

Impaired Classical Eyeblink Conditioning in Cerebellar-Lesioned and Purkinje Cell Degeneration (*pcd*) Mutant Mice

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Converging lines of evidence from rabbits, rats, and humans argue for the crucial involvement of the cerebellum in classical conditioning of the eyeblink/nictitating membrane response in mammals. For example, selective lesions (permanent or reversible) of the cerebellum block both acquisition and retention of eyeblink conditioning. Correspondingly, electrophysiological and brain-imaging studies indicate learning-related plasticity in the cerebellum. The involvement of the cerebellum in eyeblink conditioning is also supported by stimulation studies showing that direct stimulation of the two major afferents to the cerebellum (the mossy fibers emanating from the pontine nucleus and climbing fibers originating from the inferior olive) can substitute for the peripheral conditioned stimulus (CS) and unconditioned stimulus (US), respectively, to yield normal behavioral learning. In the present study, we examined the relative contribution of the cerebellar cortex versus deep nuclei (specifically

the interpositus nucleus) in eyeblink learning by using mutant mice deficient of Purkinje cells, the exclusive output neurons of the cerebellar cortex. We report that Purkinje cell degeneration (*pcd*) mice exhibit a profound impairment in the acquisition of delay eyeblink conditioning in comparison with their wild-type littermates. Nevertheless, the *pcd* animals did acquire a subnormal level of conditioned eyeblink responses. In contrast, wild-type mice with lesions of the interpositus nucleus were completely unable to learn the conditioned eyeblink response. These results suggest that both cerebellar cortex and deep nuclei are important for normal eyeblink conditioning.

Key words: Purkinje cells; mutant mice; eyeblink conditioning; learning; memory; cerebellum; interpositus nucleus; long-term depression

Although the importance of the cerebellum in classical eyeblink conditioning has been well documented during the past 15 years (McCormick et al., 1981, 1982; Lincoln et al., 1982; Clark et al., 1984; McCormick and Thompson, 1984a,b; Lavond et al., 1985; Yeo et al., 1985; Berthier and Moore, 1986, 1990; Mauk et al., 1986; Steinmetz et al., 1986, 1989; Schreurs et al., 1991; Clark et al., 1993; Krupa et al., 1993; Nordholm et al., 1993; Molchan et al., 1994), because of the difficulty in lesioning all cerebellar cortex without damage to the interpositus nucleus, the relative importance of these two structures is unclear. Both the cortex and the deep nuclei receive mossy fiber and climbing fiber projections (Chan-Palay, 1977; Courville et al., 1977; Kitai et al., 1977; Voogd, 1982; Ito, 1984; Shinoda et al., 1992; Steinmetz and Sengelaub, 1992; Mihailoff, 1994), pathways thought to convey information about peripheral conditioned stimulus (CS) and unconditioned stimulus (US), respectively (Thompson, 1986, 1990), and therefore both are potentially capable of supporting CS–US associations. Consistent with this view, long-term depression (LTD) and long-term potentiation (LTP), two forms of synaptic plasticity implicated in associative learning, have been demonstrated in the

cerebellar cortex and the deep nuclei, respectively (Ito et al., 1982; Ekerot and Kano, 1985; Racine et al., 1986; Linden, 1994; Chen and Thompson, 1995). Although some have reported that the cortex is necessary (Yeo et al., 1984, 1985), others have suggested that although the cortex is involved, it is not necessary for eyeblink conditioning (Lavond et al., 1987; Lavond and Steinmetz, 1989).

Recently, various mutant (e.g., spontaneous mutants, gene knockouts, and transgenics) mice have been used to study the neural mechanisms underlying learning and memory (for reviews, see Goldowitz and Eisenman, 1992; Grant and Silva, 1994). In essence, these animals provide an effective means to determine the importance of, for example, a particular type of neuron or a specific protein in learned behavior. Eyeblink conditioning is an ideal behavioral model for testing various mutants because the neural circuitry involved in this learning has been well characterized (Thompson, 1986, 1990).

In the present study, we first determined whether associative eyeblink conditioning is feasible (see also Aiba et al., 1994) and whether the cerebellum is crucial for this type of learning in wild-type mice. We then employed Purkinje cell degeneration (*pcd*) mutant mice to dissect the components of cerebellar circuitry involved in eyeblink conditioning. The *pcd* mutants are born with Purkinje cells, but during the course of development they are all lost by the third and fourth postnatal weeks (Mullen et al., 1976; Landis and Mullen, 1978; Goodlett et al., 1992). Because Purkinje cells are the sole output neurons of the cerebellar cortex (Ito, 1984), the *pcd* mice are completely devoid of neural outputs from the cerebellar cortex. A phaseolus vulgaris leucoagglutinin (PHA-L) tract tracing study in-

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cluded here showed that there were no aberrant projections from the cortex to the deep nuclei in *pcd* mutants. Thus, the *pcd* mice provide fully reproducible, natural lesions of the entire cerebellar cortex.

EXPERIMENT 1. EYEBLINK CONDITIONING AND CEREBELLAR LESIONS IN MICE

Materials and Methods

Subjects and surgeries

Adult male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), weighing between 22 and 27 gm, were housed individually in a colony room with a 12 hr light/dark cycle. Subjects had *ad libitum* access to food and water except during the behavioral experiment. All experiments were conducted during the light phase of the cycle. Before the experiment, animals were assigned randomly to either paired, pseudo-random, or lesioned groups.

While they were under ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) anesthesia, animals were implanted subcutaneously with four wires (Teflon-coated stainless steel, 0.003 inch bare, 0.0045 inch coated; A-M Systems, Everett, WA) to the left upper eyelid. The tips of the wires were exposed, and two of the wires were used to record differential electromyograph (EMG) from obicularis oculi, and the remaining two wires were used to deliver periorbital shock. A four-pin strip connector to which the wires were soldered was cemented to the skull of the animal with dental acrylic. Animals in the lesioned group received bilateral electrolytic lesions of the interpositus nucleus (coordinates from the bregma: 6.5 mm posterior, 1.7 mm lateral, and 3.5 mm ventral) by passing anodal current (1.5 mA for 10 sec) through an electrode (insulated with epoxy, except for 0.5 mm at the tip) just before the eyelid surgery.

Behavioral training

For eyeblink conditioning, we used the techniques developed by Skelton (1988) on rats and adapted by Aiba et al. (1994) in mice. Training took place in a small cylindrical Plexiglas container (3.75 inch diameter) that was placed inside a sound- and light-attenuating chamber (Ralph Gerbrands Company, Arlington, MA). The strip connector was attached to the mating plug of a commutator that has channels to relay EMG signals and to deliver the shock. After 1 d of habituation, animals underwent either paired or pseudo-random training. The daily paired training consisted of 100 trials grouped in 10 blocks. Each block included one CS alone (the first trial), one US alone (the sixth trial), and eight CS-US paired trials. The CS and US were a 352 msec tone (1000 Hz, 80 dB, 5 msec rise/fall time) and a coterminating 100 msec shock (100 Hz biphasic square pulses), respectively, with a 252 msec interstimulus interval and a randomized intertrial interval between 20 and 40 sec (mean = 30 sec). The US intensity used was the minimal voltage required to elicit an eyeblink/head turn response, and the US intensity was adjusted daily for each animal. Animals assigned to the paired group received 5 d of paired training followed by 4 d of CS-alone extinction trials. In pseudo-random training, animals received the same number of CS and US as in the paired condition, but they occurred at random intervals and were never paired. The pseudo-random group received 5 d of unpaired CS-US training followed by 5 d of CS-US paired training. Animals with cerebellar lesions underwent 10 d of CS-US paired training.

EMG analysis

For conditioned response (CR) analysis, the EMG signal was amplified in the band of 300–5000 Hz and sent to a window discriminator where the number of pulses above the noise envelope was sampled and stored by a computer into 189 consecutive 4 msec bins. The discriminated EMG activity was analyzed trial by trial using the following criteria. (1) The trial was considered invalid and excluded from analysis if during the 252 msec pre-CS period the average unit count per bin was higher than two (indicating high EMG activity before the CS onset), or the SD of unit counts per bin was larger than the average unit count per bin (indicating unstable EMG activity before the CS onset), or the average unit count within 28 msec (seven bins) after the CS onset was bigger than the average plus the SD of the pre-CS period (indicating short-latency startle response to the CS). (2) The CR was defined as the average unit count of any consecutive seven bins during the period from 84 msec after tone onset to the onset of the shock (total = 168 msec) that was higher than average plus SD plus one unit per bin of pre-CS period. [For the CR measurement, a constant value of one unit per bin was included as a CR

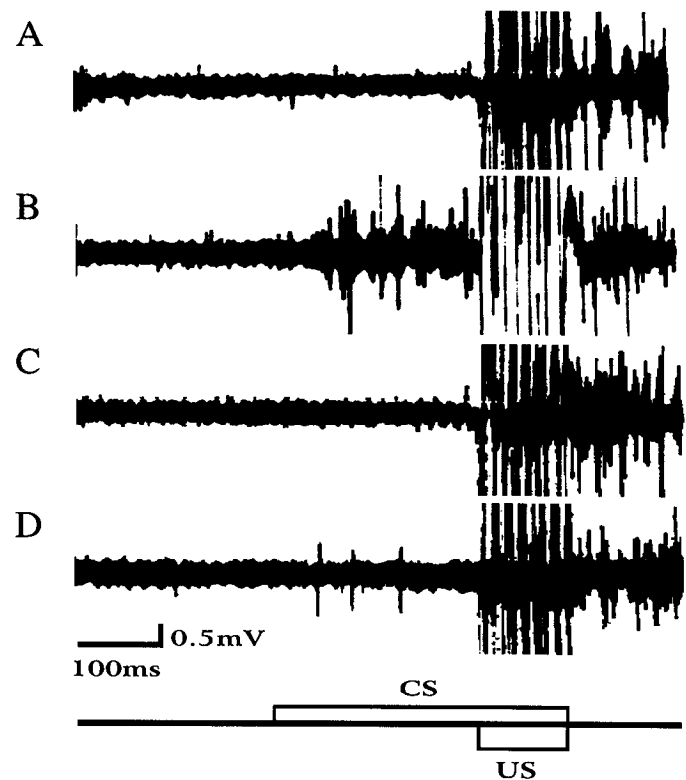


Figure 1. Typical EMG responses observed from a normal mouse on (A) day 1 and (B) day 5 and from a cerebellar-lesioned mouse on (C) day 1 and (D) day 10 of eyeblink conditioning.

threshold. This value is comparable to the CR threshold value (0.5 mm deflection in the eyelid/nictitating membrane response) used in the rabbit eyeblink conditioning paradigm.] For tone-alone trials, the period for scoring a CR was extended to the termination of the tone (total = 268 msec).

The UR amplitudes were determined from US-alone trials. Because the shock US saturates the EMG activity and because the shock offset is not instantaneous (because of capacitance), we analyzed the unit activity during the 100 msec (25 bins) period 50 msec after the termination of the US. The UR amplitude was defined as the difference in averaged unit activity during the UR period and averaged unit activity 100 msec before the US onset.

Histology

When the behavioral studies were completed, the animals were given an overdose of sodium pentobarbital and perfused with 0.9% saline followed by 10% buffered formalin. The brains were sectioned on a microtome (80 μ m sections), mounted on gelatin-coated slides, and stained with Prussian blue and cresyl violet. The slides were scanned into a computer using a 35 mm Nikon film scanner (LS-3510 AF) for lesion verification.

Results

Figure 1 represents the typical EMG activity patterns observed from a normal wild-type mouse in response to paired tone-airpuff presentations before (A) and after (B) conditioning. Before the animal acquired eyeblink CRs (day 1), there were no differences in EMG activity between pre-CS and CS periods. The saturated EMG signal during the US period is the shock artifact. After termination of the shock, there was an increase in EMG activity (most likely an unconditioned EMG response to the shock). After the animal learned (day 5), there was a substantial increase in EMG activity during the CS period, reflecting a conditioned EMG response from the eye. In the cerebellar-lesioned animal, the EMG activity during the CS period did not increase from day 1

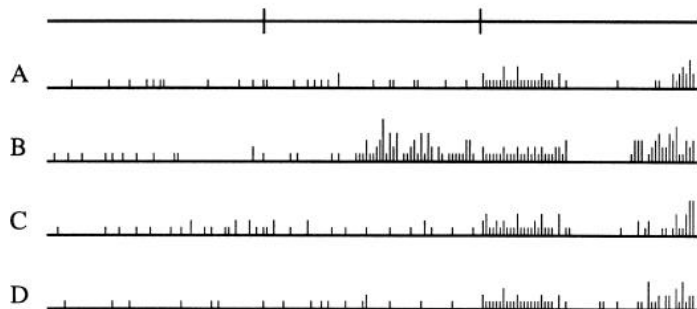


Figure 2. Examples of discriminated EMG activity from a normal mouse on (A) day 1 and (B) day 5 and from a cerebellar-lesioned mouse on (C) day 1 and (D) day 10 of eyeblink conditioning. The first and second vertical lines denote the onset of CS and US, respectively.

(C) to day 10 (D) of paired training. Examples of discriminated EMG units can be seen in Figure 2. Although the normal mouse displayed an increase in discriminated units during the CS period from day 1 (A) to day 5 (B) of training, the cerebellar-lesioned mouse showed no increase in discriminated units from day 1 (C) to day 10 (D) of training.

Figure 3 shows a transverse brain section from a typical mouse in the cerebellar-lesioned group. Cerebellar-lesioned animals sustained substantial bilateral destruction of the interpositus nucleus and the overlying cerebellar cortex.

Figure 4 presents the percentage (A) and the amplitude (B) of eyeblink CRs displayed by paired ($n = 6$), pseudo-random ($n = 6$), and lesioned ($n = 6$) groups. The percentage of CRs of the paired group increased gradually and significantly during the 5 d of training [linear trend analysis for all time points, $F_{(1,24)} = 22.76$, $p < 0.01$], reaching an asymptote of 66%. When switched to extinction trials, the previously acquired CRs decreased progressively to 7% over 4 d [linear trend analysis, $F_{(1,18)} = 21.96$, $p < 0.01$]. The animals in the pseudo-random group exhibited no reliable increase in CRs over the course of 5 d of unpaired CS–US

presentations [linear trend analysis, $F_{(1,24)} = 6.28$, $p > 0.05$]. When switched to paired trials, however, they acquired CRs in a manner similar to that of the paired group [linear trend analysis, $F_{(1,24)} = 28.81$, $p < 0.01$] (maximum of 71%). In contrast to the unlesioned paired group, the cerebellar-lesioned animals failed to show a reliable increase in CRs even with 10 d of paired training [linear trend analysis, $F_{(1,423)} = 3.71$, $p > 0.05$]. In all three groups, trends similar to those of percentage of CRs were observed in CR amplitudes.

EXPERIMENT 2: EYEBLINK CONDITIONING IN PCD MICE

Materials and Methods

Subjects, surgeries, behavioral training, and histology

Adult male *pcd* ($n = 11$) and wild-type littermate mice ($n = 10$) of C57BL/6J strain (Jackson Laboratories), weighing between 20 and 30 gm, were housed and prepared for eyeblink conditioning in a manner similar to that described in Experiment 1. Because *pcd* is a recessive autosomal mutation (Mullen et al., 1976), the actual genotypes of the wild-type mice may include both homozygotes (*wt/wt*) and heterozygotes (*wt/pcd*). Both *pcd* and wild-type animals received 10 d of CS–US paired training followed by 4 d of CS-alone extinction training. Animals were trained “blind” by the experimenters (L.C. and S.B.), and the EMG analysis was carried out as described previously. In addition, we analyzed CR onset and CR peak latencies in the following manner. (1) The CR onset latency was computed as the first time, during the period between the CS onset and the US onset, at which the average unit count of any consecutive seven bins (28 msec) was higher than the average plus SD plus one of pre-CS period. (2) The CR peak latency was calculated as the time between the CS and the US onsets at which the average unit count of any consecutive seven bins was the highest. On tone-alone trials, the period for scoring CR onset and peak latencies, extended to the tone offset. After termination of the experiment, the animals were given an overdose of sodium pentobarbital and perfused with 0.9% saline followed by 10% buffered formalin. The brains were then removed, and transverse sections were made throughout the cerebellum (20 μ m using a cryostat) and stained with cresyl violet to verify the phenotype of all animals. Transverse sections were also made at the level of the hippocampus, the basal pontine gray, and the inferior olive.

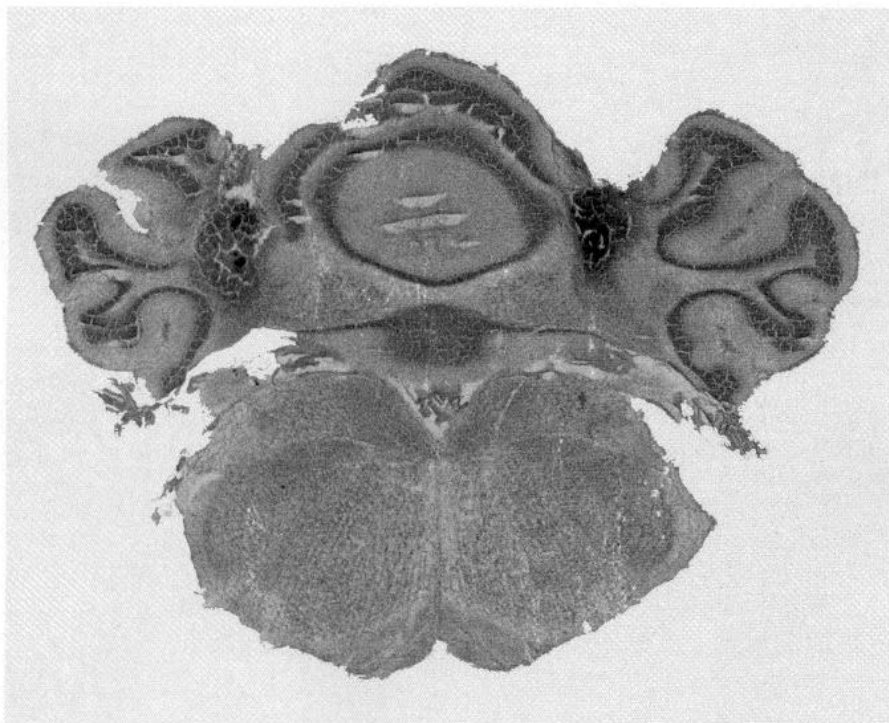


Figure 3. A transverse brain section stained with Prussian blue and cresyl violet from a typical animal with bilateral electrolytic cerebellar lesions.

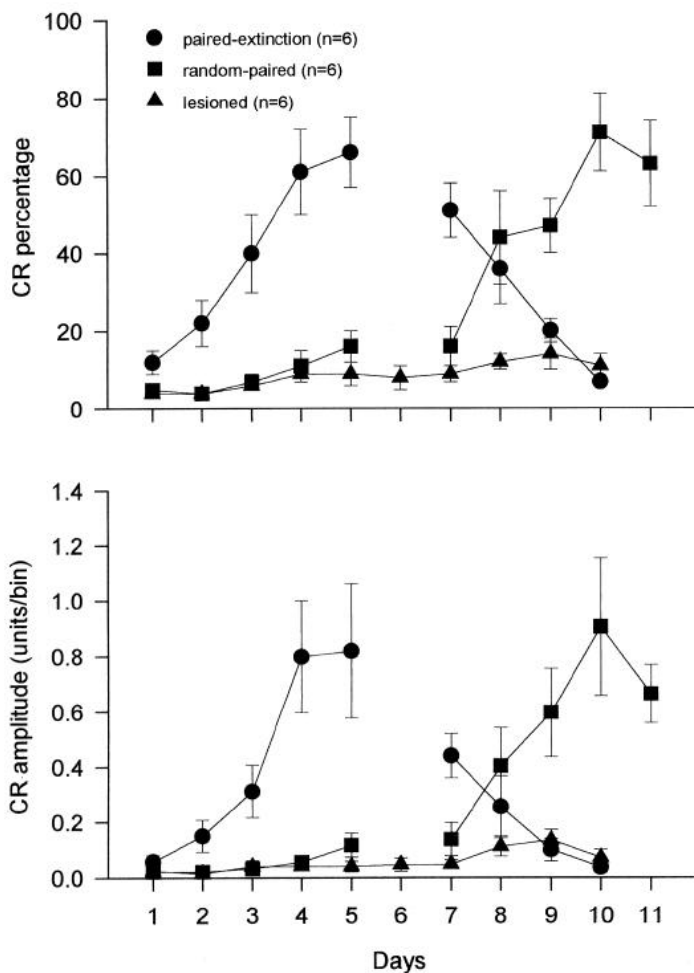


Figure 4. Percentage of CRs (top) and CR amplitudes (bottom) from paired (●), pseudo-random (■), and cerebellar-lesioned (▲) groups during acquisition and extinction training.

PHA-L tract tracing

The anterograde tracer PHA-L was used to show that there were no aberrant projections from the cerebellar cortex to the deep nuclei in the *pcd* mutants. Three *pcd* and two wild-type mice (age-matched to those used in eyeblink conditioning) were used. Under ketamine-xylazine anesthesia, a glass micropipette (tip diameter ranging between 10 and 15 μ m) was stereotaxically positioned in the molecular, Purkinje cell, and granular layers of the cerebellum (lobulus simplex). Injections of a 2.5% solution of PHA-L (Vector Labs) in 0.1 M sodium PBS, pH 7.4, were made during a 10 min period by applying a +5 μ A current from a constant current source (Stoelting, Wood Dale, IL), pulsed at 7 sec intervals. A survival time of 10–16 d was given for optimal transport of the tracer.

Fixation was performed by deeply anesthetizing the animal with sodium pentobarbital (65 mg/kg, i.p.) and perfusing with 200 ml of ice-cold 4.0% paraformaldehyde in 0.1 M sodium borate buffer, pH 9.5, after a brief saline rinse to remove the blood. The brains were then removed and postfixed overnight at 4°C in the same fixative containing 10% sucrose.

After the brain was frozen, serial 30 μ m thick sections were cut on a sliding microtome. All sections were saved in a one-in-four series. One series was then processed for immunohistochemistry with an anti-PHA-L serum raised in rabbits (Dako Labs, Carpinteria, CA), at a dilution of 1:1000. The primary antiserum was localized using a variation of the avidin-biotin-complex system described in detail by Gerfen and Sawchenko (1984). In brief, the sections were incubated for 1 hr at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Labs), and then placed in the mixed avidin-biotin-horseradish peroxidase (HRP) complex solution for the same period of time. The Vectastain Elite ABC kit (Vector, Burlingame, CA) was used, and the working solution of avidin-biotin-HRP complex was prepared by adding 90 μ l of

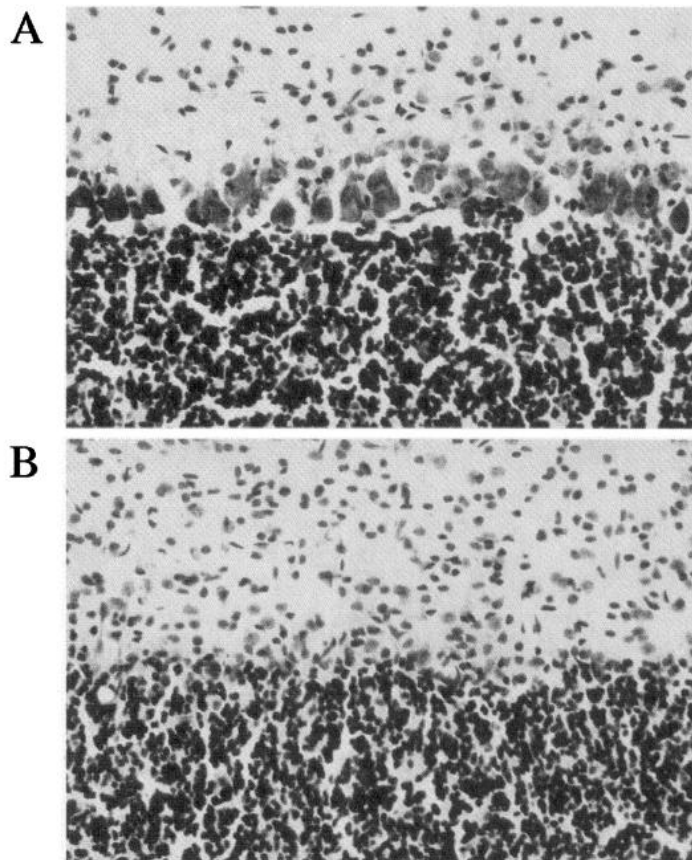


Figure 5. Transverse sections of the cerebellum stained with cresyl violet from (A) wild-type and (B) *pcd* mutant mice.

reagent A and 90 μ l of reagent B to 10 ml of 0.02 M potassium PBS, pH 7.4. The sections were recycled through the biotinylated antibody and the avidin-biotin-HRP complex and then processed for peroxidase histochemistry using a 0.05% solution of diaminobenzidine (Polyscience, Niles, IL). The sections were mounted on gelatin-coated slides, and the reaction product was intensified by placing the slides in a 0.005% aqueous solution of osmium tetroxide for 30 min, in a 0.05% aqueous solution of theoscarbohydrazide (EM Sciences, Ithaca, NY) for 15 min, and in the osmium solution for an additional 30 min. Slides were then dehydrated and coverslipped with DPX, and an adjacent series was stained with cresyl violet for reference purposes.

Results

Figure 5 shows a cerebellar section stained with cresyl violet from typical wild-type (A) and *pcd* (B) mice. As can be seen, the cerebellar section of the *pcd* mouse is completely devoid of Purkinje cells. We examined all regions of the cerebellum (anterior and posterior lobes) from *pcd* mice and found no Purkinje neurons. In contrast, a layer of Purkinje neurons is visible in the cerebellar section of the wild-type mouse. In addition to the Purkinje cells, there seems to be a slight disruption of the cerebellar granule cell layer in the *pcd* mutants. Other structures that have been implicated in eyeblink conditioning, such as the hippocampus, the interpositus nucleus, the inferior olive, and the basal pontine gray were also examined (Fig. 6). Neurons in the inferior olive seem to be smaller in size, whereas the interpositus, basal pontine gray, and hippocampus seem to be qualitatively normal in the *pcd* mutants.

Figure 7 represents coronal sections of the cerebellum from *pcd* (A) and wild-type (B) mice injected with PHA-L in the cerebellar cortex. The figures on the left depict adjacent sections stained with cresyl violet for reference purposes. As can be seen from the

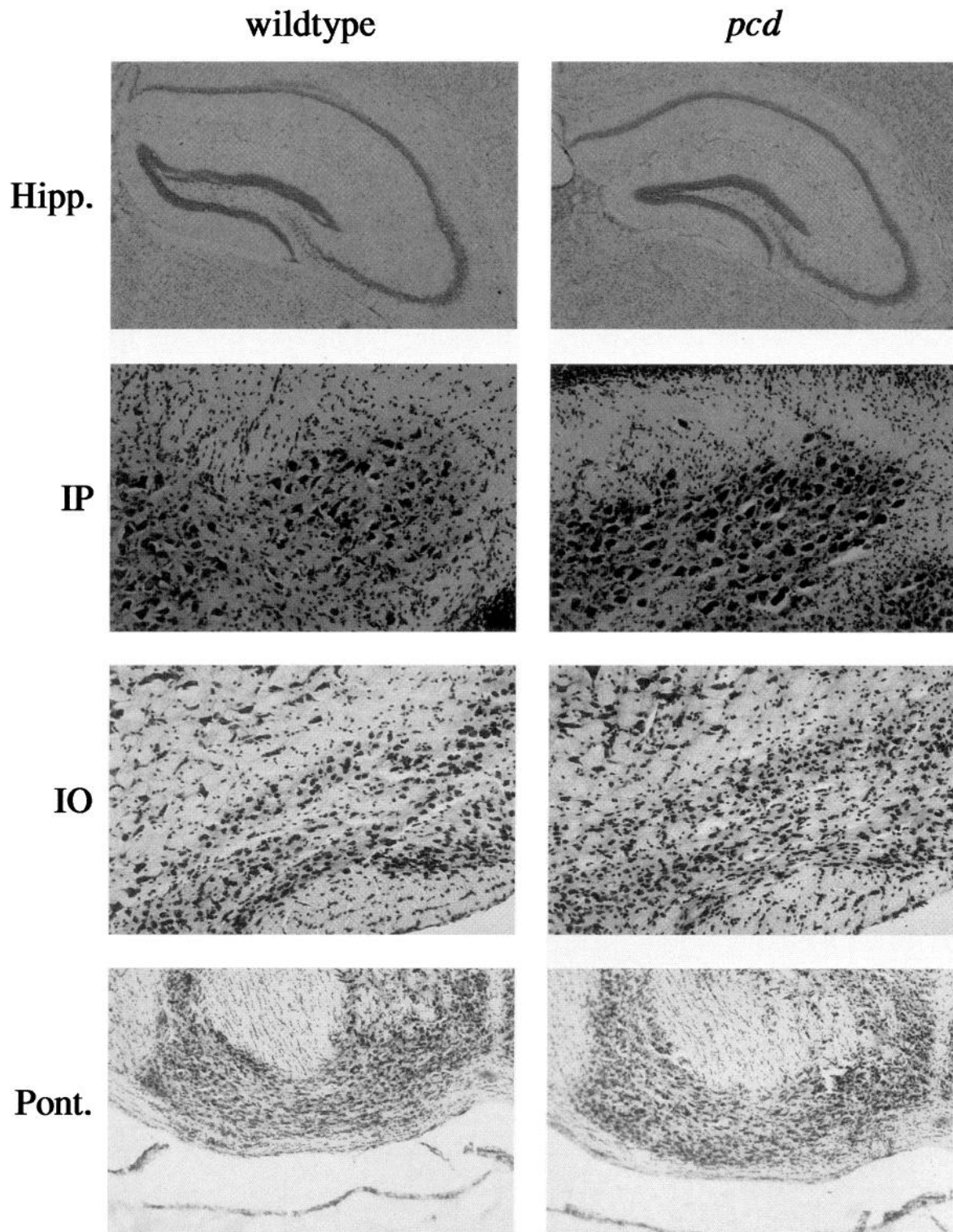


Figure 6. Transverse brain sections from wild-type and *pcd* mice. *Hipp.*, Hippocampus; *IP*, interpositus nucleus; *IO*, inferior olive; *Pont.*, basal pontine gray.

bright-field photomicrographs (figures on the right), the cerebellar section from the wild-type mouse shows intense PHA-L-immunoreactive labeling in the deep nuclei after an injection confined to the cerebellar cortex. In contrast, no PHA-L-immunoreactive fibers are present in the deep nuclei of the *pcd* mouse, even with a much larger injection in the cortex. Figure 8

shows higher magnification of the deep nuclei from *pcd* (A) and wild-type (B) animals. Once again, immunoreactive labeling is intense in the wild-type and completely absent in the *pcd* animal.

Overall, the *pcd* mice exhibited a significant impairment in the acquisition of conditioned eyelid EMG responses in comparison to the wild-type mice (Fig. 9) [one-way ANOVA on percentage of

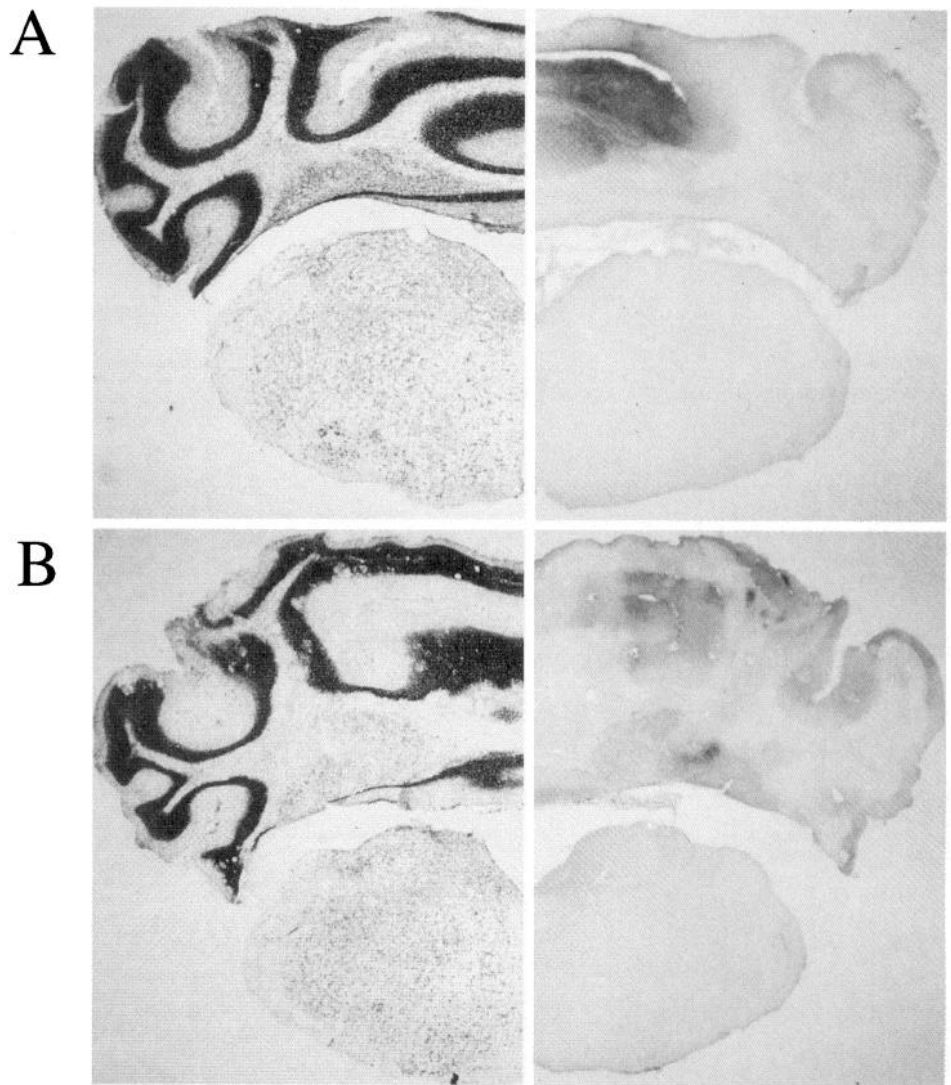


Figure 7. Transverse sections (magnified 20 \times) of the cerebellum from (A) *pcd* and (B) wild-type mice injected with PHA-L in the cerebellar cortex.

CRs with 10 d of acquisition as repeated measures: $F_{(1,20)} = 13.11$, $p < 0.01$; CR amplitudes: $F_{(1,20)} = 13.53$, $p < 0.01$]. Although both wild-type and *pcd* groups displayed gradual increases in the CR after some days, the wild-type animals acquired CRs at a significantly faster rate than the *pcd* animals did [group \times day interaction on percentage of CRs: $F_{(9,171)} = 2.49$, $p = 0.01$; CR amplitudes: $F_{(9,171)} = 2.17$, $p < 0.05$]. Thus, *pcd* mice are impaired profoundly but do show modest eyeblink conditioning. Moreover, the *pcd* mutants exhibited more shorter peak-latency CRs than the wild-type animals (Fig. 10) [group \times latency interaction: $F_{(3,63)} = 4.87$; $p < 0.01$]. During the CS period, *pcd* animals displayed significantly more CRs in the 121–180 msec range [$F_{(1,20)} = 18.12$, $p < 0.01$] and displayed significantly fewer CRs in the 181–240 msec range [$F_{(1,20)} = 6.04$, $p < 0.05$] than the wild types. Although there was a trend, the CR onset latency of *pcd* mice was not statistically shorter than the CR onset latency of wild types. The sensitivity to the shock US and the amplitudes of the URs after the shock US did not differ between *pcd* and wild-type mice (Fig. 11), and both groups exhibited extinction during the CS-alone trials (Fig. 9).

DISCUSSION

In the first study we demonstrated that associative eyeblink conditioning is feasible in mice when the CS and US are presented in

a paired manner, as did Aiba et al. (1994) using a similar procedure. The CRs were not observed in the group in which the CS and US were randomly presented. Furthermore, the acquired CRs were extinguished with repeated CS-alone presentations. As shown previously in rabbits, rats, and humans (Thompson, 1986; Skelton, 1988; Daum et al., 1993; Lavond et al., 1993), lesions of the cerebellum (specifically the interpositus nucleus) also completely abolish eyeblink learning in mice. Thus, the cerebellum is critically involved in eyeblink conditioning in mice.

The results from the second study with *pcd* mice that are devoid of Purkinje cells indicate that the cerebellar cortex plays an important role in eyeblink conditioning. The *pcd* mice, which completely lack cortical efferents to the deep nuclei (as shown by the PHA-L tract tracing), were strongly impaired in eyeblink learning; however, they still exhibited a low but significant level of eyeblink CRs during the course of training. A recent study indicated that the Purkinje cell number correlates highly with the rate of eyeblink learning in rabbits; i.e., animals with more Purkinje cells show stronger eyeblink conditioning than animals with fewer Purkinje cells (Woodruff-Pak et al., 1990). Interestingly, the CRs exhibited by the *pcd* animals tend to have shorter peak latencies than those CRs displayed by the wild types. The incomplete impairment in eyeblink conditioning and alterations in CR timing

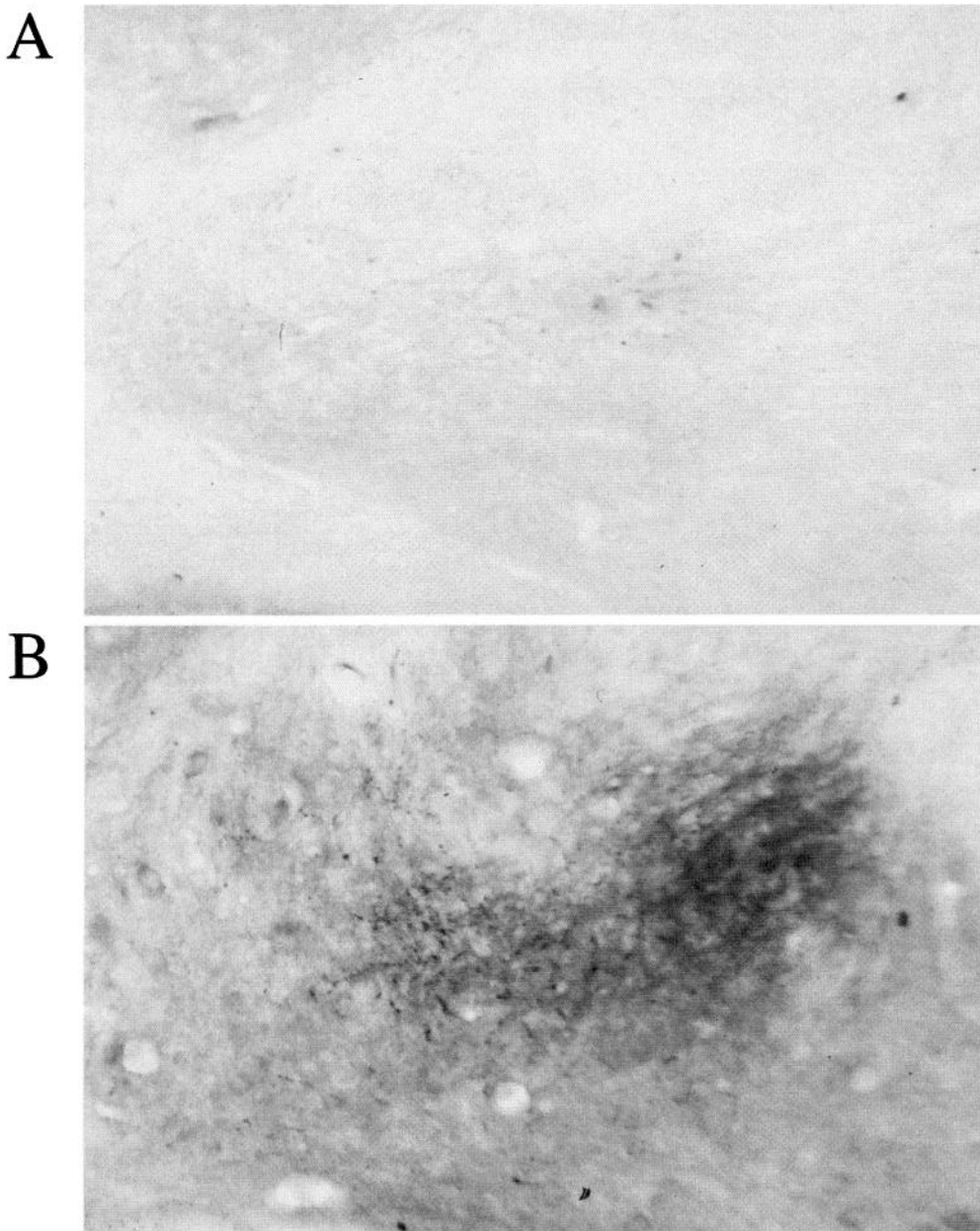


Figure 8. A higher magnification (200 \times) of the deep nuclei from (A) *pcd* and (B) wild-type mice injected with PHA-L in the cerebellar cortex.

have been reported previously in rabbits with confined and limited cerebellar cortical lesions (Lavond et al., 1987; Lavond and Steinmetz, 1989; Logan, 1991; Perrett et al., 1993). In contrast to the *pcd* mice, the interpositus-lesioned mice in the first study were completely unable to learn eyeblink conditioning. Thus, the present results are consistent with findings from rabbit lesions studies and suggest that the cerebellar cortex has important involvement in *normal* eyeblink conditioning.

When *pcd* mutants were presented with repeated CS alone trials, they exhibited extinction of acquired CRs. This finding suggests that the Purkinje cells and thus the entire cerebellar cortex (because the Purkinje cells are the sole efferent neurons of the cortex) are not necessary for the extinction of eyeblink CRs. In rabbits, however, it was reported recently that post-training lesions of the anterior lobe of cerebellar cortex prevent extinction of eyeblink CRs (Perrett and Mauk, 1995). It is possible that the differences in species and/or procedures (e.g., airpuff US used

with rabbits vs shock US used with mice) may account for this discrepancy.

The *pcd* mice are known to exhibit cerebellar symptoms such as impaired motor coordination and mild ataxia (Goldowitz and Eisenman, 1992); however, the deficit in eyeblink conditioning in *pcd* animals is probably not caused by deficits in sensory or motor factors. They did not differ from the wild types in terms of sensitivity to the shock (Fig. 8), UR performance to the US (Fig. 8), and startle response to a burst of white noise (personal observations). Furthermore, recent studies suggest that impairments in motor coordination can be dissociated from impairments in eyeblink conditioning (Chen et al., 1995; Shibuki et al., in press). The *pcd* mice are also impaired in hidden platform training, but not in visible platform training, in the Morris water maze task (Goodlett et al., 1992). Thus, the learning deficit in *pcd* mice is probably not attributable to motivational factors.

It is possible that the learning deficit observed in *pcd* mice may

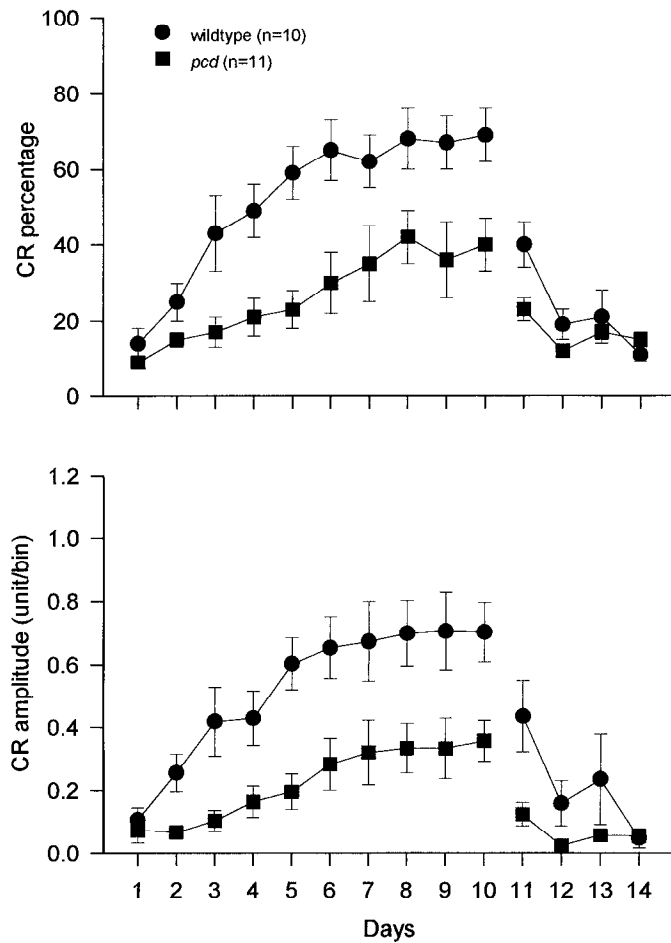


Figure 9. Percentage of CRs (top) and CR amplitudes (bottom) exhibited by *pcd* (■) and wild-type (●) mice during 10 d of acquisition and 4 d of extinction.

be caused in part by the degeneration of neuronal elements other than or in addition to the Purkinje cells. As mentioned earlier, the *pcd* mice are initially born with Purkinje cells. By the third and fourth postnatal weeks of development, however, *pcd* mutants lose their Purkinje cells (Mullen et al., 1976; Landis and Mullen, 1978; Goodlett et al., 1992). Goldowitz and Eisenman (1992) stated that “the loss of the postsynaptic partner well after the period of synaptogenesis, as occurs in the *pcd* and nervous mutants, leaves the presynaptic cell population relatively intact.” Others have reported that the *pcd* mutants show neurochemical changes in the deep cerebellar nuclei (Raffler-Tarlov et al., 1979), limited loss of inferior olivary neurons (Ghetti et al., 1987; Triarhou and Ghetti, 1991), partial degeneration of cerebellar granule cells (Ghetti et al., 1978), and retinal degeneration (Mullen and La Vail, 1975). These neural degenerations observed in *pcd* mice are considered to be secondary to Purkinje cell degeneration, and they tend to occur during a much slower time course (beginning at ~3–4 weeks of age). The *pcd* animals used in the present study were ~10 weeks old, and our histological analyses do show other neural degenerative effects in addition to the Purkinje neurons. There was a small decline in the number of granule cells in the cerebellar cortex, but because there were no Purkinje cells (thus no output from cortex), this seems unlikely to contribute to the impairment in eyeblink conditioning. We did not notice any reduction of cells in the pontine nuclei that send projections to the

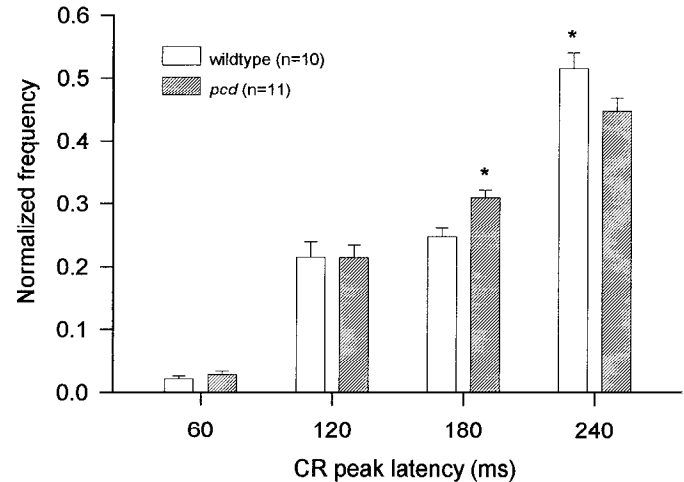


Figure 10. Normalized histogram of CR peak latencies from *pcd* and wild-type mice.

granule cells and to the interpositus nucleus. The hippocampus also seemed normal in the *pcd* mice. The olivary cells in the *pcd* animals, however, seemed to be smaller than those in the wild types. As noted earlier, the climbing fibers from the inferior olive relay US information to the cerebellar cortex and nuclei, and lesions to this structure prevent eyeblink conditioning in rabbits (McCormick et al., 1985; Yeo et al., 1986). According to Triarhou and Ghetti (1991), the number of inferior olive cells declines by 18% by postnatal day 23 and by 49% by postnatal day 300. Thus, deficits in eyeblink conditioning in *pcd* mice may be attributable, in part, to the degeneration of neurons in the inferior olive. The neurochemical changes in the interpositus nucleus (resulting from complete loss of GABAergic Purkinje cells) may also contribute to the impairment in eyeblink learning observed in *pcd* mutants. The possibility of retinal degeneration affecting eyeblink conditioning can be ruled out because this task does not require any visual cues. Recently, it has been shown that neonatal exposure to the antimetabolic agent methylazoxymethanol, which disrupts cerebellar circuitry (e.g., misalignment of Purkinje cells) without any overt dysmorphology in other brain regions (e.g., normal appearance of the inferior olive, pontine nuclei, red nuclei, and cerebellar deep nuclei), severely impaired eyeblink conditioning in rats (Freeman et al., 1995). Thus, eyeblink conditioning can be disrupted with a confined abnormality to the cerebellar cortex, as we have demonstrated with *pcd* mutant mice.

Similar impairments in eyeblink conditioning have also been reported in gene knockout mice that lack metabotropic glutamate receptors (mGluR1) (Aiba et al., 1994) and glial fibrillary acidic protein (GFAP) (Shibuki et al., in press). Both mGluR1 and GFAP mutants also show deficits in cerebellar LTD. LTD is a long-lasting decrease in parallel fiber-Purkinje cell synapses after conjoint stimulation of the parallel fibers and the climbing fibers (Ito et al., 1982; Ekerot and Kano, 1985; Linden, 1994) and has been proposed as a synaptic mechanism underlying certain aspects of cerebellar-dependent learning (Ito, 1984, 1989; Thompson, 1990; Chen and Thompson, 1995). In PKC γ mutant mice (deficient in the γ isoform of protein kinase C), cerebellar LTD is normal and eyeblink conditioning is unimpaired (Chen et al., 1995). Thus, the absence of LTD or Purkinje cells seems to produce similar deficits in eyeblink conditioning, suggesting that synaptic processes such as LTD that occur on Purkinje neurons may play a role in normal eyeblink learning.

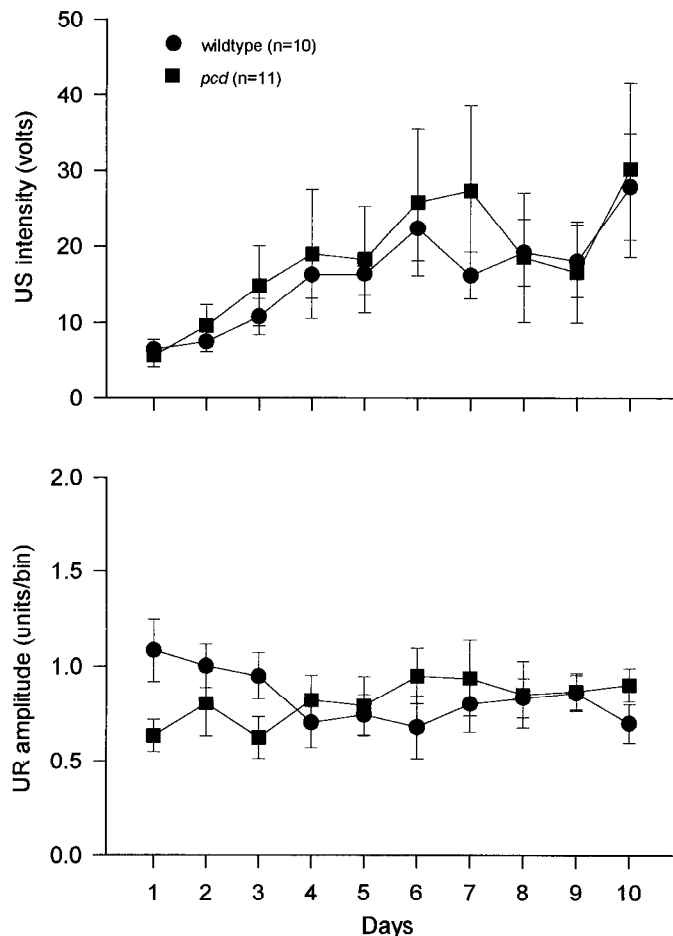


Figure 11. Sensitivity to the shock US (top) and UR (bottom) responses to the US exhibited by *pcd* (■) and wild-type (●) mice.

In addition to eyeblink conditioning (present study) and spatial abilities (Goodlett et al., 1992), the *pcd* mutants are impaired in other behavioral tasks, such as spontaneous alternation and exploration (Lalonde et al., 1987). Different cerebellar mutant mice such as *weavers* (with almost complete loss of granule cells and ~28% loss of Purkinje cells), *staggerers* (with prominent degenerations of Purkinje cells, granule cells, and inferior olive neurons), and *nervous* (with selective loss of Purkinje cells and deep cerebellar nuclei) also exhibit deficits in spontaneous alternation, exploration, and habituation (Lalonde et al., 1986, 1988a,b). Eyeblink conditioning, however, has not yet been tested in these mutants.

In summary, the present study supports the notion that the cerebellum is essential for classical eyeblink conditioning in mammals. In the absence of Purkinje cells, eyeblink learning still occurs, but it is substantially attenuated and the timing of the learned response is altered. In contrast, lesions of the interpositus nucleus completely prevent eyeblink conditioning. Thus, it seems that the cerebellar cortex is important for *normal* eyeblink conditioning. The cerebellar cortex is also important for other forms of motor learning, such as plasticity of the vestibulo-ocular reflex (VOR) (Ito, 1984, 1989), and our results indicating that the Purkinje cells are critical for eyeblink conditioning are consistent with a recently proposed computational VOR model (du Lac et al., 1995). The availability of various cerebellar mutant, gene knockout, and transgenic mice offers a new and exciting means to

explore the relationship between cerebellar circuitry, neuronal mechanisms, and behavior.

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