The Cerebellum and Red Nucleus Are Not Required for *In Vitro* Classical Conditioning of the Turtle Abducens Nerve Response

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The role of the cerebellum during motor learning is a controversial issue. Many authors have suggested that the cerebellum and its connections with the red nucleus are essential for the acquisition of the conditioned eye blink reflex. Although there is little argument that the cerebellum is an important component to the generation of the conditioned response (CR), a number of studies have suggested that the cerebellum is not essential for conditioning. Using an *in vitro* model of the classically conditioned turtle abducens nerve response, we investigated the effect of cerebellar and red nucleus lesions on the acquisition, extinction, and reacquisition of CRs. Neural discharge was recorded from the abducens nerve after a single shock unconditioned stimulus (US) was applied to the ipsilateral trigeminal nerve. When the US was paired with a conditioned stimulus (CS) applied to the posterior eighth, or auditory, nerve, a positive slope of CR acquisition was recorded in the abducens nerve. After extinction stimuli in which the CS and US were alternated, the number of CRs decreased to near zero. When the CS and US were once again paired, reacquisition at a faster rate was recorded. The CRs showed unusual timing features compared with preparations in which the cerebellum was intact; they had significantly shorter latencies and showed burst-like responses. These data demonstrate that it is possible to classically condition this *in vitro* preparation in the absence of the cerebellum and red nucleus. However, the latencies of CRs were found to be dramatically altered in the cerebellar-lesioned preparations, suggesting that the cerebellum does play a role in the timing of the CR.

Key words: classical conditioning; cerebellum; in vitro*; turtle; brainstem; red nucleus*

It has been suggested that the cerebellum and red nucleus are in the essential pathways for the acquisition and retention of the classically conditioned eye blink reflex (Rosenfield and Moore, 1983; McCormick and Thompson, 1984; Yeo et al., 1985; Berthier and Moore, 1986; Thompson, 1986; Desmond and Moore, 1991; Steinmetz et al., 1992; Clark and Lavond, 1993). Evidence suggests that cerebellar ablations prevent the acquisition of conditioned responses (CRs). Although there is little argument that the cerebellum is an important component to the generation of the CR, a number of studies have suggested that the cerebellum is not essential for conditioning (Karamian et al., 1969; Norman et al., 1977; Welsh and Harvey, 1989; Kelly et al., 1990; Yeo, 1991; Harvey et al., 1993; Keifer, 1993b; Gruart et al., 1994; Bloedel and Bracha, 1995). Welsh and Harvey (1989) suggested that during conditioning of the rabbit nictitating membrane or eye blink reflex, it was difficult to ascertain the presence of CR acquisition because motor deficits masked the expression of conditioned reflexes. In other words, their interpretation was that the animal could learn but could not generate the motor response. Performance deficits have been most clearly shown by changes in the timing of the CRs. Perrett et al. (1993) showed that the timing of the occurrence of a conditioned eye blink reflex was itself a learned behavior and that lesions of the cerebellar cortex disrupted the latency and thus performance of the conditioned reflex. They further suggested that the acquisition of the CRs occurs at sites both inside and outside of the cerebellar cortex.

Much progress in the investigation of the cellular basis of classical conditioning has been made in *in vitro* preparations of invertebrate species such as the marine mollusks *Aplysia* (Lukowiak and Sahley, 1981; Glanzman, 1995), *Pleurobranchaea* (Mpitsos and Davis, 1973), and *Hermissenda* (Farley and Alkon, 1987). Recently, an *in vitro* model of the classically conditioned vertebrate abducens nerve response was reported (Keifer et al., 1995). Using the remarkable ability of the turtle to withstand anoxia, an *in vitro* brainstem–cerebellum preparation can remain viable for extended periods of time (Hounsgaard and Nicholson, 1990; Callister et al., 1995). With this preparation, a neural discharge can be recorded from the abducens nerve, which projects to muscles controlling the extraocular muscles, after a single shock electrical stimulus is applied to the ipsilateral trigeminal nerve [the unconditioned stimulus (US)] that contains sensory fibers from the head. This discharge represents a neural correlate of the turtle eye blink reflex (Keifer, 1993a). When this stimulus is paired with a train of stimulus pulses applied to the posterior eighth, or auditory, nerve [the conditioned stimulus (CS)], after a period of time a positive acquisition slope of CRs can be measured in the abducens nerve (Keifer et al., 1995). After unpaired stimuli such as an alternate-pairing training protocol in which the CS precedes the US by 10 sec, the CRs extinguish and show reacquisition when pairing is resumed.

Because evidence suggested that this *in vitro* preparation could be classically conditioned, we were interested in addressing the controversial role of the cerebellum in classical conditioning. In

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the present study, the *in vitro* brainstem–cerebellum preparation underwent one of two treatments, (1) an ablation that removed the entire cerebellum or (2) a cerebellar ablation combined with removal of tissue containing the red nucleus. The results will show that in preparations receiving either treatment, the acquisition, extinction, and reacquisition of classically conditioned abducens nerve responses can be recorded. However, the latency of the CRs recorded in lesioned preparations was significantly shorter than those recorded in intact brainstem–cerebellum preparations. These data suggest that the cerebellum is not necessary for the acquisition of the classically conditioned abducens nerve response in this *in vitro* preparation from the turtle. However, the cerebellum does play a role in the latency of the CRs.

Portions of this paper have been published previously in abstract form (Keifer, 1993b; Anderson and Keifer, 1997).

MATERIALS AND METHODS

In vitro *preparation.* The preparation of the isolated turtle brainstem–cerebellum preparation has been described previously (Keifer, 1996). With the exception of the cerebellar and red nucleus lesions, the procedures used here were identical. Pond turtles (*Chrysemys picta*; $n = 34$; carapace length of 4–6 inches) were anesthetized by hypothermia and decapitated (Parsons and Huggins, 1965; Marcus, 1981). The brain, brainstem, and upper cervical spinal cord were quickly dissected while bathed in cold physiological saline containing (in mM): 100 NaCl, 6 KCl, 40 $NaHCO_3$, 2.6 $CaCl_2$, 1.6 $MgCl_2$, and 20 glucose. The preparation was transferred to the recording chamber in which it was continuously bathed in physiological saline oxygenated with 95% O_2/5% CO_2 and maintained at room temperature (22–24°C) at pH 7.6. The telencephalon was transected and discarded, and the dura covering the preparation was carefully dissected away. For all experiments, the cerebellum and the cerebellar peduncles containing the deep cerebellar nuclei were transected with the intention of removing the entire cerebellum. For those preparations in which the red nucleus and associated mesencephalic structures were also removed, the brainstem was transected at the level of the trochlear nerve.

Stimulation and recording procedures. Fire-polished glass suction electrodes were used for stimulation and recording of the nerves, with the tip diameter fashioned to match the size of the nerve. Neuronal discharge was recorded in the abducens nerve after a single shock stimulus was applied to the ipsilateral trigeminal nerve. This discharge is thought to represent a neural correlate of the eye blink reflex because it is similar to muscle activity recorded during eye blinks in behaving turtles (Keifer, 1993a). The abducens nerve in turtles projects to the retractor bulbi, lateral rectus, and pyramidalis muscles of the eye (Barbas-Henry and Lohman, 1986, 1988). The retractor bulbi retracts the globe of the eye into the orbit, passively raising the nictitating membrane and lowering the eyelid at the same time (Walls, 1942), whereas the pyramidalis actively moves the nictitating membrane and eyelid (Duke-Elder, 1958). The single shock stimulus to the trigeminal nerve served as the US in the present study. The CS was a train of stimulus pulses applied to the ipsilateral posterior root of the eighth nerve. This nerve has been shown to contain primarily auditory fibers (Ten Donkelaar and Nieuwenhuys, 1979; Herrick and Keifer, 1998). Extracellular signals were amplified with a bandpass of 10 Hz to 3 kHz, recorded on videocassette tape (Vetter), and reproduced on a chart recorder (Astro-Med).

Conditioning protocol. The procedures for conditioning the *in vitro* turtle preparation have been described previously (Keifer et al., 1995). A delayed-conditioning protocol in which the CS immediately precedes the US was used. The CS consisted of a 100 Hz, 1 sec train stimulus (0.1 msec duration pulses) applied to the posterior root of the eighth nerve. The amplitude of the CS ranged from 33 to 82% of the current threshold that produced activity in the abducens nerve. The CS immediately preceded a single shock US applied to the ipsilateral trigeminal nerve. The intertrial interval was 30 sec. Each pairing session consisted of 50 CS–US stimulus trials followed by a 30 min rest period during which there was no stimulation. If the preparation showed a reliable CR, an alternate-pairing session was used to test for CR extinction and possible sensitization or pseudoconditioning effects. The alternate-pairing sessions consisted of the CS followed 10 sec later by the US. The alternate pairing was continued until the CR showed extinction, and then the paired CS–US trials were resumed to test for reacquisition of the response.

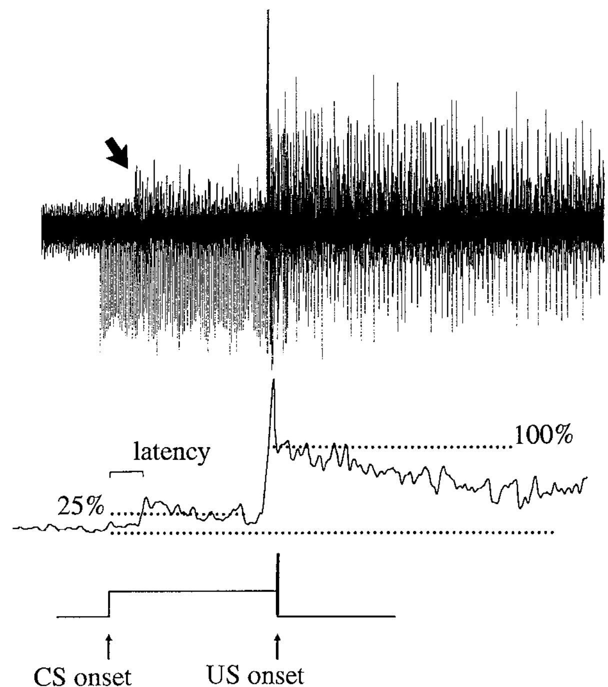

Figure 1. Illustration of the methods used to determine the CR. The *top trace* is an extracellular recording from the abducens nerve during a paired CS–US stimulus presentation in which a CR was recorded (*arrow*). The *middle trace* was generated by the National Institutes of Health Image analysis program in which the physiological records were scanned into a computer, converted into relative density plots, and integrated. A baseline level of activity (*lower dotted line*) was determined before the stimuli. The 100% level of the UR was determined by averaging several responses for 1 sec after the US. A response was considered a CR if it occurred during the CS and had an amplitude of at least 25% of the UR. Latency was measured from the start of the CS to the beginning of the CR. The *bottom trace* shows the 1 sec CS followed immediately by the single shock US.

Data analysis. A CR was defined as a neuronal discharge recorded in the abducens nerve that occurred during the conditioning stimulus and had an amplitude of at least 25% of the unconditioned response (UR) (Keifer et al., 1995). In Figure 1, a typical extracellular recording of a conditioned and unconditioned abducens nerve response is shown in the *top trace*. By generating spike density plots using the public domain National Institutes of Health Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/), we produced the integrated *trace* shown in the *middle* of Figure 1. By designating a baseline of activity and a 100% level of the UR generated from the mean of several responses, we calculated the 25% threshold (Fig. 1). The latency of the CR could also be accurately measured and compared with the UR using the National Institutes of Health Image program. The latency of the CR, the time period from the onset of the CS to the initiation of the CR, was recorded, as was the total number of CRs per block of 10 stimuli and per session (five blocks per session). Data were plotted as acquisition curves. Paired-sample t tests, regression analyses, and univariate ANOVAs were done on a PowerMac 7200 using StatView statistical software (Abacus Concepts, Calabasas, CA). All data are presented as mean ± SE except where indicated. Acquisition, extinction, and reacquisition rates were calculated as the mean number of CRs divided by the number of sessions.

Histology. The preparations were histologically processed to determine the degree of the lesions. At the end of the experiment, the tissue was fixed in 4% paraformaldehyde and later sectioned at 50 μm on a microtome. Tissue was stained with thionin to facilitate the assessment of the extent of the lesions. The degree of cerebellar cortex removal was assessed by counting the number of Purkinje cells that remained in each preparation. Previous experiments allowed us to count the total number of Purkinje cells in intact cerebella, and these data were used to evaluate the percent of Purkinje cells present in the lesion experiments. Because the total number of Purkinje cells that remained after the lesion were

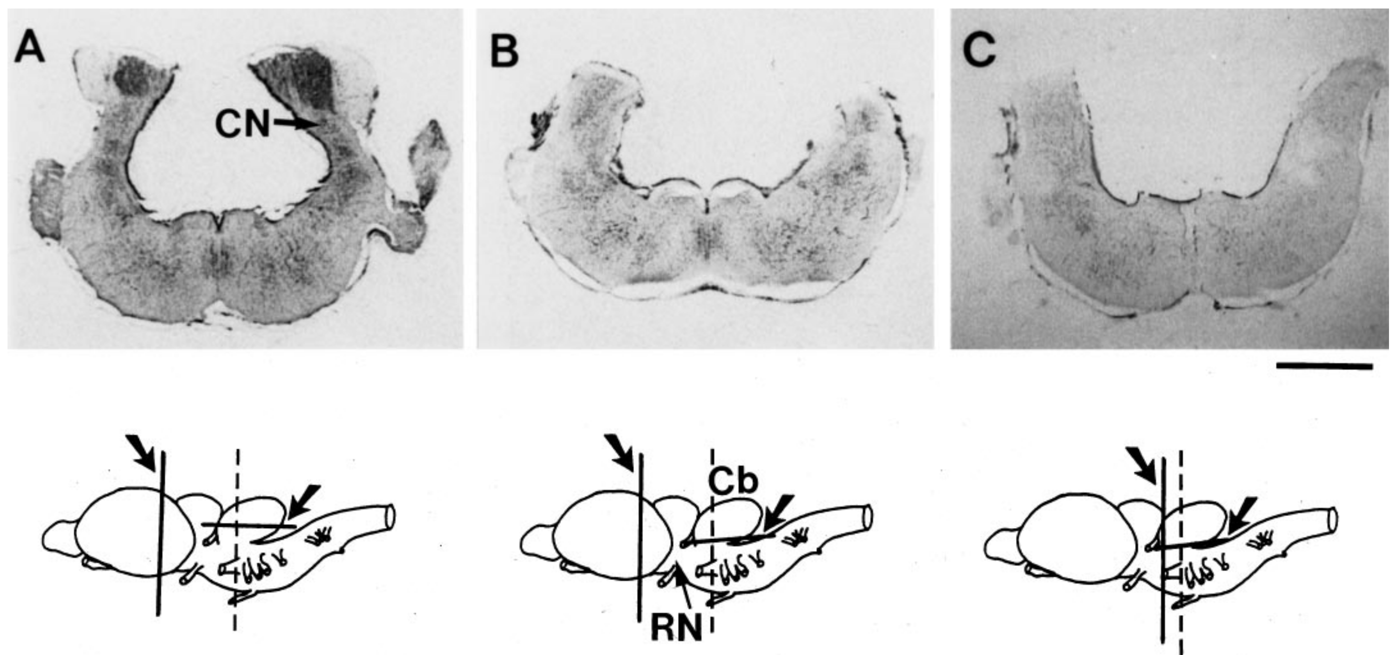

Figure 2. Photomicrographs illustrating the degree of the cerebellar lesions in three preparations that were conditioned. The schematics below each photograph refer to the location of the transections (*solid lines*). The red nucleus (*RN*) is located dorsal to the trigeminal nerve. *Cb*, Cerebellum. *A*, A section through the cerebellum taken from a conditioned preparation in which 848 Purkinje cells (~89% ablated) remained after a lesion of the cerebellar cortex. The red nucleus and deep cerebellar nuclei (*CN*) were intact in this preparation. *B*, A section from a preparation in which there were zero Purkinje cells remaining after the cerebellar lesion and in which the deep cerebellar nuclei were completely ablated bilaterally. The red nucleus in this preparation was intact. *C*, Photograph from a preparation that had complete removal of the cerebellar cortex. The deep cerebellar nuclei were completely ablated bilaterally, as was the red nucleus. All three photomicrographs were taken at the level of the trigeminal nerve (*dotted line*). Scale bar, 700 μm.

counted, the number ipsilateral to the side of the brainstem that was conditioned was about half of the total number (see Fig. 2*A*). The presence of the deep cerebellar nuclei and the red nucleus was also assessed in thionin-stained tissue for all preparations.

RESULTS

Thirty-four *in vitro* turtle brainstem preparations were examined in this study, 15 that received the cerebellar ablation alone and 19 that received combined cerebellar and red nucleus lesions. Of these 34 preparations, 19 (56%) exhibited a positive slope of CR acquisition (8 of the 15 that received the cerebellar ablation alone and 11 of the 19 that received cerebellar and red nucleus lesions). In the Results, we will first describe the extent of the lesions followed by properties of CR acquisition and extinction. Finally, differences in CR characteristics between cases with cerebellar lesions, those with cerebellar and red nucleus lesions, and intact brainstem–cerebellum preparations will be presented.

Extent of cerebellar and red nucleus lesions

The extent of the cerebellar and red nucleus lesions was examined for all cases, and the results are summarized in Figure 2 and Table 1. The degree of removal of the cerebellar cortex was assessed by counting the total number of Purkinje cells that remained after the lesion in thionin-stained sections for each preparation. In 19 preparations that received a lesion of the cerebellum and that demonstrated CR acquisition, the number of intact Purkinje cells ranged from 848 to 0 (Table 1). For comparison, the total number of Purkinje cells present in the intact turtle cerebellar cortex is 7436 ± 320 (mean ± SD; data from three animals). Thus, the cerebellar cortex lesions involved from 89 to 100% removal of the cortex. The deep cerebellar nuclei were completely intact bilaterally in only three of the 19 preparations that showed acquisition (cases 1, 4, and 8 in Table 1). Three preparations had partial lesions ipsilateral to the side that was conditioned (cases 2, 3, and 5), and six showed complete bilateral ablation of the deep cerebellar nuclei (cases 6, 7, and 9–12). Nineteen of the preparations that received a cerebellectomy additionally received a transection at the level of the trochlear nerve that resulted in complete removal of the red nucleus bilaterally. Six of the 19 preparations that demonstrated conditioning were not included in Table 1 because either there was not a complete enough data set or the data could not be completely analyzed because of spontaneous activity. Photomicrographs illustrating the extent of the cerebellar ablations from three preparations that demonstrated CR acquisition are shown in Figure 2. The photomicrograph in Figure 2*A* is taken from case 1 in which 848 Purkinje cells remained. In this case, the transection was made too superficially so that Purkinje cells along the lateral edges of the cortex, as well as the deep cerebellar nuclei, remained intact. Schematic diagrams summarizing the transections (*solid lines*) are shown below the photomicrographs for the cases shown. The rostrocaudal level at which the photomicrograph was taken is illustrated by the *dotted line*. Figure 2*B* is taken from case 6 in which there was a complete transection removing the cerebellar cortex and deep nuclei; however, the red nucleus was intact. Finally, Figure 2*C* illustrates the complete removal of the entire cerebellum and red nucleus in case 12.

Conditioning after removal of the cerebellum and red nucleus

Figure 3 shows an example of acquisition, extinction, and reacquisition of the conditioned abducens nerve response in a preparation (case 1) that received a cerebellectomy and in which the red nucleus was intact. There were 848 Purkinje cells intact after the cerebellar lesion, and these cells represent ~11% of the total

Table 1. Summary of lesions and CR parameters for preparations that received an ablation of the cerebellum and red nucleus and that showed acquisition

Case	Treatment	No. of Purkinje cells	Cerebellar nuclei	Mean latency ± SE (msec)	CRs/session (%)
1	Lesion Cb	848	Intact bilat	222 ± 109	11
2	Lesion Cb	310	Partial ipsi	350 ± 214	6
3	Lesion Cb	36	Partial ipsi	111 ± 88	28
4	Lesion Cb	34	Intact bilat	175 ± 77	8
5	Lesion Cb	0	Partial ipsi	144 ± 104	8
6	Lesion Cb	0	Complete bilat	183 ± 133	8
7	Lesion Cb/RN	54	Complete bilat	290 ± 143	13
8	Lesion Cb/RN	14	Intact bilat	61 ± 14	8
9	Lesion Cb/RN	0	Complete bilat	118 ± 93	6
10	Lesion Cb/RN	0	Complete bilat	295 ± 208	32
11	Lesion Cb/RN	0	Complete bilat	382 ± 222	8
12	Lesion Cb/RN	0	Complete bilat	483 ± 21	8

Data are from 12 of 19 preparations that showed CR acquisition, extinction, and reacquisition. Lesion Cb, Lesion of the cerebellar cortex and deep nuclei; Lesion Cb/RN, combined lesions of the cerebellum and red nucleus. Extent of cerebellar nuclei lesions were either intact bilaterally (*Intact bilat*), partial lesions on the ipsilateral side to the conditioning (*Partial ipsi*), or complete lesions bilaterally (*Complete bilat*). All of the partial ipsilateral lesions also had a complete lesion of the deep nuclei on the contralateral side. The rate of acquisition is shown in the last column as the mean percent of CRs per session during the acquisition phase of the experiment.

in intact cerebella. The deep cerebellar nuclei were also intact bilaterally in this preparation, as shown in the photomicrograph in Figure 2*A*. Figure 3*A* illustrates the percent of CRs exhibited during each pairing session. In this case, during the first pairing session, a minimal number of CRs was generated. The number of CRs per session gradually increased to 44% by the fourth pairing session, which is a mean rate of ~11% per session. After acquisition, the paired stimuli were changed to extinction stimuli consisting of alternate CS–US trials in which the CS preceded the US by 10 sec. The preparation was presented with four sessions of alternate CS–US. As can be seen in Figure 3*A*, there was a gradual extinction of CRs to 4% by the fourth extinction session (session 8). After extinction of the CRs, paired CS–US trials were resumed. This resulted in reacquisition of the CR at a faster rate than that in the initial acquisition phase, and by the third reacquisition session, a percent of CRs of 52% was observed. Figure 3*B* shows the same data presented in Figure 3*A*, but the percent of CRs was divided into five blocks of 10 stimuli plotted for every session for greater resolution in the acquisition curve. Extracellular abducens nerve recordings are shown during the different phases of the experiment (Fig. 3*a–d*). The stimulus artifacts produced by the CS are visible in the recordings as downward deflections, whereas the single shock US is visible as an upward deflection. A CR (*arrow*) is shown during the initial acquisition (*a*) and in the early phases of unpaired extinction trials (*b*). Later extinction trials resulted in no recorded CR (*c*), and reacquisition of CRs was observed when the stimuli were once again paired (*d*).

Similar findings were obtained from a different preparation (case 6) in which zero Purkinje cells remained after complete bilateral ablation of the cerebellum and deep cerebellar nuclei. The red nucleus was intact bilaterally. The photomicrograph in Figure 2*B* is taken from this case. Data from this case are shown in Figure 4. In this experiment, five pairing sessions resulted in CR acquisition to a maximum of 32%. This was a rate of 8% per session. Two extinction sessions of alternate CS–US followed, and the percent of CRs dropped to 10%. Reacquisition to 32% of CRs was obtained after resuming the CS–US pairing in sessions 8 and 9.

Figure 5 shows an example of acquisition, extinction, and reacquisition of the conditioned abducens nerve response in a preparation (case 12) that received a complete ablation of the cerebellum and deep cerebellar nuclei and a lesion at the level of the trochlear nerve that removed the entire red nucleus bilaterally. The histology from this preparation is shown in Figure 2*C*. Figure 5*A* illustrates the percent of CRs produced during each training session. The number of CRs per session gradually increased to 36% by the fourth pairing session, which is a mean rate of ~8% per session. After the alternate CS–US pairings, there was a gradual extinction of CRs to 4% by the third extinction session (session 7). Pairing of the CS–US resumed, and the preparation exhibited CRs at a faster rate (10%) than that in the initial acquisition phase. By the third reacquisition session, the percent of CRs was 24%. Figure 5*B* shows the same data presented in Figure 5*A* plotted in five blocks of 10 stimuli for every session. Extracellular abducens nerve recordings show CRs (Fig. 5*a–d*, *arrow*) during the initial acquisition (*a*) and in the early phases of unpaired extinction trials (*b*). Later extinction trials resulted in no recorded CR (*c*), and reacquisition of CRs was observed when the stimuli were once again paired (*d*).

Analysis of the data obtained from 12 preparations (Table 1) that exhibited reacquisition after acquisition and extinction of CRs showed that there were no significant differences between any of the preparations on the basis of the type of lesion ($F = 0.62$; $p = 0.54$), the total number of CRs ($F = 0.51$; $p = 0.62$), the rate of acquisition ($F = 0.04$; $p = 0.84$), and the latency of CRs ($F = 0.29$; $p = 0.87$). Therefore, data from all 12 preparations were combined into the histogram shown in Figure 6. During the initial acquisition phase of the experiments, the first pairing session resulted in very few CRs (3%). The next four acquisition sessions displayed a gradual increase in the percent of CRs to a mean of 36%, with an acquisition rate of ~8% CRs per session. After acquisition, all preparations were presented with alternate CS–US extinction trials. These sessions of alternate pairing produced extinction of the CR at a rate of 14% fewer CRs per session to a mean of 4% by the last extinction session. Presentation of paired stimuli was resumed, and CR reacquisition was obtained with a steeper slope than that occurring during the initial acquisition trials, at a rate of ~14% CRs per session. The percent of CRs also surpassed that of the initial acquisition sessions, reach-

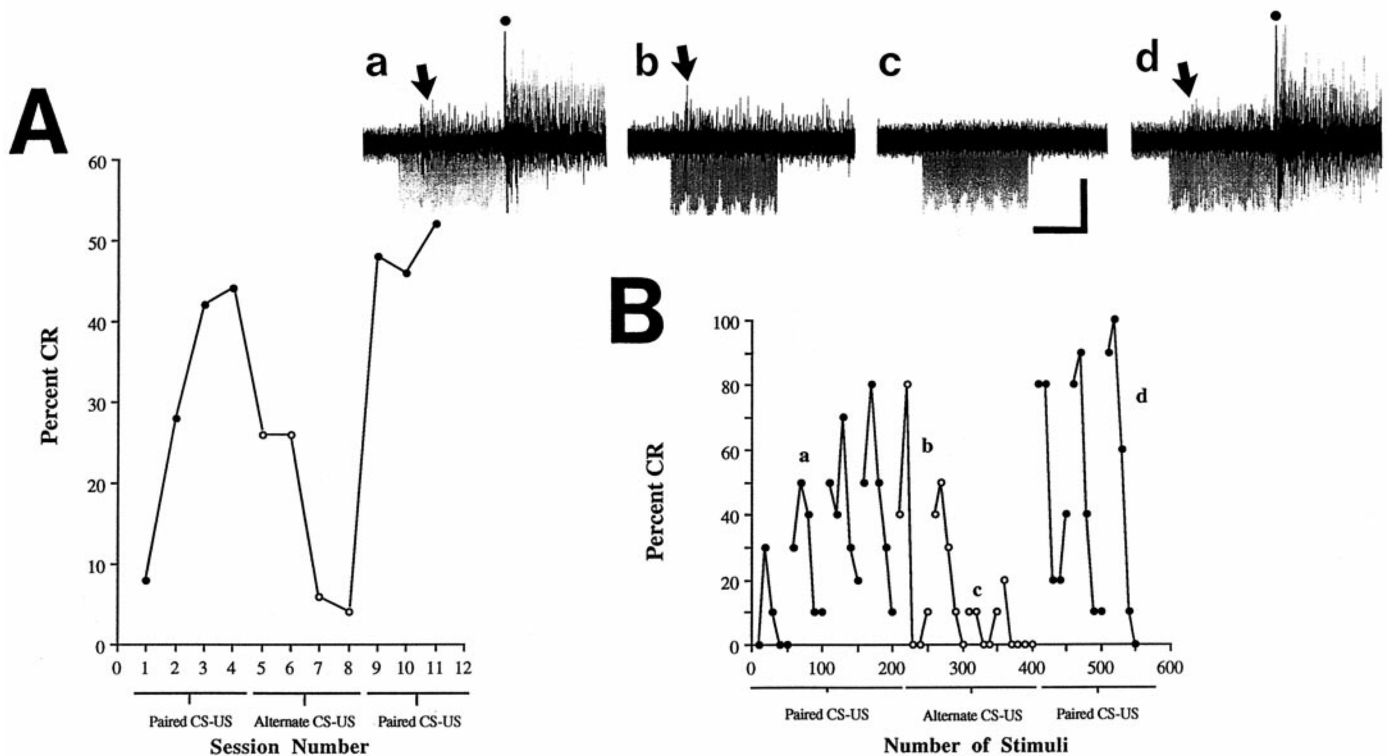

Figure 3. Acquisition curves from a preparation (case 1) in which there was an 89% removal of the cerebellar cortex. The deep cerebellar nuclei and red nucleus were intact. The photomicrograph in Figure 2*A* is from this preparation. *A*, The percent of CRs exhibited during each stimulus session. During the first pairing session (*filled circles*), a minimal number of CRs was generated. The number of CRs per session gradually increased to 44% by the fourth pairing session at a rate of ~11% CRs per session. Paired stimuli were then changed to extinction stimuli consisting of alternate CS–US trials in which the CS preceded the US by 10 sec. The preparation was presented with four sessions of alternate CS–US (*open circles*). There was a gradual extinction of CRs to 4% by the fourth extinction session (session 8; extinction rate of −9% per session). After extinction of the CR, paired CS–US trials were resumed. This resulted in reacquisition of the CR at a faster rate (14%) than that during the initial acquisition, and by the third reacquisition session, a percent CR of 52% was observed. *B*, Same data shown in *A* but with greater resolution for each session. The percent of CRs was divided into five blocks of 10 stimuli plotted for every session. For the paired CS–US trials, the preparation typically exhibited few CRs in the first block of the session, followed by a gradual acquisition of CRs during the subsequent sessions. Examples of extracellular abducens nerve recordings are shown during acquisition sessions that produced a CR (*a*; *arrow*), during early extinction trials that produced a CR (*b*; *arrow*), during extinction trials in which no CR was recorded (*c*) and during reacquisition of CRs (*d*; *arrow*). In this and all subsequent figures, the CS is indicated in the *traces* by the downward spikes; the US is indicated by the upward spike (*dot*). Calibration: 50 μV, 0.5 sec.

ing a peak of 40% CRs in the last pairing session. These data on CR acquisition from the lesioned preparations compare well with data from intact preparations (Keifer et al., 1995, compare Fig. 6 with their Fig. 4). In both groups, a mean maximum of 40–50% of the CSs generated a CR, whereas they extinguished to ~10% or less.

The data shown in Figures 3–6 demonstrate that it is possible to classically condition this *in vitro* preparation from the turtle in the absence of the cerebellum and red nucleus. Additionally, they show that unpaired stimuli presented as alternate CS–US separated by 10 sec do not support the CR. However, timing features of the CR were found to be dramatically altered in preparations that received a lesion of the cerebellum as compared with those with an intact cerebellum. These results will be presented below.

Characteristics of the abducens nerve conditioned response

Many of the CRs recorded in the present study showed unusual timing properties as compared with the CRs of intact brainstem–cerebellum preparations. An example of one of these unusual abducens nerve CRs is shown in the physiological recording in Figure 7. This CR had a relatively short latency of onset with respect to the onset of the CS of 120 msec. Short latency CRs was a typical feature of those recorded in cerebellectomized preparations (see below). Additionally, the CR waveform shown in Figure 7 illustrates a second feature of the timing of these CRs. This *trace* shows a burst response rather than the sustained discharge that is more characteristic of the majority of CRs that were recorded in these preparations (see CRs in Figs. 1, 3, 5). Thus, the abducens nerve activity had subsided by the time of the US onset. Approximately 30% of the abducens nerve CRs observed in this study showed burst-like responses. This compares with significantly fewer burst-like responses that were recorded in intact brainstem–cerebellum preparations (5%; $t = 2.6$; $p < 0.01$; data from Keifer et al., 1995).

There was a significant difference in mean latency of the CR between preparations that received lesions of the cerebellum and red nucleus and those that were intact. Among the lesioned preparations, there were no significant differences in mean latency between individual preparations or any consistent differences between latency and the extent of the lesions between the cases with cerebellar lesions and those with cerebellum and red nucleus lesions ($t = 0.48$; $p = 0.64$). Therefore, these data were combined in Figure 8*A* to show the mean onset of the CRs for the 12 preparations from which data were analyzed. These data show

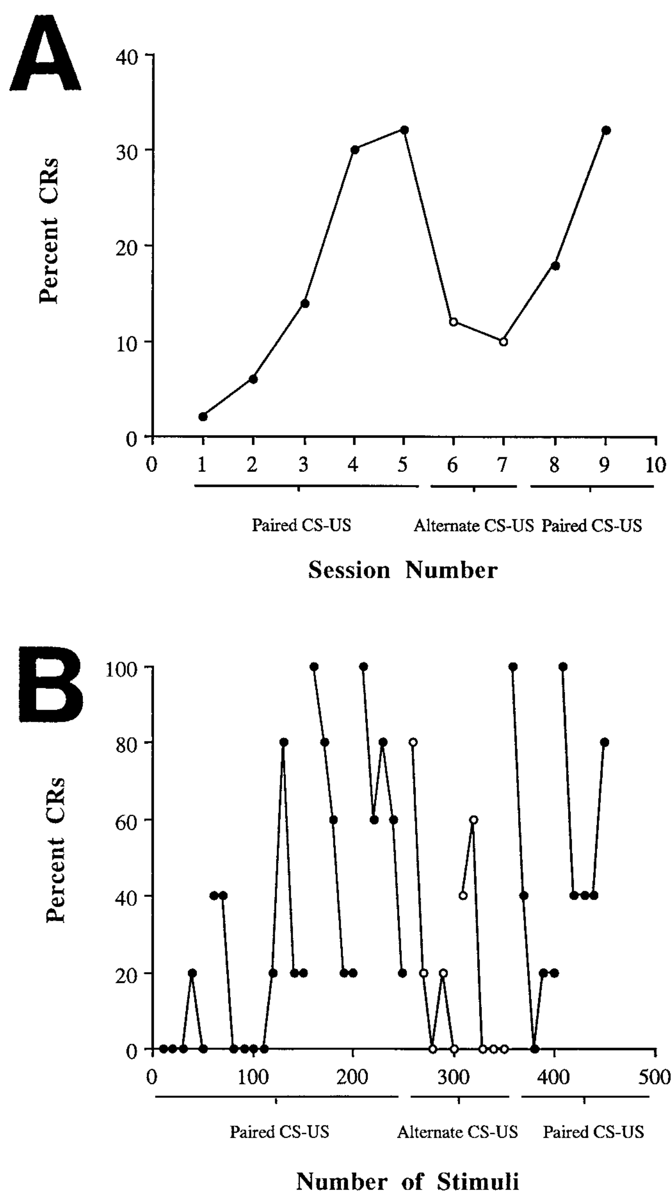

Figure 4. Acquisition curves from a preparation (case 6) in which zero Purkinje cells remained after complete bilateral ablation of the cerebellum and deep cerebellar nuclei. The red nucleus was intact bilaterally. The photomicrograph shown in Figure 2*B* is from this preparation. *A*, Five pairing sessions resulting in CR acquisition to 32%. Two extinction sessions of alternate CS–US followed, and the percent of CRs declined to 10%. Reacquisition to 32% occurred after resuming the CS–US pairing. *B*, Same data shown in *A* but with the percent of CRs divided into five blocks of 10 stimuli plotted for each session.

that the latency of the CR is significantly shorter (242 ± 8 msec) in preparations with cerebellum/red nucleus lesions than in those preparations in which the cerebellum and red nucleus were intact (392 ± 51 msec; $t = 4.22$; $p < 0.005$; intact data from Keifer et al., 1995). The mean latencies for each of the experiments reported in the present study ranged from 61 to 483 msec (Table 1).

These latency data were also combined across experiments and plotted against the session number. As can be seen in Figure 8*B*, there is a significant positive slope of CR latency over the time course of the experiments for both cerebellum- and cerebellum/red nucleus-lesioned preparations (*closed circles*; $F = 18.40$; $p < 0.005$). This was also the case for preparations in which the cerebellum and red nucleus were intact (*open circles*; $F = 115.0$; $p < 0.001$). Of the cases that received lesions, the mean CR latency was 188 msec in the second session, and this gradually increased in latency by the ninth session to a mean response latency of 336 msec. By comparison, the intact preparations had a mean latency of 215 msec in the second session and 588 msec by the ninth session. The shift in CR latency toward the onset of the US over the period of conditioning, or the slopes of the functions shown in Figure 8*B*, was significantly greater for the intact preparations than it was for the cerebellectomized preparations ($t = 91.6$; $p < 0.001$). These findings suggest that in both the intact and cerebellectomized preparations, timing mechanisms are present that act to shift the CR closer in time to the onset of the US as conditioning proceeds. This mechanism seems to function to a greater degree, however, with an intact cerebellum.

UR Suppression

Studies of classical conditioning of the rabbit eye blink reflex have suggested that a temporal association of the CS–US can in some instances result in a diminished UR. The neuronal basis for this UR suppression is not well understood, but it has been suggested that the interpositus nucleus and the red nucleus are integral components for the production of UR suppression (Canli and Donegan, 1995). Keifer et al. (1995) observed UR suppression in 11 of 32 (34%) *in vitro* turtle brainstem–cerebellum preparations, all of which demonstrated acquisition of CRs. In these cases, the UR would be completely absent and then reappear at irregular intervals. Augmentation of the UR was not observed. In the present study, UR suppression was observed in 5 (33%) of the 15 preparations that received a cerebellar lesion but that had an intact red nucleus. None of these preparations that demonstrated UR suppression, however, showed acquisition of CRs. Of the 19 preparations that received cerebellum and red nucleus lesions, 6 (32%) demonstrated UR suppression, and these also showed CR acquisition.

DISCUSSION

In this study, we used an *in vitro* preparation from the turtle to examine the role of the cerebellum and red nucleus in classical conditioning. In this discussion, we first present evidence of classical conditioning in this *in vitro* preparation. Next, we discuss the role of the cerebellum in the timing and generation of the *in vitro* classically conditioned abducens nerve response, and finally, we discuss the implications this study has for understanding the organization of the conditioned vertebrate eye blink reflex.

Evidence of *in vitro* conditioning

Evidence presented by Keifer et al. (1995) suggested that it was possible to classically condition abducens nerve reflex pathways entirely *in vitro*. The CRs in this case are hypothesized to be a model of the turtle eye blink reflex, although the possibility remains that they represent horizontal eye movements. That the CRs represent eye blinks is supported by the observation that stimulation of the posterior eighth nerve in a reduced preparation with intact peripheral attachments to the eye results in a behavioral eye blink and discharge in the retractor bulbi muscle. Horizontal eye movements have not been evoked by this method. The behavioral correlate of the abducens nerve CR will be addressed in future studies. Using a decerebrate brainstem–cerebellum preparation, we used a delayed-training protocol exactly the same as the one described here. In that study, after application of

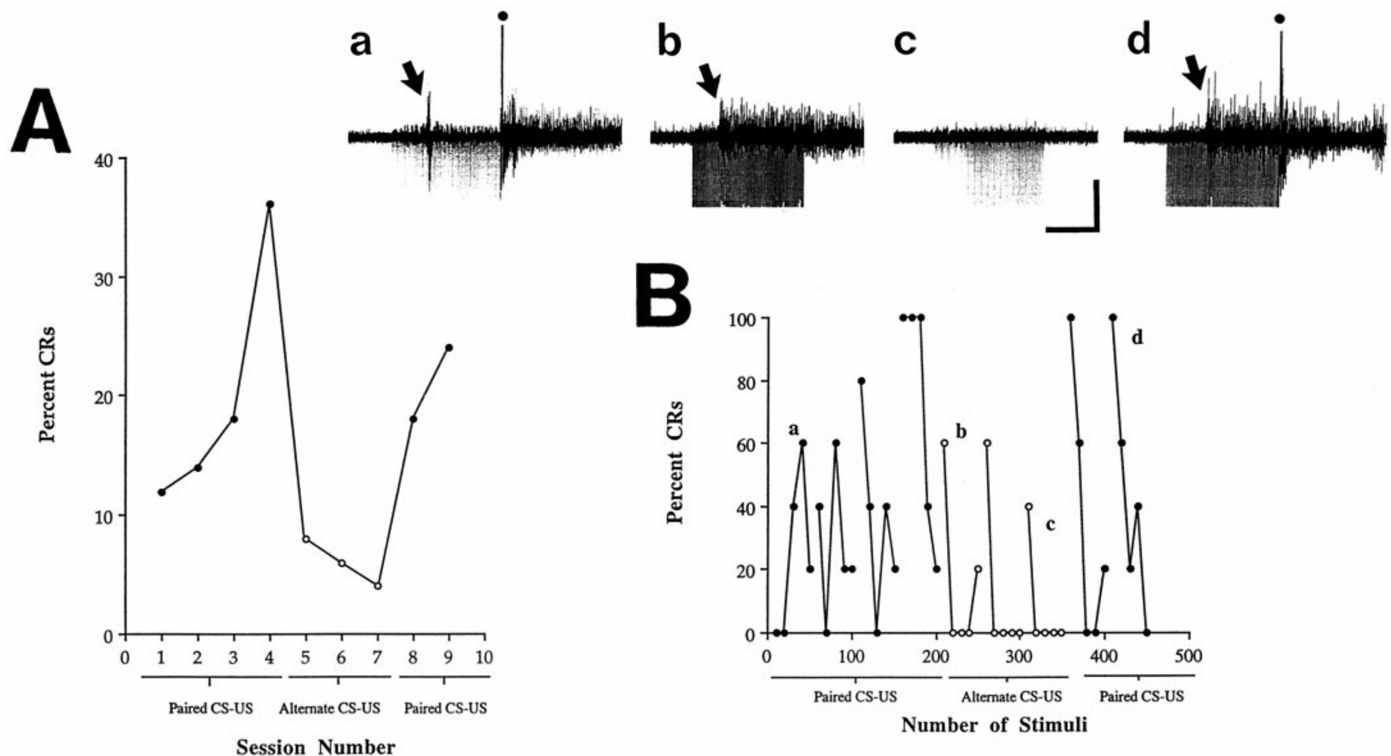

Figure 5. Acquisition curves from a preparation (case 12) in which zero Purkinje cells remained after the cerebellar ablation. The deep cerebellar nuclei and red nucleus were also completely ablated bilaterally. The photomicrograph shown in Figure 2*C* is from this preparation. *A*, Four pairing sessions resulted in CR acquisition to 36%. Three extinction sessions of alternate CS–US pairing followed, and the percent of CRs dropped to 4%. Reacquisition to 24% was obtained after CS–US pairing resumed in sessions 8 and 9, and this occurred at a faster rate than that seen during the initial acquisition. *B*, Same data shown in A but with the percent of CRs divided into five blocks of 10 stimuli plotted for each session. Examples of extracellular abducens nerve recordings are shown during acquisition sessions in which a CR was produced (*a*, *arrow*), during early extinction trials that produced a CR (*b*, *arrow*), during extinction trials in which no CR was recorded (*c*), and during reacquisition of CRs (*d*, *arrow*). Calibration: 50 μV, 0.5 sec.

paired posterior eighth and trigeminal nerve stimuli, a positive slope of CR acquisition was observed. Extinction of abducens nerve CRs was obtained when unpaired stimuli consisting of CS alone, backward pairing, or alternate CS–US were applied. Presentation of paired stimuli resulted in reacquisition of CRs generally at a faster rate than that during the initial pairing. Further evidence of conditioning was that CRs were of long latency, ranging from means of 200 to 450 msec, and thus were not reflex responses. The present study supports and extends the original observations of *in vitro* conditioning reported by Keifer et al. (1995). Importantly, more extensive testing with the alternate CS–US stimuli was performed, and these data suggest that CRs are not supported by this stimulus protocol. Thus, there is a requirement for close temporal association (<10 sec separation) of the CS and US to generate abducens nerve CRs, a hallmark of classical conditioning.

Role of the cerebellum in classical conditioning

The cerebellar theory of motor learning suggests that the Purkinje cells of the cerebellar cortex represent the site of synaptic plasticity and that the synaptic modification is dependent on the simultaneous activity of the climbing fibers (conveying information about the US) and the parallel fibers (conveying information about the CS) (see Thompson, 1986). A significant challenge to the cerebellar theory of motor learning is that classical conditioning has been achieved when the cerebellum and/or deep cerebellar nuclei were removed. A large body of evidence suggests that lesions of the cerebellum or red nucleus result in prevention or attenuation of CRs (Rosenfield and Moore, 1983; McCormick and Thompson, 1984; Yeo et al., 1985; Steinmetz et al., 1992; Clark and Lavond, 1993). Other studies, however, have questioned the interpretation of these observations (Karamian et al., 1969; Norman et al., 1977; Welsh and Harvey, 1989; Kelly et al., 1990; Yeo, 1991; Harvey et al., 1993; Keifer, 1993b; Gruart et al., 1994; Bloedel and Bracha, 1995; Chen et al., 1996; for review, see Llinás and Welsh, 1993). Welsh and Harvey (1989) and others (see Llinás and Welsh, 1993) have argued that the more general role of the cerebellum in the control and coordination of motor output overshadows the role of learning. Thus, ablations of the cerebellum produce large performance deficits that overshadow the expression of learned responses. Welsh and Harvey (1989) showed that after lesions of the ipsilateral interpositus nucleus, conditioned eye blink responses were clearly generated. However, there was a decrement in the frequency and an increased time to onset of the CR. Kelly et al. (1990) using a decerebrate–decerebellate rabbit preparation recorded acquisition, extinction, and reacquisition of the eye blink CR. They also observed a decreased frequency of the CR and an increased variability of CR onset compared with preparations with an intact cerebellum. After lesions of the cerebellar cortex in the rabbit, Perrett et al. (1993) found that eye blink CRs had short, relatively fixed latencies. They concluded that although CRs could still be generated in the absence of the cerebellum, the cerebellar cortex is necessary for the appropriate timing of the learned CRs. This interpretation of cerebellar function in the adaptive timing of CRs has been further substantiated by recent studies (Katz and

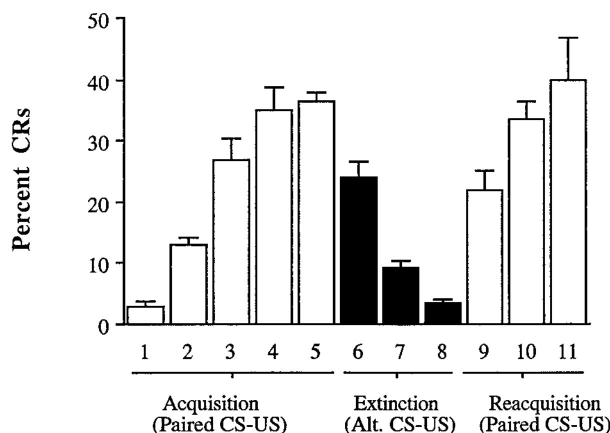

Figure 6. Mean percent of CRs ± SE for the different training sessions for all the preparations that conditioned and showed reacquisition (n = 12). *Open bars*, Paired stimuli; *solid bars*, alternate CS–US. There was a positive slope of CR acquisition during the first five pairing sessions to a mean of 36 ± 2% (mean acquisition rate of 8%). During extinction trials of alternate pairing of the CS and US, there was a gradual decrease in the number of CRs to a mean of 4 ± 0.5% (mean extinction rate of −14%). Pairing of the CS–US was resumed, and there was a steeper slope of reacquisition than that occurring during the initial acquisition trials. The percent of CRs also surpassed that of the original acquisition sessions, reaching a peak of 40 ± 4% (mean reacquisition rate of 14%).

Steinmetz, 1997; Svensson et al., 1997). To further investigate the role of the cerebellar cortex in classically conditioned eye blink reflexes, Chen et al. (1996) used a Purkinje cell degeneration mutant mouse that lacks Purkinje cells as an adult. Because the Purkinje cells are the only output neurons from the cerebellar cortex, these mice have a functionally ablated cortex. This study shows impaired eye blink conditioning in these mice. However, the mutant mice still demonstrated a positive slope of acquisition (to 40% CRs) and extinction of eye blink CRs. There was, in addition, a significant decrease in the latency of the CR. This acquisition of CRs with a decreased latency is very similar to what was observed in the decerebellate *in vitro* turtle brainstem preparation.

In the present study, we found that it is possible to classically condition the abducens nerve response in the absence of the cerebellar cortex, deep cerebellar nuclei, and red nucleus. Furthermore, in preparations having incomplete cerebellar lesions,

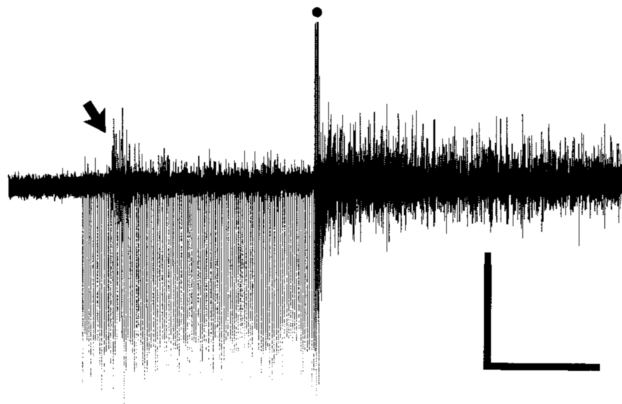

Figure 7. Extracellular recording from the sixth nerve showing the burst-like CR (*arrow*) that was generated in ~30% of the CRs observed in this study. Typically, there was a sharp onset of the CR with a duration too short to coincide with the onset of the UR. Calibration: 50 μV, 0.5 sec.

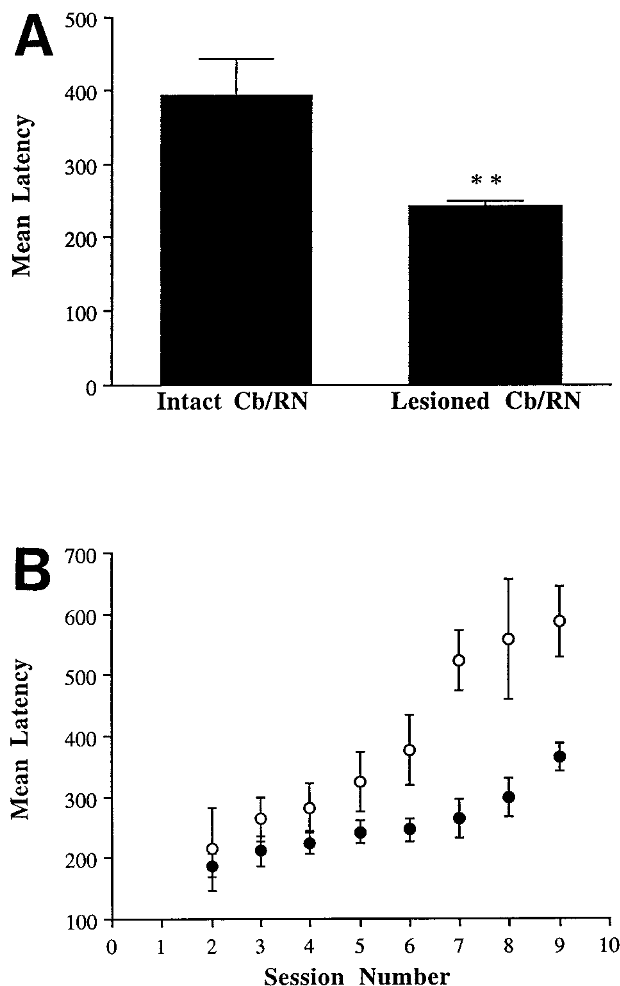

Figure 8. *A*, Quantitative data showing significant differences in the mean latency of the CR between lesioned and intact preparations. Although experimental preparations that had an 89–100% removal of the cerebellar cortex (n = 12) demonstrated CRs, the latency of the response was significantly shorter compared with the latencies of preparations in which the cerebellum and red nucleus were intact (t = 2.598; $^{**}p$ = 0.01; data from Keifer et al., 1995). *B*, Comparison of the mean latency of the CR plotted against the session number. The *open circles* represent the intact cerebellum and red nucleus preparations, and the *closed circles* represent the lesioned cerebellum and red nucleus preparations. Both types of preparations showed similar CR latencies at the start of the training sessions. However, the intact cerebellar preparations generated significantly longer latency CRs as training progressed and produced them at a faster rate than did the preparations without a cerebellum (t = 91.6; p < 0.001).

no significant differences in the number of CRs or the rate of acquisition of the CR related to the number of Purkinje cells or the presence or absence of the cerebellar nuclei were observed. There were, however, significant differences between intact and cerebellar-lesioned preparations such that the latency of the CRs was considerably shorter. Additionally, both intact and lesioned treatment groups showed a significant increase in the latency of the CR over training. The latency of the CR increased throughout the training sessions for both groups, although the intact cerebellum preparations showed a significantly faster increase in the latency as training proceeded. Presumably, in the intact animal, it is an adaptive feature of the CR to respond as close as possible to, but still precede, the UR. This is so that when the US begins, the

eyelid is closed to protect the eye. These data suggest that although the cerebellum seems to play a role in the latency of the CR, mechanisms are still present in the brainstem that gradually shift the latency of the CR toward the onset of the US as conditioning proceeds (see Moore et al., 1989).

Anatomical basis for conditioning in the turtle brainstem

If the cerebellar cortex, deep nuclei, and red nucleus are not necessary for the acquisition of abducens nerve CRs in this preparation, where then is the site of learning? Theories suggest that learning occurs at sites that receive convergent information about the CS and US (see Thompson, 1986). A recent tract tracing study in the turtle (Herrick and Keifer, 1998) provided evidence of sites of CS–US convergence within the abducens nerve eye blink reflex circuitry that may be responsible for supporting the acquisition of CRs in these lesioned preparations. Injections of either neurobiotin or fluorescein dextran into the trigeminal or posterior eighth nerve trunks resulted in terminal label directly from both nerves in the cerebellar cortex, the principal sensory trigeminal nucleus, and the principal and accessory abducens motor nuclei. These anatomical findings suggest that synaptic modifications during conditioning may occur in the reflex pathway, either in the trigeminal nucleus or at the level of the motoneurons. Anatomically, the direct connections of these pathways in the turtle may be somewhat different from those in mammals. Evidence strongly supports CS–US convergence in the pars oralis division of the spinal trigeminal nucleus in the rabbit, although the anatomy remains to be described (Nowak and Gormezano, 1990; Bracha et al., 1991; Richards et al., 1991). It is unknown whether the motoneurons controlling eye blinks in rabbits or other mammals receive convergent information as they do in the turtle. The presence of convergent inputs in the brainstem pathways controlling blinking might explain the conflicting and controversial data that conditioning can be achieved in animals with a cerebellectomy. As with all lesion experiments, the caveat remains that neural networks that are damaged may be capable of functions that they do not normally engage in when intact. However, significant mechanistic capacities of brain structures may be revealed after such lesions. Given the accumulation of data over the years, it appears that brainstem pathways may support eye blink conditioning. Moreover, an important role of the cerebellum in conditioning may be in forming the appropriate amplitude and timing components of the CR, which would correspond with its well known role in sensorimotor integration.

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