

A system of feed-forward cerebellar circuits that extend and diversify sensory signaling

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2

November 9, 2023 (this version)

Reviewed preprint version 1

August 15, 2023

Posted to bioRxiv

April 11, 2023

Sent for peer review

April 11, 2023

Harsh N. Hariani, A. Brynn Algstam, Christian T. Candler, Isabelle F. Witteveen, Jasmeen K. Sidhu, Timothy S. Balmer 

Interdisciplinary Graduate Program in Neuroscience • School of Life Sciences, Arizona State University, Tempe, AZ, 85287 • Barrett Honors College

 https://en.wikipedia.org/wiki/Open_access

 Copyright information

Abstract

Sensory signals are processed by the cerebellum to coordinate movements. Numerous cerebellar functions are thought to require the maintenance of a sensory representation that extends beyond the input signal. Granule cells receive sensory input, but they do not prolong the signal and are thus unlikely to maintain a sensory representation for much longer than the inputs themselves. Unipolar brush cells (UBCs) are excitatory interneurons that project to granule cells and transform sensory input into prolonged increases or decreases in firing, depending on their ON or OFF UBC subtype. Further extension and diversification of the input signal could be produced by UBCs that project to one another, but whether this circuitry exists is unclear. Here we test whether UBCs innervate one another and explore how these small networks of UBCs could transform spiking patterns. We characterized two transgenic mouse lines electrophysiologically and immunohistochemically to confirm that they label ON and OFF UBC subtypes and crossed them together, revealing that ON and OFF UBCs innervate one another. A Brainbow reporter was used to label UBCs of the same ON or OFF subtype with different fluorescent proteins, which showed that UBCs innervate their own subtypes as well. Computational models predict that these feed-forward networks of UBCs extend the length of bursts or pauses and introduce delays—transformations that may be necessary for cerebellar functions from modulation of eye movements to adaptive learning across time scales.

Significance statement

The cerebellum is essential for the accurate performance of behaviors ranging in complexity from stabilizing an image on the retina to playing a piano or performing a gymnastics routine. Cerebellar dysfunction disrupts the ability to produce smooth movements and leads to a disorder called ataxia. Damage to the vestibular cerebellum occurs in various disorders including medulloblastoma and leads to nystagmus, involuntary movements of the eyes that prevent normal vision. Treating disorders of motor control such as nystagmus, requires a better understanding of how representations of movements are maintained in the firing patterns of neurons in the cerebellar circuit. Here we use transgenic mice to label a type of neuron called the unipolar brush cell and revealed that these cells innervate one another and are likely to increase the length and diversity of spiking patterns in the cerebellum. These

eLife assessment

This study presents **valuable** findings about synaptic connectivity among subsets of unipolar brush cells (UBCs), a specialized interneuron primarily located in the vestibular lobules of the cerebellar cortex. The evidence supporting the claims are interesting although **incomplete** in some areas. The work will be of interest to cerebellar neuroscientists as well as those focussed on synaptic properties and mechanisms. Although several **compelling** pieces of data were presented, substantial work remains to be conducted in order for the hypothesis and predictions of the manuscript to confirm how these factors play out in the actual brain circuit and how it would impact the processing of feedback or feedforward activity that would be required to promote behavior.

Introduction

Head movement signals are encoded by the firing rate of vestibular afferents that project to the cerebellum and brainstem. The vestibular nuclei maintain a sensory representation of the head movement for tens of seconds after the vestibular signal ends through a mechanism called velocity storage (Fernandez and Goldberg, 1971 [↗](#); Raphan et al., 1979). The cerebellum dynamically modulates the activity of the cells that encode velocity storage to control vestibular responses such as compensatory eye movements (Yakushin et al., 2017 [↗](#)). It is unclear how the cerebellar circuit maintains a representation of a head movement long after the firing of the afferents cease.

Granule cells receive direct input from vestibular afferents, but cannot maintain the signal long enough to underlie velocity storage, because they produce only transient firing responses. The only other excitatory neuron type in the cerebellar cortex that could provide feed-forward excitation to prolong the signal to granule cells is the unipolar brush cell (UBC). UBCs are powerful circuit elements that amplify their input signals by extending them in time and projecting them to numerous granule cells (Berthie and Axelrad, 1994 [↗](#); Mugnaini et al., 1994 [↗](#), 2011 [↗](#); Rossi et al., 1995 [↗](#); Nunzi et al., 2001 [↗](#)). UBCs receive direct input from vestibular afferents, produce long duration spiking responses, and could therefore contribute to the maintenance of a sensory representation necessary to modulate low-frequency vestibular responses (Rossi et al., 1995 [↗](#); Mugnaini et al., 2011 [↗](#); Balmer and Trussell, 2019 [↗](#)). Although a single UBC can prolong its input for seconds, multiple UBCs connected in series could extend the signal much further. Indeed, there is some evidence suggesting that UBCs provide direct synaptic input to other UBCs (Diño et al., 2000 [↗](#); Nunzi and Mugnaini, 2000 [↗](#); van Dorp and De Zeeuw, 2015 [↗](#)).

In addition to prolonging signals, UBCs may play important roles in diversifying granule cell firing patterns, which is essential for expansion recoding models of cerebellar learning (Marr, 1969 [↗](#); Albus, 1971 [↗](#); Ito, 1982 [↗](#); Kennedy et al., 2014 [↗](#)). This diversity in UBC responses is due to different subtypes of UBCs that can be classified by their expression of specific proteins or neurotransmitter receptors (Nunzi et al., 2002 [↗](#), 2003 [↗](#); Kim et al., 2012 [↗](#); Sekerkova et al., 2014 [↗](#)) or by their electrophysiological response to synaptic stimulation: ON UBCs that express mGluR1 are excited by glutamate and OFF UBCs that express calretinin are inhibited by glutamate

(Borges-Merjane and Trussell, 2015 [↗](#)). Whether UBCs target UBCs of their own subtype, the other, or both, remains unclear, and is necessary to understand how sensory signals are extended and diversified in the granule cell layer.

The connectivity of different types of UBCs is difficult to address. Paired electrophysiological recordings of synaptically connected UBCs are challenging because of their long axons and their relative scarcity among the granule cells. Labeling UBCs of the same type with a single fluorophore provides ambiguous results- the axon terminals of UBCs look similar to UBC dendritic brushes, so a UBC axon contacting a dendritic brush of the same color would appear essentially identical to a large brush. We overcame this challenge by utilizing a Brainbow approach and by combining mouse lines to label UBC subtypes with different fluorescent proteins. We find that all UBC connectivity patterns are present and develop computational models to simulate how these feed-forward circuits dynamically transform their inputs. Interconnected UBCs prolong and diversify input spiking patterns by producing an extended burst (ON to ON), extended pause (ON to OFF), pause after a delay (OFF to ON), or burst after a delay (OFF to OFF). Thus, UBCs connected in series increase the computational power of the circuit and could underlie mechanisms of cerebellar function that require transmission delays or prolonged activity.

Results

Electrophysiological responses of GRP and P079 UBCs reveal that they are ON and OFF UBC subtypes

To label a population of UBCs that are excited by glutamate (ON UBCs), we used a mouse line that expressed Cre recombinase (Cre) under the control of the GRP (gastrin releasing peptide) promoter (Gerfen et al., 2013 [↗](#)), a promoter that had been reported to be active in mGluR1-expressing (mGluR1(+)) UBCs in a similar mouse line (Kim et al., 2012 [↗](#)). We crossed the GRP-Cre mouse to a tdTomato reporter (Ai9) (Madisen et al., 2010 [↗](#)) and targeted fluorescently labeled UBCs for whole-cell recording in acute brain slices (**Fig 1A** [↗](#)). The white matter of lobe X was stimulated with an extracellular electrode to evoke synaptic currents.

Synaptic stimulation evoked fast EPSCs and a slow inward current that began at the end of stimulation at higher frequencies, which is diagnostic of ON UBCs ($n = 15$) (**Fig 1B** [↗](#)). The latency between presynaptic stimulation and the beginning of the fast EPSC was 1.24 ± 0.29 ms (mean $\pm$ SD; $n = 13$), had a low jitter (defined as the SD of latency; 0.074 ± 0.046 ms; mean $\pm$ SD; $n = 13$), and was reliable having no failures across all cells and trials, which confirms that they were monosynaptic. The slow EPSCs peaked 70.13 ± 33.45 ms after the end of the 200 ms long 50 Hz stimulus train and their decay was well approximated by an exponential with a time constant of 358.31 ± 213.83 ms (mean $\pm$ SD; $n = 14$) (**Fig 1C** [↗](#)). In current-clamp recordings, synaptic stimulation produced a burst of action potentials in GRP UBCs that varied in duration from 510 to 900 ms ($n = 3$) (**Fig 1D** [↗](#)). The synaptic currents in GRP UBCs were confirmed to be mediated by mGluR1 and AMPA receptors because they were blocked by JNJ16259685 and GYKI53655 ($99.3 \pm 2.4\%$; paired t-test; $P=0.0068$; $n = 9$; mean $\pm$ SEM) (**Fig 1E-F** [↗](#)). While the two drugs together blocked most of the inward current (**Fig 1F** [↗](#)), their individual contribution ranged widely in different cells (3-70% block by JNJ16259685 and 30-100% block by GYKI53655). This indicates that GRP UBCs are ON UBCs and their synaptic input varies from being mediated mainly by mGluR1 to being entirely mediated by AMPA receptors.

Using the same approach, we tested the response to synaptic stimulation in a population of UBCs that expressed mCitrine in a mouse line (P079) that was generated in an enhancer trap forward genetic screen (Shima et al., 2016 [↗](#)). In all P079 UBCs recorded that responded to presynaptic stimulation, a 50 Hz train evoked a slow outward current ($n = 12$). Faster rates of synaptic stimulation produced larger outward currents (**Fig 1G** [↗](#)). The slow outward currents peaked 52.7

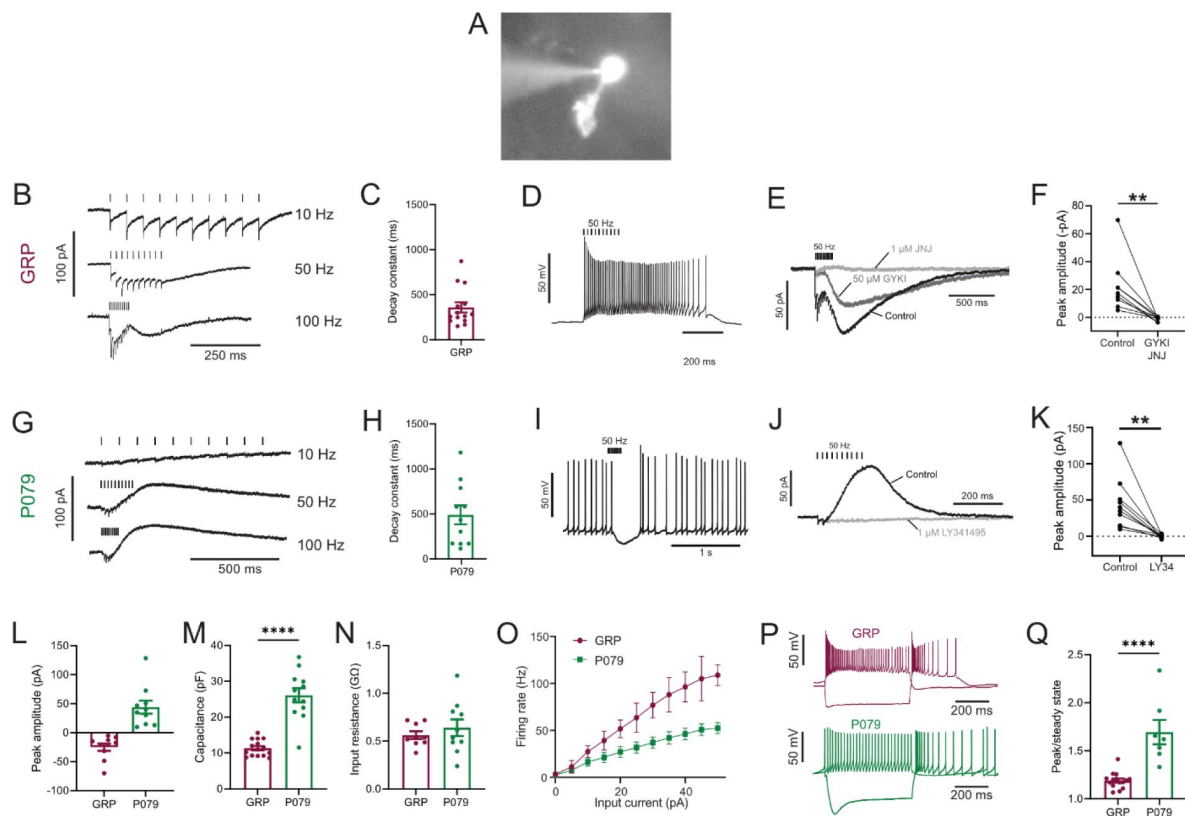


Figure 1

Physiological characterization of GRP and P079 UBCs in acute brain slices

- A) Representative image of UBC in whole cell patch clamp recording filled with Alexa Fluor 488.
- B) In GRP UBCs, mossy fiber stimulation evoked fast excitatory post synaptic currents and a slow inward current that became more prominent with faster stimulation rates.
- C) Summary of the decay constants of the slow EPSCs generated by a train of 10 stimuli at a 50 Hz rate in GRP UBCs.
- D) In current clamp, 50 Hz stimulation produced a burst of spikes that outlasted the stimulus.
- E) The fast EPSCs and part of the slow EPSC was blocked by an AMPA receptor antagonist (50 μ M GYKI53655). The remaining slow EPSC was blocked by an mGluR1 antagonist (1 μ M JNJ16259685).
- F) Inward currents in GRP UBCs were consistently blocked by AMPA receptor and mGluR1 receptor antagonists.
- G) In P079 UBCs, mossy fiber stimulation evoked slow outward currents.
- H) Summary of the decay constants of the slow outward currents generated by a train of 10 stimuli at a 50 Hz rate in P079 UBCs.
- I) In current clamp, 50 Hz stimulation generated a pause in spontaneous action potential firing in P079 UBCs.
- J) The outward current was blocked by an mGluR2 antagonist (1 μ M LY341495).
- K) Outward currents in P079 UBCs were entirely blocked by 1 μ M LY341495.
- L) Peak amplitudes of the slow currents were inward in all recorded GRP UBCs and outward in all recorded P079 UBCs.
- M) The capacitance was significantly higher in P079 UBCs compared to GRP UBCs.
- N) The input resistance was not different between GRP UBCs and P079 UBCs.
- O) Frequency-intensity curves show that GRP UBCs are more excitable and are able to fire at faster rates than P079 UBCs.
- P) Example traces showing the response of GRP and P079 UBCs to 40-pA depolarizing and -100-pA hyperpolarizing 500 ms current steps. The GRP UBCs fire at a higher rate than P079 UBCs during a 40-pA depolarizing current step. The P079 UBCs have a more prominent voltage sag in response to the -100-pA hyperpolarizing current step than the GRP UBCs.
- Q) The sag ratio was larger in P079 UBCs than GRP UBCs.
- Stimulation artifacts have been removed for clarity. Error bars are SEM.

± 46.86 ms after the end of the 200 ms long 50 Hz stimulus train and their decay was well approximated by an exponential with a time constant of 489.2 ± 350.4 ms (mean $\pm$ SD; $n = 11$) (**Fig 1H**). These outward currents hyperpolarized the cells and produced a pause in spontaneous spiking activity that varied from 0.5 to 3.9 seconds, averaging 1.72 ± 1.19 seconds (mean $\pm$ SD; $n = 10$) (**Fig 1I**). The outward current was mediated by mGluR2 receptors, as it was blocked by the mGluR2 antagonist LY341495 ($96.53 \pm 1.79\%$; paired t-test; $P = 0.0033$; mean $\pm$ SEM) (**Fig 1J-K**). These electrophysiological characteristics confirm that the P079 UBCs that can be visualized in acute brains slices are OFF UBCs.

The slow postsynaptic currents were inward in every GRP UBCs and outward in every P079 UBC (**Fig 1L**). The capacitance of GRP UBCs was less than half that of P079 UBCs, which suggests that the membrane area of the OFF UBCs is greater (GRP UBCs: 11.34 pF ± 0.59 , $n = 15$; P079 UBCs: 26.08 pF ± 1.96 , $n = 12$; t-test; $P < 0.0001$; mean $\pm$ SEM; **Fig 1M**). We did not observe a significant difference in input resistance between the two cell types (GRP UBCs: 0.56 G Ω ± 0.04 , $n = 9$; P079 UBCs: 0.64 G Ω ± 0.09 , $n = 10$; t-test; $P = 0.44$; mean $\pm$ SEM; **Fig 1N**). Current steps were injected into both types of UBCs to characterize their excitability. There was a significant interaction between the UBC type and response to current steps (mixed model ANOVA, $P < 0.0001$, $n = 17$). GRP UBCs had a higher firing rate in response to the same level of injected current compared to P079 UBCs, despite their similar input resistance, suggesting that GRP ON UBCs are more excitable and can fire at faster rates (**Fig 1O-P**). Hyperpolarizing current steps produce a sag that is mediated by the h-current (I_h) in UBCs (Kim et al., 2012). P079 UBCs had a significantly more prominent sag than GRP UBCs, measured in response to a -100 -pA current step (peak/steady state; P079: 1.69 ± 0.13 , $n = 7$; GRP: 1.19 ± 0.02 , $n = 14$; t-test; $P < 0.0001$; mean $\pm$ SEM) (**Fig 1P-Q**). This is consistent with previous work showing that OFF UBCs have a larger h-current than ON UBCs (Kim et al., 2012). In summary, electrophysiological recordings from GRP and P079 UBCs confirm that they are ON and OFF UBC subtypes, respectively.

GRP and P079 UBCs are distinct subpopulations that differ in size and distribution

Besides the functional differences in the GFP and mCitrine-expressing UBCs outlined above, these neurons also showed significant differences in size and distribution. Crossing the GRP-Cre/Ai9 mice with the P079 mice resulted in triple transgenic mice that expressed tdTomato and mCitrine in almost entirely separate subpopulations of UBCs—only 3 out of 944 UBCs that were labeled expressed both fluorescent proteins. GRP and P079 UBCs were present in the lobes of the cerebellar vermis in different densities (**Fig 2A**). More GRP UBCs than P079 UBCs were present in lobes VI-IX, with few of either type in lobes II-V. The highest density of both UBC types was in lobe X, which is where we focused the following analyses (**Fig 2B**). UBCs have convoluted dendritic brushes that are similar in appearance to their axon terminals (**Fig 2C**).

GRP UBCs have been reported to have smaller somas than UBCs that express calretinin (calretinin(+)), a calcium-binding protein that labels the OFF UBC population (Kim et al., 2012; Borges-Merjane and Trussell, 2015). We found that the cross-sectional area of GRP UBCs was significantly smaller than that of P079 UBCs (GRP: 68.75 ± 9.69 μm^2 , mean $\pm$ SD, $n = 92$; P079: 74.47 ± 12.53 μm^2 , mean $\pm$ SD, $n = 114$, t-test, $P = 0.0004$), which is consistent with our electrophysiological analysis of their membrane capacitance (**Fig 2D**). The distribution of GRP and P079 UBCs also supports their identity as ON and OFF UBCs, as the density of mGluR1(+) ON UBCs is higher in the dorsal leaflet of lobe X and the density of calretinin(+) OFF UBCs is higher in the ventral leaflet (Nunzi et al., 2002; Sekerkova et al., 2014).

GRP UBCs are a subset of mGluR1(+) UBCs

Somatodendritic expression of mGluR1 and calretinin defines two separate populations of UBCs (termed Type II and type I respectively (Nunzi et al., 2002)) and correspond to the functionally-defined ON and OFF subtypes (Borges-Merjane and Trussell, 2015). To investigate the expression

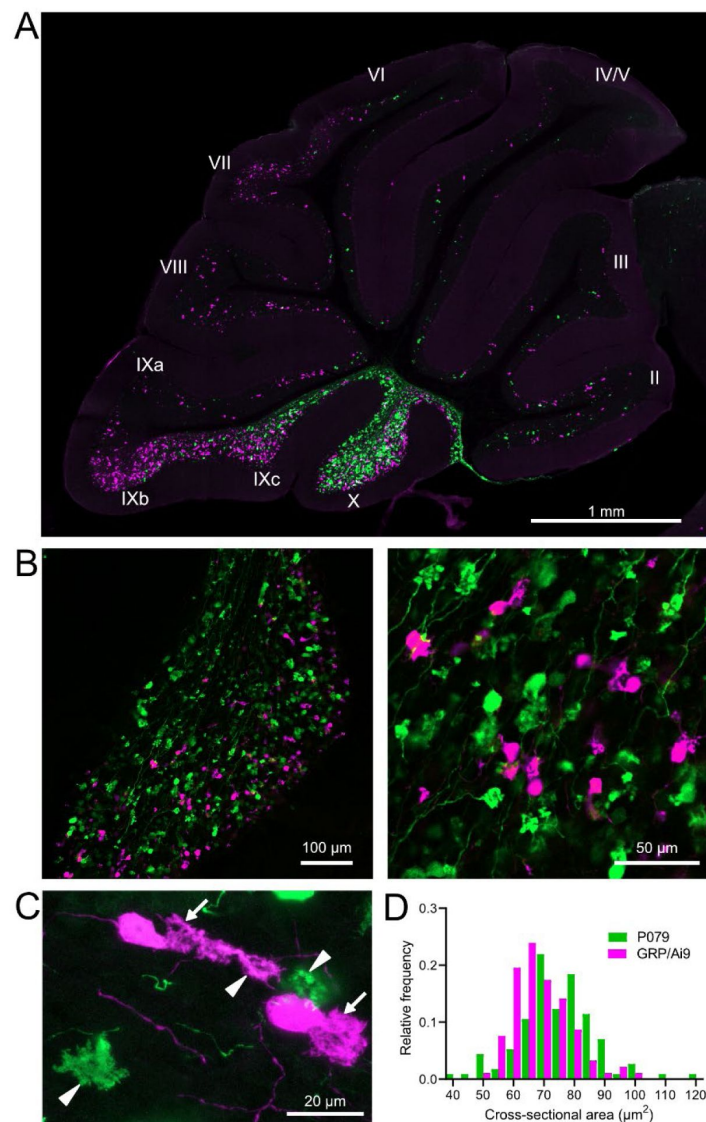


Figure 2

GRP/Ai9/P079 mouse line labels two distinct subsets of UBCs

A) Sagittal section of cerebellar vermis. Fluorescence is restricted to UBC cell types. P079 UBCs (mCitrine-green) are present in high density in lobe X and lobe IXc, while GRP UBCs (tdTomato- magenta) are present in those lobes as well as lobe IXb and in a lower density in Lobe VI-VIII. Maximum intensity projection.

B) Left- Sagittal section showing expression of mCitrine labeling P079 UBCs and tdTomato labeling GRP UBCs in lobe X. More GRP UBCs are present in the dorsal leaflet than the ventral leaflet. Right- UBCs and their axons and terminals are well labeled. Only rarely were UBCs labeled with both colors. Maximum intensity projection.

C) Magnified view of UBCs with their dendritic brushes indicated with arrows. Two P079 terminals and one GRP terminal are indicated with arrowheads. The GRP UBC at the top of the image demonstrates the challenge of differentiating the dendritic brush of a UBC from a terminal that is labeled with the same fluorophore. Maximum intensity projection.

D) In lobe X, P079 UBCs had larger somas than GRP UBCs, although there is a somewhat bimodal distribution in the P079 population.

of mGluR1 and calretinin in GRP and P079 UBCs, we immunohistochemically labeled, imaged, and counted UBCs in both ventral and dorsal areas of lobe X in 4-8 brain slices from triple transgenic mice ($n = 4$ mice). We found that 98.1% (155/158) of the GRP/Ai9 UBCs expressed mGluR1 in their dendritic brushes (**Fig 3A-B**). The GRP/Ai9/mGluR1(+) population represented 20.9% (155/741) of the total population of mGluR1(+) UBCs. 0/149 GRP/Ai9 UBCs expressed calretinin in a separate series of slices (**Fig 3C-D**). Thus, the GRP line labels about 1/5th of the ON UBCs and does not label OFF UBCs.

P079 mouse line drives mCitrine expression in the majority of calretinin(+) UBCs

Using the same triple transgenic mice and immunohistochemical approach described above, 92.2% (284/308) of the P079 UBCs were calretinin(+) (**Fig 3C-D**). The P079 UBCs represent 70.5% (284/403) of the total calretinin(+) population. We were surprised to find, however, that 61.3% (200/326) of the P079 UBCs appeared mGluR1(+). This represents 27.0% (200/741) of the total mGluR1(+) population. These P079/mGluR1(+) UBCs could be differentiated from P079/mGluR1(-) UBCs by their lower expression of mCitrine: the relative labeling intensity of the mGluR1(+) P079 UBCs, after amplification with an anti-GFP antibody, was less than half of the more common mGluR1(-) P079 UBCs (43.2% soma pixel intensity relative to mGluR1(-) P079 UBCs, $n = 34$ z-planes with at least one of each type, t-test, $P = 8.403e-6$). In addition, the cross-sectional area of the mGluR1(+) P079 somas was $60.62 \pm 13.67 \mu\text{m}^2$, mean $\pm$ SD, $n = 52$ and significantly smaller than the mGluR1(-) P079 somas ($72.48 \pm 16.90 \mu\text{m}^2$ mean $\pm$ SD, $n = 40$, t-test, $P = 0.00035$). It is likely that the P079 UBCs that express a low density of mCitrine were not able to be visualized in our electrophysiological experiments because their native fluorescence was below the level of detection. Unfortunately, the observation that some mGluR1(+) UBCs are labeled in the P079 mouse line precludes its use for expressing genes in OFF UBCs specifically.

GRP and P079 UBCs are distinct populations in dorsal cochlear nucleus

UBCs are also present in the dorsal cochlear nucleus, a cerebellum-like circuit in the auditory brainstem of mammals. Like granule cells in the dorsal cochlear nucleus, UBCs receive multisensory signals from various sources (Ryugo et al., 2003; Balmer and Trussell, 2021, 2022). In order to test whether our findings about the identity of UBCs labeled in GRP-cre and P079 mice is generalizable across UBC populations, we examined their distribution in the dorsal cochlear nucleus. We found that 97.8% (132/135) of the GRP/Ai9 UBCs expressed mGluR1 in their dendritic brushes (**Fig 4A-B**). The GRP/Ai9/mGluR1(+) population represented 34.2% (132/386) of the population of mGluR1(+) UBCs, and is therefore a subpopulation of about 1/3 of the mGluR1(+) ON UBCs. In a separate series of slices, 0/115 GRP/Ai9 UBCs expressed calretinin and is therefore useful for expressing genes in a subset of ON UBCs without expression in OFF UBCs in the dorsal cochlear nucleus.

We also found that 89.7% (140/156) of the P079 UBCs were calretinin(+) (**Fig 4C-D**). The P079 UBCs represent 85.9% (140/163) of the total calretinin(+) population. Similar to our results in the cerebellum, we found that 56.7% (127/224) of the P079 UBCs were mGluR1(+). This represents 32.9% (127/386) of the mGluR1(+) population. As in the cerebellum, these P079/mGluR1(+) UBCs were identifiable by their significantly lower intensity of mCitrine after antibody amplification (43.5% intensity compared to mGluR1(-) P079 UBCs, $n = 19$ z-planes with at least one of each type, t-test, $P = 1.352e-6$). In summary, in the dorsal cochlear nucleus, the P079 mouse line expresses mCitrine in 86% of the calretinin(+) UBCs and 33% of the mGluR1(+) UBCs and the GRP line expresses Cre in 34% of the mGluR1(+) UBC population and 0% of the calretinin(+) population. Thus, the expression pattern in the dorsal cochlear nucleus of UBCs in these two mouse lines mirrors that of the cerebellum.

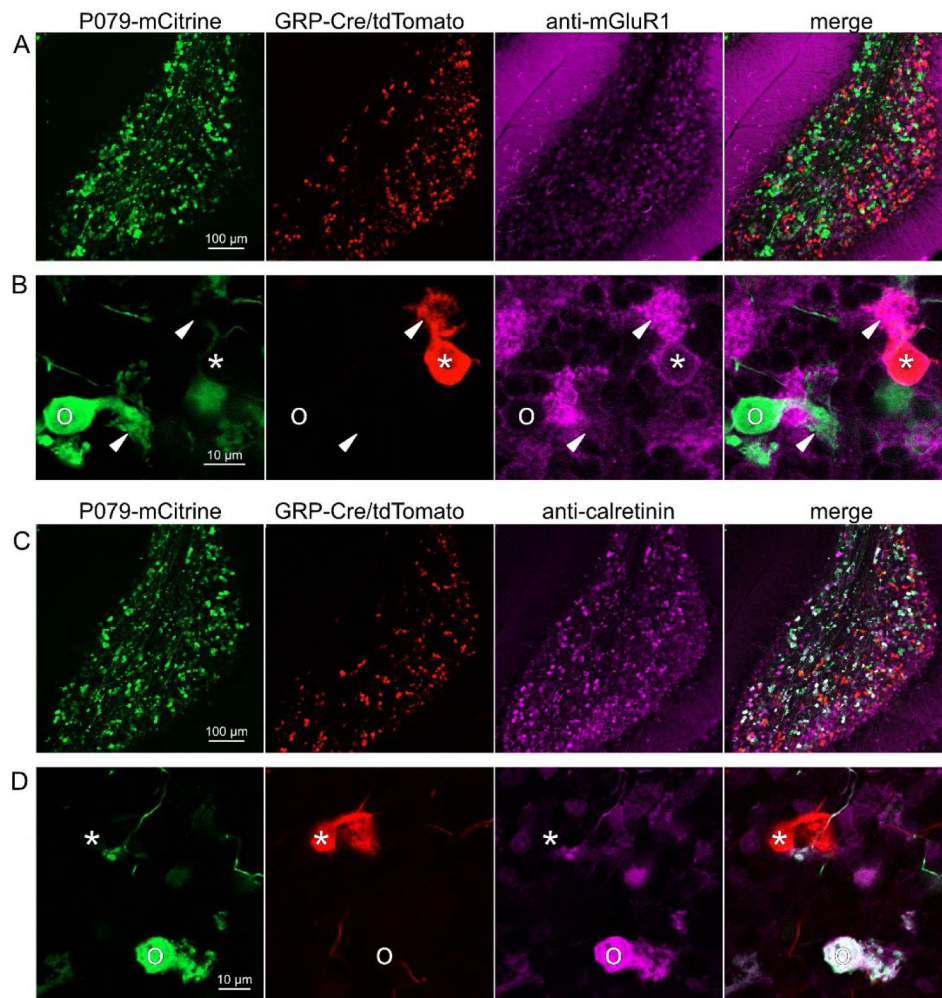


Figure 3

Expression of mGluR1 and calretinin in GRP and P079 UBCs in the cerebellum

A) Sagittal section of lobe X showing genetically expressed mCitrine (green) from the P079 mouse, tdTomato (red) from the GRP-Cre/Ai9 mouse, and immunohistochemical labeling of mGluR1 (magenta). Maximum intensity projections.

B) Example of a P079 UBC (soma indicated with O, brush indicated with arrowhead), GRP UBC (soma indicated with *, brush indicated with arrowhead). The GRP UBCs express mGluR1 in the somatic membrane and dendritic brush. Most P079 UBCs do not express mGluR1. Single image planes.

C) Sagittal section of lobe X showing genetically expressed mCitrine (green) from the P079 mouse, tdTomato (red) from the GRP-Cre/Ai9 mouse, and immunohistochemical labeling of calretinin (magenta). Maximum intensity projections.

D) Example of a P079 UBC (soma indicated with O), GRP UBC (soma indicated with *). The P079 UBCs express calretinin in their cytoplasm. GRP UBCs do not express calretinin.

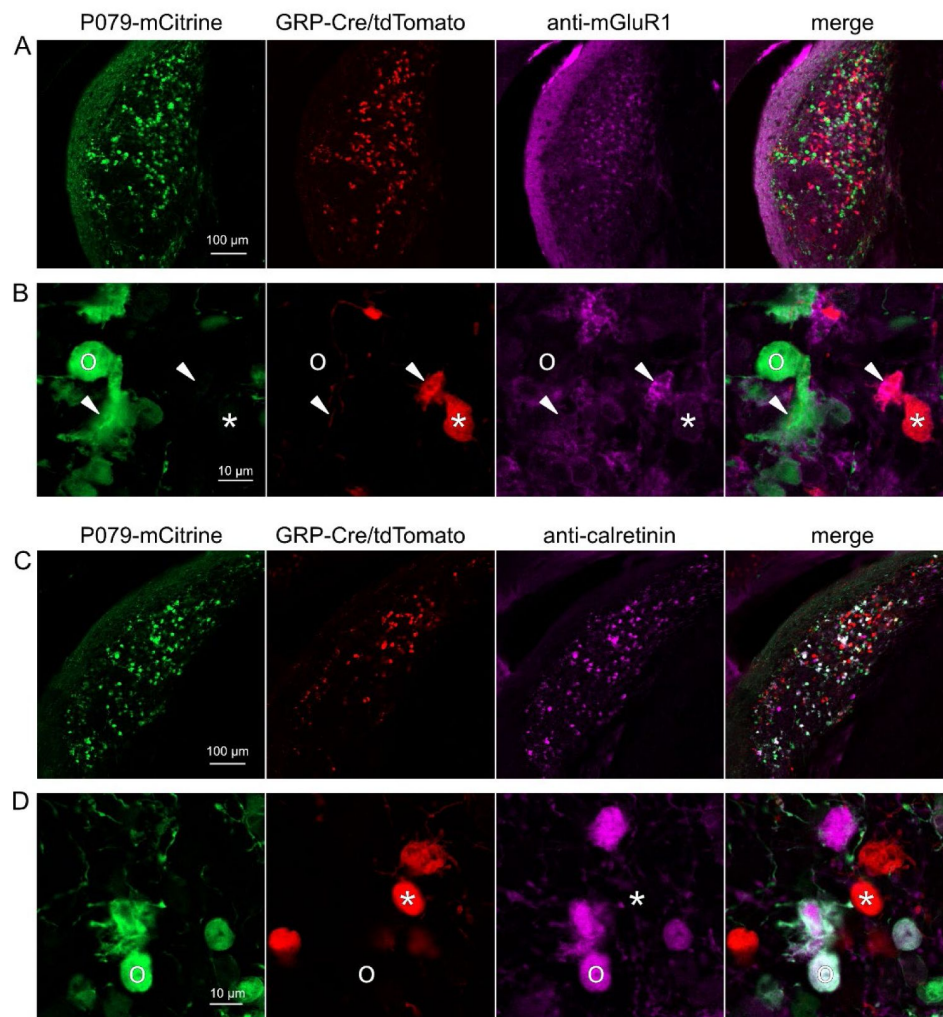


Figure 4

Expression of mGluR1 and calretinin in P079 and GRP UBCs in the dorsal cochlear nucleus

A) Coronal section of the dorsal cochlear nucleus showing genetically expressed mCitrine (green) from the P079 allele, tdTomato (red) from the GRP-Cre/Ai9 alleles, and immunohistochemical labeling of mGluR1 (magenta). Maximum intensity projections.

B) Example of a P079 UBC (soma indicated with O, brush indicated with arrowhead), GRP UBC (soma indicated with *, brush indicated with arrowhead). The GRP UBCs expresses mGluR1 in the somatic membrane and dendritic brush. Most P079 UBCs do not express mGluR1, although some that are weakly labeled do appear to express mGluR1.

C) Coronal section of the dorsal cochlear nucleus showing genetically expressed mCitrine (green) from the P079 allele, tdTomato (red) from the GRP-Cre/Ai9 alleles, and immunohistochemical labeling of calretinin (magenta). Maximum intensity projections.

D) Example of a P079 UBC (soma indicated with O), GRP UBC (soma indicated with *). The P079 UBCs express calretinin in their cytoplasm. GRP UBCs do not express calretinin.

Axonal projections between ON UBCs and OFF UBCs

Do ON and OFF UBC subtypes target one another? In the GRP/Ai9/P079 mouse line, all GRP UBCs are ON UBCs and the P079 UBCs are mostly OFF UBCs, although their OFF UBC identity must be confirmed by their expression of calretinin. Thus, we searched for red GRP UBC axon terminals that contacted green P079 UBC brushes that also expressed calretinin. These contacts were presumed to be synapses based on our previous anatomical analyses of functional synapses between mossy fibers and UBCs (Balmer and Trussell, 2019 [↗](#)). Several examples of such synaptic connections were identified, indicating that GRP ON UBCs provide synaptic input to P079 OFF UBCs (**Fig 5A-B** [↗](#)). Using the same approach, we found OFF UBC axon terminals (P079 and calretinin(+)) contacting ON UBC brushes (GRP) (**Fig 5C-D** [↗](#)). These projections were rare and we did not attempt to characterize the proportion of ON to OFF UBC connections, as both populations are subsets of ON and OFF UBCs and would be a significant underestimate. However, these results do reveal that UBC subtypes synapse on one another and may introduce complex spiking patterns.

Axonal projections from ON UBCs to other ON UBCs

To test whether ON UBCs synapse on other ON UBCs, we searched for labeled axon terminals in the GRP/Ai9 mouse that contacted brushes of ON UBCs labeled with mGluR1. Two examples are shown in **Fig 6** [↗](#). Care was taken to confirm that mGluR1 labeling that appeared to decorate the brush of a UBC could be traced to a soma with the characteristic expression in the somatic membrane that forms a circle. These examples show that GRP ON UBCs target mGluR1(+) UBCs that are not transgenically labeled in the GRP mouse line.

In a second approach to investigate synaptic connections between ON UBCs, we utilized a Brainbow2.1-Confetti reporter mouse line to label GRP ON UBCs with distinct colors (Livet et al., 2007 [↗](#); Snippert et al., 2010 [↗](#)). In the offspring resulting from the GRP-Cre/Brainbow2.1-Confetti cross, expression of the fluorescent proteins was low and it was therefore necessary to enhance with anti-GFP and anti-mCherry antibodies. In doing so, both membrane-bound mCerulean and cytoplasmic YFP were labeled with the anti-GFP antibody, and cytoplasmic tdimer2(12) was labeled with anti-mCherry. This resulted in various combinations of expression that could be identified by wavelength (green or red) and cellular localization (membrane-bound or cytoplasmic). In most labeled UBCs, multiple alleles were expressed and could be visualized in the same cells, perhaps owing to the sensitivity of the antibody amplification (**Fig 7A** [↗](#)). Several examples of GRP UBC axon terminals contacting the brushes of other GRP UBCs were identified (**Fig 7B-D** [↗](#)). Thus, GRP ON UBCs project to other GRP ON UBCs in a feed-forward excitatory circuit that is likely to prolong the duration of an excitatory signal.

Axonal projections from OFF UBCs to other OFF UBCs

To address whether OFF UBCs project axons to other OFF UBCs, we exploited the observation that the P079 line expresses mCitrine in some, but not all, calretinin-expressing OFF UBCs. Thus, when calretinin is labeled immunohistochemically using a non-green fluorophore, all OFF UBCs are revealed and their brushes can be examined for the presence of connections from mCitrine-labeled P079 OFF UBCs axon terminals. Several examples for OFF to OFF UBC synaptic connections were confirmed anatomically (**Fig 8** [↗](#)). In some cases, calretinin(+) axon terminals innervated the brushes of P079 UBCs that were also calretinin(+) (**Fig 8A-B** [↗](#)). In other cases, the P079 UBC terminal contacted calretinin(+) UBCs that were not labeled transgenically in the P079 line (**Fig 8C** [↗](#)).

Brainbow2.1-Confetti reporter mice were crossed with a line that expresses Cre in calretinin(+) neurons after induction by tamoxifen (Taniguchi et al., 2011 [↗](#)). Tamoxifen induced the expression of fluorescent proteins in a subset of these calretinin(+) OFF UBCs and were amplified with antibodies as above (**Fig 9A** [↗](#)). Several examples are shown, confirming that calretinin(+) OFF

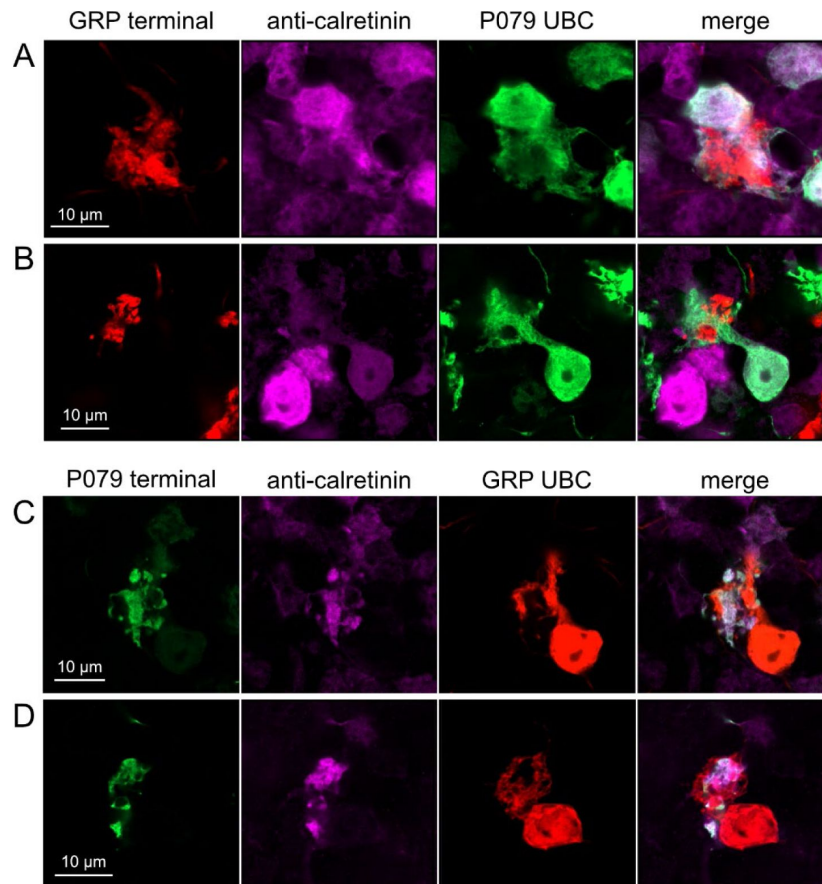


Figure 5

Anatomical evidence showing axonal projections between ON and OFF UBC subtype

A-B) ON UBC axon terminals (GRP- red) that project to OFF UBCs (P079- green), confirmed to express calretinin (magenta). Single image sections.

C-D) OFF UBC axon terminals (P079- green), confirmed to express calretinin (magenta) contacting the dendritic brushes of ON UBCs (GRP-red). Single image sections.

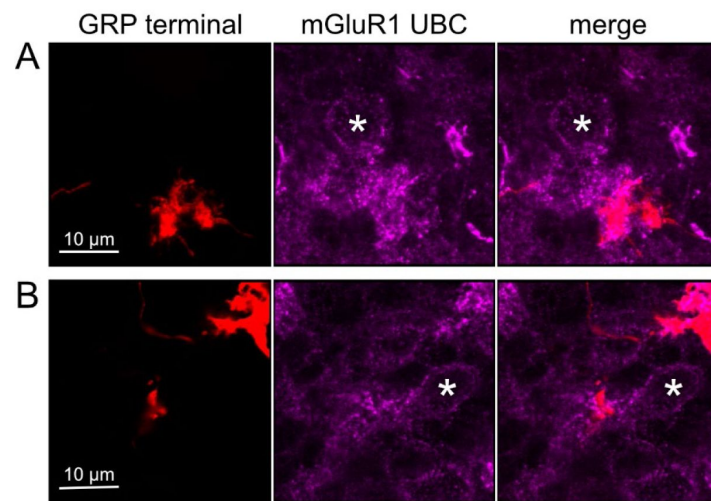


Figure 6

Anatomical evidence for synaptic connections between ON UBCs

A-B) GRP ON UBC terminals (red) contacting mGluR1(+) ON UBCs (magenta). Somas indicated with *. Single image sections.

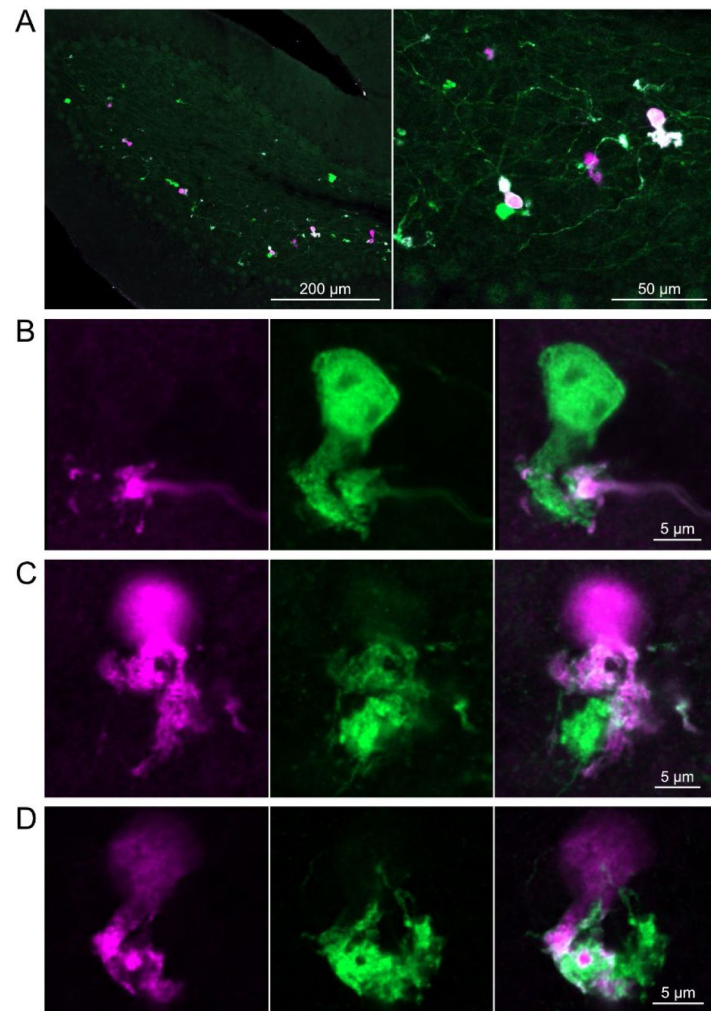


Figure 7

Brainbow reporter labels synaptic connections between GRP ON UBCs

A) GRP ON UBCs expressed fluorescent proteins that were either amplified with the anti-GFP antibody or anti- mCherry antibody. GRP UBCs and their axons and terminals were labeled. Maximum intensity projections.

B) Example of a GRP UBC expressing cytoplasmic YFP (green) that appears to be contacted by a presynaptic terminal from the axon of another GRP UBC that expresses both cytoplasmic YFP (green) and cytoplasmic tdimer2(12) (magenta). Single image sections.

C) Example of GRP ON UBC expressing cytoplasmic tdimer2(12) (magenta) and membrane-bound mCerulean (green) that appears to receive a synaptic terminal that expresses cytoplasmic YFP (green). Single image sections.

D) Example of GRP ON UBC that expresses cytoplasmic tdimer2(12) (magenta) receiving input from a GRP UBC axon that expresses cytoplasmic YFP (green). Single image sections.

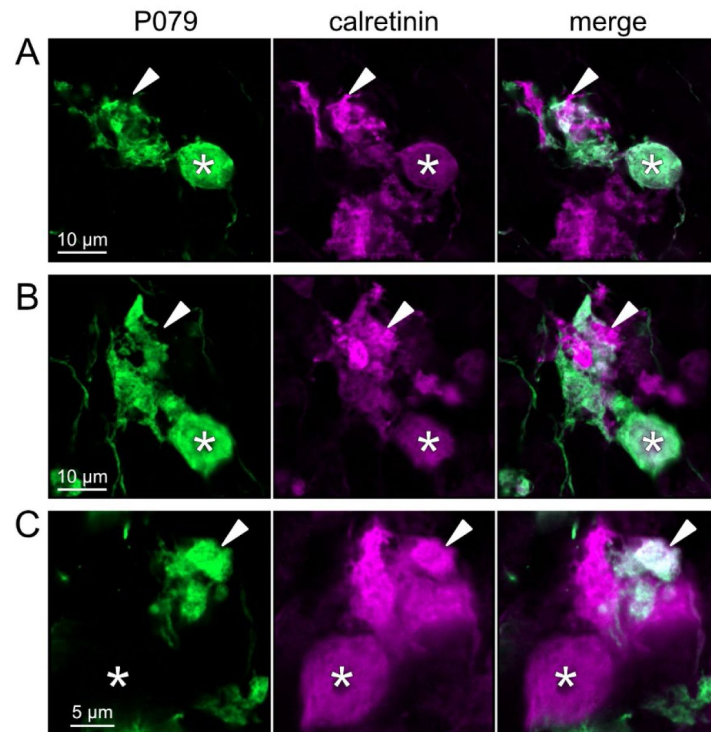


Figure 8

Anatomical evidence for synaptic connections between OFF UBCs

A-B) Examples of P079 OFF UBCs (green) that are confirmed to express calretinin (magenta) and are contacted by calretinin(+) terminals that are not labeled in the P079 line. Single image sections.

C) Example of an OFF UBC that expresses calretinin (magenta) that is contacted by a P079 (green) axon terminal that expresses calretinin (magenta).

Somas are indicated with *, terminals are indicated with arrowheads. Single image sections.

UBCs provide input to one another (**Fig 9B-D**). In one of these examples an axon terminal appeared to contact the soma of a postsynaptic UBC (**Fig 9C**), which has been previously reported to occur between extrinsic mossy fibers and UBC somata (Balmer and Trussell, 2019). In another case, an axon terminal appeared to project a narrow fiber to the edge of the dendritic brush of a postsynaptic UBC (**Fig 9D**). These presence of large or small contacts implies differences in synaptic strength, suggesting that OFF UBCs may provide strong synaptic signals to one another (**Fig 9B**) or presumably weaker signals (**Fig 9C-D**) that are subsequently integrated with other inputs.

Computational models predict transformations of spiking patterns through synaptically connected ON and OFF UBCs

To address how spiking activity may be transformed by different types of UBCs, computational models were developed to simulate the essential features of ON and OFF UBCs, including their passive electrical properties, synaptic current responses and spiking patterns. UBCs can fire spontaneously or be silent in acute brain slices (van Dorp and De Zeeuw, 2015; Kim et al., 2012). We chose to make the OFF UBCs spontaneously active in this model so that synaptic input would produce a pause in firing (OFF response) that could be easily measured. By contrast, we made ON UBCs not spontaneously active so that synaptic input would produce a burst of action potentials (ON response). Action potentials triggered glutamate concentration transients that drove the conductance of model AMPA receptors in ON UBCs and mGluR2 in OFF UBCs (see Methods). We contrasted simple cases of an excitatory input to either an ON or OFF UBC, with a more complex case in which the excitatory input drove an “intermediate” UBC which then synapsed on a second UBC. In this way, we could examine the predicted outcomes of ON to ON, ON to OFF, OFF to ON, and OFF to OFF scenarios.

Serial ON UBCs multiply burst duration

Presynaptic input to an ON UBC produced bursts of action potentials that multiplied in duration in a circuit with an intermediate ON UBC (**Fig 10A-C**). Three presynaptic spikes in a burst lasting 19.6 ms produced a burst of 12 action potentials in the first UBC, lasting 358.0 ms, and 35 action potentials in the second UBC, lasting 1175.3 ms, ~60 times longer than the initial presynaptic input (**Fig 10A-B**). The enhancement of the spiking duration both in the first and second ON UBC were more pronounced with shorter bursts of presynaptic input and began to plateau at longer durations of input (**Fig 10C**). The plateau in spiking duration occurs because the rebound AMPA receptor-mediated slow EPSC that occurs at the end of synaptic stimulation in ON UBCs reaches a maximum after ~100 ms of synaptic stimulation (Lu et al., 2017).

Intermediate ON UBCs extend the pause in postsynaptic OFF UBCs

A burst of presynaptic spikes causes a pause in spontaneous firing in OFF UBCs, due to the outward current produced by mGluR2 (**Fig 10D**). When an intermediate ON UBC was present between the presynaptic axon and the OFF UBC, the synaptic signal to the OFF UBC was extended, and therefore increased the mGluR2 current, markedly lengthening the duration of the pause in spiking (**Fig 10E-F**). Indeed, the presence of an intermediate ON UBC increased the duration of the pause in the postsynaptic OFF UBC by ~940 ms following 1-10 presynaptic action potentials (indicated by an upward shift in the curve in **Fig 10G**). The small difference in how much the pause was lengthened across the range of 1-10 presynaptic spikes is due to the intermediate ON UBC's response—even a single presynaptic spike produces a burst of action potentials that is only slightly extended by longer trains of presynaptic input. Across a population of ON UBCs, however, the size and duration of the slow inward currents that drive their firing vary and would thus extend the pause of a postsynaptic OFF UBC for different durations.

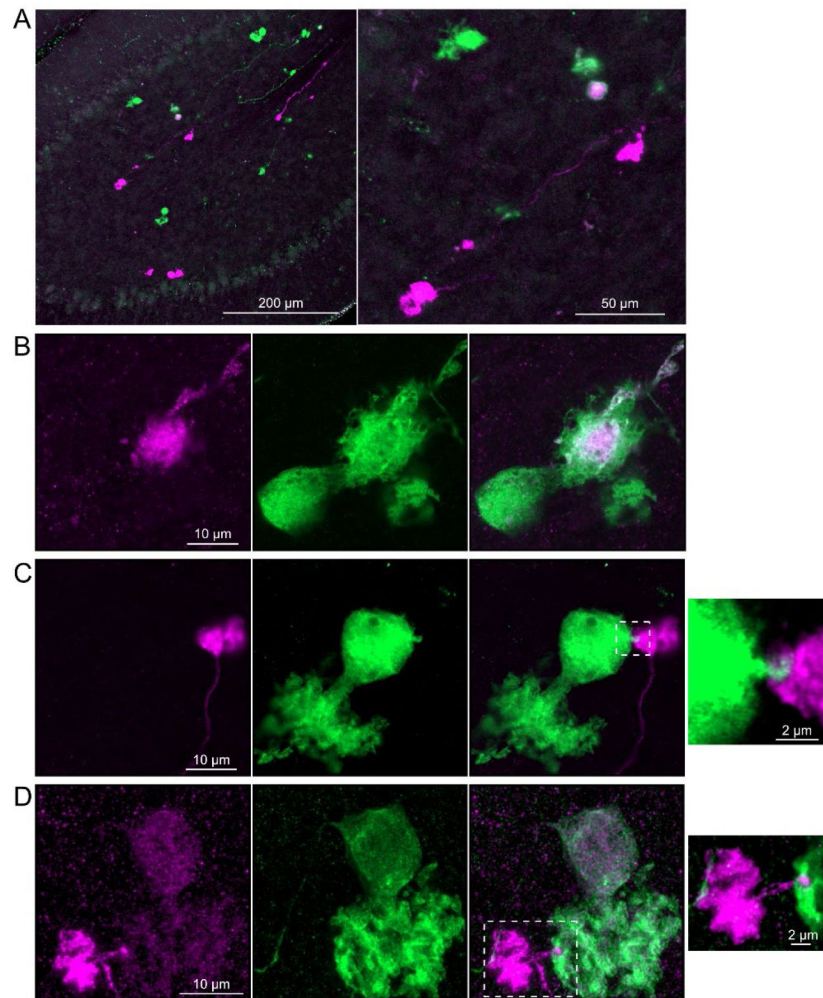


Figure 9

Brainbow reporter labels synaptic connections between OFF UBCs in calretinin-Cre mouse line

A) Calretinin-Cre UBCs expressed fluorescent proteins that were either amplified with the anti-GFP antibody or anti-mCherry antibody, revealing UBCs and their axons and terminals in lobe X. Maximum intensity projections.

B) Example of a labeled calretinin-Cre axon terminal (magenta) that contacted the brush of a calretinin-Cre UBC (green), showing that these OFF UBC project to one another.

C) Example of a calretinin-Cre UBC axon terminal (magenta) that appeared to contact a spine-like extension from the soma of another calretinin-Cre UBC (green).

D) Example of a calretinin-Cre axon terminal (magenta) that projected a small fiber that made a bouton-like synapse onto the brush of another calretinin-Cre UBC (green) that is presumed to have another unlabeled input to the majority of its dendritic brush.

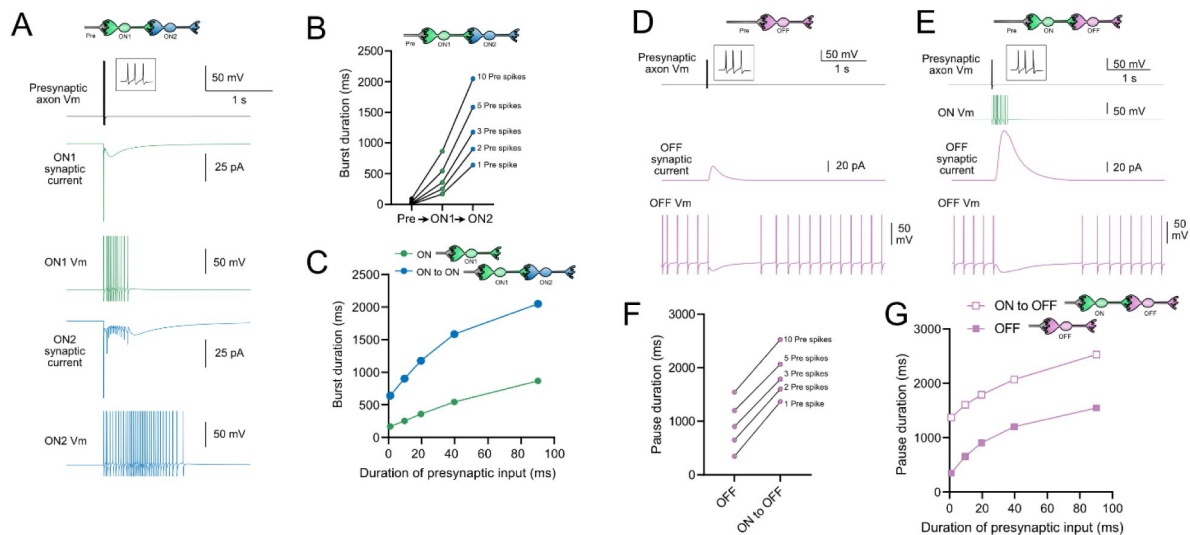


Figure 10

Model predicts that intermediate ON UBCs extend signal

- A) Model in which a presynaptic axon fired 3 action potentials (inset shows presynaptic spikes with expanded time scale) that generated an AMPA receptor-mediated synaptic current and a burst in spikes in the first ON UBC (ON1), which produced an extended AMPA receptor-mediated synaptic current in the postsynaptic UBC (ON2) and an extended burst of spikes.
- B) Presynaptic spikes were amplified to longer bursts in each subsequent ON UBC.
- C) Burst duration in the first ON UBC (green) and second ON UBC (blue) as a function of presynaptic input duration.
- D) Model in which a presynaptic axon fired 3 action potentials that generated an mGluR2-mediated synaptic current in an OFF UBC, which produced a pause in spontaneous firing lasting ~1 s.
- E) An intermediate ON UBC between the presynaptic axon and OFF UBC generated a larger and longer lasting mGluR2-mediated current that produced an extended pause in spontaneous firing.
- F) Pause duration in an OFF UBC was extended by an intermediate ON UBC.
- G) Pause duration in an OFF UBC with and without an intermediate ON UBC as a function of presynaptic input duration.

Intermediate OFF UBC produce a delayed pause in postsynaptic ON UBC

Because OFF UBCs fired spontaneously in our model, an ON UBC postsynaptic to an intermediate OFF UBC fired with an irregular spiking pattern (**Fig 11A**). Presynaptic input to the intermediate OFF UBC paused both its firing and its release of glutamate onto the postsynaptic ON UBC, which therefore produced a pause in the postsynaptic ON UBC. However, this pause only occurred after a delay of about 370 ms, due to the slow AMPA receptor mediated EPSC in the ON UBC that decayed slowly until it no longer drove spiking. The length of the delay before the pause began is related to the strength of the AMPA receptor-mediated current in the postsynaptic ON UBC. The length of the pause in the ON UBC depends on how long the intermediate OFF UBCs pauses. Thus, an intermediate OFF UBC converts the usual excitatory ON UBC response to a pause that is similar to that of the OFF UBC itself. However, the pause is distinct from that of OFF UBCs, because it is delayed and briefer (**Fig 11B**).

Serial OFF UBCs produce a delayed burst in postsynaptic OFF UBC

Finally, we considered the case of a connection between two OFF UBCs. In a circuit with a spontaneously firing OFF UBC synapsing onto another OFF UBC (labeled OFF1 and OFF2 respectively, in **Fig 11C-D**), the tonic glutamatergic input and mGluR2 inhibition prevented spontaneous spiking in the postsynaptic cell. When the intermediate OFF UBC received a burst of presynaptic input, it was inhibited by its own mGluR2 current and therefore stopped firing, thereby disinhibiting the postsynaptic OFF UBC, which then fired after a delay (**Fig 11C**). Note that this firing would be more pronounced had we incorporated calcium and TRP conductances that contribute to a ‘late onset response’ in UBCs (Locatelli et al., 2013; Subramaniam et al., 2014). The duration of the delayed firing in the second postsynaptic OFF UBC depended on the duration of presynaptic input signal to the intermediate UBC (**Fig 11D**). Moreover, the delay between the last spike in the intermediate OFF UBC and the first spike in the postsynaptic OFF UBC was contingent on the slow decay of the mGluR2 current and was 1082.7 ms in the example in **Fig 11C**.

In summary, our modeling suggests that feed-forward circuits of ON and OFF UBCs transform presynaptic signals by producing an extended spike burst (ON to ON), extended pause (ON to OFF), pause after a delay (OFF to ON), or burst after a delay (OFF to OFF) (**Fig 12**). This diversification of spiking patterns and durations may be important for maintaining sensory representations of movement and mechanisms of adaptive learning.

Discussion

GRP and P079 mouse lines label distinct UBC subtypes that form parallel and convergent pathways

Transgenic mice were generated that expressed a green fluorescent protein in OFF UBCs and a red fluorescent protein in ON UBCs in the same tissue. Virtually no neurons expressed both fluorescent proteins. Immunohistochemical analysis showed that all GRP UBCs expressed mGluR1 and most P079 UBCs expressed calretinin in both cerebellum and dorsal cochlear nucleus. GRP and P079 UBCs had different average soma sizes and distributions in the cerebellum. Whole-cell recordings in acute brain slices confirmed that the GRP UBCs produce excitatory ON responses mediated by mGluR1 and AMPA receptors, and that P079 UBCs produce inhibitory OFF responses mediated by mGluR2. Their membrane capacitance and excitability were so distinct that the distributions of these properties were almost non-overlapping. These results provide further evidence that some UBC subtypes are genetically and functionally distinct, despite the observation

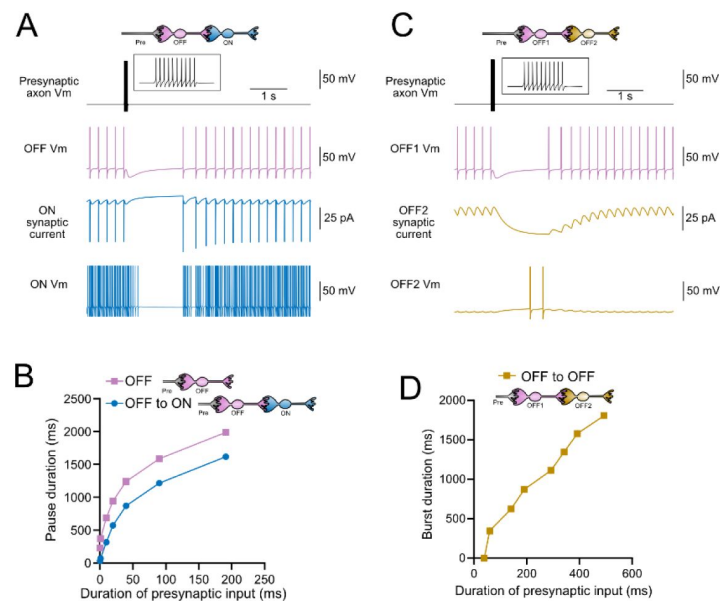


Figure 11

Model predicts that intermediate OFF UBCs delays signals

A) Model showing an intermediate OFF UBC (magenta) that produced a delayed pause in a postsynaptic ON UBC (blue). The spontaneous activity of the intermediate OFF UBC drove irregular spiking in the postsynaptic ON UBC. Presynaptic input (10 spikes shown in inset with expanded time scale) produced a pause in the intermediate OFF UBC, which stopped its release of glutamate onto the ON UBC, and produced a pause after a delay that is due to the slow decay of the AMPA receptor-mediated current.

B) The intermediate OFF UBC (magenta) paused for longer than the postsynaptic ON UBC (blue), because the pause in the ON UBC occurred after a delay and it ended as soon as the intermediate OFF UBC resumed firing.

C) Model showing an intermediate OFF UBC that produced delayed spikes in a postsynaptic OFF UBC. Spontaneous firing of the intermediate OFF UBC (OFF1- magenta) tonically inhibited the postsynaptic OFF UBC (OFF2- gold). Presynaptic input caused a pause in the intermediate OFF UBC, which disinhibited the postsynaptic OFF UBC, allowing it to fire with a delay that depended on the decay of the mGluR2 current.

D) The duration of the burst of action potentials in the postsynaptic UBC increased with longer durations of presynaptic input.

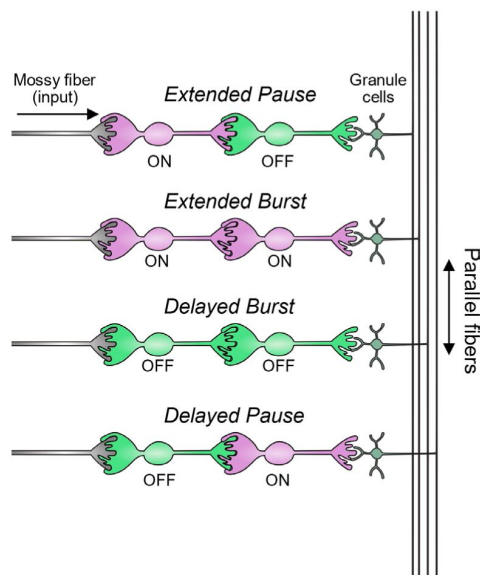


Figure 12

Summary of transformations of spiking patterns in UBC circuits

Four patterns of connectivity between UBC subtypes were anatomically defined and computationally modeled. Intermediate ON UBCs extended the usual pause or burst in firing of the postsynaptic OFF or ON UBC. Intermediate OFF UBCs delayed and inverted the response of the postsynaptic UBC, producing either a delayed burst in OFF UBCs or a delayed pause in ON UBCs.

that when the entire population of UBCs is examined, their electrophysiological responses form a continuum from entirely excited by glutamate to entirely inhibited by glutamate (Guo et al., 2021 [↗](#); Kozareva et al., 2021 [↗](#)).

The UBCs in these mouse lines expressed the fluorescent proteins throughout their axons and presynaptic terminals, which revealed for the first time that axon terminals from one UBC subtype contact the dendritic brushes of the other UBC subtype. We also showed that UBCs of the same subtype provide input to one another, by labeling GRP or calretinin-Cre UBCs with different fluorescent proteins using the Brainbow2.1-Confetti reporter line. This result was confirmed with additional approaches that utilized the incomplete labeling of mGluR1(+) ON UBCs in the GRP line and the incomplete labeling of calretinin(+) OFF UBCs in the P079 line. Earlier work reported that mGluR1(+) UBCs and calretinin(+) UBCs form parallel pathways (Nunzi et al., 2002 [↗](#)). Our current data extends the previous result by showing that the even more specific subtypes of GRP or P079 UBCs provide input to members of their own subtype. We did not estimate the density of these UBC to UBC connections, because the sparseness of labeling using these approaches made an accurate calculation impossible. Previous work using organotypic slice cultures from P8 mice estimated that 2/3 of the UBC population receives input from other UBCs (Nunzi & Mugnaini, 2000 [↗](#)), although it is unclear whether this is the case in older mice. These networks of UBCs could provide the granule layer with a circuit mechanism to diversify and extend the pattern of spiking produced by mossy fiber input.

The purpose of this study was to reveal the postsynaptic targets of UBCs with a focus on ON and OFF UBC subtypes. However, it is important to emphasize that the final output and main target of UBCs is granule cells. Most of the labeled UBC axon terminals did not synapse on UBCs and are therefore presumed to synapse on numerous granule cells. Even the axon terminals that targeted UBCs likely also contacted granule cells. Thus, UBCs are interneurons that, when positioned between mossy fibers and granule cells, modify the input to granule cells and have the capacity to synchronize the firing of large ensembles of granule cells via their branching axons and large terminals.

The approaches used here were not able to determine the existence of networks of more than 2 UBCs connected one after the other. If present, 3 or more UBCs in series could extend and transform the input in even more dramatic ways. The temporal diversity that UBC circuits generate may underlie the flexibility of the cerebellum to coordinate movements over a broad range of behaviors.

UBC circuits generate a broad range of firing patterns and may be essential for cerebellar learning

Vestibular afferents that encode head movements synapse on both UBCs and granule cells. Granule cells that receive this input are likely to fire in phase with the input signal. A single intermediate UBC would shift the phase of firing of its postsynaptic granule cells, depending on the UBC's response to glutamatergic input, which varies from nearly complete inhibition (e.g. P079 OFF UBC) to complete excitation (e.g. GRP ON UBC) (Guo et al., 2021 [↗](#); Kozareva et al., 2021 [↗](#)). For example, an intermediate ON UBC between a presynaptic input and another UBC extended the signal markedly in our models. In circuits with an intermediate OFF UBC, the postsynaptic UBCs signal was inverted: ON UBCs paused when they were no longer driven by glutamate, and OFF UBCs fired when they were no longer inhibited by glutamate. The signal that granule cells subsequently receive would be a burst or pause in firing that lasts for hundreds or thousands of milliseconds. We suggest that these transformations could provide a circuit mechanism for maintaining a sensory representation of movement for seconds, which is thought to be necessary to modulate low-frequency vestibular responses such as compensatory eye movements.

The delays introduced by an intermediate UBC were due to the slow decay of the excitatory or inhibitory synaptic currents produced by the presynaptic input. Other cellular and synaptic properties that would shape the timing of pauses, delays, and bursts, include the amount of glutamate released and the rate of its removal, the geometry of the extracellular space, glutamate receptor number and localization, short-term synaptic plasticity, currents underlying rebound spiking, and intrinsic excitability (Kim et al., 2012 [↗](#); Kinney et al., 2013 [↗](#); Locatelli et al., 2013 [↗](#); Zampini et al., 2016 [↗](#); Lu et al., 2017 [↗](#); Balmer et al., 2021 [↗](#)). The combination of numerous mechanisms that shape the signal as it is transmitted along the granule layer circuit could produce tunable delay lines essential for sensory representation on an enormous range of time scales.

Extending the duration of a sensory feedback signal may also be essential for motor learning when there is a significant delay between the motor output and the sensory feedback, for example, when throwing an object at distant target, a challenge referred to as the credit assignment problem (Sutton and Barto, 1981 [↗](#); Raymond and Lisberger, 1998 [↗](#); Suvrathan, 2019 [↗](#)). There are a variety of ways that the cerebellum could extend a signal in time, such as slow axonal conduction, long membrane time constants, inhibition followed by rebound spiking, and slow intracellular processes (Braitenberg, 1967 [↗](#); Rossi et al., 1995 [↗](#); Fiala et al., 1996 [↗](#); Hooper et al., 2002 [↗](#); D'Angelo and De Zeeuw, 2009 [↗](#); van Dorp and De Zeeuw, 2014 [↗](#)). UBCs circuits represent another mechanism that could prolong granule cell firing and contribute to the maintenance of sensory signals that underly motor learning in behaviors with delayed sensory feedback.

The diversification of spiking patterns by intermediate UBCs may contribute to the pattern separation that is necessary for theories of cerebellar learning (Marr, 1969 [↗](#); Albus, 1971 [↗](#); Ito, 1982 [↗](#); Kennedy et al., 2014 [↗](#); Zampini et al., 2016 [↗](#)). In one of the best understood cerebellum-like circuits, the electrosensory lobe of electric fish, UBCs are essential to produce temporally diverse and delayed spiking patterns that are necessary for accurate cancellation of self-generated signals (Kennedy et al., 2014 [↗](#)). The authors report that a post-inhibitory rebound that occurred in UBCs may account for the delayed spiking patterns. The presence of synaptic pathways with multiple UBCs could contribute to these long pauses and delays that may be critical for adaptive learning on time scales ranging from hundreds of milliseconds to tens of seconds.

Sources of synaptic input to UBC subtypes

The firing rates of putative UBCs recorded in vivo in the vestibular cerebellum of mammals follow head velocity and eye movements, in some cases with delays of hundreds of milliseconds (Simpson et al., 2005 [↗](#); Barmack and Yakhnitsa, 2008 [↗](#); Hensbroek et al., 2015 [↗](#)). Our data suggests that these long delays could be implemented by feed-forward circuits containing multiple UBCs. It is unknown whether UBCs that are labeled in the GRP or P079 mouse lines are a subtype that receives a specific source of input. In lobe X, ON UBCs defined by their electrophysiological response or mGluR1- expression receive input from both primary vestibular afferents and second-order mossy fibers from the vestibular nucleus, while OFF UBCs only receive vestibular nuclear input (Balmer and Trussell, 2019 [↗](#)). Understanding whether the UBCs labeled in these mouse lines receive head or eye movement signals, integrated signals that encode head orientation relative to gravity, or another type of signal, would help elucidate their specific roles in cerebellar functions.

Methods

Animals

Mice of both sexes were used from the following mouse lines and their crosses: P079 line: Et(tTA/mCitrine)P079Sbn (Shima et al., 2016 [↗](#)), GRP-Cre line: Tg(Grp-Cre)KH107Gsat (Gerfen et al., 2013 [↗](#)), Ai9 line: Gt(ROSA)^{26Sortm9(CAG-tdTomato)Hze} (Madisen et al., 2010 [↗](#)), Brainbow2.1-Confetti line: Gt(ROSA)^{26Sortm1}(CAG-Brainbow2.1)Cle/J (Snippert et al., 2010 [↗](#)), calretinin-Cre line:

Calb2^{tm2.1(cre/ERT2)Zjh/J} (Taniguchi et al., 2011 [DOI](#)). Cre-mediated recombination was induced in calretinin-Cre mice by intraperitoneal injections of 10 mg/ml tamoxifen (Sigma T5648) in corn oil at a dose of 75 mg/kg per day for 2-3 days and perfused 2-3 weeks later. Mice were bred in a colony maintained in the animal facility managed by the Department of Animal Care and Technologies and all procedures were approved by Arizona State University's Institutional Animal Care and Use Committee. Transgenic mice were genotyped by light at P0-P3 or by PCR.

Brain slice preparation

For acute brain slice electrophysiology, male and female P079 and GRP-Cre/Ai9 mice were used at ages P21-35. Animals were deeply anesthetized with isoflurane and the brain was extracted under ice-cold high-sucrose artificial cerebral spinal fluid (ACSF) containing the following (in mM): 87 NaCl, 75 sucrose, 25 NaHCO₃, 25 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 0.4 Na-ascorbate, 2 Na-pyruvate, 0.5 CaCl₂, 7 MgCl₂, bubbled with 5% CO₂/95% O₂. Parasagittal cerebellum sections 200-300 µm thick were cut with a vibratome (7000smz-2, Campden Instruments) in ice-cold high-sucrose ACSF. Slices recovered at 35°C for 30-40 minutes, in ACSF containing the following (in mM): 130 NaCl, 2.1 KCl, 1.2 KH₂PO₄, 3 Na-HEPES, 10 glucose, 20 NaHCO₃, 0.4 Na-ascorbate, 2 Na-pyruvate, 1.5 CaCl₂, 1 MgSO₄, bubbled with 5% CO₂/95% O₂ (300–305 mOsm). Slices were maintained at room temperature (~23°C) until recording. Recordings were performed from lobe X of the cerebellum within 6 hours of preparation.

Electrophysiological recordings

Acute brain slices were perfused with ACSF using a peristaltic pump (Ismatec) at 2-3 ml/min, and maintained at 32-34°C with an inline heater (Warner Instruments). ACSF contained 0.5 µM Strychnine and 5 µM SR95531 to block synaptic inhibition. The recording set up was composed of an Olympus BX51 fixed-stage microscope with Dodt gradient contrast optics (Dodt et al., 2002), and 4X and 60X (water immersion) Olympus objectives. UBCs were initially identified by soma size or transgenic fluorescence and confirmed by intracellular filling with 5 µM Alexa Fluor 568 or 488 hydrazide sodium salt (Life Technologies). Patch electrodes were pulled with borosilicate glass capillaries (OD 1.2 mm and ID 0.68 mm, AM Systems) with a horizontal puller (P1000, Sutter Instruments). Intracellular recording solution contained (mM): 113 K-gluconate, 9 HEPES, 4.5 MgCl₂, 0.1 EGTA, 14 Tris-phosphocreatine, 4 Na₂-ATP, 0.3 tris-GTP, 0.1-0.3% biocytin, 290 mOsm, pH 7.2-7.25. All recordings were corrected for a -10 mV junction potential. Data were acquired using a Multiclamp 700B amplifier and pClamp 11 software (Molecular Devices). Signals were acquired with 5-10X gain, sampled at 50-100 kHz using a Digidata (1550A, Molecular Devices) analog-digital converter, and low-pass filtered at 10 kHz, with further filtering applied offline. Patch pipettes tip resistance was 5-8 MΩ; series resistance was compensated with correction 20-40% and prediction 50-70%, bandwidth 2 kHz. Membrane potential was held constant at -70 mV in voltage clamp experiments. Electrical stimulation was performed using a concentric bipolar electrode (CBBPC75, FHC) placed in the white matter of the sagittal cerebellar slice. Stimuli were evoked using a stimulus isolation unit (Iso-Flex, A.M.P.I.) delivering 100-250 µs duration pulses of 0-90 V.

Immunohistochemistry and imaging

Mice were overdosed with isoflurane and perfused through the heart with 0.01 M phosphate buffered saline, 7.4 pH (PBS) followed by 4% paraformaldehyde in PBS. Brains were extracted from the skull and incubated in 4% paraformaldehyde in PBS overnight at 4°C. 50 µm thick sections were made on a vibratome (7000smz-2, Campden Instruments). To recover cells that were filled with biocytin during whole-cell recording, acute brain slices were fixed 1-2 h in 4% paraformaldehyde in PBS, followed by storage in PBS. Both floating 50 µm sections and 200-300 µm thick acute slices were treated with the following procedures. Sections were rinsed 3 x 5 min in PBS, blocked and permeabilized in 5% BSA, 2% fish gelatin, 0.2% Triton X-100 in PBS for >1 hr at room temperature. Sections were incubated in primary antibodies overnight in 1% fish gelatin in PBS at 4°C on an orbital shaker. Primary antibodies included chicken polyclonal anti-GFP (1:2000,

Aves Labs, GFP-1020, AB_10000240), goat polyclonal anti-mCherry (1:2000, Sicgen, AB0040, AB_2333093), mouse monoclonal anti-rat mGluR1a (1:800, BD Pharmingen, 556389, AB_396404), rabbit polyclonal anti-calretinin (1:2000, Swant, 7697, AB_10000342). Sections were rinsed 3 x 5 min in PBS, followed by secondary antibodies (1:500) and streptavidin (1:2000) that were diluted in 1% fish gelatin in PBS and incubated overnight at 4°C on an orbital shaker. Secondary antibodies included donkey anti-chicken Alexa Fluor 488 (Jackson ImmunoResearch, 715-545-155, AB_2340375), donkey anti-mouse Alexa Fluor 647 (Jackson ImmunoResearch, 715-605-151, AB_2340863), donkey anti-rabbit Alexa Fluor 647 (Jackson ImmunoResearch, 711-605-152, AB_2492288), donkey anti-rabbit Cy3 (Jackson ImmunoResearch, 711-165-152, AB_2307443), donkey anti-goat Cy3 (Jackson ImmunoResearch, 705-165-147, AB_2307351), streptavidin-Alexa Fluor 647 (ThermoFisher Scientific, S21374, AB_2336066). Sections were mounted on microscope slides (Superfrost Plus, Fisher Scientific) and coverslipped with Fluoromount-G (Southern Biotech). Images were acquired a confocal microscope (LSM800, Zeiss) with the Airyscan system that reconstructs super- resolution images from a series of images acquired under spatially structured illumination. Images are single optical planes unless otherwise specified.

Image quantification

Sagittal brain slices containing lobe X were imaged systematically across the medial-lateral axis. One area in the dorsal region and one area in the ventral region of lobe X in each selected slice was imaged using a 63x objective. The volume was 202.8 μm x 202.8 μm x 10.8 μm for each image using ~12 z- planes. Images were annotated for transgenic expression of tdTomato or mCitrine as well as mGluR1 or calretinin labeling. tdTomato, mCitrine and calretinin labeling is cytoplasmic and UBCs were easily counted if they had a labeled soma and attached dendritic brush. mGluR1 is mostly expressed in the dendrite, but labeling is also present in the soma membrane, which can be observed as a circle. mGluR1 UBCs were counted if there was labeling in the dendritic brush that was attached to an apparent mGluR1-labeled soma. This necessarily conservative method of identifying mGluR1-labeled UBCs likely resulted in an underestimation of the total number of mGluR1(+) UBCs.

The labeling intensity of mCitrine in P079 UBCs that were mGluR1(+) was compared to P079 UBCs that were mGluR1(-) by measuring the mean pixel intensity in the somas of each type of UBCs. At least one of each type was measured in the same image and same z-plane to account for differences in brightness that could be due to differences in immunohistochemical labeling, image acquisition settings, and depth in the slice (deeper cells are less well labeled due to more limited antibody penetration). For each z- plane with at least one mGluR1(+) and one mGluR1(-) P079 UBC, a difference in brightness was calculated and reported as a relative brightness of the mGluR1(+) P079 UBCs to the brighter mGluR1(-) P079 UBCs.

Computational modeling

Single compartment models of ON and OFF UBCs were built using NEURON (Hines and Carnevale, 1997 [DOI](#); Carnevale and Hines, 2006 [DOI](#)) and utilized fast voltage-gated sodium (g_{Na}) and potassium (g_{K}) conductances to produce action potentials (Destexhe et al., 1994 [DOI](#)), a slow voltage-gated potassium conductance from a model of cerebellar granule cells ($g_{\text{K-slow}}$) (D'Angelo et al., 2001 [DOI](#)), and a passive leak conductance (g_{pas}). A hyperpolarization-activated non-specific conductance (g_{H}) (Kim et al., 2012 [DOI](#); Subramaniam et al., 2014 [DOI](#)), which was added to ON and OFF UBCs a voltage sag in response to hyperpolarizing current pulses that reproduced those reported in **Figure 1** [DOI](#). Input resistance of the cells was measured using a -5 pA current pulse and adjusted to approximate GRP ON UBCs and P079 OFF UBCs by adjusting the passive leak conductance. Capacitance was measured from the membrane time constant and adjusted by changing the membrane area. Specific membrane capacitance was set to 1 $\mu\text{F}/\text{cm}^2$. **Tables 1** [DOI](#)-**2** [DOI](#) shows the variables used in ON and OFF UBCs.

ON UBC		
<i>Modeled conductance</i>	<i>g_{max} (S/cm²)</i>	<i>E_{rev} (mV)</i>
g_{Na}	0.1	50
g_K	0.03	-90
g_H	1e-5	-30
g_{pas}	2e-4	-65
g_{K-slow}	8e-4	-90
Diameter	20 μm	

Table 1

ON UBC conductances

OFF UBC		
<i>Modeled conductance</i>	<i>g_{max} (S/cm²)</i>	<i>E_{rev} (mV)</i>
g_{Na}	0.1	50
g_K	0.03	-90
g_H	3.1e-4	-30
g_{pas}	7e-5	-62
g_{K-slow}	8e-4	-90
Diameter	27 μm	

Table 2

OFF UBC conductances

Synaptic transmission was implemented by applying glutamate transients simulated with a 3-dimensional diffusion equation to an AMPA receptor model that was fit to ON UBC data (Lu et al., 2017 [↗](#)) and described in detail in our previous work (Balmer et al., 2021 [↗](#)) (**Table 3** [↗](#)-**4** [↗](#)). OFF UBCs have mGluR2 receptors that are thought to be distant from the presynaptic release sites (Jaarsma et al., 1998 [↗](#)), which we approximated by reducing the amount of glutamate released by a factor of 10 and increasing the distance by 1 μm in the diffusion equation (**Table 3** [↗](#)). To model mGluR2 currents in P079 UBCs that are mediated by G-protein coupled inwardly rectifying potassium channels, a GABA-B receptor model (Destexhe et al., 1998 [↗](#)) was modified to approximate the kinetics of our recorded currents by increasing the unbinding rate from 0.02 ms^{-1} to 0.0215 ms^{-1} (**Table 4** [↗](#)). A train of 10 presynaptic release events produced an 865 ms long burst in the ON UBC model and a 1545 ms pause in the OFF UBC model, which is within the range observed in our data set.

Conflict of interest statement

The authors declare no competing financial interests.

Acknowledgements

Funding was provided by the NIH/NIDCD R00 DC016905, Hearing Health Foundation, and National Ataxia Foundation. We thank Dr. Jason Newbern for confocal microscope use, Dr. Sacha Nelson for the P079 mouse line, and Dr. Larry Trussell for comments on the manuscript.

<i>Glutamate diffusion parameter</i>	<i>AMPA synapse</i>	<i>mGluR2 synapse</i>
Molecules released (#)	3e6	3e5
Distance (nm)	750	1750
Diffusion coefficient (cm ² /s)	0.33	0.33
Tortuosity	1.55	1.55
Volume fraction	0.21	0.21
Ambient glutamate (mM)	0.005	0

Table 3

Glutamate diffusion at AMPA and mGluR2 receptors

<i>Synaptic receptor</i>	<i>g_{max} (S/cm²)</i>	<i>E_{rev} (mV)</i>
ON UBC- AMPA	4e-9	0
OFF UBC- mGluR2	1e-8	-90

Table 4

Synaptic conductances

References

- Albus JS (1971) **A theory of cerebellar function** *Math Biosci* **10**:25–61
- Balmer TS, Borges-Merjane C, Trussell LO (2021) **Incomplete removal of extracellular glutamate controls synaptic transmission and integration at a cerebellar synapse** *eLife* **10**
- Balmer TS, Trussell LO (2019) **Selective targeting of unipolar brush cell subtypes by cerebellar mossy fibers** *eLife* **8**
- Balmer TS, Trussell LO (2021) **Trigeminal Contributions to the Dorsal Cochlear Nucleus in Mouse** *Front Neurosci* **15**
- Balmer TS, Trussell LO (2022) **Descending Axonal Projections from the Inferior Colliculus Target Nearly All Excitatory and Inhibitory Cell Types of the Dorsal Cochlear Nucleus** *J Neurosci* **42**:3381–3393
- Barmack NH, Yakhnitsa V (2008) **Functions of interneurons in mouse cerebellum** *J Neurosci* **28**:1140–1152
- Berthie B, Axelrad H (1994) **Granular layer collaterals of the unipolar brush cell axon display rosette-like excrescences. A Golgi study in the rat cerebellar cortex** *Neurosci Lett* **167**:161–165
- Borges-Merjane C, Trussell LO (2015) **ON and OFF unipolar brush cells transform multisensory inputs to the auditory system** *Neuron* **85**:1029–1042
- Braitenberg V (1967) **Is the cerebellar cortex a biological clock in the millisecond range?** *Prog Brain Res* **25**:334–346
- Carnevale NT, Hines ML (2006) **The NEURON book**
- D’Angelo E, De Zeeuw CI (2009) **Timing and plasticity in the cerebellum: focus on the granular layer** *Trends Neurosci* **32**:30–40
- D’Angelo E, Nieuws T, Maffei A, Armano S, Rossi P, Taglietti V, Fontana A, Naldi G (2001) **Theta-Frequency Bursting and Resonance in Cerebellar Granule Cells: Experimental Evidence and Modeling of a Slow K⁺-Dependent Mechanism** *J Neurosci* **21**:759–770
- Destexhe A, Contreras D, Sejnowski TJ, Steriade M (1994) **A model of spindle rhythmicity in the isolated thalamic reticular nucleus** *J Neurophysiol* **72**:803–818
- Destexhe A, Mainen ZF, Sejnowski TJ (1998) **Kinetic models of synaptic transmission** *Methods Neuronal Model* **2**:1–25
- Diño MR, Schuerger RJ, Liu Y-B, Slater NT, Mugnaini E (2000) **Unipolar brush cell: a potential feedforward excitatory interneuron of the cerebellum** *Neuroscience* **98**:625–636
- Fernandez C, Goldberg JM (1971) **Physiology of peripheral neurons innervating semicircular canals of the squirrel monkey II. Response to sinusoidal stimulation and dynamics of peripheral vestibular system.** *J Neurophysiol* **34**:661–675

- Fiala JC, Grossberg S, Bullock D (1996) **Metabotropic Glutamate Receptor Activation in Cerebellar Purkinje Cells as Substrate for Adaptive Timing of the Classically Conditioned Eye-Blink Response** *J Neurosci* **16**:3760–3774
- Gerfen CR, Paletzki R, Heintz N (2013) **GENSAT BAC cre-recombinase driver lines to study the functional organization of cerebral cortical and basal ganglia circuits** *Neuron* **80**:1368–1383
- Guo C, Huson V, Macosko EZ, Regehr WG (2021) **Graded heterogeneity of metabotropic signaling underlies a continuum of cell-intrinsic temporal responses in unipolar brush cells** *Nat Commun* **12**
- Hensbroek RA, Ruigrok TJ, van Beugen BJ, Maruta J, Simpson JI (2015) **Visuo-Vestibular Information Processing by Unipolar Brush Cells in the Rabbit Flocculus** *Cerebellum* **14**:578–583
- Hines ML, Carnevale NT (1997) **The NEURON simulation environment** *Neural Comput* **9**:1179–1209
- Hooper SL, Buchman E, Hobbs KH (2002) **A computational role for slow conductances: single-neuron models that measure duration** *Nat Neurosci* **5**:552–556
- Ito M (1982) **Cerebellar control of the vestibulo-ocular reflex--around the flocculus hypothesis** *Annu Rev Neurosci* **5**:275–296
- Jaarsma D, Dino MR, Ohishi H, Shigemoto R, Mugnaini E (1998) **Metabotropic glutamate receptors are associated with non-synaptic appendages of unipolar brush cells in rat cerebellar cortex and cochlear nuclear complex** *J Neurocytol* **27**:303–327
- Kennedy A, Wayne G, Kaifosh P, Alvina K, Abbott LF, Sawtell NB (2014) **A temporal basis for predicting the sensory consequences of motor commands in an electric fish** *Nat Neurosci* **17**:416–422
- Kim JA, Sekerkova G, Mugnaini E, Martina M (2012) **Electrophysiological, morphological, and topological properties of two histochemically distinct subpopulations of cerebellar unipolar brush cells** *Cerebellum* **11**:1012–1025
- Kinney JP, Spacek J, Bartol TM, Bajaj CL, Harris KM, Sejnowski TJ (2013) **Extracellular sheets and tunnels modulate glutamate diffusion in hippocampal neuropil** *J Comp Neurol* **521**:448–464
- Kozareva V, Martin C, Osorno T, Rudolph S, Guo C, Vanderburg C, Nadaf N, Regev A, Regehr WG, Macosko E (2021) **A transcriptomic atlas of mouse cerebellar cortex comprehensively defines cell types** *Nature* **598**:214–219
- Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, Sanes JR, Lichtman JW (2007) **Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system** *Nature* **450**:56–62
- Locatelli F, Botta L, Prestori F, Masetto S, D’Angelo E (2013) **Late-onset bursts evoked by mossy fibre bundle stimulation in unipolar brush cells: evidence for the involvement of H- and TRP-currents** *J Physiol* **591**:899–918
- Lu HW, Balmer TS, Romero GE, Trussell LO (2017) **Slow AMPAR Synaptic Transmission Is Determined by Stargazin and Glutamate Transporters** *Neuron* **96**:73–80

- Madisen L *et al.* (2010) **A robust and high-throughput Cre reporting and characterization system for the whole mouse brain** *Nat Neurosci* **13**:133–140
- Marr D (1969) **A theory of cerebellar cortex** *J Physiol* **202**:437–470
- Mugnaini E, Floris A, Wright-Goss M (1994) **Extraordinary synapses of the unipolar brush cell: an electron microscopic study in the rat cerebellum** *Synapse* **16**:284–311
- Mugnaini E, Sekerkova G, Martina M (2011) **The unipolar brush cell: a remarkable neuron finally receiving deserved attention** *Brain Res Rev* **66**:220–245
- Nunzi MG, Birnstiel S, Bhattacharyya BJ, Slater NT, Mugnaini E (2001) **Unipolar brush cells form a glutamatergic projection system within the mouse cerebellar cortex** *J Comp Neurol* **434**:329–341
- Nunzi M-G, Mugnaini E (2000) **Unipolar brush cell axons form a large system of intrinsic mossy fibers in the postnatal vestibulocerebellum** *J Comp Neurol* **422**:55–65
- Nunzi MG, Russo M, Mugnaini E (2003) **Vesicular glutamate transporters VGLUT1 and VGLUT2 define two subsets of unipolar brush cells in organotypic cultures of mouse vestibulocerebellum** *Neuroscience* **122**:359–371
- Nunzi MG, Shigemoto R, Mugnaini E (2002) **Differential expression of calretinin and metabotropic glutamate receptor mGluR1alpha defines subsets of unipolar brush cells in mouse cerebellum** *J Comp Neurol* **451**:189–199
- Th Raphan, Matsuo V, Cohen B (1979) **Velocity storage in the vestibulo-ocular reflex arc (VOR)** *Exp Brain Res* **35**:229–248
- Raymond JL, Lisberger SG (1998) **Neural learning rules for the vestibulo-ocular reflex** *J Neurosci* **18**:9112–9129
- Rossi DJ, Alford S, Mugnaini E, Slater NT (1995) **Properties of transmission at a giant glutamatergic synapse in cerebellum: the mossy fiber-unipolar brush cell synapse** *J Neurophysiol* **74**:24–42
- Ryugo DK, Haenggeli CA, Doucet JR (2003) **Multimodal inputs to the granule cell domain of the cochlear nucleus** *Exp Brain Res* **153**:477–485
- Sekerkova G, Watanabe M, Martina M, Mugnaini E (2014) **Differential distribution of phospholipase C beta isoforms and diacylglycerol kinase-beta in rodents cerebella corroborates the division of unipolar brush cells into two major subtypes** *Brain Struct Funct* **219**:719–749
- Shima Y, Sugino K, Hempel CM, Shima M, Taneja P, Bullis JB, Mehta S, Lois C, Nelson SB (2016) **A Mammalian enhancer trap resource for discovering and manipulating neuronal cell types** *Elife* **5**
- Simpson JI, Hulscher HC, Sabel-Goedknecht E, Ruigrok TJ (2005) **Between in and out: linking morphology and physiology of cerebellar cortical interneurons** *Prog Brain Res* **148**:329–340
- Snippert HJ *et al.* (2010) **Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells** *Cell* **143**:134–144

Subramaniam S, Solinas S, Perin P, Locatelli F, Masetto S, D'Angelo E (2014) **Computational modeling predicts the ionic mechanism of late-onset responses in unipolar brush cells** *Front Cell Neurosci* **8**

Sutton RS, Barto AG (1981) **Toward a modern theory of adaptive networks: expectation and prediction** *Psychol Rev* **88**:135–170

Suvrathan A (2019) **Beyond STDP—towards diverse and functionally relevant plasticity rules** *Curr Opin Neurobiol* **54**:12–19

Taniguchi H *et al.* (2011) **A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex** *Neuron* **71**:995–1013

van Dorp S, De Zeeuw CI (2014) **Variable timing of synaptic transmission in cerebellar unipolar brush cells** *Proc Natl Acad Sci U S A* **111**:5403–5408

van Dorp S, De Zeeuw CI (2015) **Forward signaling by unipolar brush cells in the mouse cerebellum** *Cerebellum* **14**:528–533

Yakushin SB, Raphan T, Cohen B (2017) **Coding of Velocity Storage in the Vestibular Nuclei** *Front Neurol* **8**

Zampini V, Liu JK, Diana MA, Maldonado PP, Brunel N, Dieudonne S (2016) **Mechanisms and functional roles of glutamatergic synapse diversity in a cerebellar circuit** *eLife* **5**

Article and author information

Harsh N. Hariani

Interdisciplinary Graduate Program in Neuroscience, School of Life Sciences, Arizona State University, Tempe, AZ, 85287

A. Brynn Algstam

Barrett Honors College, School of Life Sciences, Arizona State University, Tempe, AZ, 85287

Christian T. Candler

Interdisciplinary Graduate Program in Neuroscience, School of Life Sciences, Arizona State University, Tempe, AZ, 85287

Isabelle F. Witteveen

School of Life Sciences, Arizona State University, Tempe, AZ, 85287

Jasmeen K. Sidhu

School of Life Sciences, Arizona State University, Tempe, AZ, 85287

Timothy S. Balmer

School of Life Sciences, Arizona State University, Tempe, AZ, 85287

For correspondence: timothy.balmer@asu.edu

Copyright

© 2023, Hariani et al.

This article is distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use and redistribution provided that the original author and source are credited.

Editors

Reviewing Editor

Roy Sillitoe

Baylor College of Medicine, United States of America

Senior Editor

Barbara Shinn-Cunningham

Carnegie Mellon University, United States of America

Reviewer #1 (Public Review):

The manuscript by Hariani et al. presents experiments designed to improve our understanding of the connectivity and computational role of Unipolar Brush Cells (UBCs) within the cerebellar cortex, primarily lobes IX and X. The authors develop and cross several genetic lines of mice that express distinct fluorophores in subsets of UBCs, combined with immunocytochemistry that also distinguishes subtypes of UBCs, and they use confocal microscopy and electrophysiology to characterize the electrical and synaptic properties of subsets of so-labelled cells, and their synaptic connectivity within the cerebellar cortex. The authors then generate a computer model to test possible computational functions of such interconnected UBCs.

Using these approaches, the authors report that:

1. GRP-driven TDTomato is expressed exclusively in a subset (20%) of ON-UBCs, defined electrophysiologically (excited by mossy fiber afferent stimulation via activation of UBC AMPA and mGluR1 receptors) and immunocytochemically by their expression of mGluR1.
2. UBCs ID'd/tagged by mCitrine expression in Brainbow mouse line P079 is expressed in a similar minority subset of OFF-UBCs defined electrophysiologically (inhibited by mossy fiber afferent stimulation via activation of UBC mGluR2 receptors) and immunocytochemically by their expression of Calretinin. However, such mCitrine expression was also detected in some mGluR1 positive UBCs, which may not have shown up electrophysiologically because of the weaker fluorophore expression without antibody amplification.
3. Confocal analysis of crossed lines of mice (GRP X P079) stained with antibodies to mGluR1 and calretinin documented the existence of all possible permutations of interconnectivity between cells (ON-ON, ON-OFF, OFF-OFF, OFF-ON), but their overall abundance was low, and neither their absolute or relative abundance was quantified.
4. A computational model (NEURON) indicated that the presence of an intermediary UBC (in a polysynaptic circuit from MF to UBC to UBC) could prolong bursts (MF-ON-ON), prolong pauses (MF-ON-OFF), cause a delayed burst (MF-OFF-OFF), cause a delayed pause (MF-OFF-ON) relative to solely MF to UBC synapses which would simply exhibit long bursts (MF-ON) or long pauses (MF-OFF).

The authors thus conclude that the pattern of interconnected UBCs provides an extended and more nuanced pattern of firing within the cerebellar cortex that could mediate longer lasting sensorimotor responses.

The cerebellum's long known role in motor skills and reflexes, and associated disorders, combined with our nascent understanding of its role in cognitive, emotional, and appetitive processing, makes understanding its circuitry and processing functions of broad interest to the neuroscience and biomedical community. The focus on UBCs, which are largely restricted to vestibular lobes of the cerebellum reduces the breadth of likely interest somewhat. The overall design of specific experiments is rigorous and the use of fluorophore expressing mouse lines is creative. The data that is presented and the writing are clear. However, despite some additional analysis in response to the initial review, the overall experimental design still has issues that reduce overall interpretation (please see specific issues for details), which combined with a lack of thorough analysis of the experimental outcomes undermines the value of the NEURON model results and the advance in our understanding of cerebellar processing in situ (again, please see specific issues for details).

Specific issues:

1. All data gathered with inhibition blocked. All of the UBC response data (Fig. 1) was gathered in the presence of GABAAR and Glycine R blockers. While such an approach is appropriate generally for isolating glutamatergic synaptic currents, and specifically for examining and characterizing monosynaptic responses to single stimuli, it becomes problematic in the context of assaying synaptic and action potential response durations for long lasting responses, and in particular for trains of stimuli, when feed-forward and feed-back inhibition modulates responses to afferent stimulation. I.e. even for single MF stimuli, given the >500ms duration of UBC synaptic currents, there is plenty of time for feedback inhibition from Golgi cells (or feedforward, from MF to Golgi cell excitation) to interrupt AP firing driven by the direct glutamatergic synaptic excitation. This issue is compounded further for all of the experiments examining trains of MF stimuli. Beyond the impact of feedback inhibition on the AP firing of any given UBC, it would also obviously reduce/alter/interrupt that UBC's synaptic drive of downstream UBCs. This issue fundamentally undermines our ability to interpret the simulation data of Vm and AP firing of both the modeled intermediate and downstream UBC, in terms of applying it to possible cerebellar cortical processing in situ.

The authors' response to the initial concern is (to paraphrase), "its not possible to do and its not important", neither of which are soundly justified.

As stated in the original review, it is fully understandable and appropriate to use GABAAR/GlycineR antagonists to isolate glutamatergic currents, to characterize their conductance kinetics. That was not the issue raised. The issue raised was that then using only such information to generate a model of in situ behavior becomes problematic, given that feedback and lateral inhibition will sculpt action potential output, which of course will then fundamentally shape their synaptic drive of secondary UBCs, which will be further sculpted by their own inhibitory inputs. This issue undermines interpretation of the NEURON model.

The argument that taking inhibition into account is not possible because of assumed or possible direct electrical excitation of Golgi cells is confusing for two interacting reasons. First, one can certainly stimulate the mossy fiber bundle to get afferent excitation of UBCs (and polysynaptic feedback/lateral inhibitory inputs) without directly stimulating the Golgi cells that innervate any recorded UBC. Yes, one might be stimulating some Golgi cells near the stimulating electrode, but one can position the stimulating electrode far enough down the white matter track (away from the recorded UBC), such that mossy fiber inputs to the

recorded UBC can be stimulated without affecting Golgi cells near or synaptically connected to the recorded UBC. Moreover, if the argument were true, then presumably the stimulation protocol would be just as likely to directly stimulate neighboring UBCs, which then drove the recorded UBC's responses. Thus, it is both doable and should be ensured that stimulation of the white matter is distant enough to not be directly activating relevant, connected neurons within the granule cell layer.

Finally, the authors present three examples of UBC recordings with and without inhibitory inputs blocked, and state "Thus, these large conductances are unlikely to be significantly shaped by 1-10 ms IPSCs from feedforward and feedback GABA/glycine inhibition" and "GABA/glycinergic inhibition...has little to no effect on the slow inward current that develops after the end of stimulation". This response reflects on original concerns about lack of quantification or consideration of important parameters. In particular, while the traces with and without inhibition are qualitatively similar, quantitative considerations indicate otherwise. First, unquantified examples are not adequate to drive conclusions. Regardless, the main issue (how inhibition affects actual responses in situ) is actually highlighted by the authors current clamp recordings of UBC responses, before and after blocking inhibition. The output response is dramatically different, both at early and late time points, when inhibition is blocked. Again, a lack of quantification (of adequate n's) makes it hard to know exactly how important, but quick "eye ball" estimates of impact include: 1) a switch from only low frequency APs initially (without inhibition blocked) to immediate burst of high frequency APs (high enough to not discern individual APs with given figure resolution) when inhibition is blocked, 2) Slow rising to a peak EPSP, followed by symmetrical return to baseline (without inhibition blocked) versus immediate rise to peak, followed by prolonged decay to baseline (with inhibition blocked), 3) substantially shorter duration (~34% shorter) secondary high frequency burst (individual APs not discernible) of APs (with inhibition blocked versus without inhibition blocked), and 4) substantial reduction in number of long delayed APs (with inhibition blocked versus without inhibition blocked). Thus, clearly, feedback/lateral inhibition is actually sculpting AP output at all phases of the UBC response to trains of afferent stimulations. Importantly, the single voltage clamp trace showing little impact of transient IPSCs on the slow EPSC do not take into account likely IPSC influences on voltage-activated conductances that would not occur in voltage-clamp recordings but would be free to manifest in current clamp, and thereby influence AP output, as observed.

So again, our ability to understand how interconnected UBCs behave in the intact system is undermined by the lack of consideration and quantification of the impact of inhibition, and it not being incorporated into the model. At the very least a strong proviso about lack of inclusion of such information, given the authors' data showing its importance in the few examples shown, should be added to the discussion.

1. No consideration for involvement of polysynaptic UBCs driving UBC responses to MF stimulation in electrophysiology experiments. Given the established existence (in this manuscript and Dino et al. 2000 Neurosci, Dino et al. 2000 ProgBrainRes, Nunzi and Mugnaini 2000 JCompNeurol, Nunzi et al. 2001 JCompNeurol) of polysynaptic connections from MFs to UBCs to UBCs, the MF evoked UBC responses established in this manuscript, especially responses to trains of stimuli could be mediated by direct MF inputs, or to polysynaptic UBC inputs, or possibly both (to my awareness not established either way). Thus the response durations could already include extension of duration by polysynaptic inputs, and so would overestimate the duration of monosynaptic inputs, and thus polysynaptic amplification/modulation, observed in the NEURON model.

Author response: "UBCs receive a single mossy fiber input on their dendritic brush, and thus if our stimulation produces a reliable, short-latency response consistent with a monosynaptic input, then there is not likely to be a disynaptic input."

This statement is not congruent with the literature, with early work by Mugnaini and colleagues (Mugnaini et al. 1994 Synapse; Mugnaini and Flores 1994 J. Comp. Neurol.) indicating that UBCs are innervated by 1-2 mossy fibers, which are as likely other UBC terminals as MFs. This leaves open the possibility that so called monosynaptic responses do, as originally suggested, already include polysynaptic feedforward amplification of duration. While the authors also indicate that isolated disynaptic currents can be observed when they occur in isolation, a careful examination and objective documentation of "monosynaptic" responses would address this issue. Presumably, if potential disynaptic UBC inputs occur during a monosynaptic MF response, it would be detected as an abrupt biphasic inward/outward current, due to additional AMPA receptor activation but further desensitization of those already active (as observed by Kinney et al. 1997 J. Neurophysiol: "The delivery of a second MF stimulus at the peak of the slow EPSC evoked a fast EPSC of reduced amplitude followed by an undershoot of the subsequent slow current"). If such polysynaptic inputs are truly absent and are "rare" in isolation, some estimation of how common or not such synaptic amplification is, would improve our understanding of the overall significance of these inputs.

1. Lack of quantification of subtypes of UBC interconnectivity. Given that it is already established that UBCs synapse onto other UBCs (see refs above), the main potential advance of this manuscript in terms of connectivity is the establishment and quantification of ON-ON, ON-OFF, OFF-ON, and OFF-OFF subtypes of UBC interconnections. But, the authors only establish that each type exists, showing specific examples, but no quantification of the absolute or relative density was provided, and the authors' unquantified wording explicitly or implicitly states that they are not common. This lack of quantification and likely small number makes it difficult to know how important or what impact such synapses have on cerebellar processing, in the model and in situ.

To address this issue, the authors added the following text to the discussion section: "We did not estimate the density of these UBC to UBC connections, because the sparseness of labeling using these approaches made an accurate calculation impossible. Previous work using organotypic slice cultures from P8 mice estimated that 2/3 of the UBC population receives input from other UBCs (Nunzi & Mugnaini, 2000), although it is unclear whether this is the case in older mice."

While accurate, the addition doesn't really address the situation, which is that apparently the reported connections are rare. Adding the information about 2/3 of UBCs having UBC inputs in culture, implies the opposite might be true (i.e. that they might be quite common), which is in contrast to the authors' data, so should be reworded for clarity, which should also incorporate the considerations covered in point #2 above. I.e. if the authors do establish that none of their recordings have polysynaptic inputs, and if they determine that the number of cells that showed isolated di-synaptic inputs is indeed rare, then it suggests that these specific polysynaptic connections are in fact rare.

1. Lack of critical parameters in NEURON model.
 - A) The model uses # of molecules of glutamate released as the presumed quantal content, and this factor is constant. However, no consideration of changes in # of vesicles released from single versus trains of APs from MFs or UBCs is included. At most simple synapses, two sequential APs alters release probability, either up or down, and release probability changes dynamically with trains of APs. It is therefore reasonable to imagine UBC axon release probability is at least as complicated, and given the large surface area of contact between two UBCs, the number of vesicles released for any given AP is also likely more complex.

B) the model does not include desensitization of AMPA receptors, which in the case of UBCs can paradoxically reduce response magnitude as vesicle release and consequent glutamate concentration in the cleft increases (Linney et al. 1997 JNeurophysiol, Lu et al. 2017 Neuron, Balmer et al. 2021 eLIFE), as would occur with trains of stimuli at MF to ON-UBCs.

While the authors have not added the suggested additional parameters, their clarifications regarding the implications of existing parameters, and demonstration of reasonable fits to experimental data, and lack of substantial effect of simulating reduced vesicle release probability, provided by the authors, adequately addresses this concern.

1. Lack of quantification of various electrophysiological responses. UBCs are defined (ON or OFF) based on inward or outward synaptic response, but no information is provided about the range of the key parameter of duration across cells, which seems most critical to the current considerations. There is a similar lack of quantification across cells of AP duration in response to stimulation or current injections, or during baseline. The latter lack is particularly problematic because in agreement with previous publications, the raw data in Fig. 1 shows ON UBCs as quiescent until MF stimulation and OFF UBCs firing spontaneously until MF stimulation, but, for example, at least one ON UBC in the NEURON model is firing spontaneously until synaptically activated by an OFF UBC (Fig. 11A), and an OFF UBC is silent until stimulated by a presynaptic OFF UBC (Fig. 11C). This may be expected/explainable theoretically, but then such cells should be observed in the raw data.

The authors have added additional analysis and discussion, which adequately addresses this concern.

Reviewer #2 (Public Review):

In this paper, the authors presented a compelling rationale for investigating the role of UBCs in prolonging and diversifying signals. Based on the two types of UBCs known as ON and OFF UBC subtypes, they have highlighted the existing gaps in understanding UBCs connectivity and the need to investigate whether UBCs target UBCs of the same subtype, different subtypes, or both. The importance of this knowledge is for understanding how sensory signals are extended and diversified in the granule cell layer.

The authors designed very interesting approaches to study UBCs connectivity by utilizing transgenic mice expressing GFP and RFP in UBCs, Brainbow approach, immunohistochemical and electrophysiological analysis, and computational models to understand how the feed-forward circuits of interconnected UBCs transform their inputs.

This study provided evidence for the existence of distinct ON and OFF UBC subtypes based on their electrophysiological properties, anatomical characteristics, and expression patterns of mGluR1 and calretinin in the cerebellum. The findings support the classification of GRP UBCs as ON UBCs and P079 UBCs as OFF UBCs and suggest the presence of synaptic connections between the ON and OFF UBC subtypes. In addition, they found that GRP and P079 UBCs form parallel and convergent pathways and have different membrane capacitance and excitability. Furthermore, they showed that UBCs of the same subtype provide input to one another and modify the input to granule cells, which could provide a circuit mechanism to diversify and extend the pattern of spiking produced by mossy fiber input. Accordingly, they suggested that these transformations could provide a circuit mechanism for maintaining a sensory representation of movement for seconds.

Overall, the article is well written in a sound detailed format, very interesting with excellent discovery and suggested model.

I believe the authors have provided appropriate responses and have consequently revised the manuscript in a convincing manner. Although I am not an expert in physiology, I find the explanations and clarifications to be acceptable.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):*

The manuscript by Hariani et al. presents experiments designed to improve our understanding of the connectivity and computational role of Unipolar Brush Cells (UBCs) within the cerebellar cortex, primarily lobes IX and X. The authors develop and cross several genetic lines of mice that express distinct fluorophores in subsets of UBCs, combined with immunocytochemistry that also distinguishes subtypes of UBCs, and they use confocal microscopy and electrophysiology to characterize the electrical and synaptic properties of subsets of so-labelled cells, and their synaptic connectivity within the cerebellar cortex. The authors then generate a computer model to test the possible computational functions of such interconnected UBCs.

Using these approaches, the authors report that:

- 1. GRP-driven TdTomato is expressed exclusively in a subset (20%) of ON-UBCs, defined electrophysiologically (excited by mossy fiber afferent stimulation via activation of UBC AMPA and mGluR1 receptors) and immunocytochemically by their expression of mGluR1.*
- 1. UBCs ID'd/tagged by mCitrine expression in Brainbow mouse line P079 are expressed in a similar minority subset of OFF-UBCs defined electrophysiologically (inhibited by mossy fiber afferent stimulation via activation of UBC mGluR2 receptors) and immunocytochemically by their expression of Calretinin. However, such mCitrine expression was also detected in some mGluR1 positive UBCs, which may not have shown up electrophysiologically because of the weaker fluorophore expression without antibody amplification.*

This is correctly stated with the exception that the P079 mouse line itself expresses mCitrine. The Brainbow mouse line was used in the connectivity study by crossing it to the GRP-Cre or Calretinin-Cre lines.

- 1. Confocal analysis of crossed lines of mice (GRP X P079) stained with antibodies to mGluR1 and calretinin documented the existence of all possible permutations of interconnectivity between cells (ON-ON, ON-OFF, OFF-OFF, OFF-ON), but their overall abundance was low, and neither their absolute nor relative abundance was quantified.*

They were certainly rare to observe using our approaches, but we reasoned that the densities of such connections are not possible to estimate accurately. Please see discussion below.

- 1. A computational model (NEURON) indicated that the presence of an intermediary UBC (in a polysynaptic circuit from MF to UBC to UBC) could prolong bursts (MF-ON-ON), prolong pauses (MF-ON-OFF), cause a delayed burst (MF-OFF-OFF), cause a delayed pause (MF-OFF-ON) relative to solely MF to UBC synapses which would simply exhibit long bursts (MF-ON) or long pauses (MF-OFF).*

The authors thus conclude that the pattern of interconnected UBCs provides an extended and more nuanced pattern of firing within the cerebellar cortex that could mediate longer-lasting sensorimotor responses.

The cerebellum's long-known role in motor skills and reflexes, and associated disorders, combined with our nascent understanding of its role in cognitive, emotional, and appetitive processing, makes understanding its circuitry and processing functions of broad interest to the neuroscience and biomedical community. The focus on UBCs, which are largely restricted to vestibular lobules of the cerebellum reduces the breadth of likely interest somewhat. The overall design of specific experiments is rigorous and the use of fluorophore expressing mouse lines is creative. The data that is presented and the writing are clear. However, the overall experimental design has issues that reduce overall interpretation (please see specific issues for details), which combined with a lack of thorough analysis of the experimental outcomes severely undermines the value of the NEURON model results and the advance in our understanding of cerebellar processing in situ (again, please see specific issues for details).

Specific issues:

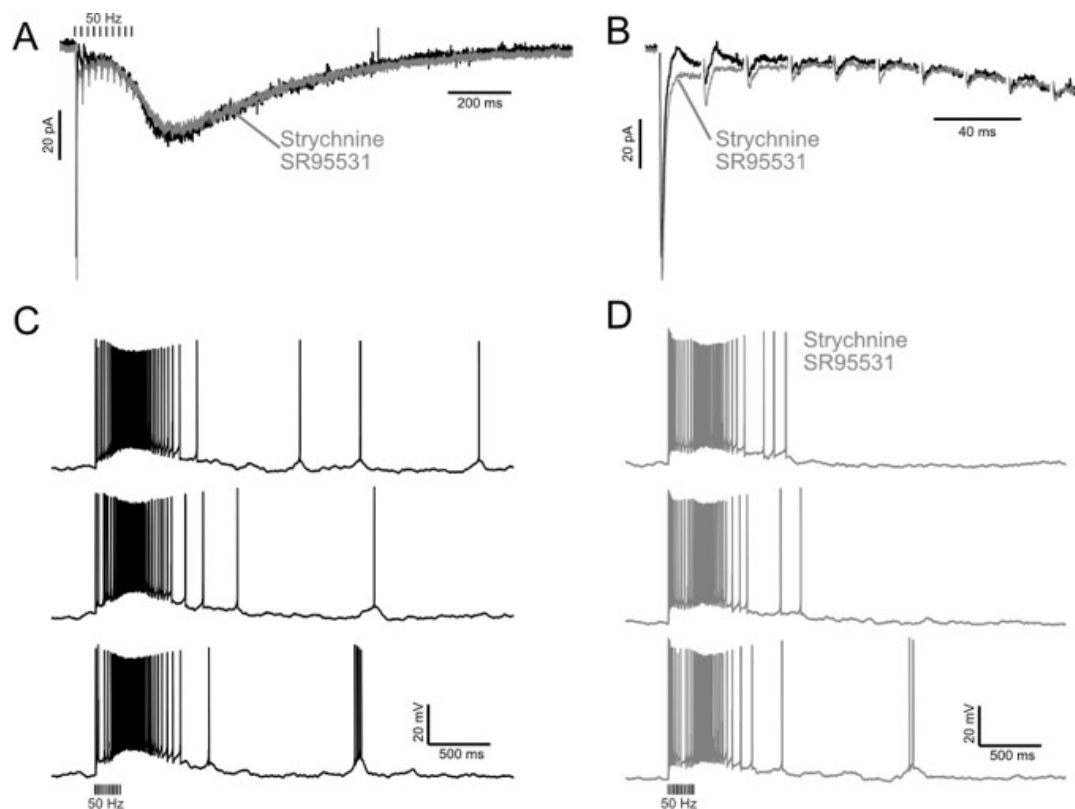
- 1. All data gathered with inhibition blocked. All of the UBC response data (Fig. 1) was gathered in the presence of GABAAR and Glycine R blockers. While such an approach is appropriate generally for isolating glutamatergic synaptic currents, and specifically for examining and characterizing monosynaptic responses to single stimuli, it becomes problematic in the context of assaying synaptic and action potential response durations for long-lasting responses, and in particular for trains of stimuli, when feed-forward and feed-back inhibition modulates responses to afferent stimulation. That is, even for single MF stimuli, given the >500ms duration of UBC synaptic currents, there is plenty of time for feedback inhibition from Golgi cells (or feedforward, from MF to Golgi cell excitation) to interrupt AP firing driven by the direct glutamatergic synaptic excitation. This issue is compounded further for all of the experiments examining trains of MF stimuli. Beyond the impact of feedback inhibition on the AP firing of any given UBC, it would also obviously reduce/alter/interrupt that UBC's synaptic drive of downstream UBCs. This issue fundamentally undermines our ability to interpret the simulation data of Vm and AP firing of both the modeled intermediate and downstream UBC, in terms of applying it to possible cerebellar cortical processing in situ.*

The goal of Figure 1 was to determine the cell types of labeled UBCs in transgenic mouse lines, which is determined entirely by their synaptic responses to glutamate (Borges-Merjane and Trussell, 2015). Thus, blocking inhibition was essential to produce clear results in the characterization of GRP and P079 UBCs. While GABAergic/glycinergic feedforward and feedback inhibition is certainly important in the intact circuit, it was not our intention, nor was it possible, to study its contribution in the present study. Leaving inhibition unblocked does not lead to a physiologically realistic stimulation pattern in acute brain slices, because electrical stimulation produces synchronous excitation and inhibition by directly exciting Golgi cells, rather than their synaptic inputs. The main inhibition that UBCs receive that are crucial to determining burst or pause durations is not via GABA/glycine, but instead through mGluR2, which lasts for 100-1000s of milliseconds. The main excitation that drives UBC firing is mGluR1 and AMPA, which both last 100-1000s of milliseconds. Thus, these large conductances are unlikely to be significantly shaped by 1-10 ms IPSCs from feedforward and feedback GABA/glycine inhibition. Recent studies that examined the duration of bursting or pausing in UBCs had inhibition blocked in their experiments, presumably for the reasons outlined above (Guo et al., 2021; Huson et al., 2023).

In Author response image 1 is an example showing the synaptic currents and firing patterns in an ON UBC before and after blocking inhibition. The GABA/glycinergic inhibition is fast, occurs soon after the stimuli and has little to no effect on the slow inward current that develops after the end of stimulation, which is what drives firing for 100s of milliseconds.

Author response image 1.

Example showing small effect of GABAergic and glycinergic inhibition on excitatory currents and burst duration. A) Excitatory postsynaptic currents in response to train of 10 presynaptic stimuli at 50 Hz before (black) and after (Grey) blocking GABA and glycine receptors. The slow inward current that occurs at the end of stimulation is little affected. B) Expanded view of the synaptic currents evoked during the train of stimuli. GABA/glycine receptors mediate the fast outward currents that occur immediately after the first couple stimuli. C) Three examples of the bursts caused by the 50 Hz stimulation in the same cell without blocking GABA and glycine receptors. D) Three examples in the same cell after blocking GABA and glycine receptors.

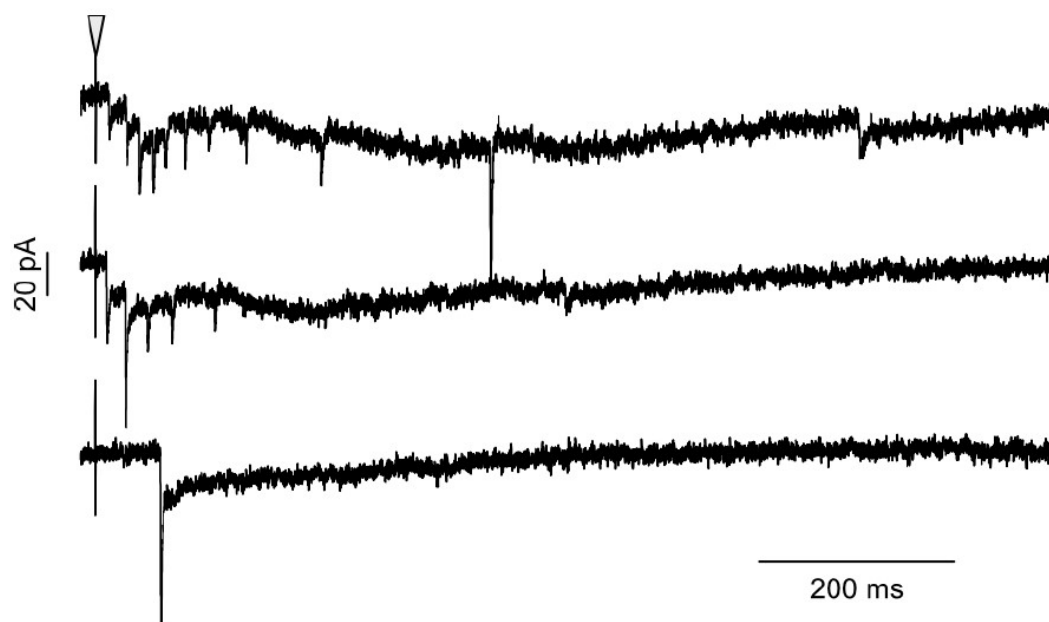


1. No consideration for the involvement of polysynaptic UBCs driving UBC responses to MF stimulation in electrophysiology experiments. Given the established existence (in this manuscript and Dino et al. 2000 Neurosci, Dino et al. 2000 ProgBrainRes, Nunzi and Mugnaini 2000 JCompNeurol, Nunzi et al. 2001 JCompNeurol) of polysynaptic connections from MFs to UBCs to UBCs, the MF evoked UBC responses established in this manuscript, especially responses to trains of stimuli could be mediated by direct MF inputs, or to polysynaptic UBC inputs, or possibly both (to my awareness not established either way). Thus the response durations could already include extension of duration by polysynaptic inputs, and so would overestimate the duration of monosynaptic inputs, and thus polysynaptic amplification/modulation, observed in the NEURON model.

We are confident that the synaptic responses shown are monosynaptic for several reasons. UBCs receive a single mossy fiber input on their dendritic brush, and thus if our stimulation produces a reliable, short-latency response consistent with a monosynaptic input, then there is not likely to be a disynaptic input, because the main input is accounted for by the monosynaptic response. In all cells included in our data set, the fast AMPA receptor-mediated currents always occurred with short latency (1.24 ± 0.29 ms; mean $\pm$ SD; $n = 13$), high reliability (no failures to produce an EPSC in any of the 13 GRP UBCs in this data set), and low jitter (SD of latency; 0.074 ± 0.046 ms; mean $\pm$ SD; $n = 13$). These measurements have been added to the results section. In some rare cases, we did observe disynaptic currents, which were easily distinguishable because a single electrical stimulation produced a burst of EPSCs at variable latencies. Please see example in Author response image 2. These cases of disynaptic input, which have been reported by others (Diño et al., 2000; Nunzi and Mugnaini, 2000; van Dorp and De Zeeuw, 2015) support the conclusion that UBCs receive input from other UBCs.

Author response image 2.

Example of GRP UBC with disynaptic input. Three examples of the effect of a single presynaptic stimulus (triangle) in a GRP UBC with presumed disynaptic input. Note the variable latency of the first evoked EPSC, bursts of EPSCs, and spontaneous EPSCs.



1. *Lack of quantification of subtypes of UBC interconnectivity. Given that it is already established that UBCs synapse onto other UBCs (see refs above), the main potential advance of this manuscript in terms of connectivity is the establishment and quantification of ON-ON, ON-OFF, OFF-ON, and OFF-OFF subtypes of UBC interconnections. But, the authors only establish that each type exists, showing specific examples, but no quantification of the absolute or relative density was provided, and the authors' unquantified wording explicitly or implicitly states that they are not common. This lack of quantification and likely small number makes it difficult to know how important or what impact such synapses have on cerebellar processing, in the model and in situ.*

As noted by the reviewer, the connections between UBCs were rare to observe. We decided against attempting to quantify the absolute or relative density of connections for several reasons. A major reason for rare observations of anatomical connections between UBCs is likely due to the sparse labeling. First, the GRP mouse line only labels 20% of ON UBCs and we are unable to test whether postsynaptic connectivity of GRP ON UBCs is the same as that of the rest of the population of ON UBCs that are not labeled in the GRP mouse line. Second, the Brainbow reporter mouse only labels a small population of Cre expressing cells for unknown reasons. Third, the Brainbow reporter expression was so low that antibody amplification was necessary, which then limited the labeled cells to those close to the surface of the brain slices, because of known antibody penetration difficulties. Therefore, we refrained from estimating the density of these connections, because each of these variables reduced the labeling to unknown degrees and we reasoned that extrapolating our rare observations to the total population would be inaccurate.

A paper that investigated UBC connectivity using organotypic slice cultures from P8 mice suggests that 2/3 of the UBC population receives UBC input, based on the observation that 2/3 of the mossy fibers did not degenerate as would be expected after 2 days in vitro if they were severed from a distant cell body (Nunzi and Mugnaini, 2000). It remains to be seen if this high proportion is due to the young age of these mice or is also the case in adult mice. Even if these connections are indeed rare, they are expected to have profound effects on the circuit, as each UBC has multiple mossy fiber terminals (Berthie and Axelrad, 1994), and mossy fiber terminals are estimated to contact 40 granule cells each (Jakab and Hamori, 1988). We have added a comment regarding this point to the discussion.

1. Lack of critical parameters in NEURON model.

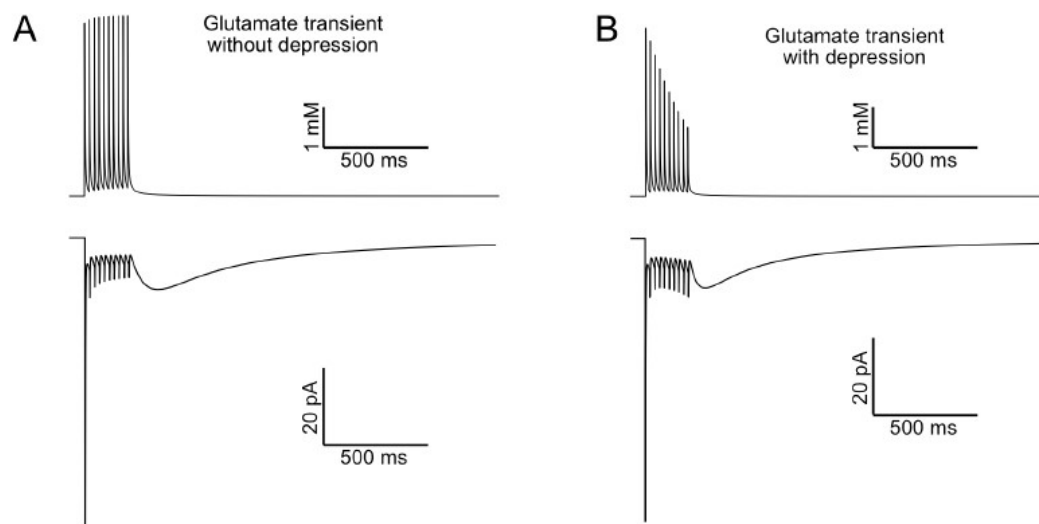
A) The model uses # of molecules of glutamate released as the presumed quantal content, and this factor is constant. However, no consideration of changes in # of vesicles released from single versus trains of APs from MFs or UBCs is included. At most simple synapses, two sequential APs alters release probability, either up or down, and release probability changes dynamically with trains of APs. It is therefore reasonable to imagine UBC axon release probability is at least as complicated, and given the large surface area of contact between two UBCs, the number of vesicles released for any given AP is also likely more complex.

B) the model does not include desensitization of AMPA receptors, which in the case of UBCs can paradoxically reduce response magnitude as vesicle release and consequent glutamate concentration in the cleft increases (Linney et al. 1997 JNeurophysiol, Lu et al. 2017 Neuron, Balmer et al. 2021 eLIFE), as would occur with trains of stimuli at MF to ON-UBCs.

A) The model produces synaptic AMPA and mGluR2 currents that reproduce those we recorded in vitro. We did not find it necessary to implement changes in glutamate release during a train as the model was fit to UBC data with the assumption that the glutamate transient did not change during the train. If there is a change in neurotransmitter release during a train, it is therefore built into the model, which has the advantage of reducing its complexity. UBCs are a special case where the postsynaptic currents are mediated mostly by the total amount of transmitter released. Most of the evoked current occurs tens to hundreds of milliseconds after neurotransmitter release and is therefore much more sensitive to total release and less sensitive to how it is released during the train. Author response image 3 shows the effect of reducing the amount of glutamate released by 10% on each stimulus in the model. Despite a significant change in the pattern of neurotransmitter release, as well as a reduction in the total amount of glutamate, the slow EPSC still decays over the course of hundreds of milliseconds.

Author response image 3.

Effect of short-term depression of neurotransmitter release. A) The top trace shows the glutamate transient that drives the AMPA receptor model used in our study. No change in release is implemented, although the slow tail of each transient summates during the train. The bottom trace shows the modeled AMPA receptor mediated current. B) In this model the amount of glutamate released is reduced by 10% on each stimulus. The duration of the slow AMPA current that develops at the end of stimulation is similar, despite a profound change in the pattern of neurotransmitter exposure.



B) The detailed kinetic AMPA receptor model used here accurately reproduces desensitization, and in fact recovery from desensitization is what mediates the slow ON UBC current. This AMPA receptor is a 13-state model, including 4 open states with 1-4 glutamates bound, 4 closed states with 1-4 glutamates bound, 4 desensitized states with 1-4 glutamates bound, and 5 closed states with 0-4 glutamates bound. The forward and reverse rates between different states in the model were fit to AMPA receptor currents recorded from dissociated UBCs and they accurately reproduced the ON UBC currents evoked by synaptic stimulation in our previous work (Balmer et al., 2021).

1. *Lack of quantification of various electrophysiological responses. UBCs are defined (ON or OFF) based on inward or outward synaptic response, but no information is provided about the range of the key parameter of duration across cells, which seems most critical to the current considerations. There is a similar lack of quantification across cells of AP duration in response to stimulation or current injections, or during baseline. The latter lack is particularly problematic because, in agreement with previous publications, the raw data in Fig. 1 shows ON UBCs as quiescent until MF stimulation and OFF UBCs firing spontaneously until MF stimulation, but, for example, at least one ON UBC in the NEURON model is firing spontaneously until synaptically activated by an OFF UBC (Fig. 11A), and an OFF UBC is silent until stimulated by a presynaptic OFF UBC (Fig. 11C). This may be expected/explainable theoretically, but then such cells should be observed in the raw data.*

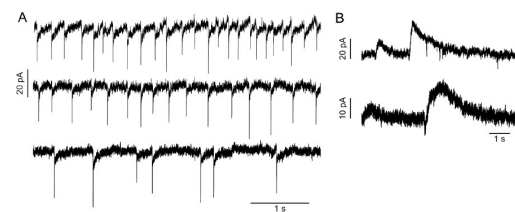
To address this reasonable concern of a general lack of quantification of electrophysiological responses we have added data characterizing the slow inward and outward currents evoked

by synaptic stimulation in GRP and P079 UBCs in the results section and in new panels in Figure 1. We report the action potential pause lengths in P079 UBCs and burst lengths in ON UBCs in the results section. However, we favor the duration of the currents to the length of burst and pause, because the currents do not depend on a stable resting membrane potential, which is itself difficult to determine in intracellular recordings of these small cells. We have added peak times and decay time constants of the slow inward and outward currents in ON and OFF UBCs in the results section and have added new panels to figure 1.

In a series of recent publications that focused on UBC firing, the authors argue that cell-attached recordings are necessary to determine accurately the burst and pause lengths, as well as spontaneous firing rates (Guo et al., 2021; Huson et al., 2023). (The trade-off of these extracellular recordings is that the monosynaptic nature of the input is nearly impossible to confirm.) Spontaneous firing rates were variable within both GRP and P079 UBCs from silent to firing regularly or in bursts, as previously reported for UBCs (Kim et al., 2012; van Dorp and De Zeeuw, 2015). For clarity, we chose to model the GRP UBCs as silent unless receiving synaptic input and P079 UBCs as active unless receiving synaptic input. As the reviewer suggests, we have observed UBCs firing in the patterns similar to those shown in the model UBCs that have input from a spontaneously active presynaptic UBC. In Author response image 4 are some examples.

Author response image 4.

Examples of UBCs that receive spontaneous input. A) Three ON UBCs that had spontaneous EPSCs, suggesting the presence of an active presynaptic UBC. B) Two OFF UBCs that had spontaneous outward currents.



Reviewer #2 (Public Review):

In this paper, the authors presented a compelling rationale for investigating the role of UBCs in prolonging and diversifying signals. Based on the two types of UBCs known as ON and OFF UBC subtypes, they have highlighted the existing gaps in understanding UBCs connectivity and the need to investigate whether UBCs target UBCs of the same subtype, different subtypes, or both. The importance of this knowledge is for understanding how sensory signals are extended and diversified in the granule cell layer.

The authors designed very interesting approaches to study UBCs connectivity by utilizing transgenic mice expressing GFP and RFP in UBCs, Brainbow approach, immunohistochemical and electrophysiological analysis, and computational models to understand how the feed-forward circuits of interconnected UBCs transform their inputs.

This study provided evidence for the existence of distinct ON and OFF UBC subtypes based on their electrophysiological properties, anatomical characteristics, and expression patterns of mGluR1 and calretinin in the cerebellum. The findings support the classification of GRP UBCs as ON UBCs and P079 UBCs as OFF UBCs and suggest the presence of synaptic connections between the ON and OFF UBC subtypes. In addition, they found that GRP and P079 UBCs form parallel and convergent pathways and have different membrane capacitance and excitability. Furthermore, they showed that UBCs of

the same subtype provide input to one another and modify the input to granule cells, which could provide a circuit mechanism to diversify and extend the pattern of spiking produced by mossy fiber input. Accordingly, they suggested that these transformations could provide a circuit mechanism for maintaining a sensory representation of movement for seconds.

Overall, the article is well written in a sound detailed format, very interesting with excellent discovery and suggested model, however, I have some comments/suggestions that may help to improve this manuscript:

- The discovery of UBCs innervating each other and their own subtypes, suggesting the presence of feed-forward networks in the cerebellum, is an incredibly fascinating and exciting finding followed by an intriguing model by authors. However, it is worth considering an alternative model as well. I acknowledge that visualizing such interactions using current tools and methods can be challenging ("The approaches used here were not able to determine the existence of networks of more than 2 UBCs connected one after the other. If present, 3 or more UBCs in series could extend and transform the input in even more dramatic ways. The temporal diversity that UBC circuits generate may underlie the flexibility of the cerebellum to coordinate movements over a broad range of behaviors."). Therefore, if this is the case in which more than 2 UBCs connected one after the other, then an alternative model PERHAPS resembles the basal nuclei, with its direct and indirect circuits, can be considered (maybe a type of circular model). The basal nuclei circuits are also regulated by modulators such as D1 dopamine receptors in the direct pathway, causing depolarization, and D2 dopamine receptors in the indirect pathway, resulting in hyperpolarization upon dopamine activation. This approach could involve using computational models to gain insight into potential alternatives within this pathway (may be a future direction).*

Thank you for this suggestion to consider the potentially similar circuit interactions in the basal nuclei. We will certainly investigate this further as we move forward with modeling the feed-forward networks in the cerebellum.

- GRP UBCs are more densely distributed in lobes VI-IX, while P079 UBCs are more densely distributed in the dorsal leaflet of lobe X in sagittal sections. While the cerebellum is well known for its characteristic stripy pattern, are UBC distributions the same in coronal/transverse section?*

UBCs of different types, based on their expression of specific proteins, have overlapping but somewhat distinct distributions in coronal sections. The densities of calretinin-expressing UBCs are higher within Zebrin II positive zones and form sagittal stripes, whereas the densities of mGluR1-expressing and PLCb4-expressing UBCs vary less but are in their highest densities at the midline (Chung et al., 2009; Sekerkova et al., 2014). The difference noted by the reviewer between the dorsal and ventral leaflets of lobe X are the most distinct that we know of in the GRP and P079 populations.

- The extension of the axons from both subtypes of UBCs show they are long enough to pass several UBCs and even projections are directed toward the white matter (e.g. Fig 9A), suggesting targeting the UBCs or granule cells in other lobules. Is it suggesting UBCs connectivity between different lobules (perhaps longitudinal connectivity)? Is there any observation or information in coronal/transverse section to visualize mediolateral connectivity?*

This is certainly worth exploring in future work. UBCs have been reported to project their axons into and across the white matter (Diño et al., 2000). To our knowledge, whether UBCs project their axons out of one lobule and into another has not been examined.

- The limitation in identifying networks involving more than two sequentially connected UBCs was briefly noted. I suggest including a paragraph describing limitations and discussing the implications of the findings would enhance the overall impact of the research and broaden our understanding of cerebellar function.
- It is a pity that there is no clear conclusion to the discussion of this very interesting study. I suggest providing the key points as a conclusion.

Thank you for these suggestions. Limitations and implications are included throughout the discussion section and we feel that the summary figure and significance statement now sufficiently convey the key conclusions of the study.

- Please make the correction in Figure 2A by relabeling it as IXa, IXb, and IXc to correct the typographical error.

Fixed

- I recommend rotating Figure 7A to align its orientation with the other figures for consistency.

Fixed

Reviewer #1 (Recommendations For The Authors):

Minor comments that should be addressed for clarity:

1. In the NEURON model, why was the reversal potential for the leak conductance and G_{max} for I_h different for the two types of UBCs. Relatedly, why is E_{rev} for GABAB -95mV if E_k is -90mV?

The h-current (I_h) was estimated from a hyperpolarizing current step in both cell types and these data have been added to the result section and as a panel in Figure 1. The conductance of I_h in the model cells were adjusted accordingly, with OFF UBCs having ~3 times that of ON UBCs and approximated the measured voltage sag, as we now describe in the methods section. The reversal potential of the model mGluR2 current (which is based on a model of GABAB) has been fixed.

1. Line 69 justification for their dual genetic approach is a bit too strong: "Paired recordings not possible". It may be difficult, but it is certainly possible.

Reworded

1. Confusing wording, only one stat for two parameters? Line 93: These currents were produced by both mGluR1 and AMPA receptors, as they were blocked by their antagonists JNJ16259685 and GYKI53655, respectively (92.86% {plus minus} 3.25; paired t-test; $P=0.0066$; $n = 9$; 95 mean {plus minus} SEM) (Fig 1D-E).

Reworded

References

Balmer TS, Borges-Merjane C, Trussell LO (2021) Incomplete removal of extracellular glutamate controls synaptic transmission and integration at a cerebellar synapse. eLife

10:e63819.

Berthie B, Axelrad H (1994) Granular layer collaterals of the unipolar brush cell axon display rosette-like excrescences. A Golgi study in the rat cerebellar cortex. *Neuroscience Letters* 167:161–165.

Borges-Merjane C, Trussell LO (2015) ON and OFF unipolar brush cells transform multisensory inputs to the auditory system. *Neuron* 85:1029–1042.

Chung SH, Sillitoe RV, Croci L, Badaloni A, Consalez G, Hawkes R (2009) Purkinje cell phenotype restricts the distribution of unipolar brush cells. *Neuroscience* 164:1496–1508.

Diño MR, Schuerger RJ, Liu Y-B, Slater NT, Mugnaini E (2000) Unipolar brush cell: a potential feedforward excitatory interneuron of the cerebellum. *Neuroscience* 98:625–636.

Guo C, Huson V, Macosko EZ, Regehr WG (2021) Graded heterogeneity of metabotropic signaling underlies a continuum of cell-intrinsic temporal responses in unipolar brush cells. *Nat Commun* 12:5491.

Huson V, Newman LN, Regehr WG (2023) A continuum of response properties across the population of Unipolar Brush Cells in the Dorsal Cochlear Nucleus. *J Neurosci* Available at: <https://www.jneurosci.org/content/early/2023/07/26/JNEUROSCI.0873-23.2023> [Accessed August 15, 2023].

Jakab RL, Hamori J (1988) Quantitative morphology and synaptology of cerebellar glomeruli in the rat. *Anatomy and embryology* 179:81–88.

Kim JA, Sekerkova G, Mugnaini E, Martina M (2012) Electrophysiological, morphological, and topological properties of two histochemically distinct subpopulations of cerebellar unipolar brush cells. *Cerebellum* 11:1012–1025.

Nunzi M-G, Mugnaini E (2000) Unipolar brush cell axons form a large system of intrinsic mossy fibers in the postnatal vestibulocerebellum. *Journal of Comparative Neurology* 422:55–65.

Sekerkova G, Watanabe M, Martina M, Mugnaini E (2014) Differential distribution of phospholipase C beta isoforms and diacylglycerol kinase-beta in rodents cerebella corroborates the division of unipolar brush cells into two major subtypes. *Brain structure & function* 219:719–749.

van Dorp S, De Zeeuw CI (2015) Forward signaling by unipolar brush cells in the mouse cerebellum. *Cerebellum* 14:528–533.