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Differential Coding Strategies in Glutamatergic and GABAergic Neurons in the Medial Cerebellar Nucleus

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The cerebellum drives motor coordination and sequencing of actions at the millisecond timescale through adaptive control of cerebellar nuclear output. Cerebellar nuclei integrate high-frequency information from both the cerebellar cortex and the two main excitatory inputs of the cerebellum: the mossy fibers and the climbing fiber collaterals. However, how nuclear cells process rate and timing of inputs carried by these inputs is still debated. Here, we investigate the influence of the cerebellar cortical output, the Purkinje cells, on identified cerebellar nuclei neurons in vivo in male mice. Using transgenic mice expressing Channelrhodopsin2 specifically in Purkinje cells and tetrode recordings in the medial nucleus, we identified two main groups of neurons based on the waveform of their action potentials. These two groups of neurons coincide with glutamatergic and GABAergic neurons identified by optotagging after Chrimson expression in VGLUT2-cre and GAD-cre mice, respectively. The glutamatergic-like neurons fire at high rate and respond to both rate and timing of Purkinje cell population inputs, whereas GABAergic-like neurons only respond to the mean population firing rate of Purkinje cells at high frequencies. Moreover, synchronous activation of Purkinje cells can entrain the glutamatergic-like, but not the GABAergic-like, cells over a wide range of frequencies. Our results suggest that the downstream effect of synchronous and rhythmic Purkinje cell discharges depends on the type of cerebellar nuclei neurons targeted.

Key words: cerebellar nuclei; cerebellum; electrophysiology in vivo; Purkinje cells; temporal coding

Significance Statement
Motor coordination and skilled movements are driven by the permanent discharge of neurons from the cerebellar nuclei that communicate cerebellar computation to other brain areas. Here, we set out to study how specific subtypes of cerebellar nuclear neurons of the medial nucleus are controlled by Purkinje cells, the sole output of the cerebellar cortex. We could isolate different subtypes of nuclear cell that differentially encode Purkinje cell inhibition. Purkinje cell stimulation entrains glutamatergic projection cells at their firing frequency, whereas GABAergic neurons are only inhibited. These differential coding strategies may favor temporal precision of cerebellar excitatory outputs associated with specific features of movement control while setting the global level of cerebellar activity through inhibition via rate coding mechanisms.

Introduction
The cerebellum plays a major role in the control, timing, and learning of skilled movements (Ito, 1984; Thach et al., 1992; Spencer et al., 2003; Bastian, 2006) via a constant dialog with the cerebral cortex (Allen and Tsukahara, 1974; Schmahmann and Pandya, 1997; Kelly and Strick, 2003; Glickstein et al., 2011;
The cerebellar nuclei generate the final output of the cerebellum by integrating inputs from inhibitory Purkinje cells (PCs) with those of the mossy and climbing fiber collaterals, the two main inputs of the cerebellum (Gauck and Jaeger, 2000; Rowland and Jaeger, 2005; Bengtsson et al., 2011; Steuber and Jaeger, 2013; Bengtsson and Jörntell, 2014) (see Fig. 1A). CN neurons seem to sum input rate from PCs and encode some part of the movement as a rate code (Thach, 1968; Armstrong and Edgley, 1984; Brooks and Thach, 2011; Abbasi et al., 2017) or follow the temporal pattern of synchronous PC discharges (Heck et al., 2007; De Zeeuw et al., 2008, 2011; Person and Raman, 2011, 2012; Sarnaik and Raman, 2018) or pauses (Shin and De Schutter, 2006; Steuber et al., 2007; Hong et al., 2016) as observed in delay eye-blink conditioning (McCormick and Thompson, 1984; Yeo et al., 1997; Rasmussen et al., 2013; Ten Brinke et al., 2015, 2017). Indeed, disrupting the temporal pattern of PCs or CN neurons discharges strongly affects motor coordination and leads to ataxia (Hoebeek et al., 2005; Walter and Khodakhah, 2006; Walter et al., 2006; Chen et al., 2010; White et al., 2014; White and Siliiote, 2017). However, whether rate-based or temporal coding strategies are associated with a specific task or with specific physiological properties of CN neuron subtypes has not been established yet as the distinction between cell types in the CN remains difficult.

CN neurons have been subdivided into six subgroups, of which five are present in the medial nucleus: glutamatergic (GABAergic) and glycinergic projection neurons, and local GABAergic and/or glycinergic cells (Czubayko et al., 2001; Uusisaari et al., 2007; Alvina et al., 2008; Uusisaari and Knöpfel, 2008, 2011; Bagnall et al., 2009; Dayadnejad et al., 2010; Zheng and Raman, 2010; Blenkinsop and Lang, 2011; Bengtsson and Jörntell, 2014; Husson et al., 2014; Ankri et al., 2015; Najac and Raman, 2015; Canto et al., 2016; Yarden-Rabinowitz and Yarom, 2017). Specific intrinsic biophysical properties (e.g., spike width, afterhyperpolarization shape and duration, firing rate) for at least 4 different CN neuron subtypes have been described using intracellular recordings (Uusisaari et al., 2007; Uusisaari and Knöpfel, 2008, 2011; Bagnall et al., 2009; Ankri et al., 2015; Najac and Raman, 2015; Canto et al., 2016; Yarden-Rabinowitz and Yarom, 2017). However, in recent in vivo studies in awake animals (Bengtsson et al., 2011; Bengtsson and Jörntell, 2014; Brown and Raman, 2018; Sarnaik and Raman, 2018), only glutamatergic projection neurons could be reliably recorded. This illustrates the difficulties in discrimination of neuronal subtypes using extracellular recordings as opposed to the recorded. This illustrates the difficulties in discrimination of neuronal subtypes using extracellular recordings as opposed to the

It's not entirely clear how the text relates to the cerebellum or the subtypes of neurons. It seems to be discussing the complexity of distinguishing between subtypes based on their physiological properties. However, it's not clear how these findings suggest that several coding strategies are multiplexed in CN neurons, depending on the cell-type targeted, allowing for a simultaneous control of firing rate and temporal discharge.
Optogenetic stimulation in awake animals (GAD2-IREs-Cre and VGLUT2-IREs-Cre). For GABA and GLUT neuron optotagging, animals were surgically implanted with 2 mm length optical fiber (Ø 105 μm, ThorLabs) in the cerebellar cortex, above the CN. A 595 nm wavelength LED driven by a DC2100 controller (M595F2, ThorLabs) was connected to the optical fiber. Each episode (2 s duration) of stimulation consisted of a train of pulses (5 ms pulse duration) at 100 Hz for 125 ms repeated at least 10 times every 5–10 s. We only included neurons that responded to optogenetic stimulation with a short latency (<20 ms) (Klapoetke et al., 2014; Mardiniy et al., 2018). The latency to optogenetic stimulation was defined as the first epoch that exceeded 3 times the SD of the baseline firing rate. Assessment of firing rate change was based on peristimulus time histogram (constructed with at least 10 trials).

Tetrode recordings in anesthetized L7-ChR2(H134R)-YFP mice. Mice were deeply anesthetized under urethane (1.9 g/kg i.p.). Tracheotomy was performed to facilitate breathing. Mice were placed in a stereotaxic frame (Narashige). Body temperature was maintained using a heating blanket (CWE, TC-1000) and monitored by a rectal probe. Craniotomy was then performed to uncover the dorsal vermis and paravermis of the cerebellar cortex. After incision of the dura, the tissue was constantly moisturized with a saline solution (NaCl 9 g/L). PCs and CN cells were recorded using custom-designed silicon probes (2×16 channels, Atlas Neuroengineering) motorized with a manipulator (Luigs and Neuman, SM5). Each probe (tetrode) was composed of 4 shanks (150 μm between shanks) bearing 4 circular iridium oxide recording sites (15 μm diameter) separated by 20 μm in a diamond shape. One probe was lowered into lobules IV and V in the vermis to record PCs. The second one was lowered through lobule VI in the paravermis to record CN neurons. Both probes were positioned using motorized micromanipulators (Luigs and Neumann).

Data acquisition was performed using a portable 32 channel acquisition system (ME32-FAI-μA, MultiChannel Systems) at 20 kHz sampling rate and 5 KHz low-pass filtering. The probes were painted with DiI for post hoc reconstruction of the recording sites.

Juxtacellular and multichannel recordings in awake GAD2-IREs-Cre and VGLUT2-IREs-Cre mice. A vertical skin cut was applied to expose skull. The skull was pretreated with Optibond All-in-one (Kerr), and a 5.5 × 4.0 mm custom-made pedestal was attached to the skull with Charisma (Heraeus Kulzer). Mice were allowed to recover at least 2 weeks after surgery and before recordings. Before electrophysiology experiments, a small cranietomy (Ø = 2.0 mm) was performed on the skull over recording targets. Mice were head fixed but allowed to move on a cylinder treadmill. GABA and GLUT cells were recorded with glass capillary (Ø = 2 mm, tip = 2 μm, filled with 2 M saline; n = 15 cells) electrodes as previously described (Ten Brinke et al., 2017) or with a 64-channel silicon probe (assy 77H-H2, Cambridge NeuroTech; n = 2 cells). The electrode was motorized with a manipulator (Luigs and Neuman, SM7) and penetrated the cerebellar nuclei with an angle of 35° from vertical axis. For glass capillary recordings, neuronal electrophysiological signals were amplified (Axon Instruments CNS, MultiClamp 700B, AutoMate Scientific US) and digitized at 20 kHz with an Axon Instruments Digitap data acquisition system. For silicon probe recordings, signals were amplified and digitized at 20 kHz with an Intan evaluation system (RHD2000, Intan Technology).

Data analysis. Spike sorting was performed using the open source software OpenElectrophy (RRID:SCR_000819) (García and Fourcaud-Trocme, 2009). Before spike sorting, stimulation artifacts were removed as described by Rowland and Jaeger (2008). Raw data for each tetrode were high-pass filtered (200 Hz) using a Butterworth filter. A manual threshold was determined by visually inspecting the signal, after which peaks crossing the threshold were detected. Spike waveforms were collected (±1 ms around the peaks) and classified after normalization at the positive peak time and amplitude using principal component analysis (PCA) using the scikit-learn package (Pedregosa et al., 2011). Spike waveforms were sorted into independent units using Gaussian Mixture Models based on the expectation maximization algorithm (Python scikit-learn package). Individual units were always confirmed by visual inspection, and we took a conservative approach and kept only well-separated clusters.

Average spike waveforms from all recorded units (cells) were classified using PCA followed by Hierarchical Clustering on Principal Components (Lé et al., 2008) in R statistical software (R Core Team, 2015) based on parameters extracted from the average spike waveforms (see Fig. 2D). Other analyses were performed using custom routines written in Python. Instantaneous firing rate (IFR) was calculated as the inverse of the interval between consecutive spikes.

Immunofluorescence. Animals were killed and perfused transcardially with 50 mL saline, followed by 100 mL of 4% PFA/0.1 M PBS, pH 7.4. Then, the brain was removed immediately and postfixed for 2 h at room temperature. The fixed brain dehydrated in 10% sucrose overnight at 4°C, then was embedded in 10% sucrose/12% gelatin, and further dehydrated in 30% sucrose solution. Next, transverse sections (40 μm) were cut with a microtome (Leica Microsystems, CM1950). Sections were incubated for 24 h with rabbit-anti-Gad65/67* (1:1000, Millipore AB1511; RRID:AB_90715). Next, sections were incubated for 12 h in 5% normal donkey serum-0.4% Triton X-100—0.01 μM PBS with A488-conjugated donkey anti-rabbit lgG (1:200, Jackson ImmunoResearch Laboratories, lot #711—545-152). All incubations were performed at 4°C with continuous gentle agitation and followed by 3 × 10 min washes in PBS. Eventually, sections were stained with DAPI and mounted with VECTASHIELD Mounting Medium (Vector Laboratories, lot #H-1000).

Statistical analyses. Spike time-locking was assessed by detecting a nonuniform distribution of spikes in the interval between successive stimulations during periodic pulse trains using the Rayleigh test (using Scipy Python package) (Millman and Aivazis, 2011; see also Najac and Raman, 2015). Kolmogorov–Smirnov tests on IFR distributions (see Fig. 6) were performed with a bin of 5 Hz. Statistical tests between groups are described in Results.

Results

Optogenetic excitation of PCs in lobule IV-V inhibit CN neurons in the medial nuclei

Using the L7-ChR2(H134R)-YFP strain of mice in which ChR2 was specifically expressed in PCs, we recorded the activity of CN neurons in anesthetized mice in the medial nucleus while optically stimulating PCs in lobules IV and V. To facilitate probe positioning in the medial CN in CD1 mice for which a complete stereotaxic atlas is not available, PC tract was traced using AAV1/2-GFP or DiI injections (N = 2 animals). We determined that, in CD1 mice, the caudodorsal medial nucleus can be targeted by inserting the silicon probe 600 μm posterior to lobules IV and V at 2.8—3.3 mm depth (Fig. 1B, bottom; n = 25 recordings sites, N = 13 animals). Electrode positioning and blue light power were physiologically assessed by simultaneously recording multunit activity in PCs and in CN neurons (Fig. 1C).

Light-induced (5 ms pulses at 40, 65, and 115 Hz) PC discharge was assessed in a group of PCs (n = 10) using multunit recordings in the PC layer in the illumination area (Fig. 1D,E; see Materials and Methods). No depolarization block was observed, and the averaged firing rate increased compared with baseline (mean ± SD, baseline: 46.5 ± 33.8 Hz; 40 Hz: 65.6 ± 40.1 Hz; 65 Hz: 65.2 ± 36.9 Hz; 115 Hz: 57.1 ± 34.3 Hz). Although individual PCs did not follow the stimulation at the highest frequency, by building interspike interval (ISI) histograms (Fig. 1D) and the histogram of the spike times between 2 pulses of light, we observed that 8 of 10 PCs were time-locked (i.e., synchronized) to PC illuminations at high frequency (Fig. 1E). Therefore, considering that illumination activates tens of PCs (see Chaumont et al., 2013), we estimate that the PC population can follow illumination at all frequencies tested. We then used tetrode recordings and isolated 39 individual cells based on their averaged spike waveforms in the caudodorsal medial nucleus (N = 13 animals; Fig. 2A–C).
Discriminating GLUT versus GABA neurons

To classify CN cells, a PCA was performed on parameters of the averaged spike waveforms (Fig. 2D, E), followed by hierarchical clustering based on the first two principal components (see Materials and Methods). The hierarchical clustering identified two groups of averaged waveforms (Group 1: 22 cells; Group 2: 17 cells; N/H11005 = 13 animals). The two groups could be discriminated by the decay phase of the spike waveform (e.g., the negative half-width; Fig. 2D, F; Table 1), with Group 1 having a faster action potential than Group 2 neurons (action potential width Group 1: 0.47 ± 0.08 ms vs Group 2: 0.65 ± 0.11 ms). Next, we analyzed the spike statistics of individual cells belonging to these two groups separately and found that Group 1 and Group 2 cells had significantly different firing rates (Fig. 2G; mean Group 1: 63.9 ± 23.3 Hz; mean Group 2: 23.2 ± 19.4 Hz; Wilcoxon rank test, p = 1.4 × 10⁻⁷), whereas the spike train regularity was similar for the two groups (mean CV Group 1: 0.58 ± 0.42 vs Group 2: 0.59 ± 0.35; Wilcoxon rank test, p = 0.37; mean CV2 Group 1: 0.43 ± 0.22 vs Group 2: 0.42 ± 0.24; Wilcoxon rank test, p = 0.36). Only 3 pairs of cells belonging to different groups were recorded on the
same tetrodes, and no pairwise spike correlation was observed between the cells of these pairs.

In a separate series of experiments in awake animals, we assessed whether the GLUT versus GABA identity of the cells could account for this segregation in two groups. Specific expression of Chrimson in GLUT or GABA CN neurons was obtained by injecting AAV9-hSyn-FLEX-ChrimsonR-tdTomato virus in the CN of VGLUT2-cre and GAD2-cre mice, respectively (Fig. 3). CN neurons were extracellularly recorded and opto-tagged in awake animals (see Materials and Methods). Opto-tagging (GLUT vs GABA) was confirmed by an instantaneous increase in firing rate under blue light illumination (mean ± SD delay after light onset GLUT: 6.8 ± 5.3 ms, n = 6; GABA: 5 ± 4.2 ms, n = 11; Fig. 3A). We determined the averaged spike waveforms of the GLUT and GABA neurons and measured the 5 most relevant parameters that were identified by PCA in the dataset recorded earlier using tetrodes (Fig. 3; Table 1; n = 15). GLUT and GABA neurons were discriminated by the same set of action potential waveform parameters that earlier discriminated the neurons recorded in anesthetized animals using tetrodes into two groups (compare

Figure 2. Tetrode recordings identify two distinct groups of CN neurons. A. Example of unit isolation: raw signal on the 4 recording sites of a single tetrode. Orange and blue dots represent spikes from the 2 units isolated by the PCA (Materials and Methods). B. Projection of the spikes from the 2 units against the first 2 principal components. C. Averaged spike waveforms of the 2 units on the 4 recording sites. D. Averaged waveforms for all the recorded units were classified using PCA followed by a hierarchical clustering on principal components (HCPC). Description of the measured parameters for the PCA and projection on the first two principal components (top right). Onset time, Time point where the spike waveform reached 5% of its positive peak; P. Rise slope, positive 10%–90% rise time; P. Half-width, spike duration at 50% of the positive peak; N. Peak amplitude, negative peak; Negative Peak time, time of negative peak; N. fall slope, 10%–90% fall time; N. Half-width, spike duration at 50% of the negative peak; N. Decay time, spike duration from negative peak to 10% of the negative peak; End time, time point at 5% of its negative peak. E. Ascending hierarchical tree highlighting 2 groups of action potential waveforms. F. Superimposed averaged spike waveforms of all units normalized to the maximum amplitude (left); mean of the averaged spike waveforms for the 2 groups (Group 1, red, n = 22; Group 2, blue, n = 17) (right) identified. Shaded area represents SD. G. Boxplot of firing rates, CV and CV2 for the 2 groups. Wilcoxon rank test, Firing rate: ***p = 1.4 × 10^{-7}; CV: p = 0.37; CV2: p = 0.36.
Also, we confirmed that GLUT cells had a higher firing rate, comparable with the Group 1 neurons (mean: 66.6 ± 39.7 Hz; n = 6) than GABA cells (mean: 29.1 ± 23 Hz; n = 11; Fig. 3D; p = 0.012, Wilcoxon rank test), which corresponded to the Group 2 neurons. No difference was observed in spike train regularity (Fig. 3D; mean CV = 0.76 ± 0.22 vs 0.90 ± 0.42; Wilcoxon rank test, p = 0.28; mean CV2, 0.61 ± 0.16 vs 0.64 ± 0.15; Wilcoxon rank test, p = 0.42). These results demonstrate that GLUT and GABA neurons can be reliably discriminated solely by their spike waveform in extracellular recordings in awake animals. For these reasons, we will refer to the Group 1 neurons recorded in anesthetized L7-ChR2(H134R)-YFP mice using tetrodes as GLUT-like neurons, and Group 2 neurons as GABA-like neurons.

### Impact of synchronous PC firing on firing rate of GLUT-like and GABA-like neurons

In the L7-ChR2(H134R)-YFP mice, we combined tetrode recordings with a set of PC illumination protocols: periodic pulse trains at 40, 65, and 115 Hz (Fig. 4A). Most CN neurons (37 of 39) were inhibited by synchronous activation of PCs (Fig. 4B). The amount of inhibition did not depend on the baseline firing rate of the CN cells (GLUT-like: r = −0.18, n = 22, p = 0.40; GABA-like: r = −0.06, n = 17, p = 0.83). When the stimulation frequency was increased (trains at 40, 65, and 115 Hz), the mean firing rate of CN neurons monotonically decreased for both GLUT-like and GABA-like cells (Fig. 4B). These results suggest that, while individual PCs do not strictly follow optogenetic stimulation at high frequency (115 Hz, see above), PC population
controls similarly GLUT-like and GABA-like neuron firing rates at all tested frequencies.

GLUT-like neurons are synchronized and entrained by synchronous PC inputs
We observed that some CN cells discharges could be time-locked to PC inputs during trains of stimulation, whereas others fired randomly during stimulation, albeit at lower rates (see two example cells in Fig. 5A–D vs Fig. 5E–H). Indeed, ISI histograms estimated during illumination displayed a peak at the illumination frequency (Fig. 5C). We then computed spike time-locking by building the histogram of the spike times between 2 illuminations during trains of stimulation pulses (Fig. 5B,F). The Rayleigh test for histogram uniformity demonstrated that spiking in GLUT-like neurons was time-locked (i.e., synchronized) to PC illuminations (p < 0.01 indicating nonuniform distribution for 22 of 22 cells at 40 Hz, 19 of 22 cells at 65 Hz, and 17 of 22 cells at 115 Hz). By contrast, most GABA-like neurons spiked with uniform probability all through the interstimulus interval, indicating that these neurons were not synchronized to PC spiking input >40 Hz (13 of 17 at 40 Hz, 10 of 17 at 65 Hz, 14 of 17 at 115 Hz). To further quantify the spiking activity during trains of illuminations, we calculated the power spectra of the autocorrelogram (restricted to the time of illumination) for each cell (Fig. 5D,H). The spectra of spiking activity of CN neurons revealed that the GLUT-like neurons output followed the dominant input frequency (Fig. 5D,I) in that they were entrained by synchronous PC activity. By contrast, the spectra of GABA-like neurons was independent of the input frequencies (Fig. 5H), indicating that GABA-like neurons were not entrained by the PC neurons, at least not at the three different frequencies used in our experiments (compare Fig. 5D,H). Consistent with this, the power spectral density values at the stimulation frequencies were significantly larger for GLUT-like neurons than for GABA-like neurons (Fig. 5F; 40, 65, and 115 Hz p = 2.6 × 10−6, p = 3.7 × 10−5 and p = 0.00076, Wilcoxon rank test).

Because fixed-frequency stimulations are physiologically unlikely, we also illuminated the PCs in an irregular manner (illumination times drawn according to a homogeneous Poisson process with average rate of 40 Hz). Because of the irregular illumination, the instantaneous rate of PCs discharges varied from 5 to 210 Hz (Fig. 6A). The distributions of IFRs for two examples of GLUT-like and GABA-like neurons during illumination and baseline are shown in Figure 6B. To test whether CN neurons were also entrained by irregular activity of PCs, we compared (using Kolmogorov–Smirnov test) the distributions of IFR of CN neurons during stimulation and the distribution of instantaneous stimulation rate of PCs. If the CN cells would be entrained by PCs, then we should expect that the two distributions will be statistically similar. In 14 of 22 GLUT-like neurons, no differences (p > 0.05) were observed between the IFR histogram during illumination and the histogram of instantaneous stimulation frequencies, whereas in the 8 remaining cells, p was <0.05 (Fig. 6B,C). This indicates that a majority of GLUT-like cells (63%) followed the PC discharges during stimulation (Fig. 6C). Conversely, in 15 of 17 GABA-like neurons, the IFR histogram during illumination was significantly different from the histogram of instantaneous stimulation frequencies (p < 0.05; for 2 GABA-like neurons p > 0.05; Fig. 6C), showing that most of these neurons did not follow the temporal PC discharge pattern during stimulation. Together, our findings demonstrate that most of the GLUT-like neurons were able to follow PC discharge in a temporally precise manner (fixed frequencies: 100% at 40 Hz, 86% at 65 Hz, 77% at 115 Hz; 63% during irregular stimulation).

Discussion
Using in vivo extracellular recordings of CN neuronal activity in anesthetized and awake mice, we showed that presumptive GLUT and GABA neurons can be discriminated using simple features of extracellular spike waveforms and respond differently to PC inputs.

Evidence for the discrimination of GLUT versus GABA neurons in CN
In our first series of experiments, using tetrodes in anesthetized mice and without prior information on cell type identity, we adopted a conservative approach, leading to a dataset of 39 identified CN cells, with only few multiple cells recorded on a single tetrode. This difficulty is likely the result of the spatial organization of the electrodes combined with the facts that CN neurons are densely packed. Silicon probes have flat shanks that do not allow a 3D positioning of the recording sites as opposed to, for
Figure 5. Differential temporal coding by GLUT-like and GABA-like neurons. Nuclear neuron discharges during trains of PC illumination at 40, 65, and 115 Hz. A–D, Example of GLUT-like neuron. A, Raw trace from a GLUT-like unit during illumination (blue bars) at 65 Hz. B, Histogram of the spike latency from stimulation onset. Gray bar represents the position of the subtracted stimulation artifact. C, ISI histograms at 40, 65, and 115 Hz. D, Power spectra of the unit autocorrelogram at the same frequencies. E–H, Same analyses for a GABA-like neuron. I, Boxplot of averaged power density for GLUT-like and GABA-like neurons at 40, 65, and 115 Hz. White line indicates median. Box limit indicates first (Q1) and third (Q3) quartiles. Whiskers represent 10th and 90th percentiles. Wilcoxon rank tests: ***p = 2.6 × 10^{-6}, ***p = 3.7 × 10^{-5}, ***p = 0.00076.
example, quartz electrodes, which have a tetrahedral architecture (Gao et al., 2012), limiting the discriminating power of such tetrodes. Nevertheless, PCA followed by a hierarchical clustering analysis allowed us to identify two clusters of spike waveforms (Group 1: narrow action potential width; Group 2: broader action potential), forming two populations of neurons with different mean firing rates. In a second series of experiments in awake animals, we specifically expressed Chrimson in GLUT and GABA neurons; and using glass pipettes and tetrodes, we identified the same properties as in the aforementioned Group 1 and Group 2 cells: GLUT neurons had a narrow spike width and high firing rate, whereas GABA neurons had a broader spike width and a low firing rate as opposed to the neocortex in which pyramidal GLUT cells have broader spikes and GABA cells have narrow spikes (Barthó et al., 2004). These findings are in agreement with in vitro intracellular recordings (Uusisaari et al., 2007). In whole-cell current-clamp configuration, Uusisaari et al. (2007) found that non-GABAergic big cells (presumably the glutamatergic projection cells) had narrow action potential widths and higher firing rate compared with GABAergic cells, having larger action potential widths and lower firing rate. Although glycinergic projection cells have also narrow spikes and similar maximum firing rates as glutamatergic projection neurons (Bagnall et al., 2009), they were identified in the ventral part of the rostral medial nucleus,
whereas our recordings were mainly located in caudal dorsal areas of the nucleus (Fig. 1B). However, given that smaller glutamatergic neurons generally fire at low frequency (Uuisisaari et al., 2007) and that our PCA analysis was based on a limited dataset, we cannot rule out that some of the cells that were recorded using tetrodes may belong to other classes of CN neurons. Still, as independent analyses of two different datasets recorded in anesthetized and awake animals identified similar groups based on biophysical parameters (spike waveform and firing rate), it is the most parsimonious to postulate that most of Group 1 GLUT-like cells are glutamatergic projection neurons of the cerebellum. We then note that, in all recording conditions, including acute brain slices in vitro (Uuisisaari and Knöpfel, 2011), anesthetized and awake animals, the same biophysical segregation (spike waveform and mean firing rate) between GABA and GLUT neurons has been observed. This indicates that these properties must be a hallmark of CN physiology as they were observed in different physiological conditions.

Nonglutamatergic cells can be split into GABAergic nucleo-olivary projection neurons, local GABA/glycinergic, and nucleocortical glycnergic neurons (Uuisisaari and Knöpfel, 2011). Glycinergic nucleocortical cells are probably mostly silent (Ankri et al., 2015) and nucleo-olivary neurons are most prominently distributed in the ventrolateral part of the cerebellar nuclei (Fredette and Mugnaini, 1991; De Zeeuw et al., 1997; Najac and Raman, 2015). Therefore, although we cannot rule out that some cells may belong to other classes of CN neurons, we postulate that most of the GABA-like cells belong to the local GABAergic group of neurons, and demonstrate that they are reliably inhibited by PC inputs in vivo.

**Different coding strategies in CN neurons of the medial nucleus**

CN neurons integrate excitatory inputs from mossy and climbing fibers, and inhibitory inputs from PCs and local interneurons. It was recently demonstrated that excitatory afferents are responsible for half of the action potentials in CN cells (Yarden-Rabinowitz and Yarom, 2017); the other half is intrinsically elicited since CN neurons are spontaneously active (Thach, 1968; Aizenman and Linden, 1999; Raman et al., 2000; Alviña et al., 2009; Sangrey and Jaeger, 2010; Radayonnejad et al., 2010; Zheng and Raman, 2010; Boehme et al., 2011; Engbers et al., 2013). Although the existence of a PC inhibitory control of CN cells is generally accepted (Ito et al., 1970; Ito, 1984; Gauck and Jaeger, 2000; Czubayko et al., 2001; Telgkamp and Raman, 2002; Pedroarena and Schwarz, 2003; Rowland and Jaeger, 2005; Blenkinsop and Lang, 2011; Person and Raman, 2011; Chaumont et al., 2013), the question of whether this inhibition gradually decreases CN neurons mean firing rate (rate code), delimits a temporal window for neuron discharge (temporal code), or both is still debated (De Zeeuw et al., 2008, 2011). CN neurons can follow ongoing movement kinematics (Thach, 1968, 1970a; Armstrong and Edgley, 1984; Rowland and Jaeger, 2005) and can be entrained by synchronous PCs, as suggested by recordings showing correlated firing of PC and CN neurons (Thach, 1970a,b; Armstrong and Edgley, 1984; McDevitt et al., 1987). Recently, several studies also demonstrated that rate and time coding strategies can be multiplexed (Sudhakar et al., 2015; Abbasi et al., 2017; Brown and Raman, 2018). Our study is in agreement with previous experiments showing that large CN neurons (presumably glutamatergic projection cells) are entrained by synchronous PCs, whereas at high frequencies GABAergic neurons are essentially inhibited (Person and Raman, 2011; Najac and Raman, 2015; Sarnaik and Raman, 2018). However, in some in vivo recordings, it was shown that synchronized PCs entrain large CN neurons without affecting their overall firing rates (Person and Raman, 2011; Sarnaik and Raman, 2018) unless climbing fiber inputs were involved (Hoebek et al., 2010; Bengtsson et al., 2011; Tang et al., 2016, 2019). This absence of an effect on CN neuron firing rates might be accounted for by the slow kinetics of the ChR2 currents in PCs that would fail to elicit a spike at each illumination at high frequency or by a reduced number of activated PCs. Notably, in an in vitro study, an inhibitory effect of increasing PC firing rate with constant synchrony has been observed (Wu and Raman, 2017). In our study, while the mean firing rates of individual PCs do not follow illumination at high frequency (i.e., mean firing rate = 57 Hz at 115 Hz), PCs are entrained at the illumination frequency up to 115 Hz at the population level (Fig. 1), and GLUT-like cells are both inhibited and entrained by blue light activation. These results suggest that PCs can fire at 115 Hz but skip some cycles as during high-frequency oscillations (de Solages et al., 2008) and that converging inputs from 30 to 50 PCs to a given CN neuron (Person and Raman, 2011) ensure its entrainment at 115 Hz. However, the lack of a strict PC entrainment at 115 Hz likely led to a loose synchronization of PC firing that may promote the concomitant inhibition of CN neurons, as observed by Person and Raman (2011) (see stimulation at 100 Hz in their Fig. 2D). Finally, the apparent difference in PC influence on CN neurons firing rate with some in vivo recordings (Person and Raman, 2011; Sarnaik and Raman, 2018) might originate in the different targeted nucleus, the medial instead of the interposed nucleus. In the medial nucleus, PC axons spread out in a wide area of the nucleus (Sugihara et al., 2009), as opposed to PC projections in the interposed nucleus (Sarnaik and Raman, 2018). This configuration likely leads to a few millisecond (2–3 ms) jitter in action potential conduction time on PC axons, which is in line with the loose synchronization mediated by ChR2 activation in PCs (Chaumont et al., 2013). These jitters may slightly desynchronize PC inputs and result in a mild inhibition of CN neurons (De Zeeuw et al., 2008), as shown by Person and Raman (2011). Although the light-induced jittering in PC inputs is not physiological, it may mimic the physiological jitter in PC synchrony when PCs are excited by clusters of activated granule cells (Heck et al., 2007; Valera et al., 2016). Since GLUT-like cells are both gradually inhibited and entrained by a population of PC inputs discharging together (de Solages et al., 2008), we conclude that they multiplex a population rate and temporal code strategies. Conversely, local GABA-like cells are inhibited by PC inputs, but not entrained at high frequencies, in agreement with in vitro recordings of GABAergic nucleo-olivary cells (Najac and Raman, 2015). As PC frequency increases, local GABA interneurons might be fully inhibited, releasing the local inhibitory brake on projection GLUT cells that are entrained by PC inputs. These differential coding strategies between CN neurons might favor temporal precision of cerebellar outputs associated with specific features of movement control in the medial nucleus.

**References**


