Latencies of Climbing Fiber Inputs to Turtle Cerebellar Cortex

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INTRODUCTION

Responses of a cortical sheet of neurons to synchronous spike initiation from a common input should occur asynchronously, with the latency correlated with the differing lengths of conduction paths. However, previous studies reported that regions of rat cerebellar cortex (Cb) responds synchronously to inferior olive stimulation (IO) (Ling and Rosenbluth 2003; Sugihara et al. 1993). Moreover, such synchronous responses may exist in behaving animals because neurons in the IO are normally synchronized (Llinas et al. 1974; Welsh et al. 1995).

In many species, the cerebellar cortex (Cb) is foliated so it is difficult to record olivocerebellar responses across much of the convoluted cortical surface and to measure the climbing fiber (CF) path that carries these signals. Physiological measurements of response latency deep in a folium are confounded by inaccuracies in localizing the microelectrode tip to the cerebellar layer and location along the cortical surface. Despite these limitations, the rat IO evokes synchronous activity in Cb, latency and CF length can be directly measured. The results indicate that CF fiber input to Cb evokes complex Purkinje cell responses whose conduction velocity is fixed outside the brain stem but varies within the Cb to synchronize responses throughout the entire Cb cortex.

METHODS

The subjects of these experiments were 17 red-ear pond turtles, Pseudemys scripta elegans (carapace length, ~15 cm), housed in an aquarium at room temperature with facilities to swim and bask on a 16/8-h light-dark cycle. Extracellular Cb recordings were made in an in vitro brain stem preparation (modified from Ariel and Fan 1993). Briefly, the animal was anesthetized with 12.5 mg thiopenthal, and the brain was surgically removed followed by immediate telencephalic ablation. The dura was removed from the cerebellum and brain stem, leaving the pia intact. The resulting preparation was placed dorsal side up onto the polyethylene mesh above a Sylgard floor on one side of a two-compartment chamber divided by a central wall of Sylgard. Normally, the Cb rests over the fourth ventricle and is connected to the brain stem rostrilaterally by discrete left and right peduncles. After cutting the left peduncle, the Cb was flipped onto the opposite compartment. A small 1-mm slit was cut in the Cb was flipped onto the opposite compartment. A small 1-mm slit was
made at the caudal Cb midline so that the Cb laid flat, ventricular side up. The caudal edges of the Cb were secured with pins into the Sylgard floor, as were the spinal cord and thalamus. Using a standard stereomicroscope, a recording pipette was advanced through the ventral Cb surface at an angle of 45° (except for the depth series experiment of Fig. 1, when the pipette was advanced orthogonally into the Cb).

The preparation was normally bathed in a room temperature media (in mM, 96.5 NaCl, 2.6 KCl, 2.0 MgCl₂, 31.5 NaHCO₃, 4.0 CaCl₂, 20 d-glucose) bubbled with 95% O₂-5% CO₂ (pH ~ 7.6, osmolarity ~ 274 mOsmol). In this media, Cb levels of ATP are maintained without increases in extracellular potassium even during anoxia (Perez-Pinzon et al. 1992). Occasionally, the preparation was bathed in modified media in which either CoCl₂ or CdCl₂ substituted for CaCl₂ to block synaptic transmission (Chen and Chesler 1991; Chesler and Rice 1991). The protocols of animal care and surgical procedures were approved by the Saint Louis University Animal Care Committee.

**Electrophysiological recording and stimulation**

Extracellular field potentials (with respect to the chorided silver ground wire on the pia side of the Cb) were recorded by intracellular amplifiers (3 KHz low-pass filtered cut-off frequency) using glass pipettes (25-μm tip diam) filled with 3 M K-acetate (resulting DC resistance ~ 1 Mohm). For each condition, 16 or 20 responses were digitized for off-line computer analysis, but in some cases, the initial response was discarded due to an artifact. The signals were filtered with a boxcar filter that did not shift the response latency. This filter improved the software’s automated detection of response peaks, providing an unbiased measure of latency from stimulus onset. Some stimulus artifacts were trimmed prior to displaying superimposed traces.

A stimulus isolator provided biphasic current pulses of 100-μs duration to the tungsten bipolar stimulating electrodes (115-μm tip separation). Using the brain stem preparation described above in the initial experiments, the path of this electrode to the inferior olive was hampered by the a fibrous pial membrane, which covered the brain stem outside of the floor and inner walls of the fourth ventricle that was covered by the Cb. Consequently, the stimulating electrode would first dimple this membrane before passing into the brain stem, which led to the inability to measure the stimulating electrode depth. Later experiments used two different modifications of the brain stem preparation to better measure the depth of the stimulating electrode. One approach was to make a parasagittal cut through the inferior olive in the contralateral brain stem and measure the effectiveness of medial surface stimulation to evoke a cerebellar response (Fig. 6, inset). A second approach was to cut the dorsal midline of pia, beginning at the obex and extending 2 mm caudally, using fine iris scissors. Splitting the brain stem in this manner allowed the stimulating electrode to bypass the pia and reach the inferior olive directly, thereby permitting an accurate measurement of electrode depth.

Using a stereomicroscope, the position of the tip of the recording pipette was also initially difficult to measure. Later experiments used a compound microscope configured for differential interference con-

![FIG. 1. Responses at 8 depths into cerebellar cortex (Cb). Each trace is the average of 20 trials. A: brain stem stimulation (50 μA, 100 μs) deep to the obex and contralateral to the recording electrode in the Cb. B: molecular layer stimulation (50 μA, 100 μs) positioned in the transverse plane medial to the recording electrode. Both stimuli evoked a large response at the middle of the Cb, presumably at the level of the Purkinje cell bodies. Ventricular surface of the Cb was visualized using differential interference contrast microscopy to position the tip of the recording pipette. Recordings were made at different depths as the electrode was advanced orthogonal to that surface, and Cb depth was directly measured by a micrometer. C: brain sketch showing electrode positions used for A. Each box of Cb grid is 0.8 mm square. D: cross-section of Cb stained for Nissl substance to reveal Cb layers.](http://jn.physiology.org/doi/10.1152/jn.00811.2004)
trast (DIC) to visualize the pipette tip adjacent to the Cb’s ventricular surface. Although variation in stimulating and recording depth led to variability in response amplitude for a given stimulus current, the response latency was unaffected. As the brains were similar in size, the positions of stimulating and recording electrodes were marked onto a common template, for which the distance from the obex to the peduncle is 11 mm and from the peduncle to the caudal end of the Cb is 8 mm. Relative to these rostrocaudal and mediolateral distances, the depth of the stimulating electrode near the IO was small (always <1 mm).

Histology

In some cases, the metal bipolar stimulating electrode was replaced with a pipette filled with pontamine sky blue (4% in 0.1 M potassium acetate). Dye injection was either by pressure pulses or negative current. Following these physiology experiments, the tissue was immersed in 4% paraformaldehyde in phosphate buffer overnight. After sinking in 30% sucrose, the tissue was frozen, and 50-µm sections were made on a sliding microtome. Sections were dried on a microscope slide, coverslipped, and photographed. Sections were counterstained (cresyl violet or neutral red) and rephotographed.

RESULTS

Extracellular potentials were consistently evoked from the in vitro turtle Cb in response to brief pulses (100 µs) of low current (10–100 µA) to the brain stem at the rostrocaudal level of the obex, but lateral and deep to the surface (Fig. 1C). The field potentials were first characterized to localize their source within the Cb layers (Fig. 1D). Depth profiles shown in Fig. 1A show that brain stem stimulation-evoked fields measured at all Cb depths as well as within the bath just ventral to the ventricular surface of the tissue. The dominant evoked signals were slow field potentials that had their maximum amplitude as a negative deflection near the mid-depth of the cerebellar cortex (negative is down on all figures). A negative deflection was also recorded far into the fourth ventricle (in this case, above the inverted Cb sheet with respect to the ground wire below the Cb sheet). This negativity may also contribute to the local fields recorded in the granule cell layer (Fig. 1A).

The response to brain stem stimulation clearly reversed as the electrode depth changed (consistent with findings of Bantli 1972). At the depth of the maximal signal, spontaneous high-frequency multi-unit spike activity was often recorded, possibly reflecting activity of Purkinje cells. The depth profile to molecular layer stimulation (Fig. 1B) was similar to that of stimulation of the brain stem, but the response latency was shorter than the responses to brain stem stimulation. The common position for the largest extracellular negativity for both brain stem and molecular layer stimulation suggests that both fields are dominated by a common depolarized cellular element whose surface constitutes most of the excitatory membranes of the Cb, namely the Purkinje cell.

Another indication that the source of these fields was the Purkinje cell comes from recordings made in a Cb that was first fixed on a vibratome and its caudal end was sliced off at a shallow angle from the ventricular side. Later, during brain stem stimulation in the recording chamber, typical field potentials were recorded when the recording pipette was placed on the cut edge adjacent to somata of Purkinje cells (visualized with DIC optics). No fields were evoked from the region of Cb that lacked Purkinje cell bodies.

In the remaining experiments, the depth of the Cb recording electrode was adjusted to the depth that yielded the largest response to the brain stem stimulation. At that Cb depth, the field potential responses to brain stem stimulation were exclusively negative deflections. The onset and peak latencies were 5.96 ± 1.29 and 9.90 ± 2.16 (SD) ms (n = 11). These responses had a half-width duration of 6.49 ± 1.25 ms, which is much longer than an extracellular spike. Although small changes in the position of molecular layer stimulation caused large changes in the size and latency of the responses (data not shown), similar changes in the electrode position in the brain stem had minimal effects (see Fig. 3). These findings suggest that these Cb field responses to brain stem stimulation were dominated by Purkinje cell responses but were not simple spikes.

With the recording electrode at its optimal depth in the Cb and the stimulating electrode at a fixed position deep to the obex, two other features of Cb response shape were observed (see both in Fig. 2A). One is that the initial negative voltage deflection was often followed by a second smaller but longer lasting negative wave with a mean peak latency of 26.41 ± 4.24 ms (Fig. 2, A and B). The second wave appears independent of the first because it did not recover for long intervals of paired pulse stimulation (Fig. 2B). This differs from the first deflection that responded to the second pulse of a paired pulse with interstimulus intervals >12 ms. However, quantifying the first deflection’s strength for short paired pulse intervals was difficult because the second deflection confounded its measurement. The second deflection was clearly depressed for a long duration so all the remaining experiments used single pulses with interstimulus intervals >3 s.

The second observation is that, at certain stimulus amplitudes, four to five small but synchronized oscillations were superimposed on the larger responses (Fig. 2, A and C; see also Figs. 3, 4, 6, and 8). The wavelets had variable latencies from preparation to preparation but lasted about 20 ms. These rhythmic events follow the large initial deflection and are similar to the complex spike wavelets shown in the initial description of climbing fiber responses of Purkinje cells (cat, Eccles et al. 1966a; turtle, Larson-Prior and Slater 1989).

To confirm that these extracellular responses were indicative of complex spike responses of Purkinje cells in this preparation, higher impedance pipettes (DC resistance ~ 4 Mohm) were advanced into the Purkinje cell layer. Figure 2C shows an isolated Purkinje cell recording, in which there was a regular spontaneous firing rate of simple spikes (1-ms positive voltage deflection) but no spontaneous complex spikes. However, >7 ms following a pulse to the brain stem (deep to the obex and contralateral to the intact peduncle), an initial 1-ms positive deflection was followed by an 18- to 30-ms series of slower spikes. In this case, the average interval between spontaneous simple spike was 71.1 ms (Fig. 3D, filled bars). If the firing of simple and complex spikes was an independent event, the average latency of a simple spike that follows a complex spike (evoked at random) would be about 35 ms. The bottom traces of Fig. 2C show that simple spike firing was suppressed following the occurrence of a complex spike. The average latency of a simple spike after a complex simple (evoked at random) was 193.1 ms (Fig. 3D, open bars). This shows that simple and complex spike firing are not independent events. Moreover, these recordings provide evidence that the field
FIG. 2. Cb recordings in response to 90-μA pulses into the contralateral brain stem deep to the obex. A: 19 superimposed traces show 2 negative deflections, on which small synchronous oscillations were observed. B: responses to 8 paired pulse stimuli of different intervals are shown using an adjacent stimulation site that evoked a prominent second deflection. Note that this second deflection did not occur in response to the 2nd pulse of the pair. However, the 1st deflection responded when the interval of the paired pulses was >12 ms. C: isolated Purkinje cell unit recording, showing suppression of simple spikes firing by occurrence of complex spike, evoked by brain stem stimulation. 1: 3.2 s of spontaneous simple spike firing. Note its regular spike frequency. 2: traces derived from 30 s of data as in 1, showing 10 superimposed 130-ms traces, each aligned by a simple spike. 3: 10 superimposed 260-ms traces, each aligned by a complex spike evoked by a 90-μA pulse. One gray spike indicates it is the 2nd simple spike after the complex spike. 4: 7 superimposed 260-ms traces, each aligned by a complex spike evoked by a 90-μA pulse. Three gray spikes represent the spontaneous simple spikes in traces that failed to evoke complex spikes. D: histogram of spontaneous simple spike latency. Filled bars, measured after another simple spike without brain stem stimulation as in C2 (n = 419). Open bars, measured after evoked complex spike as in C3 (n = 246).
potentials evoked by brain stem stimulation may represent complex spike activity evoked by Purkinje cells.

**Localization of source for Cb input to IO**

Because the dorsal surface of the caudal brain stem was accessible, the floor of the fourth ventricle was readily mapped to localize the IO that evoked Cb responses. As shown in Fig. 3, large responses were typically evoked just left of the midline (contralateral to the Cb recording). Apart from the synchronized activity evoked deep to the obex, two other responses were observed. At the level of the eighth cranial nerve and the vestibular nuclei, responses were evoked with latencies much shorter than those evoked at the level of the obex (data not shown). On the other hand, stimulation that was contralateral and rostral to the obex evoked unit activity with highly variable latencies >50 ms (see responses to stimulation sites 2–4 of Fig. 3A). These variable responses occurred together with the shorter latency responses when evoked more caudally (sites 3 and 4) or independently when stimulating more rostrally (site 2, see also Fig. 3C).

A relationship between response latency and stimulus position was observed. As shown in Fig. 3B, the response latency to stimulation further from the intact Cb peduncle was longer than that evoked closer to the peduncle. This relationship is examined in more detail in Fig. 4. By stimulating more sites in the ipsilateral brain stem, it was possible to identify the path of afferent axons from the IO across the midline and along the floor and walls of the fourth ventricle. As the stimulation sites approached the peduncle, some Cb responses exhibited more complexity, perhaps due to recruitment of other Cb afferents. However, as with data shown in Fig. 3B, the response peak initiated deep to the obex had a shorter latency when the conduction path was shorter (Fig. 4B). Peak latency measure-
ments were quantified because they were detected reliably and remained consistent for a large range of stimulus intensities above threshold. These latencies were plotted as a function of distance and fitted with a regression line of the six mean values (Fig. 4C). For this preparation, the mean conduction velocity of this signal within the brain stem was measured to be 1.68 m/s (inverse of a slope of 0.59 ms/mm). Including the similar findings measured in another five preparations (1.44, 1.18, 1.62, 3.55, 2.59 m/s), it is estimated that the mean brain stem conduction velocity is 1.76 m/s (Fig. 9A).

To determine whether these field potentials are due to climbing fiber input to the Cb, additional experiments were performed. First, in some experiments, the brain stem was cut just rostral to the peduncle to eliminate the possibility that the Cb responses from stimulation of caudal brain stem structures were mediated via rostral structures such as the red nucleus (see Keifer 1996). The Cb responses persisted after rostral brain stem ablation (data not shown). In a second set of experiments, the brain stem was bathed in a modified media in which calcium ions were substituted with other divalent cations (either cobalt or cadmium) to block synaptic transmission (Fig. 5). With the Cb still bathed in normal media, the experiment tested whether brain stem stimulation excited axons that projected directly to the Cb or excited the Cb transsynaptically. In these experiments, tectal responses to optic nerve stimulation were used to verify that the synaptic-blocking media was effective within the brain stem compartment (Fig. 5, left traces). When the tectal response was blocked, the Cb field potentials due to brain stem stimulation were unchanged (Fig. 5, 2nd row of responses). When the synaptic-blocking media was added to the Cb compartment, the Cb response stopped responding to brain stem stimulation (Fig. 5, 4th row of right response column). Such experiments show that Cb responses are primarily, if not entirely, due to direct inputs to Cb and not spinal or other inputs that are relayed within the brain stem to the Cb. The response block during Cb application is also consistent with the depth profile of these Cb fields (Fig. 1), indicating that these field potentials are not generated by the climbing fibers themselves, but by the Purkinje cell responses to climbing fiber input.

The final two experiments that localized the source of these responses required surgical manipulations of the brain stem. In some experiments, after the intact contralateral brain stem was mapped from the dorsal surface (as in Figs. 3 and 4), the brain
stem was cut through the most sensitive region in a parasagittal plane. The lateral brain stem was deflected so that the medial brain stem surface (Fig. 6, shaded) could be mapped with the stimulating electrode just beneath the cut surface. Even before stimulation, a small, ventral area of gray matter was visible on this medial surface. The recordings revealed that only stimuli in that oval region evoked Cb responses (Fig. 6, inset A). Stimulation that was rostral, dorsal, or caudal to this region failed to evoke responses even using current in excess of 100 μA. Moreover, these Cb fields were strong in response to currents as low as 10 μA.

The second experimental approach to localize the turtle’s IO involved a different surgical manipulation of the brain stem. The fibrous pia membrane was cut along the midline, caudal to the obex, which permitted accurate measurements of the depth of the tip of the metal bipolar stimulating electrodes (115-μm tip separation). Effective positions for Cb responses were about 0.9 mm below the dorsal surface of the floor of the fourth ventricle. To document that location, the bipolar electrode was replaced with a micropipette filled with pontamine sky blue. Figure 7 shows the Cb response to monopolar stimulation through the micropipette. These Cb fields were evoked with brief pulses (100 μs) of current as low as 8 μA, indicating that very little current spread from the tip. After the dye was injected and the tissue processed histologically, the pipette tip was also localized about 0.9 mm below the dorsal surface. The results are consistent with the other experiments indicating that the IO is near the ventral surface at the level of the obex.

**Synchrony of Cb response independent of the recording site**

With the ability to record responses to climbing fiber input across the entire Cb surface and to measure the conduction path, the response latency was examined as a function of Cb position. In these experiments, the stimulation electrode did not move in the brain stem but the recording pipette was repositioned to many Cb locations for a given preparation. Figure 8 shows sketches of Cb, where the numbers on a sketch corre-
spond to the sequence and location of recording positions for a given preparation. Because the pipette depth was different for each recording location, response amplitude was variable. However, off-line analysis of the peak response latency revealed that the responses evoked by each Cb position had roughly the same latency. There was no systematically change in latency with respect to the recording position mediolaterally or rostrocaudally, indicating that CF responses in turtle Cb are synchronous across the entire cortex.

Crossed and uncrossed responses were also readily examined because the Cb midline is clearly visualized as a groove in the ventricular surface. With respect to the only connected peduncle, a consistent observation (4/4 brains) was that contralateral Cb responses were evoked only within a millimeter of the midline (Fig. 8B). (These contralateral responses are nominally ipsilateral to the side of the brain stem stimulation). A lack of responsiveness to brain stem stimulation was also observed along the lateral edges on both sides of the Cb (Fig. 8B, triangles), presumably due to damage from surgery where the forceps/pins held the Cb or due to the thinning of the tissue there.

Mean conduction velocity was compared for the path within the brain stem (from the IO to the peduncle) with the path within of the Cb. Graphs of the peak response latencies and the conduction distances within the Cb were plotted (Fig. 9B) in the same format at data from experiments in which the Cb recording pipette did not move, yet the stimulating electrode was moved in the brain stem (Fig. 9A, thin lines are regression lines from 6 experiments, including example in Fig. 4C). The mean slope was 0.569 (Fig. 9A, thick line), equivalent to a spike conduction velocity of 1.76 m/s.

Within the Cb, however, the slopes of each preparation were near zero (thin lines in Fig. 9B from 3 experiments, including examples in Fig. 8), with the mean slope of 0.05 (Fig. 9B, thick line). Determination of the mean spike conduction velocity within the Cb is difficult because of inaccuracies of computing the inverse of the flat line. Certainly, the spike conduction velocity is a higher value within the Cb for those climbing fibers that terminate in the caudal end of the Cb, relative to the climbing fibers that end in the rostral Cb. The finding of invariant latencies within the Cb show that Cb responses are synchronous following stimulation of the IO.

**DISCUSSION**

Based on in vitro extracellular recordings in Cb during brain stem stimulation, the turtle IO has been localized to gray matter near the ventral surface of the brain stem at a level just rostral to the obex. The depth profile of these Cb fields indicate that they were primarily Purkinje cell responses to climbing fiber input, not fields generated by the climbing fibers themselves. Each response had a prominent short-latency negative deflec-
tion, frequently followed by another smaller deflection. Superimposed on these deflections were synchronized membrane oscillations, like complex spike wavelets of Purkinje cells. These responses were shown to be directly activated by IO because they occurred during synaptic blockade of brain stem. The response latency did not lengthen for recording sites in the Cb that were more distant from the peduncle. This result extends a finding of complex spike synchrony to a nonmammalian species and shows this synchrony for the entire Cb. Moreover, we find that stimulating different positions along the walls of the fourth ventricle leading to the peduncle did result in a change in latency that increased with longer conduction paths within the brain stem. Therefore a mechanism exists within the Cb to synchronize Cb responses to IO signals.

Olivocerebellar physiology

Previous extracellular studies of turtle Cb (Keifer 1996; Walsh et al. 1972) have not focused on the olivocerebellar circuit. Intracellular Purkinje cell recordings indicate that turtle Purkinje cells have similar membrane conductances and spike firing properties as that of other vertebrates (Hounsgaard and Midtgaard 1988). Intracellular Purkinje cell recordings in turtle show that fast Na\(^+\) spikes are found only in its soma and spread distally, whereas slower, longer Ca\(^{2+}\) spikes originate along the proximal dendrites and can be evoked there by climbing fiber input (Chan et al. 1989; Hounsgaard and Midtgaard 1988). However, those responses to climbing fiber input were only evoked by shocking the peduncle.

In frog, olivocerebellar responses were measured in vivo (Llinas et al. 1969; Straka and Dieringer 1992), again using only white matter stimulation. Although focal IO stimulation was not employed, intracellular Purkinje cell recordings did show similar complex spike responses in Purkinje cells. The response consisted of a synchronized 20-ms burst of oscillations, the first wave being the largest. The response latencies were 2–5 ms for a 4-mm conduction distance, equivalent to the turtle Cb conduction velocity in vitro.

The depth profile and response shape described in these in vitro data are similar to field potential recordings using an in vivo turtle Cb (Bantli 1974), although those responses were again evoked by stimulated the posterior border of the peduncle. Compared with other species studied, Bantli found that the turtle had the largest negativity extending into the granule cell layer, without a positivity at the level of the Purkinje cell soma. This uniqueness was attributed to the curvature of turtle Cb and climbing fiber input restricted only to the lower third of the molecular layer, where the Purkinje cell dendrites are smooth (Bangma et al. 1983; Kunzle 1985a).

IO localization in the turtle

Although identifying the IO may be difficult in lower vertebrates because their cell bodies are not densely packed, there is evidence in turtle that CF originate at the level of the obex in the ventral portion of the inferior reticular field (Kunzle 1983a; but see Schwarz and Schwarz 1980). Using retrograde and anterograde tracers, the IO was identified in the caudal rhombencephalic tegmentum in an otherwise “cytoarchitecturally indistinct area” (Kunzle 1985a; Kunzle and Wiklund 1982).
FIG. 8. Cb responses during stimulation of one location deep to the obex, as the Cb recording position was varied. Horizontally magnified sets of superimposed traces were aligned with the stimulus to reveal response latency differences. Each response within each data set represents a different Cb position of the recording electrode. Peak latency measurements are plotted below the response sets. A: stimulation strength was 85 μA. Amplitudes were variable because Cb response amplitudes are very sensitive to the electrode depth although the response latencies are not (see Fig. 1). B: stimulation strength was 90 μA. Data from this Cb were too numerous to display below so only 6 of the 13 sets of traces are shown. Error bars indicate ±SD of each sample of 19 latencies. Regression lines used in Fig. 9.
In this study, brief pulses (100 μs) of low current (10–100 μA) were presented across bipolar electrodes deep to the obex and contralateral to the intact peduncle, a position that is consistent with anatomical IO localization in turtle. Responses from this area persisted during synaptic blockade so are not due to transynaptic activation of other Cb inputs. However, when mapping the brain stem on the side of the intact peduncle, it is difficult to exclude activation of non-IO inputs to Cb. The dorsal column nuclei are superficial structures near the obex (Kunzle and Woodson 1983) but few neurons there, if any, project to the Cb (Kunzle 1983a). Rostral to the obex are neurons that project directly to Cb from the trigeminal and vestibular nuclei. There are also direct spinocerebellar projections from ventral spinal cord (Kunzle 1983b) and even from the dorsal root ganglia (Kunzle 1982). Because all these inputs terminate as mossy fibers in the granule cell layer, activation of these paths is not expected to contribute to responses that are characteristic of CF input.

Anatomical localization of CF terminals in turtle Cb is also consistent with Cb responses from IO. Whereas the Purkinje cell projections are strictly ipsilateral (Bangma et al. 1983), CF axons that terminate contralateral to the injected peduncle were only labeled close to the Cb midline (Tolbert et al. 2004). This is consistent with our finding that responses were only observed within 1 mm across the midline from the intact peduncle.

**Synchrony in the IO**

In general, current pulses can be criticized for not being physiologically normal stimuli because they generate excessive synchronized activity in neurons that normally do not fire synchronously. However, the climbing fiber output of IO is normally quite synchronous (Lang 2003). IO neurons’ gap junctions transmit subthreshold oscillations of membrane potential at ~5–10 Hz, which synchronize the IO output (Llinas et al. 1974; Long et al. 2002; Sotelo et al. 1974). In fact, the IO has a higher gap junction density than most of the brain (Condorelli et al. 1998; De Zeeuw et al. 1995).

Therefore the Cb response to electrical IO stimulation may be similar to the normal synchronous activity of climbing fibers. The large initial negative deflection of turtle Cb described above may result from a physiologically normal calcium spike initiated by CF synapses of Purkinje cell dendrites in the inner third of the molecular layer (Bantli 1974; Chan et al. 1988; Hounsgaard and Midtgaard 1989; Kunzle 1985a).

Long latency events followed the initial deflection. Such delayed responses may be similar to slow excitatory synaptic potentials recorded in turtle Purkinje cells (Larson-Prior and Slater 1989). Unlike the initial deflection, the next delayed response was weak, easily depressed by previous stimuli, and had a latency >25 ms. This delayed deflection may be analogous to the delayed reflex responses originally reported by Eccles et al. (1966b) because of its long variable latency and inability to follow repetitive stimulation.

The longest latency events were asynchronous voltage deflections with latencies of >50 ms. The long latencies suggest that the responses are probably transynaptic (although it was not examined during the synaptic block experiments) and may be similar to the slow Cb activation observed after molecular layer stimulation (Chesler and Chan 1988).

**IO synchrony + fixed latency of olivocerebellar path = complex spike synchrony**

Synchronous activation of turtle climbing fibers results in synchronous complex response within the Cb. The results in turtle were similar to that found in the mammalian Cb, where IO responses evoked by midline brain stem stimulation (between the 2 IO) generated synchronous Cb responses (Lang and Rosenbluth 2003; Sugihara et al. 1993). Synchrony was also found by multi-electrode recordings of complex spikes that occurred during tactile and visual stimulation (Lang 2001; Llinas and Sasaki 1989; Sasaki et al. 1989; Wylie et al. 1995). Spontaneous complex spike synchrony was found predominantly in rostrocaudal bands that are 0.5 mm wide in the mediolateral dimension (Fukuda et al. 2001). The length of a synchronous band was difficult to measure in the foliated Cb, although measurements were made as deep as 2.6 mm into a fissure (Sugihara et al. 1993).

The distance between the IO and the caudal end of the turtle Cb varies from 11 to 19 mm, which is comparable to the almost twofold path length difference in the rat (Sugihara et al. 1993). Therefore signals originating in the IO would be expected to
arrive at different times across the Cb surface. However, because the Cb response to IO stimulation is synchronous, mechanisms must exist to compensate for length differences in the conduction path from the IO. One possible mechanism is a systematic increase in the CF axon’s diameter. In rats, axons projecting to the top of a folium were thicker than axons only terminating deep within the fissure, a distance of about 2 mm (Sugihara et al. 1993). The measured diameter change was 28% so, given that axon diameter is linearly related to conduction velocity (Goldman and Albus 1968), this change cannot fully compensate for a nearly twofold difference in path length. A second difference was that axons projected straight to the top of a folium, while axons terminating deep within the fissure took a more tortuous route. A similar analysis can be made for the unfoliated turtle Cb (Tolbert et al. 2004). The mean diameter of primary CF axons found in the rostral end of the Cb was 12% narrower than that at the caudal end (a distance of 8 mm). Similar to the rat data (Sugihara et al. 1993), the axon diameters of the turtle CF overlap substantially.

Another compensatory mechanism may be to modify the myelination of the climbing fibers as a function of the path length from the IO to their axon terminal (Lang and Riesenbluth 2003). In rats, myelination can compensate for difference in the conduction path length during the first postnatal month. In contrast, changes in olivocerebellar conduction time in myelin-deficient rats only dropped by a third of the normal decrease. This suggests that myelination contributes significantly to the rat’s compensatory mechanism for differences in olivocerebellar path length.

In turtle, the axons below Purkinje cells mostly course sagittally with different levels of myelination (Mugnaini et al. 1974). These fibers were described as thinly and thickly myelinated with diameters from 1 to 8.5 μm, but it is not known if their myelination compensates for differences in CF path length. Apart from morphological features of climbing fibers, differences in physiological properties such as the density of Na+ channels may also play a role in compensating for differences in path length.

Role of olivocerebellar synchrony in Cb function

The IO is thought to contribute to the Cb’s role in temporal coordination of motor behaviors (Llinas et al. 1975) or the learning of complex motor behaviors (Ito 2001; Marr 1969; but see Anderson and Keifer 1997). This study focused on the former hypothesis, showing that a synchronous input from IO to Cb exists in turtle, an early stage of vertebrate evolution. The turtle Cb is dominated by inputs from vestibular and oculomotor centers (vestibular nuclei, ipsilateral accessory optic system/preptectum, and interstitial nucleus of medial longitudinal fasciculus; Bangma et al. 1983; Kunzle 1983a, 1985b). These anatomical findings are supported by extracellular physiology of visual and vestibular Cb responses, suggesting that turtle Cb is primarily involved in coordinating reflexes that stabilize eye and head position (Ariel and Fan 1993; Fan et al. 1993).

The experiments described here were limited to a single electrode recording. Until recently, measurement of higher spatial resolution of a cortical structure required arrays of multiple electrodes and complex circuitry for amplification and analysis (Fukuda et al. 2001). Now, optical imaging techniques have sufficient spatiotemporal resolution to measure synchrony of regions of the Cb surface (Cohen and Yarom 2000). In the future, measuring optical responses to IO stimulation in the turtle may improve spatial resolution to reveal details of topography and function of the entire unconvoluted Cb surface (Ariel et al. 2003).

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