Pathway-Specific Drive of Cerebellar Golgi Cells Reveals Integrative Rules of Cortical Inhibition

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Cerebellar granule cells (GrCs) constitute over half of all neurons in the vertebrate brain and are proposed to decorrelate convergent mossy fiber (MF) inputs in service of learning. Interneurons within the GrC layer, Golgi cells (GoCs), are the primary inhibitors of this vast population and therefore play a major role in influencing the computations performed within the layer. Despite this central function for GoCs, few studies have directly examined how GoCs integrate inputs from specific afferents, which vary in density to regulate GrC population activity. We used a variety of methods in mice of either sex to study feedforward inhibition recruited by identified MFs, focusing on features that would influence integration by GrCs. Comprehensive 3D reconstruction and quantification of GoC axonal boutons revealed tightly clustered boutons that focus feedforward inhibition in the neighborhood of GoC somata. Acute whole-cell patch-clamp recordings from GrCs in brain slices showed that, despite high GoC bouton density, fast phasic inhibition was very sparse relative to slow spillover mediated inhibition. Dynamic-clamp simulating inhibition combined with optogenetic MF activation at moderate rates supported a predominant role of slow spillover mediated inhibition in reducing GrC activity. Whole-cell recordings from GoCs revealed a role for the density of active MFs in preferentially driving them. Thus, our data provide empirical confirmation of predicted rules by which MFs activate GoCs to regulate GrC activity levels.

Key words: cerebellum; feedforward inhibition; granule layer; interneurons; mossy fibers; nucleocortical

Significance Statement

A unifying framework in neural circuit analysis is identifying circuit motifs that subserve common computations. Wide-field inhibitory interneurons globally inhibit neighbors and have been studied extensively in the insect olfactory system and proposed to serve pattern separation functions. Cerebellar Golgi cells (GoCs), a type of mammalian wide-field inhibitory interneuron observed in the granule cell layer, are well suited to perform normalization or pattern separation functions, but the relationship between spatial characteristics of input patterns to GoC-mediated inhibition has received limited attention. This study provides unprecedented quantitative structural details of GoCs and identifies a role for population input activity levels in recruiting inhibition using *in vitro* electrophysiology and optogenetics.

Introduction

A fundamental function of the cerebellar granule cell (GrC) is to decorrelate information conveyed via convergent multi-

modal mossy fibers (MFs), increasing utility for learned associations (Marr, 1969; Albus, 1971; Billings et al., 2014; Cayco-Gajic et al., 2017). Recent work has demonstrated that GrCs receive and respond to MFs conveying diverse information (Huang et al., 2013; Ishikawa et al., 2015), but little attention has been paid to the potential role of multimodal integration by Golgi cells (GoCs). GoCs are in a key position to regulate expansion recoding by GrCs because feedforward inhibition sets spiking threshold and thereby the number of different

Received June 7, 2018; revised Nov. 27, 2018; accepted Dec. 13, 2018.

Author contributions: S.T. wrote the first draft of the paper; S.T., J.I.G., and A.L.P. edited the paper; S.T., J.I.G., and A.L.P. designed research; S.T., J.I.G., and A.L.P. performed research; S.T., J.I.G., K.P., and A.L.P. analyzed data; S.T. wrote the paper.

This work was supported by the Japan Society for the Promotion of Science Overseas Research Fellowship and the Uehara Memorial Foundation research fellowship to S.T.; and the Klingenstein Foundation, the Boettcher Foundation Webb-Waring Biomedical Research Award, and National Institutes of Health Grant NS084996 to A.L.P., and Neuroscience Training Grant T32NS 099042 to J.I.G. Imaging experiments were performed in the University of Colorado Anschutz Medical Campus Advance Light Microscopy Core supported in part by Rocky Mountain Neurological Disorders Core Grant P30NS048154 and by National Institutes of Health/National Center for Advancing Translational Sciences Colorado CTSI Grant UL1 TR001082. Engineering support was provided by the Optogenetics and Neural Engineering Core at the University of Colorado Anschutz Medical Campus, supported in part by the National Institute of Neurological Disorders and Stroke of the National Institutes of Health Award P30NS048154. We thank

Samantha Lewis for expert technical support during the project; and Dr. Christian Rickert for assistance with dynamic-clamp design.

The authors declare no competing financial interests.

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<https://doi.org/10.1523/JNEUROSCI.1448-18.2018>

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afferents required to drive GrC firing (Marr, 1969; D'Angelo et al., 2013). Indeed, theory suggests that feedforward inhibition via GoCs performs a thresholding-like function, clamping the number of active GrCs at a relatively fixed level by engaging GoCs in a scaled manner with increasing activity from MFs (Marr, 1969; Medina et al., 2000).

GoC inhibition of GrCs has been studied extensively in slices and is characteristically diverse. Fast phasic IPSCs, a pronounced slow spillover-mediated component, and “tonic” GABA_A-receptor mediated currents are all forms of inhibition mediated by GoCs (for review, see Farrant and Nusser, 2005; Crowley et al., 2009; D'Angelo and De Zeeuw, 2009; Nieus et al., 2014). The spillover and tonic inhibitory tone within the layer would seemingly provide an ideal mechanism for widely inhibiting the vast number of GrCs without necessarily forming direct contact with each cell. Furthermore, relating GoC recruitment to the density of active MFs is critical for testing the hypothesis of dynamic thresholding in service of pattern separation.

Another challenge for GoCs is inhibiting the vast number of GrCs to regulate activity within the GrC layer. GoC axons are famously dense, but details of spatial ramification patterns that define the likelihood of local GrCs sharing inhibition remain undefined. Indeed, the problem of quantitatively addressing the distribution of inhibition from a single GoC was described by Ramon y Cajal: “When one of these axons appears completely impregnated in a Golgi preparation, it is almost impossible to follow its complete arborization . . . It is only in the incomplete impregnations of adult animals . . . that one can study the course and divisions of the axon. Ramon y Cajal 1890a” (Palay and Chan-Palay, 1974). To our knowledge, this observation remains relevant in contemporary literature where all GoC reconstructions have been incomplete (Simpson et al., 2005; Barmack and Yakhnitsa, 2008; Kanichay and Silver, 2008; Vervaeke et al., 2010, 2012; Szoboszlay et al., 2016; Valera et al., 2016).

To address these questions, we used a variety of methods to resolve GoC connectivity rules and the capacity of specific afferents to produce fast phasic and slow spillover-mediated inhibition. We performed comprehensive single-cell, high-resolution reconstruction of GoCs with quantitative morphological analysis to estimate glomerular innervation by GoCs. Optogenetic activation of specific MF afferents from the pontine or cerebellar nuclei, which differ systematically in their density, were used with electrophysiological recordings of GoCs from slices to test the prediction that the density of afferent activity engages graded inhibition to regulate GrC threshold.

Materials and Methods

Animals

All procedures followed the National Institutes of Health Guidelines and were approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee at the University of Colorado Anschutz Medical Campus. Animals were housed in an environmentally controlled room, kept on a 12 h light/dark cycle, and had *ad libitum* access to food and water. Adult mice (2–5 months old) of either sex were used in all experiments; sex was not monitored for experimental groupings. Genotypes used were C57BL/6 (Charles River Laboratories), Neuromodulator receptor1-Cre (*Ntsr1-Cre*; Mutant Mouse Regional Resource Center, STOCK Tg (*Ntsr1-cre*) GN220Gsat/Mmucd), and *GlyT2-eGFP* (Salk Institute, Tg(*Slc6a5-EGFP*)13Uze) (Zeilhofer et al., 2005). All transgenic animals were bred on a C57BL/6 background and maintained as heterozygotes. *Ntsr1-Cre* animals were genotyped for Cre, and *GlyT2-eGFP* animals were genotyped for eGFP (Transnetyx).

Virus injections

For surgical procedures, at least 1-month-old mice were anesthetized with intraperitoneal injections of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) mixture. Mice were placed in a stereotaxic apparatus, and bupivacaine (6 mg/kg) was injected along the incision line. Craniotomies were made above the cerebellar cortex (from λ : −1.9 mm, 1.1 mm lateral, 1.2 mm ventral), interposed nuclei (IN) (from λ : −1.9 mm, 1.1 mm lateral, 2.4 mm ventral), and pontine nuclei (from bregma: −3.7 mm, 0.5 mm lateral, 5.5 mm ventral). Pressure injections of virus were administered using a pulled glass pipette (7–9 μ m tip diameter). Mice were allowed to survive for >6 weeks before experiments, which we found in pilot experiments optimized expression of reporter proteins in MF terminals.

Viruses

AAV8-hSyn1-mCherry-Cre (titer: 10^{12} , University of North Carolina) and AAV2-CAG-FLEX-eGFP (titer: 10^{12} , University of Pennsylvania) were coinjected to cerebellar cortex to sparsely label neurons for morphological analysis of GoCs. AAV2-hSyn1-hChR2(H134R)-mCherry-WPRE (University of North Carolina Vector Core) were injected to WT mouse IN and pontine nuclei to induce ChR2 expression for electrophysiological recordings. AAV2-EF1a-DIO-hChR2(H134R)-mCherry-WPRE-pA was injected into the IN of *Ntsr1-Cre* mice for a subset of nucleocortical (NC) MF studies (University of North Carolina Vector Core).

Electrophysiology

Slice preparation. At least 6 weeks after virus injection, mice were deeply anesthetized with isoflurane before transcardial perfusion, and slicing in warm (37°C–40°C), oxygenated (95% O₂–5% CO₂) Tyrode's solution contained the following (in mM): 123.75 NaCl, 3.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 1.5 CaCl₂, 1 MgCl₂, and 10 glucose (Person and Raman, 2011; Ankri et al., 2014). Dissected cerebellum was sliced at 300 μ m in the coronal plane for GoC recordings and either parasagittal or coronal planes for GrC recordings on a Leica VT1000S Vibratome. Slices were transferred to an oxygenated Tyrode's solution (37°C) and incubated for 30–60 min.

In vitro recordings. One hour after slicing for GrC recordings and immediately after slicing for GoC recordings (Hull and Regehr, 2012), tissue was transferred to the recording chamber. Oxygenated Tyrode's solution (30°C) was perfused over the slice at 3 ml/min and visualized with an Axio Examiner (Carl Zeiss) equipped with xenon lamp LAMBDA DG-4 (Sutter Instruments) for optogenetic stimulation through the objective. MFs were stimulated optogenetically with 2 ms light pulses with a measured power of 18.5 mW at 473 nm (0.96–3.77 mW/mm², depending on diameter of the light cone at preparation). Pulled glass patch electrodes (GoCs: 2–3.5 M Ω ; GrCs: 4–6 M Ω ; Sutter Instruments, P-97) were filled with K-gluconate-based internal solution containing the following (in mM): 120 K-gluconate, 2 Na-gluconate, 6 NaCl, 2 MgCl₂, 1 EGTA, 4 Mg-ATP, 0.3 Tris-GTP, 14 Tris-creatine phosphate, 10 HEPES, and adjusted for pH (7.3) with KOH and osmolarity (290 mOsm) with sucrose. For all GoC recordings and a subset of GrC recordings, biocytin (0.3%; Tocris Bioscience) was added to the internal solution. Whole-cell recordings were made in current-clamp and voltage-clamp mode, low-pass filtered at 6–10 kHz, amplified with a MultiClamp 700C, partially compensated for series resistance, digitized at 10–50 kHz with a Digidata 1550, and monitored with pClamp acquisition software (Molecular Devices). Blockade of neurotransmitter receptors was achieved with bath application of 10 μ M RS-CPP (Tocris Bioscience) to block NMDARs and 10 μ M SR95531 (Tocris Bioscience) to block GABA_ARs. Data were analyzed with custom routines and with the Neuromatic package (Think-Random) in IGOR Pro (Wavemetrics; RRID:SCR_000325). We targeted recordings to locations of ChR2 expression in MFs, particularly Crus 1, 2, Lobule Simplex, and Lobule 4/5.

Dynamic clamp. Dynamic-clamp experiments were performed using a custom-built microcontroller-based dynamic-clamp system with 10 μ s input–output latency (Desai et al., 2017) (www.dynamicclamp.com). We modified the layout, customized resistors, made a custom 3D printed enclosure, and calibrated the system with details provided as an open resource from the Optogenetics and Neural Engineering Core at the

University of Colorado Anschutz Medical Campus with modifications publicly available. Simulated current timing was triggered by TTL via a Master-8 Pulse Stimulator (A.M.P.I.). We simulated both MF EPSCs and GoC-mediated IPSCs, with parameters calculated from our GrC recording dataset using optogenetic stimulation of MFs as well as from previous reports (Rossi and Hamann, 1998). Dynamic-clamp inhibitory postsynaptic conductances (IPSGs) mimicked GABA_A receptor-mediated conductances with a reversal potential of -70 mV and a linear ohmic current-voltage relation, with kinetics following a single exponential rise and decay model as follows: Fast phasic IPSGs had a 2.15 ms rise time, 2.29 ms τ_{decay} , and a peak conductance of 0.3 nS (small) or 1.2–1.5 nS (large). Slow phasic IPSGs had a 30.2 ms rise time, 630 ms τ_{decay} , and peak conductances of 0.032 or 0.32 nS. These reflected observations made in a subset of recordings in which trains of MF stimuli were made while recording IPSCs in GrCs (see Fig. 5). Both depressing and nondepressing synapses were mimicked. Depressing synapses reflected previous reports from *in vivo* recordings, where IPSGs rapidly depressed to $\sim 50\%$ after the first IPSP in a burst (Duguid et al., 2015). Dynamic-clamp mimicking MF-like excitatory postsynaptic conductances (EPSPs) were delivered at 100 Hz and depressed to a steady state of 50% across trains (Saviane and Silver, 2006). Each EPSP followed an excitatory synaptic model with peak conductance of 1.5 nS, 0.4 ms rise time, and decay time of 1.3 ms and had a net reversal potential of 0 mV, reflecting dual AMPA and NMDA receptor-mediated conductances as described previously (Walcott et al., 2011).

Tissue preparation for light microscopy

Mice were overdosed with an intraperitoneal injection of a sodium pentobarbital solution, Fatal Plus (Vortech Pharmaceuticals), and perfused transcardially with 0.9% saline followed by 4% PFA. Brains were removed and postfixed for at least 24 h and then cryoprotected in 30% sucrose. Tissue was sliced in 40 μm serial coronal sections using a freez- ing microtome and stored in 0.1 M PB.

For *post hoc* morphological analyses following slice electrophysiology, tissue was transferred to warm 4% paraformaldehyde and postfixed for <3 h then placed in 30% sucrose. Tissue was rinsed in 0.1 M PB for 30 min then treated in 0.3% Triton X-100 in 0.1 M PB for 2 h followed by three washes in PB (10 min each). To visualize biocytin, tissue was incubated with streptavidin conjugated to AlexaFluor-647 or -555 (Invitrogen) diluted 1:100 in 0.1 M PB overnight at 4°C followed by three washes in PB (20 min each).

Imaging

Confocal images were obtained using Carl Zeiss LSM 780. To reconstruct all processes and/or map all axonal boutons of GoCs, sequential images were taken with diffraction limited resolution achieved via 63 $\times$ oil objective with NA 1.4 (Plan-Apochrom 63 $\times$ /1.4 oil DIC M27 objective by Ar-Iron laser; 0.39 μm z step). This achieved a computed x, y resolution of 175 nm for the 488 nm signal and 200 nm for 561 nm signal, based on the relationship $\text{Res}_{(x,y)} = \lambda/2\text{NA}_{\text{obj}}$. For MF density analysis, images of filled cells were collected and density analyzed within $53,615 \pm 5180 \mu\text{m}^2$ of the GoC soma. Images examining MF proximity to GoCs were visualized in Zen software using transparent rendering mode. High-resolution imaging for GlyT2-GFP boutons was performed with a Nikon A1r-HD confocal with a Plan Apo 60 $\times$ oil objective, NA 1.4. Deconvolution was performed in NIS-Elements C imaging software.

Morphological analysis

GoC reconstructions were performed using Neurolucida 360 software (MBF Bioscience; RRID:SCR_001775). Reconstructed GoCs were located in vermal lobule 4/5. Processes were traced in user-guided mode. Fine-grained reconstructions captured axonal and dendritic swellings by adjusting points along processes to match thickness. Because individual GoCs spanned multiple sections, each section was individually reconstructed; then each reconstructed section was stacked using morphological landmarks visible across sections, such as the Purkinje cell layer. To define the relative extent of basal axons and dendrites, we traced these processes with attention to process thickness and contours. Axons were characterized by their smaller diameter ($\sim 0.2 \mu\text{m}$)

and were studied with boutons ($\sim 3 \mu\text{m}$ diameter). For all analyses of reconstructed GoC morphological features, we used Neurolucida Explorer. To define the GrC layer (GCL) volume occupied by the cell, we computed the convex hull volume, which calculates the volume of a convex polygon connecting the tips of the distal processes. To map boutons, we used Neurolucida 360 and placed markers on each bouton with 0.39 μm z resolution. All images were aligned, and coordinates of boutons were exported and processed in MATLAB (MathWorks; RRID: SCR_001622). Nearest boutons: All distance analyses used Euclidean distance. Nearest bouton color maps were constructed as 3D scatter plots in MATLAB. Bouton distance probabilities were computed in 0.2 μm bins and normalized by the total number of boutons. Comparisons of bouton density between single GoCs and global GlyT2-GFP label were calculated by first computing the local density of boutons for single GoCs, defined as the number of boutons within a 25 μm radius sphere of each bouton. The density of GlyT2 boutons was determined by mapping boutons in 6 GlyT2-GFP GCL samples and dividing the total bouton count by the volume of the image. Density measurements were then compared between single GoCs and the GlyT2-GFP⁺ population by computing the ratio of bouton densities in a single GoC and mean GlyT2-GFP bouton density. The ratio was used to estimate the fraction of bouton density accounted for by a single GoC. We display the distribution of density ratios in a histogram normalized by the total number of boutons with a bin size of 0.05.

Experimental design and statistical analysis

Paired and unpaired *t* tests were performed using RStudio (version 1.0.136; RStudio). Linear regression analysis was performed using Prism 7.04 software (GraphPad) and MATLAB (RRID:SCR_002798). Statistical tests are specified in the text. Electrophysiological and morphological analyses are described above.

Results

Spatial distribution of GoC processes

Rules of integration in GrCs will depend critically on the structure of feedforward inhibition within the GCL. Previous studies have proposed contradictory integrative models. Influential older work proposed that GoC axons tile the GCL in a nonoverlapping manner such that GrCs are innervated by a single GoC (Eccles et al., 1967), yet physiological measurements estimated convergence of multiple GoCs onto GrCs, but the existence of slow spillover current makes convergence estimates challenging (Rossi and Hamann, 1998). Several morphological features of GoCs, including axonal volume and bouton density, are each essential to infer the inhibitory convergence in the GCL. Therefore, we set out to define the density of GoC basal axons to test these assumptions and inform inhibitory connectivity rules in the GCL. We performed comprehensive basal arbor reconstruction to quantify GoC morphological characteristics that contribute to spatial distribution of feedforward inhibition in the GCL. To do so, we used a sparse viral labeling technique, which restricted the number of fluorescent GoCs in cerebellar cortex. A combination of low-titer Cre recombinase-expressing virus (AAV8-hSyn1-mCherry-Cre; titer: 10^2) and high-titer Cre-dependent reporter virus (AAV2-CAG-FLEX-eGFP; titer: 10^{12}) were injected into cerebellar cortex, which resulted in very sparse labeling of individual GoCs (Fig. 1A).

In keeping with previous reports, sparse GoC label revealed extensive basal axons, which were characterized by their small diameter ($\sim 0.2 \mu\text{m}$) (Palay and Chan-Palay, 1976; Holtzman et al., 2006; Barmack and Yakhnitsa, 2008; Hull and Regehr, 2012; Vervaeke et al., 2012; Ankri et al., 2015; Valera et al., 2016). An example GoC is shown in Figure 1B. This GoC extended processes $\sim 200 \mu\text{m}$ mediolaterally, $\sim 330 \mu\text{m}$ dorsoventrally, and $\sim 180 \mu\text{m}$ rostrocaudally, occupying a convex hull volume, or the volume of space occupied by the boundaries of the axonal field, as

Figure 1. Sparse viral label and comprehensive reconstruction of cerebellar GoCs. **A**, Schematic of sparse viral labeling technique where low-titer AAV8-hSyn1-mCherry-Cre 10^2 and high-titer AAV2-CAG-FLEX-EGFP 10^{12} were coinjected to cerebellar cortex. Bottom, Representative example of GoC axonal boutons. **B**, Example of a sparsely labeled GoC. Scale bar, 20 μ m. **C**, Comprehensive, 3D reconstruction of GoC displayed in 2D. Red represents axon. Cyan represents basal dendrite. Yellow represents apical dendrite. Beige represents soma. Left, Overview of the single GoC. Middle, Axon processes in GCL. Right, Apical dendrites and basal dendrites. Scale bar, 50 μ m. **D**, Total length of each process type. **E**, Convex hull volume of each process type. **F**, Maps of axonal boutons from two representative sparsely labeled GoCs. Black dots indicate the location of each bouton. Green circle indicates soma.

if a sheet were draped around it, of $\sim 5.7 \times 10^6 \mu\text{m}^3$ and overall axonal length of 26.7 mm (Neurolucida; see Materials and Methods). Therefore, this GoC basal axon encompasses a volume that contains $\sim 15,000$ GrCs and 580 MF rosettes based on estimates of GrC and MF rosette densities (Palkovits et al., 1971). GoC basal dendrites were distinguishable from axons by their larger diameter (0.3–3.2 μm) and smooth surface devoid of boutons as previously described (Palay and Chan-Palay, 1974; Hull and Regehr, 2012; Vervaeke et al., 2012; Ankri et al., 2015; Rudolph et al., 2015; Szoboszlay et al., 2016). Dendrites comingled with axons but were considerably shorter (Fig. 1C, right), with a length totaling 1.4 mm (Fig. 1D) and encompassing a computed volume of $0.41 \times 10^6 \mu\text{m}^3$ (Fig. 1E; convex hull volume; Neurolucida, see Materials and Methods). Because the total axon process was ~ 20 times longer than basal dendrites, GoC-mediated inhibition is predicted to be distributed more widely than the afferent input to a given GoC, as has been previously noted (D'Angelo et al., 2013).

The highly restricted cell labeling *in vivo* also permitted novel quantification of GoC bouton density and numbers. Axons possessed distinct bouton swellings ($\sim 3 \mu\text{m}$ diameter). We comprehensively mapped boutons of four complete GoCs located in vermal lobule 4/5 and quantified bouton density of an additional partially reconstructed GoC (Fig. 1F), which was included in a subset of analyses. Axons of individual GoCs were studded with a total of 6000–7500 boutons (5.9×10^3 to 7.46×10^3), for an average single GoC bouton density of 4.34×10^5 boutons/ mm^3 ($n = 4$ from four mice). We calculated the nearest bouton by Euclidean distance, which revealed that a majority (74%–93%) were within 4 μm of another bouton from the same cell (Fig. 2A–C; median nearest bouton, 2.18–3.39 μm ; $n = 27,402$ bou-

tons from 4 cells). The distance from each bouton to its nearest within-cell neighbor remained fairly constant across the axonal arbor (Fig. 2C), illustrated in the color map of nearest neighbors. We quantified this observation with linear regressions relating the nearest bouton distance to its position relative to the soma on a per-cell basis (ranges across cells: $R^2 = 0.0005$ –0.2, all $p < 0.01$, $F = 3.25$ –54, $df = 1893$ –7811, $n = 5$). Although the nearest boutons remained fairly constant across the axonal arbor, visual inspection of the bouton maps suggested clustering toward the soma. We therefore analyzed the number of neighbors within 10 μm of each bouton. This analysis revealed highly structured clustering, where a majority of boutons close to soma had >20 close neighbors from the same cell. This clustering dropped as a function of distance from the soma (Fig. 2D,E; $R^2 = 0.05$ –0.35, all $p < 0.01$, $F = 213$ –3100, $df = 1893$ –7811, $n = 5$).

These comprehensive bouton maps allowed us to estimate GoC overlap at the glomerulus. To do so, we first compared bouton distributions from single-cell reconstructions with those observed in *GlyT2-eGFP* mice, which label $\sim 85\%$ of GoCs (Fig. 3A) (Simat et al., 2007). Mapping boutons in these preparations revealed an overall bouton density of 6.54×10^6 boutons/ mm^3 . Distributions of nearest boutons differed slightly between single cell reconstructions and *GlyT2-eGFP* label (Fig. 3B). Not surprisingly, *GlyT2-eGFP*-labeled boutons were more closely spaced, with a prominent peak at $\sim 1.5 \mu\text{m}$ compared with the peak at 3 μm for the single neurons. Differences between single cells and the population were more pronounced when looking at local clustering, seen by measuring the distances to the closest 10 neighbors (Fig. 3C). *GlyT2-eGFP*-labeled boutons were denser, with the closest 10 boutons appearing on average $\sim 4 \mu\text{m}$ away from every other bouton from the same cell. By contrast, the nearest 10 boutons to each bouton on axons from individual

Figure 2. Clustered boutons revealed with high-resolution mapping. **A**, Bouton map of GoC with distance to the nearest bouton represented by color. **B**, Probability histogram of nearest bouton distances in five GoCs (gray) and mean of five cells (blue, shaded area represents SD). Bin width: 0.1 μm . **C**, Summary of nearest boutons as a function of distance from soma. Lines indicate linear regressions plotted for each neuron. **D**, Bouton map of the same GoC shown in **A** with the number of boutons within 10 μm radius from each individual bouton represented by color. **E**, Number of boutons within 10 μm radius from each bouton as a function of distance from the soma. Lines indicate linear regressions plotted for each neuron.

neurons averaged $\sim 7 \mu\text{m}$. These analyses allowed us to estimate how many GoC axonal arbors overlap locally. To estimate GoC axonal overlap, we examined the proportion of GlyT2-GFP-positive bouton density accounted for by a single GoC. In areas within 50 μm of a GoC soma, single GoC axons tended to account for 40%–85% of bouton density, suggesting that glomeruli here are likely dominated by a single GoC (Fig. 3D). Nevertheless, the proportion of population bouton density accounted for by a single GoC falls off with distance from the GoC soma, as indicated with significant negative slopes of regressions (Fig. 3D; $R^2 = 0.29$ – 0.66 ; $p < 0.0001$; $F = 768$ – 11600 ; $df = 1895$ – 7811 , $n = 5$).

The distribution of single GoC:GlyT2 bouton density (Fig. 3E) can be viewed as an estimate of the distribution of the number of overlapping GoCs. The peak in the distributions near 0.2 (mean

0.22 ± 0.05 for complete reconstructions) indicates that a typical degree of overlap between GoCs is ~ 5 . Assuming that 15% of GoC boutons remain unlabeled in GlyT2-GFP mice shifts the distribution slightly to peak ~ 0.18 , suggesting that between 5 and 6 overlapping GoCs is common. Notably, the broad distribution around the peaks indicates a large variance in the number of overlapping GoCs at any position, ranging from 2 to 20 as extremes.

Low-probability fast phasic feedforward inhibition in the GCL

The spatial extent of GoC processes positions them as sites of MF convergence and inhibitory divergence, motivating experiments examining how feedforward inhibition is recruited by identified cerebellar inputs. To begin to test how diverse MF afferents in-

Figure 3. Contribution of single GoCs to population distribution. **A**, GlyT2-eGFP-expressing processes in mouse GCL. Scale bar, 10 μ m. **B**, Probability histograms of nearest bouton distances for single GoCs (black) and the population of GoCs labeled with GlyT2-GFP (red). Bin width: 0.25 μ m. **C**, Probability histograms of average distances to the closest 10 boutons from each bouton for single GoCs (black) and a population of GoCs labeled with GlyT2-GFP (red). Bin width: 0.25 μ m. **D**, Ratio of single GoC bouton density to the population-level bouton density as a function of distance from GoC soma. The mean GlyT2 bouton density within a 25 μ m radius sphere was compared with the number of boutons within a 25 μ m radius sphere for each bouton in the single GoCs. Lines indicate linear regressions plotted for each neuron. **E**, Probability histogram plotting the ratio of the total GlyT2 bouton density accounted for by one GoC. Bin width: 0.05.

teract in the GCL, we first scaled down the question to address how identified sources of information recruit GrCs and feedforward inhibition via GoCs. We examined unimodal information processing of GrCs by recording postsynaptic currents evoked by optogenetic stimulation of a subset of MFs originating from neurons in the cerebellar nuclei, known as the nucleocortical (NC) pathway or those originating in the pontine nuclei. AAV2-hSyn1-hChR2-mCherry-WPRE-PA (see Materials and Methods) was injected into either the IN or pontine nuclei to express ChR2 in MFs. In a small subset of experiments, AAV2-EF1a-DIO-hChR2(H134R)-mCherry was injected into the IN of *Ntsr1-Cre* mice, to manipulate the NC pathway. MF density averaged 3673 ± 1911 rosettes/mm³ ($n = 7$ from 5 mice); thus, NC pathway inputs were sparsely spaced, as described previously (Gilmer and Person, 2017). We examined the GrC responses to MF optogenetic stimulation in an acute brain slice preparation from adult mice. We isolated EPSCs and IPSCs by holding GrCs at -70 or 0 mV, respectively, and stimulated ChR2 expressing MFs at between 20 and 60 Hz with blue light pulses delivered through the objective (Fig. 4A; 2 ms pulses; 0.96 – 3.77 mW/mm²).

EPSCs evoked from optogenetic excitation of the NC had an average amplitude of 80.6 ± 9.3 pA (Fig. 4B,F; SEM; $n = 7$) and 10%–90% rise and 37% decay times typical of AMPA-type glutamate receptor-mediated currents in GrCs, averaging 0.25 ± 0.04 ms and 1.0 ± 0.2 ms, respectively, similar to Group 2 excitatory inputs previously described (Chabrol et al., 2015). Surprisingly, however, given the large spatial convergence of MFs and subsequent divergence of inhibition within the GCL fast phasic IPSCs recorded at 0 – 3 mV holding potentials were uncommon, observed in just 5 of 12 responsive GrCs and 5 of 96 total GrCs

(Fig. 4C,G; from $N = 11$ and $N = 18$ mice, respectively). Fast phasic IPSCs were distinct from EPSCs, with smaller amplitudes and slower kinetics, averaging 40.3 ± 5.5 pA, with 0.9 ± 0.1 ms 10%–90% rise times and 2.7 ± 0.4 ms 37% decay times (Fig. 4I; $n = 5$; EPSC vs IPSC amplitude, $p = 0.007$; rise time, $p < 0.001$; decay time, $p = 0.001$; unpaired t tests). These measurements were statistically indistinguishable from spontaneous fast phasic inhibitory currents (amplitude, $p = 0.1$; rise time, $p = 0.3$; decay time, $p = 0.2$; unpaired t tests), suggesting that they originate from single GoCs rather than recruitment of multiple convergent GoCs. Moreover, as expected for feedforward inhibition, IPSC latencies and temporal variability were distinct from those of EPSCs (Fig. 4D), averaging 13.5 ± 0.9 ms versus 4.2 ± 0.3 ms for EPSCs, with latency jitter (SD of latency) averaging 3.6 ± 0.7 ms versus 0.29 ± 0.2 ms for EPSCs (Fig. 4E; $p < 0.001$; unpaired t test). Thus, fast phasic inhibition observed in GrCs following NC optogenetic stimulation had the hallmark of feedforward inhibition mediated through GoC recruitment but was nevertheless uncommon.

Given the shared sparseness of direct excitatory and disynaptic phasic inhibitory inputs to GrCs following NC stimulation, we next examined the overlap of these inputs onto single GrCs. As expected for sparse synaptic contacts, GrCs with phasic responses to NC pathway stimulation showed either EPSCs (58.3%) or fast phasic IPSCs (41.6%) but not both in our recording set (Fig. 4I). When NC EPSCs were elicited at -70 mV, no fast phasic IPSC was detectable holding the cell at 0 mV; conversely, when IPSCs were evident at 0 mV, no EPSC was detectable at -70 mV.

Figure 4. GrC responses to optogenetic activation of identified MF populations. **A**, Schematic diagram of recording configuration. Chr2-expressing NC or pontine MFs were optogenetically stimulated during GrC recordings. **B**, **C**, Overlaid EPSCs or IPSCs evoked by 2 ms optogenetic stimulation of NC MFs at -70 or 0 mV holding. **D**, Latency of EPSCs and fast phasic IPSCs ($p < 0.001$; unpaired t test). Gray error bar indicates mean. **E**, Jitter of timing of EPSCs and fast phasic IPSCs was significantly different ($p < 0.001$, unpaired t test). Error bar indicates mean. **F**, **G**, Representative traces of responses to light stimulation following NC MF stimulation at -70 mV (solid lines) and 0 mV (dashed lines). **H**, Example of slow outward current evoked after optogenetic stimulation of NC pathway at 20 Hz, $V_{\text{hold}} = 0$ mV. **I**, Summary of fast phasic current amplitudes recorded at -70 or 0 mV. **J**, Summary of charge transfer during slow outward currents evoked by NC MF stimulation. **K–M**, Same as **F**, **G** but for pontine MF stimulation. **N**, Example of slow outward current evoked after optogenetic stimulation of pontine MFs at 20 Hz, $V_{\text{hold}} = 0$ mV. **O**, Same as **I** but for pontine MF stimulation. **P**, Same as **J** but for pontine MF stimulation. $*p < 0.05$.

Slow spillover-mediated inhibition has been proposed as the primary form of inhibition within the GCL, so we next examined GrCs for slow inhibitory currents. Slow inhibitory currents were observed in all GrCs with phasic excitatory or inhibitory responses, with an average charge transfer of 124.5 ± 21 (pA · ms; IPSC area) (Fig. 4H,J). Thus, slow phasic inhibition dominates feedforward inhibitory processing from identified MF pathways, consistent with observations from electrical stimulation.

To contrast experiments studying low density of MFs from the NC pathway, we next examined GrC responses to a dense MF population originating in the pontine nuclei with optogenetics (Huang et al., 2013; Gilmer and Person, 2017). Labeled pontine MFs were dense, averaging $107,749 \pm 27,965/\text{mm}^3$ (SD) $n = 10$ in 5 mice. We made whole-cell patch-clamp recordings of GrCs in the vicinity of labeled MFs. Light stimulation evoked excitatory or fast phasic inhibitory currents in 21 GrCs in 8 mice (Fig. 4K–M): EPSCs, 55.6 ± 7.6 pA, with 0.4 ± 0.03 ms 10%–90% rise times and 1.3 ± 0.11 ms 37% decay times, 2.8 ± 0.2 ms latency ($n = 18$); IPSCs, 22.2 ± 1.2 pA, with 2.2 ± 0.4 ms 10%–90% rise times and 2.3 ± 0.5 ms 37% decay times; 12.8 ± 2.1 ms latency ($n = 8$). The fraction of overlap of phasic EPSCs and fast phasic IPSCs was higher with pontine stimulation, as expected of a denser input: EPSCs without phasic IPSCs were evoked in 62% of GrCs and exclusively phasic IPSCs were evoked in 14% of recorded GrCs, constituting 76% of responsive cells. In the remaining 24% of responsive neurons, pontine MF stimulation evoked both EPSCs and fast phasic IPSCs (5 of 21) (Fig. 4O). Thus, density of MF afferents influences the overlap between phasic excitation and inhibition. As was observed for NC fibers, how-

ever, slow inhibitory currents dominated fast phasic inhibition and were seen in all cells with phasic currents following pontine MF activation, with an average charge transfer of 88 ± 8.0 (pA · ms) (Fig. 4N,P).

To summarize, fast phasic inhibition was probabilistically recruited onto GrCs depending on the approximate density of MF terminals, but slow inhibitory currents were much more widespread, even when evoked by a sparse MF input.

Predominant role of slow spillover inhibition in regulating GrC excitability

GoC-mediated feedforward inhibition has been proposed to regulate the number of MFs necessary to recruit GrCs. We tested the efficacy of physiological levels of feedforward inhibition regulating GrC excitability, combining optogenetic stimulation of pontine MFs and dynamic clamp, mimicking physiologically realistic feedforward fast and slow phasic inhibitory conductances ($N = 12$ mice). We recorded from GrCs in the presence of SR95531 ($10 \mu\text{M}$) to block endogenous GABA_A receptors. In whole-cell current-clamp mode, we measured GrC firing in response to 20 Hz optogenetic stimulation of pontine MFs, followed by delayed fast phasic IPSCs or slow phasic IPSCs produced via the dynamic clamp, mimicking GoC-mediated feedforward inhibition. GrCs were held at -60 to -75 mV by current injection to promote MF-driven firing. We used two conductances for both fast and slow phasic currents, with the lower of the two matching physiologically measured values but the higher providing insight into the upper bound of physiological levels of inhibition.

Figure 5. Predominant role for slow feedforward inhibition in regulating GrC synaptic responses at moderate excitation frequency. **A**, Left, Schematic of recording configuration where dynamic-clamp and optogenetic MF stimulation were combined to examine the role of feedforward inhibition (**A**). Right, Representative traces showing GrC responses to 20 Hz optogenetic stimulation of pontine MFs without (top) and with (bottom) dynamic-clamp physiological fast phasic IPSPs (bottom). Bottom, The same conventions describe experiments using dynamic clamp to mimic 100 Hz excitation and 60 Hz inhibition. **B**, Black represents summary of MF opto-evoked firing rate changes with and without small fast phasic IPSPs. Red represents same but for dynamic-clamp EPSPs + IPSPs. **C**, **D**, Same as **A**, **B**, except with large fast phasic IPSPs. **E**, **F**, Same as **A**, **B**, except with small, slow phasic IPSPs. Bottom, Representative trace from GrC in response to 50 Hz light train illustrating similarity to injected conductance. **G**, **H**, Same as **A**, **B**, except with large, slow phasic IPSPs. Significant changes in firing rate were observed in 7 of 8 GrCs ($p < 0.05$, paired t tests). * $p < 0.05$.

Low-conductance fast phasic IPSPs (0.3 nS; rise time 2.15 ms, decay tau 2.29 ms), approximating a single fast phasic GoC input, were delayed by 12.8 ms relative to light in a 20 Hz light pulse 500 ms train to approximate feedforward inhibition. Across the population, this manipulation did not significantly change optogenetically driven firing compared with optogenetic stimulation alone (Fig. 5*A*, *B*; -3.9 ± 2.3 Hz from baseline, $p > 0.1$, paired t test; $n = 8$), although in one cell fast phasic IPSPs significantly reduced MF-driven firing responses (1 of 8 cell; -10.8 Hz from baseline, pontine 81.8 ± 2.0 sp/s, pontine + IPSP 71.0 ± 1.8 sp/s, unpaired t test, $n = 10$ pontine; $n = 5$ pontine + IPSP trials; $p = 0.002$). As a positive control, we next tested whether stronger phasic inhibition influenced firing rate. We increased the magnitude of the phasic stimulation to 1.2 nS, which is physiologically unlikely because fast phasic contacts are rare and this assumes 4 onto a single cell, when only 60% of GrC dendrites receive direct contacts (Jakab and Hamori, 1988; Rossi and Hamann, 1998). As expected, this manipulation reduced response frequency (Fig. 5*C*, *D*; -11.9 ± 3.4 Hz from baseline, $p < 0.05$, paired t test, $n = 8$), with significant effects seen in 4 of 8 cells ($p < 0.05$, unpaired t test, $n = 10$ pontine; $n = 5$ pontine + IPSP trials in each cells).

Because MFs are known to burst at much higher rates (>100 Hz), we also examined the role of phasic inhibitory currents to reduce excitability to higher-frequency stimuli. Optogenetic

probes are not well suited to follow such high rates, so we used a dynamic-clamp only approach to mimic both excitation and inhibition. At high rates, GoC inhibitory currents also depress, so we combined high-frequency (100 Hz; 1.5 nS peak conductance) EPSPs, which depressed to $\sim 50\%$ of their maximum amplitude after three stimuli (Saviane and Silver, 2006), and high-frequency IPSPs (60 Hz), which depressed to 50% after one stimulus (Duguid et al., 2015). Both physiological and large fast phasic inhibitory currents also significantly reduced response rates in some neurons (Fig. 5*B*, *D*; $p < 0.05$ for 4 of 11 neurons, mean rate change -0.9 ± 1.5 spikes/s, 0.3 nS peak; $p < 0.05$ 8 of 9 cells, mean rate change -19.6 ± 4.6 spikes/s, 1.5 nS peak).

In previous dynamic-clamp studies, slow spillover-like inhibitory currents attenuated GrC firing more effectively than fast phasic IPSCs (Crowley et al., 2009). We extended these experiments to explicitly test a role for feedforward inhibition, such that the slow current was delayed relative to excitation and used conductances mimicking current from a single GoC or higher conductances that reflect summated slow phasic inhibition. We first mimicked slow phasic inhibition observed during 50 Hz light trains to stimulate MFs (Fig. 5*E*, bottom), injecting IPSPs (0.032 nS) following the first light stimulation, again delayed by 12.8 ms. This experimental condition did not reduce MF driven firing rates (Fig. 5*E*; -4.4 ± 2.2 Hz from baseline, $p > 0.05$, paired t test, $n = 8$). Because multiple GoCs converge in a glomerulus, we next

Figure 6. GoC recruitment follows MF population activity levels. **A**, Schematic diagram of recording configuration. ChR2-expressing NC or pontine MFs were stimulated during GoC recordings. **B**, Biocytin-filled GoCs (cyan) recovered after recordings show proximity of RFP-expressing MFs. Scale bar, 50 μ m. Right, Putative synaptic contacts (arrows) or absence of contacts between recorded GoC and MF, associated with physiological traces in **C** and **D** shown in transparency rendering mode. **C**, Representative examples of GoC evoked firing in response to optogenetic stimulation of pontine MFs at 40 Hz. Responses varied between cells (top, bottom). **D**, Representative traces showing diversity of evoked EPSCs following optogenetic stimulation of pontine MFs at 40 Hz. $V_{\text{hold}} = -70$ mV. **E**, Traces are matched with current-clamp responses in **C**. **E**, **F**, Relationship of GoC firing rate change to stimulation rate. **E**, Nonsignificant relationships. **F**, Significant relationships. **G**, Probability distribution of initial EPSC amplitudes measured in GoCs showing significant (black; data from **F**) or nonsignificant (red; data from **E**) input–output relationships. **H**, Relationship of EPSC amplitude to ChR2-expressing MF density ($R^2 = 0.44$, $p = 0.001$, $F = 14.3$, $df = 18$, $n = 20$). **I**, Relationship of GoC firing rate change to 40 Hz stimulation as a function of ChR2-expressing MF density ($R^2 = 0.23$; $p = 0.04$; $F = 5.0$; $df = 16$; $n = 18$). **J**, Multi-EPSC-peak probability plotted as a function of initial EPSC amplitude ($R^2 = 0.41$, $p < 0.0001$, $F = 27.9$, $df = 40$, $n = 42$).

tested higher-conductance IPSPs that reflect summated slow phasic inhibition. We therefore increased conductance 10-fold capturing summation. As expected, larger slow phasic IPSPs (0.32 nS) strongly attenuated rates in most GrCs (Fig. 5*G,H*; 7 of 8 cells, $p < 0.05$, unpaired t test, $n = 10$ pontine; $n = 5$ pontine + IPSP trials in each cells).

GoC recruitment scales with MF input density

The dynamic-clamp experiments indicate that fast phasic feed-forward inhibition from GoCs regulates the threshold of GrCs (Brickley et al., 1996; Wall and Usowicz, 1997; Rossi and Hamann, 1998; Hamann et al., 2002; Mitchell and Silver, 2003). Threshold regulation was a major role for GoCs in theoretical studies proposing a role for pattern discrimination by GrCs, and predicts that GoC recruitment would scale with input density (Marr, 1969; Pellionisz and Szentágothai, 1973; Mapelli et al., 2009; Honda and Ito, 2017). Therefore, identifying rules of GoC recruitment is essential to differentiate between diverse models of GrC information processing.

To better understand the rules of GoC recruitment by identified MF inputs, we next recorded from GoCs directly, measuring evoked firing and synaptic currents following optogenetic stimulation of MFs originating in the cerebellar or pontine nuclei (Fig. 6*A*). We used *GlyT2-eGFP* mice to identify GoCs under

fluorescence, noting that this excludes a small subpopulation of GoCs from our dataset (Simat et al., 2007). We first tested the efficacy of EPSPs from both NC and pontine sources to drive firing in GoCs in current-clamp mode during trains of stimuli delivered at rates between 20 and 60 Hz (Fig. 6*C*). GoCs were spontaneously active during patching, with firing rates spanning 2.4–47.5 spikes/s. We performed linear regression analysis on firing rate changes as a function of stimulation frequency. Consistent with previous observations from electrical stimulation (Kanichay and Silver, 2008), MF stimulation rates were poor predictors of firing rate changes across the population ($R^2 = 0.03$, $p = 0.03$, $F = 4.6$, $df = 176$, $n = 178$ from 36 cells pooled; $N = 23$ mice).

This filtering property has been previously ascribed to a large afterhyperpolarization from EPSP-driven spikes (Kanichay and Silver, 2008). Nevertheless, it raises the question of how GrC population activity could remain constant if GoC recruitment is strongly filtered, prompting us to look closer at the relationship between MF activity and GoCs. Indeed, when we examined the relationship of firing rate with stimulation rate in individual neurons, we noted that some were strongly correlated with significant within-cell correlation (Fig. 6*F*; $p < 0.04$, $n = 7$), whereas many cells were not (Fig. 6*E*; $p > 0.05$, $n = 28$). We next examined whether properties of the EPSCs differed

between these groups and found a strong bias toward stronger EPSCs supporting linear input–output relations (Fig. 6G; 332.1 ± 41.5 pA, $n = 7$). Weaker EPSCs, by contrast, did not modulate GoC spike rates with increasing stimulus rates (Fig. 6G; 71.5 ± 8.1 pA, $n = 35$).

These findings indicate that EPSC amplitude determines the input-filtering properties of GoCs. To identify factors that influence the amplitude of EPSCs onto GoCs, we took advantage of the fact that optogenetic probes are coupled with a fluorophore; thus, we could directly measure the density of activated inputs. This allowed us to test the prediction that MF convergence onto GoCs from multiple inputs would summate to regulate EPSC amplitude in GoCs. Consistent with high levels of convergence of MFs onto GoCs, there was a strong linear relationship between MF density and EPSC amplitude, with higher-density MFs eliciting larger EPSCs (Fig. 6H; $R^2 = 0.44$, $p = 0.001$, $F = 14.3$, $df = 18$, $n = 20$). Despite the sparseness of NC MFs, which constitute just 1%–10% of local MFs (Gilmer and Person, 2017), optogenetic activation of the pathway at 40 Hz elicited EPSCs in 35% of GoCs recorded in the vicinity of labeled MFs (34 of 96). Denser MF populations from the pontine nuclei elicited responses in 87% of nearby GoCs (14 of 16). Not surprisingly, given the large difference in density between pontine and NC inputs, pontine inputs elicited significantly larger EPSCs ($p < 0.001$, unpaired t test, -259.5 ± 26.8 pA, $n = 70$ from 14 cells pooled, -40.7 ± 3.1 pA, $n = 131$ from 28 cells pooled, respectively). These observations suggest that GoCs integrate many inputs from diverse sources and are responsive to the level of input population activity as expected for active thresholding.

Having identified a relationship between MF input density and GoC EPSC amplitude and linearity of firing responses, we next reasoned that GoC firing responses might be sensitive to the overall level of MF activity levels (i.e., the density of active inputs). We tested this idea by analyzing the density of MFs labeled within the vicinity of the recorded GoC, and relating measurements to the magnitude of firing rate changes observed with 40 Hz stimulation. In keeping with this reasoning, we found a positive significant correlation between active input density and the magnitude of firing rate changes during stimulation (Fig. 6I; $R^2 = 0.23$; $p = 0.04$; $F = 5.0$; $df = 16$; $n = 18$).

As described previously (Kanichay and Silver, 2008; Hull and Regehr, 2012; Cesana et al., 2013; Gao et al., 2016), there was considerable diversity in GoC EPSC properties, which differed by amplitude, latency, and whether they were singular or included disynaptic feedback via ascending and parallel fiber input from GrCs. In general, EPSCs fell into three broad classes, which included (1) short latency; (2) mixed short- and long-latency producing multiple peaks per stimulus; and (3) long latency (Fig. 6D). Consistent with the view that these response classes reflect monosynaptic and disynaptic input, biocytin fills of recorded GoCs revealed ChR2-RFP-expressing MF inputs adjacent to somata and/or basal dendrites of short-latency responders, defined as those with responses occurring within 3.6 ms. Nine of 11 short-latency responders were histologically recovered. In the case of long-latency responders (those with responses >3.8 ms), no MF inputs were identified adjacent to the somata or basal dendrites (Fig. 6B; $n = 3$ of 3 recovered GoCs with long latency responses). Furthermore, bath application of the NMDA receptor antagonist CPP reduced the likelihood of multi-peaked EPSC being elicited from $37.9 \pm 14.0\%$ to $5.8 \pm 1.0\%$ ($n = 3$ cells; $N = 3$ mice), sup-

porting the view that late synaptic responses were the result of disynaptic recruitment of GrCs (Cesana et al., 2013).

Because GoC multi-peaked EPSCs are a readout of GrC recruitment, which is in turn regulated by GoC inhibitory feedback (Cesana et al., 2013), we reasoned that we could use the relationship between MF input strength and multi-peaked EPSC probability to test whether GoCs dynamically modulate MF–GrC gain as predicted in numerous models (Mitchell and Silver, 2003). GoC EPSC multi-peak probability was linearly correlated with the initial EPSC amplitude (Fig. 6J; $R^2 = 0.41$, $p < 0.0001$, $F = 27.9$, $df = 40$, $n = 42$), indicating that stronger input recruited more GrCs. Interestingly, however, there was no detectable change in multi-peak probability or number of peaks over the course of moderate frequency (40 Hz) stimulus trains ($p = 0.68$, $p = 0.65$, paired t test of early vs late train multi-peak probability or early vs late train number of peaks per stimulus). This stability of multi-peak probability was evident regardless of whether multi-peak probability was high or low at the beginning of the stimulus train. Coupled with the observation that GABA_A receptor blockade strongly enhances multi-peak probability (Cesana et al., 2013), these findings indicate that feedback inhibition within the layer stabilizes GrC excitability for a given input excitation level.

Discussion

This study used a combination of quantitative morphometry, optogenetics, and dynamic clamp to test theoretical predictions about GoCs as global regulators of GrC excitability. Theory has long posited a role for GoCs in regulating GrC population activity (Eccles et al., 1967; Marr, 1969), proposing that they respond dynamically to varying input levels to modulate all local GrC thresholds, but data testing key assumptions of this view have been lacking. Here we confirm theoretical predictions that GoC axons are ideally suited to globally inhibit neighboring GrCs; that spillover-mediated feedforward inhibition alters GrC thresholds; and that convergent afferents are essential to recruit GoC inhibition in a manner consistent with a global “listening” mechanism well suited for normalizing activity in the GCL. These data indicate that the large multimodal integrative capacity of the GoC, combined with physiological integrative rules of GrCs, set up an MF activity level-detector to regulate inhibition levels within the GCL.

GrCs have been proposed to perform pattern separation by sparsening information conveyed by MF inputs (Cayco-Gajic et al., 2017). Inhibition from wide-field interneurons has been proposed in both mammalian cerebellum and other systems to perform these computations (Pouille et al., 2009). An underlying but untested assumption is that inhibitory interneurons contact all or nearly all neurons in the field to effectively regulate population activity (Marr, 1969; Albus, 1971; Billings et al., 2014; Duguid et al., 2015; Cayco-Gajic et al., 2017). Similar roles have been proposed for cerebellar GoCs. However, although their morphology has long been appreciated as complex and suitable for widespread inhibition, quantitative analysis of axonal bouton density has not been performed, leading to conflicting speculation about whether inhibition they produce is spatially structured. Our morphometry shows that GoCs are suited to contact every nearby GrC. The ubiquity of boutons throughout the axonal arbor is important in light of potential alternative computations that could distribute inhibition to subsets of neighboring GrCs to decorrelate or temporally sculpt responses. Such findings show that cerebellar GoCs favor a blanket inhibitory process within the region.

The quantitative morphometry of reconstructed GoCs allowed us to evaluate how a population of GoCs might contribute to spatial distribution of inhibition. Although partial reconstructions of cerebellar GoCs have been published, they have most often followed labeling in brain slices, which necessarily exclude processes leaving or reentering the slice (Kanichay and Silver, 2008; Vervaeke et al., 2010, 2012; Szoboszlay et al., 2016; Valera et al., 2016). Other published reconstructions have followed *in vivo* labeling but were incomplete (Simpson et al., 2005; Barmack and Yakhnitsa, 2008). Nevertheless, these partial reconstructions support the view advanced here that GoCs are characterized by dense axonal arbors but differ in the extent and orientation of axonal fields (Simpson et al., 2005; Holtzman et al., 2006; Barmack and Yakhnitsa, 2008). Recent work has identified microcircuitry differences between GoCs with distinct molecular identities (Ankri et al., 2015). However, thus far it is unknown whether such connectivity and molecular identities extend to the morphology of GoCs. Future work should relate molecular identity to morphological characteristics to clarify distinct functional roles for GoC subtypes (Simat et al., 2007; Ankri et al., 2015; Eyre and Nusser, 2016).

The comprehensive reconstructions performed here offer insight into a GoC structural motif, where boutons from individual cells remain tightly clustered, even though the overall density of boutons falls off with distance from the soma; that is, their nearest neighbor distances remain constant, whereas the number of close neighbors drops off with distance from the soma. Comparisons of the density of GlyT2-positive GoC boutons, which reflect upward of 85% of total GoC bouton population, with those of individual cells suggest that within 50 μm of a GoC soma, a single GoC dominates the inhibitory axonal population. That dominance shifts as a function of the distance from GoC somata: The distributions of the fraction of the GlyT2 GFP-labeled bouton density accounted for by an individual GoC (Fig. 3E) indicate that a common degree of overlap is ~ 5 GoC axons because the distributions peak near 0.2, in line with physiological estimates (Rossi and Hamann, 1998). The broadness of the distributions, however, reveals that the number of overlapping GoCs can vary considerably: between 2 and 20 at extremes. These differences are likely to produce a large variety of inhibitory environments within a population of GrCs and may contribute to diversifying GrC responses to MF input. Accounting for unlabeled GoC boutons does not alter these estimates substantially. Based on estimates of glomerular density (Palkovits et al., 1971; Billings et al., 2014), our measurements would suggest that, on average, an individual GoC makes between 0.5 and 1.5 boutons per glomerulus within its axonal field.

The conclusions drawn from the reconstruction data indicate that the GoC is in a position to “globally” inhibit GrCs within range of its axonal arbors, as seen in thresholding motifs (Marr, 1969; Albus, 1971). However, examination of GrCs that were excited by optogenetic activation of MF input revealed surprisingly sparse fast phasic inhibition, even when MF inputs were dense (Fig. 4). This seeming inconsistency was resolved by analyzing GrCs for spillover-like slow inhibitory currents. This form of inhibition, which differs in kinetics and amplitude from direct phasic inhibition, was always observed in GrCs activated by MFs. Slow spillover-mediated IPSCs have been extensively studied in GrCs (Rossi and Hamann, 1998; Mitchell and Silver, 2000; Rossi et al., 2003; Duguid et al., 2012, 2015) and shown to dominate inhibitory processing in GrCs. The difference in likelihood of fast phasic and slow spillover currents, coupled with the extremely high density of GoC boutons, suggested that fast phasic inhibi-

tory events occur probabilistically due to the chance spatial proximity of GrCs dendrites relative to GoC axonal boutons. This view may be consistent with the observation that “tonic inhibition” is present in the GCL: Tonic inhibition, which lacks resolvable inhibitory current temporal modulation, may be at the end of a continuum of inhibitory current kinetics, where the distance from GoC glomerular synapses to GrC dendrite postsynaptic sites dictates kinetics.

Nevertheless, the striking differences between these IPSCs prompted further investigation into the relative physiological roles of these forms of inhibition. Previous dynamic-clamp studies have shown that slow inhibition strongly inhibits GrCs (Crowley et al., 2009; Solinas et al., 2010; Kalmbach et al., 2011; Duguid et al., 2015). We extended these studies by combining physiological levels of inhibition through a dynamic clamp with optogenetic activation of pontine MFs, offset in time from EPSPs. As expected, slow spillover-like conductances, offset in time relative to opto-EPSPs, reduced GrC responses to both moderate-frequency EPSPs and faster dynamically clamped EPSP trains.

Recent work has shown that MF-mediated feedforward inhibition through GoCs is not solely responsible for modulating GrC excitability and response timing. For instance, other sources of drive to GoCs have recently been described, including from climbing fibers and serotonin inputs (Nietz et al., 2017; Fleming and Hull, 2018). Moreover, physiological diversity of MF drive to GrCs can modulate GrC response timing (Chabrol et al., 2015), another prominent role ascribed to GoC feedforward inhibition. In addition, the duration and mixture of MFs activated can engage nonlinear recruitment patterns (Hernandez et al., 2018). Thus, future studies should examine ways in which GoCs are recruited either independent of MFs or selectively by particular subpopulations.

Another source of complexity in relating MF activity to recruitment of feedforward inhibition is the fact that EPSP-spike coupling in GoCs is heavily temporally filtered (Kanichay and Silver, 2008; Vervaeke et al., 2010). Our study corroborated reports from several groups finding that GoCs do not reliably follow increasing MF stimulation rates with high fidelity. Because our study involved optogenetic recruitment of MFs labeled with a fluorophore, we could relate GoC activation in our physiology recordings to the density and distribution of MFs activated by light. We uncovered a linear relationship between active MF density and EPSC amplitudes in GoCs elicited by light stimulation, indicative of convergence onto GoCs (Hernandez et al., 2018). Furthermore, MF density related to the firing response elicited at a fixed stimulus rate. In contrast to highly filtered EPSP-GoC firing rate relationships seen across the population and with electrical stimulation, firing rates of GoCs that received the strongest EPSCs from optogenetic stimuli (i.e., those with the highest density of MFs) linearly increased with stimulation rates. These findings relate the density of MF activity to the recruitment of GoCs in a rate-dependent manner, and suggest inhibitory mechanisms within the GCL maintain a large dynamic range by integrating across both MF firing rate and density.

In conclusion, this study relates GoC morphology and unique synaptic physiology onto GrCs to local circuit computations that function to sparsen GrC activity relative to MF inputs by reducing excitability. Our data reveal a strikingly dense inhibitory field of local GoCs, analogous to the wide-field inhibitory interneurons in insect mushroom bodies (Papadopolou et al., 2011) and show that individual GoCs integrate many MF inputs to regulate their output, driving activity that scales with input. Thus, the GoC occupies a key multimodal integrative niche within the layer

that, compared with the extremely limited extent of the GrC dendrite, allows for a broader integration of multimodal signals to regulate GrC population activity.

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