Exp Brain Res (1998) 121:115–124

REVIEW ARTICLE

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Temporal discrimination in the cerebellar cortex during conditioned eyelid responses

Received: 25 August 1997 / Accepted: 12 January 1998

Abstract Little is known about mechanisms used by the nervous system to encode time. In light of recent evidence, cerebellar cortex involvement in the learned timing of conditioned eyelid responses shows promise as an area of investigation into neural timing mechanisms. Lesion studies indicate that the cerebellar cortex is necessary for response timing, but do not rule out the possibility that response timing is encoded afferent to the cerebellum. To differentiate between precerebellar and cerebellar cortical timing mechanisms, rabbits were trained by pairing direct stimulation of mossy fibers in the cerebellum as the conditioned stimulus (CS) with an eyeshock unconditioned stimulus (US). We find that individual animals can produce diffently timed conditioned responses when trained with a mossy fiber CS that has been paired with the US at various interstimulus intervals. The fact that differently timed responses can be conditioned using constant-frequency stimulation of an invariant subset of mossy fibers as the CS suggests that timing information in the afferent input to the cerebellum is not essential. Two rabbits trained with single-pulse stimulation in the cerebellum as the CS also learned differently timed conditioned responses; suggesting that fiber recruitment during a stimulus train does not convey the necessary temporal coding to the cerebellar cortex. Together with the lesion data, these findings suggest that the learned timing of conditioned eyelid responses occurs in the cerebellar cortex.

Key words Pavlovian eyelid conditioning · Neural timing mechanisms · Long-term depression · Cerebellar granule cells · Golgi cells · Rabbit

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Introduction

Numerous investigators have proposed a role for the cerebellar cortex in the timing of movements (Braitenberg 1967; Freeman and Nicholson 1970; Eccles 1973; Fujita 1982; Brooks 1984; Moore et al. 1989; Keele and Ivry 1990; Ivry 1993; Buonomano and Mauk 1994; Fiala et al. 1996; Mauk and Donegan 1997). While there is increasing evidence that the cerebellum does indeed time movements (Soechting et al. 1976; Brooks 1984; Keele and Ivry 1990; Ivry 1993; Perrett et al. 1993; Jueptner et al. 1995), mechanisms through which it might do so remain speculative. Much of the evidence for a timing function within the cerebellum comes from studies of volitional movements, many involving humans or monkeys. However, it is difficult to investigate cellular and systems-level mechanisms through which this function may be expressed in these preparations. This is mainly due to difficulties in identifying discrete regions of the cerebellum that may be involved in relatively complex movements and in assessing activity in these discrete regions once they are identified. Pavlovian eyelid responses are simple and easily quantified movements which display learned timing (Coleman and Gormezano 1971; Mauk and Ruiz 1992). Recent evidence suggests that the anterior lobe of cerebellar cortex is important for this timing (Perrett et al. 1993; Perrett and Mauk 1995). As such, eyelid conditioning is potentially a good model system with which to investigate the role of the cerebellar cortex in the timing of movements.

Acquisition of conditioned eyelid responses depends critically on the interstimulus interval (ISI) used to train the animal. In the case of rabbits, the interval between the conditioned stimulus (CS) and unconditioned stimulus (US) must be at least 80 ms and no longer than 3–4 s (Schneiderman and Gormezano 1964; Smith 1968; Smith et al. 1969; Coleman and Gormezano 1971; Salafia et al. 1980). Within this range, conditioned responses always peak near the onset of the US (Martin and Levey 1965; Levey and Martin 1968; Smith 1968; Mauk and Ruiz 1992). Therefore, the conditioned response seems to be maximally

adaptive to protect the animal from the US. This adaptability can be demonstrated in individual animals trained to produce differently timed responses by pairing different CSs with the US at different ISIs (Mauk and Ruiz 1992; Perrett et al. 1993). Training with a short ISI will result in conditioned responses with fast latencies and steep rise times, while a different CS paired with the US at a long ISI will elicit conditioned responses with longer latencies and slower rise times. This differential timing of conditioned responses in the same animal is under strict control of the CS, is seen on a trial-by-trial basis, and is evident throughout all phases of acquisition (Mauk and Ruiz 1992). Thus, the characteristic temporal properties of conditioned eyelid responses require a neural mechanism that is capable of temporal discriminations (Coleman and Gormezano 1971; Mauk and Ruiz 1992) and these properties are not merely reflective of a behaviour occurring in time (see Keele and Ivry 1990; Ivry and Baldo 1992).

The stimuli used in eyelid conditioning engage the input fibers to the cerebellum in a characteristic manner. The CS is conveyed to the cerebellum through mossy fiber inputs (Steinmetz et al. 1986a, 1989), the US is conveyed as climbing fiber inputs (McCormick et al. 1985; Mauk et al. 1986; Steinmetz et al. 1989), and the output of the cerebellum through interpositus neurons is necessary for the integrity of the conditioned response (McCormick and Thompson 1984a; Yeo et al. 1985; Steinmetz et al. 1992). The cerebellum is implicated as a probable site for synaptic plasticity involved in the conditioned response by the convergence of these pathways within the cerebellum and a large body of empirical evidence that supports this hypothesis (for reviews, see McCormick and Thompson 1984b; Steinmetz and Thompson 1991; Yeo 1991; Thompson and Krupa 1994; Mauk 1997). Perhaps the most compelling evidence in support of this hypothesis is a study by Steinmetz and colleagues (1989) which demonstrated that eyelid responses can be conditioned using direct stimulation of mossy fibers in the pontine nuclei as the CS and climbing fibers in the inferior olive as the US.

We have previously investigated the role of the cerebellar cortex in the timing of conditioned eyelid responses in the rabbit (Perrett et al. 1993; Perrett and Mauk 1995). Cerebellar cortex lesions which included the anterior lobe spared conditioned responses but disrupted their learned timing. Irrespective of the prelesion timing, postlesion responses showed extremely short, relatively fixed latencies. Because the basic expression of inappropriately timed conditioned responses remains after anterior lobe lesions, the timing of responses does not appear to be generated efferent to the cerebellar cortex. This leaves two possibilities; timing is generated in the cerebellar cortex or in regions that project to the cerebellum via mossy fiber CS inputs. Since mossy fibers send collaterals to the interpositus nucleus (McCrea et al. 1977; Murakami et al. 1981; Steinmetz and Sengelaub 1992; Gould et al. 1993), it seems likely that timing information would be conveyed there also, which is inconsistent with the characteristic short-latency responses we have observed following cerebellar cortex lesions. This suggests that timing does not occur afferent to the cerebellum. However, we have tested this possibility more directly by stimulating mossy fibers in the middle cerebellar peduncle as the CS. If timing is mediated by synaptic plasticity at a site afferent to the cerebellum and conveyed there through the activation of different subsets of mossy fibers during the CS, stimulation of a constant subset of mossy fibers as the CS should not result in differently timed conditioned responses. However, if timing occurs through mechanisms within the cerebellar cortex, mossy fiber stimulation should still support differently timed responses. We find that rabbits trained with direct stimulation of mossy fibers within the cerebellum as the CS can learn differently timed conditioned responses.

Materials and methods

Animals and surgical procedures

Eight male New Zealand albino rabbits (*Oryctolagus cunniculus*) weighing 2–3 kg were used in this study. Treatment of the animals and surgical procedures were in accordance with an approved animal welfare protocol. Each rabbit was prepared with a bolt cemented to, and extending vertically from, the skull and a chronic stimulating electrode using sterile surgical techniques and halothane anesthesia (1–2% mixed in oxygen, with acepromazine 5 mg/kg as a preanesthetic).

Rabbits were placed in custom-built stereotaxic head holder such that the bregma suture was 1.5 mm above the lambda suture, and a hole was drilled into the skull overlying the cerebellum. An epoxylite-coated, stainless steel microelectrode (Frederic Haer) with approximately 100 μm of tip exposed was positioned (coordinates: 3.0 mm anterior to lambda suture, 7.0 mm lateral to the midsagital plane, and 14 mm ventral to lambda at the midsagital point) and fixed with dental acrylic. These coordinates were chosen to place the tip of the electrode among the mossy fiber afferents of the left middle cerebellar peduncle within the cerebellum. A plastic socket with wire connections to the implanted stimulating electrode and to a ground screw placed in the skull was also cemented with the electrode and headbolt to form a single headstage. At this time animals were also prepared with a small suture loop (6–0) in the central portion of the left upper eyelid and two wire electrodes that were positioned in the anterodorsal and posterodorsal aspects of the left eye. These electrodes were sterilized stainless steel wires (0.0178 cm) inserted subdurally using a 23-gauge needle. Intravenous fluids, antibiotics, and analgesics were administered postsurgery as needed, and at least 1 week was allowed for recovery.

Conditioning procedures

The training protocol consisted of pairing mossy fiber stimulation as the CS with an eyeshock US at different ISIs within the same animal (Fig. 1A). The ISIs used were: 125 ms, 150 ms, 200 ms, 240 ms, 250 ms, 500 ms, 750 ms, and 1000 ms. Animals were trained at one ISI for 2 days after a criterion of 80% conditioned responding was reached in one session, extinguished for 2 days after less than 30% conditioned responding occurred in one session, then retrained using the same stimuli at a different ISI. This was repeated as long as the stimulation would support conditioned responses (up to five different ISIs in one rabbit). The initial ISI used was varied for each animal and the order of subsequent ISIs used for conditioning was randomly selected.

The CS was a train of 0.1-ms pulses delivered at 50 Hz and coterminating with the US. A stimulation threshold for eliciting a visible response (generally a "shudder" or flinch) was determined in

each naive rabbit by increasing the current incrementally from a minimum of 60 μA. The stimulation intensity used for conditioning was one-half this threshold to a maximum of 200 μA (the range for all rabbits was 70–200 μA).

In addition, for three of these rabbits, the ability of single-pulse stimulation (0.1 ms) as the CS to support differently timed conditioned responses was tested (Fig. 3, top). These rabbits were initially conditioned with a stimulus train to determine whether the stimulation electrode would support conditioning, and it was subsequently determined whether single-pulse stimulation would support conditioning within any range of ISIs.

The US was a 50-ms train of constant-current cathodal pulses (1 ms) delivered through the wire electrodes by a World Precision Instruments stimulus isolator at a frequency of 200 Hz. Shock intensity (0.5–3 mA) was adjusted for each animal to induce a robust eyeblink without significant activation of the surrounding facial musculature.

Daily training sessions consisted of 12 blocks of nine trials each in which the trials were delivered every 30 s (54 min session). During acquisition training, each block consisted of eight paired CS/US trials and one CS-alone trial that was used for analysis. During extinction training, each block was comprised of 13 unpaired trials. The initial trial in each session was a CS-alone trial such that 109 trials (96 paired, 13 CS-alone, or 109 CS-alone) were presented daily.

Data acquisition and analysis

During daily training sessions, each rabbit was immobilized in a Plexiglas restrainer inside a sound-attenuating chamber, and a phototransistor potentiometer was attached to the animal's head bolt. Movements of the unrestrained eyelid were measured using this microtorque potentiometer connected to the suture in the eyelid by light-gauge (0.0178 cm) stainless steel wire. The potentiometer arm was counterbalanced to produce no rotation when disconnected. Movement of the upper eyelid was transduced into voltage signals that were amplified and digitized at 1 kHz using an R/C electronics RC-200 analog-to-digital converter. Two thousand points (2 s) were collected for each trial and stored on hard disk for subsequent analysis using custom software.

Because the reflex unconditioned response is generally superimposed on the peak of the conditioned response during paired trials, the data presented includes only the CS-alone trials. For consistency, means for percentage responding displayed on extinction curves includes trials which would be CS-alone during acquisition (i.e., every ninth trial). Eyelid responses greater than 0.3 mm and with latencies to onset between 40 ms and 1 s after CS onset were scored as conditioned responses. Trials were excluded from analysis if the onset of a response larger than 0.3 mm occurred in a 200-ms baseline period or in the first 40 ms after CS onset. Amplitude, latency to peak, and latency to onset were determined for each conditioned response according to the following algorithms. The mean slope of the response (over a 20-ms window) was determined for each point (1-ms, resolution). Latency to onset was defined as the initial point of upward deflection in the response slope that was 2 SDs above baseline slope variation. Latency to peak was the time between the onset of the CS and the peak of the response. Amplitude was defined as the difference between the peak of each response during the 1800 ms after CS onset and the mean eyelid position for the 200-ms baseline period.

Statistical analysis consisted of within-subjects, one-way ANOVAs for the latency-to-onset and latency-to-peak data for each animal. Means of the last 30 conditioned responses on CS-alone trials for each measure, for each ISI, were used for statistical analysis. Multiple comparisons were made using the Tukey test, and two-tailed distributions with a minimum criterion of $P<0.05$ were used for all tests.

Histology

Marking lesions were made at the end of training by passing 100 μA of constant current for 20 s through the stimulating electrode. Animals were then killed with an overdose of pentobarbital sodium (80 mg/kg i.v.) and immediately perfused intracardially with 0.5 l of 10% formalin. The brains were carefully removed and stored for several days in fresh 10% formalin. Cerebella were subsequently embedded in an albumin-gelatin mixture and these blocks were fixed by exposure to formaldehyde fumes (40%) until firm and stored in 10% formalin. The entire cerebellum was subsequently sectioned using a freezing microtome (80-mm sections) and slices were mounted on slides. The location of the marking lesion was revealed following cresyl violet and Prussian blue staining.

Results

The basic finding of this experiment is that direct stimulation of mossy fibers in the cerebellum as the CS can support different, appropriately timed conditioned responses when it is paired with the US at different ISIs. Seven out of eight animals tested were able to learn at least three differently timed conditioned responses as assessed by significant differences in latencies to peak (Fig. 1; one animal did not acquire conditioned responses). One

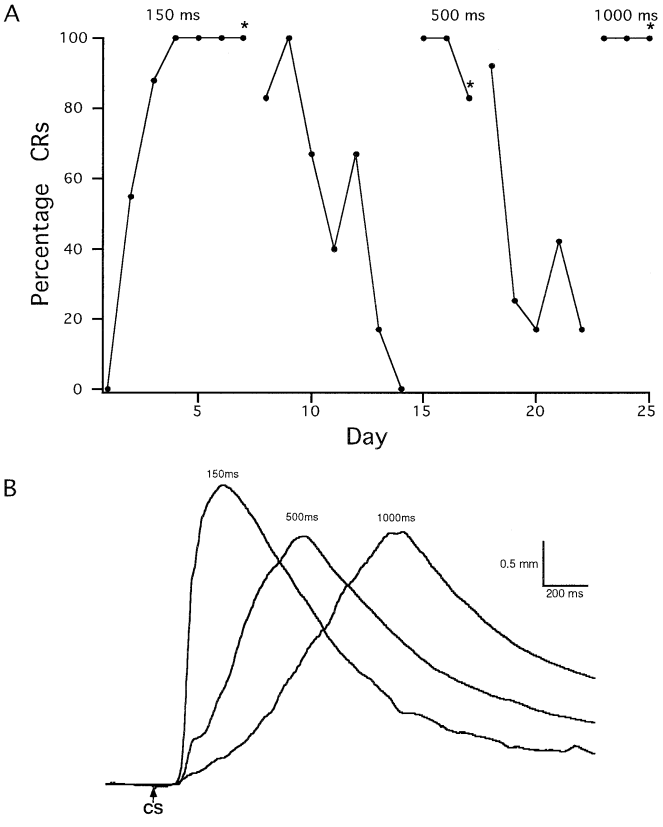


Fig. 1A, B Mossy fiber stimulation as the conditioned stimulus (*CS*) will support differently timed conditioned responses (*CRs*) in the same animal. **A** Acquisition and extinction rates illustrating the experimental protocol. Animals repeatedly received acquisition and extinction training at different interstimulus intervals (ISIs). **B** Examples of differently timed responses conditioned by pairing mossy fiber stimulation with the unconditioned stimulus (US) at three different ISIs. *Arrow* indicates CS onset and *time above each trace* is the ISI used to condition that response. Traces are means of CS-alone trials taken from the training sessions indicated by *asterisks* in **A**

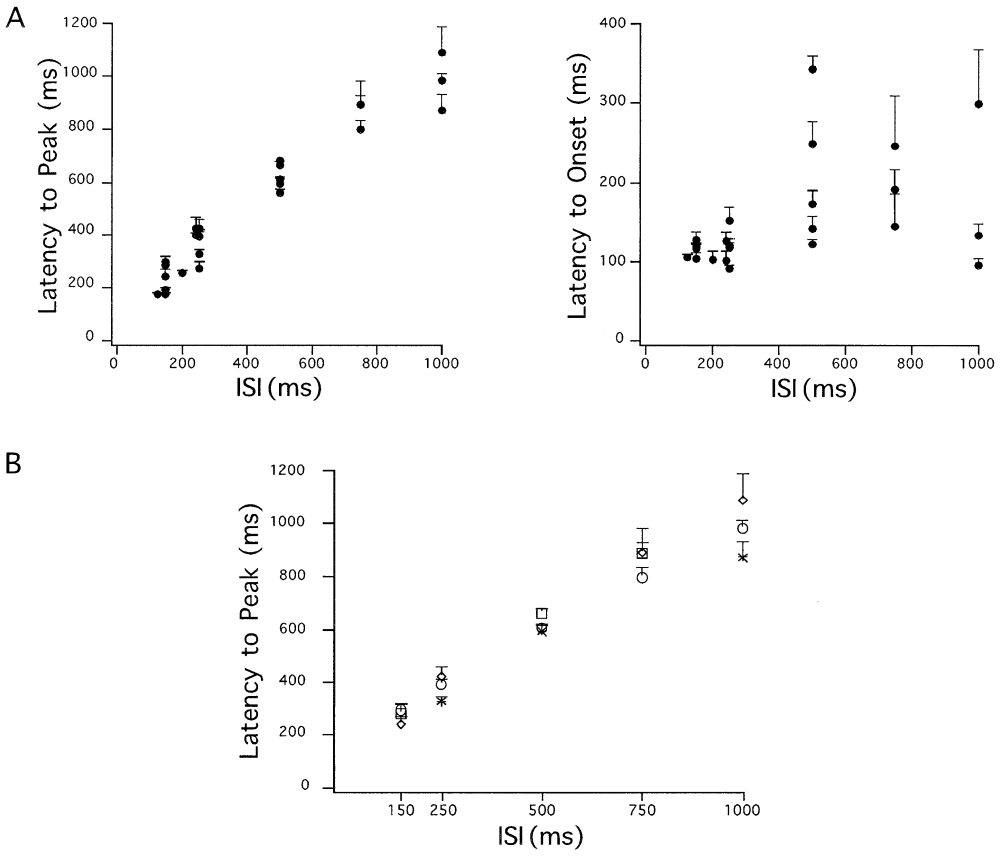


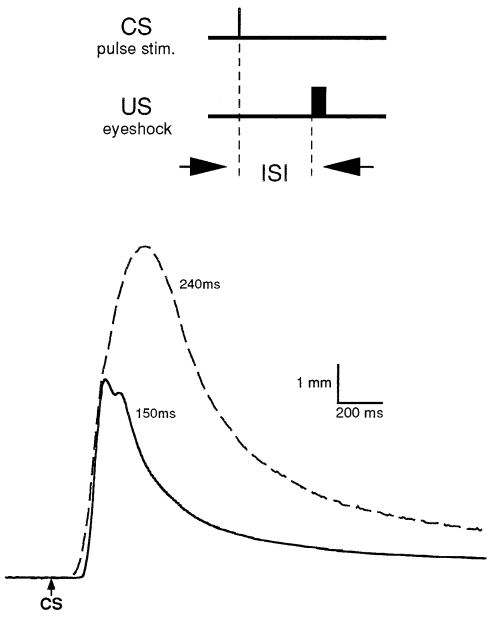
Fig. 2A, B Conditioned responses peak near the time when the US is presented. **A** Latency to peak and onset for all ISIs used across all animals in the study. **B** Response latencies to peak at all training ISIs for animals in which the tip of the stimulating electrode was in the middle cerebellar preduncle (n=4). Different symbols represent different animals (i.e., the animal denoted by the *circle* was trained at five different ISIs). Each point is the mean latency to peak or onset of CS-alone trials on the last day of training at that particular ISI. *Error bars* SEM

animal learned five differently timed responses and, in all cases, the stimulating electrode became ineffective before a limit on the number of differently times responses that could be learned was found. Consistent with rabbit eyelid conditioning using an external CS, latencies to peak in the present experiment were always similar to the ISI used to train those conditioned responses (Fig. 2; Martin and Levey 1965; Levey and Martin 1968; Smith 1968; Mauk and Ruiz 1992). Rates of acquisition and extinction, asymptomatic levels of responding, and amplitudes of conditioned responses (data not shown) were qualitatively similar to these measures of conditioning in rabbits trained with auditory tone CSs in studies from this laboratory (Mauk and Ruiz 1992; Perrett et al. 1993; Perrett and Mauk 1995) and from previous studies in which mossy fiber stimulation was used as a CS (Steinmetz et al. 1986a, b, 1989; Rosen et al. et al. 1989; Steinmetz 1990).

The acquisition and extinction rates from one animal are shown in Fig. 1. This animal exhibited 100% savings when acquiring at a new ISI following extinction of responses acquired to a previous ISI. Savings refers to the commonly observed phenomena in which reacquisition following extinction occurs more rapidly than initial acquisition. In the present experiment, savings was more robust than that usually observed when conditioning involves external CSs. This is consistent with, and has been well-documented in, an extensive literature on the use of mossy fiber stimulation as a CS (Steinmetz et al. 1986a, b; Rosen et al. 1989; Steinmetz 1990). For example, Steinmetz et al. (1986a) report a 90.7% response rate on the first day of reacquisition following 4 days of extinction training and 2 days of explicitly unpaired training. In the present experiment, the mean percentage responding on the 1st day of acquisition at a new ISI following extinction of a previous ISI when a stimulus train was used as the CS was 79.6% (n=12, range 25–100%). This rate in the one pulse experiment was 56.7% (n=6, range 17–77%). A possible explanation for this robust savings effect is that, because the same fibers are activated for each ISI, the CSs for each different ISI in this experiment are much more similar than external CSs, usually involving different modalities or differential CSs of the same modality (for example, different frequency auditory tones), that are normally used to assess savings.

Simple one-way ANOVAs done on the latency to peak data for the different ISIs used to train each animal revealed a significant difference ($P<0.001$) in latencies to peak for all seven rabbits that acquired conditioned responses. Subsequent Tukey tests revealed a significant difference ($P<0.05$) in peak latencies for all pairs of ISIs tested in all seven animals with the exception of one ISI pair. This ISI pair was 240 ms trained with one pulse stimulation and 250 ms conditioned with a stimulus train in one animal.

Simple one-way ANOVAs on the latency-to-onset data revealed a significant difference ($P<0.001$) for six out of the seven animals. Tukey tests reveal an almost equal number of ISI pairs (16) which didn't have significantly



	onset (ms)	peak (ms)	onset (ms)	peak (ms)
ISI	125 ms		200 ms	
#84	96.2±3.7	180.8±4.3	105.9±5.7	250.4±4.2
ISI	150 ms		240 ms	
#86	116.4±2.7	180.6±4.1	113.8±5.2	407.5±17.9
#87	129.9±6.4	234.3±11.2	124.6±5.7	376.7±11.2

Fig. 3 Rabbits learn differently timed responses when trained with single-pulse stimulation as the CS. The schematic at the *top* illustrates the conditioning paradigm. The ISI is effectively a trace interval. Trace intervals less than 125 ms and greater than 240 ms did not support conditioning. *Middle traces* are means of CS-alone trials from one training session. *Arrow* indicates CS onset and *time next to each trace* is the ISI used to condition that response. The electrode tip in this rabbit was localized to the anterior lobe of cerebellar cortex. The *table* displays the mean latency to onset and peak±SEM for both ISIs in each of three animals trained with one-pulse stimulation. Animals 84 and 87 had stimulating electrodes in the cerebellum

different onset latencies to the number that did (18). However, significant differences in onset latency were increasingly likely as the difference between ISI pairs increased. For example, 10 of 16 pairs which did not show significant differences in onset latencies were separated by 250 ms or less, while 12 of 18 which did show significant differences were separated by more than 250 ms. These findings are consistent with a previous study (Mauk and Ruiz 1992) which used auditory tone CSs to condition differently timed responses in individual rabbits. Mauk and Ruiz (1992) concluded that latencies to peak were more likely to be significantly different than latencies to onset and that animals were increasingly likely to generate conditioned responses with statistically different timing as the difference between ISIs increased.

To control for the possibility that timing might be encoded by the mossy fiber afferents through a mechanism such as fiber recruitment during the stimulus train, three of these animals were also trained at a number of different ISIs using single pulses as the CS. We anticipated that, if animals could learn with a single-pulse CS, the range of ISIs that would support learning would be greatly restricted and, thus, a number of ISIs would have to be tested to determine this range. Therefore, four animals were initially tested with a stimulus train as the CS at an ISI that was known to support learning to determine that the electrode was functional and placed such that it would support conditioning as a CS. Three of these animals acquired conditioned responses with a train CS and, subsequently, could acquire conditioned responses to a single-pulse CS if the ISI used to train them was between 125 and 240 ms. Each animal was trained at two ISIs from the extremes of this range. Within animals, one-way ANOVAs for the latency-to-peak data for both ISIs for these rabbits were significantly different ($P<0.001$), while latencies to onset were not significantly different (Fig. 3).

Histological analysis of the marking lesions revealed that electrode tips in four animals were in the middle cerebellar peduncle within the cerebellum (Fig. 4). Of the remaining four animals, two placements were in the medial vestibular nucleus directly ventral to the cerebellum, one placement was within anterior lobe cerebellar cortex, and the other placement was in the cerebellar white matter dorsomedial to the interpositus nucleus. The electrode tip in the animal which did not acquire conditioned responses (eight training sessions 872 trials, with a 750-ms ISI) was localized to the medial vestibular nucleus (Fig. 4).

None of the animals trained with one-pulse stimulation had electrode tips localized within the middle cerebellar peduncle (n=3; locations: medial vestibular nucleus, anterior cortex, and medial cerebellar white matter). The electrode in the anterior lobe cortex would be expected to activate granule cells (along with other cortical cell types) directly. Since we hypothesize that timing arises from cortical mechanisms in which granule cell activity is integral (see Discussion), the misplacement of the stimulating electrode in this case actually is a more direct test of that hypothesis. Because the stimulating electrode in this case is one synapse removed from the brainstem mossy fiber afferents, it becomes more difficult to invoke brainstem mechanisms to explain the proper response timing in this animal. It was probably fortuitous in this case that the electrode was placed in anterior lobe cortex, a region which the lesion data (Perrett et al. 1993; Perrett and Mauk 1995) has implicated as being important for eyelid conditioning. Electrode placements in other cortical lobes may not have supported conditioning. The electrode in the medial cerebellar white matter would be expected to activate Purkinje cell axons descending to the deep nuclei, collaterals from these axons reinnervating the cortex and, most likely, mossy fiber and climbing fiber inputs to the cortex (Ito 1984). Again, because this electrode was in a position in the cerebellum to possibly activate granule cells, the most straightforward explanation for its ability to support different, appropriately timed responses, in light of the lesion data, is through cortical mechanisms. The simplest explanation for the two animals with electrode placements in the medial vestibular

Fig. 4 Placement of stimulating electrodes (n=8) is indicated by symbols on schematic representations of coronal cerebellum/brainstem slices displayed in caudal to rostral fashion *from upper left to lower right*, respectively. Four electrode tips were in the middle cerebellar peduncle (*crosses*) two others were within the cerebellum (anterior lobe cortex and white matter dorsomedial to the interpositus nucleus), while the remaining two were localized to the medial vestibular nucleus. Seven rabbits learned differently timed conditioned responses, while one animal, which received stimulation of the medial vestibular nucleus (*filled triangle*), did not acquire conditioned responses. Three animals were trained with single-pulse stimulation as the CS and were able to learn differently timed conditioned responses (*filled circles*) (*I* interpositus nucleus, *D* dentate nucleus. *F* fastigial nucleus, *MP* middle cerebellar peduncle, *M* medial vestibular nucleus)

nuclei is that one electrode (in the animal that learned) activated mossy fibers projecting to the cerebellum, while the other (in the animal that did not learn) did not. While the fiber and cell types activated by the electrodes placed in the cerebellum in the two animals in the one-pulse study were not relatively homogeneous, as were those activated by the middle cerebellar peduncle electrodes, the conditioning of differently timed responses in these animals is consistent with the basic hypothesis that timing occurs in the cerebellar cortex.

Discussion

We found that direct, constant-frequency stimulation of mossy fibers within the cerebellum as the CS (n=4) could support differently timed conditioned responses within the same animal. Latency measures shifted when individual animals were trained at different ISIs and, thus, latencies to onset and to peak were related to the ISI used to train the animal. In no case, when the electrode tip was within the cerebellum (n=6), did animals fail to learn differently timed responses.

If timing is generated afferent to the cerebellum, it must be conveyed to the cerebellar cortex through mossy fiber coding for differently timed responses. This coding could include the activation of different subsets of mossy fibers at different times during the CS (Moore et al. 1989; Desmond and Moore 1991). In the present study, direct stimulation as the CS presumably activates a fairly constant subset of mossy fibers at an invariant frequency throughout its duration (Asanuma 1981; Yeomans 1990; Tehovnik 1996). Therefore, the appropriate timing seen in this experiment appears to occur in the absence of any temporally coded mossy fiber inputs. Combined with the earlier finding that cerebellar cortex lesions abolish the timing of conditioned eyelid responses (McCormick and Thompson 1984a; Perrett et al. 1993; Perrett and Mauk 1995), this result suggests that learned conditioned response timing is generated by mechanisms within the cerebellar cortex. Based on the lesion results, we would argue that this occurs in the anterior lobe of cerebellar cortex.

It is possible that the stimulation protocol used in this study (0.1-ms pulses at 50 Hz coterminating with the US) could activate different subsets of mossy fibers at different times during the CS through fiber recruitment during the train. To control for this, it was demonstrated in two animals that one-pulse (0.1 ms) stimulation within the cerebellum could support differently timed responses. Any possible recruitment of mossy fibers by a single pulse of such short duration could not account for the variation in latencies to peak observed in these animals. Electrode tips in these animals were in the anterior lobe

of cerebellar cortex and the cerebellar white matter dorsomedial to the interpositus nucleus. The range of ISIs that would support conditioning in these animals was greatly restricted compared with animals conditioned with a stimulus train; however, the latencies to peak for responses trained at the extremes of this range were significantly different and varied appropriately with the ISI. This finding is consistent with trace conditioning studies in which animals were trained with short CS intervals (Schneiderman 1966; Power et al. 1997). This finding is also consistent with an experiment reported in a recent abstract (Svensson et al. 1997) in which short test stimuli applied directly to the middle cerebellar peduncle could elicit conditioned eyelid responses in decerebrate ferrets trained with electrical forelimb stimulation as a CS. These authors suggest "that precerebellar brainstem sites are not required for the maintenance of CS-evoked activity and that neurons that maintain CS evoked activity, which elicits the CR, are located in the cerebellum." Both the one-pulse data from the present study and the data from Svensson et al. (1977) strengthen the hypothesis that the temporal discrimination involved in learned response timing occurs in the cerebellar cortex.

Another plausible mechanism for the generation of proper response timing when stimulating electrodes placed within the cerebellum are used as the CS is through "reverberating" activation of the deep nuclei by brain structures that project mossy fibers to the cerebellum. In this view, deep nuclei that are activated during the CS may project to brain sites such as the pontine nuclei, which then project back to the cerebellum to form a reverberating circuit. Reverberation between the deep nuclei and the pontine nuclei could recruit different or additional portions of the input pathway with each cycle that occurs during the ISI. In this model, response timing is set by interactions between active brainstem inputs and the deep nuclei and is not dependent on cortical mechanisms. However, the lesion data (Perrett et al. 1993; Perrett and Mauk 1995) suggest that any contribution of such a mechanism to response timing is not substantial. Animals which received lesions of the anterior lobe cortex, sparing the interpositus nuclei as indicated by the retention of conditioned responses, could no longer delay those conditioned responses to peak when the US would be presented. Instead, irregardless of the ISI used for that particular CS, the latency to peak of postlesion responses was in the range of 100–150 ms. Because the reverberating circuit of deep nuclei and brainstem structures was still intact in these animals (as assayed by function and through histological examination), the timing of conditioned responses should not be disrupted in these animals under those assumptions.

The results from this study are consistent with those from a previous study (Steinmetz 1990) in which response timing was examined in rabbits using stimulation of mossy fibers in the pontine nucleus region as the CS. In that study, conditioned responses were timed differently within different groups of rabbits trained with different ISIs. When ISIs were shifted to a constant 250 ms for all animals, response latencies shifted appropriately; that is, latencies to peak shifted close to 250 ms for all animals. However, because Steinmetz (1990) was mainly interested in the acquisition of conditioned responses over various ISIs using a stimulation CS and not the specific neural mechanism(s) for timing responses trained at different ISIs, there are several important differences between that study and the present one. First, stimulation within the brainstem could have activated regions other than the cerebellum which contributed to the timing of responses seen by Steinmetz. Because the goal of the present study was to minimize this possibility, we stimulated within the cerebellum. Second, Steinmetz used a robust conditioning protocol of 200-Hz stimulation to assess the limits of the ISI function. Because we were concerned that high-frequency stimulation might allow temporal coding through fiber recruitment, we used 50-Hz stimulation and, in the extreme case, showed that one-pulse stimulation could support differently timed responses. Third, in the present study up to five differently timed conditioned responses were learned by a single animal (and at least three in all animals), while two differently timed conditioned responses were learned by each animal in the Steinmetz (1990) study. The present study demonstrates that animals can learn as varied a range of differently timed responses from training with a mossy fiber CS as with an external CS (Mauk and Ruiz 1992) and indicates that a putative cerebellar cortex timing mechanism is extremely sensitive.

How might the cerebellar cortex time conditioned responses? Various investigators have proposed tapped delay lines (Freeman and Nicholson 1970; Moore et al. 1989; Desmond and Moore 1991), arrays of elements with different biophysical or biochemical time constants (Bullock et al. 1994; Fiala et al. 1996), and arrays of elements that oscillate at different frequencies (Fujita 1982; Gluck et al. 1990) as timing mechanisms which involve the cerebellar cortex. However, there is little evidence that the nervous system actually implements any of these mechanisms, particularly not in the time frame of hundreds of milliseconds to seconds seen with eyelid conditioning. We suggest that timing can emerge out of the synaptic properties and interconnections of neurons in the cerebellar cortex (Mauk and Donegan 1997; Fig. 5). Subsets of active granule cells can be time-varying during constant mossy fiber input (or in the extreme case, following a single-pulse stimulation) due to Golgi-granule cell feedback loops. In this view, time is encoded as a population vector of granule cell activity. Conditioned responses are delayed to peak coincident to the presentation of the US due to a decrease in Purkinje cell activity at that time (Gilbert and Thach 1977; Berthier and Moore 1986). We hypothesize that this decrease results from long-term depression (LTD; i.e. Linden and Connor 1995) of connections from the subset of granule cells encoding that particular time. Therefore, granule cell populations encode not only the type of stimuli being presented as the CS but also the time since its onset. Large-scale computer simulations of mossy fibers, granule cells, and Golgi

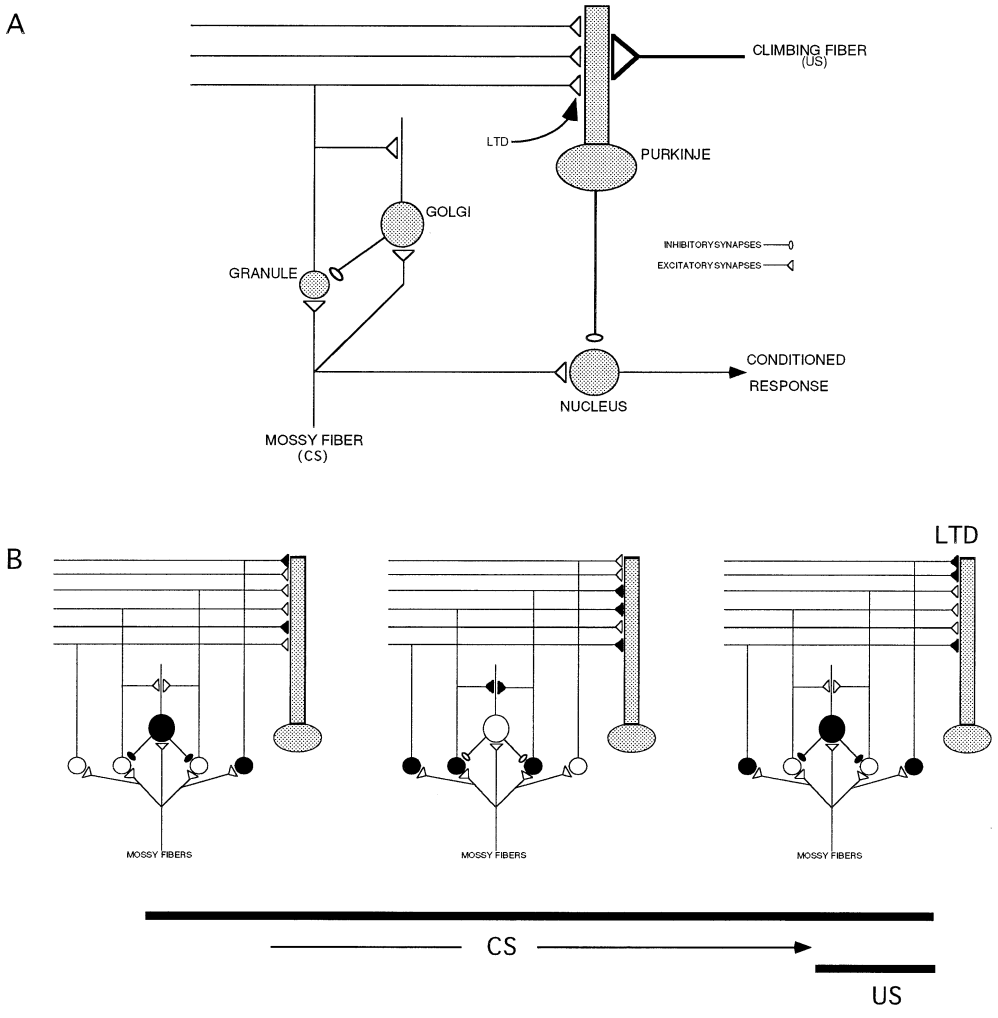


Fig. 5 **A** Schematic diagram of a simplified cerebellar circuity. We suggest that the cerebellar cortex discriminates time through the activation of different subsets of granule cells during the CS. Different subsets are activated during a constant mossy fiber input due to feedback inhibition from Golgi cells. The subset active when the US is presented has its synaptic connections with Purkinje cells involved in the eyeblink weakened through cerebellar long-term depression (*LTD*). This leads to an appropriately timed disinhibition of nucleus cells, generating a correctly timed conditioned response. **B** Time-varying view of a simplified cortical circuit, including four granule cells, a Golgi cell, and a Purkinje cell, depicting the activation of different subsets of granule cells during the presentation of a mossy fiber stimulation CS. LTD is induced (*far right*) at the synapses active when the US is presented. *Empty symbols* represent active cells and synapses, while *filled symbols* represent inactive cells and synapses

cells demonstrate that such a mechanism is feasible (Buonomano and Mauk 1994). The strength of this mechanism is that timing arises out of the established synaptic connectivity of the cerebellar cortex and does not require the imposition of hypothetical neuronal firing properties or mechanisms specifically in order to generate the observed behavior.

Note that the timing mechanism proposed above requires synaptic plasticity in the cerebellar cortex. The requirement for synaptic plasticity can be inferred from the fact that conditioned response timing is dependent on the ISI used to train the animal and, thus, is learned. These data are consistent with the hypothesis that the synaptic plasticity responsible for learned timing is LTD of granule cell synapses at Purkinje cells in the cerebellar cortex (Perrett et al. 1993; Raymond et al. 1996; Mauk 1997; Mauk and Donegan 1997). Numerous studies report LTD at this synapse (see Sakurai 1987; Ito 1989; Hirano 1990; Konnerth et al. 1992; Hemart et al. 1995; Lev-Ram et al. 1995; Linden and Connor 1995; Schreurs et al. 1996) and it has long been proposed as a neural mechanism involved in eyelid conditioning (McCormick and Thompson 1984b; Ito 1989; Steinmetz and Thompson 1991; Yeo 1991; Aiba et al. 1994; Thompson and Krupa 1994; Schreurs et al. 1996). LTD at these synapses could allow the cerebellar cortex to discriminate and selectively reinforce specific times during a CS – leading to an appropriately timed pause in Purkinje cell activity, disinhibition of interpositus neurons, and an appropriately timed response.

The elucidation of neural timing mechanisms will require very difficult experiments, undoubtedly involving recordings from numerous neurons in behaving animals. However, evidence that the timing of conditioned eyelid responses occurs in the anterior lobe of cerebellar cortex encourages examination of timing mechanisms in this region. The ability to condition differently timed responses in the same animal will greatly facilitate such investigations (see Mauk and Ruiz 1992). Indeed, the model presented above generates a number of testable predictions concerning granule and Purkinje cell activity during a CS: (1) different subsets of granule cells should be active during different CSs; (2) different subsets of granule cells should be active at different times during a particular CS; (3) the activity in each cell should follow the onset of the stimulus in a consistent manner across trials in well-trained animals; and (4) pauses in Purkinje cell activity should precede the peak of the response elicited by different CSs.

In summary, we have observed that direct mossy fiber stimulation in the cerebellum as a CS can support differently timed conditioned responses in the same animal. This observation, when considered with the earlier lesion data (Perrett et al. 1993), is consistent with the hypothesis that neural mechanisms within the cerebellar cortex correctly time conditioned eyelid responses. Thus, as has been suggested (see Brooks 1984; Thach et al. 1992; Ivry 1993; Raymond et al. 1996), the cerebellar cortex may be specialized to discriminate and modify specific temporal components of movements.

Acknowledgements I thank Garrett Kenyon, Prakash Kara, and Laura Schrader for helpful comments. All experiments were done in collaboration with Michael D. Mauk in his laboratory and I gratefully acknowledge his advice and encouragement. This work was supported by Scholar's Awards from the McKnight Endowment for Neuroscience and the National Down Syndrome Society, as well as by the NIMH.

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