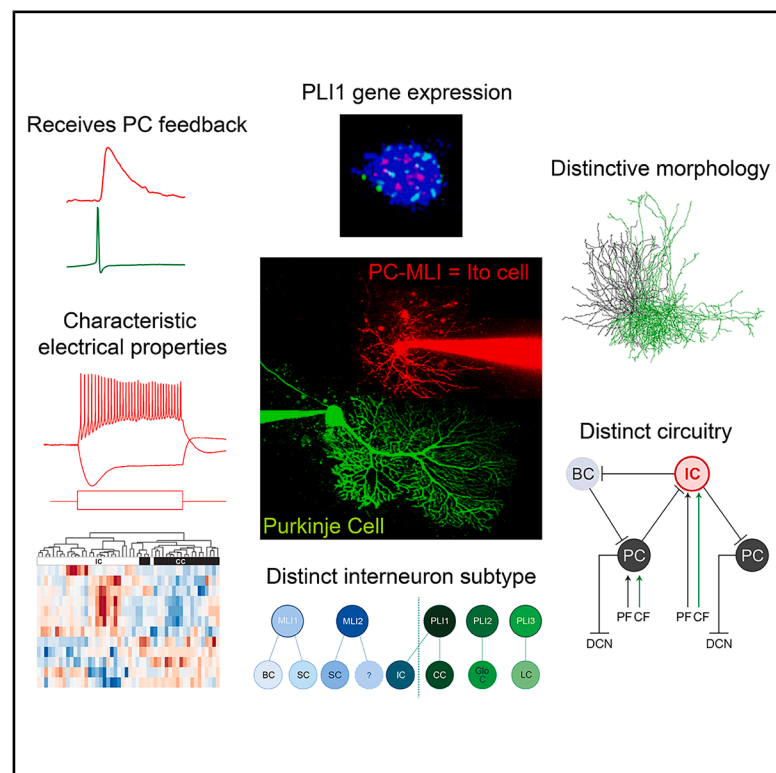


Defining a subclass of cerebellar molecular layer interneurons by their Purkinje cell feedback

Graphical abstract



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In brief

Kim & Augustine describe the anatomical, electrophysiological, molecular and connectomic properties of a distinct type of cerebellar molecular layer neuron. As recipients of inhibitory feedback from Purkinje cells, these interneurons play a specialized role in controlling cerebellar output.

Highlights

- MLIs receiving PC input (PC-MLIs) are a distinct population of cerebellar MLIs
- They exhibit defining structural, electrical, molecular, and connectomic properties
- Their unique properties may allow these cells to participate in motor learning



Article

Defining a subclass of cerebellar molecular layer interneurons by their Purkinje cell feedback

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SUMMARY

A subset of molecular layer interneurons (MLIs), previously termed Purkinje cell-input MLIs (PC-MLIs), receive inhibitory feedback from Purkinje cells (PCs), the sole output neurons of the cerebellar cortex. Here we have characterized the properties of these cells. Compared to other MLIs and Purkinje layer interneurons, they have distinct axonal and dendritic morphology, intrinsic electrical properties, and expression of marker genes. These interneurons also display distinctive circuit connectivity: they are the only MLI that receives feedback inhibition from PCs and receive minimal inhibition from basket or stellate cells. In turn, they mainly inhibit PCs and basket cells. These interneurons do not form electrical synapses with other neurons but receive the same excitatory inputs—from parallel fibers and climbing fibers—as other MLIs. These properties allow them to shape the output of the cerebellar cortex differently from other interneurons and may account for their proposed role in cerebellar motor learning.

INTRODUCTION

The cerebellar cortex plays many fundamental roles in brain information processing and behavior.¹ Molecular layer interneurons (MLIs) control the output of the cerebellar cortex by providing strong inhibitory input to Purkinje cells (PCs), the only output neurons of the cerebellar cortex.² Traditionally, MLIs have been classified into two anatomically distinct subtypes: basket cells (BCs) that inhibit PC somata and stellate cells (SCs) that inhibit PC dendrites.³ These MLIs play an essential role in cerebellar computations; for example, activating MLIs generates movement commands by suppressing PC firing,⁴ while silencing MLI activity interrupts cerebellar motor control.⁵ Therefore, it is critical to understand the precise organization and function of MLI circuits.

In addition to feedforward inhibition of PCs by MLIs, there is a monosynaptic, inhibitory feedback circuit from PCs to MLIs.^{6,7} This feedback inhibition is rapid and GABAergic. The PC-to-MLI feedback circuit is relatively short-range, with a distance of 200 μ m or less between the somata of presynaptic PCs and postsynaptic MLIs, and up to 2 PCs converge upon a single MLI.⁷ *In vivo* recordings of MLI activity and computational simulations of this feedback circuit suggest that it may play an important role in cerebellar learning by synchronizing neighboring PCs.⁷

Although MLIs outnumber PCs by approximately 10 to 1,⁸ only a small fraction of MLIs receive feedback from PCs.⁷ Here, we provide a detailed characterization of the subpopulation of MLIs that receive PC feedback. Based on their morphological, physiological and synaptic circuit properties, these interneurons are not BCs or SCs. Their physiological properties, expression of marker genes, and synaptic connectivity also indicate that they

differ from the two previous molecularly defined MLI populations, termed MLI1 and MLI2.⁹ Further, they are not candelabrum cells, the principal interneuron of the nearby Purkinje layer.¹⁰ We conclude that they represent a distinct type of MLI that we have named “Ito cells,” in honor of Masao Ito, a distinguished cerebellar physiologist who anticipated the defining property of Ito cells by being the first to demonstrate that PCs are inhibitory.¹¹ The specialized circuit connectivity of Ito cells, compared to that of other MLIs, must enable Ito cells to play a distinctive role in cerebellar network function and could account for the proposed contribution of Ito cells to cerebellar learning.⁷

RESULTS

Previous work established the presence of a local inhibitory feedback circuit in the cerebellar cortex, from PC axon collaterals to MLIs, throughout the entire cerebellum, including both lateral and vermis regions.^{6,7} Activation of this monosynaptic circuit generates inhibitory postsynaptic currents (IPSCs), but in only a subset of MLIs. This can be seen in the whole-cell patch clamp recordings shown in Figure 1A, where action potentials in individual PCs did not evoke IPSCs in BCs or SCs. Instead, IPSCs were observed only in a subset of MLIs that were previously termed “PC-MLIs”⁷ (Figures 1A and S1A). The goal of this paper is to establish that PC-MLIs are a distinct subtype of MLI that differs in many ways from other MLIs. Because they are a distinct subtype of MLI that receives PC inhibitory feedback, we have named them “Ito cells” (ICs) in honor of Masao Ito.⁺ The receipt of PC feedback is the defining characteristic of ICs. (*It is important to note that Prof. Ito gave us permission to use his name for this purpose).



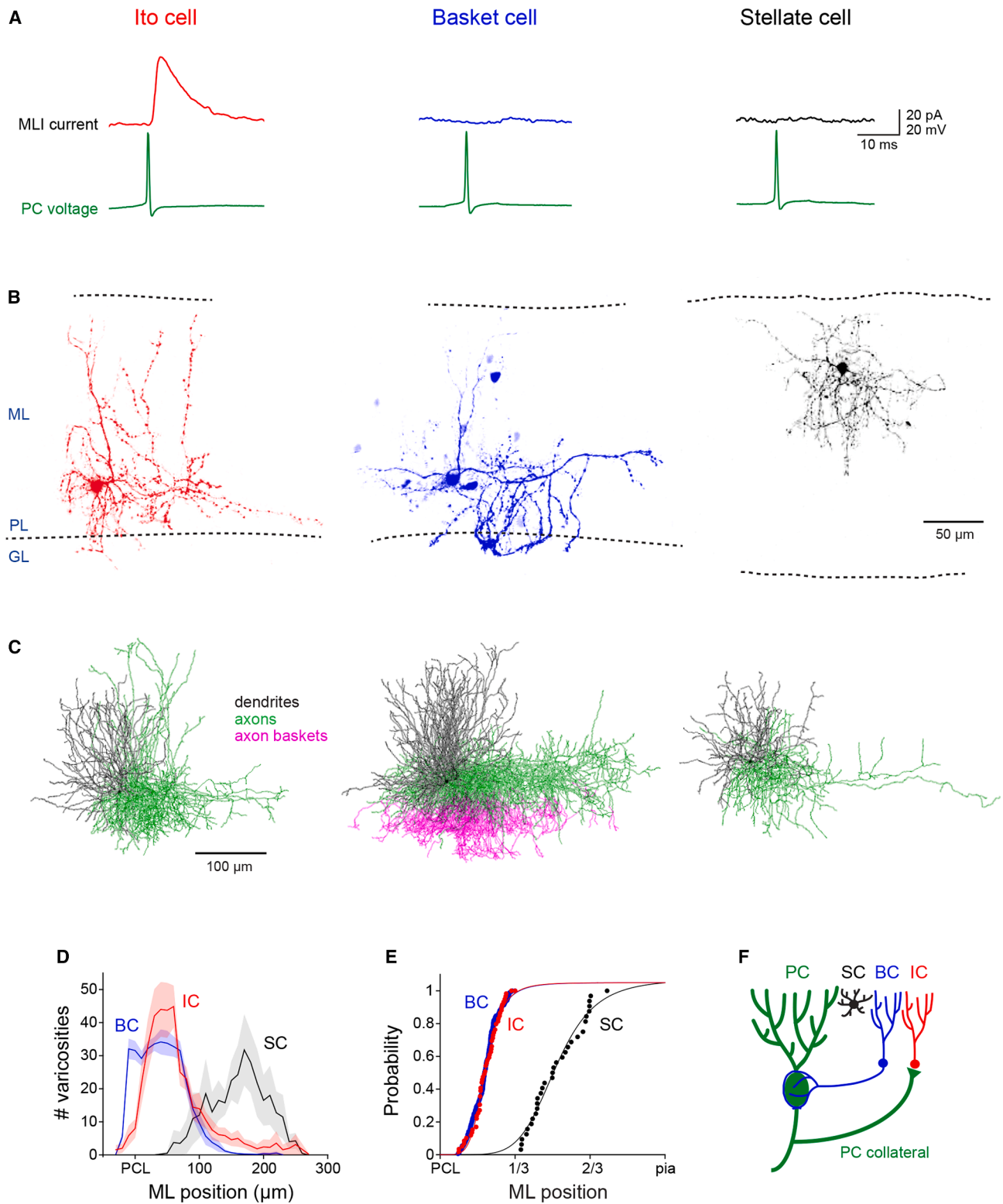


Figure 1. Comparison of morphological properties of ICs and other MLIs

(A) Ito cells (ICs) were identified based on their receipt of inhibitory feedback from Purkinje cells (PCs): in dual patch-clamp recordings, spontaneous APs in presynaptic PCs (bottom) induced an outward IPSC in a postsynaptic IC (left top), but not in basket cells (BCs, middle) or stellate cells (SCs, right).

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Ito cells are morphologically distinct

We began by comparing the morphology of ICs to that of the other MLIs. For this purpose, the structures of these cells were visualized by iontophoretically injecting neurobiotin from a patch pipette into individual MLIs (Figure 1B). BCs were defined both by their location within the inner 1/3 of the molecular layer and their axonal basket structures, while SCs were located in the outer 2/3 of the molecular layer.² ICs were defined by their receipt of PC feedback and were also located within the inner 1/3 of the molecular layer⁷ but were distinguished by their IPSC responses to PC photostimulation (Figure S1A). Every BC examined had axonal baskets that surrounded PCs, while SCs had no baskets and a more radial, star-like appearance (Figure 1B).^{3,12–14} IC axons also formed no axonal baskets. More generally, IC processes were morphologically distinct from the other types of MLIs; these distinctions are readily visualized in Figure 1C, where the structures of multiple labeled ICs, BCs and SCs are superimposed and aligned by their cell bodies. While SCs had relatively short and radiating dendrites (black in Figure 1C), BC dendrites ascended across the entire molecular layer, toward the pial surface; Ito cell dendrites were rather similar to those of BCs.^{3,12} The smooth, thick axons of BCs (green in Figure 1C) ran parallel to the PC layer and generated several thick descending collaterals (12.4 ± 0.8 per cell) that formed pinceau around PC axon hillocks and pericellular baskets around PC somata (magenta in Figure 1C).^{3,12} In contrast, IC axons were relatively slender and beaded (Figure S1B). Branches of IC axons often ascended vertically through the entire molecular layer (Figure 1C left), while BC and SC axon branches were typically shorter. Quantification revealed that ICs had a larger number of thin, beaded axons compared to those of BCs (Figure S1C). The axonal fields of ICs also were more broadly distributed in the vertical direction (Figures 1D and S1D) and somewhat more narrowly distributed in the horizontal direction (Figure S1E). Like BCs, the cell bodies of ICs were found within the first 100 μm of the inner molecular layer (Figure 1E, blue and red), with a median distance of 60 μm between their somata and the center of the PC layer. In summary, their structures and their location within the molecular layer—in addition to their receipt of feedback inhibition from PCs—distinguish ICs from BCs and SCs (Figure 1F).

Ito cells have distinct electrical properties

We next used whole-cell patch clamp recordings to compare the electrical signaling properties of ICs to those of other MLIs. In these experiments, SCs were identified by their location within the outer 2/3 of the molecular layer. Within the inner third of the molecular layer, ICs again were differentiated from BCs based on their IPSC responses to optogenetic stimulation of PCs (Figures 1A and S1A).

Quantitative analysis of 20 intrinsic electrical properties¹⁵ revealed multiple differences in both the passive and active electrical properties of the three MLI types (Table S1). The mean resting membrane potential of ICs was -64.0 ± 0.8 mV, which was significantly more negative than that of BCs or SCs (Figure S2A). While all 3 types of MLIs exhibited fast-spiking properties (Figure 2A; ref. 14), most MLIs—including ICs—did not spontaneously fire action potentials (APs) under our experimental conditions. There were several notable differences between ICs and the other MLIs in the APs evoked by depolarizing current pulses (Figure 2B, left). AP amplitude was larger in ICs than in BCs (Figure 2C), while the undershoot (afterhyperpolarization, or AHP) that follows APs was smaller for ICs than SCs (Figure 2D). ICs also had significantly longer latencies to generate an AP in response to depolarizations (Figures S2B and S2C).

Several other differences between ICs and the other MLIs were apparent in their repetitive firing of APs in response to prolonged depolarizations (Figure 2A). For a given depolarizing current, mean AP frequency tended to be higher in ICs and SCs compared to BCs (Figure S2D). However, the maximum AP frequency of ICs was ~ 60 – 80 Hz; this was lower than that of the other MLIs, which fired APs at frequencies greater than 80 Hz in response to 90 pA currents (Figure S2D) and can fire up to 300 Hz in response to larger currents.¹⁶

During prolonged depolarization of ICs, AP frequency slightly accelerated (Figure 2A, left), indicating negative frequency adaptation (Table S1). This behavior was not observed in BCs and was reduced in SCs (Table S1). The amplitude of APs also progressively declined during repetitive activity (Figure 2B, right), attenuating the ratio of the amplitudes of the last/first APs (Figure S2E). This adaptation of AP amplitude was significantly greater in ICs than in the other MLIs (Figure S2E). In addition, during repetitive activity, the APs of ICs broadened much more than the APs of BCs and SCs (Figure 2B, right; Figure S2F). Such spike broadening yielded an increase in the ratio of AP rise/decay slopes that was significantly greater for ICs compared to the other MLIs (Figure 2E). Finally, AP threshold and AHPs changed during prolonged depolarization (Figure 2A), leading to a “build up” of depolarization that was significantly greater for ICs than for BCs or SCs (Figure 2F).

One of the most prominent differences in the electrical signaling of ICs was their response to hyperpolarizing current pulses. Application of such currents to ICs yielded a hyperpolarization of the membrane potential that was not sustained even though the applied current was constant (Figure 2A, left). Such a “depolarizing sag” in the membrane potential was significantly larger in ICs than in BCs or SCs (Figure 2G). Following the end of hyperpolarizing current pulses, there was also a rebound depolarization (Figure 2A); in approximately 10% of ICs (but not BCs or SCs), this depolarizing afterpotential was sufficiently large to

(B) Examples of neurobiotin-filled IC (red), BC (blue) and SC (black); all images were reconstructed from optical sections collected via two-photon microscopy and aligned in the sagittal plane. ML, molecular layer; PL, Purkinje cell layer; GL, granule cell layer. Scale bar, 50 μm .

(C) Superimposition of images of multiple reconstructed ICs (left; $n = 7$), BCs (middle; $n = 18$), and SCs (right; $n = 4$). Process identity is indicated by color scale in inset; cells are oriented with PL at bottom. Scale bar, 100 μm .

(D) Distribution of axonal varicosities of BCs ($n = 18$), SCs ($n = 4$), and ICs ($n = 7$) within the molecular layer. Lines indicate mean values and shading indicates ± 1 SEM.

(E) Relative location of MLI soma within ML. Both BCs and ICs were distributed within the inner third of the ML, while SCs were in the outer ML.

(F) Diagram of synaptic circuits between PCs and MLIs; for clarity, feedforward inhibition of PCs by ICs and SCs is not shown.

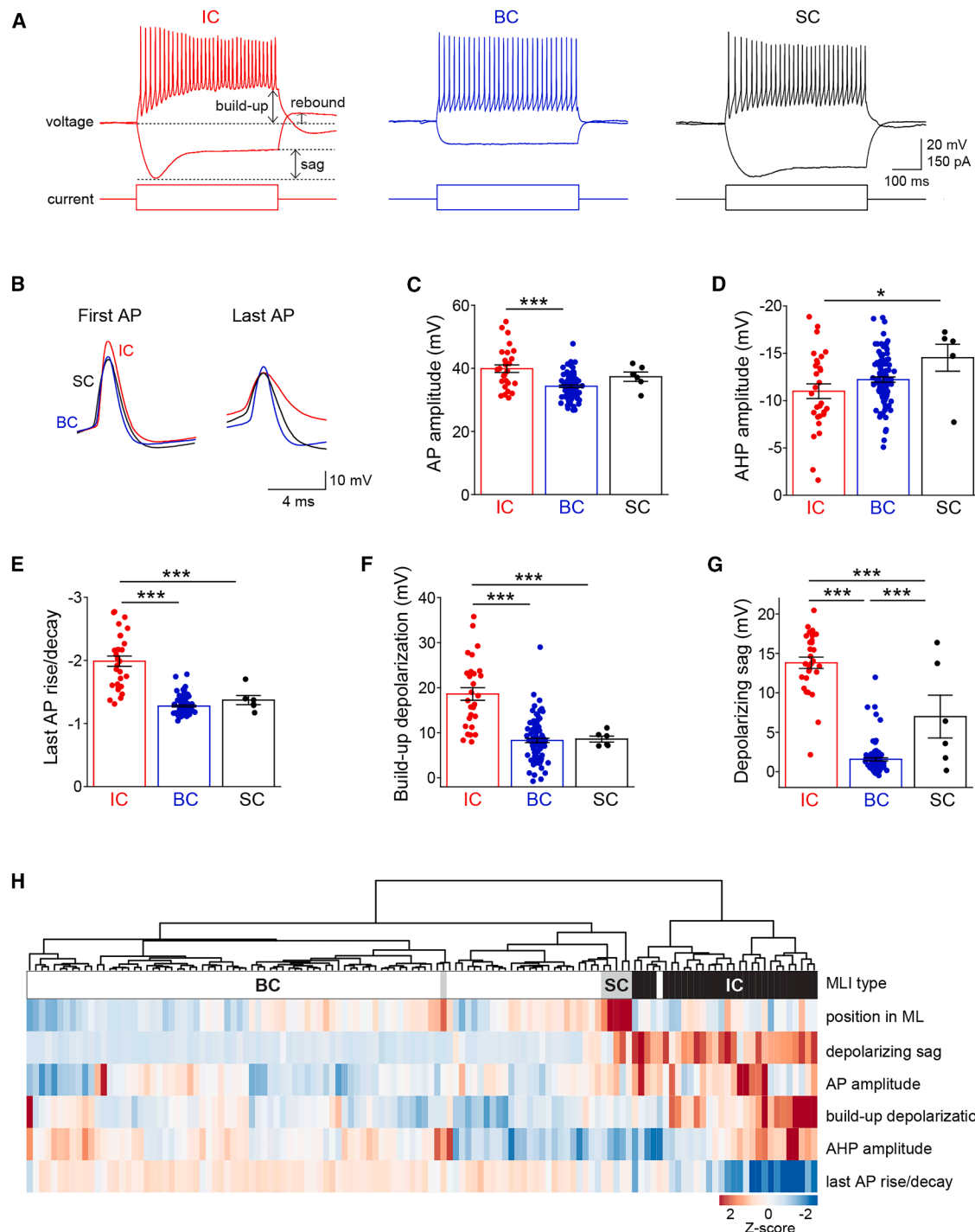


Figure 2. Comparison of electrical properties of ICs and other MLIs

(A) Responses of MLIs to depolarizing and hyperpolarizing current pulses (500 ms duration). Depolarizing sag in response to hyperpolarizing current pulses was measured as the difference between the peak hyperpolarization and the potential at the last time point of the current pulse. Build-up depolarization, due to the gradual shift in membrane potential during repetitive firing, was quantified as the difference between the resting membrane potential and the membrane potential immediately preceding the last AP.

(B) Comparison of waveforms of first and last APs in trains recorded in an IC (red), a BC (blue) and an SC (black). APs were aligned to their peak times.

(C–G) Diagnostic electrical properties of ICs. ICs have (C) larger APs, (D) smaller AP after-hyperpolarization (AHP), (E) higher rise-to-decay ratio in the last AP in a train, and (F) greater build-up depolarization during an AP train. (G) ICs produced larger depolarizing sags in response to a hyperpolarizing current (–45 pA),

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evoke an AP. Both responses arose from the hyperpolarization-activated cation current^{17,18} whose properties will be considered in detail in the following section.

To determine which intrinsic electrical properties are most uniquely associated with ICs, Orange 3 software¹⁹ was used to evaluate how much each property listed in Table S1 contributed to predicting the subtype of individual MLIs. This analysis identified six characteristic properties for MLI classification; unsupervised hierarchical clustering,²⁰ based on these six properties, grouped MLIs with similar features (Figure 2H). These groupings closely correlated with the independent identification of ICs based on the presence (ICs) or absence (BCs, SCs) of PC feedback inhibition (Figure 2H, top). In conclusion, ICs have distinct intrinsic electrical properties compared to BCs or SCs. Indeed, these distinctions are sufficient to reliably differentiate individual ICs from the other MLIs.

To assess whether these electrophysiological differences persist at warmer temperatures, we measured intrinsic properties in the same cells at 25°C and 32°C (Figures S3A–S3G). Temperature sensitivity was assessed using the Q_{10} coefficient, which reflects the change in each property for a 10°C increase in temperature. While some properties were largely unaffected, several—including depolarizing sag, action potential amplitude, threshold, input resistance, and AHP—were significantly reduced ($Q_{10} < 1$), whereas the frequency of both spontaneous and depolarization-evoked APs increased ($Q_{10} > 1$) (Figure S3H). Despite these changes, Q_{10} values were similar across cell types; this indicates that relative differences between MLI subtypes are preserved at near-physiological temperature.

Ito cells have prominent hyperpolarization-activated cation current

The prominent “depolarizing sag” observed in ICs in response to membrane hyperpolarization (Figure 2A) suggests the presence of a hyperpolarization-activated cation current (I_h ^{17,18}). Indeed, when voltage-clamped ICs were hyperpolarized to potentials more negative than the holding potential of -83 mV, I_h was apparent as a slowly developing inward current (Figure 3A, left). Treatment with ZD7288 (10 μ M), a selective I_h blocker,¹⁸ eliminated this slow inward current and defined it as I_h (Figure S4A). ZD7288 reduced I_h by $84.6 \pm 0.1\%$ in ICs ($n = 3$), $85.5 \pm 0.1\%$ in BCs ($n = 3$), and $89.3 \pm 0.1\%$ in SCs ($n = 2$), with no significant differences across groups. This drug also abolished the depolarizing sag response measured under current-clamp conditions, revealing that the sag is caused by I_h (Figure S4B). The magnitude of I_h increased with larger hyperpolarizations (Figure 3B, red), another characteristic feature of I_h .¹⁷ This was due to a hyperpolarization-dependent increase in the underlying conductance (Figure 3C); the maximum value of this conductance was 0.97 ± 0.1 nS (Figure 3D; $n = 17$). I_h was significantly smaller in both BCs (Figures 3A and 3B,

blue) and SCs (Figures 3A and 3B, black), as were the underlying conductances (Figures 3C and 3D). Repolarizing the membrane potential from -163 mV back to the holding potential produced inward tail currents that reflected the slow deactivation of I_h (Figure 3A). Tail current density was also significantly larger in ICs than in other MLIs (IC: -1.7 ± 0.4 , BC: -0.7 ± 0.03 , SC: -1.2 ± 0.5 pA/pF; $p < 0.001$ comparing ICs to BCs). This tail current was blocked by ZD7288, as was the associated rebound depolarization (Figures S4A and S4B). Thus, one characteristic property of ICs is their large hyperpolarization-activated cation current, which leads to both the depolarizing sag and rebound depolarization in response to membrane hyperpolarization.

Ito cells lack electrical synapses

Examination of the traces in Figure 3A indicates that the input resistance—evident as the initial jump in membrane leak current (I_{leak}) prior to the activation of I_h —also differed between MLI subtypes. Measurement revealed that the mean input resistance of BCs was lower than that of other MLIs (Figure 3E). Given that MLIs can be connected by electrical synapses,^{13,21–23} which reduce their input resistance,^{9,24,25} we hypothesized that BCs have lower resistance because they form electrical synapses. We tested this hypothesis in two ways. First, we asked whether mefloquine (MFQ), a gap junction blocker that inhibits electrical synapses,²⁴ alters the input resistance of ICs or BCs. Although gap junction blockers have side effects, we used MFQ because a previous study²¹ found that MFQ treatment has minimal side effects on MLIs and yields results in line with those obtained from connexin-36 knockout mice.²⁶ While MFQ significantly increased the input resistance of BCs, IC input resistance was unchanged (Figure 3H). This indicates that BCs possess gap junctions, while ICs do not.

As an independent test of the hypothesis, we used the recording pipette to introduce into MLIs neurobiotin, which can pass from cell-to-cell via the gap junctions found at electrical synapses.²² We found that neurobiotin introduced in one BC often spread to neighboring cells to yield small clusters of tracer-coupled MLIs (circles in Figure 3G, lower; Figure S4C; $n = 22$). The same was true for one SC with low input resistance. In contrast, ICs (Figure 3G, upper left; $n = 7$) and SCs with high input resistance (Figure 3G, upper right; $n = 4$) displayed no discernible tracer coupling. Combining results from all three types of MLIs, we observed an inverse relationship between the number of tracer-coupled cells per cluster and their input resistance (Figure 3H). This shows that higher input resistance is associated with less coupling and, presumably, fewer electrical synapses. Tracer coupling increased in proportion to the membrane conductance (inverse of input resistance), as expected if the amount of gap junction permeability determined tracer spread (Figure S4D). Together, our results lead us to

compared to other MLIs. Bars in C–G represent mean values and error bars are ± 1 SEM. Sample sizes: IC = 29, BC = 93, SC = 6. Asterisks indicate significant differences determined by ANOVA, followed by Tukey’s test (* $p < 0.05$, *** $p < 0.001$).

(H) Hierarchical clustering of MLIs based on their position in the molecular layer and the five electrical properties shown in C–G. Dendrogram clustered according to z-scores of properties (color scale bar), with each column representing one cell. At the top is an independent identification of MLI type, based on the presence (IC) or absence (BC, SC) of responses to PC photostimulation.

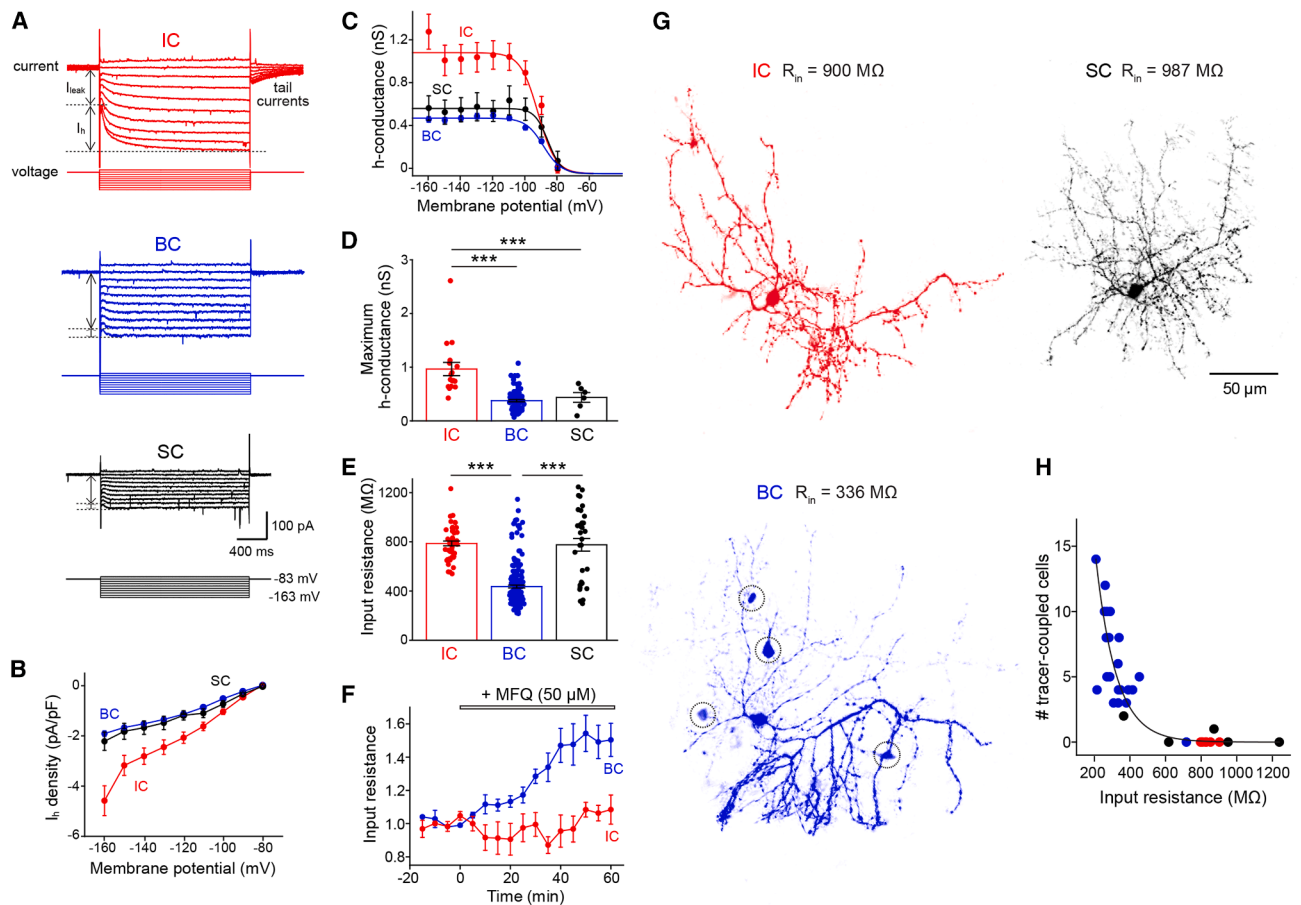


Figure 3. Comparisons of h-current and electrical synapses across MLIs

(A) Superimposed current responses (top) to a series of voltage-clamp hyperpolarizations (bottom) in representative ICs, BCs and SCs. Hyperpolarization-activated current (I_h) and leak current (I_{leak}) components of responses to the largest hyperpolarizations are indicated at left.

(B) Voltage dependence of I_h in MLIs. ICs have significantly larger I_h -current density, measured as the time-dependent inward current evoked by hyperpolarization and normalized by cell capacitance; points indicate mean values and error bars indicate ± 1 SEM. Sample sizes: IC = 17, BC = 87, SC = 6.

(C) I_h -conductance increases with greater membrane hyperpolarization. Curves indicate fits of the Boltzmann equation. Points indicate mean values and error bars indicate ± 1 SEM. Sample sizes: IC = 17, BC = 87, SC = 6.

(D–F) (D) Maximum values of I_h -conductance, determined from the measurement of I_h -conductance in individual cells. Sample sizes: IC = 17, BC = 87, SC = 6. (***) $p < 0.001$ (E) Measurement of input resistance in individual MLIs. BCs have significantly lower mean input resistance than other MLIs. Sample sizes: IC = 49, BC = 209, SC = 33. (***) $p < 0.001$ (F) Blocking gap junctions with MFQ selectively increased the normalized input resistance of BCs ($n = 10$), but not ICs ($n = 4$). Symbols indicate mean values and error bars indicate ± 1 SEM. Chemical synapses were blocked by kynurenic acid (2 mM) and gabazine (5 μ M) throughout experiment.

(G) Images of neurobiotin-filled IC (red), BC (blue) and SC (black). Neurobiotin spread only from the BC to neighboring MLIs (circles). Input resistance (R_{in}) of each MLI is indicated. Scale bar, 50 μ m.

(H) Inverse relationship between input resistance and number of neurobiotin-coupled cells. Point color indicates measurement in individual ICs (red), BCs (blue), and SCs (black). Smooth curve indicates exponential fit to data points.

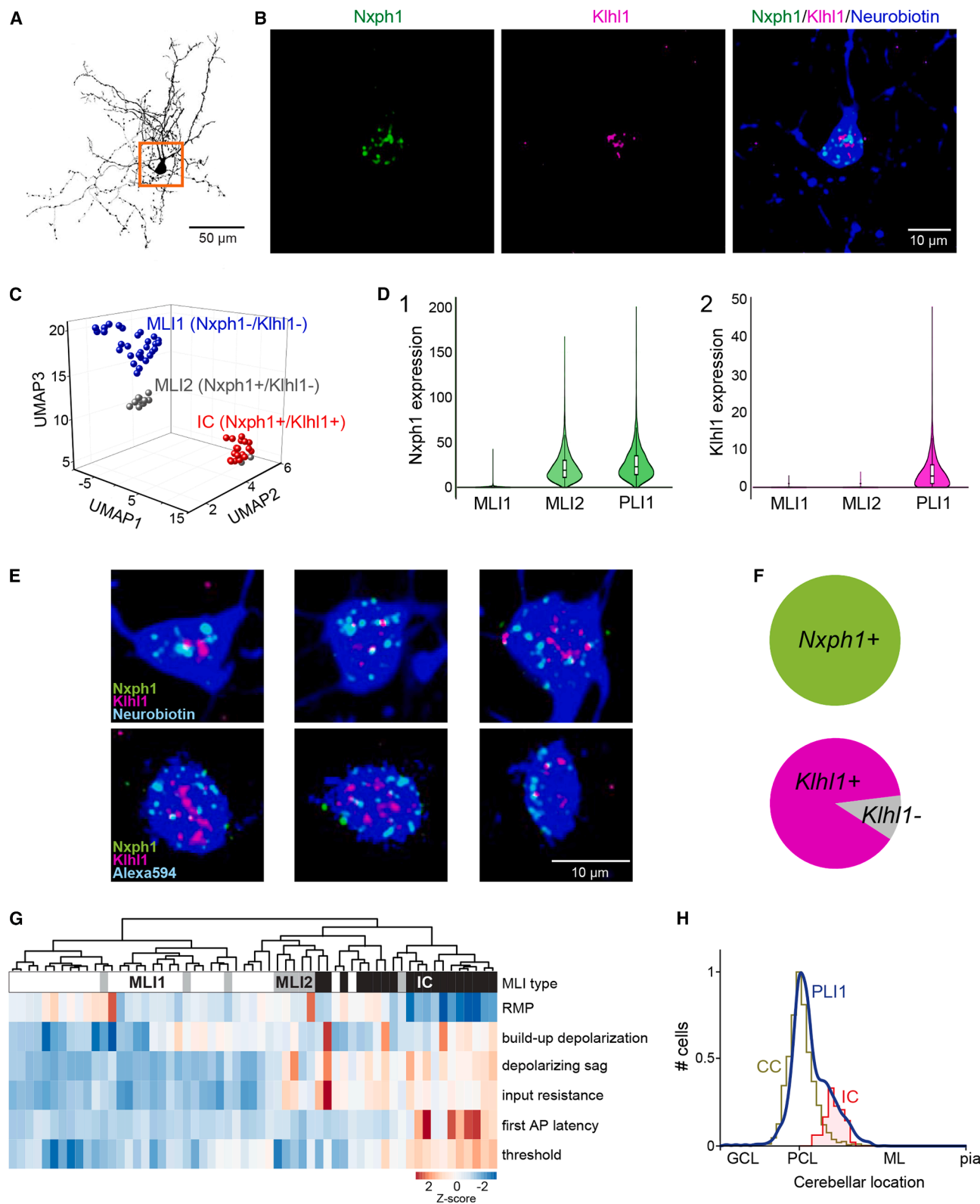
conclude that MLIs with low input resistance (chiefly BCs) are electrically coupled via gap junctions, while ICs and other MLIs with high input resistance are not.

In summary, ICs are distinct from BCs and SCs: They lack detectable electrical synapses and possess the highest density of I_h . ICs also exhibit numerous other morphological (Figures 1B–1E) and electrical (Figure 2H) properties that further distinguish them from other MLIs. Most importantly, unlike BCs and SCs, ICs receive feedback inhibition from PCs (Figure 1A). Taken together, these findings establish ICs as a separate subtype of MLI that differs from both BCs and SCs. Still more evidence that ICs form a discrete population

comes from considering their marker gene expression and synaptic circuitry, as will be done in the following sections.

Genetic profile of Ito cells distinguishes them from MLI1 and MLI2 cells

As an alternative to classification according to their anatomical properties, different populations of MLIs can also be distinguished according to their patterns of gene expression.^{9,27} Type 1 MLIs (MLI1) consist of BCs (MLI1_1) and some SCs (MLI1_2) that form electrical synapses with each other and provide feedforward inhibition to PCs. In contrast, type 2 MLIs (MLI2) consist of other morphologically heterogeneous MLIs that are found throughout



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the molecular layer and inhibit MLI1 cells.^{9,27} To determine whether ICs are MLI1-type or MLI2-type interneurons, we used single-molecule fluorescence *in situ* hybridization (smFISH)^{9,27} to assess the expression of specific gene markers in 60 physiologically phenotyped MLIs within the inner third of the molecular layer. After characterizing their electrical properties, these neurons were filled with neurobiotin or Alexa Fluor 594 (Figure 4A) to locate them during subsequent post-hoc smFISH imaging (Figure 4B). This approach allowed us to directly correlate neuronal gene expression and electrical properties.

In these experiments, photostimulation with fluorescently tagged ChR2 was incompatible with the multichannel fluorescence imaging used for the smFISH detection of gene expression. Thus, we shifted to a surrogate means of identifying ICs, based on the high input resistance and prominent depolarizing sag of these cells (Figures S5B and S5C). To validate this approach, a subset of *Klhl1*+ ICs—defined via the ratio of their input resistance and depolarizing sag—was also tested for PC connectivity by assessing IPSCs evoked by PC photostimulation. In every case (5/5), IPSCs were observed in response to PC photostimulation. Their high input resistance and large depolarizing sag also distinguished ICs from MLI1 and MLI2 cells that were identified by their expression of marker genes (Figure 4C). This indicates that this alternative strategy is an adequate surrogate means of identifying ICs.

The first marker gene that we examined was neurexophilin (*Nxph1*), a secreted protein that is rarely expressed by MLI1-type interneurons but is found in MLI2-type cells (Figure 4D1).^{9,27,28} Among the 60 cells examined, all ICs (21 cells) expressed *Nxph1* (Figures 4E and 4F); this indicates that ICs are not MLI1 cells. MLI1 cells, identified by their absence of *Nxph1* expression, also differed from ICs in many of their intrinsic electrical properties (Figures S5A–J). As a result, unsupervised hierarchical clustering of these properties readily segregated MLI1 cells from ICs (Figure 4G). Thus, ICs clearly are not MLI1-type interneurons.

To determine whether ICs are MLI2 cells, we considered a second marker gene, the actin-organizing protein kelch-like 1 (*Klhl1*). This gene is rarely expressed in MLI1 or MLI2 cells (Figure 4D2) but is abundant in nearby type-1 PC layer interneurons (PLI1), which also express *Nxph1* (Figure 4D1 and 4D2) and receive inhibitory input from PCs.¹⁰ We observed that 19 out of 21 ICs examined expressed *Klhl1*. Further, the intrinsic electrical

properties of MLI2 cells that were identified by their gene expression (Figure S5A) also differentiated MLI2 cells from ICs. While they shared numerous electrical properties with ICs, MLI2 cells had lower input resistance (Figure S5B), less depolarizing sag (Figure S5C), a more depolarized resting potential (Figure S5D), shorter latency to AP firing (Figure S5E), and a smaller depolarization required to reach threshold (Figure S5F). As a result, unsupervised hierarchical clustering of their intrinsic electrical properties positioned MLI2 cells intermediate between MLI1 cells and ICs (Figure 4G). Collectively, these results indicate that ICs are neither MLI1-type nor MLI2-type interneurons.

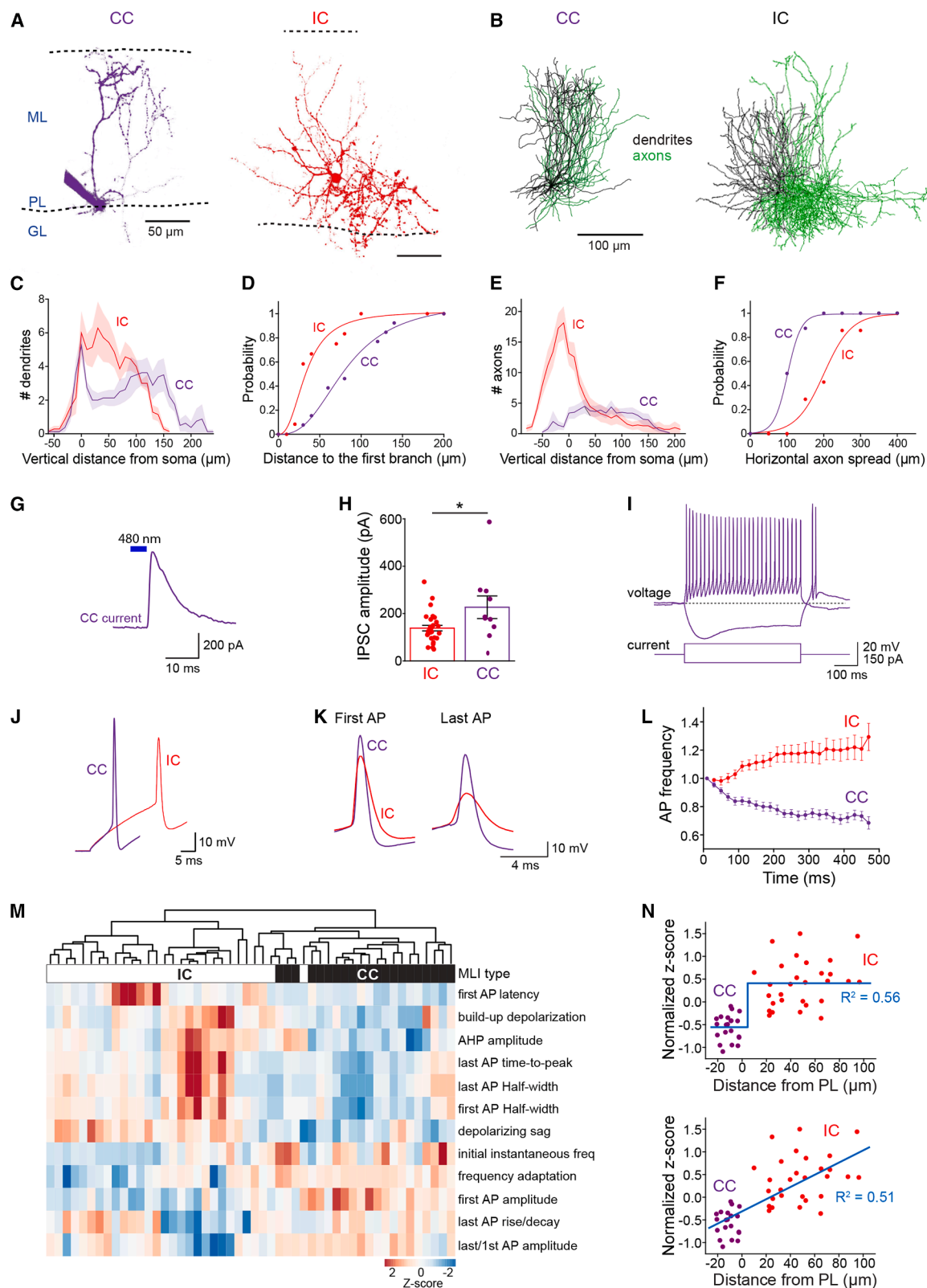
Ito cells are not candelabrum cells

Robust expression of both *Nxph1* and *Klhl1* is common to both ICs and PLI1 interneurons (Figures 4D–4F). PLI1 cells include candelabrum cells (CCs), which were originally defined by having somata that are restricted to the PC layer.²⁹ Our replotting of the smFISH results in Figure 3E of Orsino et al. (2022)¹⁰ shows that while CCs (defined by the expression of oxytocin receptors; Figure 4H, olive) are indeed found exclusively within the PC layer, other undefined PLI1 interneurons are located in the lower 1/3 of the molecular layer (Figure 4H, blue). Based on their position within the lower 1/3 of the molecular layer (Figure 4H, red), as well as their expression of the PLI marker, *Klhl1*, we propose that ICs may be the previously undefined PLI1 cells within the molecular layer.

Could ICs be “displaced” CCs? We addressed this possibility by comparing the properties of ICs to those of CCs, defined as neurons with small somata nestled between those of PCs within the PC layer.^{10,29} The morphology of dye-filled CCs (Figure 5A, purple) was distinctively different from that of ICs (Figure 5A, red) and was consistent with previous reports^{10,29}: CCs had one or two thick primary dendrites that ascended vertically toward the pia and arborized within the upper molecular layer, along with several short dendrites that projected into the granule cell layer. Alignment of the structures of multiple CCs (Figure 5B, left) made clear that CCs differ from ICs (Figure 5B, right). Compared to ICs, the vertical dendritic field of CCs within the inner molecular layer was less dense (Figure 5C) and the initial branching of their primary dendrites was farther from their somata (Figure 5D). CCs possessed thin, beaded axons that ascended vertically through the molecular layer (Figures 5A and 5B), as observed for some ICs (Figures 1B and S1B). However, CCs

Figure 4. Marker gene expression in ICs and other MLIs

- (A) Image of a neurobiotin-filled IC. Red square indicates area enlarged in B. Scale bar, 50 μ m.
- (B) smFISH imaging with probes for *Nxph1* (green, 488 nm excitation, left) and *Klhl1* (magenta, 647 nm excitation, center). Merged image, including neurobiotin staining (blue, 561 nm excitation, right panel), shows that the IC expressed both *Nxph1* and *Klhl1*. Scale bars, 10 μ m.
- (C) UMAP comparison of IC, MLI2 and MLI1 cells based on input resistance and depolarizing voltage sag in response to hyperpolarizing currents.
- (D) Comparison of (1) *Nxph1* and (2) *Klhl1* expression in MLI1, MLI2, and PLI1 cells. Violin plots of data from Kozareva et al. (2021),⁹ analyzed on the Broad Institute Single Cell Portal (https://singlecell.broadinstitute.org/single_cell/study/SCP795/).²⁸
- (E) Examples of ICs expressing both *Nxph1* and *Klhl1*. IC somata were labeled (blue) with either neurobiotin (upper panels) or Alexa Fluor 594 (lower panels); *Nxph1* probe is green, while *Klhl1* probe is magenta. Scale bar, 10 μ m.
- (F) Summary of marker gene expression in ICs: all ICs expressed *Nxph1* and 90% also expressed *Klhl1*.
- (G) Hierarchical clustering of MLIs according to the six electrical properties indicated on the right (from Figure S5). Dendrogram clustered according to z-scores of properties (colored scale bar), with each column representing one cell. At the top is an independent identification of MLI type, based on *Nxph1* and *Klhl1* expression.
- (H) Normalized distribution of candelabrum cells (CCs; olive) and PLI1 interneurons (blue), taken from data in Figure 3E of Orsino et al. (2022).¹⁰ Distribution of ICs (red) from Figure 1E can account for the subpopulation of PLI1 neurons found within the molecular layer, assuming that ICs are approximately 1/3 as abundant as CCs.



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had a lower density of axons than did ICs (Figures 5B and 5E), and their axonal field was narrower (Figures 5B and 5F). The field of the ascending axonal branches of CCs often overlapped with their dendritic field, much more than for ICs (Figure 5B).^{10,29} In summary, the morphology of ICs and CCs was quite different: ICs do not look like candelabra.

We next compared the electrical properties of ICs and CCs. Like ICs, all CCs generated rapid and large IPSCs (227 ± 48 pA) in response to PC photostimulation (Figures 5G and 5H). Similar to ICs, CCs were fast-spiking cells (Figure 5I). Both types of interneuron had high input resistance (Figure S6A), comparable resting membrane potentials (Figure S6B), and prominent I_h (Figures S6C and S6D). However, there were numerous differences between the AP firing properties of ICs and CCs. Nearly half of CCs fired APs spontaneously ($10/21$; 4.7 ± 2.3 Hz) under our experimental conditions, whereas ICs seldom did so ($6/29$; 2.8 ± 0.6 Hz). CCs also more frequently (33%) exhibited rebound firing after a hyperpolarization (Figure 5I), compared to ICs (10%). APs were larger in CCs than in ICs (Figures 5J and S6E), with larger AHPs (Figures 5J and S6F), shorter durations (Figures 5K and S6G), and shorter latencies (Figures 5J and S6H). Peak AP frequency was also greater in CCs (Figure S6I). Unlike ICs, CCs tended to exhibit positive AP frequency adaptation during prolonged depolarization (Figure 5I), with a progressive reduction in AP frequency that contrasted with the negative frequency adaptation of ICs (Figures 5L and S6J).

During CC repetitive activity, APs broadened (Figures 5K, S6G, and S6K) and their amplitude decreased, leading to a reduced last/first AP amplitude ratio in CCs (Figure S6L); all of these parameters were significantly smaller than in ICs. CCs also displayed significantly less build-up depolarization (Figure S6M) and depolarizing sag (Figure S6N) compared to ICs. Finally, unsupervised hierarchical clustering based on these electrical properties sorted ICs and CCs into distinct groups with good separation (Figure 5M).

Given the spatial proximity of ICs and CCs, is it possible that they reflect an interneuron continuum rather than being two discrete subtypes? To address this point, in Figure S7 we plotted the 12 distinctive electrophysiological parameters shown in Figure 5M as a function of the position of the neuron relative to the top of the PC layer. We then fit these data with two different functions: (1) a linear regression, reflecting the continuum model; and (2) a step function, based on the two discrete subtype model. For most of these properties (10/12), the step function model fit the data better or equally well, as indicated by their correlation coefficients. The two exceptions were the time-to-peak for the last AP in a train (Figure S7D) and AP frequency adaptation (Figure S7I), where the correlation coefficients of the linear regression fits were slightly better than those of the step function fits. We then normalized all 12 parameters according to their z-scores and merged them together for each cell. The resulting analysis (Figure 5N) makes clear that the intrinsic electrical properties of CCs and ICs differ bimodally, according to whether they reside in the Purkinje cell layer (PL) or the ML. Thus, CCs and ICs are different subtypes of PLI1 interneurons that are in different layers of the cerebellar cortex.

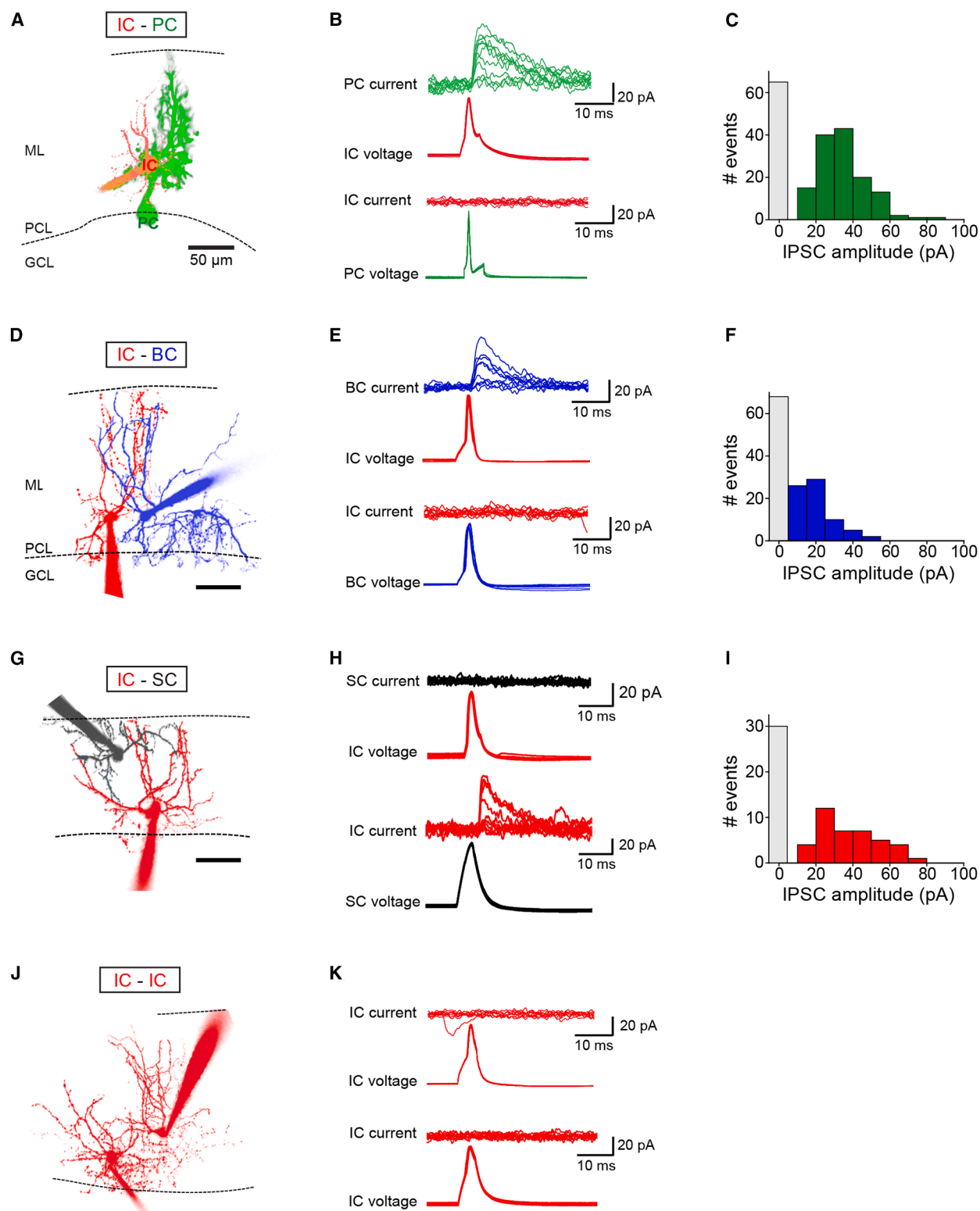
In conclusion, despite sharing both marker gene expression and receipt of PC input, ICs differ from CCs in their morphological and electrical properties, as well as in their locations (Figure 5). As will be shown next (Figures 6A–6C), ICs also provide feedforward inhibition to PCs, while CCs very rarely do.¹⁰ Therefore, ICs represent a distinct type of MLI: rather than simply being a displaced CC, ICs are different from CCs, as well as being different from BCs, SCs, MLI1s and MLI2s.

ICs to cell inhibitory circuit organization

We next characterized the inhibitory circuits that ICs form with PCs and MLIs by using patch-clamp recordings to determine the synaptic interactions between pairs of neurons.

Figure 5. Comparison of ICs and CCs

- (A) Examples of labeled CC (left, filled with Alexa dye) and IC (right, filled with neurobiotin). Solid structure in the lower left of the CC image is the dye-filled tip of the patch pipette. Scale bars, 50 μ m.
- (B) Superimposition of images of multiple reconstructed CCs (left; $n = 8$) and ICs (right; $n = 7$). Process identity is indicated by color scale in inset; cells are oriented with PCL at bottom. Scale bar, 100 μ m.
- (C) Spatial distribution of dendrites for ICs (red) and CCs (purple). Lines indicate mean values and shading indicates ± 1 SEM.
- (D) Cumulative probabilities for position of initial dendritic branch points for ICs (red) and CCs (purple).
- (E) Spatial distribution of number of IC (red) and CC (purple) axons. Lines indicate mean values and shading indicates ± 1 SEM.
- (F) Cumulative probabilities for horizontal spread of axons for ICs (red) and CCs (purple).
- (G) Example of an IPSC evoked in a CC by widefield photostimulation of PCs.
- (H) Mean amplitudes of IPSCs evoked in ICs (red) and CCs (purple) in response to widefield photostimulation of PCs. Points indicate values measured in individual cells, bars indicate mean values, and error bars show ± 1 SEM ($n = 29$ for ICs and 10 for CCs; $*p < 0.05$, Student's t test).
- (I) Representative voltage responses (top) of a CC to depolarizing and hyperpolarizing current pulses (bottom).
- (J) Examples of APs evoked by depolarizing current pulses in an IC (red) and a CC (purple). Traces are set to the same resting level and are aligned to the start of depolarization.
- (K) Comparison of waveforms of first and last APs in trains recorded in an IC (red) and a CC (purple). APs were set to the same resting level and aligned to their peak times.
- (L) Time-dependent changes in instantaneous AP frequency, contrasting the positive AP frequency adaptation in CCs and negative adaptation in ICs. Points indicate mean values and error bars are ± 1 SEM ($n = 29$ for ICs and 21 for CCs).
- (M) Hierarchical clustering of CCs and ICs according to the 12 electrical properties indicated at right (from Figure S6). Dendrogram clustered according to z-scores of properties (color scale bar), with each column representing one cell. At the top is independent identification of MLI type, based on their location in either the PL (CCs) or the ML (ICs).
- (N) Distribution of average normalized z-scores for 12 electrical properties of individual CCs and ICs across the PL and ML. A step function (upper) provides a better fit than a linear model (lower), as indicated by their correlation coefficients (R^2).



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Purkinje cells

The signature feature of ICs is their receipt of feedback inhibition from PCs (Figure 1A). Because the spatial organization and functional attributes of this feedback circuit have been characterized previously,^{6,7} here we will only address the ability of ICs to provide feedforward inhibition to PCs. Connectivity from ICs to their postsynaptic PCs was examined in oblique slices (~30° off sagittal plane), which was found to best preserve connections between ICs and their postsynaptic PCs (Figure 6A) and is consistent with our measurement that the primary axons of ICs are oriented approximately 9° ($8.8 \pm 2.4^\circ$; $n = 7$) off the sagittal plane. Inhibitory synaptic inputs from ICs to PCs were detected in ~10% of paired recordings (3/31). In these cases, stimulation of ICs produced outward IPSCs (Figure 6B, top). These IPSCs exhibited a short latency (1.03 ± 0.1 ms) and were observed in response to 70% of IC stimuli (success rate = 0.7 ± 0.06). Mean IPSC amplitude was 32.0 ± 3.9 pA at a holding potential of -53 mV (Figure 6C; 135 events in 3 paired recordings), corresponding to a synaptic conductance of 0.94 ± 0.12 nS. As previously reported,⁷ the connection between ICs and PCs was non-reciprocal, because stimulation of PCs receiving inhibitory input from ICs never evoked IPSCs in the ICs (Figure 6B, bottom).

Molecular layer interneurons

ICs also provide inhibitory input to neighboring BC/MLI1_1 cells: in 7 out of 18 experiments (Figure 6D), APs in ICs evoked IPSCs in a BC/MLI1_1 cell (Figure 6E, upper). These IPSCs were highly variable in amplitude (Figure 6F), with a CV of 0.37 ± 0.04 and a success rate of 0.5 ± 0.07 ($n = 7$). The underlying synaptic conductance was 0.53 ± 0.07 nS. The latency of these synaptic responses was approximately 1 ms, indicating that they are likely to be monosynaptic. IPSCs produced in BC/MLI1_1 cells in response to IC stimulation were quantitatively similar to those observed for inhibitory connections between other MLIs³⁰ (Table S2). Conversely, ICs did not receive inhibitory input from BC/MLI1_1 cells: stimulation of cells that received IC inhibition never evoked an IPSC in their IC partners (0/18 pairs; Figure 6E, lower).

In recordings from 13 pairs of ICs and SCs (Figure 6G), stimulation of ICs never evoked an IPSC in an SC (Figure 6H, upper). Thus, even though their axons spread throughout the ML (Figure 1D), ICs apparently do not innervate SCs. In one case, stimulation of an SC evoked IPSCs in an IC (Figure 6H, lower). These IPSCs were relatively large (Figure 6I), with a mean amplitude of 38.3 pA and a mean conductance of 1.1 nS. Notably, these IPSCs exhibited a relatively long latency (2.2 ms). This suggests that the IPSCs did not result from a direct connection between SCs and ICs; we propose that these were produced by rebound firing of PCs (SC-to-

PC-to-IC) or disinhibition of PCs (SC-to-MLI-to-PC-to-IC). We also found no connections between the 2 pairs of ICs examined (Figures 6J and 6K), though the small sample size does not allow us to make any robust conclusions.

Taken together, these results indicate that the main inhibitory input received by ICs comes from PCs. While ICs inhibit neighboring BC/MLI1_1 cells, no other direct synaptic connections (electrical or chemical) with other MLIs were detected in our recordings. Thus, our results suggest that IC circuits may lack reciprocal connections, in contrast to the extensive electrical and chemical synaptic connections that form complex, clustered networks among other MLIs.^{13,23}

Ito cell excitatory circuit organization

MLIs receive excitatory input from parallel fibers (PFs), the axons of local granule cells, as well as from climbing fibers (CFs), whose axons originate in the inferior olive.^{31,32} We next asked whether ICs also receive such excitatory input.

Extracellular stimulation of either PFs or CFs evoked excitatory postsynaptic currents (EPSCs) in ICs (Figures 7A and 7B). The mean amplitudes of EPSCs evoked by minimal stimulation were 27.6 ± 6.9 pA for PF input ($n = 7$) and 157 ± 34 pA for CF input ($n = 8$). Similar to the PF synapses of other MLIs,^{31,32} EPSCs produced in ICs in response to a second PF stimulus (50 ms interval) were larger than the EPSCs produced by the first stimulus (Figure 7A). Thus, PF inputs onto ICs exhibited synaptic facilitation. In contrast, EPSCs produced by CF stimulation showed the opposite behavior, indicating the synaptic depression that is characteristic of CF synapses of MLIs^{31,32} (Figure 7B). The peak amplitudes of EPSC evoked by PF stimulation were graded with stimulus intensity, with PF-EPSCs gradually increasing in amplitude as stimulus intensity increased (Figure 7C; $n = 7$). In contrast, CF stimulation elicited all-or-none responses with EPSC amplitude exhibiting a step-function relationship with stimulus intensity (Figure 7D; $n = 8$). In all of these properties, PF and CF inputs onto ICs resemble those found at other MLIs.

One characteristic of CF synapses is that they activate MLIs via volume transmission mediated by spillover of glutamate.³¹ We asked whether this was the case at IC excitatory synapses by applying the glutamate uptake blocker, *threo*- β -benzyloxyaspartate (TBOA) (DL-TBOA; 50 μ M). TBOA prolonged the duration of both types of EPSCs, presumably due to the slowing of glutamate removal (Figures 7E and 7F). However, while TBOA did not alter the amplitude of EPSCs induced by PF stimulation (Figure 7E), it significantly enhanced the amplitude of CF-induced EPSCs (Figure 7F). This indicates that the CF input of ICs is mediated by glutamate spillover, while PF excitation of ICs is mediated by conventional synaptic transmission. Therefore, ICs receive excitatory inputs

Figure 6. Analysis of connectivity between ICs and other inhibitory neurons

(A) Image of a dye-filled IC-PC pair. Scale bar, 50 μ m.
(B) Superimposed recordings of synaptic transmission between the neurons shown in (A). APs in the presynaptic IC (IC voltage) usually evoked IPSCs in the postsynaptic PC (PC current), while APs in the PC (PC voltage) evoked no responses in the IC (IC current).
(C) IPSC amplitudes (green) in 3 IC-PC pairs (200 events). Gray bar indicates the number of trials where no IPSCs were evoked in response to IC stimulation.
(D–F) Similar experiments for recordings of synaptic transmission between ICs and BCs (7 pairs) and (G–I) synaptic transmission between ICs and SCs. Scale bar, 50 μ m.
(J) Image illustrates patch clamp recordings from a pair of ICs. Scale bar, 50 μ m.
(K) No connections were detected between ICs ($n = 2$ pairs).

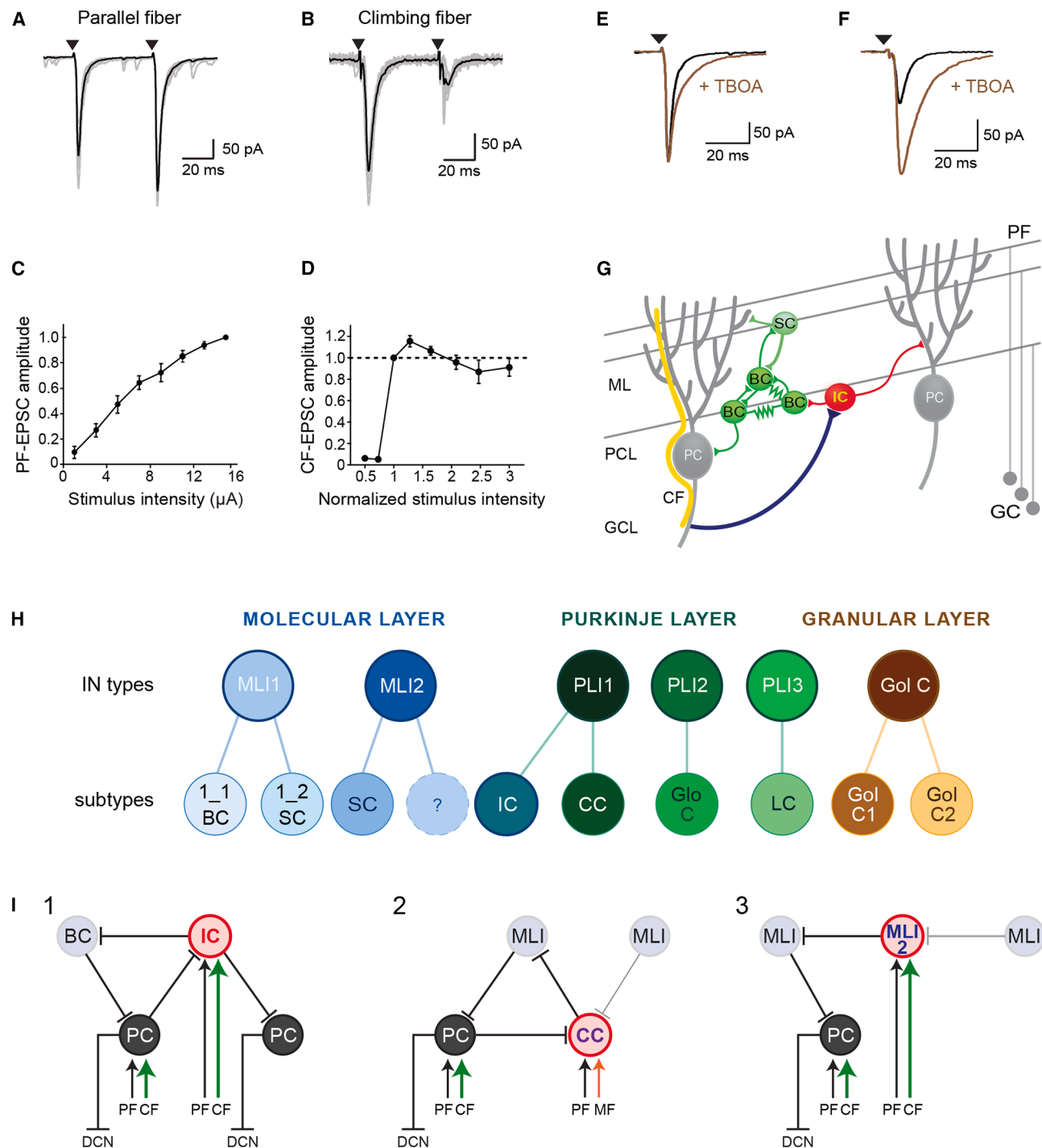


Figure 7. Excitatory inputs of ICs

(A) EPSCs evoked in an IC by two stimuli (50 ms interval, at arrowheads) applied to PFs. Response to the second stimulus was larger than the response to the first stimulus, indicating synaptic facilitation. Ten individual responses (gray) and average EPSC (black) are shown. Holding potential was -73 mV.

(B) EPSCs evoked in an IC by two stimuli (50 ms interval, at arrowheads) applied to CFs. Response to the second stimulus was smaller than the response to the first stimulus, indicating synaptic depression. Ten individual responses (gray) and average EPSC (black) are shown.

(C) Amplitude of PF-EPSCs recorded in ICs increased in a graded fashion with increasing stimulus intensity ($n = 7$).

(D) Amplitude of CF-EPSCs recorded in ICs increased in an all-or-none fashion with increasing stimulus intensity ($n = 8$). Symbols in (C) and (D) indicate mean values and error bars indicate ± 1 SEM.

(E) Treatment with the glutamate uptake blocker, TBOA (50 μ M), prolonged PF-EPSCs recorded in ICs, but did not change EPSC amplitude.

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from both PF and CF through the same synaptic mechanisms that excite other MLIs.^{31–33}

DISCUSSION

Previous work has demonstrated synaptic connections from PCs to MLIs and has characterized the basic properties of this inhibitory feedback circuit.^{6,7} Here, we have established that the neurons receiving PC feedback are a distinct subtype of MLI that we have named the “Ito cell”. Compared to other MLIs, ICs have morphological, electrophysiological, molecular, and connectivity properties (Table S3) that define them as a different subtype of MLI, regardless of whether they are classified according to anatomical criteria (BCs, SCs) or transcriptomic criteria (MLI1, MLI2). They also differ from CCs, the primary type of PCL1 interneuron. Identifying the characteristic properties of ICs aids understanding of the functional significance of these interneurons.

Ito cells are different from BCs and SCs

ICs are not BCs or SCs because of multiple differences in structure, electrical properties, and inhibitory synaptic connectivity (Table S3). ICs are structurally distinct from BCs: ICs do not form basket structures around PC somata, instead producing numerous thin, beaded axon branches (Figures 1C and S1B). Also, more IC axons extend vertically into the molecular layer in comparison to BC axons. This suggests that ICs may form synaptic contacts with vertically oriented PC dendritic arbors throughout the molecular layer, while their horizontal axons within the inner part of the molecular layer innervate BCs. Their location and axonal structure also differentiate ICs from SCs (Figures 1B–1E).

ICs have intrinsic electrical properties that clearly differentiate them from the traditional MLIs (Table S3). ICs have passive electrical properties that are different from those of BCs and SCs (Figure 3E; Figure S2A). They also exhibit many differences in AP firing properties (Figure 2) and *I_h* density (Figures 3B–3D), indicating that the complement of voltage-gated ion channels in ICs quantitatively differs from that of BCs and SCs.³⁵ Indeed, the electrical properties of ICs alone are sufficient to reliably differentiate ICs from BCs and SCs (Figure 2H).

Finally, while excitatory synaptic inputs to ICs are very similar to those of BCs and SCs, IC inhibitory circuits are unique in comparison to those of BCs and SCs (Figure 7G). Most importantly, ICs receive PC feedback while SCs and BCs do not (Figure 1A).⁷ In addition, while BCs are inhibited by ICs, SCs and other BCs,² SCs are not inhibited by ICs (Figures 6G and 6H) yet are inhibited by BCs and SCs.² Conversely, ICs generally do not receive inhibitory input from BCs or SCs. One exception was an SC-IC pair where stimulation of the SC evoked an IPSC in the IC (Figure 6H); the long latency of this response suggested a disynaptic connection, rather than a direct SC-to-IC synapse.

Ito cells are different from MLI1 and MLI2 cells

Multiple differences in molecular, electrical, and inhibitory connectivity properties demonstrate that ICs also are not MLI1 or MLI2 cells (Table S3). Surprisingly, our fluorescence *in situ* hybridization (FISH) results showed that ICs do not correspond to either of these molecularly defined interneuron types. ICs express a MLI2/PLI1 gene marker (*Nxph1*; Figure 4D1), showing that they are not MLI1 (Figure 4E). Further, they express *Klhl1* (Figure 4E), which is strongly expressed in PLI1 interneurons but not in MLI1 or MLI2 cells (Figure 4D2).¹⁰ We found that more than 90% of ICs expressed *Klhl1*, and the two ICs that did not exhibit expression could have been false negatives due to the poor signal/noise ratio of single-molecule labeling. Such a high fraction of *Klhl1* expression is not characteristic of MLI2 cells but is typical of PLI1 cells (Figure 4D2).^{9,28}

By examining the intrinsic electrical properties of individual, molecularly defined MLI1 and MLI2 cells, we also found that these properties differ from those of ICs. The electrical properties of ICs are readily differentiated from those of MLI1 cells and there are also numerous quantitative differences between the electrical properties of ICs and MLI2 cells (Figures 4G and S5). Further, the synaptic connectivity of ICs differs from that of MLI1 and MLI2 cells: while MLI2 cells receive weak inhibition from MLI1 interneurons,³⁴ ICs are not inhibited by BC/MLI1_1 (Figures 6D and 6E). Further, both MLI1 and MLI2 cells are found throughout the entire width of the ML,^{9,27} while ICs (Figure 1E) and the PC axon collaterals that innervate them⁶ ascend into only a fraction of the width of the molecular layer.

Ito cells are different from CCs

Our results indicate that CCs and ICs are two different subtypes of PLI1 cells. As members of the same interneuron type, they share numerous properties. Both express *Nxph1* and *Klhl1* and have similar input resistances, resting potentials, and *I_h* (Figures S6A–S6D). Both receive inhibitory input from PCs, which is somewhat stronger for CCs than for ICs (Figure 5H), and both inhibit MLIs (Figures 6D–6F).

However, there are several important distinctions between ICs and CCs. First, ICs do not have the candelabrum morphology that defines CCs (Figures 5A–5F). They also exhibit numerous differences in their intrinsic electrical properties (Figures 5M and S6) that collectively are organized in a discontinuous, step-function distribution across the PL and ML (Figure 5N). The excitatory input received by the two types of cells also differ: while ICs are excited by PFs and CFs, CCs receive excitatory input from PFs and mossy fibers (MFs) but receive improbable and weak excitation from CFs.¹⁰ Finally, there are quantitative differences in their inhibitory influence on PCs: while IC inhibition of PCs was observed frequently in our paired recordings (Figures 6A–6C), stimulation of CCs is reported to rarely inhibit PCs and PCs represent only 0.3% of CC output synapses.¹⁰

(F) TBOA (50 μ M) treatment significantly increased both the amplitude and duration of CF-EPSCs recorded in ICs.

(G) Local MLI circuits within the cerebellar cortex. Solid lines represent chemical synapses between neurons, while zigzag symbols indicate electrical synapses.

(H) Diagram of interneuron types and their subtypes in the cerebellar cortex, based on current single-cell transcriptomics. (I) Comparison of circuit connectivity of (1) ICs, (2) CCs,¹⁰ and (3) MLI2 cells.^{33,34} Both ICs and CCs receive inhibitory input from PCs, while ICs are the only interneurons that do not receive inhibitory input from other MLIs. While the excitatory inputs to ICs and MLI2 cells are similar, CCs do not receive CF input and instead are directly excited by MFs.

Classifying cerebellar interneurons

The distinctive properties of ICs (Table S3) indicate that these cells are an addition to the several other subtypes of cerebellar interneuron previously identified via single-cell transcriptomics (Figure 7H).^{9,28} MLI1 interneuron subtypes include BCs, SCs, and potentially other MLIs yet to be discerned.^{9,28} MLI2 interneurons are morphologically heterogeneous and include cells that resemble SCs, as well as many others that are likely to represent one or more additional subtypes (indicated by a question mark in Figure 7H). PLI1 neurons include at least 2 subtypes, CCs and ICs, with CCs residing within the PL while ICs are in the nearby inner third of the ML. Thus far, there is one known subtype of PLI2 cell—the glycinergic globular cell—and one subtype of PLI3 cell, Lugaro cells. Golgi cells within the granule cell layer consist of at least 2 molecularly distinct subtypes,⁹ Golgi1 and Golgi2, though anatomical studies suggest even greater diversity.³⁶

Advances in single-cell and single-nuclei transcriptomics have revolutionized efforts to classify neuron types throughout the brain. However, it has often proven challenging to reconcile the resulting molecular classification schemes with earlier schemes based on morphological and/or physiological criteria.^{9,28,37–39} The same is true for cerebellar MLIs: the MLI1/MLI2 classification scheme does not neatly account for the morphological properties of MLIs and, at present, cannot predict the differences in intrinsic electrical properties that we have observed. For the case of cortical interneurons, greater clarity has come from combining transcriptomic, anatomical, and electrophysiological approaches in the same individual neurons.^{40–42} It is likely that a similar approach will improve our understanding of cerebellar interneuron diversity.

Ito cells are not electrically coupled

We have provided multiple lines of evidence indicating a lack of IC electrical coupling. These are: (1) high input resistance of ICs relative to BCs and some SCs (Figure 3E); (2) a gap junction blocker, MFQ, increased the input resistance of BCs but not ICs (Figure 3F); (3) lack of dye coupling between ICs, under conditions that yielded ample tracer coupling between BCs (Figure 3G); and (4) a correlation between input resistance and tracer coupling (Figure 3H). In addition, our analysis of cerebellar single-cell transcriptomic data²⁸ reveals that while the genes for two proteins that form gap junctions, connexin-36 (*Gjd2*) and connexin-31 (*Gjb3*), are expressed in MLI1_1 neurons (and in PLI3 neurons), neither is found in PLI1 neurons (or in MLI2 neurons). Thus, ICs do not express gap junction genes or proteins. Taken together, the most parsimonious conclusion is that ICs are not electrically coupled. This contrasts with BC/MLI1_1 neurons, which are electrically coupled^{13,23} (Figures 3F and 3G) and do express *Gjd2*/*Gjb3*.²⁸ Their lack of electrical coupling may help restrict PC-mediated inhibitory feedback to individual ICs.

Physiological role of Ito cells

Previous work has suggested that feedback inhibition of ICs (then called PC-MLIs) by PCs enables more efficient cerebellum-mediated motor learning.⁷ Our detailed analysis of the cellular properties of ICs not only shows that they constitute

an additional interneuron subtype but also extends our understanding of the potential functions of these interneurons. ICs have a relatively large hyperpolarization-activated current (I_h) that plays diverse roles in regulating neuronal excitability and synaptic integration⁴³ and serves as a target for cerebellar neuromodulators.¹⁸ While the role of I_h in ICs is not yet clear, one possibility is to drive IC rebound firing in response to the substantial inhibitory input that they receive from presynaptic PCs.

Notably, ICs appear to be the only interneuron that both receives PC feedback inhibition and provides substantial feedforward inhibition to PCs. This arrangement places ICs within a PC-IC-PC disinhibitory loop (Figure 7I1, right) that may contribute to synchronizing the activity of neighboring PCs.⁷ In addition, IC-mediated inhibition of BC/MLI1_1 cells provides a potential route for polysynaptic PC inhibition (PC-to-IC-to-BC-to-PC), suggesting that ICs could influence PC output through both disinhibitory and inhibitory pathways. These circuit properties may enable ICs to modulate cerebellar output based on feedback from PCs, independent of other MLI networks.

Despite some shared features, IC circuits also differ from those of CCs and MLI2s. All three cell types inhibit BC/MLI1_1 cells, leading to disinaptic disinhibition of PCs (Figure 7I).^{10,34} However, apparently unlike ICs, CCs and MLI2s also receive weak inhibitory inputs from other MLIs^{10,34}; this may enable these interneurons to sense the local MLI network (Figures 7I2 and 7I3). Like ICs, CCs also receive inhibitory input from PCs; MLI2s do not directly receive PC inhibitory input (Figure 7I3). While ICs and MLI2 receive similar excitatory input, CCs differ in receiving excitation from MFs but receive minimal CF input (Figure 7I2). Overall, IC, CC and MLI2 cells have distinct circuits that can produce a wide repertoire of functional outputs due to their specific spatial organization, synaptic connectivity, and patterns of excitatory input.

Limitations of the study

While we have provided multiple lines of evidence demonstrating that ICs are a subtype of PLI1 interneurons, a paper published after ours was submitted has instead proposed that MLI2 cells are the targets of PC feedback inhibition.⁴⁴ The difference between the two studies comes from how MLI2 cells were identified. Lackey et al. (2026) used high input resistance and lack of electrical coupling to identify MLI2 cells⁴⁴; however, these criteria do not distinguish between MLI2 cells and PLI1 cells. We combined single-cell electrophysiology with smFISH in ICs, allowing us to compare multiple intrinsic electrical properties and expression of marker genes in ICs with those of MLI2 cells. We found that the expression of the marker gene, *Klhl1*, in ICs (Figure 4F) more closely resembles that of PLI1 cells than MLI2 cells (Figure 4D2). In addition, numerous electrophysiological criteria demonstrated that ICs were not MLI2 cells (Figure 4G). Thus, existing evidence indicates that ICs are PLI1 neurons rather than MLI2 cells. Beyond the taxonomic question of whether ICs are a subtype of PLI1 or MLI2 interneuron, it is clear that ICs are a discrete subtype of interneuron: they receive PC feedback inhibition and, in turn, provide feedforward inhibition to PCs. This distinction is of fundamental importance for cerebellar function.⁷ Future efforts to understand IC function would

be greatly facilitated by the production of a mouse line that uniquely expresses Cre recombinase in ICs.

A second limitation is that the small sample size for some paired recordings (Figure 6) impairs our ability to make conclusions about IC connections with each other and with SCs. This limitation arises because of the relative sparseness of ICs and the absence of a means of visually identifying ICs in living cerebellar slices. We have already noted that 2 paired recordings (Figures 6J and 6K) are insufficient to conclude that ICs do not form homotypic inhibitory synapses. In addition, our results also leave some uncertainty regarding SC-to-IC connectivity: in 13 paired recordings, in only one case did we observe IPSCs in an IC in response to SC excitation (Figures 6J–6L), and we consider this not to represent a monosynaptic connection because of the relatively long latency of the IPSCs. Larger sample sizes will be needed to firmly establish both of these points.

In conclusion, the distinctive intrinsic electrical properties and synaptic circuit organization of ICs suggest that this additional subtype of MLI plays specialized roles in controlling cerebellar information processing. These properties could account for the proposed role of Ito cells in cerebellar motor learning.⁷ Future efforts that identify genetic tools for selective identification of ICs and manipulation of their activity will significantly enhance our ability to define the roles of these interneurons in cerebellar function.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for materials and data should be directed to, and will be fulfilled by, the lead contact, George Augustine (george_augustine@till.org.sg).

Materials availability

This study did not generate any new materials.

Data and code availability

- All animal data reported in this paper will be made available upon request by the [lead contact](#).
- All original code generated in the analysis of this study is available online at https://figshare.com/articles/software/Claustrum_Codes/30435856.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

J.K. was responsible for experimental work and data analysis. Both authors were responsible for experimental design and manuscript writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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REFERENCES

- Kim, L.H., Heck, D.H., and Sillitoe, R.V. (2024). Cerebellar functions beyond movement and learning. *Annu. Rev. Neurosci.* 47, 145–166. <https://doi.org/10.1146/annurev-neuro-100423-104943>.
- Kim, J., and Augustine, G.J. (2021). Molecular layer interneurons: key elements of cerebellar network computation and behavior. *Neuroscience* 462, 22–35. <https://doi.org/10.1016/j.neuroscience.2020.10.008>.
- Palay, S.L., and Chan-Palay, V. (1974). *Cerebellar Cortex; Cytology and Organization* (Springer).
- Heiney, S.A., Kim, J., Augustine, G.J., and Medina, J.F. (2014). Precise control of movement kinematics by optogenetic inhibition of Purkinje cell activity. *J. Neurosci.* 34, 2321–2330. <https://doi.org/10.1523/JNEUROSCI.4547-13.2014>.
- Jelitali, M., Puggioni, P., Ishikawa, T., Rinaldi, A., and Duguid, I. (2016). Dendritic excitation-inhibition balance shapes cerebellar output during motor behaviour. *Nat. Commun.* 7, 13722. <https://doi.org/10.1038/ncomms13722>.
- Witter, L., Rudolph, S., Pressler, R.T., Lahlaf, S.I., and Regehr, W.G. (2016). Purkinje cell collaterals enable output signals from the cerebellar cortex to feed back to Purkinje cells and interneurons. *Neuron* 91, 312–319. <https://doi.org/10.1016/j.neuron.2016.05.037>.
- Halverson, H.E., Kim, J., Khilkevich, A., Mauk, M.D., and Augustine, G.J. (2022). Feedback inhibition underlies new computational functions of cerebellar interneurons. *eLife* 11, e77603. <https://doi.org/10.7554/eLife.77603>.
- Korbo, L., Andersen, B.B., Ladefoged, O., and Møller, A. (1993). Total numbers of various cell types in rat cerebellar cortex estimated using an unbiased stereological method. *Brain Res.* 609, 262–268. [https://doi.org/10.1016/0006-8993\(93\)90881-m](https://doi.org/10.1016/0006-8993(93)90881-m).
- Kozareva, V., Martin, C., Osorno, T., Rudolph, S., Guo, C., Vanderburg, C., Nadaf, N., Regev, A., Regehr, W.G., and Macosko, E. (2021). A transcriptomic atlas of mouse cerebellar cortex comprehensively defines cell types. *Nature* 598, 214–219. <https://doi.org/10.1038/s41586-021-03220-z>.
- Osorno, T., Rudolph, S., Nguyen, T., Kozareva, V., Nadaf, N.M., Norton, A., Macosko, E.Z., Lee, W.C.A., and Regehr, W.G. (2022). Candelabrum cells are ubiquitous cerebellar cortex interneurons with specialized circuit properties. *Nat. Neurosci.* 25, 702–713. <https://doi.org/10.1038/s41593-022-01057-x>.

11. Ito, M., Yoshida, M., and Obata, K. (1964). Monosynaptic inhibition of the intracerebellar nuclei induced from the cerebellar cortex. *Experientia* 20, 575–576. <https://doi.org/10.1007/BF02150304>.
12. Ramón y Cajal, S. (1995). *Histology of the Nervous System of Man and Vertebrates* (Oxford University Press).
13. Rieubland, S., Roth, A., and Häusser, M. (2014). Structured connectivity in cerebellar inhibitory networks. *Neuron* 81, 913–929. <https://doi.org/10.1016/j.neuron.2013.12.029>.
14. Sultan, F., and Bower, J.M. (1998). Quantitative Golgi study of the rat cerebellar molecular layer interneurons using principal component analysis. *J. Comp. Neurol.* 393, 353–373. [https://doi.org/10.1002/\(SICI\)1096-9861\(19980413\)393:3<353::AID-CNE7>3.0.CO;2-0](https://doi.org/10.1002/(SICI)1096-9861(19980413)393:3<353::AID-CNE7>3.0.CO;2-0).
15. Graf, M., Nair, A., Wong, K.L.L., Tang, Y., and Augustine, G.J. (2020). Identification of mouse claustral neuron types based on their intrinsic electrical properties. *eNeuro* 7, ENEURO.0216-20.2020. <https://doi.org/10.1523/ENEURO.0216-20.2020>.
16. Bao, J., Reim, K., and Sakaba, T. (2010). Target-dependent feedforward inhibition mediated by short-term synaptic plasticity in the cerebellum. *J. Neurosci.* 30, 8171–8179. <https://doi.org/10.1523/JNEUROSCI.0276-10.2010>.
17. Pape, H.C. (1996). Queer current and pacemaker: the hyperpolarization-activated cation current in neurons. *Annu. Rev. Physiol.* 58, 299–327. <https://doi.org/10.1146/annurev.ph.58.030196.001503>.
18. Saitow, F., and Konishi, S. (2000). Excitability increase induced by beta-adrenergic receptor-mediated activation of hyperpolarization-activated cation channels in rat cerebellar basket cells. *J. Neurophysiol.* 84, 2026–2034. <https://doi.org/10.1152/jn.2000.84.4.2026>.
19. Demsar, J., C.T., Erjavec, A., Gorup, C., Hocevar, T., Milutinovic, M., Mozzina, M., Polajnar, M., Toplak, M., Staric, A., Stajdohar, M., et al. (2013). Orange: data mining toolbox in python. *J. Mach. Learn. Res.* 14, 2349–2353.
20. Metsalu, T., and Vilo, J. (2015). ClustVis: a web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. *Nucleic Acids Res.* 43, W566–W570. <https://doi.org/10.1093/nar/gkv468>.
21. Alcamí, P. (2018). Electrical synapses enhance and accelerate interneuron recruitment in response to coincident and sequential excitation. *Front. Cell. Neurosci.* 12, 156. <https://doi.org/10.3389/fncel.2018.00156>.
22. Mann-Metzer, P., and Yarom, Y. (1999). Electrotonic coupling interacts with intrinsic properties to generate synchronized activity in cerebellar networks of inhibitory interneurons. *J. Neurosci.* 19, 3298–3306.
23. Kim, J., Lee, S., Tsuda, S., Zhang, X., Asrican, B., Gloss, B., Feng, G., and Augustine, G.J. (2014). Optogenetic mapping of cerebellar inhibitory circuitry reveals spatially biased coordination of interneurons via electrical synapses. *Cell Rep.* 7, 1601–1613. <https://doi.org/10.1016/j.celrep.2014.04.047>.
24. Cruikshank, S.J., Hopperstad, M., Younger, M., Connors, B.W., Spray, D.C., and Srinivas, M. (2004). Potent block of Cx36 and Cx50 gap junction channels by mefloquine. *Proc. Natl. Acad. Sci. USA* 101, 12364–12369. <https://doi.org/10.1073/pnas.0402044101>.
25. Curti, S., Davoine, F., and Dapino, A. (2022). Function and plasticity of electrical synapses in the mammalian brain: role of non-junctional mechanisms. *Biology* 11, 81. <https://doi.org/10.3390/biology11010081>.
26. Alcamí, P., and Marty, A. (2013). Estimating functional connectivity in an electrically coupled interneuron network. *Proc. Natl. Acad. Sci. USA* 110, E4798–E4807. <https://doi.org/10.1073/pnas.1310983110>.
27. Wang, W.X., and Lefebvre, J.L. (2022). Morphological pseudotime ordering and fate mapping reveal diversification of cerebellar inhibitory interneurons. *Nat. Commun.* 13, 3433. <https://doi.org/10.1038/s41467-022-30977-2>.
28. Tarhan, L., Bistline, J., Chang, J., Galloway, B., Hanna, E., and Weitz, E. (2023). Single Cell Portal: an interactive home for single-cell genomics data. Preprint at bioRxiv. <https://doi.org/10.1101/2023.07.13.548886>.
29. Lainé, J., and Axelrad, H. (1994). The candelabrum cell: a new interneuron in the cerebellar cortex. *J. Comp. Neurol.* 339, 159–173. <https://doi.org/10.1002/cne.903390202>.
30. Vincent, P., and Marty, A. (1996). Fluctuations of inhibitory postsynaptic currents in Purkinje cells from rat cerebellar slices. *J. Physiol.* 494, 183–199. <https://doi.org/10.1113/jphysiol.1996.sp021484>.
31. Szapiro, G., and Barbour, B. (2007). Multiple climbing fibers signal to molecular layer interneurons exclusively via glutamate spillover. *Nat. Neurosci.* 10, 735–742. <https://doi.org/10.1038/nn1907>.
32. Coddington, L.T., Rudolph, S., Vande Lune, P., Overstreet-Wadiche, L., and Wadiche, J.I. (2013). Spillover-mediated feedforward inhibition functionally segregates interneuron activity. *Neuron* 78, 1050–1062. <https://doi.org/10.1016/j.neuron.2013.04.019>.
33. Santos-Valencia, F., Lackey, E.P., Norton, A., Wardak, A., Gaynor, C.S., Ediger, S., Hemelt, M.E., Nguyen, T.M., Lee, W.C.A., Brunel, N., et al. (2026). Climbing fibres recruit disinhibition to enhance Purkinje cell calcium signals. *Nature* 653, 455–464. <https://doi.org/10.1038/s41586-026-10220-4>.
34. Lackey, E.P., Moreira, L., Norton, A., Hemelt, M.E., Osorno, T., Nguyen, T.M., Macosko, E.Z., Lee, W.C.A., Hull, C.A., and Regehr, W.G. (2024). Specialized connectivity of molecular layer interneuron subtypes leads to disinhibition and synchronous inhibition of cerebellar Purkinje cells. *Neuron* 112, 2333–2348.e6. <https://doi.org/10.1016/j.neuron.2024.04.010>.
35. Molineux, M.L., Fernandez, F.R., Mehaffey, W.H., and Turner, R.W. (2005). A-type and T-type currents interact to produce a novel spike latency-voltage relationship in cerebellar stellate cells. *J. Neurosci.* 25, 10863–10873. <https://doi.org/10.1523/JNEUROSCI.3436-05.2005>.
36. Schilling, K., Oberdick, J., Rossi, F., and Baader, S.L. (2008). Besides Purkinje cells and granule neurons: an appraisal of the cell biology of the interneurons of the cerebellar cortex. *Histochem. Cell Biol.* 130, 601–615. <https://doi.org/10.1007/s00418-008-0483-y>.
37. Harris, K.D., Hochgerner, H., Skene, N.G., Magno, L., Katona, L., Bengtsson-Gonzales, C., Somogyi, P., Kessaris, N., Linnarsson, S., and Hjerling-Leffler, J. (2018). Classes and continua of hippocampal CA1 inhibitory neurons revealed by single-cell transcriptomics. *PLoS Biol.* 16, e2006387. <https://doi.org/10.1371/journal.pbio.2006387>.
38. Tasic, B., Menon, V., Nguyen, T.N., Kim, T.K., Jarsky, T., Yao, Z., Levi, B., Gray, L.T., Sorensen, S.A., Dolbeare, T., et al. (2016). Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nat. Neurosci.* 19, 335–346. <https://doi.org/10.1038/nn.4216>.
39. Yao, Z., van Velthoven, C.T.J., Nguyen, T.N., Goldy, J., Sedeno-Cortes, A.E., Baftizadeh, F., Bertagnoli, D., Casper, T., Chiang, M., Crichton, K., et al. (2021). A taxonomy of transcriptomic cell types across the isocortex and hippocampal formation. *Cell* 184, 3222–3241.e26. <https://doi.org/10.1016/j.cell.2021.04.021>.
40. Cadwell, C.R., Palasantza, A., Jiang, X., Berens, P., Deng, Q., Yilmaz, M., Reimer, J., Shen, S., Bethge, M., Tolias, K.F., et al. (2016). Electrophysiological, transcriptomic and morphologic profiling of single neurons using Patch-seq. *Nat. Biotechnol.* 34, 199–203. <https://doi.org/10.1038/nbt.3445>.
41. Gouwens, N.W., Sorensen, S.A., Baftizadeh, F., Budzillo, A., Lee, B.R., Jarsky, T., Alfiler, L., Baker, K., Barkan, E., Berry, K., et al. (2020). Integrated morphoelectric and transcriptomic classification of cortical GABAergic cells. *Cell* 183, 935–953.e19. <https://doi.org/10.1016/j.cell.2020.09.057>.
42. Kendrick, R.M., Linderman, S.W., and Owen, S.F. (2024). Transcriptomically-measured gene expression predicts physiological variation across single neurons in humans and mice. Preprint at bioRxiv 2024. <https://doi.org/10.1101/2024.08.26.609746>.
43. Robinson, R.B., and Siegelbaum, S.A. (2003). Hyperpolarization-activated cation currents: from molecules to physiological function. *Annu. Rev. Physiol.* 65, 453–480. <https://doi.org/10.1146/annurev.physiol.65.092101.142734>.

44. Lackey, E.P., Norton, A., Moreira, L., Gaynor, C.S., Lee, W.C.A., and Regehr, W.G. (2026). Purkinje cell collaterals preferentially target a subtype of molecular layer interneuron. *J. Neurosci.* 46, e1384252026. <https://doi.org/10.1523/JNEUROSCI.1384-25.2026>.
45. Longair, M.H., Baker, D.A., and Armstrong, J.D. (2011). Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics* 27, 2453–2454. <https://doi.org/10.1093/bioinformatics/btr390>.
46. Johnson, K.W., Morin, S.M., Wroblewski, V.J., and Johnson, M.P. (2019). Peripheral and central nervous system distribution of the CGRP neutralizing antibody [¹²⁵I] galcanezumab in male rats. *Cephalalgia* 39, 1241–1248. <https://doi.org/10.1177/0333102419844711>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Neurobiotin	Vector labs	SP-1120
Alexa Fluor® 594	Thermo Fisher Scientific	A10438
Streptavidin-conjugated AlexaFluor® 647	Thermo Fisher Scientific	S21374
GABAzine	Sigma-Aldrich	S106
Mefloquine	Sigma-Aldrich	M2319
ZD7288	Sigma-Aldrich	Z3777
DL-TBOA	Tocris Bioscience	1223
HCR RNA-FISH probe set for Nxp1	Molecular Instruments	Cat# Custom; Lot# RTC845
HCR RNA-FISH probe set for Kihl1	Molecular Instruments	Cat# Custom; Lot# RTF340
HCR B1 Amplifier 488	Molecular Instruments	Lot#S030426, S030526
HCR B2 Amplifier 647	Molecular Instruments	Lot#S032826, S025826
HCR RNA-FISH Buffers (Pretreat, Probe, Wash, Amp)	Molecular Instruments	N/A
Adenosine-5'-triphosphate Di-sodium hydrate	Sigma-Aldrich	A7699
Ascorbic acid	Nacalai Tesque	03420-52
Calcium chloride	Sigma-Aldrich	C1016
Disodium phosphocreatinine	Sigma-Aldrich	P7936
EGTA	Sigma-Aldrich	E3889
Glucose	Sigma-Aldrich	616338
Guanosine 5'-triphosphate sodium salt hydrate	Sigma-Aldrich	G8877
HEPES	Sigma-Aldrich	H3375
Isoflurane	Chemical Iberica	26675-46-7
Kynurenic acid	Sigma-Aldrich	K3375
Magnesium chloride	Sigma-Aldrich	M8266
Monosodium phosphate dihydrate	Sigma-Aldrich	71505
Myo-inositol	Sigma-Aldrich	I5125
Potassium chloride	Sigma-Aldrich	P9541
Potassium gluconate	Sigma-Aldrich	P1847
Potassium hydroxide	Kanto Chemical	3234400
Sodium bicarbonate	Nacalai Tesque	31213-15
Sodium chloride	Sigma-Aldrich	S7653
Sodium pyruvate	Sigma-Aldrich	P2256
Sucrose	Sigma-Aldrich	S7903
Experimental models: Organisms/strains		
Pcp2-cre mice; B6.129-Tg(Pcp2-cre)2Mppin/J	Jackson Laboratory	004146; RRID:IMSR_JAX:004146
Pcp2-cre mice; B6.Cg-Tg(Pcp2-re)3555Jdhu/J	Jackson Laboratory	010536; RRID:IMSR_JAX:010536
loxP-STOP-loxP-channelrhodopsin2-eYFP mice (B6;129S-Gt(ROSA)26Sor ^{tm32(CAG-COP4-H134R/EYFP)Hze/J})	Jackson Laboratory	012569; RRID:IMSR_JAX:012569
C57BL/6JInv mice	Jackson Laboratory	000664; RRID:IMSR_JAX:000664
Software and algorithms		
Clampfit 11.3	Molecular devices	RRID:SCR_011323
Fiji/ImageJ	NIH	RRID:SCR_003070
Spyder Python IDE (v5.4.3)	https://www.spyder-ide.org/	RRID:SCR_000161
Orange Data Mining	Demsar et al. ¹⁷	https://orange.biolab.si/
ClustVis	https://biit.cs.ut.ee/clustvis/	RRID:SCR_017133
Origin	Origin Lab	RRID:SCR_014212

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

To generate mice selectively expressing ChR2 in Purkinje cells, PCP2-cre transgenic mice [(Pcp2-cre)2Mpin/J or (PCP2-cre)3555Jdhu/J; Jackson Labs] were crossed with mice expressing floxed ChR2-H134R [B6;129S-Gt(ROSA)26Sor^{tm32(CAG-COP4*H134R/EYFP)Hze/J}].⁷ For FISH experiments, C57BL/6JInv (Jackson Labs) mice were used. Mice were maintained under a 12h light/dark cycle with free access to food and water. All procedures were conducted according to the Guidelines of the Institutional Animal Care and Use Committees of Nanyang Technological University (A24028) and Temasek Life Sciences Laboratory in Singapore (TLL-24-001). Mice aged P24–P77 were used; the intrinsic electrical properties of ICs did not differ across this age range and it has also been reported that their synaptic inputs from PCs are consistent across this age range.⁷ Our experiments used both male and female mice and no sex-associated differences were observed.

METHOD DETAILS

Brain slice recording

To prepare cerebellar slices, mice were deeply anesthetized using isoflurane, followed by decapitation euthanasia; the brains were then promptly removed. Sagittal cerebellar vermis sections (250 μ m) were cut (Leica VT1200D vibratome) in ice-cold oxygenated cutting solution containing (in mM): 250 sucrose, 26 NaHCO₃, 10 glucose, 4 MgCl₂, 3 myo-inositol, 2.5 KCl, 2 sodium pyruvate, 1.25 NaH₂PO₄, 0.5 ascorbic acid, 0.1 CaCl₂, 1 kynurenic acid. After cutting, slices were kept at 34°C for 1 h in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 24 NaHCO₃, 1 NaH₂PO₄, 2.5 KCl, 2.5 CaCl₂, 2 MgCl₂, 10 glucose, 0.4 ascorbic acid (pH 7.4 when equilibrated with 95% O₂/5% CO₂). Whole-cell patch clamp recordings were then conducted at room temperature as previously described.⁷ Pipettes were filled with a solution containing (in mM): 130 K-gluconate, 10 KOH, 2.5 MgCl₂, 10 HEPES, 5 EGTA, 4 Na₂ATP, 0.4 Na₃GTP, 5 disodium phosphocreatine (pH 7.3, ~295 mOsm). Alexa 594 (50 μ M) or 0.5% neurobiotin was added in the internal solution to image cell morphology. IPSCs were recorded under voltage clamp with a holding potential of –53 mV, unless otherwise indicated. Electrical responses were acquired using a Multiclamp 700B amplifier with Digidata 1440A interface (Molecular Devices). Membrane potentials were corrected for the liquid junction potential of –13 mV.

In most experiments, ICs were identified by their IPSC responses to PC photostimulation. In these experiments PCs were photostimulated either by wide-field excitation from a mercury arc lamp (465–495 nm) or by small spots of laser light (405 nm), as previously described.⁷ To examine excitatory synaptic inputs to ICs, these inputs were electrically stimulated (0.5–30 μ A, 100 μ s duration) via a glass stimulating electrode filled with bath solution. Parallel fibers were stimulated by placing this electrode in the molecular layer, while climbing fibers were stimulated by placing the electrode near the PC layer. When examining excitatory synaptic responses (but not when photostimulating PCs), slices were treated with the GABA_A receptor antagonist gabazine (SR-95531, 5 μ M; Sigma) to avoid contamination by inhibitory synaptic responses. In all cases, whole-cell patch clamp recordings were used to detect IC responses to PC photostimulation.

MLI staining and morphology measurements

To visualize marker coupling and analyze the morphology of MLIs, 0.5% neurobiotin (Vector Labs) was added to the internal solution and iontophoretically injected into the neurons with a current (500 pA, 250 ms, 1 Hz) for 10 min during electrophysiological recordings. After injection, the electrode was carefully removed and the brain slices were incubated at room temperature for 1–2 h to allow sufficient time for neurobiotin diffusion. Slices were fixed overnight in 4% paraformaldehyde and washed with phosphate buffered saline (PBS). After washing, slices were incubated in PBS containing 5% normal goat serum and 0.3% Triton X-100 for 1 h, followed by the incubation for 1 h with streptavidin-conjugated Alexa Fluor 647 (1:1000, Invitrogen). Slices were then rinsed, mounted on VWR Superfrost Plus microscope slides, and coverslipped with VWR micro cover glass using a polyvinyl alcohol mounting medium containing DABCO antifade (Sigma-Aldrich).

The morphology of individual neurons and neurobiotin-coupled cells was imaged using an Olympus FV1000MPE two-photon microscope equipped with a 25 $\times$ water-immersion objective (NA 1.05) at 1–3 $\times$ zoom using FluoView software. Images were acquired at 512 $\times$ 512 pixels (0.994 μ m/pixel at 1 $\times$ zoom) with Z-stacks collected at 1 μ m intervals. Z-stacks were processed in Fiji (ImageJ) to generate maximum-intensity projections, reduce background, and adjust brightness and contrast. For reconstruction of MLI morphologies, tracing was performed using the Simple Neurite Tracer (SNT) plugin in Fiji,⁴⁵ employing its semi-automated path-finding function to trace dendrites and axons. Reconstructions were visually verified for accuracy, and the reconstructed MLI images were superimposed and aligned by their cell bodies, with the y axis corresponding to the vertical axis of the molecular layer and the x axis aligned along the Purkinje cell layer. The longer axon was positioned toward the right. To quantify potential synaptic sites, the number of axonal varicosities was measured in 10 μ m steps along the vertical axis of the molecular layer, parallel to the Purkinje cell layer. Axonal varicosities were defined as focal, bead-like swellings along the axon that were clearly distinguishable from the thin axonal shaft and did not correspond to branch points. Only discrete enlargements ($\geq 1.5\times$ that of the adjacent axonal shaft) that were continuous with the axon were counted.

Dye-coupled cells were identified by somatic fluorescence distinguishable from background fluorescence and confirmed by 3D orthogonal reconstructions showing morphology and size consistent with a neuronal soma (~10 μ m).

Single-molecule fluorescence *in situ* hybridization

To detect specific gene expression in ICs, floating slice hybridization chain reaction (HCR) was performed based on the protocol described at <https://doi.org/10.17504/protocols.io.bck7iuzn>.¹⁰ Mice were anesthetized via intraperitoneal (i.p.) administration of ketamine (100 mg/kg) and xylazine (10 mg/kg), and were transcardially perfused with ACSF to minimize background fluorescence associated with hemoglobin.⁴⁶ Whole-cell patch clamp recordings were first performed to identify the type of MLI, with Alexa 594 dye or neurobiotin added in the internal recording solution to allow post hoc identification of the cell. Slices were then fixed overnight in 4% paraformaldehyde and washed with PBS. HCR probe sets and matching fluorescent hairpins (B1-647/B2-488 or B1-488/B2-647, Molecular Instruments) were added to detect neurexophilin 1 (*Nxph1*) and Kelch-like protein 1 (*Klhl1*). In the case of neurobiotin labeling, after completing HCR, slices were washed with PBS and neurobiotin staining was performed using streptavidin-conjugated Alexa Fluor 405 or 594. After mounting, slices were imaged using a confocal laser scanning microscope (Olympus FV3000) equipped with a 60 $\times$ oil-immersion objective. HCR probe/hairpin signals were acquired at 488 nm and 647 nm, and cell detection signals at 561 nm or 405 nm. Images were processed using Fiji software.

Data analysis

The location of patched MLIs was determined by measuring the distance from the center of PC soma (center of the PC layer) to the center of the MLI soma, then normalizing this distance relative to the thickness of the inner third of the molecular layer. To characterize the electrophysiological properties of interneurons, current-clamp recordings were performed and hyperpolarizing and depolarizing current pulses (500 ms duration) were applied in increments of 15 pA. 46 electrophysiological properties were analyzed using Clampfit 11.3 (Molecular Devices) and a custom-written Python program run in Spider within Anaconda.¹⁵ Resting membrane potential was measured as the potential level recorded during spike-free periods, in the absence of any applied current. Depolarizing sag was measured during a hyperpolarizing current step to -45 mV. To avoid problems associated with using constant-amplitude current pulses to compare neurons with different input resistances, we compared the excitability properties of neurons by using their current threshold (often called rheobase in older literature).¹⁵ This was defined as the minimum depolarizing current that evoked an action potential. To further account for differences in passive electrical properties, we also calculated the threshold current density by dividing the current threshold by the cell capacitance. Subsequent comparisons of electrical properties were based on the current threshold. Most action potential properties were compared at twice the threshold current; the exceptions were action potential amplitude and threshold voltage, which were measured at the threshold current. AP threshold was defined as the membrane potential when the AP upstroke reached 10% of its maximum slope. AP amplitude was determined by calculating the difference between the AP threshold and peak. AHP amplitude was measured as the difference between the threshold and the most negative voltage during the AP undershoot. AP half-width was determined by measuring AP width at half the maximal AP amplitude. The spike latency was defined as the time from the onset of depolarization to the peak of the first AP. Build-up depolarization, resulting from the gradual shift in membrane potential during repetitive firing, was defined as the difference between the resting membrane potential and the membrane potential just before the last AP. AP waveform was measured via the 10–90% rise and decay times of the AP, as well as the ratio of the rise slope to the decay slope. AP frequency adaptation, which reflects time-dependent changes in AP frequency during a sustained depolarization, was calculated as the ratio of the inter-spike intervals (ISIs) the first two and last two APs in a train ($1 - \text{first ISI}/\text{last ISI}$).

The depolarizing voltage sag measured in response to hyperpolarizing current pulses was quantified by calculating the difference between the peak hyperpolarization and potential at the last point of the current pulse (Figure 2A). Hyperpolarization-activated inward current, I_h , was examined in voltage-clamp recordings while applying hyperpolarizing voltage steps from -83 mV to -163 mV. I_h amplitude was measured as the difference between the steady-state current at the last time point of the hyperpolarization and the minimum current recorded within the first 100 ms of the hyperpolarization (Figure 3A). Leak current was determined from the current measured within the initial 100 ms of a hyperpolarizing voltage pulse. Input resistance (R_{in}) was calculated from the slope of a linear fit to the relationship between membrane potential and applied current, which was derived from voltage responses to a series of current pulses. When measuring the depolarizing sag response to hyperpolarizing currents, membrane potential was measured at the peak of hyperpolarization, before the depolarizing sag developed. Data were analyzed with Clampfit software and expressed as mean $\pm$ SEM. Statistical significance was assessed using ANOVA, followed by post hoc Tukey's test.

The types of MLIs were classified based on their intrinsic electrical properties. To determine which properties were most predictive of MLI subtype, we assessed feature importance using neural network learning in Orange 3 in Figure 2H.¹⁹ MLIs with similar features were grouped via unsupervised hierarchical clustering, using ClustVis software.²⁰

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean $\pm$ SEM, where the mean represents the center value and SEM represents the dispersion measure. All data were analyzed using Student's unpaired t test for comparisons between two groups, or one-way ANOVA followed by Tukey's post hoc analysis for multiple comparisons, using Origin (OriginLab). Differences were considered statistically significant at $p < 0.05$ ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). The specific statistical tests, sample sizes (n , representing the number of cells analyzed), and significance levels are indicated in the corresponding figures and figure legends.