

Acquisition phase-specific contribution of climbing fiber transmission to cerebellum-dependent motor memory

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Abstract

Climbing fiber (CF) transmission from the inferior olivary triggers complex spikes (Cs) in Purkinje cells (PCs) driven by a burst of calcium spikes. In the context of motor learning, especially the compensatory optic response, CF transmission serves as instructive signals selectively conveyed to PCs. While the significance of CF input in motor memory formation is widely acknowledged, a comprehensive understanding of its distinct contribution across different temporal windows, spanning from the initial phase of learning to the retrieval period, remains incomplete. Therefore, we aimed to investigate the necessity of CF-induced instructive signals in motor learning by assessing their roles in memory acquisition, consolidation, and retrieval. We employed optogenetics to selectively inhibit CF transmission during targeted time windows. Consequently, the absence of CF-induced instructive signals during motor learning impairs memory acquisition. However, when these signals were suppressed during the consolidation and retrieval period, there was neither a loss of long-term memory nor prevention of memory retrieval. Our results highlight that CF transmission plays a specialized and critical role primarily in memory acquisition, rather than in subsequent processes.

eLife assessment

This study presents potentially **valuable** insights into the role of climbing fibers in cerebellar learning. The main claim is that climbing fiber activity is necessary for optokinetic reflex adaptation, but is dispensable for its long-term consolidation. There is evidence to support the first part of this claim, though it requires a clearer demonstration of the penetrance and selectivity of the manipulation. However, support for the latter part of the claim is **incomplete** owing to methodological concerns, including unclear efficacy of longer-duration climbing fiber activity suppression.

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Introduction

The cerebellar architecture, characterized by the presence of multiple synapses connecting various types of neurons, enables precise adjustments within intricate neural networks (Boyden et al., 2004 [↗](#)). Within this framework, the climbing fiber (CF) transmission derived from the inferior olive (IO) projecting to the cerebellar cortex is generally recognized as the primary pathway for relaying instructive signals to correct errors and enhance motor performance, including oculomotor behaviors (Albus, 1971 [↗](#); Frens et al., 2001 [↗](#); Ito, 1972 [↗](#); Marr, 1969 [↗](#)).

The optokinetic reflex (OKR), a compensatory optic response evoked by an unstable visual field, is a representative example of cerebellum-dependent motor learning (Cahill & Nathans, 2008 [↗](#); Schweigart et al., 1997 [↗](#)). Successful adaptation to stabilize retinal images in the presence of mismatched visual input requires the engagement of multiple neural substrates. Complex spikes (Cs) in floccular Purkinje cells (PCs) accompanied by excitatory inputs of CF are known to appear in response to unpredicted errors in motor performance (Clopach et al., 2014 [↗](#); Eccles et al., 1966 [↗](#); Goossens et al., 2004 [↗](#); Palmer et al., 2010 [↗](#)). Rotational visual stimulation induces Cs discharge in floccular PCs, reflecting CF-induced Cs generation in response to error signals generated from errors in oculomotor performance (Frens et al., 2001 [↗](#); Graf et al., 1988 [↗](#)). Thus, such fine-tuning of Cs in PCs is expected to control the amount of learning (Yang & Lisberger, 2014 [↗](#)).

A previous study employing a modified behavioral paradigm that intentionally suppresses the involvement of Cs has suggested that simple spike (Ss) alone is sufficient to drive cerebellar motor learning (Ke et al., 2009 [↗](#)). In contrast, others have demonstrated that pharmacological lesion and optogenetic inactivation of the IO impair oculomotor learning (Pham et al., 2020 [↗](#)) and prevent eye blink conditioning (Kim et al., 2020 [↗](#); Medina et al., 2002 [↗](#); Welsh & Harvey, 1998 [↗](#)). However, due to limitations in spatial and temporal specificity, these findings have not conclusively determined the necessity of CF in learning. Moreover, while the general perspective of the contribution of CF is primarily focused on the acquisition of memory during the active learning period, it remains unclear whether CF is dispensable in the consolidation phase, despite its continuous role in shaping PC output, which consists of CF-driven Cs with a rate of approximately 1 Hz, reaching the medial vestibular nucleus. To address this knowledge gap, we investigated the necessity of CF-induced instructive signals carried for motor learning by evaluating their contributions to memory acquisition, consolidation, and retrieval. Using optogenetics, we selectively inhibited CF signals in the flocculus, a lobe-like cerebellar structure that is mainly responsible for ocular reflex, during different phases of motor learning. Our findings demonstrate compelling evidence for the acquisition phase-specific contribution of climbing fiber transmission in motor learning.

Results

Optogenetic inhibition of climbing fiber transmission derived from the inferior olive

We first sought to specifically manipulate CF signals in the cerebellar cortex, generally known to be derived from the IO, by injecting AAV1-CaMKII α -eNpHR 3.0-EYFP or AAV1-CaMKII α -EGFP into the IO of wild-type mice (Figure 1A [↗](#), B). This allowed for the visualization of the expression of IO neurons and CF terminals in the brainstem nucleus and flocculus. Furthermore, selectively segregated expression of these regions enabled us to manipulate the terminals without

interrupting the soma of IO neurons, which, when damaged, can bring critical malfunction of motor performance and ocular reflexes, because the IO is the prominent center of sensory integration (Pham et al., 2020 [↗](#); Shaikh et al., 2017 [↗](#); Van Der Giessen et al., 2008 [↗](#)).

To validate the optogenetic inhibition of CF transmission, we adopted whole-cell patch-clamp recordings of PCs in acute cerebellar slices using a 593 nm-yellow laser. The stimulation electrode was placed on the granule cell layer near the PC (**Figure 1C** [↗](#)). The stimulation intensity was calibrated to minimally evoke CF excitatory postsynaptic current (EPSC). Once the stimulation intensity was determined, CF EPSC was induced under a laser turned on or off to investigate whether optogenetic inhibition could suppress synaptic transmission. When the laser illumination was applied, the amplitude of the EPSC was robustly decreased. In contrast, when the laser was turned off, suppression was disengaged. Laser illumination did not yield inhibition of CF EPSC in the GFP group (**Figure 1D** [↗](#) and **1E** [↗](#)). Furthermore, we verified the potential reduction of Cs firing rate in PCs of awake mice in vivo by inhibiting CF signals. In summary, these findings indicate the feasibility of optogenetic interventions in blocking CF transmission.

Inhibition of climbing fiber transmission during memory acquisition

An evaluation of CF's direct contribution in a region-specific manner has not been performed. Thus, after confirming the viability of optogenetic inhibition of CF, we investigated whether the elimination of CF transmission in the cerebellar flocculus affects cerebellar motor learning. When images on the retina are unstable due to mismatched visual inputs, CF signals are conventionally perceived to be responsible for sending error signals to the cerebellar cortex to improve ocular reflexes (Bloedel & Bracha, 1998 [↗](#); Frens et al., 2001 [↗](#); Zang & De Schutter, 2019 [↗](#)).

Using the same mouse model shown in **Figure 1** [↗](#), we surgically implanted an optical fiber in flocculus to suppress CF transmission in vivo during OKR (**Figure 2A** [↗](#)). Head-fixed mice were placed in a rotating drum painted with a black striped pattern to provide visual input, and its rotating motion elicited an unstable retinal image. Hence, in the beginning, the trace of the mouse's eye movement represented in a sine curve was only partially overlapping with the trace of the drum's kinetics, but as the mouse underwent more sessions of OKR learning, the trace of eye movement became almost identical (**Figure 2Bi** [↗](#)). Notably, the group with inhibited CF transmission exhibited only slight changes or no improved trace of eye movements (**Figure 2Bii** [↗](#)). Overall, the results demonstrated that CF transmission is required for motor memory acquisition.

This indicates that CF transmission is essential for cerebellar motor learning by presenting two contrasting learning curves. The control group exhibited an increasing trend in the learning curve, whereas the NpHR group's learning curve was almost flat, which reflects no change of gain (**Figure 2C** [↗](#)). The gain value through the learning sessions was distinctly increased in the control group, whereas the NpHR group showed no significant change of gain (**Figure 2D** [↗](#)). Furthermore, from 0 to 50 min period of learning, the control group demonstrated almost a doubling in gain, whereas the NpHR group showed little or almost no change of gain (**Figure 2E** [↗](#)). These results indicate that CF transmission is required for the proper acquisition of motor memory.

Inhibition of climbing fiber transmission during memory consolidation and retrieval phase

When memories are initially acquired, they are generally unstable; hence, it requires some time to shape a stabilized long-term memory (Attwell et al., 2002 [↗](#)). Therefore, memories undergo multiple processes that allow distinct shifts from short-term to long-term memory, leading to the construction of adapted behavior (Shutoh et al., 2006 [↗](#)). Having confirmed the active

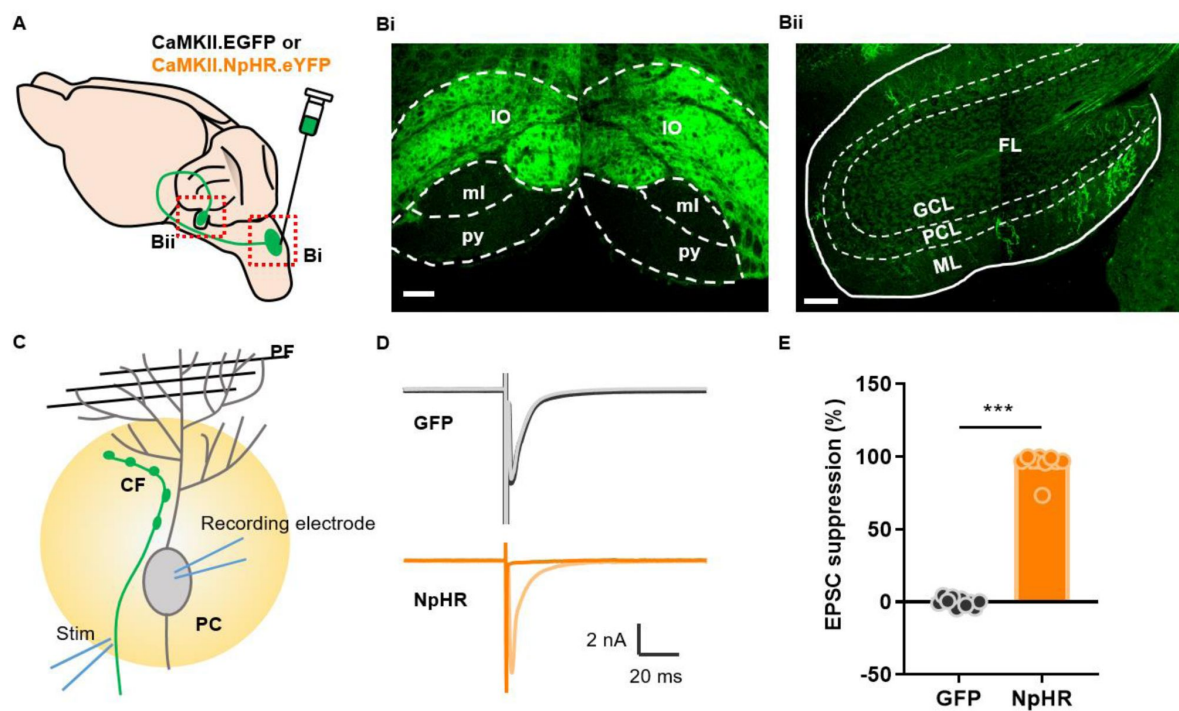


Figure 1.

Optogenetic inhibition of climbing fiber transmission derived from the inferior olive.

- A. Schematic diagram showing virus injection for optogenetic modulation of CF.
- B. Bi. A virus is expressed specifically only in the IO region (IO, inferior olive; py, pyramidal tract; ml, medial lemniscus). Scale bar, 100 μ m. Bii. Virus expression along CF in the cerebellar flocculus region. (GCL, granule cell layer; PCL, Purkinje cell layer; ML, molecular layer; FL, flocculus). Scale bar, 100 μ m.
- C. A scheme of optogenetic suppression using a yellow laser (593 nm, 3–5 mW) while recording CF EPSCs.
- D. Representative traces of CF EPSCs of GFP (top) and NpHR group (bottom) with (dark line) and without (light line) optostimulation.
- E. Quantitative analysis of suppression of CF EPSCs (n = 9 cells for each group, *** p < 0.001; Unpaired t test).

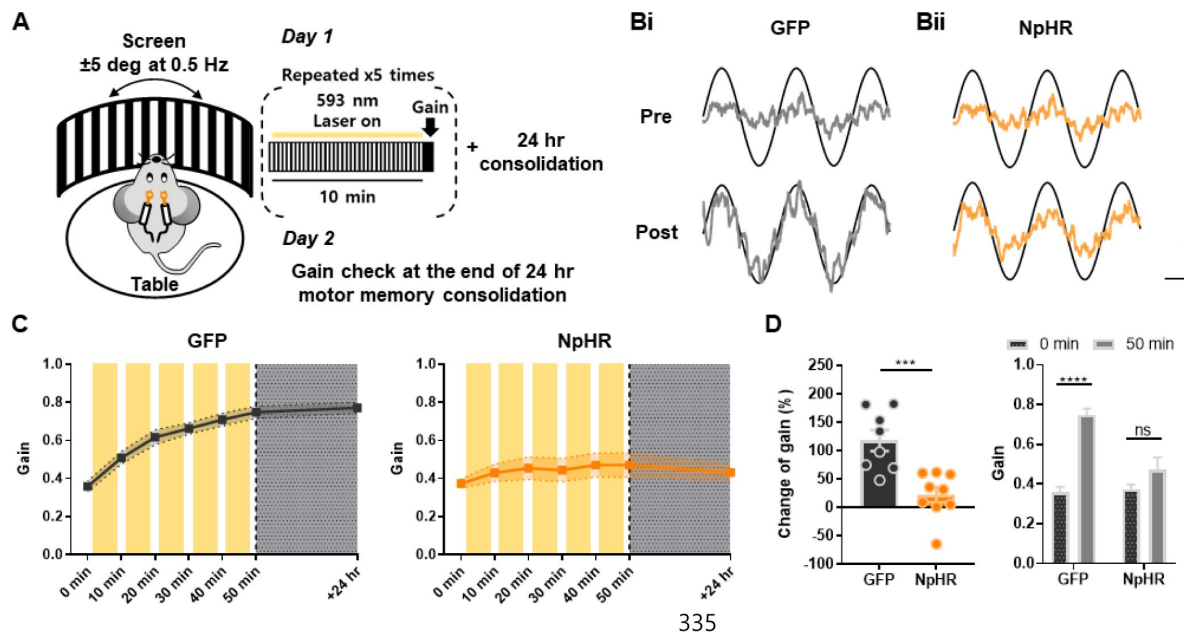


Figure 2.

Inhibition of climbing fiber transmission during memory acquisition of OKR.

A. Illustration of the optokinetic reflex behavioral test (left) and experimental scheme (right). For training, visual stimulation was given by constant rotation of the screen, while the table, where a mouse's head is fixed, remained stationary. For day 1, a mouse was trained for a total of 50 minutes, and on day 2, an amount of sustained memory was validated via gain check.

B. Representative traces of the screen (black sinusoidal curve) and eye movements (grey sinusoidal curve) divided into GFP (i) and NpHR (ii) groups. Top traces (pre) were acquired before learning, and bottom traces (post) were obtained after 50 min of learning. The vertical scale bar represents 10 degrees per second and the horizontal scale bar represents 0.5 seconds.

C. The learning curve from 0 min to 50 min, and +24 hr period. Change of gain is indicated at each time point (+10 min increment). Yellow boxes indicate opto-stimulation (12.5 mW for 10 min/session, total of 5 sessions). The left graph represents GFP (n = 8) and the right graph indicates the NpHR (n = 9) group.

D. Comparison of gain changes between GFP and NpHR groups (GFP, n = 8 mice; NpHR, n = 9 mice). Percentages of gain increment from 0 min to 50 min were calculated. The change of gain of the GFP group was significantly larger than that of the NpHR group (left; Unpaired t test, p = 0.0007). The gain of the GFP group was significantly increased from 0 min to 50 min (right; Paired t test, **** p < 0.0001), while the NpHR group showed no significant improvement of gain from 0 min to 50 min (right; Paired t test, p = 0.1046).

involvement of CF transmission during memory acquisition, we sought to understand if its role is limited to the acquisition period or extends to memory transfer during the consolidation phase for long-term storage. We implemented identical surgical and behavioral procedures as previously conducted. However, instead of optogenetically inhibiting the transmission during memory acquisition, we suppressed CF activity in two distinct optogenetic manipulation schemes: one targeting short-term inhibition (30 min) and the other for long-term inhibition (6 hr) (**Figure 3A** and **3D**). This approach was based on findings that long-term memories associated with cerebellum-dependent behaviors are vulnerable to disruption within specific time windows of consolidation, spanning from minutes to hours (Cooke et al., 2004; Titley et al., 2007). Learning curves confirmed the successful gain enhancement, validating the integrity of memory acquisition (**Figure 3B** and **3E**). Once the gain was robustly enhanced, optogenetic inhibition of CF transmission during consolidation did not yield any change in the maintenance of memory, irrespective of the duration of manipulation. Consequently, long-term memory was well-established in both NpHR and GFP groups (**Figure 3C** and **3F**). In addition, we verified whether CF signaling is required during the retrieval phase (**Figure 3G**). When mice, fully trained in the optokinetic reflex, displayed increased gain, disrupting CF signaling did not affect the retrieval of motor memory (**Figure 3H**). Thus, our findings suggest that CF activity is less likely to be essential for memory consolidation and retrieval. Its predominant effectiveness during the learning phase suggests a more concentrated role in memory acquisition.

Discussion

We examined the role of CF activity in different temporal phases of motor learning by employing optogenetic tools to specifically disrupt cortical CF transmission. Our results suggest that CF transmission is important for enhancing gain during memory acquisition but serves no critical role in conducting memory transfer during the consolidation period and recalling a maintained memory. In this study, we deliberated on these implications to expand the perspective of CF transmission in cerebellar learning. Cs is formed in response to excitatory synaptic input from the CF and is known to carry instructive signals that eventually reduce behavioral errors and enhance cerebellum-dependent motor performance (Albus, 1971; Hull, 2020; Ito, 1972; Marr, 1969). Classic theories emphasize that CF-driven Cs activity is strongly associated with motor learning; however, for various reasons, including the uncertainty of regional and temporal specificity, incomplete understanding of CF signaling remains a concern.

Once the memory is properly acquired following cerebellum-dependent learning, it is suggested to be transferred to the vestibular nuclei for long-term storage (Jang et al., 2020; Shutoh et al., 2006). By providing optogenetic stimulation to inhibit CF activity at different time points, we were able to distinctly characterize the contrasting effects of CF inhibition in different phases of memory. Our results indicated that CF transmission was pivotal to establishing strong and functional learning outcomes. However, during the process of memory transfer, it was not as essential as it was during the active learning period to acquire an increase of gain. Therefore, it is suggested that CF signaling is one of the mechanisms required for memory acquisition, but not for the transfer or retrieval of memory.

The process of cerebellum-dependent motor learning culminates in systems consolidation involving the medial vestibular nuclei (MVN), which contains post-circuitry of floccular PCs, for long-term memory formation (Shutoh et al., 2006). Because CF signaling is one of the critical factors for shaping floccular PC firing, we expected that it would indirectly modulate the activity of MVN, and thus, it would eventually affect the consolidation and retrieval of memory. However, our results found no evident influence on long-term memory formation and retrieval, which also implies the absence of a direct impact of CF signaling on MVN activity. Previous studies have suggested that intrinsic plasticity in PCs is essential for memory consolidation (Jang et al., 2020) and that the functional role of granule cells is pivotal for retrieving motor memory (Wada et al.,

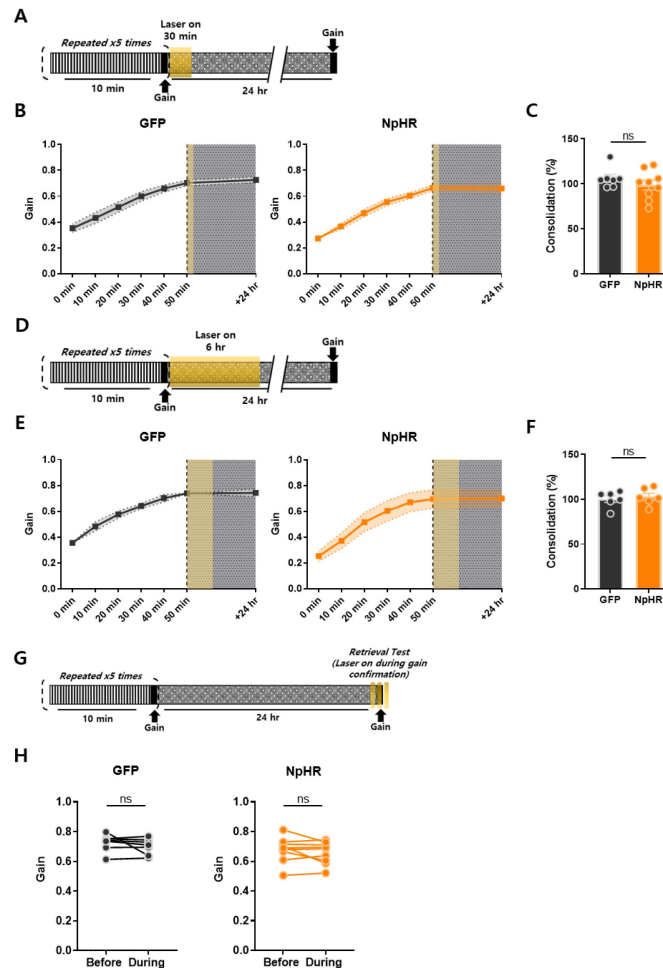


Figure 3.

Inhibition of climbing fiber transmission during memory consolidation and retrieval phase.

A. Illustration of the experimental scheme. 10 min of optokinetic reflex training was repeated 5 times, and then opto-stimulation (12.5 mW) was given at 0 min post-learning for 30 min.

B. The learning curve from 0 min to 50 min, and +24 hr period. Change of gain is indicated at each time point (+10 min increment). A yellow box indicates opto-stimulation at 0 min post-learning. The left graph represents GFP and the right graph indicates the NpHR group.

C. Comparison of consolidation percentage between GFP and NpHR groups (GFP group, n = 7 mice; NpHR group, n = 9 mice). The percentage of sustained memory after 24 hr was calculated. No difference was observed between the groups (p = 0.3173; Unpaired t test).

D. Illustration of the experimental scheme. 10 min of optokinetic reflex training was repeated 5 times, and then opto-stimulation (12.5 mW) was given at 0 min post-learning for 6 hr.

E. The learning curve from 0 min to 50 min, and +24 hr period. Change of gain is indicated at each time point (+10 min increment). A yellow box indicates opto-stimulation at 0 min post-learning. The left graph represents the GFP group, and the right graph indicates the NpHR group.

F. Comparison of gain changes between GFP and NpHR groups (GFP group, n = 6 mice; NpHR group, n = 6 mice). Percentages of gain increment from 0 min to 50 min were calculated. No difference was observed between the groups (Unpaired t test, p = 0.6405).

G. Illustration of the experimental scheme. Following proper motor learning on day 1, the remaining gain value was checked on day 2, after testing 1st gain retrieval without opto-stimulation, 2nd gain retrieval was tested with opto-stimulation to validate the effect of CF inhibition on gain retrieval (opto-stimulation was given 3 times for 24 seconds each at 12.5 mW).

H. Gain retrievals before and during opto-stimulation were compared. The left graph represents the GFP group, and the right shows the NpHR group (GFP group, n = 6 mice; NpHR group, n = 8 mice). There was no significant variation between the groups (NpHR group, p = 0.2439; GFP group, p = 0.3180; Paired t test).

2014 [↗](#)). Consequently, interfering with CF activity during consolidation did not induce significant alterations, indicating a more pronounced role of CF in memory acquisition. Accordingly, we believe there are distinct and segregated functions of substructures in the cerebellar circuit for memory processing.

The underlying mechanism of disrupted motor memory through robust suppression of CF signaling may be described as long-term depression (LTD) of CF-PC synapse, characterized by a distinct reduction in the depolarization peak of Cs ([Hansel & Linden, 2000](#) [↗](#)). CF firing typically supplements the induction of parallel fiber-Purkinje cell (PF-PC) LTD, facilitated by calcium influx through voltage-gated channels, when errors like retinal slip trigger Cs ([Coesmans et al., 2004](#) [↗](#)). However, the presence of CF-LTD alters the waveform of Cs and attenuates CF-evoked large dendritic calcium transients ([Weber et al., 2003](#) [↗](#)). Therefore, in the present study, the attempt at optogenetic manipulation to abolish CF activity during the learning period reproduced CF-LTD, impacting PF-PC LTD, susceptible to changes of CF-evoked calcium transients ([Han et al., 2007](#) [↗](#)), potentially disrupting PF-PC plasticity and subsequently impairing gain enhancement.

In conclusion, this study revealed that mice with suppressed CF signaling demonstrated a highly specific impairment of gain increase for memory acquisition. Our evidence provides a clear view of the role of CF in cerebellum-dependent motor learning; however, the underlying mechanisms remain to be fully elucidated. Future investigations on the dynamics of CF transmission in live neurons using fluorescent probes may reveal detailed mechanisms including transient calcium dynamics.

Materials and Methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Virus	AAV9.CaMKII α .EGFP	Addgene	RRID: Addgene_50469	Plasmid #50469
Virus	AAV1.CaMKII α .eNpHR3.0.EYFP	Addgene	RRID: Addgene_26971	Plasmid #26971
Chemical compound, drug	Zoletil	Virvac		
Chemical compound, drug	Rompun	Bayer		
Chemical compound, drug	Dexamethasone	Samyang Pharmaceutical		
Chemical compound, drug	Meloxicam	Boehringer Ingelheim		
Software, algorithm	ZEN	Zeiss	RRID: SCR_013672	
Software, algorithm	Fiji	http://fiji.sc	RRID: SCR_002285	
Software, algorithm	Patchmaster	HEKA	RRID: SCR_000034	
Software, algorithm	Igor Pro	WaveMetrics	RRID: SCR_000325	
Software, algorithm	GraphPad Prism	GraphPad	RRID: SCR_002798	

Animals and Stereotaxic Surgery for Virus Injection

All experimental procedures were approved by the Seoul National University Institutional Animal Care and Use Committee. First, wild-type mice aged 7–10 weeks were anesthetized using intraperitoneal injections of a Zoletil/Rompun mixture (30 mg / 10 mg/kg). For optogenetic expression in CF, the virus was injected into the IO 2–3 weeks before the behavioral test, as previously described (Kimpo et al., 2014 [DOI](#); Roh et al., 2020 [DOI](#)). Briefly, bilateral injections were made at the midpoint between the edge of the occipital bone and the C1 cervical vertebra. The glass pipette was set at a 55° angle from the vertical and 7° from the midline. After approaching a 3 mm depth, a virus solution containing 100–200 nL of AAV1-CaMkII α -eNpHR3.0-EYFP or AAV9.CaMkII α -EGFP was injected with a Picopump at 5 nL/s. The pipettes were left in place for 10 min before they were removed to minimize backflow.

Stereotaxic Surgery for Optic Cannula Implantation

After the mice were fully recovered (a few days to a week) from the first surgery of virus injection, a second operation of optic cannula implantation (Ø1.25 mm Ceramic Ferrule, L=3.0 mm) near cerebellar flocculus and head fixation was conducted. Unlike the fiber optic cannula, head fixation parts were hand-made. Before the surgery, mice were anesthetized by intraperitoneal injection of Zoletil 50 (Virbac, 15 mg/kg) and xylazine (Rompun, Bayer, 15 mg/kg) mixture. Mice were given 48 hours to recover after surgery.

Confirmation Viral Expression

After completion of all the experiments, sampling using cardiac perfusion is performed. The brain was then extracted and coronal sections were made at 30 µm intervals. Images were acquired and processed using a confocal microscope (Zeiss LSM 7 MP, Carl Zeiss, Jena, Germany) and Zen software (Zeiss). The location of virus expression was confirmed by comparison with the brain atlas (Paxinos and Franklin's The Mouse Brain in Stereotaxic Coordinates 4th Edition by George Paxinos).

Optical suppression of CF terminals in the cerebellar cortex in vivo

To manipulate CF transmission-induced Cs in PCs, AAV1.CaMKIIα.eNpHR3.0.eYFP or AAV8.CaMKIIα.eGFP was bilaterally injected into the IO. A glass micro-pipette was used to inject 200 nL per site. To optically suppress the CF terminals and record CF-PC synaptic response, a small cranial window was made above the cerebellar cortex lobule IV/V a week after the viral injection. After 2 weeks from the viral injection, mice underwent single-unit recordings. The recordings were performed as described in Neural data acquisition and analysis. Cs responses were acquired during 10 seconds of yellow on and off sessions 20 times to test the effect of CF terminal suppression on CF-PC synaptic transmission.

Neural data acquisition and analysis

Single-unit recordings were performed while mice were head-fixed and awake in the recording rack. A Digital Lynx system (Neuralynx, Bozeman, MT) was used to amplify, band pass filter (0.1–8000 Hz for simple spikes, 10–200 Hz for complex spikes), and digitize the electrode recordings. A silicon neural probe (Cambridge NeuroTech) was used to probe and acquire Cs signals. Cs signals were collected at a 32 kHz sampling rate. Cs waveforms were manually selected based on visual inspection of the averaged appearance of characteristic negative Ca^{2+} peaks.

Slice preparation, electrophysiology, and optogenetic validation

An acute brain slice preparation and electrophysiological experiments were carried out as previously described (Lee et al., 2023 [DOI](#)). First, mice aged 9–10 weeks with AAV1.CaMKIIα.eNpHR3.0.WPRE-EYFP injection into their inferior olivary neuron 2–3 weeks before the preparation were anesthetized by isoflurane and briefly decapitated. Then, 250 µm-thick sagittal slices of the cerebellar vermis were obtained from the mice using a vibratome (VT1200S, Leica). The ice-cold cutting solution contained 75 mM sucrose, 75 mM NaCl, 2.5 mM KCl, 7 mM MgCl₂, 0.5 mM CaCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 25 mM glucose with bubbled 95% O₂ and 5% CO₂. The slices were immediately transferred into the artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose with bubbled 95% O₂ and 5% CO₂. They were allowed to recover at 32 °C for 30 min and at room temperature for 1 hour. All recordings were performed within 8 hr of recovery.

Brain slices were placed in a submerged chamber with perfusion of ACSF for at least 10 min before recording. We used OptoPatcher (HEKA Elektronik) holding recording pipette (2–3 MΩ) filled with internal solution containing 9 mM KCl, 10 mM KOH, 120 mM K-gluconate, 3.48 mM

MgCl₂, 10 mM HEPES, 4 mM NaCl, 4 mM Na₂ATP, 0.4 mM Na₃GTP, and 17.5 mM sucrose (pH 7.25). The stimulation electrode was placed on the granule cell layer near the PC. The stimulation isolator injected a brief current pulse to the stimulation electrode and was controlled using PatchMaster software (HEKA Elektronik). Stimulation intensity (6–30 μ A) was calibrated to minimally evoke CF EPSC. Regarding the laser on trial, a 593.5 nm irradiation from a yellow laser was applied 5 s before CF stimulation. The eEPSC amplitude was averaged over three lasers on and off trials. Electrophysiological data were acquired using an EPC10 patch-clamp amplifier (HEKA Elektronik) and PatchMaster software (HEKA Elektronik) with a sampling frequency of 20 kHz, and the signals were filtered at 2 kHz. All electrophysiological recordings were acquired from the central cerebellar vermis. The amplitude of EPSC was analyzed using Igor Pro (WaveMetrics).

Optokinetic reflex behavior test

First, two sessions of acclimation were conducted. During each session, the mouse was restrained using a custom-made restrainer for 20 min both with and without light for habituation to the recording environment. Following the procedure reported by Stahl et al. (Stahl, 2002 [Stahl, 2002](#)), a calibration was performed. This allowed for the conversion of the dynamics of pupil to eye rotation. Next, three basal oculomotor performances including (OKR), vestibulo-ocular reflex in the dark (dVOR), and vestibulo-ocular reflex in the light (IVOR) were measured. During the recording of eye movements, it was necessary to control pupil dilation that was induced by an absence of light in dark condition. Thus, physostigmine salicylate solution (Eserine; Sigma Millipore) was given to mice under brief isoflurane anesthesia. The concentration of eserine solution was increased from 0.1%, 0.15%, and 0.2% based on the pupil size. Basal oculomotor performances were described in gains. For the actual learning protocol, we adopted OKR and it consisted of five training sessions (10 min per session), six checkup points, and 24 hr of consolidation period. During the training sessions, the mice were visually stimulated with a sinusoidally rotating drum (± 5 degrees). Completely trained mice were placed back into their home cage, which was stored in the dark condition until the last gain checkup points.

Gain Analysis

Gains acquired from basal oculomotor performances and OKR learning were calculated as the ratio of evoked eye movements to the movement of the screen or turn table as visual or vestibular stimuli, respectively. A custom-built LabView (National Instrument) analysis tool was used for all the calculations

Statistical analysis

Graph plotting and statistical analysis were performed using GraphPad Prism (GraphPad Software Inc, CA, USA). The hypothesis was tested by using paired and unpaired t-tests between sample pairs using GraphPad Prism. Statistical significance was set at $p < 0.05$. Asterisks denoted in the graph indicate the statistical significance. * indicates $p < 0.05$, ** < 0.01 , *** < 0.001 , and **** < 0.0001 . The test name and statistical values are presented in each figure legend.

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Competing interest

The authors declare that they have no conflicts of interest.

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Reviewer #1 (Public Review):

Summary:

The study by Seo et al highlights knowledge gaps regarding the role of cerebellar complex spike (CS) activity during different phases of learning related to optokinetic reflex (OKR) in

mice. The novelty of the approach is twofold: first, specifically perturbing the activity of climbing fibers (CFs) in the flocculus (as opposed to disrupting communication between the inferior olive (IO) and its cerebellar targets globally); and second, examining whether disruption of the CS activity during the putative "consolidation phase" following training affects OKR performance.

The first part of the results provides adequate evidence supporting the notion that optogenetic disruption of normal CF-Purkinje neuron (PN) signaling results in the degradation of OKR performance. As no effects are seen in OKR performance in animals subjected to optogenetic irradiation during the memory consolidation or retrieval phases, the authors conclude that CF function is not essential beyond memory acquisition. However, the manuscript does not provide a sufficiently solid demonstration that their long-term activity manipulation of CF activity is effective, thus undermining the confidence of the conclusions.

Strengths:

The main strength of the work is the aim to examine the specific involvement of the CF activity in the flocculus during distinct phases of learning. This is a challenging goal, due to the technical challenges related to the anatomical location of the flocculus as well as the IO. These obstacles are counterbalanced by the use of a well-established and easy-to-analyse behavioral model (OKR), that can lead to fundamental insights regarding the long-term cerebellar learning process.

Weaknesses:

The impact of the work is diminished by several methodological shortcomings.

Most importantly, the key finding that prolonged optogenetic inhibition of CFs (for 30 min to 6 hours after the training period) must be complemented by the demonstration that the manipulation maintains its efficacy. In its current form, the authors only show inhibition by short-term optogenetic irradiation in the context of electrical-stimulation-evoked CSs in an ex vivo preparation. As the inhibitory effect of even the eNpHR3.0 is greatly diminished during seconds-long stimulations (especially when using the yellow laser as is done in this work (see Zhang, Chuanqiang, et al. "Optimized photo-stimulation of halorhodopsin for long-term neuronal inhibition." *BMC biology* 17.1 (2019): 1-17.), we remain skeptical of the extent of inhibition during the long manipulations. In short, without a demonstration of effective inhibition throughout the putative consolidation phase (for example by showing a significant decrease in CS frequency throughout the irradiation period), the main claim of the manuscript of phase-specific involvement of CF activity in OKR learning can not be considered to be based on evidence.

Second, the choice of viral targeting strategy leaves gaps in the argument for CF-specific mechanisms. CaMKII promoters are not selective for the IO neurons, and even the most precise viral injections always lead to the transfection of neurons in the surrounding brainstem, many of which project to the cerebellar cortex in the form of mossy fibers (MF). Figure 1Bii shows sparsely-labelled CFs in the flocculus, but possibly also MFs. While obtaining homogenous and strong labeling in all floccular CFs might be impossible, at the very least the authors should demonstrate that their optogenetic manipulation does not affect simple spiking in PNs.

Finally, while the paper explicitly focuses on the effects of CF-evoked complex spikes in the PNs and not, for example, on those mediated by molecular layer interneurons or via direct interaction of the CF with vestibular nuclear neurons, it would be best if these other dimensions of CF involvement in cerebellar learning were candidly discussed.

<https://doi.org/10.7554/eLife.95838.1.sa1>

Reviewer #2 (Public Review):**Summary:**

The authors aimed to explore the role of climbing fibers (CFs) in cerebellar learning, with a focus on optokinetic reflex (OKR) adaptation. Their goal was to understand how CF activity influences memory acquisition, memory consolidation, and memory retrieval by optogenetically suppressing CF inputs at various stages of the learning process.

Strengths:

The study addresses a significant question in the cerebellar field by focusing on the specific role of CFs in adaptive learning. The authors use optogenetic tools to manipulate CF activity. This provides a direct method to test the causal relationship between CF activity and learning outcomes.

Weaknesses:

Despite shedding light on the potential role of CFs in cerebellar learning, the study is hampered by significant methodological issues that question the validity of its conclusions. The absence of detailed evidence on the effectiveness of CF suppression and concerns over tissue damage from optogenetic stimulation weakens the argument that CFs are not essential for memory consolidation. These challenges make it difficult to confirm whether the study's objectives were fully met or if the findings conclusively support the authors' claims. The research commendably attempts to unravel the temporal involvement of CFs in learning but also underscores the difficulties in pinpointing specific neural mechanisms that underlie the phases of learning. Addressing these methodological issues, investigating other signals that might instruct consolidation, and understanding CFs' broader impact on various learning behaviors are crucial steps for future studies.

<https://doi.org/10.7554/eLife.95838.1.sa0>

Author response:***eLife assessment***

This study presents potentially valuable insights into the role of climbing fibers in cerebellar learning. The main claim is that climbing fiber activity is necessary for optokinetic reflex adaptation, but is dispensable for its long-term consolidation. There is evidence to support the first part of this claim, though it requires a clearer demonstration of the penetrance and selectivity of the manipulation. However, support for the latter part of the claim is incomplete owing to methodological concerns, including unclear efficacy of longer-duration climbing fiber activity suppression.

We sincerely appreciate the thoughtful feedback provided by the reviewer regarding our study on the role of climbing fibers in cerebellar learning. Each point raised has been carefully considered, and we are committed to addressing them comprehensively. We acknowledge the importance of addressing methodological concerns, particularly regarding the efficacy of long-term suppression of CF activity, as well as ensuring clarity regarding penetrance and selectivity of our manipulation. To this end, we have outlined plans for substantial revisions to the manuscript to adequately address these issues.

Public Reviews:

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The study by Seo et al highlights knowledge gaps regarding the role of cerebellar complex spike (CS) activity during different phases of learning related to optokinetic reflex (OKR) in mice. The novelty of the approach is twofold: first, specifically perturbing the activity of climbing fibers (CFs) in the flocculus (as opposed to disrupting communication between the inferior olive (IO) and its cerebellar targets globally); and second, examining whether disruption of the CS activity during the putative "consolidation phase" following training affects OKR performance.

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We appreciate the thorough review and recognize both the strengths and weaknesses highlighted.

We concur with the reviewer's assessment of the novelty of our approach, particularly in specifically perturbing the activity of CF in the flocculus and examining the effects during different phases of learning. Also the usage of OKR behavior paradigm adds strength to our study by providing a well-established model for investigating cerebellar learning processes.

Regarding concerns about the efficacy of long-term optogenetic inhibition and the specificity of viral targeting, we are committed to addressing these issues through additional experiments. Specifically, we aim to demonstrate sustained inhibition of CF transmission by verifying the maintenance of inhibition throughout the putative consolidation phase. This may involve monitoring CF activity during the irradiation period in vivo. Furthermore, we plan to provide further characterization of viral targeting to ensure specificity of our approach.

Additionally, we recognize the importance of discussing alternative mechanisms of CF involvement in cerebellar learning. Hence, we will expand the manuscript to provide more comprehensive discussion of these dimensions of CF function to provide a clearer understanding of the broader implications of our findings.

Reviewer #2 (Public Review):

Summary:

The authors aimed to explore the role of climbing fibers (CFs) in cerebellar learning, with a focus on optokinetic reflex (OKR) adaptation. Their goal was to understand how CF activity influences memory acquisition, memory consolidation, and memory retrieval by optogenetically suppressing CF inputs at various stages of the learning process.

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consolidation, and understanding CFs' broader impact on various learning behaviors are crucial steps for future studies.

We appreciate the reviewer's recognition of the significance of our study in addressing the fundamental question of the role of CF in adaptive learning within the cerebellar field. The use of optogenetic tools indeed provides a direct means to investigate the causal relationship between CF activity and learning outcomes.

To address concerns regarding the effectiveness of CF suppression during consolidation, we plan to conduct further in-vivo recordings. These will demonstrate how reliably CF transmission can be suppressed through optogenetic manipulation over an extended period.

In response to the concern about potential tissue damage from laser stimulation, we believe that our optogenetic manipulation was not strong enough to induce significant heat-induced tissue damage in the flocculus. According to Cardin et al. (2010), light applied through an optic fiber may cause critical damage if the intensity exceeds 100 mW, which is eight times stronger than the intensity we used in our OKR experiment. Furthermore, if there had been tissue damage from chronic laser stimulation, we would expect to see impaired long-term memory reflected in abnormal gain retrieval results tested the following day. However, as shown in Figures 2 and 3, there were no significant abnormalities in consolidation percentages even after the optogenetic manipulation.

Finally, we appreciate the reviewer's recognition of the challenges involved in pinpointing specific neural mechanisms. We plan to expand the discussion to address these complexities and outline future research directions.

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