

Synchronous climbing fiber activity enables instructive signaling for cerebellar learning through modulation of disinhibitory circuits

Received: 16 October 2023

Accepted: 13 March 2026

Published online: 14 May 2026

 Check for updates

Changjoo Park ^{1,2,15,20}, Zhen Yang ^{3,20}, Abdulraheem Nashef ^{3,20},
Jawon Gim ^{2,4,20}, Sangkyu Bahn ^{2,5,20}, Gyu Hyun Kim ^{4,6,20}, Ke Zhang ^{7,8,16,20},
Laurence Cathala ^{3,20}, Sungho Hong ^{9,20}, Yoonseok Im ^{1,2,10,17},
Sang-Hoon Lee ^{4,18}, Kisuk Lee ^{11,12,19}, Min-Soo Kim ¹³, Don B. Arnold ¹⁴,
Kea Joo Lee ⁴ , Jason M. Christie ³  & Jinseop S. Kim ^{1,2} 

Motor learning relies on signals that instruct adaptive plasticity following errors. In the cerebellum, climbing fibers (CFs) provide these instructions to Purkinje cells (PCs). Yet CFs fire continuously, even without errors, requiring molecular layer interneuron (MLI) inhibition of PCs to counteract CF excitation and prevent maladaptive plasticity. Here, to identify how this regulatory inhibition is contextually suppressed to selectively permit error-driven learning in mice, we combined connectomics, functional recordings, computational modeling and behavioral manipulations. We discovered that CFs target not only PCs but also a specific MLI subtype that inhibits PC-targeting MLIs, creating serial disinhibition. These disinhibitory MLIs integrate multiple CFs, causing increased activation with CF synchrony. This stronger disinhibitory drive allows larger CF-evoked calcium responses in PCs. Disruption of MLI-to-MLI inhibition prevents CF-instructed motor learning, confirming the necessity of this disinhibitory pathway. Therefore, population synchrony selectively enables CF-driven plasticity through disinhibitory network interactions, demonstrating that instructive signaling is a product of circuit-level processing.

Adaptive motor control requires neural circuits that can modify their spiking output pattern in response to past performance errors. The cerebellum is a brain area central to this process¹, relying on climbing fiber (CF) input from the inferior olive to guide the acquisition of adaptation behavior². Classic theories^{3–5} propose that CFs specifically signal motor errors to instruct corrective plasticity induction in post-synaptic Purkinje cells (PCs). Yet it is well known that CFs discharge persistently across a diversity of motor behaviors, including those correctly performed^{6–8}. This presents a fundamental paradox: if CFs are always active, what determines the efficacy of their input to drive corrective changes in response to motor errors? The answer may lie not in CF activity itself, but in how cerebellar circuits conditionally

process these signals. Thus, understanding the circuit substrates that gate CF influence on PCs is essential for explaining how continuous CF activity can evoke distinct circuit responses to selectively drive learning depending on network state.

A potential solution lies in the dense inhibitory network surrounding PCs. Molecular layer interneurons (MLIs) provide powerful dendritic inhibition that opposes CF-triggered plasticity at PC synapses. Prior evidence shows that concurrent MLI-mediated inhibition suppresses CF-evoked calcium transients and blocks long-term depression induction at parallel fiber (PF) inputs^{9–11}. The behavioral relevance of this inhibitory control has been demonstrated through studies showing that MLI-targeted manipulations can disrupt various forms

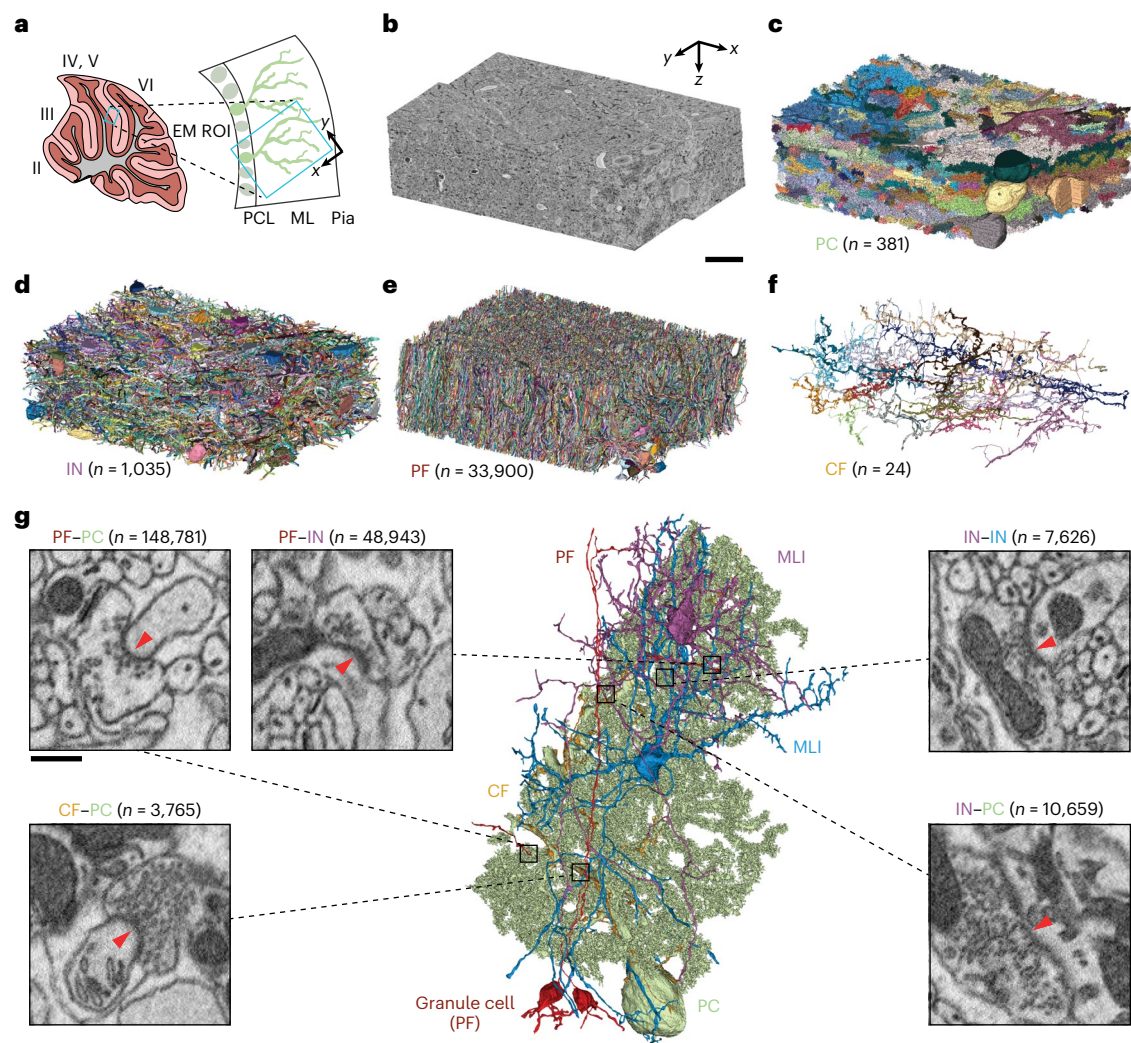


Fig. 1 | Dense reconstruction of cerebellar molecular layer circuits.

a, A mid-sagittal section of lobule IV/V of an adult mouse at P56 was sampled (cyan box), which mainly includes the molecular layer and partially the PC layer. **b**, Aligned EM volume (175 × 122 × 50 μm³) with defined axes. Scale bar, 25 μm. **c–f**, Complete 3D reconstructions of major cell types: PCs (**c**), MLIs (or INs)

(**d**), PFs (**e**) and CFs (**f**), with cell counts indicated. **g**, Representative synaptic connections between neuron types identified through semi-automated detection. The red arrowheads indicate synaptic contacts pointing from post- to presynaptic structures. The numbers indicate total synapses per connection type. Scale bar, 0.5 μm. ROI, region of interest.

of cerebellar learning^{12,13}. This suggests a core principle of cerebellar function in the service of learning: whether CF signals potently instruct motor adaptation depends not necessarily on the information they carry, but on the inhibitory context in which they arrive. Such conditional processing requires specialized inhibitory organization.

Recent studies have revealed that MLIs segregate into functionally distinct subtypes: one preferentially inhibits PCs (MLI1), while another targets PC-inhibiting MLIs (MLI2), establishing parallel pathways for direct inhibition and disinhibition^{14–16}. This organization mirrors disinhibitory circuits in forebrain regions that conditionally relieve inhibitory constraints on learning-induced plasticity^{17,18}. Beyond their structured connectivity, MLIs are responsive to CF activity¹⁹, creating dynamic engagement of the inhibitory network including feedforward PC disinhibition^{20,21}. This responsiveness becomes particularly important given that strong electrical coupling of olivary neurons produces synchronized CF spiking across their population during sensorimotor events^{22,23}, raising the possibility that synchrony levels could differentially engage these parallel inhibitory pathways.

Here, using complementary connectomics and physiological approaches, we uncover the circuit mechanisms underlying conditional

CF signaling. We demonstrate that CFs preferentially innervate disinhibitory MLIs, which integrate convergent inputs from multiple CFs, making their activation proportional to the level of CF synchrony. This specialized inhibitory connectivity creates a substrate through which population-level CF activity selectively gates plasticity at PC synapses through control of PC calcium signaling and is required for CF-mediated adaptive learning. Consequently, our findings shift the conceptual framework away from viewing CFs strictly as channels encoding motor errors toward understanding them as inputs whose instructive value emerges from their population activity and modulation of inhibitory network dynamics.

Results

Molecular layer volume reconstruction

The molecular layer of the cerebellar cortex serves as the critical zone where CF inputs, MLI inhibition and PF excitation converge on PC dendrites to generate conditional instructive signals. We reconstructed this circuit to identify the connectivity enabling such selective signal transmission. Using serial block-face electron microscopy (EM)²⁴, we imaged a volume of lobule IV/V from a mouse that captured the molecular

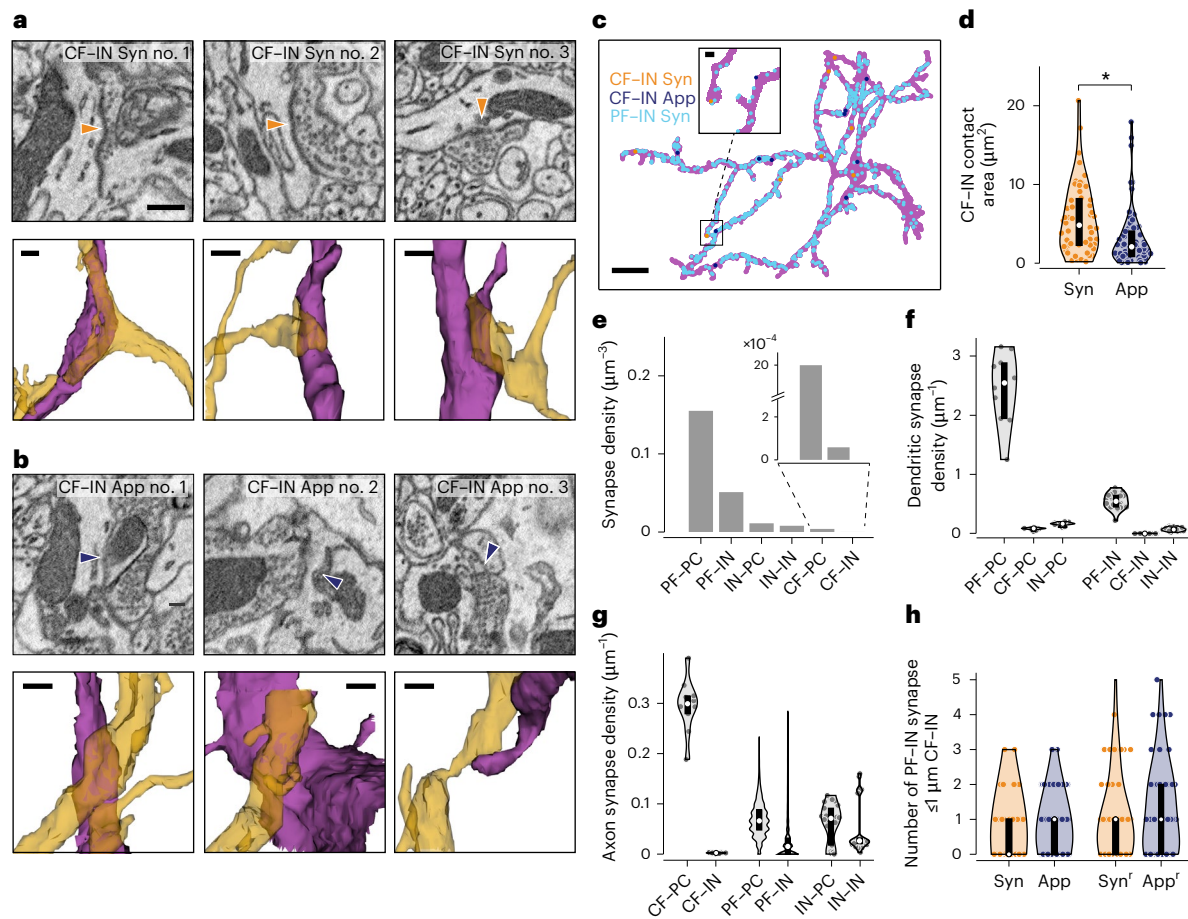


Fig. 2 | CF synapses with MLIs. **a**, Representative EM images and 3D reconstructions showing CF-MLI synapses with presynaptic vesicle clusters and postsynaptic densities. **b**, CF-MLI appositions containing vesicle clusters but lacking postsynaptic specializations. Color-coded arrowheads mark contact sites (orange, synapses; dark blue, nonsynaptic appositions). **c**, Reconstruction of an MLI showing both synaptic and nonsynaptic CF contacts alongside PF synapses. Scale bars, 0.5 μm (**a,b**) and 20 μm (**c**). **d**, CF-MLI synapses occupy larger membrane areas than nonsynaptic appositions ($n = 53$ synapses versus $n = 75$ appositions, $P = 0.00055$, unpaired two-sided t -test; box plots show

median $\pm$ quartiles; $^*P < 0.05$). **e**, Synapse density per unit volume for all connection types. **f,g**, Synapse density along dendritic (**f**; $n = 10$ PCs, $n = 26$ INs) or axonal (**g**; $n = 10$ CFs, $n = 7,367$ PFs, $n = 26$ INs) processes for each connection type (no statistical comparisons performed; box plots show median $\pm$ quartiles). **h**, Distance distributions between CF-MLI contacts and nearest PF-MLI synapses. Observed synaptic (Syn) and nonsynaptic appositions (App) show no enrichment compared to randomized spatial distributions, which serve as a control (Syn', App'; $P = 0.233$; $n = 53$ for Syn', $n = 75$ for App'; one-way ANOVA test; box plots show median $\pm$ quartiles).

layer and a portion of the PC layer (Fig. 1a,b and Data availability). We analyzed the image volume using a semi-automated reconstruction pipeline²⁵ comprising machine-learning segmentation followed by extensive manual proofreading. The reconstruction covered an estimated 60.3% of the entire volume (Methods). Morphology-based classification of reconstructed segments identified canonical cell types including PCs, MLIs (or INs), PFs and CFs (Fig. 1c–f and Data availability).

Next, we mapped connectivity using automated synapse detection, which we previously showed to achieve >95% accuracy for a small sample of the dataset²⁶. We extensively proofread the identified synapses, ensuring detection of all connection types (Methods). This yielded 219,774 synapses comprising connections between PCs, MLIs (or INs), CFs and PFs (Fig. 1g). For detailed connectivity analyses, we selected 10 PCs (Supplementary Fig. 1) and 26 MLIs (Supplementary Fig. 2) with intact somata or large reconstructed volumes ($>5 \times 10^8$ and $>6 \times 10^7$ voxels, respectively), along with 10 CFs (Supplementary Fig. 3) innervating these PCs and all 33,900 PFs. The remaining cell fragments were retained for supplementary analyses. Unlike a recent study that sampled connectivity across a larger volume²⁷, our reconstruction within this compact region enabled detection of complete local circuits, including rare but functionally critical connections.

CF-MLI synaptic communication

Our EM volume revealed principles of cellular organization. Notably, CFs predominantly formed planar arbors oriented along the faces of PC dendrites and only occasionally exhibiting the conventionally described helical winding pattern^{28–30} (Extended Data Fig. 1a,b). Since MLIs innervate the same dendritic domains of PCs, we tested whether this CF arrangement facilitates interactions with MLIs. Indeed, angular distributions confirmed both CF and MLI dendrites aligned with PC dendritic planes while PFs projected in an orthogonal orientation (Extended Data Fig. 1c–f). The extent of proximity suggested contacts and functional interactions between CFs and MLIs warranting closer examination.

We therefore systematically cataloged all CF-MLI appositions within our volume, identifying 997 close membrane contacts including 128 autodetected synapses. We manually inspected these 128 sites for ultrastructural hallmarks of synapses, including presynaptic vesicle clusters and postsynaptic densities. We identified structural evidence of direct CF-MLI synapses at 53 locations (Fig. 2a and Supplementary Fig. 4). The remaining 75 contacts had presynaptic vesicle clusters but lacked overt postsynaptic specializations (Fig. 2b and Supplementary Fig. 5). Both contact types were distributed across MLI dendrites (Fig. 2c). Notably, CF-MLI synapses occupied larger

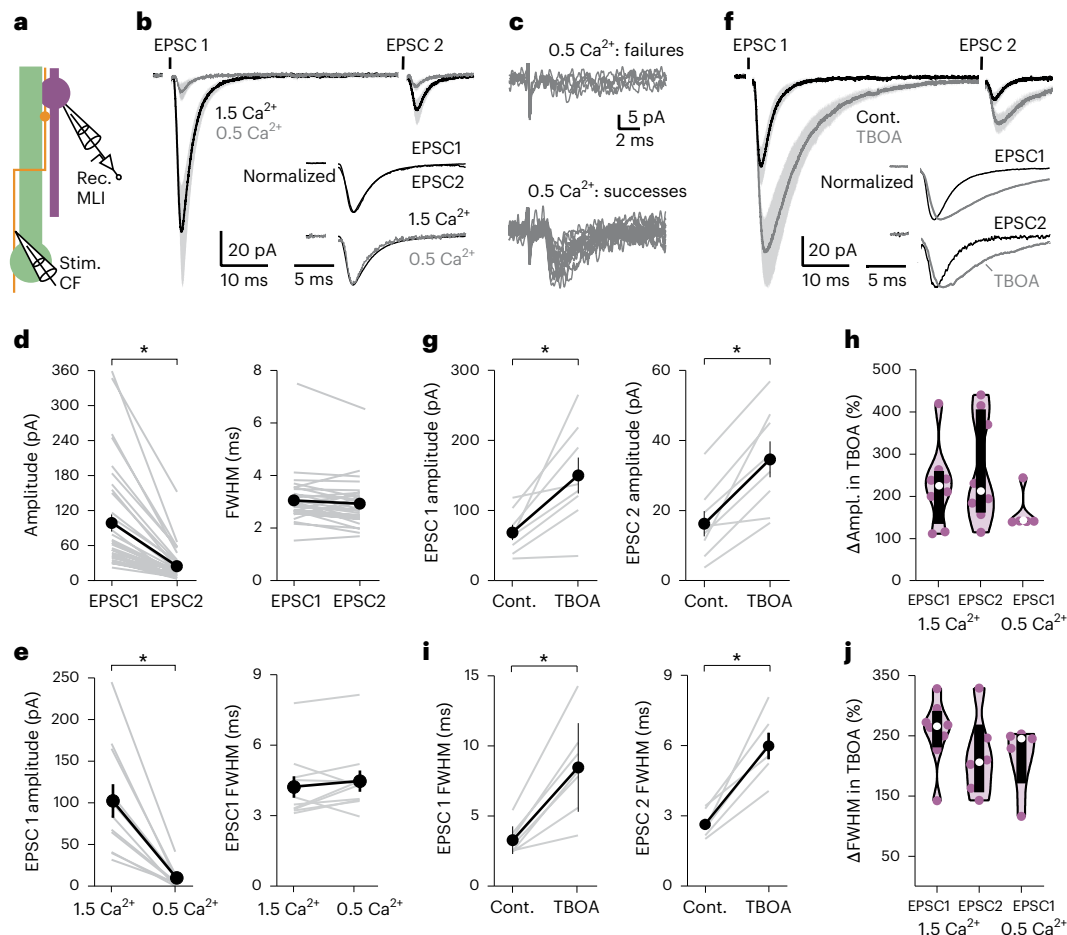


Fig. 3 | Functional identification of CF–MLI synaptic transmission. a, Configuration for whole-cell recordings from MLIs (Rec. MLI) in acute cerebellar slices with CF stimulation (Stim. CF). **b,** Population-averaged CF-evoked EPSCs recorded from MLIs ($n = 8$ from 7 mice, mean $\pm$ s.e.m.) using paired-pulse stimulation (20 Hz) in physiological and lowered external calcium (1.5 mM and 0.5 mM, respectively). The insets compare normalized average EPSCs with different quantal contents (top, EPSC1 and EPSC2 in 1.5 mM Ca^{2+} ; bottom, EPSC1 in 1.5 versus 0.5 mM Ca^{2+}). **c,** Individual CF stimulation trials recorded from an MLI in low P_{r} conditions (0.5 mM Ca^{2+}) displaying failed (top) and successful (bottom) transmission events demonstrating quantal-like synaptic properties. **d,** Paired CF-evoked EPSCs exhibit strong paired-pulse depression in physiological calcium (left: $75.1\% \pm 1.6\%$ decrease, $P = 0.0001$, $n = 36$ cells from 31 mice) while their durations remain similar (right, $P = 0.0555$, $n = 29$ cells from 23 mice; both two-sided Wilcoxon tests; *, $P < 0.05$). **e,** When external calcium is lowered, EPSC1 amplitude decreases between 1.5 mM Ca^{2+} and 0.5 mM Ca^{2+} (left, $91.4\% \pm 1.6\%$, $P = 0.001$, $n = 11$ cells from 9 mice) without change to the duration of the responses (right, $P = 0.09$, both two-sided Wilcoxon tests, both mean $\pm$ s.e.m.). **f,** Population-averaged CF-evoked EPSCs recorded from MLIs ($n = 8$ from 8 mice, mean $\pm$ s.e.m.) in 1.5 mM Ca^{2+} and following glutamate uptake blockade (50 μM

TBOA). The insets compare normalized average EPSCs (top, EPSC1; bottom, EPSC2) in both conditions. **g, h,** TBOA increases EPSC amplitudes of both EPSC1 ($P = 0.0078$, $n = 8$ cells from 8 mice) and EPSC2 ($P = 0.0078$, $n = 8$ cells from 8 mice, both two-sided Wilcoxon tests) in 1.5 mM Ca^{2+} (**g**) as well as EPSC1 in 0.5 mM Ca^{2+} (not shown; $P = 0.03$, $n = 7$ cells from 6 mice, two-sided Wilcoxon tests) with similar fold changes between conditions (**h**; for EPSC1 versus EPSC2 in 1.5 mM Ca^{2+} , $P = 0.46$, two-sided Wilcoxon test and for EPSC1 in 1.5 mM Ca^{2+} versus 0.5 mM Ca^{2+} , $P = 0.435$, two-sided Mann–Whitney U test; plot shows median $\pm$ quartiles). **i, j,** TBOA prolongs the duration of both EPSC1 ($P = 0.0078$, $n = 8$ cells from 8 mice) and EPSC2 ($P = 0.0312$, $n = 6$ cells from 6 mice; both two-sided Wilcoxon tests) in 1.5 mM Ca^{2+} (**i**) as well as EPSC1 in 1.5 mM Ca^{2+} versus 0.5 mM Ca^{2+} (not shown; $P = 0.0625$, $n = 5$ cells from 4 mice; two-sided Wilcoxon tests) with similar fold changes despite different quantal contents (**j**; for EPSC1 versus EPSC2 in 1.5 mM Ca^{2+} , $P = 0.2188$; for EPSC1 0.5 mM Ca^{2+} , $P = 0.124$, both two-sided Mann–Whitney U tests; plot shows median $\pm$ quartiles). In plots **d–i**, individual MLIs are represented by gray lines with mean $\pm$ s.e.m. (black circles). In plots **h** and **j**, individual cells (purple circles) are represented along with median $\pm$ quartiles. Cont., Control; $\Delta\text{Ampl.}$, change in amplitude; ΔFWHM , change in full width at half maximum.

membrane areas than nonsynaptic appositions (Fig. 2d) indicating that these may represent anatomically distinct contact types rather than a continuum of structural variation. Compared to all other synaptic connection types, CF–MLI synapses were the rarest in per-unit volume (5.5×10^{-5} synapses per μm^3 ; Fig. 2e) and per-unit length of pre- and postsynaptic processes (Fig. 2f,g).

The presence of vesicle clusters at CF–MLI appositions lacking postsynaptic densities raises the possibility that these contacts could support spillover-mediated transmission, where glutamate released from CFs diffuses to activate nearby AMPA receptors, a mechanism previously described for CF–MLI communication^{19,21}. Given that spillover diffusion is spatially limited ($<1 \mu\text{m}$)^{31–33}, we examined whether CF–MLI

contacts localize near PF–MLI synapses (Fig. 2c). While we found both CF–MLI synapses and nonsynaptic appositions within close proximity of PF inputs, this co-occurrence was not above chance levels. Unlike a prior study³⁴, we found that CF–MLI contacts showed no type bias near PF–MLI synapses nor enrichment relative to randomly selected locations on MLI dendrites (Fig. 2h and Extended Data Fig. 2b,c). The absence of spatial enrichment near PF synapses neither supports nor definitively excludes spillover, suggesting that if these nonsynaptic contacts mediate spillover, it occurs without structural optimization relative to existing PF synapses.

To functionally validate these anatomically identified CF–MLI contacts, we performed whole-cell recordings from MLIs in adult cerebellar

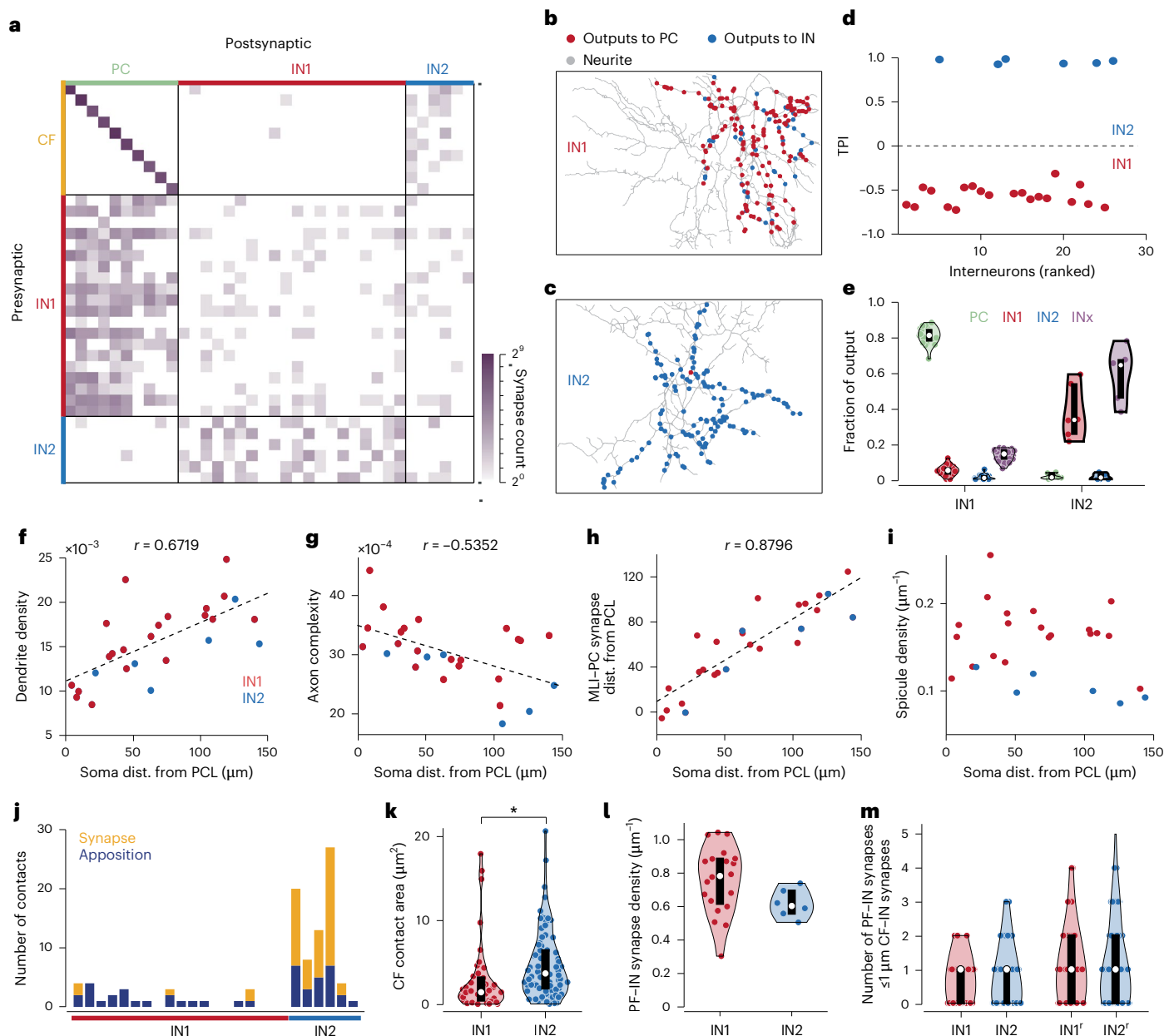


Fig. 4 | MLI network organization reveals functionally specialized interneuron populations. **a**, A weighted adjacency matrix showing the synaptic connectivity between reconstructed neurons, with color intensity representing synapse number on log scale. **b, c**, Representative MLI reconstructions with synaptic outputs color-coded by target for PC synapses (red) and MLI synapses (blue), demonstrating that IN1 cells primarily target PCs (**b**) while IN2 cells preferentially target other MLIs (**c**). **d**, TPI distribution defining two distinct MLI populations based on synaptic output patterns. **e**, Quantification of synaptic target distribution for IN1 ($n = 20$ cells) and IN2 ($n = 6$ cells) populations (no statistical comparisons performed; box plots show median $\pm$ quartile). INx indicates MLI fragments with undetermined classification. **f–i**, Relationship between MLI soma distance (dist.) from PCL boundary and dendrite density (**f**), axon complexity (**g**), average MLI–PC synapse distance (**h**) and spicule density (**i**). The red and blue dots indicate individual IN1 and IN2 cells, respectively, with linear fits as dashed lines. **j**, The distribution of CF contact types (synapses versus nonsynaptic appositions) on individual IN1 and IN2 cells. **k**, CF contact area comparison between IN1 and IN2 cells ($n = 41$ CF–IN1 contacts, $n = 87$ CF–IN2 contacts, $P = 0.026$, unpaired two-sided t -test; box plots show median $\pm$ quartiles; *, $P < 0.05$). **l**, Density of PF synapses on IN1 versus IN2 cells ($n = 20$ IN1 cells, $n = 6$ IN2 cells, $P = 0.0726$; two-sided Mann–Whitney U tests; box plots show median $\pm$ quartiles). **m**, Spatial relationship between CF contacts and PF–MLI synapses compared to random dendritic locations. IN1' and IN2' indicate randomized controls and each dot represents one CF–IN contact ($P = 0.0647$, $n = 41$ CF–IN1' contacts, $n = 87$ CF–IN2' contacts, one-way ANOVA test; box plots show median $\pm$ quartiles).

slices using minimal electrical stimulation to activate single CFs (Fig. 3a and Extended Data Fig. 3a,b; Methods). In most MLIs (37 of 54 cells), we observed excitatory postsynaptic currents (EPSCs) with a broad range of amplitudes, fast kinetics characteristic of AMPA-receptor-mediated synaptic transmission and a low paired-pulse ratio (PPR = 0.25 ± 0.02) characteristic of high release probability (P_R) transmission (Fig. 3b,d and Extended Data Fig. 3c) consistent with previous reports^{19,21}. Despite substantial amplitude reduction from EPSC1 to EPSC2 ($75.1 \pm 1.6\%$

decrease), EPSC durations remained identical (Fig. 3b,d), indicating that kinetics are independent of quantal content, providing initial evidence against glutamate accumulation from multivesicular release or spillover activation of AMPA receptors in contrast to observations made at CF–PC synapses³⁵. To confirm this interpretation, we reduced external calcium to lower P_R , reasoning that decreased release should dramatically reduce glutamate pooling. This manipulation significantly decreased EPSC1 amplitude ($91.42 \pm 1.62\%$ reduction; Fig. 3e). It also

revealed transmission failures (Fig. 3c); failure rates in responsive MLIs (7 of 11) ranged from 0.03 to 0.58 (mean of 0.48 ± 0.12). Such quantal features are diagnostic of putative single-vesicle events, providing evidence for direct synaptic connections. Importantly, lowering calcium did not alter EPSC kinetics (Fig. 3b,e), confirming that CF–MLI waveform kinetics are independent of quantal content and not shaped by glutamate pooling.

We then examined CF–MLI EPSCs in the presence of glutamate transporter blocker TBOA, reasoning that blocking clearance should exacerbate pooling for large quantal content as greater transmitter is available for spillover. However, TBOA (50 μ M) enhanced both EPSC amplitude and decay in physiological and low calcium to identical extents (Fig. 3f–j), despite differences in P_R and quantal content. To conclude, the stability of the EPSC time course, and the consistent effects of TBOA on amplitude and decay across different release conditions, argue against glutamate pooling in shaping the CF–MLI communication. These functional properties align with the distinct anatomical contact types we observed, suggesting specific point-to-point CF–MLI signaling, providing the first demonstration of rare but functional direct synapses between CFs and MLIs.

Disinhibitory MLI hierarchy

We next analyzed MLI network structure to determine how CFs engage specific components of the inhibitory circuitry. Complete connectivity matrices, weighted by the number of synapses between neuron pairs, were constructed from our dense EM volume to document synaptic connections among CFs, MLIs and PCs (Fig. 4a). This revealed that MLIs segregated into two distinct groups based on their postsynaptic targets: one group primarily targeted PCs (for example, Fig. 4b), while the other preferentially innervated MLIs (for example, Fig. 4c). To quantify this observation, we calculated a target preference index (TPI; Methods), allowing us to classify MLIs objectively based on their synaptic outputs. The resulting TPI values exhibited a clearly bimodal distribution, rather than a continuum, delineating two functionally specialized populations that we termed interneuron 1 (IN1) (TPI < 0, PC preferring) and IN2 (TPI > 0, MLI preferring) (Fig. 4d and Extended Data Fig. 4a). Among the 26 MLIs analyzed, this classification yielded 20 IN1 and 6 IN2 cells. The same subtype ratio was observed when we analyzed an additional 137 partial MLI fragments (–3.5:1; 109 IN1 versus 28 IN2), indicating a stable subtype distribution within the dataset (Extended Data Fig. 4b).

The functional specialization between IN1 and IN2 MLIs was pronounced. IN1 cells directed the majority of their synaptic outputs

(80.6%) toward PCs, and only a minority (19.4%) toward other MLIs. Conversely, IN2 cells displayed nearly exclusive targeting of other MLIs (97.4%), predominantly innervating IN1 cells (37.2% of total outputs), with very sparse direct PC innervation (2.6%) (Fig. 4e). This hierarchical

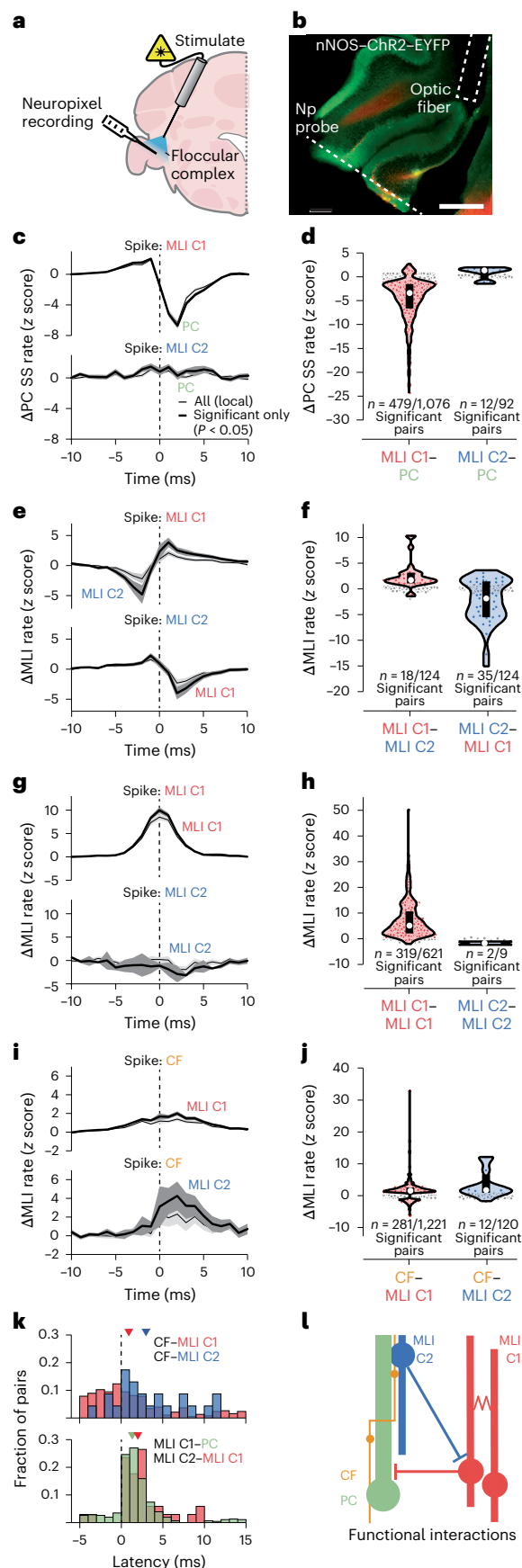


Fig. 5 | Functional classification reveals disinhibitory MLI circuitry.

a, Experimental approach combining Neuropixels recordings with MLI optotagging in the cerebellar flocculus. **b**, Histological image showing Neuropixels probe tract (dye labeled in red; MLIs expressing Chr2-YFP in green). Scale bar, 400 μ m. **c**, STAs (mean $\pm$ s.e.m.) showing PC simple spike (SS) modulation by Cluster_1 (C1) and Cluster_2 (C2) MLIs. Gray, all nearby PCs (Extended Data Fig. 5); black, significant interactions only (using two-sided paired t -test comparing firing rates during the response time to the baseline firing rate: 40–50 ms before the trigger spike). **d**, Population analysis of MLI–PC interactions. Each dot represents one MLI–PC pair; significant interactions in black outline (median $\pm$ quartiles) and pairs with nonsignificant interactions in gray. **e, f**, Directional, inter-cluster MLI–MLI connectivity analyzed as in **c** and **d**, revealing preferential Cluster_2 to Cluster_1 synaptic inhibition. The box plot represents median $\pm$ quartiles in **f, g, h**. **g, h**, Intra-cluster MLI–MLI synchrony analyzed as in **e** and **f**. Cluster_1 pairs show strong correlation (reflecting presumptive gap junctional coupling) while Cluster_2 pairs exhibit weak synchrony. **i, j**, CF-triggered responses (mean $\pm$ s.e.m.) demonstrate selective excitation of Cluster_2 MLIs (**i**) and distributions of significant interactions (median $\pm$ quartiles) are shown in **j**. **k**, Response latency distributions. Top: CF-triggered Cluster_2 responses are delayed relative to Cluster_1, which are predominantly zero-aligned. Bottom: latencies for PC–Cluster_1 and Cluster_1–Cluster_2 interactions. **l**, Summary circuit diagram illustrating functional connectivity between identified cell types. Data in **c–k** were obtained from six mice.

arrangement, in which IN2 cells inhibit IN1 cells that in turn inhibit PCs, establishes a clear disinhibitory motif within the molecular layer circuitry. Importantly, our results align with recent findings demonstrating biased connectivity between transcriptionally defined MLI1 and MLI2 subtypes^{14,15}, indicating that our connectivity-based IN1/IN2 classification identifies the same functional populations. By contrast, traditional morphological classifications (basket versus stellate cells) showed no relationship to our IN1/IN2 distinction. Morphological features such as soma position, dendrite density and axon complexity varied continuously between IN1 and IN2 (Fig. 4f–h) and failed to predict connectivity patterns. While dendritic and somatic spicule density showed some separation between IN1 and IN2 (Fig. 4i), overlapping distributions prevented unambiguous classification. This indicates that molecular identity and connectivity, rather than other anatomical features, define functionally relevant MLI subtypes.

CFs target disinhibitory MLIs to create parallel signaling pathways

To understand how CFs engage this inhibitory network, we examined CF connectivity with MLIs. While maintaining canonical one-to-one arrangements onto PCs, CFs displayed striking subtype selectivity in their direct connectivity with MLIs, forming synapses almost exclusively onto IN2 cells (Fig. 4a,j). Specifically, substantial CF innervation was identified in 5 out of 6 IN2 cells (9.6 ± 6.2 synapses per IN2), whereas minimal CF innervation was detected in only 3 of 20 IN1 cells ($1-3$ synapses each, totaling just 15% of IN1s). Beyond established synapses, IN2 cells also received more nonsynaptic, vesicle-containing CF appositions than IN1 cells, with approximately half (55%) of all IN2 contacts constituting synapses compared to the rare (12%) synaptic contacts on IN1 cells (Fig. 4j), even though the spatial relation of IN1 and IN2 to CF are essentially the same (Extended Data Fig. 4c,d). CF contact types together on IN2 cells occupied larger membrane areas than those on IN1 cells (Fig. 4k), though this difference was absent when synaptic and nonsynaptic contacts were analyzed separately (Extended Data Fig. 2a), indicating that the larger average contact size of IN2 reflects its higher proportion of synaptic contacts rather than intrinsic size differences.

Both MLI subtypes received similar densities of conventional PF synapses (Fig. 4l) and neither showed enrichment of CF contacts near PF–MLI synapses compared to the other type or random dendritic locations (Fig. 4m and Extended Data Fig. 2d,e), confirming that the spatial independence between CF and PF inputs applies regardless of MLI subtype. We conclude that selective CF–IN2 connectivity establishes a parallel signaling pathway: direct CF-mediated excitation of PCs coincides with CF-driven suppression of IN1-mediated inhibition through activation of IN2 cells. Such circuit organization provides a structural mechanism by which CF activity could dynamically relieve inhibitory constraints on PC excitability, potentially gating the induction of plasticity and learning.

CFs and MLIs form divergent and convergent pathways to PCs

To test the functional implications of this inhibitory circuit architecture, we obtained high-density extracellular recordings in the floccular complex (Fig. 5a,b) while mice performed the vestibulo-ocular reflex (VOR). The VOR is well-defined sensorimotor behavior that drives stabilizing eye movements in response to head motion that reliably engages the floccular region. Using Neuropixels probes in *Nos1^{Chr2}* mice, which express Chr2 exclusively in MLIs^{11,36}, we unambiguously identified MLI units ($n = 136$) through optotagging (Extended Data Fig. 5a). We further distinguished PC units in these same recordings by their characteristic complex spikes (rate, 1.68 ± 0.05 Hz; $n = 166$) and CF activity was inferred from these responses. Simple spiking was definitively identified in a subset of these PCs ($n = 101$) that showed characteristic postcomplex spike pauses. As expected from MLI–PC connectivity, optogenetic MLI activation significantly reduced simple spike firing in these cells ($77.5\% \pm 0.1\%$ reduction, $P < 0.05$; Extended Data Fig. 5b,c). This

approach yielded simultaneous recordings from 10.5 ± 1.9 MLIs, 7.8 ± 1.3 PCs (simple spiking) and 12.8 ± 2 CFs per session (Extended Data Fig. 5d), enabling analysis of their network interactions during motor behavior.

We analyzed MLI functional connectivity patterns using spike-triggered averaging (STA)³⁷. Most MLI–PC pairs showed strong, short-latency (<3 ms) suppression of simple spiking in nearby PCs (mean decrease: 1.55 ± 0.09 s.d.; Extended Data Fig. 6a–c), while a subset lacked PC inhibition despite similar proximity. MLI–MLI interactions exhibited two patterns: infrequent inhibitory interactions between MLI pairs (Extended Data Fig. 6d–f) and prevalent short-timescale synchronous activity (Extended Data Fig. 6g–i). To determine whether the structural heterogeneity identified by TPI in our connectome analysis corresponded to functional differences, we tested whether inhibitory connectivity patterns could distinguish MLI subtypes. Unsupervised clustering using MLI–PC and MLI–MLI inhibition patterns alone yielded concordance with a previously established supervised classification scheme for the subset of MLIs that could be classified by that method¹⁴ (Extended Data Fig. 7a,b). Incorporating rapid-timescale MLI–MLI interactions as a third feature substantially improved classification, producing two distinct clusters with strong alignment to the predefined MLI functional categories (concordance, 94.8%; $P < 0.001$; randomly assigned clusters concordance of $78.1\% \pm 2.2\%$; Extended Data Fig. 7b,c). Notably, our unsupervised approach classified all opto-tagged MLIs into Cluster_1 ($n = 121$) and Cluster_2 ($n = 15$) (Extended Data Fig. 7d), resulting in a biased ratio reminiscent of the IN1:IN2 distribution observed in our connectome analysis.

MLIs in the two clusters displayed distinct interaction patterns that helped define their circuit functions. Cluster_1 MLIs provided the primary inhibitory drive to PCs (suppression of -2.04 ± 0.11 spikes s^{-1}) and that Cluster_2 showed negligible PC inhibition (Fig. 5c,d). Directional analysis of MLI–MLI pairs revealed asymmetric connectivity. Cluster_2 MLIs preferentially inhibited those in Cluster_1 (28.2% of pairs; mean latency, 1.97 ms) (Fig. 5e,f) with connectivity in the opposite direction masked by cross-correlation artifacts or correlated circuit interactions. Asymmetric MLI–MLI connectivity was further distinguished by strong within-Cluster_1 synchrony (3.8 ± 0.25 spikes s^{-1}), which was absent among Cluster_2 MLIs (-0.37 ± 0.29 spikes s^{-1} ; Fig. 5g,h), implying gap junctional coupling exclusively among Cluster_1 MLIs. These collective properties identify Cluster_1 as IN1 (PC-inhibiting cells) and Cluster_2 as IN2 (MLI-inhibiting cells), designations that align with the connectivity patterns of transcriptionally defined MLI1 and MLI2 subtypes^{14,15}, respectively.

CF inputs drive responses in disinhibitory MLIs through convergent integration

We next examined how CF inputs differentially engage these MLI populations. Our connectome analysis revealed selective CF targeting of IN2 (MLI2). Functional recordings confirmed this structural prediction. Cluster_2 (MLI2) cells showed increased firing following CF activation (Fig. 5i,j) with latencies consistent with monosynaptic transmission (3 ± 0.91 ms; Fig. 5k). By contrast, Cluster_1 (MLI1) cells rarely responded to CFs (Fig. 5i,j) and when they did, generally showed near-instantaneous responses indicative of indirect circuit effects (latency of 0.92 ± 0.25 ms; Fig. 5k). Thus, both structural and functional evidence establishes a disinhibitory hierarchy where CFs simultaneously excite PCs and MLI2 cells, the latter suppressing electrically coupled MLI1 cells that inhibit PCs (Fig. 5l).

Using slice recordings, prior reports have shown that MLIs can integrate input from multiple CFs^{19,21}. Such convergence was evident in our IN2 reconstructions: nearly all cells received innervation from multiple CF branches, with each branch forming several synaptic connections (Fig. 6a,b). Our functional recordings corroborated this anatomical observation, with individual Cluster_2 (MLI2) cells responding to more than one CF on average (Fig. 6c,d). Since CFs originate from electrically coupled olivary neurons^{23,38–40}, we examined whether converging CF

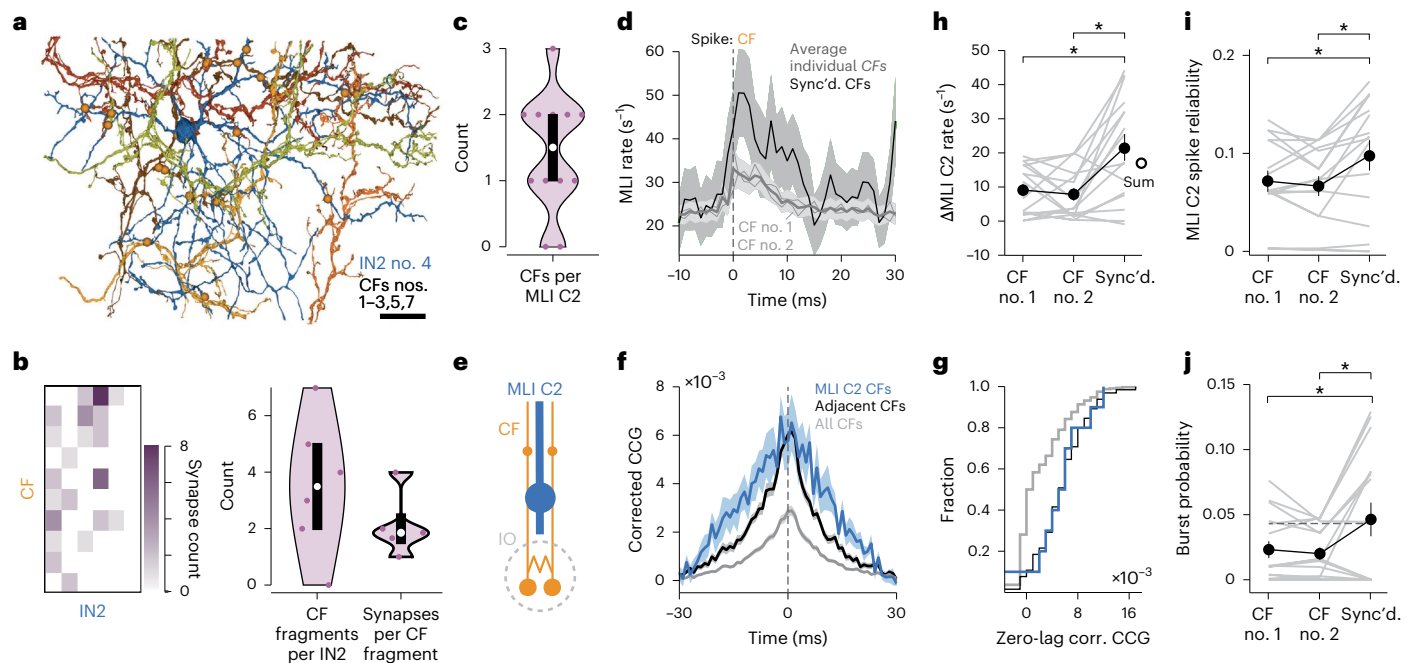


Fig. 6 | Population CF activity drives MLI2 responses through convergent innervation. **a**, EM reconstruction of an IN2 with synapse (orange dots) from multiple CF fragments. **b**, Left: adjacency matrix showing CF fragments contacting each IN2 (modified from Fig. 4a). Right: quantification of CF fragment counts per IN2 ($n = 6$ cells) and apposition counts per CF ($n = 10$ processes; both median $\pm$ quartile). **c**, The number of distinct CFs that activate each Cluster_2 MLI, determined from Neuropixels recordings ($n = 12$ cells; median $\pm$ quartiles). **d**, The change in firing rate of Cluster_2 MLIs to CF input (mean $\pm$ s.e.m.). Gray, individual CF responses; black, synchronous CF activation. **e**, Circuit diagram illustrating convergent CF-MLI connectivity. **f**, CF synchrony analysis with CCGs comparing synchrony between CFs converging on the same Cluster_2 MLI,

neighboring CF pairs and all CF pairs (mean $\pm$ s.e.m.). **g**, Cumulative distribution of spike timing differences for the same conditions. **h**, Cluster_2 MLI responses to individual CFs and their synchronous activation. Gray lines, each CF-MLI pair tested ($n = 16$); solid dots, population mean $\pm$ s.e.m.; open dot, predicted linear sum ($P = 0.001$ for both, two-sided paired t -tests; *, $P < 0.05$). **i**, Response reliability increases with convergent CF activation ($P = 0.006$ and $P = 0.002$ for CF 1 versus Sync'd and CF 2 versus Sync'd, respectively, two-sided paired t -tests, $n = 16$ pairs, mean $\pm$ s.e.m.). **j**, Burst probability analysis showing enhanced bursting with synchronous CF input ($P = 0.03$ for CF 1 versus Sync'd and $P = 0.02$ for CF 2 versus Sync'd, two-sided paired t -tests, $n = 16$ pairs, mean $\pm$ s.e.m.). Data in **c–j** were obtained from six mice. Sync'd, synchronized; corr., correlation.

inputs fire synchronously. Indeed, CF pairs targeting the same Cluster_2 (MLI2) cell frequently fired within several milliseconds of each other (90% of tested pairs; Fig. 6e,g). However, the level of coordinated firing was comparable to that of randomly selected neighboring CF pairs (84.6%, $P = 0.84$; Fig. 6f,g) indicating that synchrony between MLI2-targeting CFs simply reflects the inherent olivary coupling.

The functional impact of this convergence was clear: coactivation of multiple CFs produced larger increases in the firing of Cluster_2 (MLI2) cells compared to individual CFs alone, with responses summing linearly ($P < 0.002$; Fig. 6d,h). This enhanced excitation increased the reliability of Cluster_2 MLI responses to CF input ($P < 0.006$; Fig. 6i). Moreover, synchronous CF input moderately increased the probability of Cluster_2 MLI burst firing by 2.1-fold ($P < 0.05$, paired t -test; Fig. 6j). Thus, MLI2s act as coincidence detectors that translate population CF activity into scaled output; the more CFs fire together, the stronger the MLI2 response.

CF synchrony controls PC dendritic calcium signaling through disinhibitory gating

CF-evoked calcium influx in PC dendrites triggers motor learning plasticity, but this process is normally suppressed by MLI inhibition^{9–11,41}. We used computational modeling constrained by our anatomical data to examine how CF synchrony overcomes this constraint through MLI2-mediated disinhibition amid plausible PF activity (Fig. 7a; Methods). These simulations revealed that increased CF synchrony proportionally enhanced MLI2 spiking activity, which suppressed MLI1 output through their direct inhibitory connectivity (Fig. 7b,c). By incorporating a compartmental PC model with biophysically constrained calcium dynamics^{42,43}, we found that this CF synchrony-driven reduction in

MLI1 activity released PCs from inhibition, allowing enhanced calcium responses to CF input: when a single CF activated a PC, the resulting dendritic calcium signal scaled with the number of simultaneously active CFs elsewhere in the network (Fig. 7b–e). This synchrony-dependent enhancement occurred in both spiny and aspiny dendritic compartments (Fig. 7f).

To experimentally test whether CF synchrony recruits disinhibition to enhance PC dendritic calcium signals, we reanalyzed data from our prior study using in vivo two-photon microscopy to image GCaMP6f-expressing PC dendrites in lobule Crus II during cued licking behavior (Fig. 7g; Methods)⁹. Since PC dendritic calcium events are CF driven^{44,45}, we identified isolated calcium events in individual PCs during licking and quantified CF population synchrony for those events as the number of simultaneous events in the remaining population (Fig. 7h). Dendritic calcium events increased in amplitude with greater CF synchrony (Fig. 7i–m), consistent with synchrony-driven relief of MLI inhibition.

Next, to causally determine whether the synchrony effect requires modulation of active MLI circuits, we leveraged the fact that MLIs in these *Kit^{Cre}* mice (a driver line providing genetic access to all MLIs⁴⁶) expressed the chemogenetic inhibitor hM4D (Fig. 7g). By suppressing MLI activity uniformly regardless of CF synchrony levels, clozapine N-oxide (CNO) administration increased the mean amplitude of all CF-evoked calcium events during licking ($\Delta F/F$ 0.237 ± 0.002 and 0.363 ± 0.032 , control and CNO, respectively; $P = 0.0197$, paired t -test) (Fig. 7i,j), confirming the suppressive role of MLI inhibition in this context⁹. Importantly, linear regression revealed that CNO significantly reduced the slope relating CF synchrony to calcium event amplitude (Fig. 7i–l,n), demonstrating that functional MLI circuitry is required

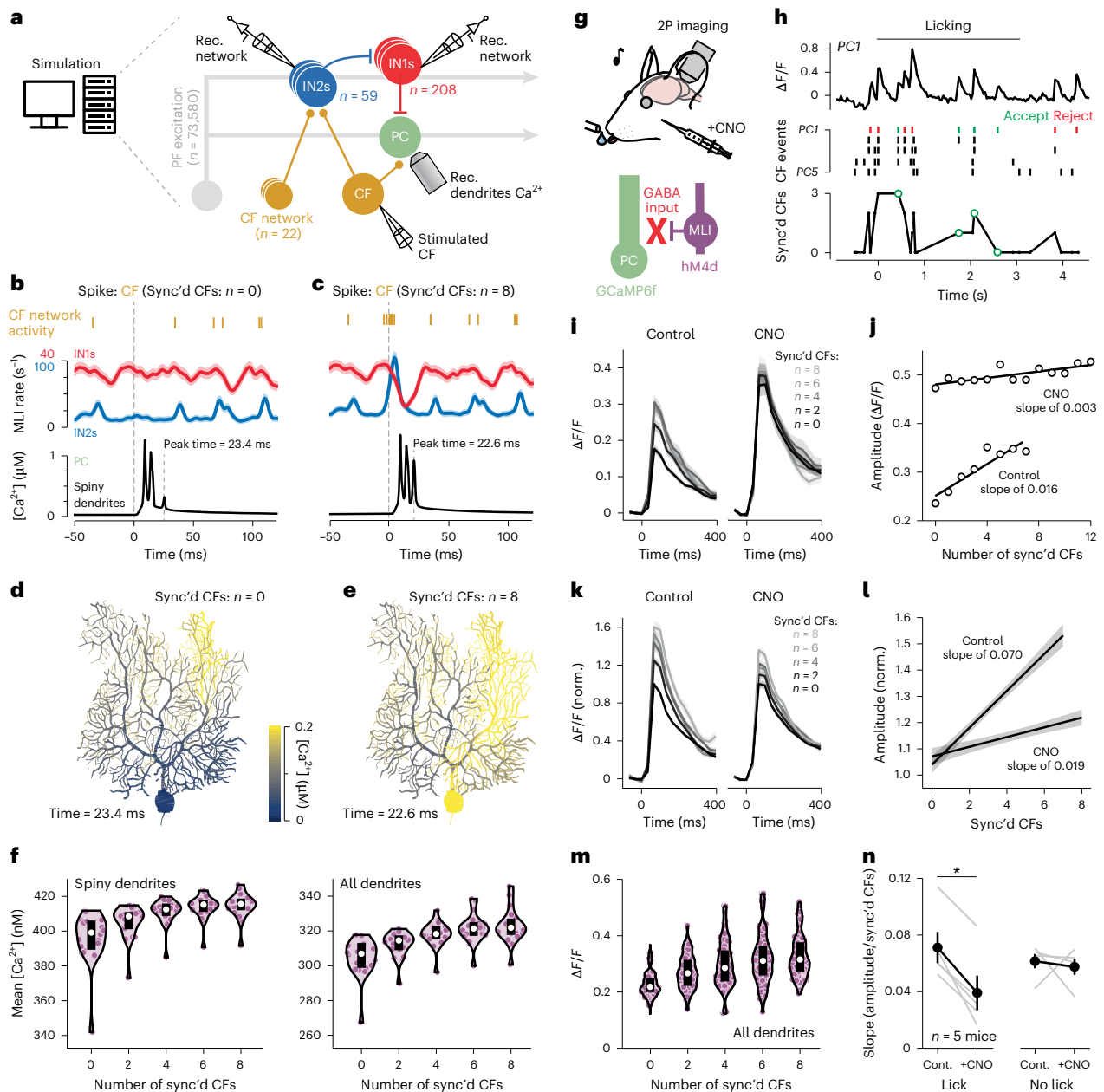


Fig. 7 | CF synchrony controls PC calcium dynamics through disinhibitory gating. **a**, A model schematic showing interconnected IN1s and IN2s receiving PF excitatory drive, with IN2s integrating convergent CF inputs as well as a test PC which receives input from a single test CF. Rec., recording in the simulation. **b, c**, Population firing rates of IN1 and IN2 cells (top) and dendritic calcium response in the test PC (bottom) following isolated activation of the test CF (**b**) or synchronous activation of additional eight CFs in the network (**c**) (mean $\pm$ s.e.m.). **d, e**, Color-coded CF-evoked dendritic calcium concentrations in the test PC following isolated activation of the test CF (**d**) or with synchronous activity from other CFs (**e**). **f**, Calcium concentration averaged over 0–30 ms, plotted for the number of synchronous CFs in both spiny and aspiny PC dendritic compartments (each dot represents a separate simulation run). Mean increases with synchrony (0 versus 2, 0 versus 4, 0 versus 6, 0 versus 8, 2 versus 4, 2 versus 6, 2 versus 8, all $P < 0.005$; $P = 0.037$ for 4 versus 8; otherwise $P > 0.05$, one-way ANOVA followed by one-sided Mann–Whitney U -tests; median $\pm$ quartiles. Significance not illustrated on plot). **g**, Experimental setup: two-photon imaging of GCaMP6f-expressing PC dendrites during cued licking in mice where MLIs express hM4D for chemogenetic suppression with intraperitoneal CNO administration. **h**, The analysis procedure illustrated for an example PC. Isolated CF-evoked calcium events are identified during licking, with the CF synchrony level quantified by counting simultaneous

events in the remaining PC population (four example PCs shown for simplicity). For each mouse ($n = 5$), events were obtained from 74 and 104 PCs in control and CNO, respectively. **i, j**, Representative PC showing mean CF-evoked calcium events ($\pm$ s.e.m.) sorted by CF synchrony level in control and CNO conditions (**i**) with corresponding linear regression fits to the data (**j**). **k, l**, Population data ($n = 5$ mice) showing the averaged calcium events (range, 440–3,460 and 1,049–4,204 in control and CNO per condition, respectively; mean $\pm$ s.e.m.) normalized to the amplitude of asynchronous events (**k**) and linear regression fits (shading: 95% confidence interval) to the normalized (norm.) amplitude-synchrony relationship for all PCs in control and CNO conditions (**l**). **m**, Peak amplitude of all PCs across animals with increasing numbers of synchronous CFs (dots, individual PCs from 5 mice; median $\pm$ quartiles). Larger events correlate with increased synchrony (0 versus 2, 0 versus 4, 0 versus 6, 0 versus 8, 2 versus 6, 2 versus 8, all $P < 0.05$, ANOVA with two-sided Dunnett's post-tests. Significance not illustrated on plot). **n**, Quantification of slope changes (mean $\pm$ s.e.m.; $n = 5$ mice) between control (cont.) and CNO conditions, measured in separate sessions, during licking and nonlicking epochs ($P = 0.004$ and $P = 0.70$, respectively, two-sided paired t -tests; *, $P < 0.05$). Images in **d** and **e** adapted from ref. 43 under a Creative Commons license CC BY 4.0. Image in **g** reproduced from ref. 9 under a Creative Commons license CC BY 4.0.

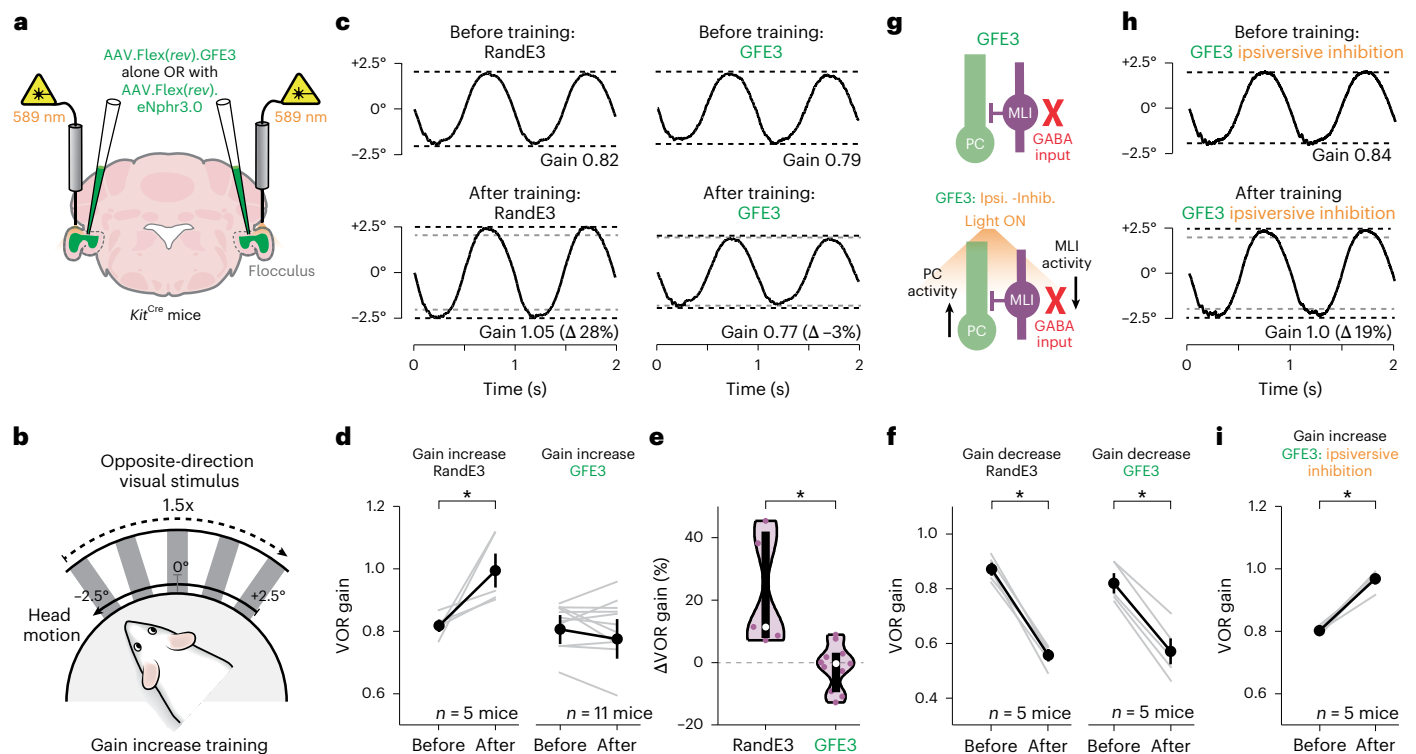


Fig. 8 | Elimination of MLI-mediated disinhibition prevents CF-instructed adaptive learning. a, The experimental approach. **b**, A schematic of gain-increase training involving pairing sinusoidal head turns with opposite-direction visual motion (60 min sessions). **c**, Representative VOR-evoked eye movements recorded from either a control (left) or GFE3-expressing (right) mouse before and after gain-increase training. **d**, VOR gain measurements in control and GFE3-expressing mice during gain-increase training (left and right, $P = 0.0141$ and $P > 0.99$, respectively, ANOVA with two-sided Bonferroni's post-tests; mean $\pm$ s.e.m.). **e**, Change in VOR gain relative to baseline for each mouse shown in **d** ($P = 0.0188$, two-sided unpaired *t*-test; median $\pm$ quartiles). **f**, VOR gain

measurements during gain-decrease training in control and GFE3-expressing mice (left and right, $P < 0.0001$ and $P < 0.0001$, respectively, two-way ANOVA with two-sided Bonferroni's post-tests; mean $\pm$ s.e.m.). **g**, Circuit diagram illustrating GFE3-mediated elimination of MLI-to-MLI inhibition and optogenetic rescue through eNPHR3.0-mediated MLI suppression. **h**, Representative VOR eye movements from a GFE3-expressing mouse before and after gain-increase training without (left) and with (right) optogenetic MLI suppression during ipsiversive head turns in separate sessions. **i**, Optogenetic MLI suppression during training rescued VOR gain-increase learning in GFE3-expressing mice ($P = 0.0004$, two-sided paired *t*-test; mean $\pm$ s.e.m.).

for CF synchrony to enhance PC dendritic calcium signaling. During nonlicking periods when Crus II MLIs are relatively inactive^{9,47,48}, the synchrony–amplitude relationship remained unchanged in CNO compared to control (Fig. 7n), confirming minimal effect when MLIs are not strongly inhibiting PCs. Notably, even with MLI activity chemo-genetically suppressed, calcium event amplitude still increased with CF synchrony across all behavioral contexts, pointing to an additional enhancing effect probably reflecting direct summation of presynaptic CF activity at PC dendrites⁴⁵ (Fig. 7i–l,n), independent of the disinhibitory pathway. Together, these findings establish that cerebellar circuitry implements a biological coincidence detector where CF synchrony determines the magnitude of plasticity-inducing dendritic calcium signals through recruitment of MLI2-mediated disinhibition.

Learning deficits in the absence of MLI-mediated disinhibition of PCs

To determine whether this PC disinhibitory pathway is required for cerebellar learning, we used a molecular-genetic approach to eliminate inhibitory signaling between MLIs. We injected adeno-associated virus (AAV)-containing Cre-dependent, gephyrin-targeted E3 ligase GFE3 (ref. 49) into the flocculi of *Kit^{Cre}* mice (Fig. 8a and Extended Data Fig. 8a; Methods). A subset of mice additionally received Cre-dependent eNPHR3.0 for later rescue experiments (Fig. 8a; see below). Whole-cell recordings from acute slices confirmed that GFE3-expressing MLIs had greatly reduced spontaneous inhibitory postsynaptic currents (sIPSCs; Extended Data Fig. 8b,c), consistent with disrupted MLI–MLI inhibition. PCs from GFE3-expressing regions displayed sIPSC rates comparable

to controls (Extended Data Fig. 8b,d), demonstrating intact MLI-to-PC inhibition. Thus, GFE3 specifically eliminated MLI-to-MLI inhibition, which is predominantly mediated by MLI2 cells, functionally removing the CF-driven disinhibitory pathway.

The flocculus is required to adaptively recalibrate the VOR when image motion is encountered during head rotations as this signals a performance error. We next assessed VOR learning in all GFE3-expressing mice using gain-increase training, where sinusoidal head turns are repeatedly paired with opposite-direction visual motion (Fig. 8b), a form of learning that depends on CF-induced PC calcium signaling and plasticity^{50–54}. Control-injected mice exhibited normal gain-increase learning, while GFE3-expressing mice (with or without eNPHR3.0) failed to adapt to the pairing stimulus (Fig. 8c–e and Extended Data Fig. 9c). Importantly, baseline VOR performance was identical between groups (1 Hz VOR gain of 0.82 ± 0.02 and 0.83 ± 0.02 , RandE3 and GFE3, respectively, $P = 0.44$; Mann–Whitney test) confirming that the observed deficits were not due to impaired basic motor function. These results suggest that PC disinhibition is essential for CF-induced learning as its elimination prevents relief of PC inhibitory constraints despite the animals experiencing sensory feedback of an underperforming VOR. Notably, GFE3-expressing mice acquired normal gain-decrease learning in response to same-direction visual–vestibular pairings (Fig. 8f and Extended Data Fig. 9a,b), highlighting the specificity of the deficit to CF-dependent learning mechanisms^{50–53}.

To directly examine whether the learning deficit resulted specifically from the loss of PC disinhibition, we tested whether optogenetic MLI suppression could bypass the GFE3-induced loss of MLI–MLI

inhibition and restore gain-increase VOR adaptation in GFE3 and eNpHR3.0 co-expressing mice. To activate inhibitory eNpHR3.0, light pulses were delivered to both flocculi through bilaterally implanted optical fibers, timed to the ipsiversive phase of peak head velocity (Fig. 8b,g and Extended Data Fig. 9d,e), when CF activity normally drives learning^{11,51,55}. Under these pairing conditions, the mice normally adapted their VOR by increasing the gain (Fig. 8h,i). These results demonstrate that CF-dependent cerebellar learning critically requires the MLI2-mediated disinhibition of PCs.

Discussion

Our findings demonstrate that inhibitory microcircuits gate cerebellar instructive signals through a mechanism dependent on CF synchrony. By selectively innervating disinhibitory MLI2 cells that integrate convergent CF inputs, the circuit ensures that only synchronous CF activity can overcome inhibitory constraints on plasticity induction. When CFs fire synchronously, MLI2 cells are strongly activated, suppressing the activity of PC-targeting MLI1 cells and promoting large dendritic calcium transients in PCs to initiate synaptic plasticity. This arrangement resolves the longstanding paradox of continuous CF firing: individual CFs discharge during both correct and incorrect movements, but only their synchronized population activity triggers adaptive learning. Thus, instructive signals emerge not from the informational content carried by individual CFs, but from the structured inhibitory network dynamics that conditionally gate their effectiveness.

Disinhibitory MLI circuits

Our identification of logic of MLI network organization reveals how these circuits are involved in conditional control over CF-driven plasticity. The inhibitory pathway from MLI2 cells onto PC-targeting MLI1 cells provides a mechanism for selectively relieving PC inhibition during motor errors^{9,11}. This disinhibitory motif parallels circuit architectures observed across multiple brain regions⁵⁶, most notably, forebrain VIP interneurons, which similarly target other inhibitory populations to gate plasticity at principal neurons^{17,18,57,58}. Like disinhibitory cortical VIP cells^{59–61}, our data show that cerebellar MLI2s receive long-range evaluative inputs, in this case from olivary CFs corroborating recent work of other groups^{34,62}, enabling context-dependent circuit modulation.

Our connectivity-based classification of MLIs, confirmed by functional recording, aligns closely with recently defined MLI1/MLI2 populations^{14,15}, supporting the notion that functional connectivity, rather than morphological criteria alone¹⁶, best defines interneuron subtypes in the cerebellar molecular layer. Despite clearly distinguishing IN1 and IN2 subtypes based on connectivity, finer-scale functional or anatomical distinctions, especially within the IN1 (MLI1) population, may yet be identified (for example, unique connectivity features of MLI1.1 basket cells)¹⁵. Future work will be required to determine how interaction patterns between MLI subtypes change in response to adaptive stimuli and how their connectivity through multisynaptic pathways influences net PC disinhibition in these contexts, fully elucidating how differential MLI recruitment shapes conditional circuit operations during behavior.

CF–MLI communication

Our connectomic analysis challenges the traditional view that CFs exclusively innervate PCs by revealing direct CF–MLI synapses, with pronounced selectivity toward MLI2 cells. Although these direct connections were rare within our volume, their clear subtype specificity argues against random connectivity, suggesting instead specialized developmental mechanisms. This rarity probably explains their limited anatomical description in ultrastructural examinations³⁰. Notably, our identification of direct CF–MLI2 synapses contrasts with a recent study concluding that CF–MLI2 signaling occurs primarily through glutamate spillover³⁴, possibly reflecting methodological differences in our approaches. The convergence of multiple CFs onto individual

MLI2 cells, each forming multiple synaptic contacts, suggests these connections are optimized for reliable, synchronous signal transmission rather than merely serving modulatory roles.

Our functional recordings confirmed point-to-point synaptic transmission between CFs and MLIs, characterized by a broad range of amplitudes, fast kinetics and quantal properties. Our finding that EPSC kinetics were invariant across different levels of quantal content excludes spillover mechanisms involving release-dependent glutamate pooling from multiple release sites. Interestingly, we identified nonsynaptic CF–MLI appositions containing vesicle clusters but lacking postsynaptic specializations. The pronounced TBOA sensitivity of CF–MLI EPSCs suggests our recordings may capture composite responses from both direct synapses and these appositions. This interpretation is consistent with prior observations of nonsynaptic CF–MLI communication^{19,21,63} while extending them to reveal a possible dual transmission mode. Our anatomical findings show that IN2 (MLI2) cells receive both contact types support this model. Such a dual mechanism could ensure robust CF–MLI2 activation while maintaining the temporal precision needed for coincidence detection.

CF-mediated instructive signaling and learning

CFs instruct cerebellar learning, including VOR adaptation, by triggering calcium-dependent plasticity, with calcium levels in PCs determining the magnitude and direction (that is, a continuum spanning long-term depression to long-term potentiation) at PF synapses^{41,54,64}. However, continuous firing across diverse motor behaviors creates a regulatory challenge. Without additional gating⁶⁵, persistent CF activity could drive maladaptive plasticity during accurately performed movements, though distributed plasticity mechanisms throughout the cerebellar cortex may provide additional safeguards in conjunction with CF-mediated processes⁶⁶. While MLI inhibition curtails PC excitation, including CF-evoked activity and calcium signaling^{67–70}, the mechanisms permitting conditional release from this inhibition were unclear, despite behavioral evidence—most notably in the flocculus during the VOR—that identical CF activation induces learning in some contexts but not others^{11,51,71}.

We posit that CF synchrony is a critical variable controlling instructive signaling efficacy. Although synchronous CF discharges have previously been associated with movement initiation and muscle coordination^{22,72}, their mechanistic contribution to behaviorally relevant computation remained unclear. Our findings demonstrate a previously unrecognized role for CF synchrony: selective engagement of disinhibitory circuits that gate cerebellar plasticity. Our results showed that increasing CF synchrony proportionally enhances PC calcium responses by recruiting the MLI2-mediated disinhibitory pathway, whereas asynchronous CF firing fails to overcome inhibitory constraints imposed by MLI1 cells. Notably, even with complete MLI suppression, we observed a residual synchrony-dependent enhancement of PC calcium signals, establishing an additional CF synchrony mechanism independent of disinhibition. This may reflect summation of presynaptic CF activity at PC dendrites that varies with the population activity of olivary neurons^{45,73}, though the precise mechanism and its interaction with the disinhibitory pathway require further investigation.

Collectively, our findings indicate that instructive CF signaling is determined by circuit computations decoding CF population synchrony rather than individual CF firing patterns. More broadly, these results suggest that CF synchrony itself, regulated by intrinsic or extrinsic inputs such as nucleo-olivary inhibition that suppresses olivary gap junctional coupling^{40,74,75}, may serve as part of a hierarchical gating mechanism by which subcortical circuits contribute to dynamic control of cerebellar learning.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions

and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41593-026-02268-2>.

References

- Raymond, J. L. & Medina, J. F. Computational principles of supervised learning in the cerebellum. *Annu. Rev. Neurosci.* **41**, 233–253 (2018).
- Silva, N. T., Ramírez-Buritica, J., Pritchett, D. L. & Carey, M. R. Climbing fibers provide essential instructive signals for associative learning. *Nat. Neurosci.* **27**, 940–951 (2024).
- Marr, D. A theory of cerebellar cortex. *J. Physiol.* **202**, 437–470 (1969).
- Albus, J. S. A theory of cerebellar function. *Math. Biosci.* **10**, 25–61 (1971).
- Ito, M. Neural design of the cerebellar motor control system. *Brain Res.* **40**, 81–84 (1972).
- Kitazawa, S., Kimura, T. & Yin, P. B. Cerebellar complex spikes encode both destinations and errors in arm movements. *Nature* **392**, 494–497 (1998).
- Heffley, W. et al. Coordinated cerebellar climbing fiber activity signals learned sensorimotor predictions. *Nat. Neurosci.* **21**, 1431–1441 (2018).
- Kostadinov, D., Beau, M., Blanco-Pozo, M. & Hausser, M. Predictive and reactive reward signals conveyed by climbing fiber inputs to cerebellar Purkinje cells. *Nat. Neurosci.* **22**, 950–962 (2019).
- Gaffield, M. A., Rowan, M. J. M., Amat, S. B., Hirai, H. & Christie, J. M. Inhibition gates supralinear Ca^{2+} signaling in Purkinje cell dendrites during practiced movements. *eLife* **7**, e36246 (2018).
- Callaway, J. C., Lasser-Ross, N. & Ross, W. N. IPSPs strongly inhibit climbing fiber-activated $[\text{Ca}^{2+}]_i$ increases in the dendrites of cerebellar Purkinje neurons. *J. Neurosci.* **15**, 2777–2787 (1995).
- Rowan, M. J. M. et al. Graded control of climbing-fiber-mediated plasticity and learning by inhibition in the cerebellum. *Neuron* **99**, 999–1015 (2018).
- Boele, H. J. et al. Impact of parallel fiber to Purkinje cell long-term depression is unmasked in absence of inhibitory input. *Sci. Adv.* **4**, eaas9426 (2018).
- Wulff, P. et al. Synaptic inhibition of Purkinje cells mediates consolidation of vestibulo-cerebellar motor learning. *Nat. Neurosci.* **12**, 1042–1049 (2009).
- Lackey, E. P. et al. Specialized connectivity of molecular layer interneuron subtypes leads to disinhibition and synchronous inhibition of cerebellar Purkinje cells. *Neuron* **112**, 2333–2348 (2024).
- Kozareva, V. et al. A transcriptomic atlas of mouse cerebellar cortex comprehensively defines cell types. *Nature* **598**, 214–219 (2021).
- Wang, W. X. & Lefebvre, J. L. Morphological pseudotime ordering and fate mapping reveal diversification of cerebellar inhibitory interneurons. *Nat. Commun.* **13**, 3433 (2022).
- Kepecs, A. & Fishell, G. Interneuron cell types are fit to function. *Nature* **505**, 318–326 (2014).
- Letzkus, J. J., Wolff, S. B. & Luthi, A. Disinhibition, a circuit mechanism for associative learning and memory. *Neuron* **88**, 264–276 (2015).
- Szapiro, G. & Barbour, B. Multiple climbing fibers signal to molecular layer interneurons exclusively via glutamate spillover. *Nat. Neurosci.* **10**, 735–742 (2007).
- Arlt, C. & Häusser, M. Microcircuit rules governing impact of single interneurons on purkinje cell output in vivo. *Cell Rep.* **30**, 3020–3035 (2020).
- Coddington, L. T., Rudolph, S., Vande Lune, P., Overstreet-Wadiche, L. & Wadiche, J. I. Spillover-mediated feedforward inhibition functionally segregates interneuron activity. *Neuron* **78**, 1050–1062 (2013).
- Welsh, J. P., Lang, E. J., Sugihara, I. & Llinas, R. Dynamic organization of motor control within the olivocerebellar system. *Nature* **374**, 453–457 (1995).
- Long, M. A., Deans, M. R., Paul, D. L. & Connors, B. W. Rhythmicity without synchrony in the electrically uncoupled inferior olive. *J. Neurosci.* **22**, 10898–10905 (2002).
- Denk, W. & Horstmann, H. Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. *PLoS Biol.* **2**, e329 (2004).
- Kim, J. S. et al. Space-time wiring specificity supports direction selectivity in the retina. *Nature* **509**, 331–336 (2014).
- Park, C., Gim, J., Lee, S., Lee, K. J. & Kim, J. S. Automated synapse detection method for cerebellar connectomics. *Front. Neuroanat.* **16**, 760279 (2022).
- Nguyen, T. M. et al. Structured cerebellar connectivity supports resilient pattern separation. *Nature* **613**, 543–549 (2023).
- Eccles, J. C. *The Cerebellum as a Neuronal Machine* (Springer, 2013).
- Apps, R. & Garwicz, M. Anatomical and physiological foundations of cerebellar information processing. *Nat. Rev. Neurosci.* **6**, 297–311 (2005).
- Palay, S. L. & Chan-Palay, V. *Cerebellar Cortex: Cytology and Organization* (Springer, 1974).
- Cathala, L., Holderith, N. B., Nusser, Z., DiGregorio, D. A. & Cull-Candy, S. G. Changes in synaptic structure underlie the developmental speeding of AMPA receptor-mediated EPSCs. *Nat. Neurosci.* **8**, 1310–1318 (2005).
- Otis, T. S., Wu, Y. C. & Trussell, L. O. Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. *J. Neurosci.* **16**, 1634–1644 (1996).
- DiGregorio, D. A., Nusser, Z. & Silver, R. A. Spillover of glutamate onto synaptic AMPA receptors enhances fast transmission at a cerebellar synapse. *Neuron* **35**, 521–533 (2002).
- Santos-Valencia, F. et al. Climbing fibres recruit disinhibition to enhance Purkinje cell calcium signals. *Nature* <https://doi.org/10.1038/s41586-026-10220-4> (2026).
- Wadiche, J. I. & Jahr, C. E. Multivesicular release at climbing fiber–Purkinje cell synapses. *Neuron* **32**, 301–313 (2001).
- Kim, J. et al. Optogenetic mapping of cerebellar inhibitory circuitry reveals spatially biased coordination of interneurons via electrical synapses. *Cell Rep.* **7**, 1601–1613 (2014).
- Fetz, E. E. & Cheney, P. D. Postspike facilitation of forelimb muscle activity by primate corticomotoneuronal cells. *J. Neurophysiol.* **44**, 751–772 (1980).
- Llinas, R., Baker, R. & Sotelo, C. Electrotonic coupling between neurons in cat inferior olive. *J. Neurophysiol.* **37**, 560–571 (1974).
- Devor, A. & Yarom, Y. Electrotonic coupling in the inferior olivary nucleus revealed by simultaneous double patch recordings. *J. Neurophysiol.* **87**, 3048–3058 (2002).
- De Zeeuw, C. I., Wentzel, P. & Mugnaini, E. Fine structure of the dorsal cap of the inferior olive and its GABAergic and non-GABAergic input from the nucleus prepositus hypoglossi in rat and rabbit. *J. Comp. Neurol.* **327**, 63–82 (1993).
- Coesmans, M., Weber, J. T., De Zeeuw, C. I. & Hansel, C. Bidirectional parallel fiber plasticity in the cerebellum under climbing fiber control. *Neuron* **44**, 691–700 (2004).
- Ma, M. et al. Molecular layer interneurons in the cerebellum encode for valence in associative learning. *Nat. Commun.* **11**, 4217 (2020).
- Zang, Y., Dieudonne, S. & De Schutter, E. Voltage- and branch-specific climbing fiber responses in purkinje cells. *Cell Rep.* **24**, 1536–1549 (2018).
- Ozden, I., Sullivan, M. R., Lee, H. M. & Wang, S. S. Reliable coding emerges from coactivation of climbing fibers in microbands of cerebellar Purkinje neurons. *J. Neurosci.* **29**, 10463–10473 (2009).

45. Gaffield, M. A., Bonnan, A. & Christie, J. M. Conversion of graded presynaptic climbing fiber activity into graded postsynaptic Ca^{2+} signals by purkinje cell dendrites. *Neuron* **102**, 762–769 (2019).
46. Amat, S. B. et al. Using c-kit to genetically target cerebellar molecular layer interneurons in adult mice. *PLoS ONE* **12**, e0179347 (2017).
47. Gaffield, M. A. & Christie, J. M. Movement rate is encoded and influenced by widespread, coherent activity of cerebellar molecular layer interneurons. *J. Neurosci.* **37**, 4751–4765 (2017).
48. Astorga, G. et al. Concerted interneuron activity in the cerebellar molecular layer during rhythmic oromotor behaviors. *J. Neurosci.* **37**, 11455–11468 (2017).
49. Gross, G. G. et al. An E3-ligase-based method for ablating inhibitory synapses. *Nat. Methods* **13**, 673–678 (2016).
50. Raymond, J. L. & Lisberger, S. G. Neural learning rules for the vestibulo-ocular reflex. *J. Neurosci.* **18**, 9112–9129 (1998).
51. Kimpo, R. R., Rinaldi, J. M., Kim, C. K., Payne, H. L. & Raymond, J. L. Gating of neural error signals during motor learning. *eLife* **3**, e02076 (2014).
52. Boyden, E. S., Katoh, A. & Raymond, J. L. Cerebellum-dependent learning: the role of multiple plasticity mechanisms. *Annu. Rev. Neurosci.* **27**, 581–609 (2004).
53. Kakegawa, W. et al. Optogenetic control of synaptic AMPA receptor endocytosis reveals roles of LTD in motor learning. *Neuron* **99**, 985–998 (2018).
54. Bonnan, A., Rowan, M. M. J., Baker, C. A., Bolton, M. M. & Christie, J. M. Autonomous Purkinje cell activation instructs bidirectional motor learning through evoked dendritic calcium signaling. *Nat. Commun.* **12**, 2153 (2021).
55. Nguyen-Vu, T. D. et al. Cerebellar Purkinje cell activity drives motor learning. *Nat. Neurosci.* **16**, 1734–1736 (2013).
56. Schneider-Mizell, C. M. et al. Inhibitory specificity from a connectomic census of mouse visual cortex. *Nature* **640**, 448–458 (2025).
57. Pfeffer, C. K., Xue, M., He, M., Huang, Z. J. & Scanziani, M. Inhibition of inhibition in visual cortex: the logic of connections between molecularly distinct interneurons. *Nat. Neurosci.* **16**, 1068–1076 (2013).
58. Williams, L. E. & Holtmaat, A. Higher-order thalamocortical inputs gate synaptic long-term potentiation via disinhibition. *Neuron* **101**, 91–102 (2019).
59. Pi, H. J. et al. Cortical interneurons that specialize in disinhibitory control. *Nature* **503**, 521–524 (2013).
60. Lee, C. et al. Whole-brain mapping of long-range inputs to the VIP-expressing inhibitory neurons in the primary motor cortex. *Front. Neural Circuits* **17**, 1093066 (2023).
61. Wall, N. R. et al. Brain-wide maps of synaptic input to cortical interneurons. *J. Neurosci.* **36**, 4000–4009 (2016).
62. Fakharian, M. A., Shoup, A. M., Hage, P., Elseweifi, H. Y. & Shadmehr, R. A vector calculus for neural computation in the cerebellum. *Science* **388**, 869–875 (2025).
63. Coddington, L. T., Nietz, A. K. & Wadiche, J. I. The contribution of extrasynaptic signaling to cerebellar information processing. *Cerebellum* **13**, 513–520 (2014).
64. Piochon, C. et al. Calcium threshold shift enables frequency-independent control of plasticity by an instructive signal. *Proc. Natl Acad. Sci. USA* **113**, 13221–13226 (2016).
65. Najafi, F. & Medina, J. F. Beyond ‘all-or-nothing’ climbing fibers: graded representation of teaching signals in Purkinje cells. *Front. Neural Circuits* **7**, 115 (2013).
66. Gao, Z., van Beugen, B. J. & De Zeeuw, C. I. Distributed synergistic plasticity and cerebellar learning. *Nat. Rev. Neurosci.* **13**, 619–635 (2012).
67. Mittmann, W., Koch, U. & Hausser, M. Feed-forward inhibition shapes the spike output of cerebellar Purkinje cells. *J. Physiol.* **563**, 369–378 (2005).
68. Brunel, N., Hakim, V., Isope, P., Nadal, J. P. & Barbour, B. Optimal information storage and the distribution of synaptic weights: perceptron versus Purkinje cell. *Neuron* **43**, 745–757 (2004).
69. Kitamura, K. & Hausser, M. Dendritic calcium signaling triggered by spontaneous and sensory-evoked climbing fiber input to cerebellar Purkinje cells in vivo. *J. Neurosci.* **31**, 10847–10858 (2011).
70. Dizon, M. J. & Khodakhah, K. The role of interneurons in shaping Purkinje cell responses in the cerebellar cortex. *J. Neurosci.* **31**, 10463–10473 (2011).
71. Nguyen-Vu, T. B. et al. A saturation hypothesis to explain both enhanced and impaired learning with enhanced plasticity. *eLife* **6**, e20147 (2017).
72. Welsh, J. P. Functional significance of climbing-fiber synchrony: a population coding and behavioral analysis. *Ann. N. Y. Acad. Sci.* **978**, 188–204 (2002).
73. Mathy, A. et al. Encoding of oscillations by axonal bursts in inferior olive neurons. *Neuron* **62**, 388–399 (2009).
74. Best, A. R. & Regehr, W. G. Inhibitory regulation of electrically coupled neurons in the inferior olive is mediated by asynchronous release of GABA. *Neuron* **62**, 555–565 (2009).
75. Lefler, Y., Yarom, Y. & Uusisaari, M. Y. Cerebellar inhibitory input to the inferior olive decreases electrical coupling and blocks subthreshold oscillations. *Neuron* **81**, 1389–1400 (2014).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2026

¹Department of Biological Sciences, Sungkyunkwan University, Suwon, South Korea. ²Laboratory of Computational Neuroscience, Korea Brain Research Institute, Daegu, South Korea. ³Department of Physiology and Biophysics, University of Colorado School of Medicine, Aurora, CO, USA. ⁴Neural Circuits Research Group, Korea Brain Research Institute, Daegu, South Korea. ⁵Cognitive Science Research Group, Korea Brain Research Institute, Daegu, South Korea. ⁶Department of Biomedical Sciences, Korea University, Seoul, South Korea. ⁷Integrative Biology and Neuroscience Graduate Program, Florida Atlantic University, Jupiter, FL, USA. ⁸International Max Planck Research School for Brain and Behavior, Max Planck Florida Institute for Neuroscience, Jupiter, FL, USA. ⁹Center for Memory and Glioscience, Institute for Basic Science, Daejeon, South Korea. ¹⁰Department of Physics and Astronomy, Seoul National University, Seoul, South Korea. ¹¹Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA, USA. ¹²Princeton Neuroscience Institute, Princeton University, Princeton, NJ, USA. ¹³School of Computing, Korea Advanced Institute of Science and

Technology, Daejeon, South Korea. ¹⁴Department of Biological Sciences, Dornsife College, University of Southern California, Los Angeles, CA, USA.

¹⁵Present address: Princeton University, Princeton, NJ, USA. ¹⁶Present address: Sanofi S.A., Cleveland, OH, USA. ¹⁷Present address: Samsung Electronics, Hwaseong-Si, South Korea. ¹⁸Present address: Brain Research Core Facilities of Korea Brain Research Institute, Daegu, South Korea. ¹⁹Present address: Zetta AI LLC, Sherrill, NY, USA. ²⁰These authors contributed equally: Changjoo Park, Zhen Yang, Abdulraheem Nashef, Jawon Gim, Sangkyu Bahn, Gyu Hyun Kim, Ke Zhang, Laurence Cathala, Sungho Hong. ✉e-mail: relaylee@kbri.re.kr; jason.m.christie@cuanschutz.edu; jinseopskim@skku.edu

Methods

Animals

Animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of the Korea Brain Research Institute, the Max Planck Florida Institute for Neuroscience and the University of Colorado School of Medicine. C57BL/6J (wild type), *Nos1^{Chr2}* and *Kit^{Cre}* mice were maintained on a 12-h light–dark cycle at with ad libitum access to food and water.

Sample preparation, EM imaging and reconstruction

Cerebellar tissue from a 56-day-old male wild-type mouse was processed for serial block-face EM following standard protocols⁷⁶. The tissue was infiltrated with epoxy resin with carbon powder⁷⁷ and sectioned along the sagittal axis. Imaging was performed using a Merlin VP field emission scanning electron microscope (Carl Zeiss) equipped with 3View2 in-chamber ultramicrotome and backscattered electron detector (Gatan). Images were acquired at 12 nm XY resolution with 50-nm section thickness. Each block face was captured in 2×3 tiles ($5,000 \times 5,000$ pixels each) with 10–20% overlap between tiles. A total of 1,000 consecutive sections were imaged, resulting in six image stacks of 1,000 images each (one stack per tile position).

Each of the six tile stacks was aligned separately using ImageJ with TrakEM2 plugin⁷⁸ and custom MATLAB scripts, then merged into a single volume. Rotational drift from TrakEM2 alignment was corrected, and tiles were welded by selecting inner pixels. The final registered volume comprised $14,600 \times 10,200 \times 1,000$ voxels, corresponding to a physical volume of approximately $175 \times 122 \times 50 \mu\text{m}^3$.

To automate reconstruction, we employed a modified three-dimensional (3D) U-Net^{79,80} to segment neurons using cellular membranes as boundaries. Eight subvolumes from various locations served as training data, annotated by experts using VAST software⁸¹. The trained network segmented the entire volume to generate putative neuronal fragments. The artificial intelligence-aided segmentation was proofread using custom software with interactive GUI and background process management systems²⁵. Proofreading proceeded progressively, with each neuronal fragment represented by one segment after completion.

Cell type classification

For this report, we classified reconstructed neurons into four general types (PC, CF, PF and MLI) based on morphology. We further distinguished three MLI subtypes (termed IN1, IN2, INx to avoid confusion with transcriptional MLII/MLI2 designations) based on connectivity. Not all reconstructed neurons were classified, cells severely truncated by image borders were designated as undecidable owing to insufficient morphological features. While the cerebellar cortex contains additional rare inhibitory neuron types (for example, Lugaro cells and Candelabrum cells), their presence in our dataset is uncertain. Given their sparse distribution, any such cells present would be classified as MLIs.

Since it was difficult to find the exact number of missing cells that are not reconstructed, we estimated the reconstruction completeness based on a probabilistic analysis of cell types at various locations from the EM volume. Considering the fact that we reconstructed PF starting at the center of the EM volume and progressed outward, we sampled five equal-sized subvolumes ($6.14 \times 6.14 \times 6.14 \mu\text{m}^3$), which were sampled at various distances from center. From each subvolume, 100 background and 100 foreground voxels were randomly sampled. Cell type identity of unsegmented voxels was determined by examining EM morphology. Reconstruction (recon) completeness was calculated using equation (1), where x is the sampled unsegmented voxels, y is the sampled segmented voxels, $m = 0.3975$ (total unsegmented fraction) and $n = 0.6025$ (total segmented fraction)

$$\text{recon} = \frac{ny}{mx + ny} \quad (1)$$

Reconstruction completeness, estimated from volumetric sampling, was near total for PCs (99.6%), MLIs (97.7%) and CFs (100%), with partial coverage of PFs (76.9%; rarely including granule cell somata or ascending axon) due to their high density and volume boundary truncation.

Morphological and spatial analysis

Neuronal segments required separation into soma, dendrite, dendritic spine and axon components for independent analysis. PC somata and dendrites were separated based on the subvolumes used for proofreading. PC axons were not captured in our imaging volume. Smaller MLI compartments were isolated using manually defined bounding boxes, with multiple boxes sometimes required to exclude dendritic processes.

Erosion sufficient to remove spine necks was applied to PC dendrites, eliminating spines while preserving shaft-derived components. The skeletonization of PC dendrites required prior spine removal as the TEASAR⁸² algorithm yields accurate centerline representations only when applied to spine-free shafts. The presence of spines and spine–spine contacts would otherwise generate artifactual branches. The TEASAR algorithm was applied directly to spine-containing MLI dendrites, where spines appeared as characteristically short branches on the resulting skeleton. These spine-originated branches were identified and removed through length-based thresholding, yielding the dendritic shaft skeleton.

Spatial organization within the molecular layer was quantified through multiple measures. To establish MLI positions, we first defined the PC layer (PCL) boundary. The PCL plane was computed using least-squares fitting through the centroids of PC soma–dendrite transitions. While cerebellar foliar curvature can create nonplanar PCL boundaries, the boundary appeared approximately planar within our imaging volume. MLI soma positions were quantified as perpendicular distances from this reference plane.

Angular distributions of surrounding elements were analyzed within $2\text{-}\mu\text{m}$ shells around PC dendrites, created by dilating shaft volumes and subtracting the original shaft. For each voxel within these shells, we computed displacement vectors from the nearest skeleton point. To standardize measurements across the complex dendritic geometry, we established local coordinate systems at each skeleton point comprising (1) the tangential direction along the skeleton, (2) a horizontal direction perpendicular to the tangent and parallel to average PC dendritic plane and (3) a vertical direction orthogonal to both. Displacement vectors were projected onto planes perpendicular to the local tangent and binned by the angle relative to horizontal and vertical axes. Cell type-specific angular distributions were computed independently for each PC and averaged across the population.

Semi-automated synapse detection

We identified synapses using an automated method²⁶ followed by manual proofreading for specific connection types. Contacts between neuron segments were first extracted by detecting segment ID changes across six-neighborhood voxels. Six connection types were analyzed: PF–PC, PF–MLI, CF–PC, CF–MLI, MLI–PC and MLI–MLI.

Given the large number of contacts, we employed artificial intelligence for classification. Two 3D U-Nets separately predicted synaptic contact and vesicle cloud probabilities. The large serial block-face EM volume was divided into subvolumes for parallel processing. Vesicle clouds were segmented using a watershed algorithm variant, with candidates identified from synaptic contact probabilities. Size thresholds of 200 voxels (contacts) and 1,000 voxels (vesicle clouds) eliminated noise. A support vector machine classified candidates based on probability distributions, using a -0.2 decision boundary to minimize false negatives. Contacts were confirmed as synapses when vesicle clouds were detected within 5 voxels. Synapse direction was determined from vesicle cloud position.

The system achieved an F1 score of 0.955 on a test volume ($11.9 \times 11.9 \times 12.4 \mu\text{m}^3$, 508 ground-truth labels). Despite the high accuracy, all MLI–MLI and MLI–PC synapses underwent manual verification owing to uncertainty about inhibitory synapse variability in the training set. Synapses from MLI dendrites or onto MLI axons were discarded as false positives. CF–MLI synapses required special attention as they were not included in training sets, being previously considered non-existent. Of 128 artificial intelligence-detected CF–MLI contacts, 61 were false by prompt expert consensus. The remaining 67 underwent expert evaluation by 4 reviewers using standard criteria: membrane darkening and proximal presynaptic vesicles. Each reviewer scored contacts from 0 to 3 (false positive, probably false, probably true or true positive). Synapses ($n = 53$) were confirmed when cumulative scores reached ≥ 6 . The rest were determined as nonsynaptic appositions ($n = 75$). Contact size was calculated accounting for voxel anisotropy ($12 \times 12 \times 50 \text{ nm}^3$). Contact faces in X/Y directions contributed 600 nm^2 each, while Z -direction faces contributed 144 nm^2 . Total contact size equaled the sum of all contact face areas.

MLI subtype classification and connectivity analysis

MLI subtypes (IN1, IN2 and INx) were classified based on the TPI, a measure of whether a given neuron preferentially targets PCs or other MLIs. TPI was calculated using equation (2) where a is the number of output synapses onto MLIs and b is the number of output synapses onto PCs. TPI values range from +1 (exclusively targeting MLIs) to −1 (exclusively targeting PCs). TPI values were calculated for MLIs having at least 30 output synapses. Cells with $\text{TPI} < 0$ were classified as IN1, those with $\text{TPI} > 0$ as IN2. MLIs with insufficient synapses for reliable TPI calculation were classified as INx.

$$\text{TPI} = \frac{a - b}{a + b} \quad (2)$$

Surgery

For surgical procedures, mice (6–23 weeks old, both sexes) were anesthetized with isoflurane (3–5% for induction, <1.5% for maintenance in O_2) and placed in a stereotaxic frame (Kopf). Pre-emptive analgesia was administered subcutaneously: buprenorphine (0.35 mg kg^{-1}), carprofen (5 mg kg^{-1}) and dexamethasone (3 mg kg^{-1}). Anesthesia depth was monitored by toe-pinch reflex and body temperature was maintained at 37°C using a heating pad. A stainless-steel head post was secured to the exposed skull using dental cement (Metabond, Parkell). Small craniotomies ($<0.5 \text{ mm}$) were then made for stereotactic injections and/or optical fiber implants. Larger craniotomies were used for imaging windows as described previously⁹. Mice were monitored daily for signs of distress after the surgeries. Experiments generally commenced ≥ 14 days postsurgery to ensure full recovery.

For viral injections targeting the flocculi of *Kit^{Cre}* mice, the microinjection coordinates (in mm from Bregma) were medial/lateral (M/L) = ± 2.33 , anterior/posterior (A/P) = -5.68 and dorsal/ventral (D/V) = 2.7 – 2.8 mm (angle of 5°). AAVs included among the following: AAV9-EF1 α -Flex(*loxP*)rev-GFE3-eGFP, AAV1-EF1 α -Flex(*loxP*)rev-RandE3-2A-GFP, AAV1-EF1 α -Flex(*loxP*)rev-EGFP and AAV5-EF1 α -Flex(*loxP*)rev-eNphr3.0-HA. For two-photon imaging experiments, viral injections included AAV1-CAG-Flex(*loxP*)-ChR2-HA-2A-hM4D for MLI manipulation, combined with AAV1-Pcp2.4-FLPo and AAV1-CAG-Flex(FRT)-GCaMP6f for PC-specific calcium imaging as previously described⁹. Virus solution was injected at a slow rate (-0.05 – $0.1 \mu\text{l min}^{-1}$) by pressure, for a total volume of 0.2 – $0.5 \mu\text{l}$ per site. For eNphr3.0-expressing animals, optical fibers (200 μm , NA 0.22 with $\varnothing 1.25 \text{ mm}$ ferrules; Thorlabs) were bilaterally implanted in each side of the cerebellum to deliver light to the virally transduced region of flocculus with the following coordinates: M/L = $\pm 3.35 \text{ mm}$, A/P = 5.65 mm , D/V = $1.9 \pm 0.1 \text{ mm}$ (angle of -16°).

For Neuropixels recordings in *Nos1^{Chr2}* mice, a unilateral optical was implanted at the following coordinates to target the left flocculus:

M/L = $\pm 2.315 \text{ mm}$, A/P = 5.60 mm , D/V = 3.1 mm (angle -8°), with the fiber tip resting on the floccular surface. A silver ground wire was also cemented to the skull. In these mice, a second surgery was performed before the first recording in which a small craniotomy was made through the left temporal bone overlying the parafoveolus, taking care to preserve the semicircular canals. The dura was left intact, and the opening was sealed with silicone elastomer (Kwik-Cast, WPI) until use.

In vivo Neuropixels recording and analysis

On recording days, the mice were lightly anesthetized ($<1.5\%$ isoflurane) and the silicone plug removed. After a brief recovery, the mice were then head-fixed in the recording apparatus. A Neuropixels 1.0 probe (IMEC) was advanced into the floccular complex (comprising either or both the flocculus and parafoveolus) with a motorized micromanipulator (MPM, New Scale Technologies). Electrophysiological signals were digitized at 30 kHz and recorded using OpenEphys software, referenced to the ground wire. For optotagging, light from a DPSS 473-nm laser (MBL-F-473-200 mW, CNI) was routed through an acousto-optic modulator (OptoElectronics Tech), fiber port and patch cable (Thorlabs) to the implanted optical fiber. Square pulses (10 ms, 50 Hz, 3–5 mW) were generated by custom LabVIEW software that communicated with NI DAQ boards. The resulting commands were logged alongside electrophysiology using bar-coded synchronization pulses from a microcontroller (Arduino Mega). Light pulses for optotagging were delivered during stationary periods after VOR trials.

All units were sorted offline with Kilosort⁸³ and then manually curated in Phy2 (<https://github.com/cortex-lab/phy>). Opto-tagged units were identified manually by the experimenter. For better visualization, we removed 2 ms after light delivery and at the end of the light pulse to avoid photoelectric artifacts. To classify opto-tagged MLIs into functional subtypes, we employed unsupervised hierarchical clustering. For each MLI unit, we first computed spike-triggered averages (STAs) to characterize its functional connectivity with PCs and other MLIs. Three feature vectors were extracted: (1) the mean of the two strongest PC responses (1–10 ms postspike), accounting for both direct inhibition and potential disinhibition; (2) the mean of the two largest MLI–MLI effects (trough STA values at 1–5 ms postspike), usually representing synaptic inhibition; and (3) the mean of the two strongest MLI–MLI synchrony values (peak STA values within $\pm 3 \text{ ms}$ of the trigger spike), capturing gap junctional coupling. These three feature vectors were concatenated for each unit, and pairwise correlation coefficients were computed using MATLAB's corrcoef function, yielding an $n \times n$ correlation matrix. We then applied hierarchical clustering using MATLAB's linkage function to construct a linkage tree values ($Z \in \mathbb{R}^{n \times 3}$) and partitioned the resulting dendrogram into two clusters using the cluster function.

To analyze the temporal relationships between CF pairs, we computed cross-correlograms (CCGs) by aligning spike times between units which accounts for firing rate modulations. To distinguish true synchrony from coincident firing due to local rate fluctuations, we calculated the expected chance cofiring using a spike triplet method⁸⁴. For each observed spike triplet (T1, T2 and T3), we estimated the temporal uncertainty of spike T2 by defining a window from halfway between T1 and T2 to halfway between T2 and T3. This created a probability distribution for spike occurrence (equation (3)). The rate-corrected CCG was then calculated by subtracting the null CCG from the raw CCG (equation (4)) where Pr represents the probability of a complex spike (CS) occurring at time t , Δt is the time bin used for synchrony calculation and the likelihood quantifies the probability that CS_i (or $\overline{\text{CS}}_i$ under the null hypothesis) fires in temporal proximity to a CS_j spike. This correction isolated significant temporal relationships beyond those expected from firing rate covariations.

$$\widehat{\text{CS}}_i(t) = \frac{2\Delta t}{T_{i+1} - T_{i-1}} \quad (3)$$

$$CCG(t) = \frac{\Pr(CS_1(t), |, CS_2(0) = 1) - \Pr(\bar{CS}_1(t), |, CS_2(0) = 1)}{\Delta t}. \quad (4)$$

We identified burst events triggered between CFs and MLIs using a previously established criteria⁸⁵. For each spike in our MLI recordings, we counted spikes occurring within a 15 ms window. If ≥ 3 spikes were detected with mean interspike intervals, less than half the overall mean interspike interval, this constituted a burst. Burst significance was determined by comparing spike counts within equivalent windows to Poisson-distributed spike trains (1,000 iterations) matched for firing rate. To calculate burst probability following CF input, we created a binary time series marking burst occurrences and computed the complex spike-triggered average of these events. Similarly, we quantified MLI response reliability to single or coincident CF inputs using established methods⁸⁶. The STA of MLI activity within 1–10 ms of CF occurrence was convolved with a 7-ms Gaussian window. Response reliability was calculated as the mean pairwise correlation coefficient of these convolved firing patterns across trials, providing a measure of response consistency to CF activation.

Two-photon calcium imaging and CF synchrony analysis during licking behavior

Two-photon, in vivo calcium imaging data were obtained from a previously published dataset and the details are described therein⁹. Briefly, GCaMP6f⁸⁷ fluorescence was imaged from PC dendrites in lobule Crus II at 30 Hz using a resonant scanning two-photon microscope while expert, head-fixed mice licked for water rewards in response to auditory cues. MLIs in these *Kit^{Cre}* mice expressed hM4D for chemogenetic suppression, with imaging conducted in separate sessions either in control or after intraperitoneal CNO (the cognate agonist of hM4D) administration (5 mg kg⁻¹).

Raw fluorescence traces were motion corrected and PC dendrites segmented as previously described⁹. We removed two mice owing to the small numbers of identified PCs, leaving five mice. For this current analysis we identified CF-evoked calcium transients using the OASIS algorithm⁸⁸, which performed denoising and inference. Calcium signals were referenced to, and divided by, baseline as they were converted to $\Delta F/F$. To ensure analysis of isolated events, we selected calcium transients that met two criteria: (1) no CF event in the same PC during the preceding 300 ms and (2) no subsequent event within 66 ms. These temporal constraints excluded overlapping or clustered responses that could confound amplitude measurements. For each isolated event in a given PC, we quantified CF population synchrony by counting all simultaneous events in the remaining PCs (including overlapping or clustered events), as we were interested in the total level of network activity during that moment. Events were considered ‘synchronous’ if their peaks occurred within the same imaging frame (33 ms window at 30 Hz acquisition). Given the slow frame rate of image acquisition, this quantification could not delineate millisecond precision. Thus, we defined CF synchrony level as the number of coactive PCs during each event, ranging from 0 (isolated event) to $N-1$ (where N is the total PCs monitored). Events were categorized by behavioral state (licking versus nonlicking periods) and pharmacological condition (control versus CNO). We performed linear regression analysis on the relationship between calcium event amplitude and synchrony level for each experimental condition, fitting slopes to quantify how synchrony influenced calcium signal magnitude. To account for differences in the numbers of imaged PCs between mice and conditions, these fits limited to one-half the total number of cells in each field of view. For population analyses of calcium amplitudes (Fig. 7m), outliers were identified and removed using the ‘rmoutliers’ function in MATLAB based on the distribution of $\Delta F/F$ values across all PCs.

VOR behavior and analysis

The VOR was elicited using a custom-built apparatus inside a light-tight enclosure, as previously described⁴¹. For learning experiments, baseline

VOR performance was assessed in darkness-induced isolation. Pilocarpine (2% ophthalmic solution; Patterson Veterinary Supply) was applied briefly (<1 min) to limit pupil dilation ensuring accurate tracking in darkness. During the training period (60 min), visual–vestibular pairing was achieved by moving the grating either out-of-phase (gain-increase training) or in-phase (gain-decrease training) with the vestibular stimulus. VOR performance was measured before training and immediately afterward. For photo-suppressing eNphr3.0-expressing MLIs in these behaving mice, we used light from a 589-nm DPSS laser (MGL-F-589-200mW; CNI) which was routed through acousto-optic modulator and fiber port to the patch cable. Light pulses were triggered by stage position to synchronize optogenetic suppression with head movements. In these experiments, angular eye position was calculated using a previously published method⁸⁹.

During Neuropixels recordings, visual–vestibular contexts were presented in pseudo-randomized 2-min epochs: darkness, stationary grating or gratings moving in phase or out of phase with head rotation (that is, gain-up or gain-down stimuli). For these experiments, pilocarpine was omitted and eye movement tracking was unused. Custom LabVIEW software (National Instruments) synchronized stage motion, visual stimuli and electrophysiological data acquisition via DAQ boards (National Instruments). Neural activity recorded during these stimuli and periods of behavioral quiescence was pooled for analysis.

Acute slice preparation and electrophysiological recording

For whole-cell patch-clamp recording of CF–MLI EPSCs, cerebellar parasagittal slices (200- μ m thick) were prepared from adult mice (P39–129) as described previously⁹⁰. Briefly, mice were killed by decapitation and their brains were rapidly removed and placed in ice-cold solution containing (in mM) 230 sucrose, 2.5 KCl, 0.5 CaCl₂, 4 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃ and 25 glucose. Slices were cut from the dissected cerebellar vermis using a vibratome (VT1200S, Leica Biosystems), incubated at 32 °C for 30 min in recovery solution containing (in mM) 85 NaCl, 75 sucrose, 2.5 KCl, 0.5 CaCl₂, 4 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃ and 25 glucose, then maintained at room temperature for up to 8 h in recording solution containing (in mM) 125 NaCl, 2.5 KCl, 1.5 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃ and 25 glucose. During recordings, 10 μ M SR-95531 and 0.3 μ M strychnine were added to block GABA_A and glycine receptors, respectively. All solutions contained 0.5 mM ascorbic acid and were continuously bubbled with carbogen (95% O₂/5% CO₂). In experiments where the external calcium concentration was lowered (that is, 1.5 to 0.5 mM), the magnesium concentration was correspondingly increased (1 to 2 mM).

Whole-cell voltage-clamp recordings were made from MLIs located in the inner two-thirds of the molecular layer at 31–33 °C using a Multiclamp 700 amplifier (Molecular Devices) with fire-polished borosilicate glass patch electrodes (6–8 M Ω resistance) filled with internal solution containing (in mM) 115 K-MeSO₄, 40 HEPES, 6 NaOH, 1 EGTA, 0.035 CaCl₂, 4.6 MgCl₂, 0.3 NaGTP, 4 NaATP, 5 phosphocreatine and 0.03 Alexa 488 (pH 7.3; ~305 mOsm). EPSCs were recorded at –70 mV holding potential (uncorrected for liquid junction potential +6 mV), filtered at 10 kHz and digitized at 100 kHz using a Digidata 1440 A converter with pClamp 10 software (Molecular Devices). Series resistance averaged 19 M Ω (uncompensated) and was monitored throughout experiments. CFs were stimulated at 0.1 Hz using a glass electrode filled with recording solution placed in the granule cell layer or molecular layer, delivering 50- μ s pulses (5–88 V; Digitimer Ltd).

Data analysis was performed using Neuromatic⁹¹ within Igor Pro (WaveMetrics). EPSC peak amplitudes were measured as the difference between baseline and mean amplitude over a 100- μ s window centered on the response peak. Rise time was measured from 10% to 90% of peak amplitude, and decay kinetics were assessed by response duration, quantified as full-width at half-maximum (FWHM) amplitude.

For sIPSC recordings in MLIs and PCs, mice were anesthetized with ketamine/xylazine (100/10 mg kg⁻¹) and transcardially perfused with ice-cold slicing solution containing (in mM) 87 NaCl, 75 sucrose, 25

NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, and 10 glucose. Slices were cut and incubated at 34 °C in recording solution containing (in mM) 128 NaCl, 26.2 NaHCO₃, 2.5 KCl, 1 NaH₂PO₄, 1.5 CaCl₂, 1.5 MgCl₂, and 11 glucose, then maintained at room temperature. During recordings, 20 μM NBQX and 20 μM (R)-CPP were added to block AMPA and NMDA receptors, respectively. Whole-cell recordings from PCs (identified by morphology) or MLIs (identified by location or GFP expression in virally transduced cells) with borosilicate pipettes (2–4 MΩ for PCs; 3–5 MΩ for MLIs) filled with internal solution containing (in mM) 150 CsCl, 10 HEPES, 1 EGTA, 0.1 CaCl₂, 4.6 MgCl₂, 2 MgATP and 0.3 NaGTP (pH 7.3; ~300 mOsm). Spontaneous IPSCs were recorded at –70 mV with liquid junction potentials corrected offline.

Computational modeling

We constructed a cerebellar network model comprising 1 PC, 208 MLI1 cells, 59 MLI2 cells, with inputs from 73,580 PFs and 22 CFs. Cell numbers, properties and connectivity parameters were derived from our connectome data including the proportions of PFs following those of the 8 possible connectivity patterns to PC, IN1 and IN2. Gap junctions were restricted to MLI1–MLI1 pairs, with each MLI1 randomly connected to approximately 4.5 other MLI1s via gap junctions on distal dendrites¹⁴.

All simulations were implemented in the NEURON simulation environment^{92,93}. Models were adapted from published works and tuned to match the electrophysiological properties^{42,43} of the network. The PC model retained its original parameters⁴³, except for PF and CF synaptic conductances, which were adjusted to achieve physiological firing rates. For MLI models, we retained the original active mechanisms and parameters⁴² but implemented simplified morphologies based on our reconstructions. MLI models were simplified and consisted of a cylindrical soma (diameter, 5.3 μm; length, 17 μm) connected to four proximal dendrites (diameter, 0.57 μm; length, 20 μm), each branching into two distal dendrites (diameter, 0.325 μm; length, 180 μm for MLI1 and 100 μm for MLI2).

PF firing was modeled as independent Poisson processes at 4 Hz, corresponding to active movement conditions. CF firing was correlated within groups: eight additional CFs (including the PC-projecting CF) fired with variable synchrony levels and Gaussian jitter of 2 ms. Complete synchrony of the CFs approximated observed correlation coefficients (~0.15) in our experiments. Additional CF groups of two to four cells fired synchronously but independently, with intergroup intervals of ~100 ms. Control networks were constructed with alternative CF–MLI connectivity patterns. Simulations were repeated 20 times for random PF inputs. More details on the network model, compartment model of neurons and other simulation parameters can be found in the shared code repository.

Statistics and reproducibility

All statistical tests used, whether they are one- or two-sided, the exact *P* values and sample sizes, and measures of variance are indicated in the figure legends. Data distributions are shown using violin plots with individual data points. Parametric tests were applied after normality and homogeneity of variance were assessed. Nonparametric tests were used where appropriate. No statistical method was used to predetermine sample size. For connectomic analyses, all available reconstructed neurons and synapses were included. For electrophysiological and behavioral experiments, sample sizes were selected based on established standards in the field and are consistent with previous studies^{9,11–13}. No data were excluded from the analyses. During electrophysiological and behavioral experiments, conditions were tested in random order and investigators were blinded to animal or sample allocation when feasible. Animals were assigned to groups based on genotype or viral manipulation and stimuli followed predefined protocols.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The 3D EM images, proofread segmentation, neuronal 3D rendering dataset and cell type and synapse information used in this study are freely available without restriction via the BossDB (<https://bossdb.org>) repository at <https://bossdb.org/project/park2023>. Source data are provided with this paper.

Code availability

The analysis codes, simulation parameters and codes and software used for this study are freely available without restriction via GitHub at https://github.com/cns-kim-lab/park_cerebellar_disinhibition.

References

- Briggman, K. L., Helmstaedter, M. & Denk, W. Wiring specificity in the direction-selectivity circuit of the retina. *Nature* **471**, 183–188 (2011).
- Xu, Z. X. et al. Elevated protein synthesis in microglia causes autism-like synaptic and behavioral aberrations. *Nat. Commun.* **11**, 1797 (2020).
- Cardona, A. et al. TrakEM2 software for neural circuit reconstruction. *PLoS ONE* **7**, e38011 (2012).
- Çiçek, Ö., Abdulkadir, A., Lienkamp, S. S., Brox, T. & Ronneberger, O. 3D U-Net: learning dense volumetric segmentation from sparse annotation. In *International Conference on Medical Image Computing and Computer-Assisted Intervention Part II* (eds Ourselin, S. et al.) 424–432 (Springer, 2016).
- Lee, K., Zung, J., Li, P., Jain, V. & Seung, H. S. Superhuman accuracy on the SNEMI3D connectomics challenge. Preprint at <https://arxiv.org/abs/1706.00120> (2017).
- Kubota, Y., Sohn, J. & Kawaguchi, Y. Large volume electron microscopy and neural microcircuit analysis. *Front. Neural Circuits* **12**, 98 (2018).
- Sato, M., Bitter, I., Bender, M. A., Kaufman, A. E. & Nakajima, M. TEASAR: tree-structure extraction algorithm for accurate and robust skeletons. In *Proc. Eighth Pacific Conference on Computer Graphics and Applications* (eds Barsky, B. A. et al.) 281–289 (IEEE Computer Society, 2000).
- Pachitariu, M. et al. Kilosort: realtime spike-sorting for extracellular electrophysiology with hundreds of channels. Preprint at *bioRxiv* <https://doi.org/10.1101/061481> (2016).
- Herzfeld, D. J., Joshua, M. & Lisberger, S. G. Rate versus synchrony codes for cerebellar control of motor behavior. *Neuron* **111**, 2448–2460 (2023).
- Legédy, C. R. & Salzman, M. Bursts and recurrences of bursts in the spike trains of spontaneously active striate cortex neurons. *J. Neurophysiol.* **53**, 926–939 (1985).
- Nashef, A., Cohen, O., Perlmuter, S. I. & Prut, Y. A cerebellar origin of feedforward inhibition to the motor cortex in non-human primates. *Cell Rep.* **39**, 110803 (2022).
- Chen, T. W. et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* **499**, 295–300 (2013).
- Friedrich, J., Zhou, P. & Paninski, L. Fast online deconvolution of calcium imaging data. *PLoS Comput. Biol.* **13**, e1005423 (2017).
- Stahl, J. S., van Alphen, A. M. & De Zeeuw, C. I. A comparison of video and magnetic search coil recordings of mouse eye movements. *J. Neurosci. Methods* **99**, 101–110 (2000).
- Biane, C. et al. Developmental emergence of two-stage nonlinear synaptic integration in cerebellar interneurons. *eLife* **10**, e65954 (2021).
- Rothman, J. S. & Silver, R. A. NeuroMatic: an integrated open-source software toolkit for acquisition, analysis and simulation of electrophysiological data. *Front. Neuroinform.* **12**, 14 (2018).
- Hines, M. L. & Carnevale, N. T. NEURON: a tool for neuroscientists. *Neuroscientist* **7**, 123–135 (1997).

93. Hines, M. L. & Carnevale, N. T. The NEURON simulation environment. *Neural Comput.* **9**, 1179–1209 (1997).

Acknowledgements

This research was supported by Korea Brain Research Institute Basic Research Programs 23-BR-04-04 (to J.S.K.), 16-BR-02 and 25-BR-01-03 (to K.J.L.) funded by the Korean Ministry of Science and ICT; National Research Foundation of Korea grants RS-2024-00411768 (MSIT) and 2019R1A6A1A10073079 (Ministry of Education) (to J.S.K.); Center for Memory and Glioscience, Institute for Basic Science of Korea grants IBS-R001-D2 (to S.H.); NIH grants NS146784, R01NS118401, R01NS112289 and R01NS114430 (to J.M.C.) as well as NS115610 (to D.B.A); and the Life Science Research Foundation and Amgen (to A.N.). EM images were acquired at the Brain Research Core Facilities of Korea Brain Research Institute. We thank J. Shin, J. Kim, J. Yoon, H. Suh, D. Yoo, H. Kim, J. Kang, D. Cho and C. Ham for reconstruction proofreading and annotation; S. Yu, J. Park and S.-C. Yu for CF–MLI synapse verification; and J.-C. Rah, J. Y. Kwon, and K. Yamamoto for valuable discussions. We thank members of the Christie and Person laboratories at the University of Colorado School of Medicine for helpful comments as well as S. Du Lac and T. Kodama for their helpful input regarding surgical targeting of the mouse flocculus. The Optogenetics and Neural Engineering Core at the University of Colorado School of Medicine provided engineering support. The Integrative Biology and Neuroscience Graduate Program and International Max Planck Research School for Brain and Behavior are jointly administered by the Max Planck Florida Institute for Neuroscience and Florida Atlantic University.

Author contributions

Supervision and writing: J.M.C., K.J.L., and J.S.K. conceived and designed the study. K.J.L. and J.S.K. initiated and carried out the EM investigation. J.M.C. and J.S.K. supervised the overall scientific investigation, with J.M.C. leading functional recordings in addition to behavioral manipulation and J.S.K. leading connectomic reconstruction and analysis. C.P., J.M.C. and J.S.K. wrote the manuscript with input from additional authors. Structural analysis: C.P. conducted most of the scientific investigation related to the volume reconstruction. G.H.K. and S.-H.L. prepared samples and performed EM imaging under the supervision of K.J.L. S.B. registered images and developed initial skeletonization methods that Y.I. completed. J.G. segmented images using neural networks

designed and trained by K.L. and J.G. C.P. conducted most volume reconstruction analyses, and together with J.G., classified neurons and detected synapses. C.P. verified CF–MLI synapses. Y.I. analyzed cellular organization. J.G. and S.B. developed reconstruction pipeline software, with J.G. training and managing proofreaders. S.H. built the network model with input from C.P. and conducted all simulations. M.-S.K. advised on data handling. Functional and behavioral analysis: Z.Y. conducted all in vivo Neuropixels recordings and processed the data, performed calcium imaging analysis and contributed to slice recordings validating GFE3 with assistance from K.Z. A.N. analyzed the in vivo recordings with assistance from Z.Y. and contributed to analysis of calcium imaging data. Behavioral experiments were performed by K.Z. L.C. designed, performed and analyzed brain slice recordings examining CF–MLI communication. Reagents were provided by D.B.A.

Competing interests

K. Lee declares financial interests in Zetta AI, a company providing connectomic reconstruction services. Methods and code used in this research have informed subsequent developments at Zetta AI. Zetta AI had no role in the design, execution, analysis or funding of the present work. M.-S. Kim declares financial interests in GraphAI, which is unrelated to this work. The other authors declare no competing interests.

Additional information

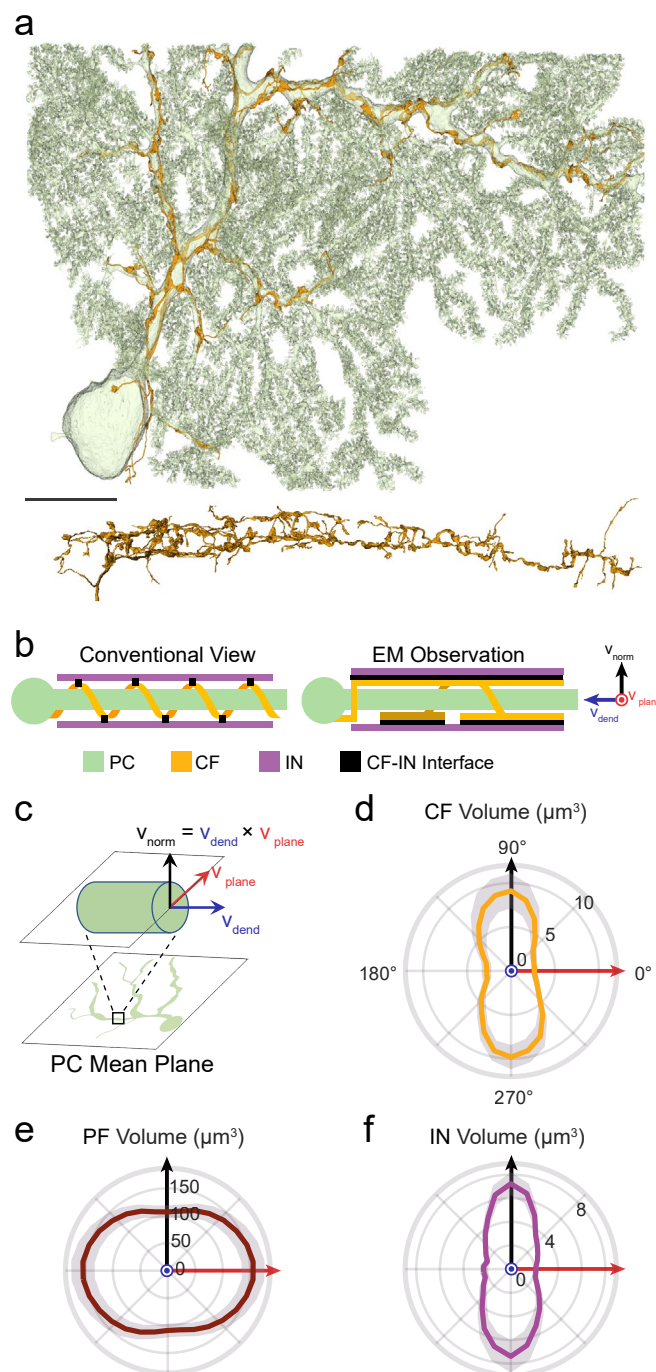
Extended data is available for this paper at <https://doi.org/10.1038/s41593-026-02268-2>.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41593-026-02268-2>.

Correspondence and requests for materials should be addressed to Kea Joo Lee, Jason M. Christie or Jinseop S. Kim.

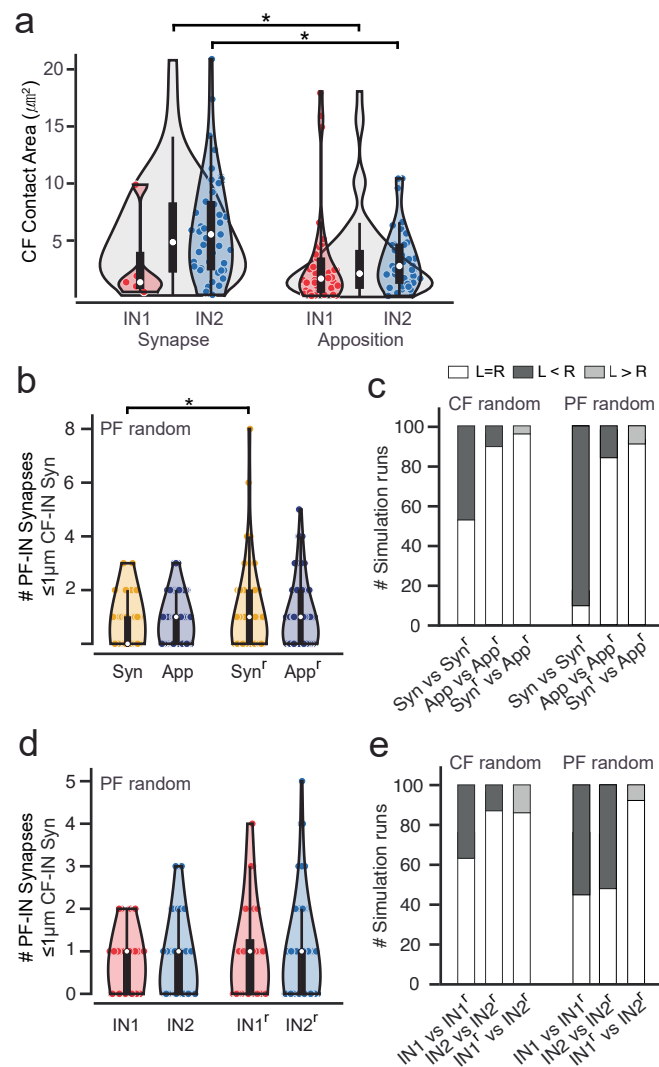
Peer review information *Nature Neuroscience* thanks Chris de Zeeuw and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.



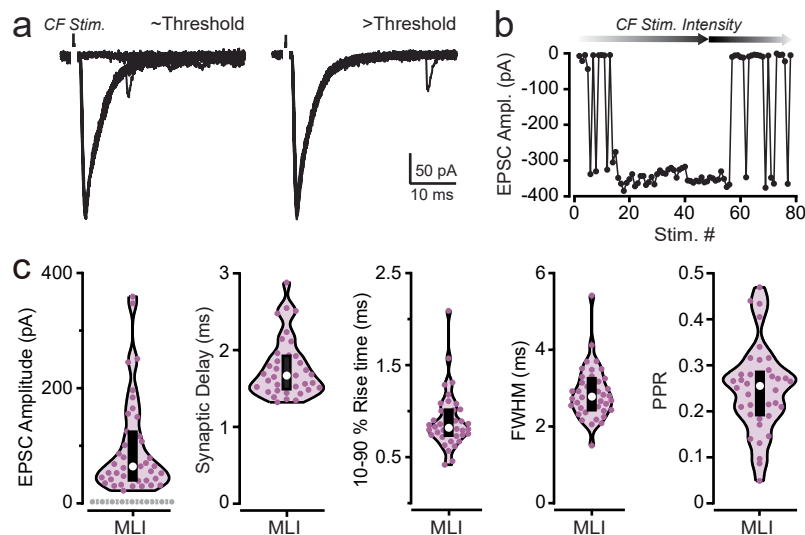
Extended Data Fig. 1 | Planar CF arborization facilitates extensive CF-MLI interfaces. **a**, 3D reconstruction of a PC (green) and its presynaptic CF (orange) viewed from XY (top) and XZ (bottom) planes. Scale bar: 25 μm . **b**, Schematic comparing expected helical CF wrapping (top) versus observed planar CF arborization (bottom) relative to MLI positions. The interfaces between membranes (red) are potential regions of interaction. **c**, Local coordinate system

for dendritic analysis: $\mathbf{v}_{\text{dend}}$ (local tangential vector of the dendritic skeleton), $\mathbf{v}_{\text{plane}}$ (a vector in the mean plane of the PC dendrites), $\mathbf{v}_{\text{norm}}$ (the normal vector defined by the cross product $\mathbf{v}_{\text{dend}} \times \mathbf{v}_{\text{plane}}$). The same coordinate system was depicted in (b) for reference. **d–f**, Angular volume distributions of CF terminals (d), PFs (e), and MLI dendrites (f) around PC dendrites. $\mathbf{v}_{\text{norm}}$ direction = 90° (mean over $n = 10$ PCs $\pm$ s.e.m.).



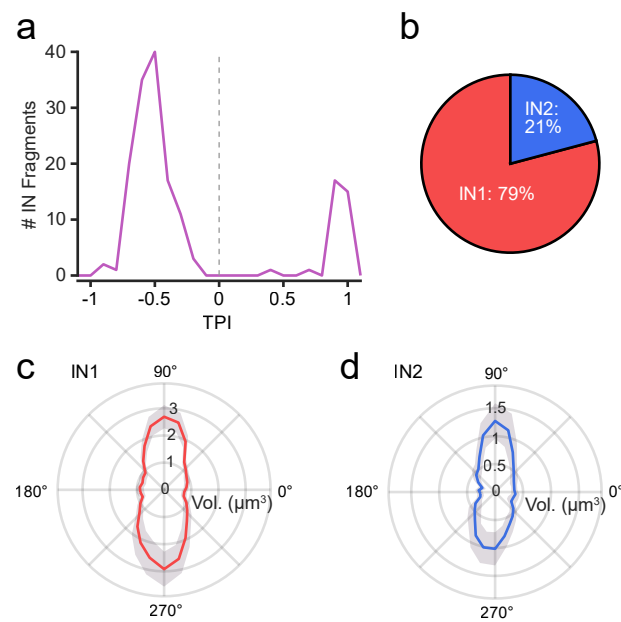
Extended Data Fig. 2 | CF-MLI synapses are large and independent of PF-MLI synapses. **a**, Comparison of contact areas of synapses and non-synaptic appositions between CFs and MLIs (gray) with further differentiation based on IN type (red and blue). Each dot represents an individual contact site ($n = 5$ IN1 and $n = 47$ IN2 synapses (Syns) and $n = 36$ IN1 and $n = 39$ IN2 appositions (Apps); adjusted P values are: $P = 0.00055$ for Syn vs App; $P = 0.337$ for IN1 Syn vs IN2 Syn; $P = 0.994$ for IN1 App vs IN2 App; $P = 0.999$ for IN1 Syn vs IN1 App; $P = 0.008$ for IN2 Syn vs IN2 App; one-way ANOVA followed by one-sided Tukey-Kramer tests; box plots: median $\pm$ quartiles). **b**, Spatial relationship between CF contacts and PF-MLI synapses. Observed synaptic and non-synaptic appositions are repeated from Fig. 2h for comparison. Their respective random controls Syn' and App' were calculated by randomizing PF-MLI synapse locations instead of CF-MLI, for a single set of Syn' and App'. The difference between Syn vs Syn' originates

from the fact that PF-MLI random point can be chosen inside the CF-MLI contact areas ($n = 52$ IN Syn^(r)s and $n = 75$ IN App^(r)s; adjusted P values are: $P = 0.677$ for Syn vs App; $P = 0.710$ for Syn' vs App'; $P = 0.021$ for Syn vs Syn'; $P = 0.736$ for App' vs App'; one-way ANOVA followed by one-sided Tukey-Kramer tests; box plots: median $\pm$ quartiles). **c**, Proportions of the statistical test results for 100 different sets of Syn' and App', respectively for PF-MLI and CF-MLI synapse location randomization. **d, e**, Same as (**b, c**) for the same contact sites grouped differently by IN1 and IN2 (repeated from Fig. 4m) and their random dendritic locations IN1' and IN2' for PF-MLI randomization (**d**, $n = 41$ IN1^(r) contacts and $n = 86$ IN2^(r) contacts; $P = 0.469$ for one-way ANOVA test; box plots: median $\pm$ quartiles; **c, e**, $P < 0.05$ for each pair in a respective one-sided test for $L < R$ or $L > R$; $P > 0.05$ for each pair in both sides of one-sided tests for $L = R$; one-sided t-tests; no adjustments for multiple comparisons).



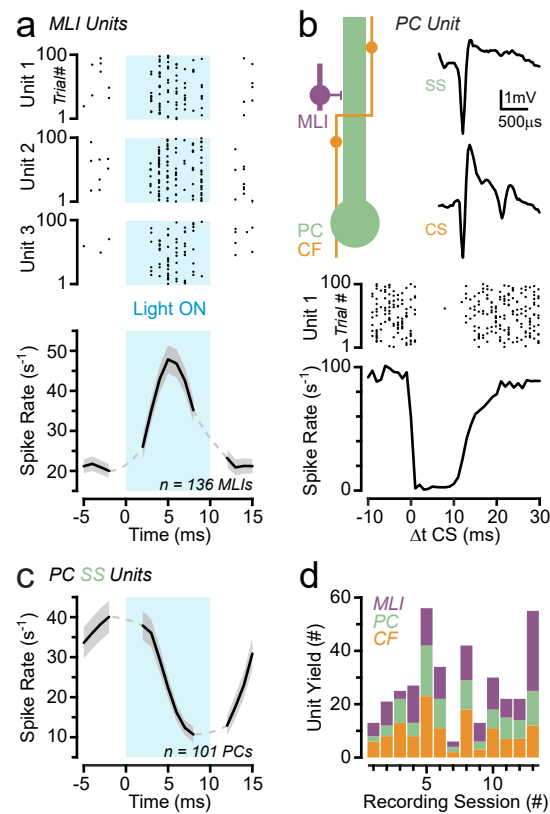
Extended Data Fig. 3 | CF-MLI EPSC characterization. **a**, Representative MLI responses to CF stimulation demonstrating minimal stimulation procedure used to recruit single CF inputs. Threshold stimulation produced all-or-none CF EPSCs with intermittent failures, while increasing stimulation intensity decreased failure rates without affecting EPSC amplitude, confirming single CF activation. **b**, EPSC peak amplitude plotted as a function of stimulus intensity for the same MLI, demonstrating the all-or-none recruitment characteristic of single fiber

stimulation. **c**, Statistical summary of CF-evoked EPSCs across recorded MLIs ($n = 36$ from 31 mice), including peak amplitude, synaptic delay, kinetics (10–90% rise time and full width at half maximum amplitude; FWHM), and paired-pulse ratio (PPR, EPSC2/EPSC1). Each dot represents one MLI; gray dots in amplitude plot indicate cells without detectable CF-evoked responses. Data shown as median $\pm$ quartiles.



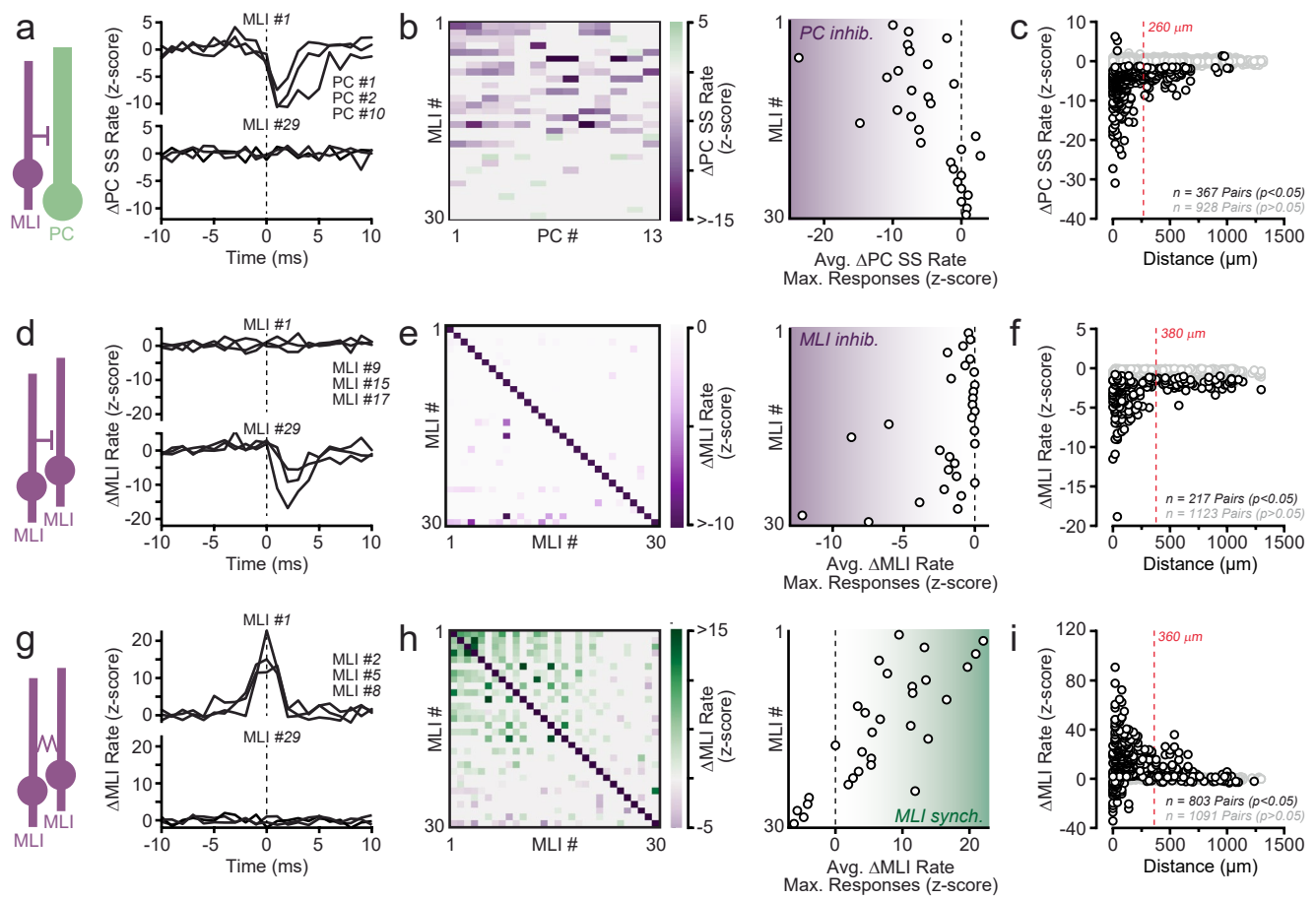
Extended Data Fig. 4 | Analysis of MLI fragments and organization of MLI subtypes relative to CF innervation. a,b, Distribution of TPI values for partially reconstructed MLI fragments (**a**), confirming bimodal distribution between IN1

($n = 109$) and IN2 ($n = 28$) subtypes (**b**). **c,d**, Angular distributions of IN1 (**c**) and IN2 (**d**) fragments around PC dendrites relative to CF terminals, analyzed using the local coordinate system defined in Extended Data Fig. 1c.



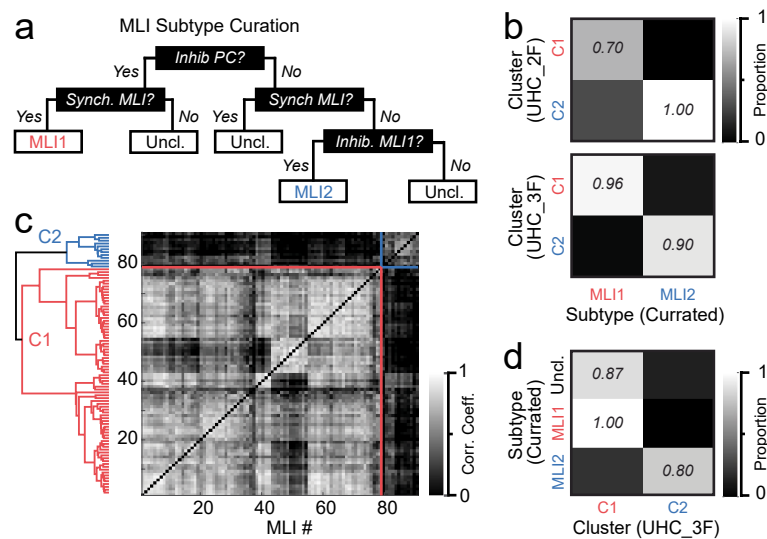
Extended Data Fig. 5 | Functional identification of cell types. **a**, Optotagging of floccular MLIs showing time-locked responses to light pulses. Top: raster plots from three example units from the same mouse; bottom: population PSTH from all identified MLIs from 6 mice (mean $\pm$ s.e.m.). **b**, PC identification criteria. Top: circuit diagram and traces from a PC unit showing characteristic simple spikes

and complex spike (CS) waveforms (averaged). Bottom: raster plot of simple spikes aligned to the occurrence of CSs and corresponding PSTH demonstrating post-CS pause from the same unit. CFs are inferred from CS activity. **c**, Population mean ($\pm$ s.e.m.) of PC simple spike activity during optogenetic MLI stimulation (6 mice). **d**, Recording yield per session for each identified cell type (6 mice).



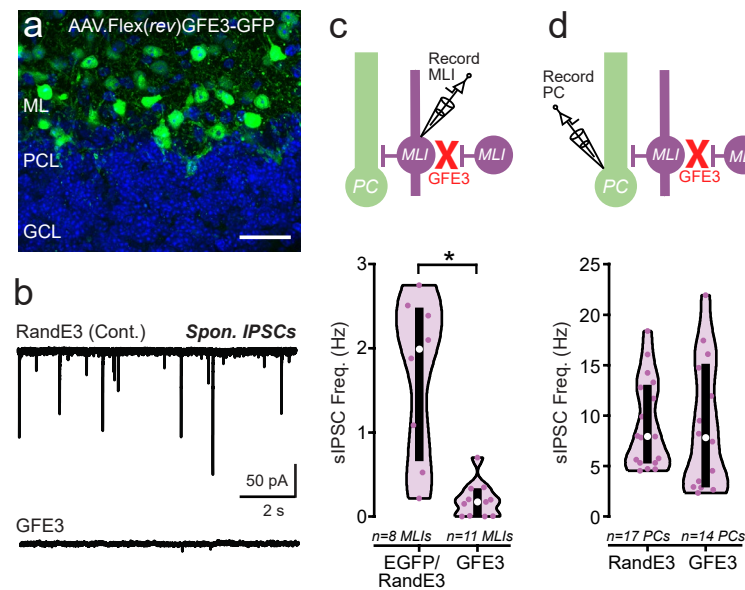
Extended Data Fig. 6 | MLI functional connectivity patterns. **a**, Spike-triggered averages (STAs) show contrasting MLI-PC functional interactions from a single recording session. Top: an MLI inhibiting multiple PCs; bottom: a neighboring MLI with negligible influence on the same nearby PCs. **b**, Left: Connectivity matrix from the recording session with all MLI-PC pairs color-coded by change in simple spike firing to each MLI (z-scored). Right: summary showing each MLI's mean inhibitory strength, calculated from interactions with its two strongest PC targets, sorted by the strength of MLI-MLI synchrony. **c**, Relationship between MLI-PC distance, inferred from Neuropixels electrode positions, and inhibitory strength. Each dot represents one MLI-PC pair; significant interactions (for each pair, $P < 0.05$; two-sided paired t-test of response time against baseline firing) in

black and non-significant interactions (for each pair, $P > 0.05$; two-sided paired t-test; no adjustments for multiple comparisons for both) in gray. Red dotted line indicates the distance threshold encompassing 75% of significant interactions, approximating the effective range of MLI-PC connectivity. **d-f**, MLI-MLI inhibitory interactions analyzed as in (**a-c**): example STAs from a single session (**d**), connectivity matrices from the same session showing all pairs and average interaction strength per MLI (**e**), and population distance-strength relationship (**f**). **g-i**, MLI-MLI synchronous interactions analyzed as in (**d-f**): example STA with zero-lag peaks (**g**), synchrony matrix with average synchrony strength per MLI (**h**), and population distance-synchrony relationship (**i**). Data (**a,b,d,e,g,h**) obtained from the same mouse; data (**c,f,i**) obtained from 6 mice.



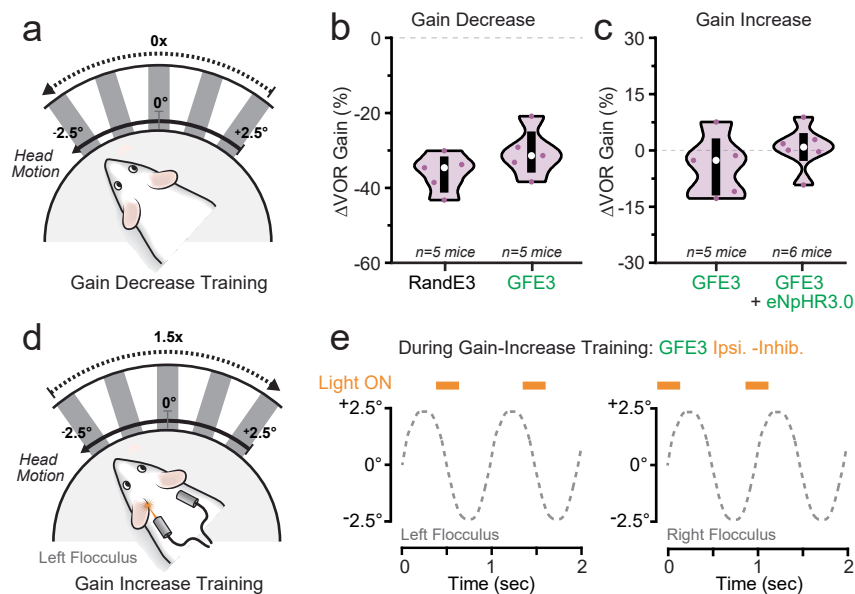
Extended Data Fig. 7 | Unsupervised clustering of MLI subtypes. **a**, Decision tree for supervised classification method based on¹⁴. **b**, Confusion matrices comparing unsupervised hierarchical clustering (UHC) to supervised classification for the subset of classifiable MLI units ($n = 96$ units). Top: using two features (2F; MLI-PC and MLI-MLI inhibition). Bottom: using three features

(3F; adding MLI-MLI synchrony). **c**, Dendrogram showing hierarchical clustering and final assignments for MLIs that met supervised classification criteria. **d**, Confusion matrix extending the analysis to all 136 optotagged MLIs, revealing how units not captured by supervised classification distribute between Cluster_1 ($n = 121$) and Cluster_2 ($n = 15$). Data (b-d) obtained from 6 mice.



Extended Data Fig. 8 | GFE3-mediated elimination of MLI-to-MLI inhibitory transmission. **a**, Fluorescence image from cerebellum of a *Kit^{Cre}* mouse injected with AAV containing Cre-dependent GFE3-eGFP, showing MLI transduction in the molecular layer. Scale bar: 25 μ m. **b**, Representative traces of sIPSCs recorded from MLIs expressing either control construct (top) or GFE3 (bottom). **c**, Quantification of sIPSC rates in floccular MLIs transduced with Cre-dependent AAVs containing control constructs (GFP or RandE3) versus GFE3 ($P < 0.0001$;

two-sided unpaired t-test; >2 mice in each condition). **d**, sIPSC rates in floccular PCs remain unchanged following GFE3-mediated elimination of inhibitory transmission onto MLIs ($P = 0.9284$; two-sided unpaired t-test; data obtained from $n = 7$ and 6 mice, RandE3 and GFE3, respectively), demonstrating preservation of MLI-to-PC inhibition while selectively disrupting MLI-to-MLI inhibitory signaling. Individual cells shown as gray circles with median $\pm$ quartiles in black.



Extended Data Fig. 9 | Preserved gain-decrease learning in GFE3-expressing mice and optogenetic rescue paradigm. **a**, Gain-decrease VOR training was accomplished by in-phase visual-vestibular pairing (60 min sessions). **b**, VOR gain measurements showing GFE3-expressing mice acquire gain-decrease adaptation comparable to control (RandE3) mice ($P = 0.1732$; two-sided unpaired t-test). **c**, Comparison of learning deficits in mice expressing GFE3 alone versus GFE3

with eNpHR3.0, demonstrating equivalent impairment of gain-increase learning ($P = 0.2936$; two-sided unpaired t-test). In plots, dots represent individual animal with median $\pm$ quartiles in black. **d,e**, Optogenetic rescue during gain-increase training. Schematic showing opposite-direction visual-vestibular pairing with bilateral light delivery (**d**) timed to the peak velocity phase of ipsiversive head motion for MLI suppression (**e**).

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☐ ☒ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☐ ☒ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	Gatan Microscopy Suite 2.3 & Zeiss SmartSEM 5.06 for 3D SBEM imaging; Custom modified TrakEM2 1.3 for EM image alignment; VAST 1.4.1 for EM training set manual segmentation; Custom-build softwares for EM reconstruction written in C++ (GCC 7.x/g++ 7.3), MySQL 5.7, and MATLAB versions 2015b to 2019a; Custom-build Synapse Detection softwares written in MATLAB 2022a with Caffe 1.0; OpenEphys v1.0 and custom codes written in LabView for Neuropixel recording; pClamp 10 for whole-cell recording.
Data analysis	Custom codes in MATLAB 2015b to 2025a for EM anatomical analyses; Kilosort 3 and Phy2 (https://github.com/cortex-lab/phy) for Neuropixel unit sorting; custom codes written in MATLAB 2023b for Neuropixel unit clustering, temporal relation analysis, and calcium imaging analyses; Neuromatic and Igor Pro for whole-cell recording analyses; NEURON simulator 8.2 with Python for neural network simulation.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The 3D EM images, proofread segmentation, and neuronal 3D rendering dataset, and cell type and synapse information used in this study are freely available without restriction are publicly available at the BossDB (<https://bossdb.org>) repository <https://bossdb.org/project/park2023>. The analysis codes, simulation parameters and codes, and software used for this study are freely available without restriction in the following GitHub repository: https://github.com/cns-kim-lab/park_cerebellar_disinhibition.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Reporting on race, ethnicity, or other socially relevant groupings

Population characteristics

Recruitment

Ethics oversight

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Data exclusions

Replication

Randomization

Blinding

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involvement in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involvement in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	C57BL/6J (WT), Nos1Chr2 and KitCre mice were used. Refer to Methods section for details.
Wild animals	No wild animals used in this study.
Reporting on sex	Mouse sex was reported in Methods section.
Field-collected samples	No field-collected sample was used in this study.
Ethics oversight	All animal procedures complied with institutional and national guidelines and were approved by the appropriate oversight committees (IACUC). See Methods for details.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	No plant was used in this study.
Novel plant genotypes	No plant was used in this study.
Authentication	No plant was used in this study.