Title:
Topography and Response Timing of Intact Cerebellum stained with Absorbance Voltage-Sensitive Dye

Abbreviated Title for Running Head:
Turtle Cerebellar Response Topography to Olive Stimulation

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ABSTRACT

Physiological activity of the turtle cerebellar cortex (Cb), maintained in vitro, was recorded during microstimulation of inferior olive. Previous single electrode responses to such stimulation showed similar latencies across a limited region of Cb, yet those recordings lacked spatial and temporal resolution and the recording depth was variable. The topography and timing of those responses were reexamined using photodiode optical recordings. Because turtle Cb is thin and unfoliated, its entire surface can be stained by a voltage–sensitive dye and transilluminated to measure changes in its local absorbance.

Microstimulation of the inferior olive evoked wide-spread depolarization from the rostral to the caudal edge of the contralateral Cb. The time course of responses measured at a single photodiode matched that of single microelectrode responses in the corresponding Cb locus. The largest and most readily-evoked response was a sagittal band centered ~0.7 mm from the midline. Focal white-matter microstimulation on the ventricular surface also activated sagittal bands, whereas stimulation of adjacent granule cells evoked a radial patch of activation. In contrast, molecular layer microstimulation evoked transverse beams of activation, centered on the rostrocaudal stimulus position, which traveled bi-directionally across the midline to the lateral edges of the Cb. A timing analysis demonstrated that both inferior olive and white-matter microstimulation evoked responses with a nearly simultaneous onset along a sagittal band, while molecular-layer microstimulation evoked a slowly propagating wave traveling approximately 25 cm/s. The response similarity to inferior olive and white-matter microstimulation suggest that the responses to white-matter microstimulation are dominated by activation of its climbing fibers. The Cb’s role in the generation of precise motor control may result from these temporal and topographic differences in orthogonally oriented pathways. Optical recordings of the turtle’s thin flat Cb can provide insights into that role.

Key Words:  Synchrony, Optical Recordings, Inferior Olive, Climbing Fibers, Purkinje Cells
INTRODUCTION

The cerebellar cortex (Cb) has a neuronal structure which is highly conserved across vertebrate species (Larsell 1967) and plays a central role in sensorimotor processing. It has three layers overlying white matter whose sole output neuron, Purkinje cells (PC), are excited by the ascending axons of the granule cells (Cohen and Yarom 1998; Huang et al. 2006; Isope and Barbour 2002; Tolbert et al. 2004), parallel fibers (Eccles et al. 1966b) and climbing fibers (Eccles et al. 1966a) which carry signals from the contralateral inferior olive (IO) (Dow 1942). Recent hypotheses have focused on Cb’s role in behavioral timing (Kistler et al. 2000; Kitazawa and Wolpert 2005; Xu et al. 2006; Yarom and Cohen 2002), however controversy surrounds reports that olivocerebellar responses (Sugihara et al. 1993) are synchronous (see Aggelopulos et al. 1995; Baker and Edgley 2006a; b; and the responses of Lang et al. 2006). One approach to understand the timing of physiological responses of cerebellar cortex has been to record multiple responses, first performed on ~2 mm² of turtle Cb (8 X 8 electrode array recording extracellular fields Bantli 1972). However, a confounding factor was that response strength and polarity varied with electrode depth (Bantli 1974). Simultaneous recordings of single rat PCs (8 x 12 array, 166 µm spacing, Fukuda et al. 2001; 4 X 8 array, 250 µm spacing, Sasaki et al. 1989) were limited to small Cb regions, due to its deeply-foliated nature of mammalian Cb.

To study the topography and timing of the entire Cb, we study in vitro turtle cerebellum, a thin unfolded neural sheet (~1-mm thick from pia to its ventricular surface resting over the fourth ventricle). It is connected to each side of the brainstem by a single cerebellar peduncle. The turtle brain is resistant to hypoxia, permitting topographic analysis of the entire cerebellum in vitro with its brainstem attached. In vitro brainstem neurons are also responsive to natural sensory stimuli: visual patterns imaged on retina (Ariel and Fan 1993; Fan et al. 1993) and head rotation (Fan et al. 1997). The climbing fiber input to turtle Cb was previously investigated using a single micropipette to record extracellular fields following IO microstimulation (Ariel 2005). It was found that moving the stimulus site along the fourth ventricle wall from the IO toward the peduncle shortened response latency within the Cb. However, response latency was independent of the recording site within the Cb. It was suggested that an invariant latency of the Cb following IO stimulation is caused by changes in its conduction velocity within the entire
cerebellar cortex but not within the brainstem. However, the poor spatial resolution of extracellular field recordings and their variability with electrode depth led to concerns about this conclusion (Baker and Edgley 2006a).

Because the turtle Cb is thin, a novel approach is developed here to simultaneously record physiological responses across the entire Cb surface using voltage-sensitive dye recording techniques developed by Saltzberg and Grinvald (Baker et al. 2005; see modern reviews by Ebner and Chen 1995; Saltzberg et al. 1977; Wu et al. 1998). This technique permits us to measure physiological activity with sub-millisecond resolution from a two-dimensional sheet of cortical tissue during afferent stimulation. Fluorescent optical recordings from Cb have been reported in vivo (Chen et al. 1996; 1998; neutral red fluorescence Kim et al. 1989; flavoprotein autofluorescence Reinert et al. 2004) and in vitro (fluorescent voltage-sensitive dyes in rodents Arata and Ito 2004; Cohen and Yarom 1999; 2000b). Here, the entire turtle brain was stained in voltage-sensitive absorbance dye and then absorbance changes in transilluminated light were measured (Konnerth et al. 1987). We find that the timing and topography of Cb responses to electrical stimulation reveal a distinct orthogonal structure of radial, transverse and sagittal pathways within this simple vertebrate cerebellar cortex.
METHODS

Common red-ear pond turtles, *Trachemys scripta elegans*, were housed at room temperature in an aquarium on a 16/8-h light-dark cycle, where they could swim and bask at will. The carapace lengths of the turtles were 15-18 cm, whose cerebella were $4.89 \pm 0.55$ mm wide and $3.81 \pm 0.50$ mm long ($n = 24$). However, in some experiments which examined responses at different layers of intracerebellar microstimulation, the cerebella of 30-cm turtles were employed. The turtles were handled and housed in accordance with N.I.H. guidelines. The protocols of animal care and surgical procedures were approved by the Saint Louis University Animal Care Committee.

The Turtle Brainstem Preparation

The surgery and the turtle preparation have been presented in detail previously (Fan et al. 1993; Rosenberg and Ariel 1990) so only the new technical aspects of optical recordings are now described. The brain was removed from the anesthetized animal, its telencephalon was quickly ablated and the brain stem was placed in a Petri dish of physiological media. One cerebellar peduncle was cut and the dura and choroid plexus were then carefully removed around the cerebellum to facilitate unimpeded staining. The entire tissue was immersed 30-60 min in a voltage-sensitive oxonol dye solution (1mg/2.5mL of oxygenated physiological media). Several oxonol dyes yielded similar results, but experiments shown here only used RH482 (supplied as NK3630 from Nippon Kankoh, Japan or JPW 1132 from L. Loew at Univ. CT). The polarity of the absorbance changes using this dye depended on the wavelengths of the illumination, indicating that these dyes signal changes in membrane voltage and not intrinsic optical signals due to light scattering (Brown and Ariel 2007).

After staining, the intact tissue was rinsed in control oxygenated physiological media for an additional 15 min, to remove excess dye. The stained tissue was then transferred to a recording chamber on a fixed-stage pedestal above the optical condenser of a standard upright Olympus BX microscope (BX-51, Olympus, Tokyo JP). The floor of this plexiglass two-level recording chamber was made of #1 thickness optical cover glass to maintain optical quality during transillumination of the tissue (Fig. 1A), The back section was 5-mm deep to accommodate the larger brainstem, while the Cb was placed in a 3-
mm deep front section. The entire floor of the recording chamber was covered by a 1 mm layer of Sylgard to facilitate pinning and immobilization of the tissue during recording.

The preparation was first placed in the back section of the recording chamber with the brainstem dorsal side up. The cerebellar cortex (Cb) was deflected and pinned to the front section of the recording chamber, ventricular surface up. Because the Cb curves down slightly in vivo as it covers the brainstem, the edges of the in vitro Cb were stretched slightly while being pinned to obtain a relatively flat and uniform surface. In some experiments, the whole preparation was flipped so that the brainstem was ventral side up, and the pial surface of the Cb faced upward. In this latter case, the molecular layer was more readily accessible.

For the accurate registration of Cb topography with the photodiode-array’s signals, photomicrographs of the transilluminated tissue was captured by a digital camera attached to the trinocular port of the microscope (Fig. 1B). Insertions of stimulating electrodes through the ventricular surface were unimpeded, whereas the fibrous pial membrane was not penetrable. To allow unimpeded access to the molecular layer through the pial surface, small patches of pia were removed. Also, when the pia faced upward, the midline raphe (or groove) could not be visualized so the accurate identification of the Cb’s orientation was limited.

The physiological media entered the recording chamber by gravity flow and was removed by suction. A 7-mm wide strip of #1 cover glass was placed directly over the Cb to minimize optical distortion of the moving surface of physiological media, leaving 2 mm between the Cb’s surface and the overlying cover glass. Distortion was also prevented during the 1-5 s recordings by closing two pinch valves on the inflow and outflow chamber tubing. Finally, the entire recording apparatus was stabilized on a vibration-isolation table.

**Optical Recordings**

Light from a 150-W tungsten-halogen bulb powered with 8.5 A from a regulated DC power supply was directed through a standard condenser to trans-illuminate the tissue in the recording chamber using full-field Köhler illumination. In some cases, a point source of light illuminated the array with and without
the presence of intervening Cb to evaluate light scattering by the tissue. Those data indicate that the Cb significantly increased the light scatter from the diode sensing the point source but only to the immediately adjacent diodes.

The transilluminating light was restricted to 715nm ± 35nm by an interference filter (Chroma Technology, Brattleboro VT) so that a decrease in absorbance by the voltage-sensitive dye RH482 is equivalent to a membrane depolarization. The image of the tissue was formed through a dual port adaptor onto either the optical element of a Motic-1000 digital camera or a 464-photodiode-array (WuTech H-469IV PDA, Gaithersburg MD) using one of two microscope objectives (2x PlanApo N NA 0.08 or 5x UMplanFL NA 0.15, Olympus, Tokyo JP). At those magnifications, each diode detects 0.06 and 0.024 mm² of Cb, respectively. Given 645 Purkinje cell bodies per mm² in turtle Cb (see Ariel et al. 2005), each diode may image 39 and 15 somata yet the dendritic trees of many more PCs.

Before the actual recording, the Cb is illuminated for 1 s while the signal from each photodiode (time constant of ~20 sec) was adjusted by electronically subtracting its dc offset. 4096 voltage points of each of 464 photodiodes were sampled at 1.6 KHz to generate 2.51 sec traces. To orient the photodiode-array data set, it was compared to the images of the microscope’s digital camera stored just prior to the optical recording. A low magnification photograph of the preparation was made to record the position of stimulating electrodes outside the field of view of the microscopic objectives.

**Olivary and Intracerebellar Microstimulation and Electrophysiological Recording.** Straight tipped tungsten bipolar microelectrodes (125 µM tip separation) were advanced at 45° angle into the IO. Manipulating stimulating electrodes into the Cb was more difficult due to the overlying cover glass. Consequently, a right angle bend was made 1.5 mm from the tip of each bipolar microelectrodes and the shaft was oriented parallel and just below the cover glass and then advanced perpendicular to the Cb surface. These bipolar electrodes delivered biphasic current pulses (AM-Systems 2100, Carlsborg WA) that varied between 4 pulses at 1.7 Hz and 27 pulses at 23 Hz. All stimulating pulses were 100 µs long using 5-200 µA.
In some experiments, extracellular field potentials were recorded for comparison with the optical data (Ariel 2005). A glass recording micropipette (25-µm tip diameter, filled with 3 M K-acetate, DC resistance <1 Mohm) was bent at three right angles and then advanced into the Cb under the cover glass. Potentials were recorded with respect to a chlorided silver ground wire in the recording chamber. The presence of the pipette tip did not change the local optical signals, indicating no effects of any potassium diffusion. Signals were amplified using a 3 KHz low-pass filtered cut-off frequency and recorded by the NeuroPlex software (RedShirt Imaging, Fairfield CT).

**Analysis of Optical Signals**

Absorbance changes were first analyzed using NeuroPlex software. The raw recordings of the diodes were displayed as 464 individual traces. Each trace was associated with a value of resting light intensity (RLI) measured for that diode without stimulation but with the same illumination of the tissue in the same position. The RLI values of the Cb stained with RH482 did not vary significantly over the course of these experiments (up to 36 hours). The NeuroPlex software automatically computed the relative optical signal (ΔI/I, the change in light intensity transmitted through the preparation divided by the absolute light intensity) for each trace and saved the raw data as a binary file of the RLI and 464 voltage traces. Assuming that regional variations in light intensity transmitted through the tissue was similar before and after staining, the optical signal can be assumed to be linearly related to the fractional change in dye absorption (−ΔA/A; the change in light absorbance of the dye divided by the steady state absolute absorbance of the dye), which relates to the change in membrane voltage (Saltzberg et al. 1977). All absorbance changes were electrically evoked as the turtle Cb has low spontaneous spike activity when studied in vitro (Fan et al. 1993) and no spontaneous waves were observed without electrical stimulation of this preparation. Also, based on the RLI, diodes were excluded from processing if they did not image the biological tissue or they had resting light levels that were too low to transmit meaningful signals.

**Averaging and Temporal Filtering.** Each diode of the WuTech H-469IV PDA exhibits a very long time constant (~20 s) which permitted a baseline drift in the unfiltered signal. This drift was often similar for all the 464 diodes, suggesting that it resulted from a common optical variable, such as changes of the
lamp output or its power supply. Even after these variables were minimized, it was often necessary to
remove slow artifactual drift by applying a minimal digital filter to each diode’s trace. Filtering had very
minimal effect on the response onset timing and amplitude though response duration was slightly
shortened. In this experimental sample, response duration did not correlate with any stimulus parameters.

Detection of Responsive Diodes. Recordings were analyzed offline by Matlab (MathWorks, Natick MA)
to quantify response latency and amplitude. To detect a subset from the 464 diodes that exhibited
significant responses, two methods were employed: wavelet detection of the risetime latency (maximal
slope after response onset), and amplitude detection of the time-to-peak latency. Of the two methods,
wavelet detection was more reliable because it was less sensitive to the shape of the response peak. In
both cases, the traces were routinely viewed for errors in diode detection.

Response Latency. Spatial analyses were made of the responsive diodes in each experiment. First,
responses were averaged for the standard recording (four responses in each 2.51 s trace) and, if possible,
so too were consecutive records of responses evoked under similar stimulus conditions. A single data set
was considered statistically synchronous if the slope of the linear regression line that fit those data was
not significantly different from zero. Differences between experimental groups were determined by
ANOVA. If the $F$ ratio indicated significant differences, a Fisher’s LSD (protected $t$) test was performed
to establish differences between individual sample means.

Quantifications of the responsive photodiodes included their response latencies, their peak
amplitudes and their distance from the stimulating point within the cerebellum (or from the peduncle for
IO microstimulation, see Ariel, 2005). Latencies were plotted against the conduction distance on latency-
distance plots and then fitted to a line by least squares regression. In case there were artifactual optical
signals from diodes immediately adjacent to the stimulating point, those fits were also performed with
those diodes excluded but the results were the same. The correlation coefficient of the fitted line
determined if the slope was significantly different than a zero-slope.

The response pattern was viewed as a grayscale latency array. The diodes were displayed as
gray boxes whose darkness was scaled as a function of their response latency. Diodes with the longest
response latencies were displayed as darkest and those with the shortest latencies were lightest. Latency
profiles that showed a regular change in the darkening of the boxes across the array indicated that the latency changed along that vector as the response slowly propagated.

**Peak Amplitude of Response Fields.** From the same diodes detected for the conduction latency analysis, a threshold was chosen that would select a large yet contiguous region of responsive diodes. A custom Matlab script was used to fit an ellipse to those diodes and the major and minor axes used to quantify the response topography. Response orientation was measured when the length of its major axis was more than twice the length of the minor axis. Orientation data were excluded from preparations in which the pia faced up to the microscope objective, thereby obscuring the midline raphe. The major axes of the remaining data set were plotted together to show the orientation of the response fields, relative to the midline of the cerebellar cortex.

The response fields were viewed graphically as a *dot-amplitude array* as follows. All the diodes that imaged the tissue were strongly filtered to allow measurement of their peak amplitudes without latency information. The diode with the largest response amplitude was displayed on a hexagonal array as a dot filling the space occupied by that diode. The dot sizes of the remaining diodes were scaled smaller based on their amplitudes. To display the orientation of the response field graphically, a threshold was set of several standard deviations above the noise level, to identify an elliptical field. The axes of that ellipse were then plotted on the same *dot-amplitude array*. In some cases, the response fields were slightly curved or the response orientation was not exactly aligned with or orthogonal to the midline raphe. This may have been due to the unavoidable stretching of the tissue when pinned down to the chamber’s floor.

A final format to display response fields uses the NeuroPlex software to generate *pseudocolor images* as used in several figures below. Those images were created with a minimal high pass filter to remove non-physiological baseline drifts for display purposes only. Also, a standard color threshold of 75% (as noted by the black line on the color scale) was established to remove noise so that the response field can be viewed as an overlay on the Cb image. The NeuroPlex system was also used to convert these 464 traces into pseudocolor movies as Supplemental Data. Each of the 464 diodes was sampled 1600 times per sec while the optical responses would really only last tens of milliseconds. The resulting video
files, using the standard mpeg video format of 30 frames per sec, appear approximately 53 \((1600/30)\) times slower than the real events (see Supplemental Data). Responses in real-time pseudocolor movies would only show an absorbance change in a single frame (not shown).
RESULTS

Topographic changes of voltage-sensitive dye signals were recorded from the entire extent of the cerebellar cortex with millisecond resolution in response to single microamp current pulses. Figure 2D shows an array of traces from the photodiodes that image a Cb placed ventricular side up in the chamber. Unlike Figure 1, this and all remaining figures are displayed oriented with the caudal Cb on top, the cut peduncle on the bottom left corner and peduncle attached to the brainstem in the lower right corner (see low magnification view in the initial frames of the movie ‘Sfig2-IO-response.mpg’ of the Supplemental Data). In Figure 2A, the tissue’s mean light level reveals an outline of the Cb, indicating that most photodiodes collected light from the transilluminated tissue. Using the highlighted diode in Figure 2A, Figure 2B shows the time course of the absorbance changes for a typical trial. This trace exhibits clear transient depolarizations for each of four stimulating pulses presented to the IO (see stimulus trace below; 50 µA, 100 µs). Because this unfiltered signal was recorded with the equivalent of a very long time constant (~20 s), both transient and sustained response components can be observed following each 100 µs pulse. Figure 2C shows the response to higher frequency stimulation. These data demonstrate that the techniques are capable of recording sustained DC-shifts in membrane potential, yet retain sufficient temporal resolution to capture transient events.

RESPONSE TOPOGRAPHY

An example of the topography of Cb responses to IO microstimulation is shown in Figure 2D. White traces show the average of four responses (as shown in Fig. 2B), overlaid on an image of the digital camera (as shown in Fig. 1B). The region of Cb responsiveness to IO microstimulation was clearly constrained to the cerebellar hemisphere that remained attached to the brainstem. Those Cb responses were best evoked by stimuli presented to the IO on the side contralateral to the attached peduncle. Even following IO microstimulation using higher currents, the activated region of the Cb never crossed the midline raphe of the cerebellum, although it expanded to activate much of the attached hemisphere (not shown).
The response latency and waveform of the optical signal (Fig. 2E) was compared to the averaged electrical trace recorded through a glass micropipette (Fig. 2F) inserted into the Cb at the same location imaged by the diode highlighted in Fig. 2A. Although the two measures of the response have a similar latency, the wider bandwidth of the micropipette recording reveals the characteristic oscillations that are reminiscent of the complex spikes recorded from Purkinje cells. These oscillations are not apparent in the optical recording.

To examine the topography of the Cb responses to IO microstimulation, the response amplitude of each diode was quantified, as displayed in the dot-amplitude array shown in Figure 3A (see Methods). In this case, the data used for Figure 2 is replotted using a dot at each diode location with diameter proportional to the amplitude of the response. From a region of responsive diodes (shown in green) and an ellipse was computer-fit to display the major axis (red) and minor axis (blue), as shown on a sketch of an average Cb-brainstem profile (Fig. 3A). The inset shows the same response from that IO microstimulation as a pseudocolor image made by the Neuroplex software (equivalent to a frame from the pseudocolor movie ‘Sfig3-IO-response.mpg’ of the Supplemental Data). Note that, in both displays, the major axis is of the responsive region is parallel to the midline raphe (the vertical line bisecting the Cb profile).

Similar dot-amplitude analyses were generated from our sample of 28 responses to IO microstimulation, as summarized in Figure 3B. The midline raphe of each Cb was aligned at the rostral boundary and the major axis of the responsive region of each experiment was displayed as a red line. The positions of these axis lines differed slightly from one another because the width and length of each tissue varied slightly. However, it is clear from the topography that IO microstimulation evoked its strongest response approximately 0.7 mm lateral to, but aligned with, the midline raphe. Notably, each of these responses extends fully in the rostrocaudal dimension of the entire Cb.

Because responses to IO microstimulation did not cross the midline, one may suspect that the unresponsive hemisphere may be damaged by cutting the peduncle on that side. The responsiveness of that hemisphere was tested using intracerebellar microstimulation. In the example shown in Figure 4, the bipolar electrode tips were visually placed on the ventricular surface of the hemisphere opposite the
attached peduncle of the turtle Cb (see ‘Sfig4-stim-image-thru-scope.jpg’ in the Supplemental Data). Direct microstimulation of that surface, at the thin white matter (WM) layer evoked strong responses (Fig. 4D) in the hemisphere that was unresponsive to IO microstimulation (Fig. 4A). Not only was that unattached hemisphere functional, but the response region was also sagittally oriented (Fig. 4D, red line) like the IO response (Fig. 4B, red line; also compare video files Sfig6-IO.mpg and Sfig6-WM.mpg in the Supplemental Data). Responses to white matter were recorded in many Cb using different medial-to-lateral stimulus positions along the rostral aspect of the Cb. The resulting summary of the topography of responses is displayed in Figure 4F. The major axis lines indicate that WM microstimulation results in responses that are sagittally oriented and reach the caudal edge of the Cb.

After recording responses to white matter stimulation (Fig. 4D), the bipolar stimulating microelectrode was then advanced deep into the cortex towards the pial side. When the tips were well into the molecular layer (ML), microstimulation was applied. The response region that originated at the stimulating point was now oriented transversely and traveled in both directions (Fig. 4G,H; and see video file Sfig6-ML.mpg in the Supplemental Data). Similar experiments of ML microstimulation were repeated at several positions in different Cb. The resulting summary of the topography of responses is displayed in Figure 4I. The major axis lines are clearly oriented in the transverse plane. Their position depended on the rostrocaudal locus of microstimulation, although in all cases their transverse responses readily crossed the midline from either side and, in many cases, extended to the lateral edges of the tissue in both directions.

STIMULATION DEPTH
Stimulation at or near the two Cb surfaces evoked responses with very different spatial properties. Initial experiments of microstimulation of middle Cb layers indicated that responses contained both sagittal and transverse topographies. As this thin flat tissue is only secured by pins on its edges, there may have been movement of the Cb during the penetration of the bipolar electrode into the tissue, making the accurate placement of the tips of the stimulating electrodes difficult in this preparation. A possibility was also
considered that current was spreading radially along the electrode track. Therefore, these experiments were repeated using larger turtles (up to 30 cm carapace length) because the Cb was about 35% thicker in these animals {standard 15 cm carapace: 834 ± 114 µm; larger 30 cm carapace: 1130 ± 121 µm}. The bipolar stimulating electrode was advanced radially in 100 µm steps, either from the ventricular or pial surface, to determine a depth that would neither evoke a sagittal or transverse response.

Figure 5 shows responses to microstimulation in the Cb of a large turtle. The left column shows pseudocolor response images when the ventricular surface faced up (Fig. 5A-top); the right column shows results from the same tissue when the pial surface faced up (Fig. 5B-top). (See magnified views of the electrode placement in Supplemental Data: Sfig5A-WM-stim.jpg & Sfig5B-Pia-stim.jpg). The second row shows responses to bipolar microstimulation at or near the white matter and was roughly oriented sagittally (parallel to the midline marked with a yellow line; Fig. 5A-WM, Fig. 5B-700 µM below the pia). The third row shows small patch-like responses to stimuli in the granule cell layer (Fig. 5A-400 µM below WM; Fig. 5B-500 µM below the pia), using the same low current level that produced the larger responses from depths at the WM or ML. The bottom row shows responses to ML microstimulation (Fig. 5A-600 µM below WM; Fig. 5B-Pia) that are oriented transversely (orthogonal to the midline, yellow line of second row). Six video files show these 6 responses in the Supplemental Data.

Although low currents were used to localize stimulation within single Cb layers, the patch-like patterns were not always circular. Therefore, occasionally, minimal currents were employed to examine the shape of the responses evoked by the electrode tips which were estimated to be within the granule cell layer. Figure 5C shows an example of a response to very low current (15 µA) presented at the same depth that evoked the small patch-like response of Figure 5A (75 µA pulses at 400 µM below WM). The left pseudocolor image of Figure 5C, processed identically to that of Figure 5A, shows a smaller patch-like response than that of Figure 5A. However, the response to 15 µA was reanalyzed using a color threshold reduced by half (37% instead of 75%, right pseudocolor image of Fig. 5C and see Sfig5C-low-stim-low-threshold.mpg in the Supplemental Data). The resulting image shows that weak sagittal and transverse components, even in response to this lower stimulus current. In general, response fields to stimuli near the ventricular surface were oriented sagittally; to stimuli near the pial surface were oriented transversely
and to stimuli elsewhere within the Cb’s depth exhibited a patch-like response as well as a mixture of weak sagittal and/or transverse fields.

RESPONSE TIMING

Because the Cb is thought to play a role in the timing of complex motor behaviors, the spatio-temporal dynamics of Cb responses were examined (Figs 6 and 7). Each row of Figure 6 shows a time sequence of the responses to IO, MN or ML microstimulation as performed in Figure 4, depicted here as a series of pseudocolor frames. The full sequences are found in Sfig6-IO.mpg, Sfig6-WM.mpg, and Sfig6-ML.mpg in the Supplemental Data. The left column displays the frame (0.625 ms) with the first indication of the response, which occurred at different latencies following stimulation. Each frame’s latency is shown in the upper left corner. The fastest response occurred following ML microstimulation (bottom row), with activity appearing at the locus of stimulation with negligible delay (‘0’ ms). The response to WM microstimulation was delayed by 7 frames (4.38 ms) but also occurred near the tip position of the stimulating electrodes (middle row). Responses to IO microstimulation (top row) had the longest latency, appearing 15 frames (9.38 ms) after the first response frame of ML microstimulation. The larger delay in the response to IO microstimulation (relative to WM) is consistent with its stimulating point being many millimeters away in the brainstem.

Subsequent frames of Figure 6 (columns 2,3,4) depict activity recorded approximately 7 ms, 18 ms, and 50 ms after the frame shown in column 1. See also the video files of Fig. 6 in Supplemental Data). As shown, ML responses began at the tips of the microelectrode, and then traveled slowly away from the stimulating point in both directions until they reached the lateral edges of the Cb. In contrast, WM and IO response regions began later, and then increased in strength uniformly within their response fields.

To quantify the temporal dynamics shown in Figure 6, risetime latencies of each diode within the response region were computed directly from selected diode traces sampled at 1.6 KHz and displayed as a grayscale latency arrays in Fig. 7. White boxes depict zero latency diodes; black boxes depict the longest latency diodes. Only diodes in the response field were analyzed; the remaining diodes are indicated as
small dots. For ML responses (Fig. 7G), the response diodes that had the shortest latencies (white or light gray boxes) were closest to the stimulating point (red dot). The latency gradually increased with distance from that point while traveling along a transverse plane (e.g. increasing dark gray boxes). This finding is consistent with the activation of parallel fibers in the ML in the transverse plane. Similar analyses were performed that measured peak latencies, instead of risetime latencies, but the conclusions are the same (see Fig. 8C below).

In contrast to ML responses, each responsive diode following WM microstimulation had a short latency, irrespective of its position in its sagittal response region (see Fig. 7D). Uniform response latency along this flat tissue is unexpected because one assumes that there is a fixed conduction velocity for the sagittally oriented axons that conduct up to 6 millimeters. Yet the response latencies, although somewhat variable, did not vary with distance so were considered statistically synchronous. Like the WM responses, responses to IO microstimulation were also nearly synchronous as shown in its grayscale latency array (Fig. 7A corresponding to the traces in Fig. 4A and top row of Fig. 6). Although Figures 7A,D,G use a common grayscale, the diodes of Figure 7A were darker than those in Figure 7D, indicating a longer mean latency. This may be expected because the conduction distance from the IO to the Cb is longer. However, the sagittal response field did not show any pattern of increasing latencies, indicating that the response field was statistically synchronous, like that of Figure 7D.

To quantify the timing of IO, WM and ML responses, the latencies shown in the grayscale latency arrays were plotted as a function of the distance from the stimulating position (WM and ML) or from the peduncle (IO, see Ariel 2005). The latency-distance plots for the data in the left column of Figure 7 are displayed in the middle column of Figure 7. Following ML microstimulation, latency gradually increased with distance traveled (Fig. 7H). In contrast, IO and WM latency-distance plots (Fig. 7B,E) did not show a strong trend. These data were fit with least square regression lines for our sample of 28 IO, 20 WM and 28 ML microstimulations (Fig. 7C,F,I; respectively). Lines with slopes that were not significantly different from zero were replaced with a flat dotted line. None of the responses to ML microstimulation had non-zero slopes (Fig. 7I). In contrast, the WM and IO groups had many latency-distance fits whose slopes were not significantly different from zero. A statistical analysis of the slopes of
these three groups by the one-way ANOVA method indicates that ML slopes are different from IO and WM responses (p < 0.01), although IO and WM slopes are not different from one another.

The mean of the regression fits to the IO, WM, and ML latency-distance plots are shown as the wide striped line in Figure 7C, F and I. From the slopes, one can estimate the conduction velocities of these responses from these data. The mean slope of the latency-distance plot for ML microstimulation was 4.1 s/m, indicating that parallel fibers conduct at 0.24 m/s. Given an average Cb width of 4.89 mm, the time difference of activation from one lateral edge to the other is 20 ms. Estimating response conduction velocities from the mean slopes for IO and WM microstimulation (0.69 and 0.49 s/m) is more inaccurate because many latency-distance slopes were statistically flat (11/28 and 8/20, respectively, for p = 0.05). Moreover, the fraction of flat slopes is dependent on the level of statistical significance required. For example, the IO and WM data sets would contain 5 and 4 additional flat lines, respectively, using p = 0.005; thereby lowering the mean slope by ~0.12 s/m. Based on these two p values, the range of time difference from rostral to caudal edges of an average Cb is 2.1 - 2.6 ms for IO responses and 1.5 - 1.9 ms for WM responses. When compared to the 20 ms time difference of ML responses between the lateral edges, the time difference of IO and WM responses are much smaller or, in many cases, statistically synchronous. Our interpretation of these data is that the responses to IO and WM stimulation can be considered synchronous across the Cb cortex.

The response onsets (y-intercepts of the regression fits of latency-distance plots) for the three stimulation conditions also exhibit statistically significant differences (p < 0.05) in the (Fig. 7C,F,I). Responses following IO microstimulation began 1.2 ms later than that of WM microstimulation. This difference maybe be due to the conduction delay made by the several millimeters travel distance from the IO position in the brainstem (see Ariel 2005). Note also in turn that WM responses at zero distance from the bipolar stimulating electrode were 3.2 ms later than that of ML responses, suggesting that the different synaptic mechanisms mediate the two responses within the Cb.

The conclusion that WM and ML responses can be differentiated based on their synchrony and topography motivated the re-examination of non-spherical responses that were observed following mid-depth Cb stimulation in the granule cell layer of standard length turtles. Such Cb response fields were
complex, as displayed in Figure 8a. The shape of the response field suggested to us that these data could be divided into two distinct regions for analysis, although some diodes near the point of stimulation may belong to both regions. The latencies of a clearly transverse region (outlined in red) were compared to that of the responsive diodes in a region that was oriented more sagittally (outlined in blue). The data in each region were plotted as blue squares and red circles on the same *latency-distance plot* and each data set was fitted with a least squares linear regression. It is clear that the red line, corresponding to data from the transverse region, has a slope indicating a slowly-conducting response; while the blue line of the sagittal region is statistically flat, indicating its response synchrony. The sagittal region exhibited a short-latency response (Fig. 8A, blue), presumably due to current spread to the WM. At the same time, the transverse response began to spread transversely (Fig. 8A, red), slowly increasing its latency much like an ML response. (See mixed response in video file Sfig8A.mpg in Supplemental Data).

Note that a similar approach can be used to interpret the patch-like data shown in Figure 5A (middle row, 400 µM below WM). There, the response field to granule cell stimulation was not perfectly circular so may be contaminated with sagittal and transverse signals. From the adjacent *grayscale latency array*, a component of the response forming a sagittal band is synchronous, while a different component forms a small transverse beam that increases its latency as it conducts slowly from the stimulating point. This phenomenon is also apparent in the *grayscale latency array* of Figure 5C (right panel). The two topographic regions, sagittal and transverse, exhibited different latency profiles: synchronous and slowly conducting, respectively. This observation supports the conclusion from the topography that even low-intensity granule cell layer microstimulation for 30-cm turtle Cb may not evoke pure patch responses.

Figure 8B shows a *grayscale latency array* of the response to ML microstimulation close to the Cb midline. In this case, it was easy to subdivide the response into two regions for analysis: to the left and to the right of the stimulating point (Fig. 8B, red dot). From these regions, two *latency-distance plots* were generated and fit by a least squares linear regression. The two regression lines had similar slopes (5.29 and 6.16 s/m), indicating that the conduction speed in each direction was equally slow (~0.17 m/s). (See conduction in both directions in video files Sfig8B.mpg and Sfig8B-ML-highpass-filter.mpg in Supplemental Data).
We considered whether the technique used to measure the response timing might influence the response latency. Figure 8C compares the two approaches used in this study; a wavelet method that identifies the time of the maximal response onset slope (‘rise time’), and an amplitude method that identifies the time of the maximal response amplitude (‘peak time’). For these optical data, the latter method required more temporal filtering of the photodiode signals. In the same Cb, these two methods were compared for responses to ML and IO microstimulation (Fig. 8C, also video files Sfig8C-IO.mpg and Sfig8C-ML.mpg in Supplemental Data). The blue symbols indicate measurements using the wavelet method, while the red symbols represent data using the peak time method. Detection of the peak time obviously occurred after the time of response onset, but linear regression slopes of these two methods were very similar (Fig. 8C, black lines). The response fields, as indicated by the small grayscale latency arrays around the two plots, indicate that the topography of responses detected by each method is similar, although the peak time method selected fewer responsive diodes.

Occasionally, specific Cb regions decreased their light absorbance in response to ML microstimulation (especially to trains of pulses). These decreases indicate net membrane hyperpolarization in these Cb regions (plotted as red diodes on a modified grayscale latency array; Fig. 8D; see traces in Sfig8D-colored-by-polarity.jpg in Supplemental Data). Two insets show the waveforms of two diode traces that are marked by ‘x’ in central array. The top diode exhibited a standard depolarizing waveform while the lower diode responded with a hyperpolarization. The hyperpolarizing regions appear to straddle the depolarizing transverse beam and its onset latency differs from the depolarization region (note the gray and red scales differ, Fig. 8D). The hyperpolarization is visible in a pseudocolor scale without a color threshold (Supplemental data, compare Sfig8D-ML.mpg to Sfig8D-ML-no-threshold.mpg).
DISCUSSION

Using in vitro optical recordings of voltage-sensitive activity of the entire turtle cerebellar cortex, robust responses were measured during cerebellar and brainstem stimulation. Topographically, the Cb exhibits clear orthogonal responses to microstimulation of its two surfaces: a) transverse response fields to pia stimulation due to activation of the molecular layer of parallel fibers oriented transversely to PCs, and b) sagittal responses to the white matter stimuli, perhaps as a consequence of Cb afferents oriented sagittally along the ventricular surface of this flat cerebellar cortex. Similar to the white matter response, microstimulation of the contralateral inferior olive evoked its strongest response in a sagittal band of activation, centered approximately 0.7 mm from the midline. Temporal analysis demonstrated that either IO or WM microstimulation evoked Cb responses with a nearly simultaneous onset across a large area, while microstimulation of the molecular layer evoked a slow wave traveling approximately 25 cm/s. These results support the view that the contralateral cerebellar cortex responds synchronously to IO input. Moreover, the similarity of both timing and topography of IO or WM evoked responses suggest that WM responses are dominated by the climbing fibers within the white matter. Using this approach, the finer topography and neural processing of separate sensorimotor channels may be revealed in this simple unfoliated cerebellar cortex.

Using the Turtle as a Model System to Study of Olivocerebellar Responses

The use of the reptilian brain for study of the cerebellum has been well established (Larsell 1932; Llinas et al. 1968). It has specific advantages in that its flat, thin profile makes a natural slice for whole-tissue optical recordings using voltage-sensitive absorbance dyes. Its thickness and PC density are quite uniform, except for a narrow lateral region distal to the peduncle (see Ariel et al. 2005). Climbing fiber input, from the IO, is readily stimulated (Ariel 2005; Straka and Dieringer 1992) near the obex where axons from the contralateral IO coalesce to form the dense bundle of olivocerebellar axons that travel together to the peduncle. A brief current pulse to this locus thereby evokes a synchronized climbing fiber input to the Cb.
In other species, there is evidence that IO normally provides a synchronous output due to gap junctions observed there (Sotelo et al. 1974) which may mediate electrotonic coupling between IO neurons (Chorev et al. 2007; Devor and Yarom 2002; Leznik and Llinas 2005; Llinas et al. 1974; Long et al. 2002). Complex spike synchrony was observed in sagittally oriented groups of PCs but there was almost no cross-correlation of complex spikes of PCs oriented transversely (Sasaki et al. 1989). Synchrony of sagittally oriented cells was enhanced during motor behaviors (Welsh et al. 1995; Wylie et al. 1995) while perturbing IO activity disrupted rhythmic tongue movements (Placantonakis et al. 2004). Synchrony has also been observed in conduction time measurements of complex spikes evoked by stimulating olivocerebellar axons near the IO (Sugihara et al. 1993). However, measurements of small conduction times and estimates of small conduction distances in highly foliated mammalian Cb has resulted in disagreement as to whether the olivocerebellar conduction time is uniform or not (Aggelopulos et al. 1995; Baker and Edgley 2006a; and rebuttal by Baker and Edgley 2006b; but see response by Lang et al. 2006). For example, Aggelopulos et al. presented latency-distance plots of complex spike responses to IO stimulation (Fig. 3A of Aggelopulos et al. 1995) based on 285 extracellular recordings from three deeply anesthetized cats combined with anatomical estimates of conduction distance derived from histological sections from fixed brains. In spite of possible inaccuracies of these anatomical estimates in this large, highly foliated structure, the cat data can readily be compared to that of the flat turtle Cb (see Fig. 7C). In the present study, however, true synchronous responses were often independently observed in many individual turtle brains whose latency-distance plots were not statistically different than zero (i.e., exhibiting synchrony).

In our previous study (Ariel 2005), single electrode recordings indicated that a mechanism exists within the Cb to compensate for differences in the conduction distance resulting in nearly synchronous responses to IO microstimulation. Baker and Edgley (Baker and Edgley 2006a) questioned the spatiotemporal measurements of those data based on the concern that population field potentials may have spread spatially across the tissue to confound the temporal accuracy. They also suggested that measuring peak latency is less temporally accurate than measuring onset latency. Furthermore, they questioned
whether different populations of olivocerebellar axons were involved when different brainstem locations were stimulated.

These issues have been addressed in the present study. First, the responses of voltage-sensitive dyes are extremely local, with a spatial resolution limited only by the resolution of the diode-array and light scattering by the tissue. In relation to measuring across 5 mm of Cb, light scattering is negligible because adjacent diodes were activated sequentially along the transverse plane of the Cb due to ML stimulation. Light scattering also did not dramatically change the response topography when the pia was up or down in the recording chamber. Second, the photodiode changes are recorded simultaneously and presented as a fractional change in dye absorption (−ΔA/A) so that the amplitude and timing of the recordings from each diode are directly comparable. From these multiple simultaneous recording sites, both onset and peak latency showed similar results (Fig. 8C). Finally, a single current pulse to the IO appeared to activate most if not all olivocerebellar inputs to the contralateral Cb, insuring that a uniform set of Cb inputs were being activated. Having addressed the concerns of Baker and Edgley (Baker and Edgley 2006a), the data presented above still show that olivocerebellar inputs arrive to the cerebellar PCs with nearly the same latency.

Assuming that there are synchronous responses among the neurons in the IO, it is still not known how olivocerebellar inputs arrive to the cerebellar PCs synchronously. In turtle, mechanisms that compensate for olivocerebellar conduction distance were shown to be localized within the Cb, and not between the IO and the cerebellar peduncle (Ariel 2005). Distance compensation may require regional myelination, as suggested for axons between thalamus and cortex (Salami et al. 2003) and postnatal changes that augment the olivocerebellar synchrony observed in adult rats (Lang and Rosenbluth 2003).

**Functional Topography**

Responses to IO stimulation are oriented sagittally in the medial contralateral hemisphere. A region approximately 0.7 mm from the midline was most excited by microstimulation of the IO contralateral to the only intact peduncle. Larger currents did activate much of the contralateral hemisphere, creating a response field that did not meet a 2-fold criterion of major:minor axis ratio set to evaluate the response
orientation. Small single brief current pulses (10-100µA) elicited optical signals with strong excitatory response fields that were oriented sagittally and never encroached on the midline raphe of the Cb. It is not known if the lack of a strong response in the lateral Cb reflects a measurement bias of this technique or a meaningful biological difference in the tissue.

Similar near-threshold stimulations of white matter in the in-vitro guinea pig Cb would evoke optical signals limited to just part of a single folia (Rokni et al. 2007). An analysis of the major:minor axis ratio of those responses revealed differences in their response orientation. A subset of those guinea pig Cb responses were oriented sagittally, suggesting that those specific white matter loci activated local bundles of climbing fibers, while other locations affected mossy fibers. In vivo rodent optical recordings using the neutral red dye also exhibited sagittal bands (100-300 µm wide) in response to either electrical stimulation of the vibrissae area of the ipsilateral face or directly in the contralateral IO (Chen et al. 1996; Hanson et al. 2000).

Responses to molecular layer stimulation transverses the entire Cb. Parallel fibers are the primary axons in the molecular layer and mainly terminate on the dendritic trees of PCs. Their axons are among the thinnest found in vertebrates (Wyatt et al. 2005). Conduction velocities of mammalian parallel fibers are 0.18–0.27 m/s although their lengths are not proportional to brain size. In turtles, parallel fibers have a mean diameter of 0.64 ±0.12 µm and have been observed to transverse nearly the entire Cb (Tolbert et al. 2004), forming approximately 100 synapses every millimeter. The optical responses following ML stimulation are consistent with continuous activation of the Cb along a narrow transverse beam from lateral edge to lateral edge. Their mean conduction velocity was 0.24-m/s, supporting the report that parallel fiber velocity is very conserved across species (Wyatt et al. 2005).

Hyperpolarizing responses were observed to ML stimulation, but only occasionally. Thus, unlike the depolarizing responses in this preparation, inhibitory networks within the Cb may be more labile in the in vitro conditions of this preparation. Hyperpolarizing response fields are also described in the guinea pig Cb. Like turtle (Fig. 8D), they are also oriented in the transverse plane straddling the beam of excitation. Those mammalian inhibitory regions were also more short-lived than the excitatory response in that in vitro Cb preparation (Cohen and Yarom 2000a).
Observing inhibition while recording voltage may be difficult because the inhibition that uses a shunting mechanism (large conductance increases near the resting membrane potential) does not produce a large membrane voltage change even though there is a large synaptic current. Thus, measuring inhibitory fields in cerebellar cortex would require neuronal membrane that is tonically depolarized. That requirement would make it even more difficult to measure in this preparation than in mammals for two reasons. First, these experiments use the turtle, a cold-blooded vertebrate species with low metabolism and naturally low level of neural activity. Second, this preparation uses the turtle’s adaptive ability to lower its metabolism even further to survive long dives and a dormant state in water under frozen ponds for many winter months. Recordings from a variety of turtle brainstem regions have demonstrate the very low tonic synaptic and spike activity in this in vitro preparation (Fan et al. 1993; Fan et al. 1997; Jones and Ariel 2008; Rosenberg and Ariel 1990). Responses in mammalian tissue are more active, even in vitro, so have exhibit more depolarized neuronal membranes upon which inhibition can often be measured as a hyperpolarization (Cohen and Yarom 2000a).

Although hyperpolarizations were not commonly measured in these experiments, it is still likely that inhibition is presence in turtle Cb (Midtgaard 1992a; b). Cb inhibitory fields were initially described electrophysiologically as two bands lateral to the excitatory parallel fiber beam (Eccles et al. 1966b), which are believed to limit the natural spread of those beams (Santamaria et al. 2007) and play a role in sharpening the response timing of PC responses (Mittmann et al. 2005). Optical recordings of transverse inhibitory bands indicate two distinct circuits in vitro (Cohen and Yarom 2000a) while in vivo recordings resulted in sagittal bands (Gao et al. 2006). Responses to granule cell layer microstimulation may evoke an excitatory patch. The repeating modules of Cb each receive excitatory input from mossy fibers, which in turn stimulate granule cells (Walsh et al. 1972). Axons from granule cells ascend to the molecular layer, synapse with PCs during the ascent and bifurcate to form parallel fibers (Huang et al. 2006; Tolbert et al. 2004). Stimulating mossy fibers activates a local patch of PCs via ascending axons of granule cells (Bower and Devor 2003; Cohen and Yarom 1998; Rokni et al. 2007), as shown by correlated activity of granule cells and their overlying PCs
Similar patches of Cb activation were observed during tactile stimulation (Bower and Woolston 1983) in intact rats.

In the present study, microstimulation within the granule cell layer also activated limited circular regions, from a single diode up to a dozen diodes. It is not know whether optical signals of these patches are due to local activation of stained granule cells or trans-synaptic activation of their overlying PCs from the ascending granule cell axons. The evoked patches were often contaminated by weak responses that were either oriented in the transverse or sagittal planes. Clearer patches were observed in larger animals that have a thicker cerebellar cortex. This suggests that, when weakly oriented responses occurred, current spread from the granule cell layer to WM and ML axons also evoked synaptic activation of PCs.

**What are these optical recordings measuring?**

For optical recordings, light could be absorbed by dye anywhere along its radial path through the Cb. To identify the source of the absorbance signal, stained intact Cb was cut transversely into 1-mm thick slices and subjected to optical recordings during microstimulation of different Cb layers (Brown and Ariel 2007). Visibly, those slices were stained throughout the layers, although the molecular layer was darker than the granule cell layer. Optical response from those slices following focal ML stimulation exhibited slowly traveling excitatory waves emanating from the stimulus site and confined to ML, consistent with the transverse beams reported in this present study. ML responses are not due to parallel fiber depolarization because the optical signals were absent during substitution of Co$^{2+}$ for Ca$^{2+}$ in the media (Brown and Ariel 2007). Thus, ML responses probably results from the local depolarization of PC dendritic membrane, as suggested by the report that simultaneous optical and intracellular recordings from turtle PCs exhibit a similar response shape (Lu et al. 2003).

The source of optical signals during WM stimulation is less clear. An optical response to antidromic PC activation would be immediate and brief, as the dyes and photodiodes used in this optical technique have enough temporal resolution to resolve single action potentials. Yet, WM responses were delayed and lasted tens of milliseconds. These WM responses were also not observed when synaptic transmission was blocked by Co$^{2+}$ substitution (Brown and Ariel 2007). Both these findings suggest that
WM responses reflect depolarization of postsynaptic membrane, either of granule cells activated by mossy fibers or of PCs activated by climbing fibers. The first option is inconsistent with guinea pig Cb optical recordings to WM stimulation in which patches attributed to mossy fibers occurred after very short latencies (Rokni et al. 2007). On the other hand, there are several lines of evidence that suggest that the source for WM responses is from PC membrane depolarization due to activation by climbing fibers. First, WM responses have a similar long-latency synchronous response as that of IO responses (Fig. 7). Also, the sagittal response topography of WM and IO are similar (Figs. 3 and 4). Finally, similar studies in sagittal turtle Cb slices show that responses to WM microstimulation are observed in the PC and molecular layers caudal to the site of stimulation (Brown and Ariel 2007).

Optical signals originating in the ML may be measuring the PC postsynaptic membrane because that membrane’s area is much larger than that of parallel fibers or ML interneurons. Another large source of ML membrane may be glial cells, which are now known to respond to ectopic vesicular glutamate release or spillover from climbing fiber synapses (Matsui and Jahr 2004; Matsui and Jahr 2003). For neural membrane in the ML, the PC dendritic area is much larger than the PC soma area. Moreover, fast Na⁺-spikes in turtle PCs are generated only in the soma while slow Ca²⁺-spikes occur throughout the dendrites (Hounsgaard and Midtgaard 1988). These regenerative Ca²⁺-spikes spread rapidly through the spiny dendrites, the proximal dendrites and the cell body in response to climbing fiber stimulation (Chan et al. 1989). The same response spread was observed in optical recordings from single PC dendrites filled with a Ca²⁺-sensitive dye in a slice of guinea pig (Ross et al. 1990).

These optical responses may also be using different mechanisms following IO, WM and ML stimulation. The pharmacology of turtle PCs indicates that slow-EPSPs evoked by ML stimulation were blocked by one glutamate receptor antagonist while slow-EPSPs evoked by climbing fibers persisted (Gabbiani et al. 1994; Larson-Prior and Slater 1989). The present study shows differences in onset latency: responses to ML stimuli were immediate while responses to WM stimuli were significantly delayed. If this delayed response is mediated by climbing fibers onto PC dendrites, it may be mediated by slower metabotropic glutamate receptors and not result ionotropic receptors (Dzubay and Otis 2002).
Alternatively, climbing fibers might cause a metabotropic glutamate receptor release of endocannabinoids that in turn mediate a decrease in GABA onto PCs (Duguid et al. 2007).

Delayed optical responses after WM and IO stimulation may also involve a coupled neuronal network which contributes to sagittal response synchronization. Electrical coupling between Purkinje cells has not been observed (Pakhotin and Verkhratsky 2005), even though Sotelo and Llinas first reported gap junctions in the molecular layer of Cb many years ago (Sotelo and Llinas 1972). Recently, however, physiological evidence indicates that electric coupling synchronizes activity in ML interneurons (Mann-Metzer and Yarom 2000) and anatomical studies show that ML interneurons are restricted to single sagittal bands (Sillitoe et al. 2008). A sagittal interneuronal network may therefore contribute to a synchronization observed in WM and IO responses in this study.

In the present study, optical recording techniques have served as a useful tool to reveal the normal spatial and temporal features of responses of the entire Cb. However, to understand the underlying mechanisms of synchronous responses to IO stimulation, modification of the Cb network by selective drug application may reveal the underlying mechanisms of response timing in the cerebellar cortex.

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FIGURE LEGENDS

Figure 1. In vitro turtle brainstem-cerebellum preparation stained with the voltage-sensitive dye RH482.

A) Photographs of a turtle preparation in the recording chamber during ambient room illumination. The camera was in the front of the microscope directed toward the microscope stage. The oxygenated media enters from the hypodermic needle at the lower right, passes over the tissue and then is aspirated from the back compartment of the chamber (not shown). The cerebellar cortex is rotated away from the brainstem by cutting its left peduncle and pinning it ventricular side up to the Sylgard floor of the front shallow compartment of the chamber. The brainstem was pinned dorsal side up in the middle deeper compartment which also contains the ground wire. For optical clarity, the cerebellar cortex is covered with a rectangle of cover slip glass which is held in place by a lead weight in the back. B) The transilluminated cerebellar cortex was also photographed through the 2x objective of the microscope via a port that maintains a fixed alignment with the photodiode-array. The midline raphe is observed between the rostral and caudal midpoints (see arrows). A glass micropipette was placed into the cerebellum so that its tip, bent at 90° but no longer visible deep in the tissue, can be positioned near the Purkinje cell layer. The shank of that pipette (A, upper right) had another bend to avoid touching the cover glass, the chamber walls, and the microscope objective. The upper left of A shows the shank of a bipolar stimulating electrode whose tip was inserted in the contralateral inferior olive, near the obex of the floor of the fourth ventricle. These photographs show the caudal Cb down, as it appears when view in front of the microscope. All subsequent figures display the cerebellum with its rostral end down, as viewed through the microscope oculars.

Figure 2. Analysis of cerebellar responses to Inferior Olive microstimulation. A) The baseline Resting Light Intensities of each diode are first measured for each preparation prior to stimulation, displayed here in grayscale. In this case using a 2x microscope objective, the cerebellum does not fill the field so its outline is apparent (same preparation as in Fig. 1). All subsequent recordings are divided by these resting intensities (I), so that each diodes response is normalized to its relative absorbance, ΔA/A, to adjust for
different amounts of staining within each tissue and among different preparations. Absorbance traces of each diode ($\Delta A/A$ measured for 155 ms) are displayed below in D. Flat gray traces are from diodes that do not correspond to the transilluminated tissue. B) A single diode trace (from the diode outlined in A) that represents the standard raw data format (after temporal filtering) with responses to 4 current pulses (50 $\mu$A, 100 $\mu$s to the contralateral Inferior Olive). C) As in B except showing responses to 26 current pulses at 12 Hz. These responses summate temporally. D) Spatial display of white traces for diodes that imaged the cerebellum, superimposed on a monochrome image of the tissue (from Fig. 1B). Short gray lines represent diode positions in the array that did not image the cerebellum. Each trace is an average of 4 responses as shown in B. Some of the diodes have a noisier baseline because the $\Delta A/A$ computation uses a small value of resting light intensity where the tissue was thicker. A curved line indicates the midline raphe. E) A mean absorbance trace of the single diode ($N = 16$) is shown on the same expanded time scale as F) the mean extracellular voltage traces ($N = 20$) recorded through the micropipette seen in Figure 1B. Electrical responses to Inferior Olive microstimulation often can exhibit oscillations suggestive of synchronized complex spikes. In contrast, optical responses have a longer time-to-peak.

Figure 3. Topography of cerebellar responses to Inferior Olive microstimulation. A) Graphical representation of amplitude responses of Figure 2D, displayed as a dot-amplitude array having the dot diameter is scaled to the peak relative absorbance change. These dots are displayed on a standard preparation drawing of a typical cerebellum and brainstem with cut peduncle and eighth cranial nerve (nVIII). A bipolar electrode stimulated the Inferior Olive at the yellow dot in the fourth ventricle near the obex. Diodes are selected (green) if their response was greater than approximately 2 standard deviations of the prestimulus baseline. A best-fit ellipse is computed for those selected diodes (major axis, red line; minor axis, blue line). B) The major ellipse axes of responses to inferior olive stimuli are displayed as red lines on the standard preparation drawing. The lines were aligned with a common point of the rostral cerebellar midline (red dot), although the caudal end of the cerebellar midline (light-blue circles) varied because the preparation size was not uniform. A ratio criterion for the major to minor axes of 2:1 was used to exclude a few circular datasets for which responses to IO microstimulation was too strong so
filled the attached hemisphere. Note that the major axes are sagittal and lateral to the midline (vertical striped line).

Figure 4. Topography of cerebellar responses depends on the stimulating location, using the same stimulus parameters (100 μA, 100μs pulse) in the same preparation (C). Responses in A,D,F are displayed as described for Figure 2D. A) Responses of the diode-array to microstimulation of the Inferior Olive, marked by a red dot in C. D) Responses of the diode-array to White Matter microstimulation on the rostral ventricular surface of the hemisphere contralateral to the attached peduncle. Note that the shank of the stimulating electrode in C and its yellow outline in D and G display optical parallax due to the overlying cover glass. G) Responses of the diode-array to microstimulation of the Molecular Layer deep to the surface stimulation described for D. B,E,H) Using dot-amplitude arrays, whose diameters are scaled to the peak absorbance change, the topography of each response is displayed as its major (red) and minor (blue) elliptical axes. The responses spanned the full extent of the cerebellar cortex. F,I) Major ellipse axes (red) drawn on a standard preparation (as in Fig. 3B). The individual cerebella had variable lengths, as indicated by the position of the light-blue circles on the midline raphe. The responses to white matter stimuli (F, 14 experiments) were parasagittal but their medial lateral position depended on the microstimulation site (blue dots). Molecular layer stimuli evoked transverse responses (I, 11 experiments) aligned with the rostral-caudal position of its microstimulation.

Figure 5. Depth analysis stimulating at three levels of a cerebellum of a 30-cm long turtle. The top row shows low-magnification photographs of the preparation viewing the ventricular (A) and pial surface (B). Depth of stimulation (50 and 75 μA) was measured from the white matter (WM) or pial surface, respectively. In the second row, yellow lines represent the midline based on the raphe or estimated from blood vessel pattern, respectively. Responses shown in the second row are to WM stimuli; in the third row to granule cell layer stimuli; and in the bottom row to molecular layer stimuli. Data for A recorded on the day of the surgery/staining: data for B recorded the next day. Pseudocolor images were generated with same filter and threshold values. C) Pseudocolor images as in middle row of 5A except granule cell
layer stimulation reduced to 15 µA. The left image was processed as in 5A. The right image was processed with threshold value of 37% (half that of the standard 75% of the left image, as noted by the black line on the color scale for each image). *Grayscale latency arrays* show latency computed for data represented in *pseudocolor image* to its left. The stimulation sites are indicated by red dots.

**Figure 6.** Series of *pseudocolor images* showing the timing of the optical responses to IO, WM, and ML microstimulation. Data from same experiment shown in Figure 4 A,D,G; respectively. Numbers indicate millisecond latency after the stimulating pulse.

**Figure 7.** Latency analysis of optical responses. **A,D,G** Three rows of *grayscale latency arrays* show latency following IO, WM, and ML microstimulation, computed for data in the three rows A,D,G of Figure 4; respectively. See also *pseudocolor images* in Fig. 6. The microstimulation sites in D and G are indicated by red dots. **B,E,H** Latency-distance plots of data shown in A,D,G; respectively. **C,F,I** Lines of linear regression fits of B,E,H and all other latency-distance plots in IO, WM, and ML samples; respectively. The inverse of the slope of the fits correspond to the velocity of the traveling response. Solid lines were statistically different than zero-slope (p < 0.05). Dotted lines indicate fits that are statistically indistinguishable from a zero-slope. Wide striped line indicates the mean slope for that sample.

**Figure 8.** Examples of different spatial-timing analyses. **A** The *grayscale latency array* on the left shows a mixed response to a mid-depth stimulation (700 µm below pia of Cb from a 15-cm long turtle; same tissue as C but day 2), with a sagittal region outlined in black and a transverse region outlined in red. The vertical line is an estimate of the midline raphe, as all examples in this figure came from experiments with the Cb placed pial side up. The *latency-distance plot* on the right shows the respective timing of the blue and red regions. **B** The *grayscale latency array* shows bidirectional transverse responses to ML stimulation. Separate latency-distance plots were generated below for responses traveling from the stimulus point to the left and right. Both components of the response travel at a similar speed in each
C) Comparison of responsive diode detection using wavelet analysis (blue, ‘rise time’) versus maximal response amplitude (red, ‘peak time’). Separate latency-distance plots show data for ML microstimulation (left) and IO microstimulation (right). Insets above the plots show grayscale latency arrays when detecting peak time, and insets below the plots show grayscale latency arrays when detecting the rise time. This result used the same tissue as A but from day 1. D) A modified grayscale latency array showing depolarizing diodes in shades of gray and hyperpolarizing diodes in shades of red. The upper left inset has a depolarizing response trace from the gray diode marked with an ‘x’. The lower left inset has a hyperpolarizing response trace from the marked red diode. The stimulus was a train of six pulses, as shown below the lower left inset. The microstimulation site is indicated by a red dot.
SUPPLEMENTAL FIGURE LEGENDS

Figure Sfig2-IO-response.mpg: Pseudocolor movie superimposed on photograph of stained turtle brain preparation. A red dot flashes at the onset of the inferior olive microstimulation.

Figure Sfig3-IO-response.mpg: Pseudocolor movie superimposed on photograph of turtle cerebellum, ventricular side up. A red dot flashes in the upper left at the onset of the inferior olive microstimulation. A black line in the color bar on the right indicates the pseudocolor threshold.

Figure Sfig4-stim-image-thru-scope.jpg: Photograph of stained turtle cerebellum, ventricular side up, transilluminated by the 150 W tungsten-halogen bulb through the microscope condenser. The magnified view of Figure 4C shows the placement of the bipolar stimulating electrode on the ventricular surface of rostrocaudal fiber bundles (white matter). The edge of the cover glass is visible on the left side, resulting in a displaced unfocused second image of the electrode. Unlike the true color of figure 4C photographed in ambient illumination, the color of this transilluminated tissue is distorted.

Figure Sfig5A-WM-stim.jpg: Photograph of cerebellum, showing magnified view of top photograph of Figure 5A. Refer to legend of Sfig4-stim-image-thru-scope. Note that this cerebellum is larger because it was removed from a 30 cm turtle.

Figure Sfig5A-WM.mpg: Pseudocolor movie superimposed on photograph of turtle cerebellum, ventricular side up. White matter microstimulation occurs when the red dot flashes in the upper left corner. A black line in the color bar on the right indicates the pseudocolor threshold.

Figure Sfig5A-400-bl-WM.mpg: Pseudocolor movie. Refer to legend of Sfig5A-WM, except the stimulation is 400 µm below the surface.

Figure Sfig5A-600-bl-WM.mpg: Pseudocolor movie. Refer to legend of Sfig5A-WM, except the stimulation is 600 µm below the surface.

Figure Sfig5B-Pia-stim.jpg: Photograph of cerebellum. Refer to legend of Sfig5A-WM-stim.jpg, except this is a magnified view of top photograph of Figure 5B.

Figure Sfig5B-700-bl-Pia.mpg: Pseudocolor movie. Refer to legend of Sfig5A-WM, except the cerebellum is pial side up and the stimulation is 700 µm below the surface.

Figure Sfig5B-500-bl-Pia.mpg: Pseudocolor movie. Refer to legend of Sfig5A-WM, except the cerebellum is pial side up and the stimulation is 700 µm below the surface.

Figure Sfig5B-Pia.mpg: Pseudocolor movie. Refer to legend of Sfig5A-WM, except the cerebellum is pial side up and the stimulation is 700 µm below the surface.

Figure Sfig5C-low-stim-low-threshold.mpg: Pseudocolor movie. Refer to legend of Sfig5A-WM, except the stimulation intensity is 15 µA, 400 µm below the surface. The movie was generated using a pseudocolor threshold half that of Figure 5A,B. Note the black line in the color bar on the right indicates the lower threshold.

Figure Sfig6-IO.mpg: Pseudocolor movie superimposed on photograph of the cerebellum, ventricular side up. A red dot flashes in the upper left at the onset of the inferior olive microstimulation.

Figure Sfig6-WM.mpg: Pseudocolor movie superimposed on photograph of the cerebellum, ventricular side up. A red dot flashes in the upper left at the onset of the white matter microstimulation.
Figure Sfig6-ML.mpg: Pseudocolor movie superimposed on photograph of the cerebellum, ventricular side up. A red dot flashes in the upper left at the onset of the molecular layer microstimulation.

Figure Sfig8A.mpg: Pseudocolor movie superimposed on photograph of the cerebellum, pial side up. A red dot flashes at the onset of microstimulation within the cerebellum.

Figure Sfig8B-ML.mpg: Pseudocolor movie superimposed on photograph of the cerebellum, pial side up. A red dot flashes at the onset of the molecular layer microstimulation.

Figure Sfig8B-ML-highpass-filter.mpg: Pseudocolor movie. Refer to legend of Sfig5A-WM, except the movie was generated using a high pass filter to demonstrate the bidirectional motion of the response.

Figure Sfig8C-IO.mpg: Pseudocolor movie superimposed on photograph of the cerebellum, pial side up. A red dot flashes in the upper left at the onset of the inferior olive microstimulation.

Figure Sfig8C-ML.mpg: Pseudocolor movie superimposed on photograph of the cerebellum, pial side up. A red dot flashes in the upper left at the onset of the molecular layer microstimulation.

Figure Sfig8D-colored-by-polarity.jpg: Spatial display of traces for diodes that imaged the cerebellum, pial side up, superimposed on a monochrome image of the tissue. Blue lines represent positions of depolarizing responses; orange lines represent positions of hyperpolarizing responses. The transverse place of the tissue was not placed horizontal in the chamber so, unlike Figure 8D, this display is not corrected for that misalignment.

Figure Sfig8D-ML.mpg: Pseudocolor movie superimposed on photograph of the cerebellum, pial side up. A red dot flashes in the upper left at the onset of the molecular layer microstimulation.

Figure Sfig8D-ML-no-threshold.mpg: Pseudocolor movie. Refer to legend of Sfig8D-ML, except the movie was generated without a pseudocolor threshold, thereby revealing depolarizing and hyperpolarizing responses.
A
Cut-End
Contra
Peduncle

B
Cut-End
Contra
Peduncle

1 mm
A B

400 µM below WM

75 µA

1 mm

50 µA

700 µM below Pia

11

15 µA 400 µM below WM Analysis at low threshold