm posterior, 1.5 mm lateral to bregma. A custom-built head-plate was glued and cemented to the skull. Baytril (10 mg/kg) and buprenorphine (0.1 mg/kg) were injected subcutaneously at the end of surgery to prevent infection, inflammation, and discomfort. Experiments were performed ~4 weeks after surgery for soma inactivation and ~8 weeks for axonal inactivation.

**Behavior**

Water restriction started 1-1.5 weeks before the behavioral training. Animals received progressively lower amounts of drinking water until stabilizing at 1 ml per day. Weight was monitored daily to ensure loss of no more than 30% of the starting body weight. Mice were trained to perform the lever-press task 1 session per day for 14 days. The lever consisted of a piezoelectric flexible force transducer (LCL-113G, Omega Engineering) attached to a brass rod. The lever position was continuously recorded using LabJack and Ephus (controlled by MATLAB), which worked with custom software running on LabVIEW (National Instruments) that monitored threshold crossing. Dispatcher software controlled the behavioral setup (Z. Mainen and C. Brody, controlled by MATLAB). Mice used their left paw to grasp a lever attached to a force transducer while resting their right paw on a stationary block. A 6 kHz tone marked a cue period (up to 10 s), during which a successful lever press was rewarded with water (~10 μL per trial) paired with a 500 ms, 12 kHz tone, and followed by an inter-trial interval (ITI, variable duration of 8-12 s). A successful lever-press movement was defined as crossing two thresholds (~1.5 mm and ~ 3 mm below the resting position) within 200 ms. Failure to press the lever passing the two thresholds during the cue period triggered a loud white-noise sound and the start of an ITI. Lever presses during ITIs were neither rewarded nor punished. Each session consisted of 100 trials.

**Lever-press movement analysis**

Lever displacement traces (voltage recordings from the force transducer) were downsampled from 10 kHz to 1 kHz, then filtered using a four-pole 10 Hz low-pass Butterworth filter, after which the velocity of the lever was determined by smoothing the difference of consecutive points with a moving average window of 5 ms. The envelope of the lever velocity

was then extracted using a Hilbert transform, and movement bouts were defined by the envelope crossing a threshold of 4.9 mm per second. Each movement bout was extended by 75 ms on either side. Bouts separated by less than 500 ms were considered continuous. Movement start and end times were defined as the points at which the lever exceeded or fell below the thresholds defined by rest periods before and after the movement bouts. Thresholds were defined as the resting position plus the 99th percentile of the noise distribution, in turn, defined as the difference between the Butterworth smoothed trace and the original trace. For reaction time analysis, the trials in which mice moved the lever within 100 ms before the cue start time were excluded from analysis. Movement correlations within sessions were calculated using the median of all pairwise correlations of rewarded movements that started after cue onset within a single session. Movement correlations across sessions were found using the median correlations of all possible pairs of movements between sessions.

**Chemogenetic inactivation**

Clozapine-N-Oxide (CNO, Enzo Life Sciences) was dissolved in sterile saline to a 2.5 mg/mL concentration. For soma inactivation, CNO was injected intraperitoneally at a 10 mg/kg body weight dose 45 minutes before behavioral training. For axonal inactivation, 30 minutes before behavioral training, 300ul CNO was injected locally by drilling a hole over the M1 mark and at a depth of 500um below pia. Only animals that had hM4Di expression within the thalamus confirmed by histology were included in the analysis. Investigators were blinded to the animal's performance when examining hM4Di expression.

**Histology**

Mice were anesthetized and transcardially perfused with ice-cold 0.1 M PBS (pH 7.4), followed by perfusion with ice-cold 4% paraformaldehyde (PFA) solution. Isolated brains were

postfixed overnight at 4 °C in 4% PFA and cryoprotected in 30% sucrose solution for at least 48 hours at 4 °C. Microtome-cut (Thermo Scientific Microm HM 430) 40-50 μm free-floating brain (coronal) sections were collected in PBS and stored at 4 °C. Slices were mounted with a CC mounting medium (Sigma-Aldrich) or DAPI (Electron Microscopy Sciences). Slices were imaged using a fluorescence microscope (Zen and ApoTome.2, Zeiss). For visualizing axonal expression of hM4Di, coronal sections were blocked in a solution consisting of 2% normal goat serum, 1% BSA, and 0.3% Triton X-100 in 1 × PBS for 1 hour at room temperature, followed by overnight incubation at 4 °C with primary antibodies (1:400 Rabbit anti-mCherry, Abcam) diluted in the blocking solution. After washing, sections were incubated in Alexa Fluor-conjugated secondary antibodies (1:200 Goat anti-rabbit 594, Thermo Fisher Scientific) for 1.5 hours at room temperature. For all experiments, we verified the expression of the injection constructs (hM4di or tdTomato) in the desired brain region.

**Identifying monosynaptic inputs to M1 L2/3 neurons**

We analyzed data from Muñoz-Castañeda et al. (2021), representing rabies virus-based monosynaptic retrograde labeling of direct inputs to M1 L2/3 excitatory neurons. We examined only data using Cux2-Cre and Sepw1-Cre lines, which represent putative excitatory M1 L2/3 neurons.

Chapter 2 is co-authored with Dr. Assaf Ramot and Prof. Takaki Komiyama.

# Chapter 3 Results

## Inactivation of the motor thalamus impairs the execution of learned movement

To investigate the functional role of thalamic inputs to M1 during the expert stage of motor learning, hM4Di, a ligand activated inhibitory GPCR (or tdTomato as control), was injected into the right motor thalamus of Vglut2-Cre mice[8]. After four weeks of virus expression, mice were trained with the lever-press task for two weeks as they became experts. On the 14th day, CNO was administered through intraperitoneal (IP) injection 45 minutes before behavioral training. Mice included in both experimental and control groups have learned the lever task before CNO application on Day 14, as demonstrated by a gradual increase in the number of rewards over sessions, as well as a gradual decrease in the cue to movement, cute to reward, movement onset to reward.

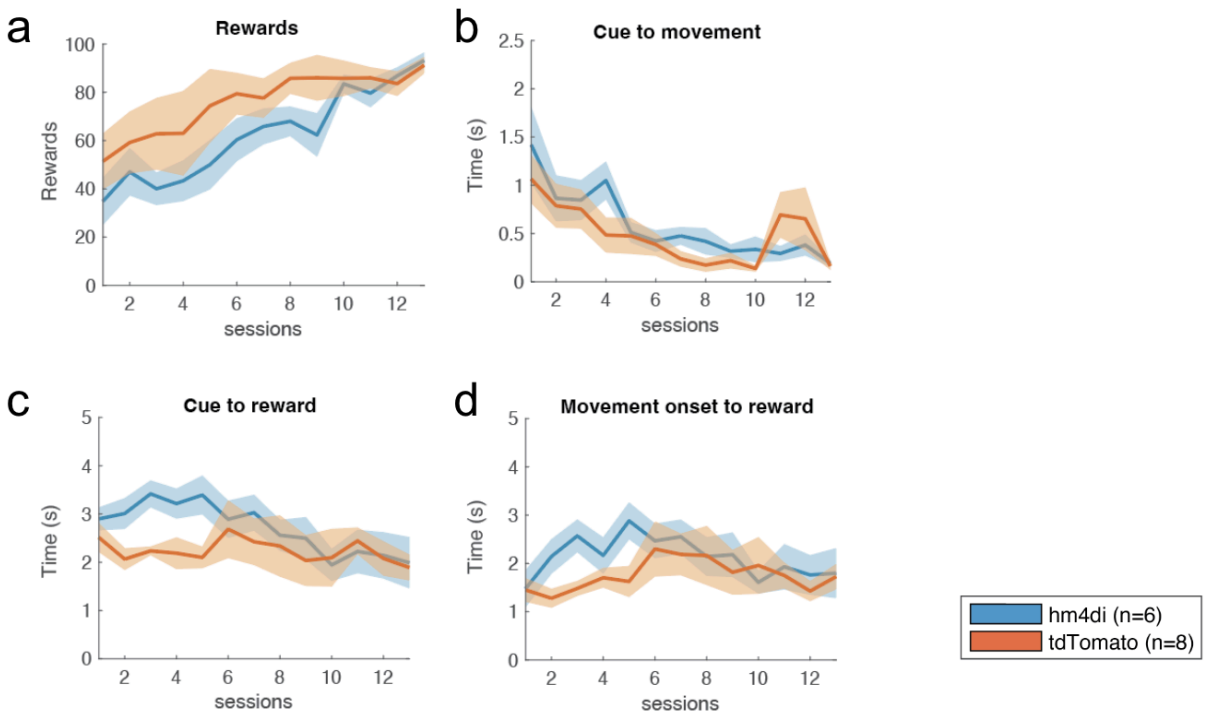


Figure 3.1: Mice in both experimental and control groups learned the lever-press task before CNO application on Day 14. a. Increase in the number of rewards across training; b. Decrease in the time between cue to first movement by mice; c. Decrease in the time from cue to reward; d. Decrease in the time from Movement onset to reward.

Upon CNO application on Day 14 to inactivate the motor thalamus, there is a significant impairment in the execution of the learned lever-pressing movement. This includes a significant decrease in the number of rewards received in the hM4Di group compared to the tdTomato control, as well as a significant increase in cue to reward and movement onset to reward. Although the difference between hM4Di group and tdTomato control in cue to movement did not reach statistical significance, it took longer for the mice in the hM4Di group to initiate their first movement after cue.

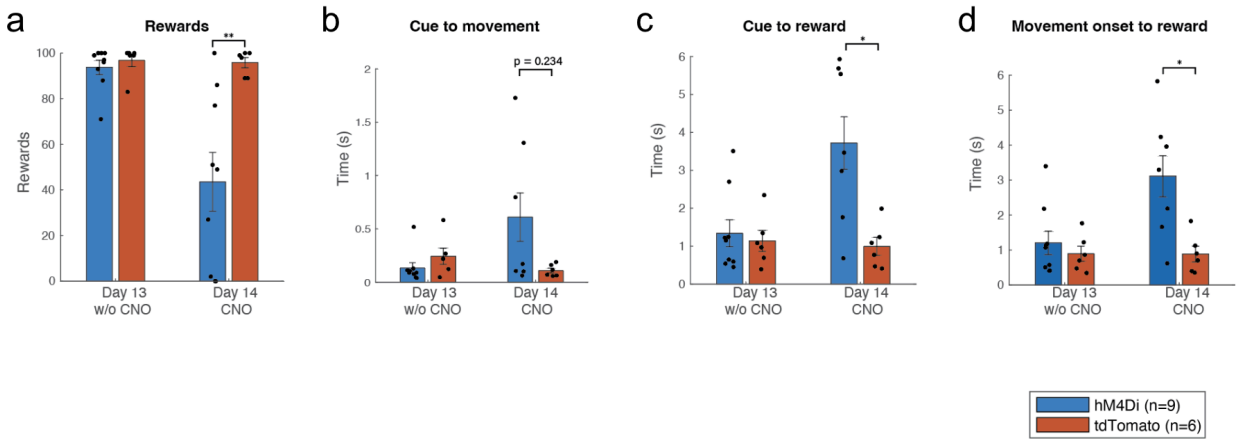


Figure 3.2: Inactivation of the motor thalamus impairs the execution of learned movement. a. Significant decrease in the number of rewards received in the hM4Di group upon application of CNO on Day 14; b. Increase in the time from cue to movement in hM4Di group upon application of CNO on Day 14; c. Significant increase in the time from cue to reward in hM4Di group upon application of CNO on Day 14; d. Significant increase in the time from movement onset to reward in hM4Di group upon application of CNO on Day 14.

For all the mice included in our analysis, virus expression of hM4Di (or tdTomato) was validated with histology.

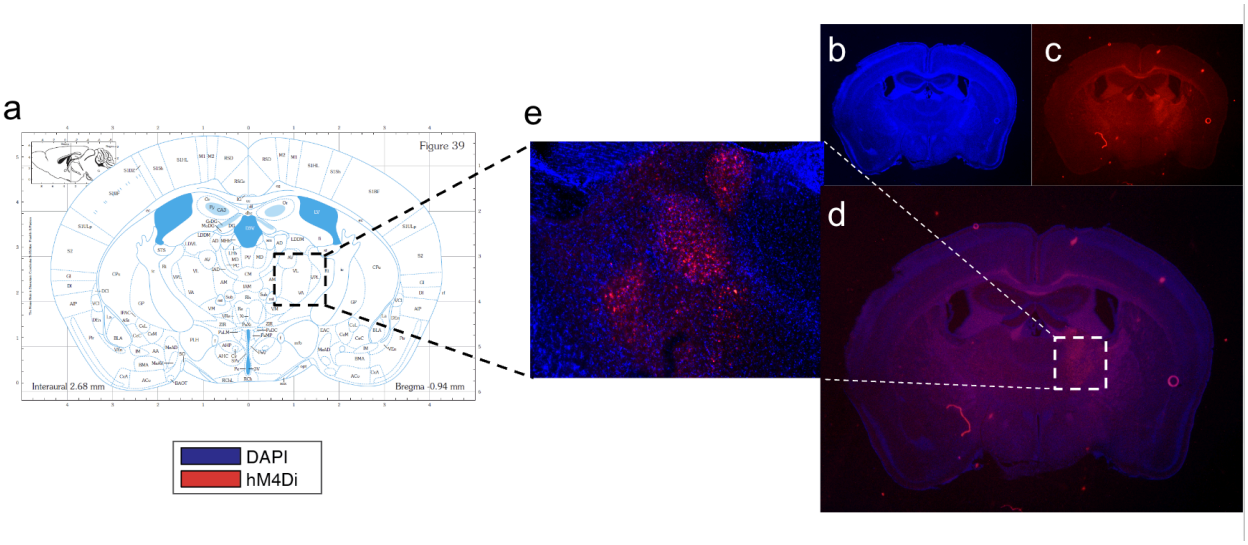


Figure 3.3: Histological validation of hM4Di expression in the motor thalamus. a. Mouse brain coronal section indicating the stereotaxic location of the motor thalamus (VA, VL); b. Fluorescence image of coronal brain slice under DAPI channel; c. Fluorescence image of the same coronal brain slice under RED channel; d. Overlay of DAPI channel and RED channel images; e. Zoom in view showing expression of hM4Di in the motor thalamus.

**No obvious behavioral effect with local CNO application**

Given that the thalamus also projects to other brain regions, such as the secondary motor cortex, somatosensory cortex, etc, inactivation of the thalamus is not specific. Therefore, we hope to inactivate thalamocortical axons innervating M1 to identify the functional properties of these inputs during the expert stage of motor learning. To inactivate thalamocortical inputs, hM4Di (or tdTomato as control) was injected into the right motor thalamus of Vglut2-Cre mice[8]. After eight weeks of virus expression, mice were trained with the lever-press task for two weeks as they became experts. On the 14th day, CNO was applied locally over M1 30 minutes before behavioral training. Mice included in both experimental and control groups have learned the lever task before CNO application on Day 14, as demonstrated by a gradual increase in the number of rewards over sessions, as well as a gradual decrease in the cue to movement, cute to reward, movement onset to reward.

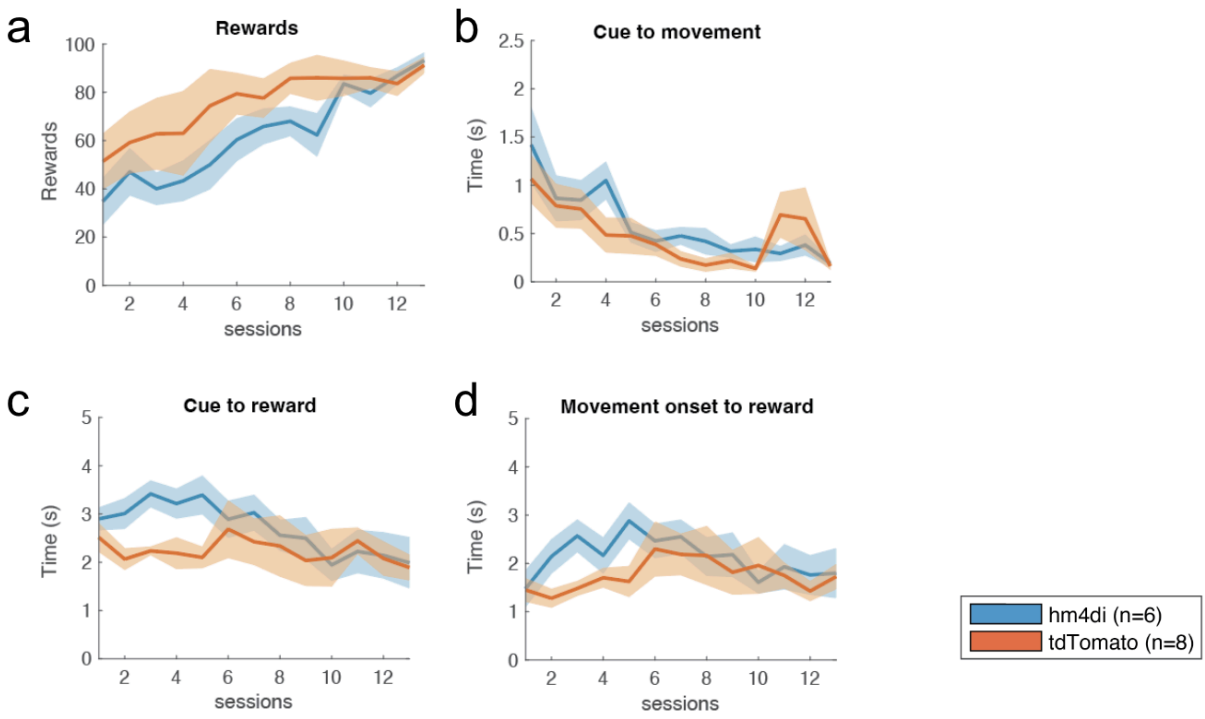


Figure 3.4: Mice in both experimental and control groups learned the lever-press task before CNO application on Day 14. a. Increase in the number of rewards across training; b. Decrease in the time between cue to first movement by the mouse; c. Decrease in the time from cue to reward; d. Decrease in the time from movement onset to reward.

Upon CNO application on Day 14 to inactivate thalamocortical inputs, there was no significant behavioral effect observed in the hM4Di group. This could potentially be due to several technical limitations, which will be discussed in detail in the next chapter.

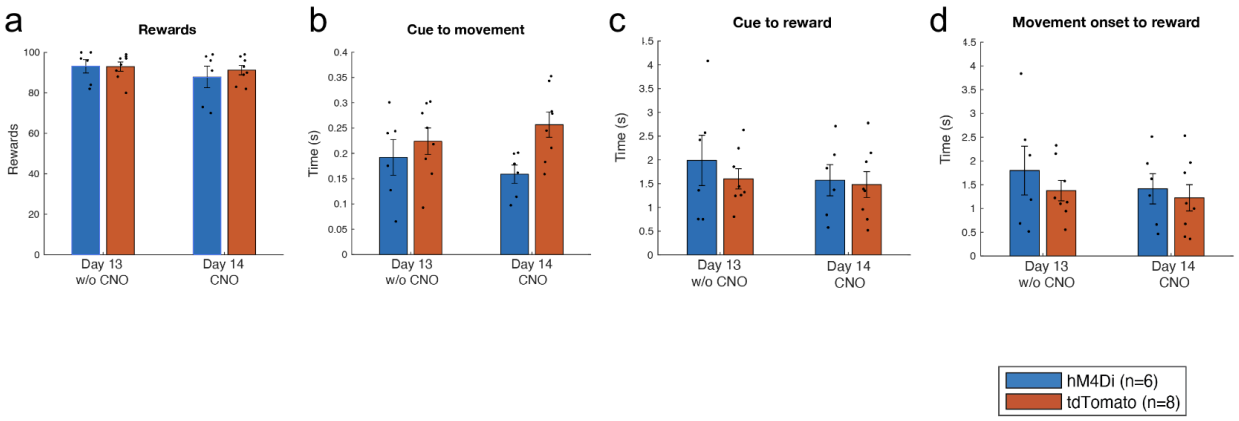


Figure 3.5: No obvious behavioral effect with local CNO application. a. No difference between the hM4Di group and tdTomato control in the number of rewards received upon application of CNO on Day 14; b. No difference in cue to movements upon application of CNO on Day 14; c. No difference in cue to reward upon application of CNO on Day 14; d. No difference in movement onset to reward upon application of CNO on Day 14.

For all the mice included in our analysis, the virus expression of hM4Di (or tdTomato) was validated in both the motor thalamus and M1 with antibody staining.

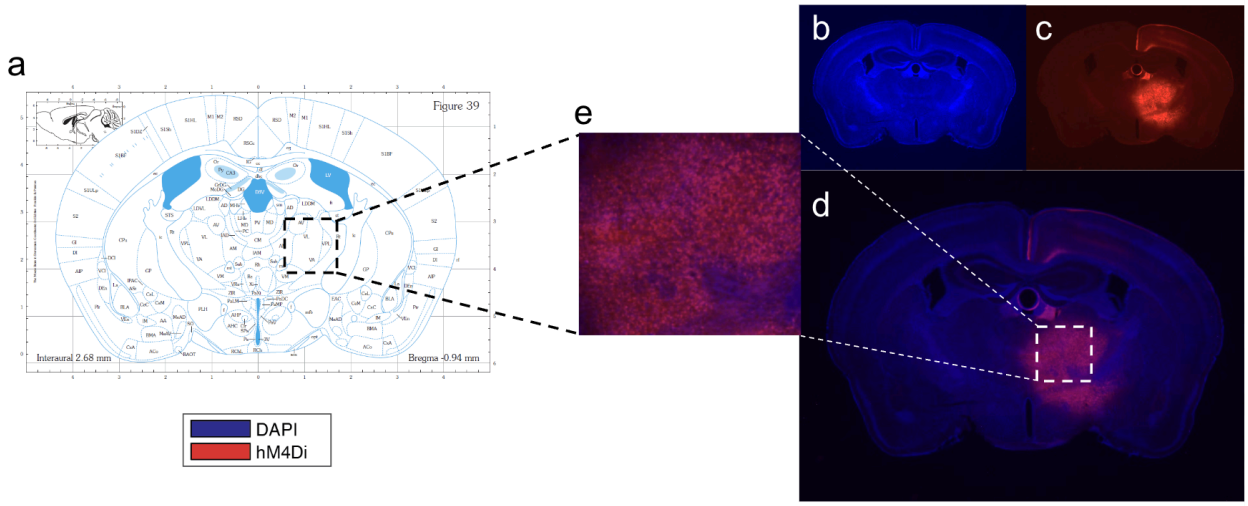


Figure 3.6: Histological validation of hM4Di expression in the motor thalamus with immunostaining. a. Mouse brain coronal section indicating the stereotaxic location of the motor thalamus (VA, VL); b. Fluorescence image of coronal brain slice under DAPI channel; c. Fluorescence image of the same coronal brain slice under RED channel; d. Overlay of DAPI channel and RED channel images; e. Zoom in view showing expression of hM4Di in the motor thalamus.

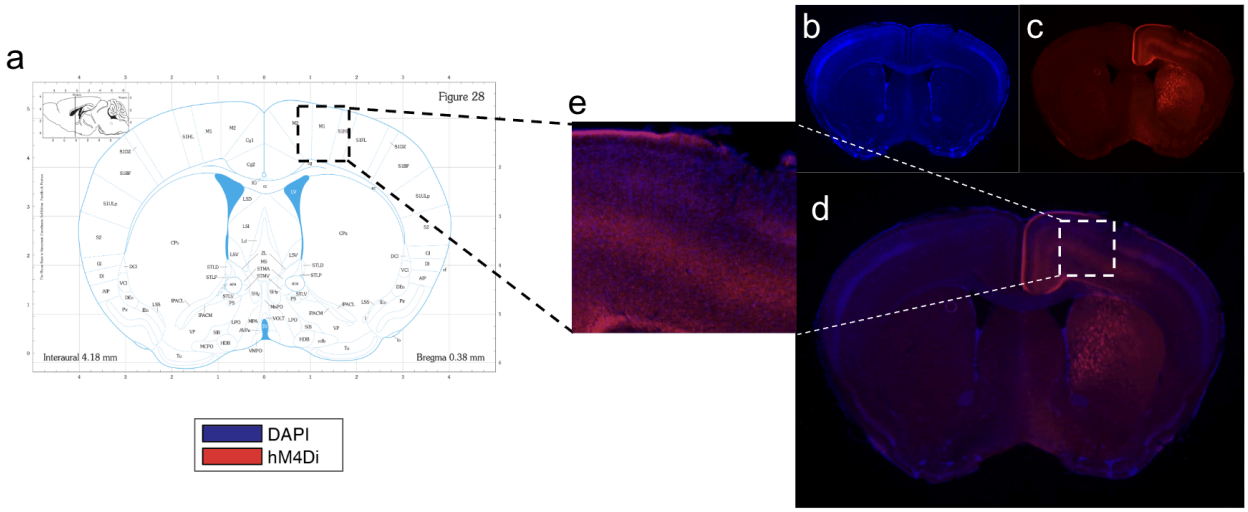


Figure 3.7: Histological validation of hM4Di expression in M1 with immunostaining. a. Mouse brain coronal section indicating the stereotaxic location of the primary motor cortex (M1); b. Fluorescence image of coronal brain slice under DAPI channel; c. Fluorescence image of the same coronal brain slice under RED channel; d. Overlay of DAPI channel and RED channel images; e. Zoom in view showing expression of hM4Di in M1.

Chapter 3 is co-authored with Dr. Assaf Ramot, Xinyi C. Wang, and Prof. Takaki Komiyama.

# Chapter 4 Discussion

## Inactivation of the motor thalamus impairs the execution of learned movement

The results above demonstrated that inactivation of the motor thalamus impairs the execution of learned movement at expert stage. Specifically, chemogenetic inactivation does not abolish movement completely. Instead, mice were still able to move and attempt to press the lever for water rewards. However, the movement is less efficient, as demonstrated by a significant increase in reaction time and the time from cue to reward. Our results suggest that thalamocortical inputs serve as the main driver behind the skill-specific M1 Layer 2/3 ensemble activity during the expert stage of motor learning.

## Potential caveats of the axonal inactivation experiment

First of all, the efficacy of local CNO application might be responsible for the non significant results that were observed in the axonal inactivation experiment. Cortical CNO injection is very shallow. Therefore, the amount of CNO injected might get easily washed out to the surface of the brain. To resolve this issue, the volume/concentration of CNO injected could be increased. CNO could also be injected at multiple cortical sites to cover a larger area of M1 by employing a cranial window with multiple pores. The time between CNO injection and behavioral training could also be decreased, as the mice included in our analysis recovered well from anesthetics. Secondly, even though the expression of hM4Di was confirmed upon immunostaining within M1, it is difficult to quantify the amount of thalamic axons that were effectively expressing hM4Di and subsequently inactivated by CNO. To resolve this, hM4Di injection with less dilution might be considered. Last but not least, there is high variability between the behavior of each individual mouse. Some mice did not show an increase in their

lever-press movement correlation within or across sessions. Further analysis should exclude mice in both experimental and control groups with a high variability in their movement using a well-defined criteria.

**Future Direction**

First of all, it remains unclear how the dynamic and activity pattern of M1 Layer 2/3 neurons are affected by abolishing thalamic inputs during the expert stage of motor learning. Therefore, imaging the activity of M1 Layer 2/3 neurons with two-photon microscopy on Day 13 and/or Day 14 of training while inactivating thalamic activity with DREADD will allow us to further understand the role that thalamic inputs play in the brain circuitry for generating voluntary movements. Secondly, we argue that this functional role of thalamocortical inputs as the main driver behind the skill-specific M1 Layer 2/3 ensemble activity is unique to the expert stage of motor learning. Given that we did not observe a significantly higher decoding accuracy of rewarded movement by thalamic inputs during the beginner stage, inactivation of thalamic activity with DREADD on Day 2 and/or Day 3 of training showing no such significant impairment in movement execution will further support our result.

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