

Classical conditioning using stimulation of the inferior olive as the unconditioned stimulus

(learning/neural plasticity/acquisition/extinction/rabbit)

MICHAEL D. MAUK, JOSEPH E. STEINMETZ, AND RICHARD F. THOMPSON

Department of Psychology, Jordan Hall, Stanford University, Stanford, CA 94305

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ABSTRACT We show that conditioned eyelid responses develop when the unconditioned stimulus is electrical stimulation of the dorsal accessory nucleus of the inferior olive. When compared to conditioning using a standard unconditioned stimulus (air puff), the conditioning produced in this manner appears quite normal: the responses develop at a similar rate, are of comparable magnitude and topography, and demonstrate a steep interstimulus interval function; and response topography varies according to the interstimulus interval. These data indicate that activation of neurons in the dorsal accessory olive is a sufficient condition for a stimulus to be an effective unconditioned stimulus. Previous experiments indicate the dorsal accessory olive is necessary in that lesions have effects functionally equivalent to removal of the unconditioned stimulus. These data indicate that the dorsal accessory olive forms a portion of the pathway conveying information about the occurrence of an unconditioned stimulus to sites of synaptic plasticity responsible for conditioning.

A major goal in the neurobiological study of associative learning is to identify response pathways and to locate regions within them that undergo plasticity responsible for the development of conditioned responses (1–4). For classical conditioning, it is generally assumed that the development of this plasticity requires a convergence of input from the two stimuli involved—the conditioned stimulus (CS) and the unconditioned stimulus (US). Since, by definition, conditioned response pathways are activated by the CS, the regions within them most likely to undergo plasticity should be those receiving input from pathways activated by the US. Portions of the conditioned response pathway necessary for the expression of conditioned eyelid responses in the rabbit have been described (5–13). We reasoned that the localization of regions of plasticity within this conditioned response circuit would be facilitated by the identification of pathways conveying information about the occurrence of the US. We report the identification of a portion of such a US pathway for classical conditioning of eyelid responses in the rabbit.

It is now well established that the cerebellum and red nucleus form part of the conditioned response pathway in rabbit eyelid conditioning. Lesions in these structures prevent the acquisition of conditioned responses (5) and lesions in trained animals produce complete and immediate abolition of conditioned responses (6–8). Rubrobulbar fibers have been identified that project to the motor nuclei responsible for eyelid responses—facial and accessory abducens (9, 10)—and lesions of this tract abolish conditioned eyelid responses (11). Studies suggest that the mossy fiber input to the cerebellum is also involved in the conditioned response pathway. Destruction of the middle cerebellar peduncle—a large source of mossy fibers—produces immediate abolition

of conditioned responses (12). Also, conditioning has been observed when the CS consists of electrical stimulation of various nuclei that provide mossy fiber input to the cerebellum (13, 14). These data, in concert with known anatomical projections, allow the following characterization of the conditioned response pathway. Mossy fibers activated by CSs provide input to the cerebellum which in turn projects to the contralateral red nucleus via the superior cerebellar peduncle. Finally, the red nucleus sends crossed projections to the motor nuclei responsible for eyelid responses.

Lesion experiments focusing on the US pathway indicate that the dorsal accessory nucleus of the inferior olivary complex (DAO)—a source of climbing fiber input to the cerebellum—is a portion of the US pathway. This suggestion is based on the demonstration that the DAO is necessary for the development and maintenance of conditioned eyelid responses. Lesions in the DAO do not immediately abolish conditioned responses, rather they produce effects functionally equivalent to the omission of the US (15). In untrained animals the lesions prevent acquisition whereas in trained animals the effect is a decline in conditioned responses that parallels the extinction seen in intact subjects presented only the CS. In the present experiments we show that stimulation of the DAO can serve as an effective US. These data, in conjunction with lesion data, indicate that the DAO forms a portion of the US pathway and suggest that activation of this pathway is a sufficient and necessary condition for a stimulus to support the development and maintenance of conditioned skeletal muscle responses.

MATERIALS AND METHODS

Male, albino rabbits were anesthetized with halothane and prepared with the following: (i) epoxyite insulated, stainless steel microelectrodes (10–20 μm of tip exposed) implanted in the rostro-medial portion of the DAO, (ii) an electromyogram (EMG) recording electrode made of stainless steel wire (0.22 mm) implanted within the muscles responsible for upper eyelid closure (obicularis oculi), and (iii) a headstage designed to accommodate preamps and connectors attached to stimulation devices. Localization of the stimulating electrodes in the inferior olive was aided by delivering 0.1-msec anodal pulses through the electrode and monitoring the field potentials elicited in the cerebellar cortex. Electrodes were cemented in the region yielding the lowest threshold for eliciting a response in the cerebellum. An example is shown in Fig. 3. Following recovery from surgery, preliminary testing was conducted to assess the response of the animal to stimulation. Stimulation consisted of 100-msec trains of 0.1-msec constant-current (cathodal) pulses. Stimulation elicited a variety of responses; most typical were lateral or upward head movements, forepaw movements, and eyelid

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Abbreviations: DAO, dorsal accessory olive; CS, conditioned stimulus; US, unconditioned stimulus; ISI, interstimulus interval; EMG, electromyogram.

closure. Initial studies demonstrated a close correspondence between the unconditioned responses elicited by stimulation and the conditioned responses that developed with training. Because a primary intent of these experiments is to compare conditioning produced by DAO stimulation with that produced by the US commonly used in the conditioned eyelid preparation (an air puff directed at the cornea), the behavioral data presented here are from animals with reasonably discrete eyelid responses.

Stimulation frequency and intensity were adjusted so as to yield as discrete an eyelid response as possible. We found that higher frequencies (250–400 Hz), and thus lower currents, resulted in responses (both conditioned and unconditioned) that were more discrete than those elicited by lower frequencies. Highest stimulation parameters used were 400 $\mu\text{A}/400$ Hz and the lowest were 60 $\mu\text{A}/60$ Hz. The detailed behavioral analysis was limited to animals in which the stimulation elicited eyelid movement and did not appear to be aversive. Animals that met these criteria ($n = 8$) were then trained using standard classical conditioning procedures. Conditioning trials consisted of the presentation of a tone CS (1 kHz, 85 dB sound pressure level, 350-msec duration) followed 250 msec later by stimulation of the DAO (100-msec train duration). The intensity of the stimulation was set just above threshold for eliciting eyelid responses. Thus, in each animal the unconditioned response was a just-detectable eyelid twitch (see Fig. 2A). Daily training sessions involved 108 trials, 12 of which were tone-alone test trials. Control animals ($n = 9$) were trained in the same manner with the exception that the US was an air puff directed at the cornea (0.22 kg/cm², 100 msec). Acquisition of conditioned responses in both stimulation and air puff-trained animals was assessed over four consecutive daily sessions. Subsequently, most animals received three sessions of extinction training in which tone and DAO stimulation were presented but never paired. In four of these animals, interstimulus interval (ISI) functions were then determined by administering eight additional sessions; four using a 50-msec ISI and four using 150-msec ISI. Lastly, the effects of cerebellar interpositus nuclei lesions were assessed in four subjects following acquisition. These lesions were ipsilateral to the trained eye and were made by passing 2 mA of direct anodal current (90–120 sec) through electrodes chronically implanted in the interpositus nuclei. These animals were allowed 3 days to recover and then given four additional training sessions. An additional 15 animals were trained with DAO stimulation as the US (either eyelid closure or head turn unconditioned response) to provide further documentation of effective US stimulation sites in the DAO.

Following completion of training, animals were sacrificed with an overdose of sodium pentobarbital, and marking lesions were created at the tips of the electrodes by passing 100 μA of anodal direct current for 5–7 sec. The animals were then perfused through the heart with 10% (vol/vol) formalin (2–3 liters). The brains were then removed, and the placement of the electrodes was assessed using standard histological procedures.

RESULTS

Acquisition. Acquisition of conditioned eyelid responses in animals trained with DAO stimulation and air puff was compared over 4 days of training using four response measures: amplitude, area, and percentage of conditioned responses as well as trials required to reach a criterion of eight conditioned responses in nine consecutive trials. For all measures the rate of acquisition of both groups was virtually identical (Fig. 1). As can be seen in Fig. 2, stimulation elicited small unconditioned responses (Fig. 2A) while training resulted in the development of robust conditioned responses

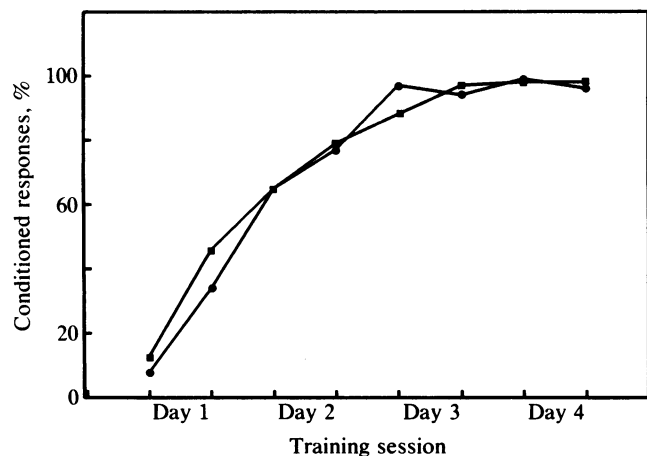


FIG. 1. A comparison of acquisition of conditioned eyelid responses expressed as percent responses for animals trained with stimulation of the DAO (■) or with an air puff (●) as the US. The four daily training sessions are represented as eight half sessions. The conditioned response criterion was an EMG response of at least 10 μV elicited by the CS.

(Fig. 2B). The latency, amplitude, and topography of the conditioned responses produced by DAO stimulation were also quite similar to those observed in air puff-trained animals

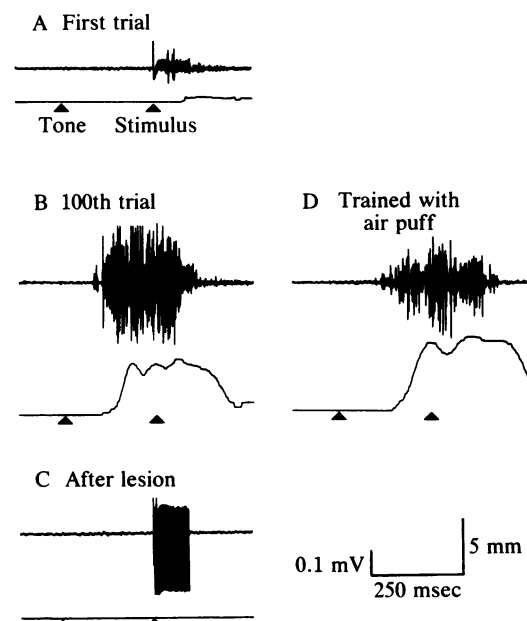


FIG. 2. Examples of eyelid responses from individual training trials. For each example the top trace is an eyelid EMG recording, and the bottom trace represents movement of the upper eyelid recorded with a potentiometer. Responses in A–C are from the same animal in which the US was DAO stimulation. The response in D is from a separate animal trained using air puff. In each trace the left arrow indicates tone onset, and the right arrow indicates onset of the particular US. The response observed in the first training trial is shown in A, the 100th trial of the same day is shown in B, and C depicts the absence of a response several days later following an electrolytic lesion in the cerebellum. The small, unconditioned response elicited by the DAO stimulation is visible in A. The much larger conditioned response that developed with training is apparent in B. Abolition of both the conditioned response (compare B and C) as well as the unconditioned response (compare A and C) by cerebellar lesion is quite apparent. For unknown reasons, the stimulation artifact increased with time in this animal. The trace in C represents only stimulation artifact. Comparing B and D illustrates the similarity of the conditioned responses that develop using the two USs: DAO stimulation (B) and air puff (D).

(compare Fig. 2 *B* and *D*). As with conditioning using air puff, lesions of the cerebellar nuclei (anterior interpositus) abolished the conditioned responses (Fig. 2*C*). The lesions also abolished the small unconditioned responses elicited by DAO stimulation—compare Fig. 2 *A* and *C*. Thus, the conditioned-response pathway involved using DAO stimulation appears to be the same as that involved with standard unconditioned stimuli.

Several statements can be made about the placement of the electrodes in relation to acquisition (Fig. 3). (i) For every animal in which conditioning was observed, the electrode tip was found to be positioned within, or just rostral to, the dorsal accessory nucleus of the inferior olive. (ii) In animals in which training was attempted and was unsuccessful, the electrodes tips were positioned dorsal and/or lateral to the DAO in surrounding reticular regions. (iii) The optimal electrode placement for discrete eyelid responses was in the rostro-medial portion of the DAO. In the animals that developed especially discrete eyelid responses using low-stimulation currents, the electrodes were positioned in this region. (iv) In general, electrodes in animals that elicited discrete eyelid responses but with higher stimulation intensities were positioned just rostral and/or medial to the ideal eyelid region. (v) When the electrodes were positioned lateral and caudal to the ideal eyelid region, more towards the center

of the DAO, lower-stimulation intensities were required to produce conditioning but the responses involved head movements rather than eyelid closure. The optimal location for a head-turn response was approximately 1.5 mm lateral to the optimal eyelid response locus.

ISI. ISI analysis of conditioning using DAO stimulation as the US highlights the similarity of conditioning based on DAO stimulation and conditioning using conventional external USs (Fig. 4). With air puff or periorbital shock as the US, ISIs less than 100 msec (including simultaneous and backwards pairing) do *not* result in conditioning. The optimal ISI range is 150–500 msec and conditioning gradually deteriorates with longer ISIs such that by 2000 msec little or no conditioning is observed (16). Furthermore, the topography of the response varies with the ISI such that the response peak occurs near US onset (17). Although only three ISIs were tested here, the results are consistent with those found using standard USs. In all four rabbits tested, no conditioning was observed using 50-msec ISI, whereas robust-conditioned responses developed at both 150 and 250 msec. It can also be seen that the latency of the conditioned responses varied systematically within ISI. These data provide additional support for the associative nature of conditioning using DAO stimulation as the US and illustrate the similarity of conditioning based on DAO stimulation and conventional USs.

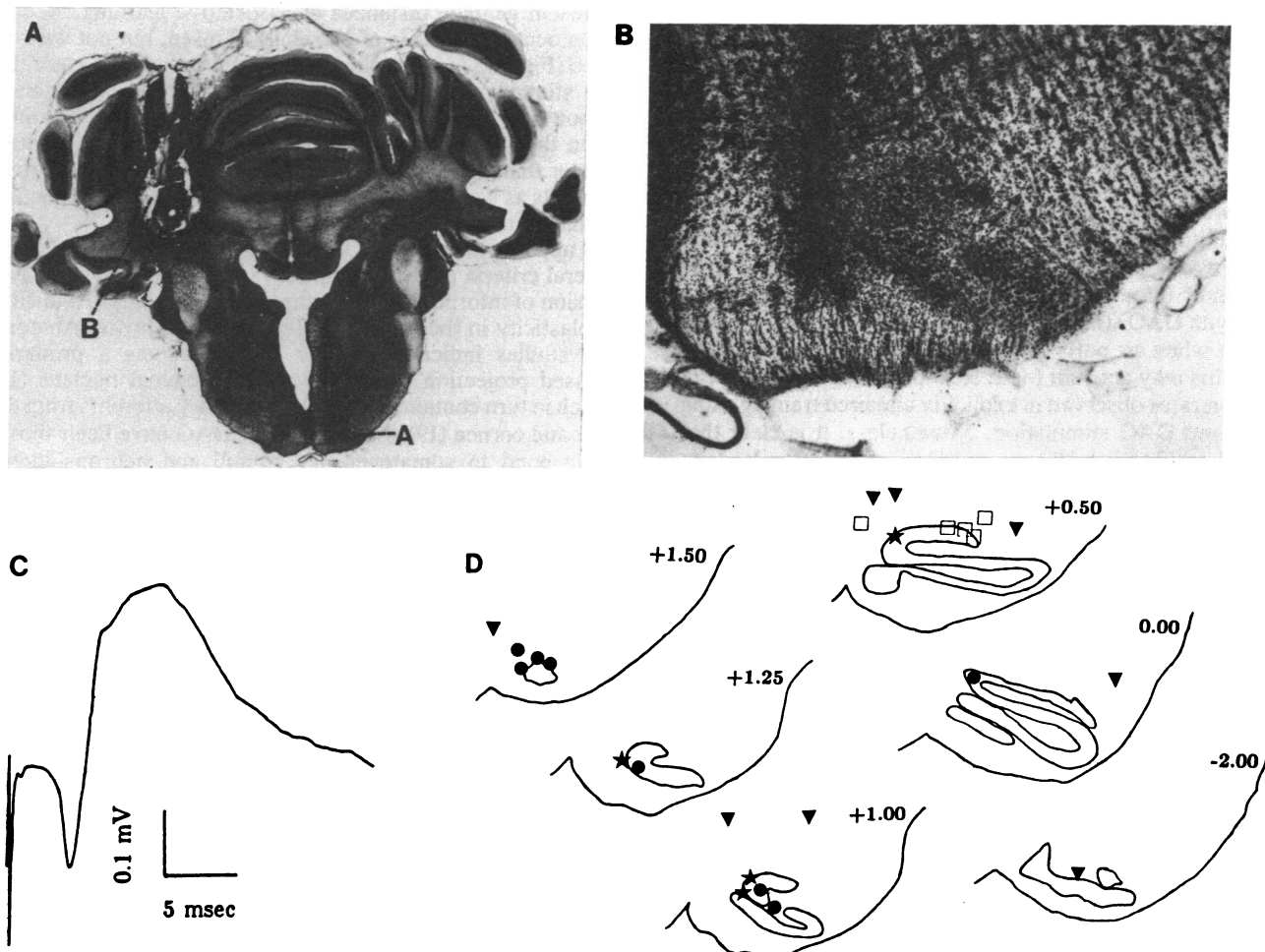


FIG. 3. The photomicrographs are taken from the animal whose conditioned responses are shown in Fig. 2 *A–C*. (A) Placement of the stimulating electrodes (A) and the electrolytic lesion in the cerebellar nuclei that resulted in abolition of the conditioned responses (B). (B) Placement of the stimulating electrode at higher magnification. The dark, C-shaped region is the dorsal accessory nucleus and the principle nucleus of the inferior olive. Training in this animal resulted in particularly discrete conditioned eyelid responses. (C) Field potential recorded in all animals. Placements resulting in conditioned eyelid responses are indicated by circles; placements resulting in particularly discrete eyelid responses are indicated by stars. Triangles denote placements in which no conditioned responses developed, and open squares indicate placements that resulted in conditioned head movements (behavioral data not reported).

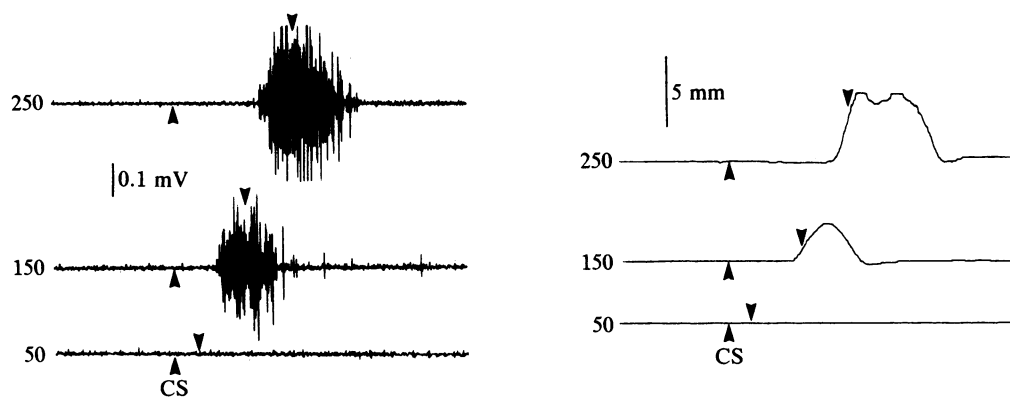


FIG. 4. Shown here are eyelid responses from one of the animals in which the effects of ISI was studied. Each EMG response in the left column is taken from the last day of training using the indicated ISI. (Right) Same eyelid responses as measured with a potentiometer attached to the upper eyelid (upward deflection indicates eyelid closure). In each trace the upward pointing arrow indicates CS onset, and the downward arrow indicates where the US normally occurs (these are CS-alone trials). Note the robust conditioned responses observed using ISIs of 150 and 250 msec and the absence of responses when the ISI is 50 msec. Also note that the latency of the response varies according to the ISI such that the response peak occurs near US onset.

Extinction. Rapid extinction of conditioned responses was observed during explicitly unpaired presentation of tone and DAO stimulation (Fig. 5). The rate of extinction was considerably more rapid than that of subjects that had been trained with the air puff US. However, the rate of extinction was similar to that in animals trained with air puff but for whom extinction training involved presentation of only CS-alone trials (Fig. 5). It may be that presentation of the (aversive) air puff during extinction training prolongs extinction via processes such as short-term sensitization. Consistent with this notion, we have observed less-rapid response decrement in animals initially trained with DAO stimulation but in which extinction was produced by unpaired presentation of tone and air puff (data not shown). Thus, whether animals are trained with DAO stimulation or air puff, response decrement is slower when air puffs are presented during the extinction phase. This may account for at least part of the difference in extinction rates observed in explicitly unpaired training using air puff and DAO stimulation. Nonetheless, it is clear that unpaired presentation of tone and DAO stimulation leads to extinction of conditioned responses.

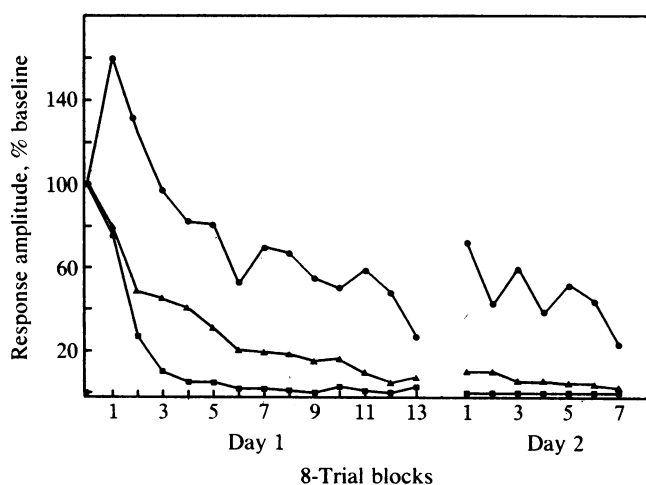


FIG. 5. Comparison of extinction of conditioned responses seen in animals trained with DAO stimulation and animals trained with air puff. Extinction for the DAO animals (■) and one group of air puff animals (●) consisted of unpaired presentation of the CS and US. For the second air puff group, extinction was produced by presentation of only the CS (▲).

DISCUSSION

The conditioned responses produced by DAO stimulation represent genuine instances of associative learning. Acquisition occurs with ISIs of 150 and 250 msec, but not with 50 msec (Fig. 4). Also, explicitly unpaired presentations of the two stimuli results in rapid extinction of the conditioned responses (Fig. 5). Thus, conditioned responses develop only when the CS precedes DAO stimulation by short intervals greater than approximately 100 msec. Further, the effective intervals as well as the rates of acquisition and extinction are similar to those observed using standard USs.

It appears that the DAO is part of a pathway that satisfies several criteria expected of pathways involved in the transmission of information about the occurrence of a US to sites of plasticity in the conditioned-response pathway. Anatomical studies indicate that the DAO receives a primarily crossed projection from the spinal trigeminal nucleus (18) which in turn contains terminals of primary afferents from the face and cornea (19). Neurons in the DAO have been shown to respond to somatosensory stimuli and neurons in the rostro-medial portion respond specifically to stimuli applied to the face (20). It is also clearly established that the inferior olivary nuclei are the source of the cerebellar climbing fiber input (21). Thus, projections have been identified that form a pathway between the primary sensory neurons activated by the US and a portion of the conditioned response pathway (the cerebellum). Consistent with these anatomical findings, previous lesion studies have demonstrated that the DAO is necessary, and the present experiments indicate that activation of the DAO is sufficient for the development and maintenance of conditioned responses.

That the DAO-climbing fiber system is both necessary and sufficient as a US pathway suggests that this pathway may be exclusively involved in conveying US information to sites of plasticity in the conditioned response circuit. Other pathways are, of course, engaged when a US such as a corneal air puff is presented to an animal. However, the influence of these other pathways on the development and maintenance of conditioned responses appears at most to be modulatory. We suggest that our data are consistent with the hypothesis that the DAO pathway is the only US pathway required for the development of conditioned responses in this paradigm and that activation of DAO neurons is a necessary and sufficient condition for a stimulus to support the development of conditioned skeletal muscle responses.

Central to the present experiments is the demonstration that activation of climbing fibers is, indeed, the critical feature that makes DAO stimulation an effective US. Possible alternatives include activation of afferents to the DAO and activation of fibers of passage. However, several lines of evidence suggest that climbing fiber activity alone makes the stimulation effective. First, the stimulation elicits climbing fiber field potentials in the cerebellar cortex (Fig. 3C). Second, the consistent and systematic relationship between electrode placement and the resultant conditioned responses implicates activation of DAO neurons rather than fibers of passage. Finally, if the stimulation activates DAO afferents such as the spinal trigeminal nucleus, then it should be aversive, and it should elicit reflex responses that are unaffected by cerebellar lesions. As we noted above, the stimulation is not aversive to the animals, and the small reflex responses elicited by stimulation are abolished by cerebellar lesions (Fig. 2).

In an early study, Brodgen and Gantt (22) reported that electrical stimulation of the cerebellar cortex could serve as an effective US for conditioning of discrete, skeletal muscle responses in dogs. It seems possible that the critical element in their experiments was activation of climbing fibers. Indeed, theoretical treatments of cerebellar involvement in motor learning have hypothesized that the climbing fiber activity may induce plasticity within the cerebellum when it occurs in conjunction with mossy fiber input (28–31). Electrophysiological evidence in support of these theories has been presented (23, 24). Also, climbing fibers appear to play a role in adaptation of the vestibulo-ocular reflex (25, 26) although a different role has been postulated in recovery from motor abnormalities induced by labyrinthine lesions (27). Our observation that climbing fibers participate in a US pathway is consistent with the spirit of these theories and is, to our knowledge, the first empirical support derived from analysis of associative learning.

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