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

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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 transformations may be necessary for numerous functions from controlling eye movements to learning new behaviors.

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 ± SD; n = 13), had a low jitter (defined as the SD of latency; 0.074 ± 0.046 ms; mean ± 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 ± 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 ± 2.4%; paired t-test; P=0.0068; n = 9; mean ± 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 ± 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 ± 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 ± SD; n = 10) (Fig 1I). The outward current was mediated by mGluR2 receptors, as it was blocked by the mGluR2 antagonist LY341495 (96.53 ± 1.79%; paired t-test; P=0.0033; mean ± 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 ± SEM; Fig 1M). We did not observe a significant difference in input

122 resistance between the two cell types (GRP UBCs: 0.56 GΩ ± 0.04, n = 9; P079 UBCs: 0.64 GΩ ± 0.09, n =
123 10; t-test; P = 0.44; mean ± SEM; Fig 1N). Current steps were injected into both types of UBCs to
124 characterize their excitability. There was a significant interaction between the UBC type and response to
125 current steps (mixed model ANOVA, P<0.0001, n = 17). GRP UBCs had a higher firing rate in response to
126 the same level of injected current compared to P079 UBCs, despite their similar input resistance,
127 suggesting that GRP ON UBCs are more excitable and can fire at faster rates (Fig 1O-P). Hyperpolarizing
128 current steps produce a sag that is mediated by the h-current (Ih) in UBCs (Kim et al., 2012). P079 UBCs
129 had a significantly more prominent sag than GRP UBCs, measured in response to a -100-pA current step
130 (peak/steady state; P079: 1.69 ± 0.13, n = 7; GRP: 1.19 ± 0.02, n=14; t-test; P<0.0001; mean ± SEM) (Fig
131 1P-Q). This is consistent with previous work showing that OFF UBCs have a larger h-current than ON
132 UBCs (Kim et al., 2012). In summary, electrophysiological recordings from GRP and P079 UBCs confirm
133 that they are ON and OFF UBC subtypes, respectively.

134

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

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

145 GRP UBCs have been reported to have smaller somas than UBCs that express calretinin
146 (calretinin(+)), a calcium-binding protein that labels the OFF UBC population (Kim et al., 2012; Borges-
147 Merjane and Trussell, 2015). We found that the cross-sectional area of GRP UBCs was significantly
148 smaller than that of P079 UBCs (GRP: 68.75 ± 9.69 μm^2, mean ± SD, n = 92; P079: 74.47 ± 12.53 μm^2,
149 mean ± SD, n = 114, t-test, P = 0.0004), which is consistent with our electrophysiological analysis of their
150 membrane capacitance (Fig 2D). The distribution of GRP and P079 UBCs also supports their identity as
151 ON and OFF UBCs, as the density of mGluR1(+) ON UBCs is higher in the dorsal leaflet of lobe X and the
152 density of calretinin(+) OFF UBCs is higher in the ventral leaflet (Nunzi et al., 2002; Sekerkova et al.,
153 2014).

154

155 ***GRP UBCs are a subset of mGluR1(+) UBCs***

156 Somatodendritic expression of mGluR1 and calretinin defines two separate populations of UBCs
157 (termed Type II and type I respectively (Nunzi et al., 2002)) and correspond to the functionally-defined
158 ON and OFF subtypes (Borges-Merjane and Trussell, 2015). To investigate the expression of mGluR1 and
159 calretinin in GRP and P079 UBCs, we immunohistochemically labeled, imaged, and counted UBCs in both
160 ventral and dorsal areas of lobe X in 4-8 brain slices from triple transgenic mice (n = 4 mice). We found
161 that 98.1% (155/158) of the GRP/Ai9 UBCs expressed mGluR1 in their dendritic brushes (Fig 3A-B). The

162 GRP/Ai9/mGluR1(+) population represented 20.9% (155/741) of the total population of mGluR1(+)
163 UBCs. 0/149 GRP/Ai9 UBCs expressed calretinin in a separate series of slices (Fig 3C-D). Thus, the GRP
164 line labels about 1/5th of the ON UBCs and does not label OFF UBCs.

165

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

167 Using the same triple transgenic mice and immunohistochemical approach described above,
168 92.2% (284/308) of the P079 UBCs were calretinin(+) (Fig 3C-D). The P079 UBCs represent 70.5%
169 (284/403) of the total calretinin(+) population. We were surprised to find, however, that 61.3%
170 (200/326) of the P079 UBCs appeared mGluR1(+). This represents 27.0% (200/741) of the total
171 mGluR1(+) population. These P079/mGluR1(+) UBCs could be differentiated from P079/mGluR1(-) UBCs
172 by their lower expression of mCitrine: the relative labeling intensity of the mGluR1(+) P079 UBCs, after
173 amplification with an anti-GFP antibody, was less than half of the more common mGluR1(-) P079 UBCs
174 (43.2% soma pixel intensity relative to mGluR1(-) P079 UBCs, n = 34 z-planes with at least one of each
175 type, t-test, P = 8.403e-6). In addition, the cross-sectional area of the mGluR1(+) P079 somas was 60.62
176 ± 13.67 μm^2, mean ± SD, n = 52 and significantly smaller than the mGluR1(-) P079 somas (72.48 ± 16.90
177 μm^2 mean ± SD, n = 40, t-test, P = 0.00035). It is likely that the P079 UBCs that express a low density of
178 mCitrine were not able to be visualized in our electrophysiological experiments because their native
179 fluorescence was below the level of detection. Unfortunately, the observation that some mGluR1(+)
180 UBCs are labeled in the P079 mouse line precludes its use for expressing genes in OFF UBCs specifically.

181

182 ***GRP and P079 UBCs are distinct populations in dorsal cochlear nucleus***

183 UBCs are also present in the dorsal cochlear nucleus, a cerebellum-like circuit in the auditory
184 brainstem of mammals. Like granule cells in the dorsal cochlear nucleus, UBCs receive multisensory
185 signals from various sources (Ryugo et al., 2003; Balmer and Trussell, 2021, 2022). In order to test
186 whether our findings about the identity of UBCs labeled in GRP-cre and P079 mice is generalizable
187 across UBC populations, we examined their distribution in the dorsal cochlear nucleus. We found that
188 97.8% (132/135) of the GRP/Ai9 UBCs expressed mGluR1 in their dendritic brushes (Fig 4A-B). The
189 GRP/Ai9/mGluR1(+) population represented 34.2% (132/386) of the population of mGluR1(+) UBCs, and
190 is therefore a subpopulation of about 1/3 of the mGluR1(+) ON UBCs. In a separate series of slices,
191 0/115 GRP/Ai9 UBCs expressed calretinin and is therefore useful for expressing genes in a subset of ON
192 UBCs without expression in OFF UBCs in the dorsal cochlear nucleus.

193 We also found that 89.7% (140/156) of the P079 UBCs were calretinin(+) (Fig 4C-D). The P079
194 UBCs represent 85.9% (140/163) of the total calretinin(+) population. Similar to our results in the
195 cerebellum, we found that 56.7% (127/224) of the P079 UBCs were mGluR1(+). This represents 32.9%
196 (127/386) of the mGluR1(+) population. As in the cerebellum, these P079/mGluR1(+) UBCs were
197 identifiable by their significantly lower intensity of mCitrine after antibody amplification (43.5% intensity
198 compared to mGluR1(-) P079 UBCs, n = 19 z-planes with at least one of each type, t-test, P = 1.352e-6).
199 In summary, in the dorsal cochlear nucleus, the P079 mouse line expresses mCitrine in 86% of the
200 calretinin(+) UBCs and 33% of the mGluR1(+) UBCs and the GRP line expresses Cre in 34% of the

201 mGluR1(+) UBC population and 0% of the calretinin(+) population. Thus, the expression pattern in the
202 dorsal cochlear nucleus of UBCs in these two mouse lines mirrors that of the cerebellum.

203

204 ***Axonal projections between ON UBCs and OFF UBCs***

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

217

218 ***Axonal projections from ON UBCs to other ON UBCs***

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

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

237

238 ***Axonal projections from OFF UBCs to other OFF UBCs***

239 To address whether OFF UBCs project axons to other OFF UBCs, we exploited the observation
240 that the P079 line expresses mCitrine in some, but not all, calretinin-expressing OFF UBCs. Thus, when

241 calretinin is labeled immunohistochemically using a non-green fluorophore, all OFF UBCs are revealed
242 and their brushes can be examined for the presence of connections from mCitrine-labeled P079 OFF
243 UBCs axon terminals. Several examples for OFF to OFF UBC synaptic connections were confirmed
244 anatomically (Fig 8). In some cases, calretinin(+) axon terminals innervated the brushes of P079 UBCs
245 that were also calretinin(+) (Fig 8A-B). In other cases, the P079 UBC terminal contacted calretinin(+)
246 UBCs that were not labeled transgenically in the P079 line (Fig 8C).

247 Brainbow2.1-Confetti reporter mice were crossed with a line that expresses Cre in calretinin(+)
248 neurons after induction by tamoxifen (Taniguchi et al., 2011). Tamoxifen induced the expression of
249 fluorescent proteins in a subset of these calretinin(+) OFF UBCs and were amplified with antibodies as
250 above (Fig 9A). Several examples are shown, confirming that calretinin(+) OFF UBCs provide input to one
251 another (Fig 9B-D). In one of these examples an axon terminal appeared to contact the soma of a
252 postsynaptic UBC (Fig 9C), which has been previously reported to occur between extrinsic mossy fibers
253 and UBC somata (Balmer and Trussell, 2019). In another case, an axon terminal appeared to project a
254 narrow fiber to the edge of the dendritic brush of a postsynaptic UBC (Fig 9D). These presence of large
255 or small contacts implies differences in synaptic strength, suggesting that OFF UBCs may provide strong
256 synaptic signals to one another (Fig 9B) or presumably weaker signals (Fig 9C-D) that are subsequently
257 integrated with other inputs.

258

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

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

273

274 ***Serial ON UBCs multiply burst duration***

275 Presynaptic input to an ON UBC produced bursts of action potentials that multiplied in duration
276 in a circuit with an intermediate ON UBC (Fig 10A-C). Three presynaptic spikes in a burst lasting 19.6 ms
277 produced a burst of 12 action potentials in the first UBC, lasting 358.0 ms, and 35 action potentials in
278 the second UBC, lasting 1175.3 ms, ~60 times longer than the initial presynaptic input (Fig 10A-B). The
279 enhancement of the spiking duration both in the first and second ON UBC were more pronounced with
280 shorter bursts of presynaptic input and began to plateau at longer durations of input (Fig 10C). The

281 plateau in spiking duration occurs because the rebound AMPA receptor-mediated slow EPSC that occurs
282 at the end of synaptic stimulation in ON UBCs reaches a maximum after ~100 ms of synaptic stimulation
283 (Lu et al., 2017).

284

285 ***Intermediate ON UBCs extend the pause in postsynaptic OFF UBCs***

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

298

299 ***Intermediate OFF UBC produce a delayed pause in postsynaptic ON UBC***

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

310

311 ***Serial OFF UBCs produce a delayed burst in postsynaptic OFF UBC***

312 Finally, we considered the case of a connection between two OFF UBCs. In a circuit with a
313 spontaneously firing OFF UBC synapsing onto another OFF UBC (labeled OFF1 and OFF2 respectively, in
314 Fig 11C-D), the tonic glutamatergic input and mGluR2 inhibition prevented spontaneous spiking in the
315 postsynaptic cell. When the intermediate OFF UBC received a burst of presynaptic input, it was inhibited
316 by its own mGluR2 current and therefore stopped firing, thereby disinhibiting the postsynaptic OFF UBC,
317 which then fired after a delay (Fig 11C). Note that this firing would be more pronounced had we
318 incorporated calcium and TRP conductances that contribute to a 'late onset response' in UBCs (Locatelli
319 et al., 2013; Subramaniyam et al., 2014). The duration of the delayed firing in the second postsynaptic

320 OFF UBC depended on the duration of presynaptic input signal to the intermediate UBC (Fig 11D).
321 Moreover, the delay between the last spike in the intermediate OFF UBC and the first spike in the
322 postsynaptic OFF UBC was contingent on the slow decay of the mGluR2 current and was 1082.7 ms in
323 the example in Fig 11C.

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

329

330 **DISCUSSION**

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

332 Transgenic mice were generated that expressed a green fluorescent protein in OFF UBCs and a
333 red fluorescent protein in ON UBCs in the same tissue. Virtually no neurons expressed both fluorescent
334 proteins. Immunohistochemical analysis showed that all GRP UBCs expressed mGluR1 and most P079
335 UBCs expressed calretinin in both cerebellum and dorsal cochlear nucleus. GRP and P079 UBCs had
336 different average soma sizes and distributions in the cerebellum. Whole-cell recordings in acute brain
337 slices confirmed that the GRP UBCs produce excitatory ON responses mediated by mGluR1 and AMPA
338 receptors, and that P079 UBCs produce inhibitory OFF responses mediated by mGluR2. Their membrane
339 capacitance and excitability were so distinct that the distributions of these properties were almost non-
340 overlapping. These results provide further evidence that some UBC subtypes are genetically and
341 functionally distinct, despite the observation that when the entire population of UBCs is examined, their
342 electrophysiological responses form a continuum from entirely excited by glutamate to entirely inhibited
343 by glutamate (Guo et al., 2021; Kozareva et al., 2021).

344 The UBCs in these mouse lines expressed the fluorescent proteins throughout their axons and
345 presynaptic terminals, which revealed for the first time that axon terminals from one UBC subtype
346 contact the dendric brushes of the other UBC subtype. We also showed that UBCs of the same subtype
347 provide input to one another, by labeling GRP or calretinin-Cre UBCs with different fluorescent proteins
348 using the Brainbow2.1-Confetti reporter line. This result was confirmed with additional approaches that
349 utilized the incomplete labeling of mGluR1(+) ON UBCs in the GRP line and the incomplete labeling of
350 calretinin(+) OFF UBCs in the P079 line. Earlier work reported that that mGluR1(+) UBCs and calretinin(+)
351 UBCs form parallel pathways (Nunzi et al., 2002). Our current data extends the previous result by
352 showing that the even more specific subtypes of GRP or P079 UBCs provide input to members of their
353 own subtype. We did not estimate the density of these UBC to UBC connections, because the sparseness
354 of labeling using these approaches made an accurate calculation impossible. Previous work using
355 organotypic slice cultures from P8 mice estimated that 2/3 of the UBC population receives input from
356 other UBCs (Nunzi & Mugnaini, 2000), although it is unclear whether this is the case in older mice. These
357 networks of UBCs could provide the granule layer with a circuit mechanism to diversify and extend the
358 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)$^{26Sortm9(CAG\text{-}tdTomato)Hze}$ (Madisen et al., 2010), Brainbow2.1-Confetti line: Gt(ROSA)26Sortm1$^{(CAG\text{-}Brainbow2.1)Cle/J}$ (Snippert et al., 2010), calretinin-Cre line: Calb2$^{tm2.1(cre/ERT2)Zjh/J}$ (Taniguchi et al., 2011). 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.

439 *Brain slice preparation*
440 For acute brain slice electrophysiology, male and female P079 and GRP-Cre/Ai9 mice were used at ages
441 P21-35. Animals were deeply anesthetized with isoflurane and the brain was extracted under ice-cold
442 high-sucrose artificial cerebral spinal fluid (ACSF) containing the following (in mM): 87 NaCl, 75 sucrose,
443 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 0.4 Na-ascorbate, 2 Na-pyruvate, 0.5 $CaCl_2$, 7 $MgCl_2$,
444 bubbled with 5% CO_2/95% O_2. Parasagittal cerebellum sections 200-300 µm thick were cut with a
445 vibratome (7000smz-2, Campden Instruments) in ice-cold high-sucrose ACSF. Slices recovered at 35°C
446 for 30-40 minutes, in ACSF containing the following (in mM): 130 NaCl, 2.1 KCl, 1.2 KH_2PO_4, 3 Na-HEPES,
447 10 glucose, 20 $NaHCO_3$, 0.4 Na-ascorbate, 2 Na-pyruvate, 1.5 $CaCl_2$, 1 $MgSO_4$, bubbled with 5% CO_2/95%
448 O_2 (300–305 mOsm). Slices were maintained at room temperature (~23°C) until recording. Recordings
449 were performed from lobe X of the cerebellum within 6 hours of preparation.
450
451 *Electrophysiological recordings*
452 Acute brain slices were perfused with ACSF using a peristaltic pump (Ismatec) at 2-3 ml/min, and
453 maintained at 32-34°C with an inline heater (Warner Instruments). ACSF contained 0.5 µM Strychnine
454 and 5 µM SR95531 to block synaptic inhibition. The recording set up was composed of an Olympus BX51
455 fixed-stage microscope with Dodt gradient contrast optics (Dodt et al., 2002), and 4X and 60X (water
456 immersion) Olympus objectives. UBCs were initially identified by soma size or transgenic fluorescence
457 and confirmed by intracellular filling with 5 µM Alexa Fluor 568 or 488 hydrazide sodium salt (Life
458 Technologies). Patch electrodes were pulled with borosilicate glass capillaries (OD 1.2 mm and ID 0.68
459 mm, AM Systems) with a horizontal puller (P1000, Sutter Instruments). Intracellular recording solution
460 contained (mM): 113 K-gluconate, 9 HEPES, 4.5 $MgCl_2$, 0.1 EGTA, 14 Tris-phosphocreatine, 4 Na_2-ATP,
461 0.3 tris-GTP, 0.1-0.3% biocytin, 290 mOsm, pH 7.2-7.25. All recordings were corrected for a -10 mV
462 junction potential. Data were acquired using a Multiclamp 700B amplifier and pClamp 11 software
463 (Molecular Devices). Signals were acquired with 5-10X gain, sampled at 50-100 kHz using a Digidata
464 (1550A, Molecular Devices) analog-digital converter, and low-pass filtered at 10 kHz, with further
465 filtering applied offline. Patch pipettes tip resistance was 5-8 MΩ; series resistance was compensated
466 with correction 20-40% and prediction 50-70%, bandwidth 2 kHz. Membrane potential was held
467 constant at -70 mV in voltage clamp experiments. Electrical stimulation was performed using a
468 concentric bipolar electrode (CBBPC75, FHC) placed in the white matter of the sagittal cerebellar slice.
469 Stimuli were evoked using a stimulus isolation unit (Iso-Flex, A.M.P.I.) delivering 100-250 µs duration
470 pulses of 0-90 V.
471
472 *Immunohistochemistry and imaging*
473 Mice were overdosed with isoflurane and perfused through the heart with 0.01 M phosphate buffered
474 saline, 7.4 pH (PBS) followed by 4% paraformaldehyde in PBS. Brains were extracted from the skull and
475 incubated in 4% paraformaldehyde in PBS overnight at 4˚C. 50 µm thick sections were made on a
476 vibratome (7000smz-2, Campden Instruments). To recover cells that were filled with biocytin during
477 whole-cell recording, acute brain slices were fixed 1-2 h in 4% paraformaldehyde in PBS, followed by
478 storage in PBS. Both floating 50 µm sections and 200-300 µm thick acute slices were treated with the
479 following procedures. Sections were rinsed 3 x 5 min in PBS, blocked and permeabilized in 5% BSA, 2%

480 fish gelatin, 0.2% Triton X-100 in PBS for >1 hr at room temperature. Sections were incubated in primary
481 antibodies overnight in 1% fish gelatin in PBS at 4˚C on an orbital shaker. Primary antibodies included
482 chicken polyclonal anti-GFP (1:2000, Aves Labs, GFP-1020, AB_10000240), goat polyclonal anti-mCherry
483 (1:2000, Sicgen, AB0040, AB_2333093), mouse monoclonal anti-rat mGluR1a (1:800, BD Pharmingen,
484 556389, AB_396404), rabbit polyclonal anti-calretinin (1:2000, Swant, 7697, AB_10000342). Sections
485 were rinsed 3 x 5 min in PBS, followed by secondary antibodies (1:500) and streptavidin (1:2000) that
486 were diluted in 1% fish gelatin in PBS and incubated overnight at 4˚C on an orbital shaker. Secondary
487 antibodies included donkey anti-chicken Alexa Fluor 488 (Jackson ImmunoResearch, 715-545-155,
488 AB_2340375), donkey anti-mouse Alexa Fluor 647 (Jackson ImmunoResearch, 715-605-151,
489 AB_2340863), donkey anti-rabbit Alexa Fluor 647 (Jackson ImmunoResearch, 711-605-152,
490 AB_2492288), donkey anti-rabbit Cy3 (Jackson ImmunoResearch, 711-165-152, AB_2307443), donkey
491 ant-goat Cy3 (Jackson ImmunoResearch, 705-165-147, AB_2307351), streptavidin-Alexa Fluor 647
492 (ThermoFisher Scientific, S21374, AB_2336066). Sections were mounted on microscope slides
493 (Superfrost Plus, Fisher Scientific) and coverslipped with Fluoromount-G (Southern Biotech). Images
494 were acquired a confocal microscope (LSM800, Zeiss) with the Airyscan system that reconstructs super-
495 resolution images from a series of images acquired under spatially structured illumination. Images are
496 single optical planes unless otherwise specified.
497

498 *Image quantification*

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

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

517

518 *Computational modeling*

Single compartment models of ON and OFF UBCs were built using NEURON (Hines and Carnevale, 1997; Carnevale and Hines, 2006) and utilized fast voltage-gated sodium (g_{Na}) and potassium (g_K) conductances to produce action potentials (Destexhe et al., 1994), a slow voltage-gated potassium conductance from a model of cerebellar granule cells ($g_{K\text{-}slow}$) (D'Angelo et al., 2001), and a passive leak conductance (g_{pas}). A hyperpolarization-activated non-specific conductance (g_H) (Kim et al., 2012; Subramaniyam et al., 2014), which was added to ON and OFF UBCs a voltage sag in response to hyperpolarizing current pulses that reproduced those reported in Figure 1 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 µF/cm^2. Tables 1-2 shows the variables used in ON and OFF UBCs.

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.

Table 1- ON UBC conductances

ON UBC		
Modeled conductance	*gmax (S/cm^2)*	*Erev (mV)*
g_{Na}	0.1	50
g_K	0.03	-90
g_H	1e-5	-30
g_{pas}	2e-4	-65
$g_{K\text{-}slow}$	8e-4	-90
Diameter	20 µm	

Table 2- OFF UBC conductances

OFF UBC		
Modeled conductance	*gmax (S/cm^2)*	*Erev (mV)*
g_{Na}	0.1	50
g_K	0.03	-90
g_H	3.1e-4	-30
g_{pas}	7e-5	-62

$g_{K\text{-}slow}$	8e-4	-90
Diameter	27 µm	

545

546

547 **Table 3- Glutamate diffusion at AMPA and mGluR2 receptors**

Glutamate diffusion parameter	*AMPA synapse*	*mGluR2 synapse*
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

548

549 **Table 4- Synaptic conductances**

Synaptic receptor	*gmax (S/cm^2)*	*Erev (mV)*
ON UBC- AMPA	4e-9	0
OFF UBC- mGluR2	1e-8	-90

550

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555

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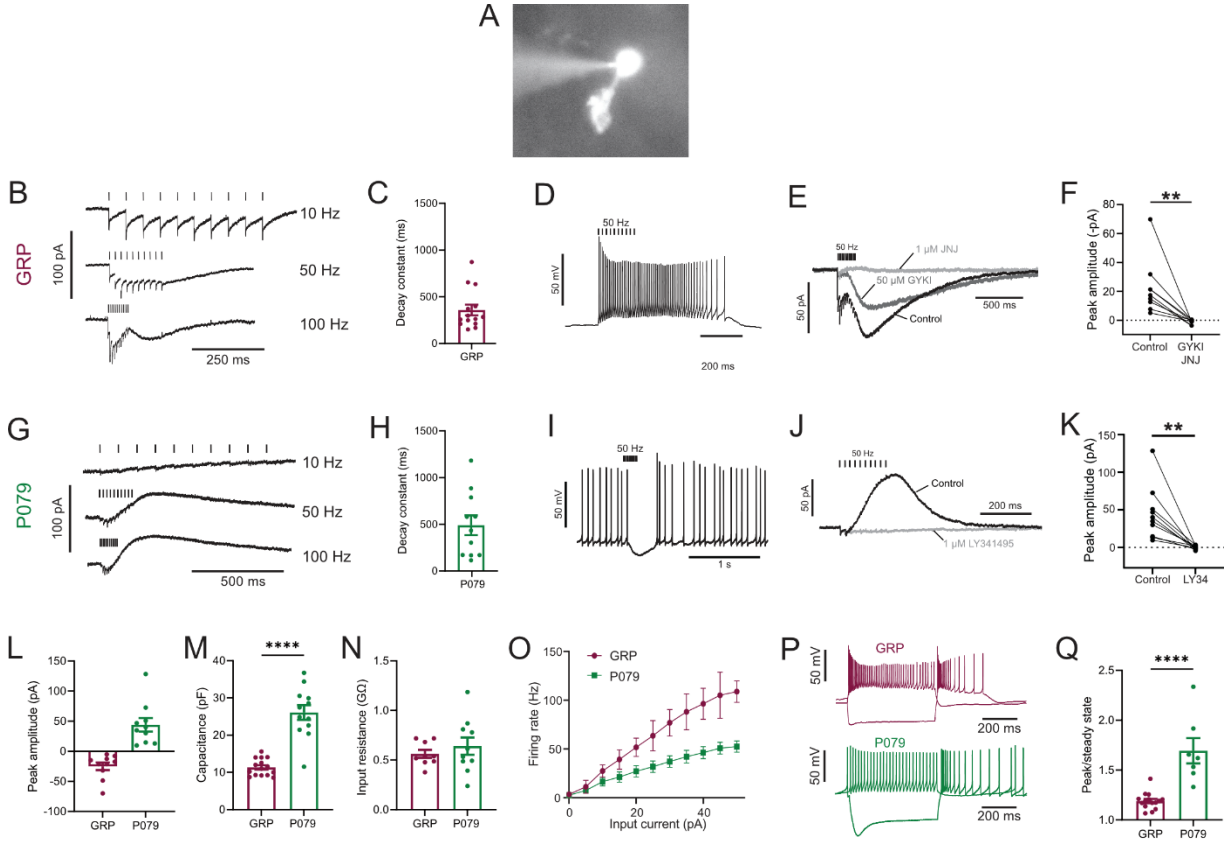
681

682

683 **FIGURES**



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685 *Figure 1- Physiological characterization of GRP and P079 UBCs in acute brain slices*

686 A) Representative image of UBC in whole cell patch clamp recording filled with Alexa Fluor 488.
687 B) In GRP UBCs, mossy fiber stimulation evoked fast excitatory post synaptic currents and a slow inward current
688 that became more prominent with faster stimulation rates.
689 C) Summary of the decay constants of the slow EPSCs generated by a train of 10 stimuli at a 50 Hz rate in GRP
690 UBCs.
691 D) In current clamp, 50 Hz stimulation produced a burst of spikes that outlasted the stimulus.
692 E) The fast EPSCs and part of the slow EPSC was blocked by an AMPA receptor antagonist (50 μM GYKI53655). The
693 remaining slow EPSC was blocked by an mGluR1 antagonist (1 μM JNJ16259685).
694 F) Inward currents in GRP UBCs were consistently blocked by AMPA receptor and mGluR1 receptor antagonists.
695 G) In P079 UBCs, mossy fiber stimulation evoked slow outward currents.
696 H) Summary of the decay constants of the slow outward currents generated by a train of 10 stimuli at a 50Hz rate
697 in P079 UBCs.
698 I) In current clamp, 50 Hz stimulation generated a pause in spontaneous action potential firing in P079 UBCs.
699 J) The outward current was blocked by an mGluR2 antagonist (1 μM LY341495).
700 K) Outward currents in P079 UBCs were entirely blocked by 1 μM LY341495.
701 L) Peak amplitudes of the slow currents were inward in all recorded GRP UBCs and outward in all recorded P079
702 UBCs.
703 M) The capacitance was significantly higher in P079 UBCs compared to GRP UBCs.
704 N) The input resistance was not different between GRP UBCs and P079 UBCs.
705 O) Frequency-intensity curves show that GRP UBCs are more excitable and are able to fire at faster rates than P079
706 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.

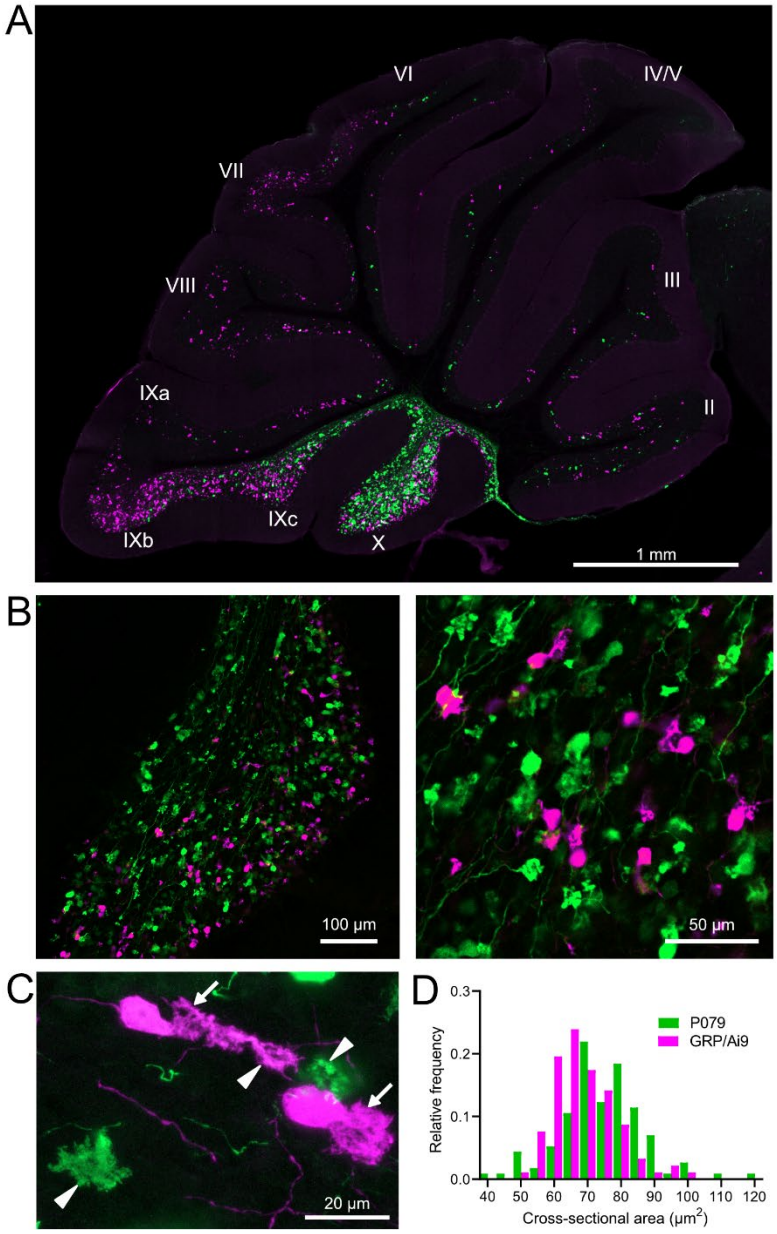


Figure 2- GRP/Ai9/P079 mouse line labels two distinct subsets of UBCs

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.

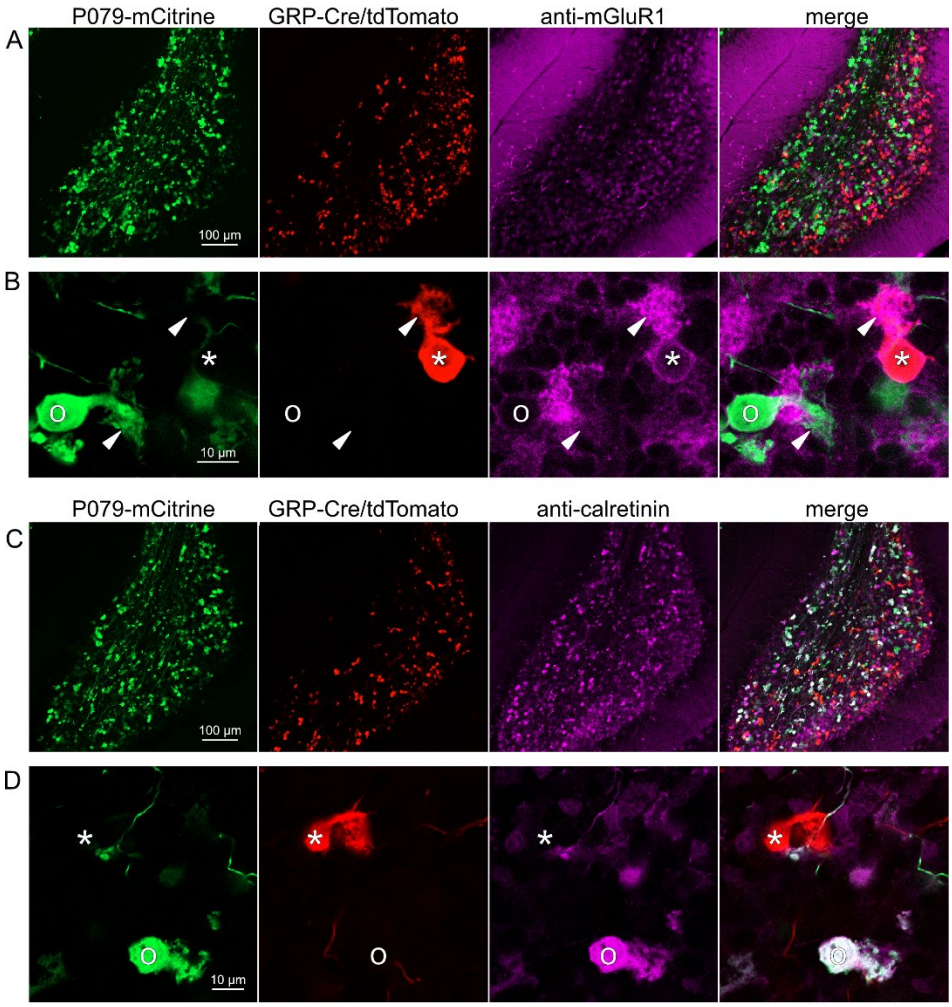


Figure 3- Expression of mGluR1 and calretinin in GRP and P079 UBCs in the cerebellum
A) Sagittal section of lobe X showing genetically expressed mCitrine (green) from the P079 mouse, tdTomato (red) from the GRP-Cre/Ai9 mouse, and immunohistochemical labeling of mGluR1 (magenta). Maximum intensity projections.
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 expresses 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.

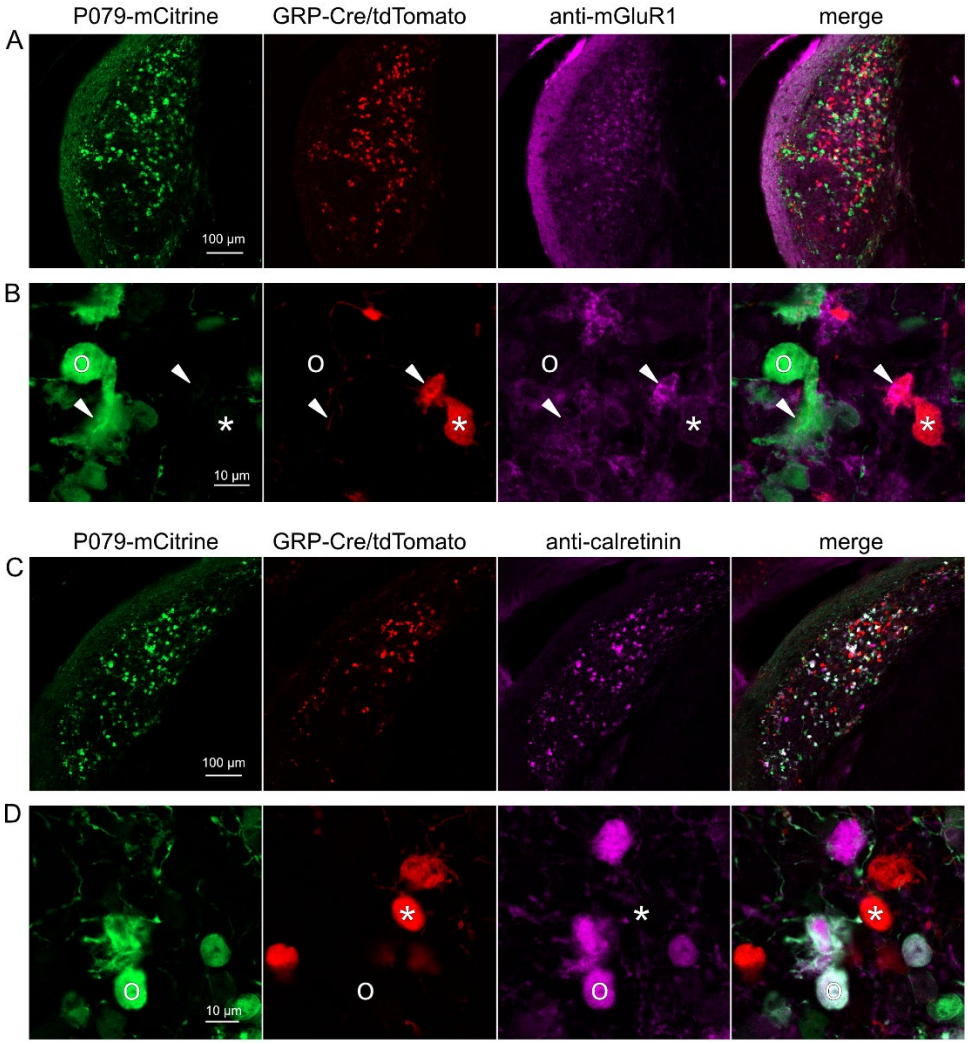


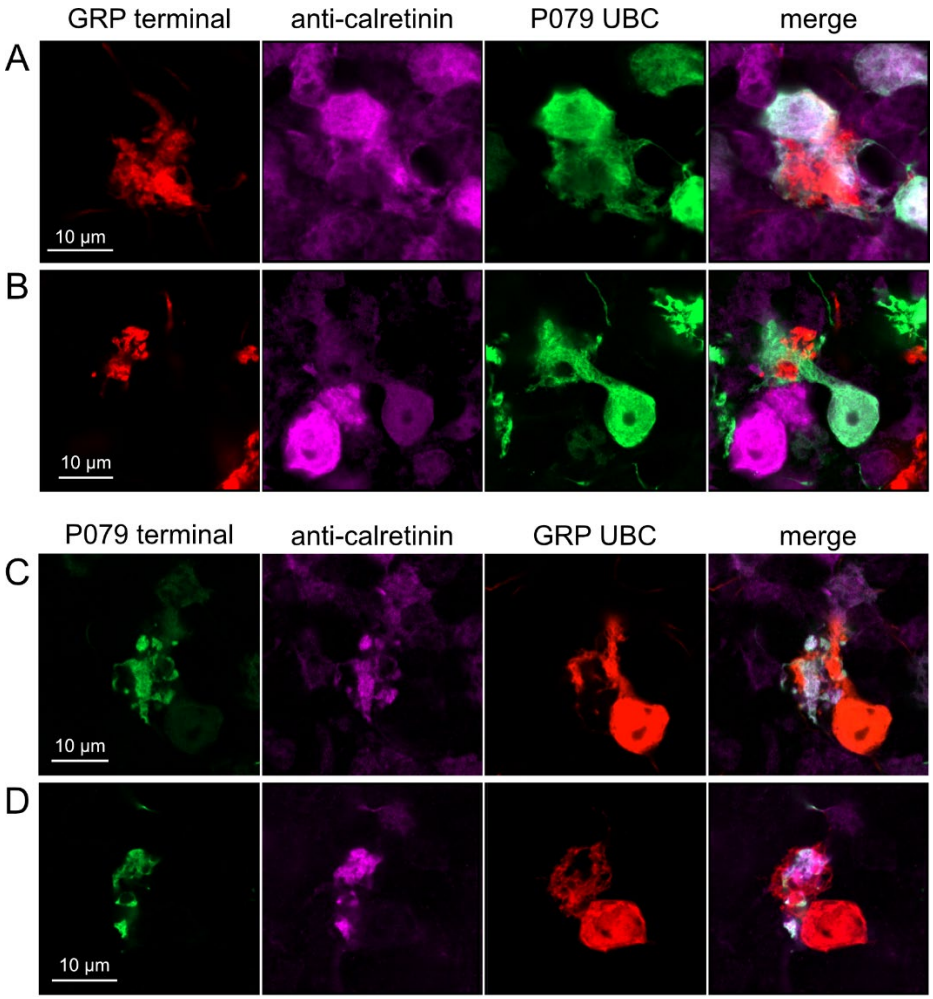
Figure 4- Expression of mGluR1 and calretinin in P079 and GRP UBCs in the dorsal cochlear nucleus

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.

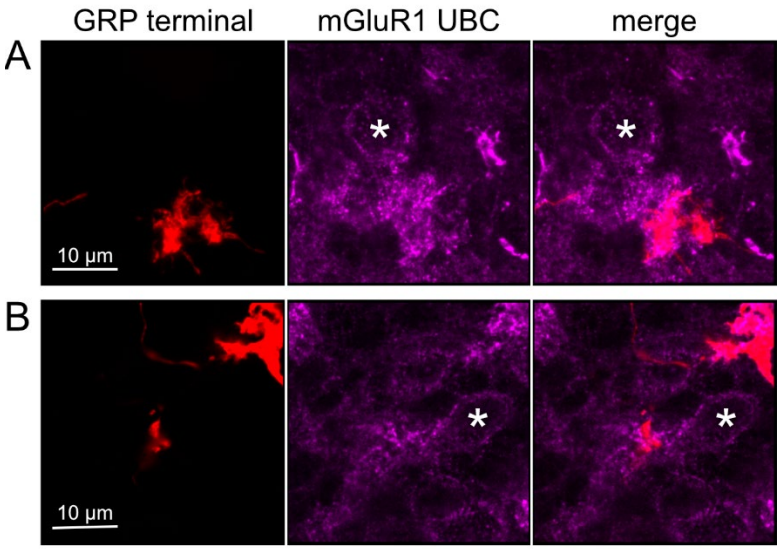
C) Coronal section of the dorsal cochlear nucleus showing genetically expressed mCitrine (green) from the P079 allele, tdTomato (red) from the GRP-Cre/Ai9 alleles, and immunohistochemical labeling of calretinin (magenta). Maximum intensity projections.

755 D) Example of a P079 UBC (soma indicated with O), GRP UBC (soma indicated with *). The P079 UBCs express
756 calretinin in their cytoplasm. GRP UBCs do not express calretinin.
757



758

759 *Figure 5- Anatomical evidence showing axonal projections between ON and OFF UBC subtype*
760 A-B) ON UBC axon terminals (GRP- red) that project to OFF UBCs (P079- green), confirmed to express calretinin
761 (magenta). Single image sections.
762 C-D) OFF UBC axon terminals (P079- green), confirmed to express calretinin (magenta) contacting the dendritic
763 brushes of ON UBCs (GRP-red). Single image sections.
764



765

766 *Figure 6- Anatomical evidence for synaptic connections between ON UBCs*
767 A-B) GRP ON UBC terminals (red) contacting mGluR1(+) ON UBCs (magenta). Somas indicated with *. Single image
768 sections.
769

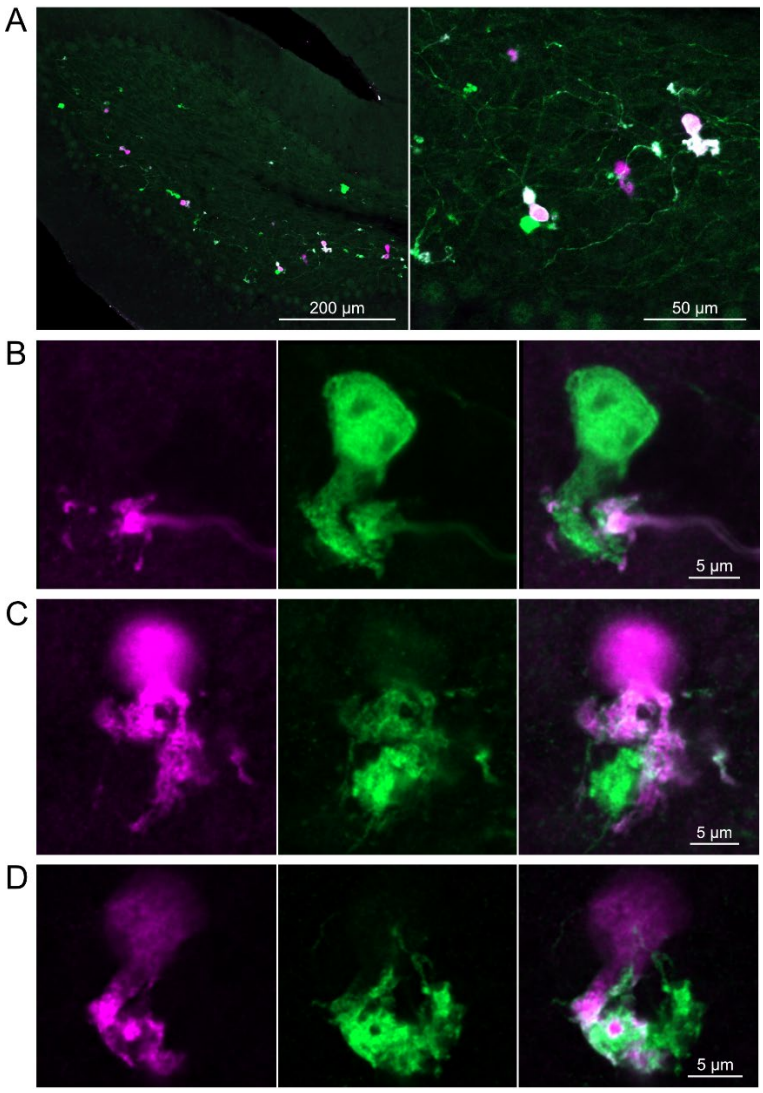


Figure 7- Brainbow reporter labels synaptic connections between GRP ON UBCs
A) GRP ON UBCs expressed fluorescent proteins that were either amplified with the anti-GFP antibody or anti-mCherry antibody. GRP UBCs and their axons and terminals were labeled. Maximum intensity projections.
B) Example of a 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.

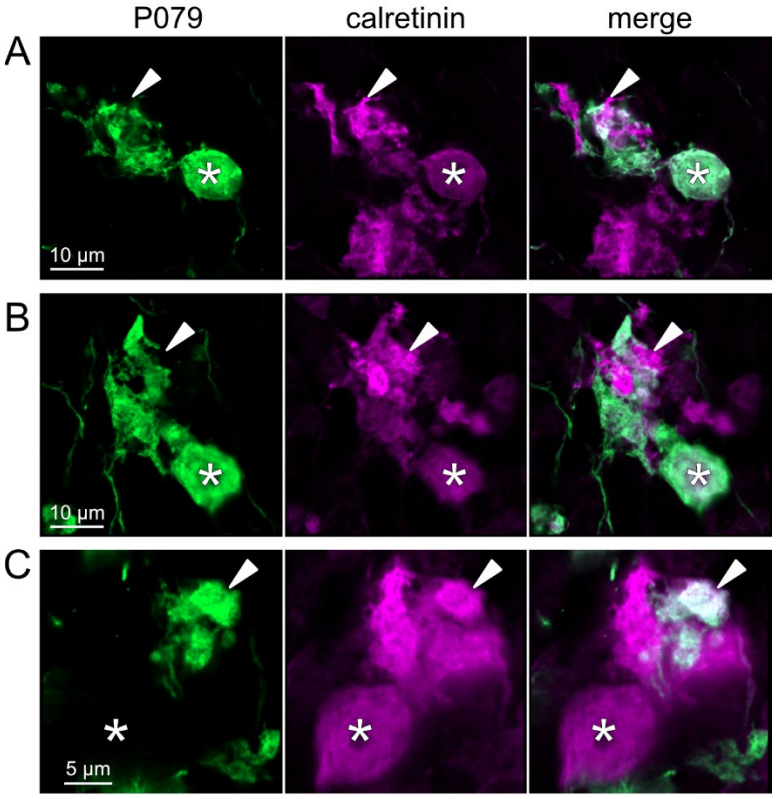


Figure 8- Anatomical evidence for synaptic connections between OFF UBCs

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.

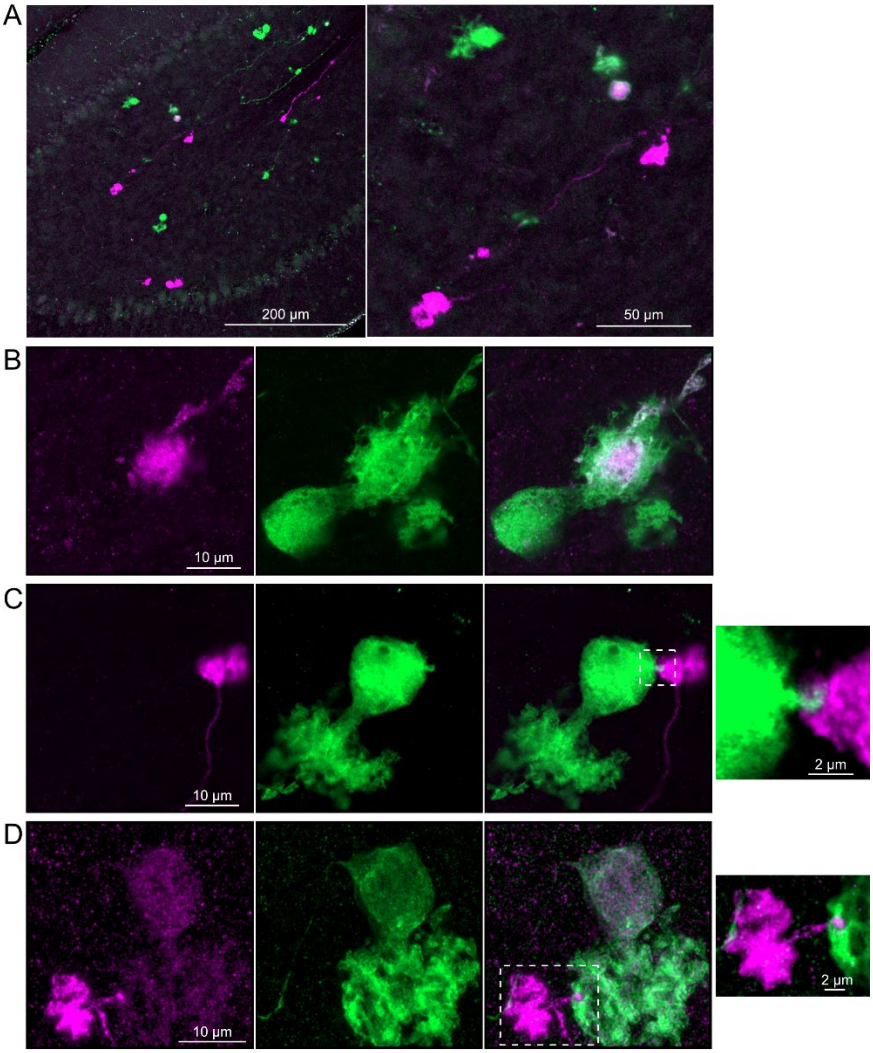


Figure 9- Brainbow reporter labels synaptic connections between OFF UBCs in calretinin-Cre mouse line
A) Calretinin-Cre UBCs expressed fluorescent proteins that were either amplified with the anti-GFP antibody or anti-mCherry antibody, revealing UBCs and their axons and terminals in lobe X. Maximum intensity 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.

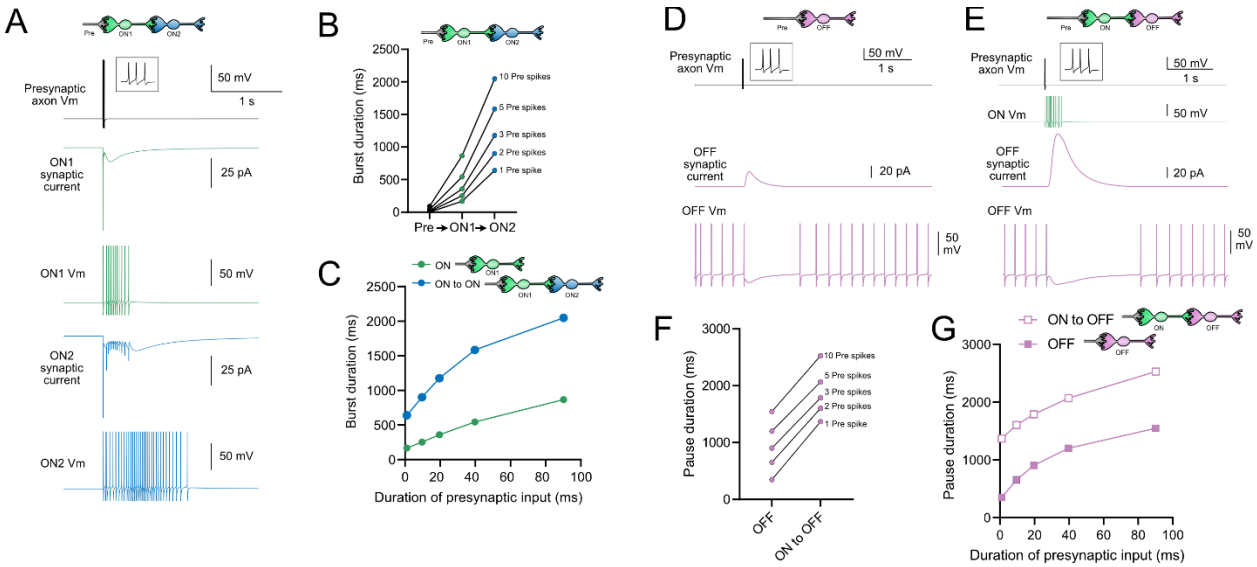


Figure 10- Model predicts that intermediate ON UBCs extend signal

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.

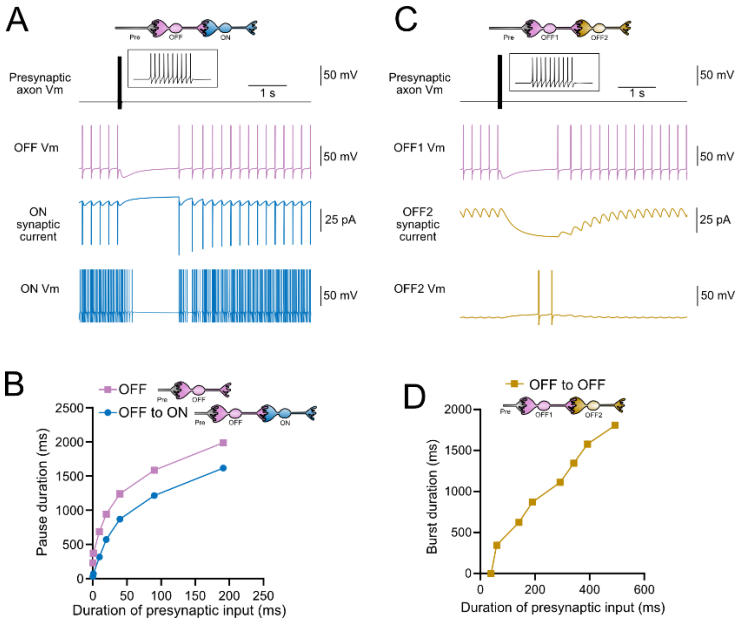


Figure 11- Model predicts that intermediate OFF UBCs delays signals

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.

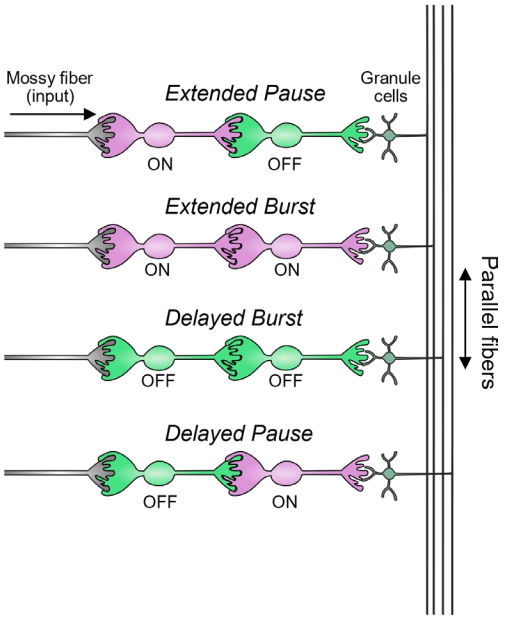


Figure 12- Summary of transformations of spiking patterns in UBC circuits
Four patterns of connectivity between UBC subtypes were anatomically defined and computationally modeled. Intermediate ON UBCs extended the usual pause or burst in firing of the postsynaptic OFF or ON UBC. Intermediate OFF UBCs delayed and inverted the response of the postsynaptic UBC, producing either a delayed burst in OFF UBCs or a delayed pause in ON UBCs.