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Accessory Abducens Nucleus and Conditioned Eye Retraction/ Nictitating Membrane Extension in Rabbit[1]

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Abstract

The role of accessory abducens nucleus neurons in the conditioned eye retraction/nictitating membrane extension response was defined in the rabbit. Horseradish peroxidase injections into the retractor bulbi muscle showed that accessory abducens nucleus is the principal location of its motorneurons. Single and multiple unit recording in accessory abducens indicated that these motor neurons show a marked responsiveness to corneal and periorbital stimulation and fire in close correlation with conditioned, unconditioned, or spontaneous eye retraction/nictitating membrane extension. Complete lesions of accessory abducens showed, at most, a partial reduction of the conditioned and unconditioned eye retraction response. Section of the extraocular muscles, other than retractor bulbi, also caused a partial reduction of the eye retraction response. Accessory abducens lesions, combined with extraocular muscle section, were necessary to dramatically reduce the eye retraction response permanently. These experiments demonstrated that accessory abducens is a primary controller of eye retraction through its axons to retractor bulbi. The other extraocular muscles act in concert with retractor bulbi to elicit conditioned and unconditioned eye retractions.

Analysis of the neural substrates of associative learning in mammalian brain is a complex problem. The use of a relatively simple behavioral paradigm in which the stimuli to be associated are easily controlled, in which at least one behavioral output is readily measured, and in which physiological recordings may be made in the animal during and after learning will facilitate progress in understanding the learning process. The rabbit nictitating membrane (NM) preparation, which was originally described by Gormezano et al. (1962), has many characteristics making it desirable for use in physiological and anatomical studies of learning (Thompson, 1976; Disterhoft et al., 1977). It and closely related eyeblink conditioning (Woody and Brozek, 1969) have been adapted by several groups as "model systems" in which to study the systems neurophysiology of learning.

A typical approach is to pair an auditory conditioned stimulus (CS) with a corneal air puff or periorbital shock unconditioned stimulus (US). In most animals, nictitating membrane conditioned responses (CRs) begin occurring reliably in the first training session (Disterhoft et al., 1977). Single neurons may be studied during the course of initial training (Kraus and Disterhoft, 1982; Berger et al., 1983).

The final output motor neurons must be known, in order to characterize the conditioned reflex arc. Cegavske et al. (1976) showed that stimulation of the abducens nerve caused NM extension in the rabbit. They also showed that the NM (or third eyelid) was physically forced out across the cornea in a passive fashion when the eyeball was retracted. Their multiple-unit recordings from the abducens nucleus correlated extremely well with NM extension, further suggesting that the output motor neurons controlling eye retraction were in the abducens nucleus.

We began the studies reported here to test the hypothesis that there might be separate groups of abducens motor neurons sending their axons to the lateral rectus (which we supposed was involved just in lateral rotation of the eye) and to retractor bulbi (involved in eye retraction). The series of experiments which followed and which we report here showed that accessory abducens (Acc ABD), rather than abducens, plays a prominent role in conditioned and unconditioned eye retraction as the major source of axons to the retractor bulbi muscle. But the other extraocular muscles, acting in coordination, also contribute significantly to eye retraction and may mediate relatively normal responses when Acc ABD has been lesioned. Preliminary reports of portions of these studies have been made (Disterhoft and Shipley, 1980; Quinn et al., 1982; Disterhoft and Weiss, 1984).

Materials and Methods

Subjects and surgical procedures. Subjects were all young adult male rabbits 6 to 8 weeks old and weighing 1 to 1.5 kg. New Zealand White albino rabbits were routinely used. Some Dutch rabbits were used in the anatomical tracing experiments. Thorazine (8 mg/kg, i.m.) was administered 30 min before surgery. Sodium pentobarbital (30 mg/kg) was given through the marginal ear vein at the beginning of surgery and supplemented if necessary.

All subjects were implanted with restraining head bolts (6-32 × ¾ inch nylon machine screws) embedded in dental acrylic and attached to the skull with stainless steel screws. The head bolts were attached with the head held in the standard rabbit stereotaxic plane (Fifkova and Marsala, 1967), lambda 1.5 mm inferior to bregma. For Acc ABD recording and lesion experiments, the skull was cleared around lambda and a trephine hole was made anterior and lateral to it. A plastic tube was placed over the skull defect which would be filled with sterile saline and capped between experiments. Two sutures were placed in each eyelid of the left eye to hold them open during behavioral training so that NM extension or eye retraction responses could be measured. If eye retraction was to be measured (Quinn et al., 1984), the cartilaginous NM was infiltrated with Xylocaine, grasped with a tissue forceps, and cut with sharp scissors near the medial canthus.

Acc ABD was located for physiological recording and lesion placement by antidromic activation of the abducens nerve with a chronic bipolar stimulating electrode constructed from used tungsten recording electrodes. The electrode was implanted surgically so that it rested directly above the abducens nerve as the nerve ran toward the orbit after exiting the brainstem. The placement was about +9 mm from lambda, 2 mm from the midline and

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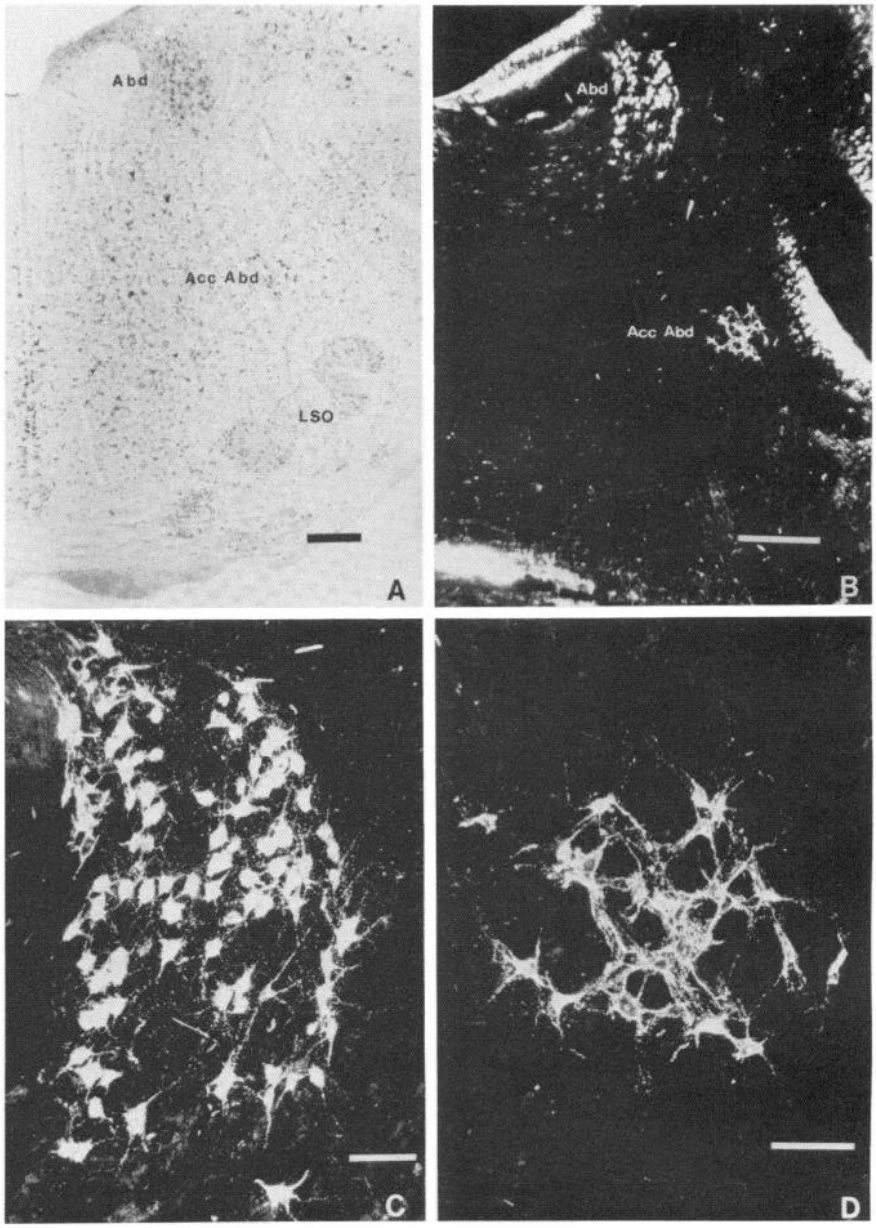


Figure 1. Location of accessory abducens nucleus in rabbit brainstem. *A*, Coronal section through rabbit brainstem. Abducens nucleus (*Abd*), accessory abducens nucleus (*Acc ABD*), and lateral superior olive (*LSO*) are indicated. Fascicles of nerve VI may be seen exiting toward the base of the brainstem. *Bar* = 0.5 mm. *B*, Polarized light photomicrograph at the same level as the section in *A*. HRP was injected into the retractor bulbi muscles. Cells in the Acc ABD nucleus are heavily labeled. *Bar* = 0.5 mm. *C*, Higher power view of abducens shown in *B*. *Bar* = 0.1 mm. *D*, Higher power view of Acc ABD shown in *B*. *Bar* = 0.1 mm.

19 to 20 mm from the skull surface with the skull in the stereotaxic plane. The final vertical placement of the electrode was determined by monitoring NM extension to short bursts of electrical stimulation (0.1 msec, 200 Hz). Lowest thresholds were in the range of 50 to 500 μA and remained constant for a 1- to 2-week period after implantation.

When Acc ABD single-neuron recording was to be done, the atlas, axis, and C3 and C4 vertebrae were fused to the occipital bone to increase stability (Fuchs and Luschei, 1970). A midline incision was made, stainless steel screws were placed in the cervical laminae or spinous processes and in the occipital bone, all of the screws were tied together with stainless steel suture wire, and the whole assembly was embedded in dental acrylic while the rabbit's neck was in a comfortable position. The muscle and skin were sutured together after liberal use of an antibiotic ointment. The rabbits recovered from and tolerated this procedure well. We found that we consistently lost Acc ABD single neurons after air puff presentation without this procedure.

All rabbits were injected with 150,000 units of Bicillin, intramuscularly, after surgery. All wounds were covered with an antibiotic ointment. Animals with cervical fusion received Bicillin daily after surgery.

Anatomical tracing experiments. Fifteen male Dutch and 6 male albino rabbits were used. The retractor bulbi is composed of four slips which insert into the sclera on the back of the eyeball posterior and internal to the insertions of the recti muscles. The retractor bulbi was exposed by incising the sclera, collapsing the eyeball, and cutting the insertions of the other extraocular muscles. A 50% solution of horseradish peroxidase (HRP) (Sigma type VI) in 2% dimethylsulfoxide was injected into the retractor bulbi under visual control through an operating microscope. Enough HRP was injected to turn the muscle bellies brown (10 to 20 μl, total). Procedures for perfusions and tetramethylbenzidine (TMB) reactions were those described by Mesulam (1978).

As will be described under "Results," we found retrograde transport of HRP to both abducens and Acc ABD after retractor bulbi injections. To test for the possibility that abducens motor neurons may have been labeled by leakage of HRP from retractor bulbi to the lateral rectus muscle, we cauterized the branch of the abducens nerve to the lateral rectus (as well as much of the muscle itself) before doing the retractor bulbi injections in three rabbits.

As a final anatomical characterization of Acc ABD cells as motor neurons, we tested them for acetylcholinesterase activity. Frozen sections of rabbit brainstem were reacted for the presence of acetylcholinesterase (Van Ooteghem and Shipley, 1984).

Behavioral preparation. Behavioral procedures were similar to those we have used in previous studies of rabbit NM conditioning (Disterhoft et al., 1977; Kraus and Disterhoft, 1982). Two days after surgical preparation, the animals were habituated to restraint in a stereotaxic device for 1- to 2-hr periods for two successive days. Their heads were bolted to the frame and their bodies were enclosed in a cloth bag. The experiments were controlled,

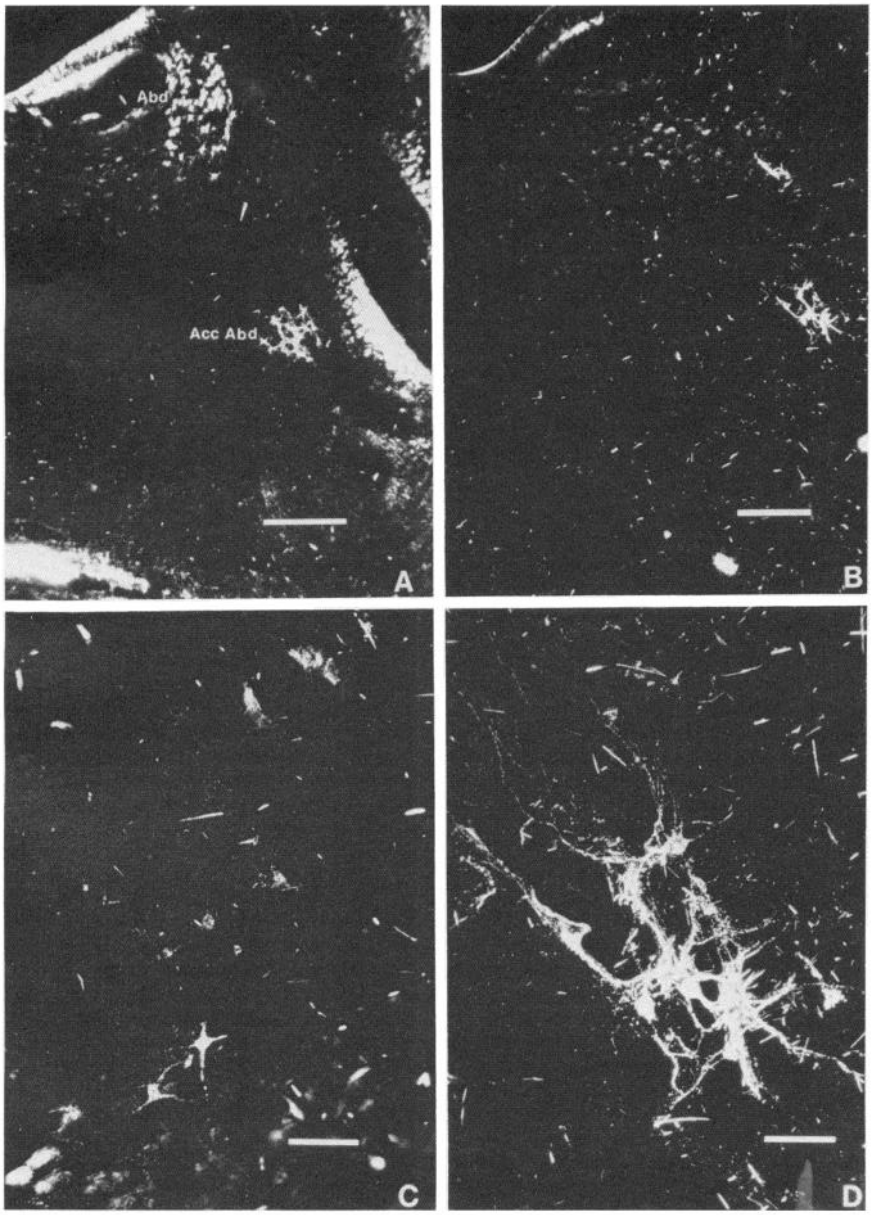


Figure 2. Anatomical control experiment demonstrating that accessory abducens almost exclusively projects to retractor bulbi. *A*, Normal: Polarized light view of coronal section similar to Figure 1*A* showing HRP labeling after retractor bulbi injection in a noncauterized rabbit. Both abducens (*Abd*) and accessory abducens (*Acc ABD*) are heavily labeled. *Bar* = 0.5mm. *B*, Experimental: Polarized light view showing retrograde HRP transport from retractor bulbi after the abducens fibers going to lateral rectus had been cauterized. Labeling in abducens is very weak, with only one or two cells showing the profile-filling commonly seen in these preparations (see *A*). The labeling in the Acc ABD nucleus is of normal intensity. *Bar* = 0.5 mm. *C*, Polarized light view of labeling in abducens shown in Figure 3*B*. This section contained the greatest number of labeled cells of any section through abducens. *Bar* = 0.1 mm. *D*, Polarized view of Acc ABD after cauterization. Labeling is normal for Acc ABD (cf. Fig. 1*D*). *Bar* = 0.1 mm.

and unit and behavioral data were taken on-line by a microprocessor system. Behavioral data were also recorded trial by trial manually as the experiments progressed. Conditioned stimuli were 400-msec, 85-dB white noise stimuli delivered through a closed headphone (Kraus and Disterhoft, 1981). Unconditioned stimuli were corneal or periorbital air puffs presented for 150 msec. Their intensity was 5 psi when measured at the solenoid delivery value. Tone CS onset preceded puff US onset by 250 msec and both terminated simultaneously. Intertrial intervals varied from 30 to 60 sec and averaged 45 sec. In some of the Acc ABD lesion experiments, only a US was presented. In these cases, 200-msec-long air puffs or shock trains of 0.1-msec, 200-Hz constant current pulses were presented. Shocks were delivered through wound clips placed anteroventral and posteroventral to the canthi of the eye.

NM extension was measured by an infrared light reflection transducer which gave a sensitive record of the NM sweep without being attached directly to the membrane (Disterhoft et al., 1977). As the experiments progressed, we developed a technique for measuring eye retraction directly to allow better quantification of lesion effects (Quinn et al., 1984). This device consisted of a contact lens to which a film strip with a linear grating was attached. The film strip was placed between a photocell and a light-emitting diode and gave a voltage output linearly related to contact lens movement when the eyeball was retracted.

Single-neuron recording. Single neurons were recorded in five rabbits. Epoxylite-coated tungsten microelectrodes with impedances of 1 to 2 megohms measured at 1000 Hz were used for extracellular single-neuron recording. A Bak AC-coupled preamplifier and impedance tester was used with an amplitude-time window discriminator for unit identification. Spikes which were being counted were displayed on a storage oscilloscope at a fast sweep time to ensure that single units were studied. The digital outputs of the discriminator were used as computer inputs.

Electrodes were introduced into the brain by a micromanipulator attached to a Narashige stereotaxic frame. All of our recording placements used lambda as the reference point. Each electrode was "zeroed" in the, *X*, *Y*, and *Z* planes with an optical device. This procedure allowed us to maintain the electrode tip in a known position within the stereotaxic planes when electrodes had to be changed.

The Acc ABD nucleus was located with stereotaxic coordinates aided by antidromic field potentials from the VIth nerve stimulating electrode. Single, 0.1-msec constant current pulses just above the level required to elicit NM extension (when presented in a short 200-Hz train) were used. As will be mentioned under "Results," all Acc ABD neurons responded vigorously to periorbital trigeminal stimulation. This marked responsiveness was used in addition to the field potential to indicate that Acc ABD neurons were being recorded. All electrode tracks were histologically reconstructed to confirm their position in or just adjacent to the Acc ABD nucleus.

Lesions. Animals to be lesioned were first trained to asymptote ($N = 11$) or were well habituated to restraint in the stereotaxic frame ($N = 6$). Behavioral

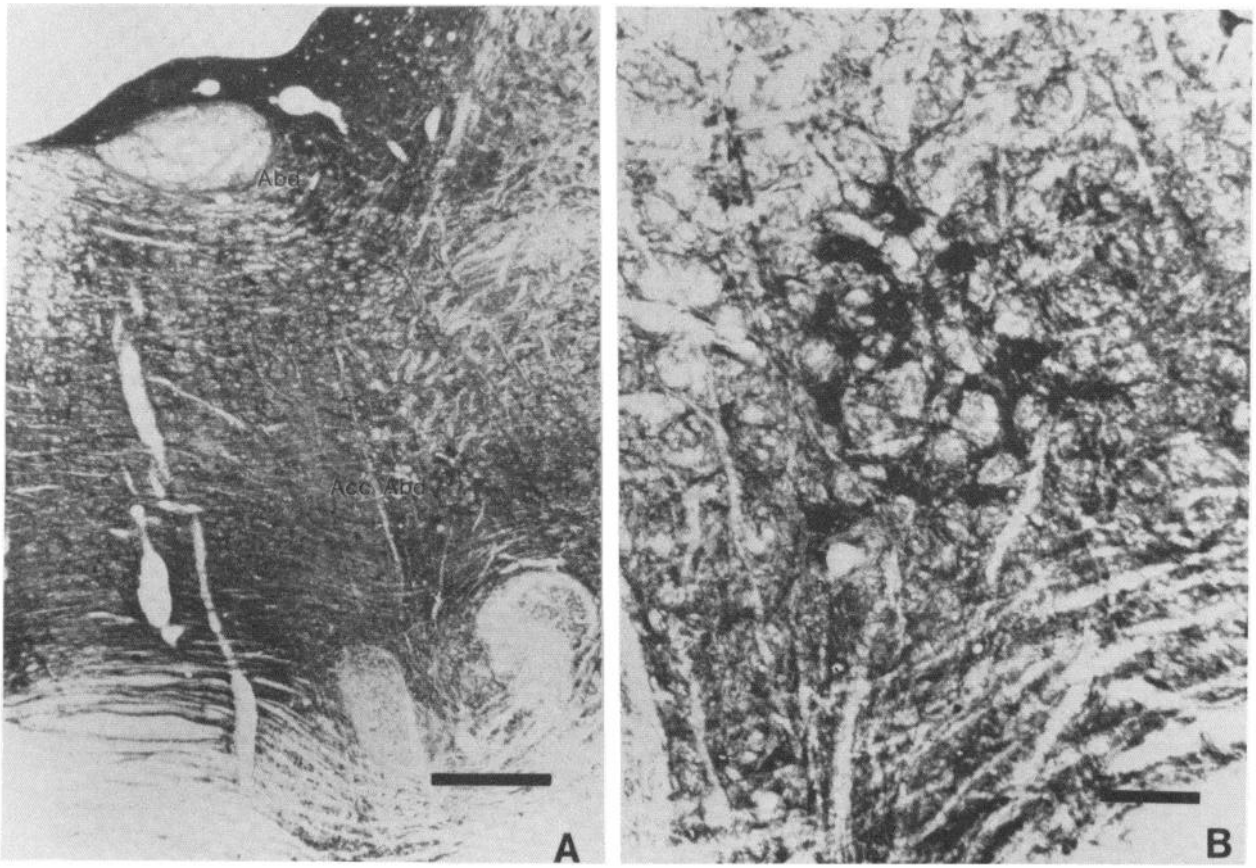


Figure 3. Neurons in both abducens (*Abd*) and Acc ABD are acetylcholinesterase positive. *A*, Coronal section through the brainstem at the level of abducens and Acc ABD. Lateral superior olive is clearly visible. (See Fig. 1*A* for comparable cresyl section.) *Bar* = 0.5 mm. *B*, Higher power view of *A* showing the neurons in Acc ABD intensely stained for acetylcholinesterase. *Bar* = 0.1 mm.

asymptote was 85 to 90% CRs in a 100-trial session and a well defined CR. This generally took three 100-trial sessions (Disterhoft et al., 1977). A prelesion conditioned or unconditioned NM extension or eye retraction was measured. Then the Acc ABD was located with the physiological recording procedures described above, using lower impedance electrodes (300 to 500 kilohms). An electrolytic lesion was made by passing 30 sec of DC current at 0.5 to 1.5 mA through the recording electrode at the center of the nucleus. The VIth nerve field potential was checked after the lesion. If a noticeable field potential remained, the electrode tip was moved slightly and a second lesion was made.

Behavioral measurements were made immediately before the lesion and 30 min after it. Behavioral measurements were continued for several days after the lesion was made (up to 14 days) to follow the time course of lesion effects. The animals were then sacrificed with an overdose of sodium pentobarbital and perfused through the heart with 10% formalin.

Frozen sections, 50 μm thick, were cut through the brainstem in the stereotaxic plane and stained with cresyl violet. The brain sections were examined at high power to determine whether all Acc ABD neurons had been eliminated. The position of Acc ABD on the unlesioned side was an important aid in determining lesion completeness since Acc ABD is a small group of cells within the reticular formation. Representative sections were drawn with the aid of a Tri-simplex projector.

Extraocular muscles were sectioned with ($N = 2$) or without ($N = 4$) Acc ABD lesions in some animals. This procedure was done with the aid of an operating microscope by incising the conjunctiva and locating the scleral attachments of the recti and oblique muscles. Each muscle belly was dissected free of connective tissue, and a loop of suture was passed around it. The muscle belly was then severed as far posteriorly as it could be visualized and the attachment to the sclera was detached. Average eye retraction responses were recorded at least 2 days after surgery to periorbital shocks presented to the intact and operated eyes. Eye retraction amplitude to 10, 15, or 20 shock presentations were compared for the intact and muscle-detached eyes.

In one animal, the completeness of the Acc ABD lesion was checked by injecting 20 μl of 50% HRP into the retractor bulbi on the same side after the lesion and behavioral testing. The rabbit was sacrificed after 2 days and the brain was processed for HRP retrograde transport with the TMB reaction as described above for the anatomical experiments.

Results

Anatomical tracing. The Acc ABD nucleus had the most heavily and reliably retrogradely labeled population of cells after HRP injections into retractor bulbi. This nucleus is a concentrated group of large, darkly staining cells ventral to abducens and just dorsal to lateral superior olive (Fig. 1*A*). Axons from it course dorsally through the main abducens and exit the brainstem with the VIth cranial nerve. When reacted with the TMB method, Acc ABD motor neurons could be seen to form a tightly packed nucleus of large, multipolar neurons with extensively branched dendritic trees (Fig. 1*D*). The nucleus was spherically shaped and about 0.5 mm in diameter. Note that the sections in Figures 1, 2, and 3 were cut perpendicular to the bottom of the brainstem rather than stereotaxically. In the rabbit stereotaxic plane, Acc ABD is ventral and anterior to abducens (Gray et al., 1981).

Abducens motor neurons were also labeled after retractor bulbi injections (Fig. 1, *B* and *C*), but they may have been labeled as a result of HRP leakage to lateral rectus after the injection (the TMB reaction method we used was extremely sensitive). In order to test this hypothesis, we dissected out the branch of the abducens nerve to lateral rectus, cauterized it and much of the lateral rectus muscle, and then injected retractor bulbi with HRP. Our consistent finding in three such experiments was that ABD uptake was severely limited while Acc ABD labeling was complete (Fig. 2). Since Figure 2*C* shows the abducens section with the most retrogradely labeled cells from one of the experiments, very few abducens neurons send their axons to retractor bulbi.

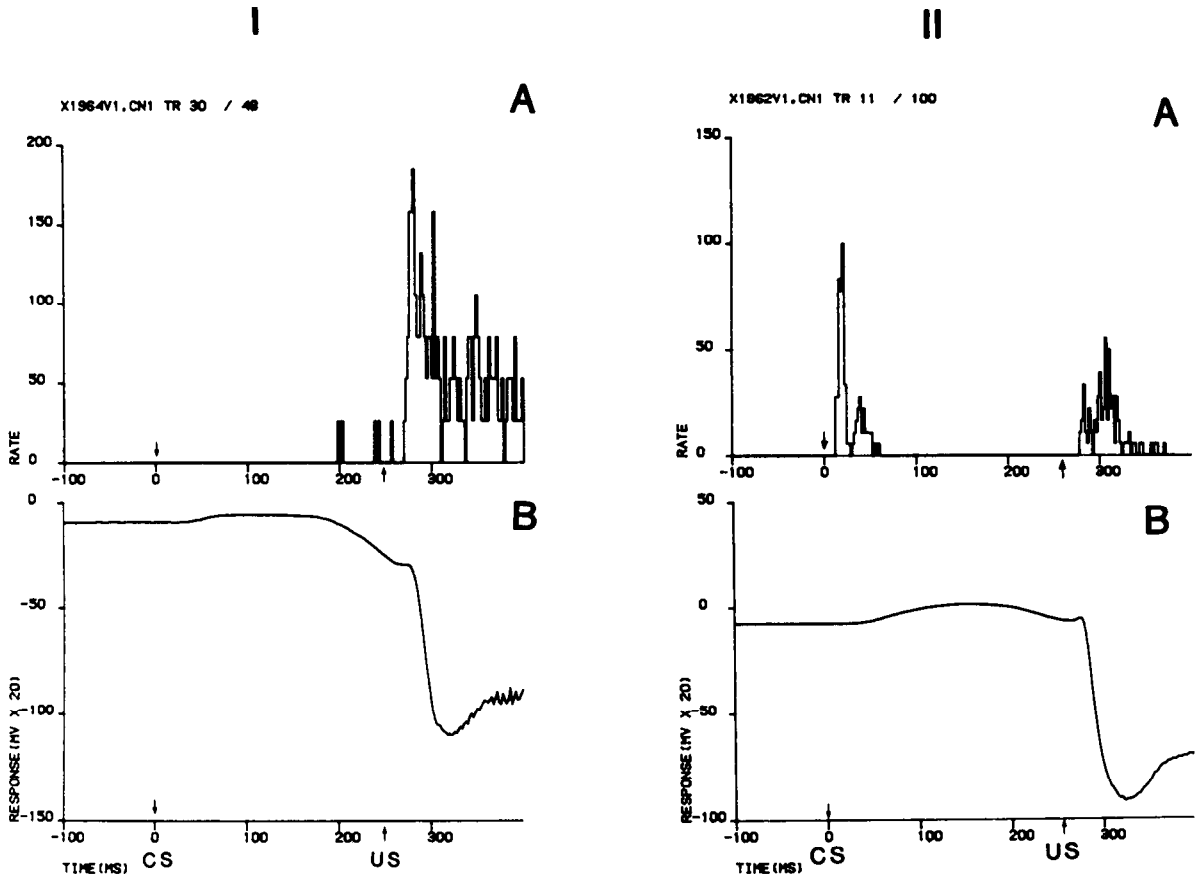


Figure 4. Behavioral and Acc ABD cellular responses during paired presentation of tone CS and airpuff US. *I*, Unit recorded after conditioning. *A*: Peristimulus histogram for single unit from the VIth nerve field potential region recorded for 20 trials. Note the lack of spontaneous activity and the clear correlation of neuronal activity with the conditioned and unconditioned NM responses (extension is downward) shown in *B* for the same trials. Tone CS was presented at 0 msec; air puff US was presented at 250 msec. *II*, Unit recorded before conditioning occurred. *A*: This well isolated unit showed one or two spikes of short latency after CS onset plus a second burst of activity which was correlated with NM extension shown in *B*. This neuron was histologically located in reticular formation medial to the Acc ABD nucleus.

Motor neurons in cranial motor nuclei are acetylcholinesterase positive (Koelle, 1963). When we processed sections through Acc ABD for acetylcholinesterase, we found that Acc ABD motor neurons were strongly acetylcholinesterase positive (Fig. 3). The abducens motor neurons in these sections were also acetylcholinesterase positive (Fig. 3*A*).

Single-neuron recording. We recorded 13 well isolated single neurons within the Acc ABD field potential region in the conditioned and unconditioned rabbit. We have also made about 100 electrode tracks through Acc ABD on which VIth nerve evoked field potentials and multiple units were recorded in the nucleus.

We were not able to routinely confirm that our Acc ABD single neurons sent their axons out of the abducens nerve with antidromic driving, but Figure 8*A* does show an example of an antidromically driven Acc ABD neuron. Apparently, the large field potential in this concentrated nuclear region tended to swamp the extracellular action potential out, even when our cells were well isolated (i.e., 150- to 400-μV spikes on a 25- to 50-μV background). This phenomenon was previously reported in abducens by Fuchs and Luschei (1970).

Neurons in Acc ABD had essentially no background firing rate. Single- and multiple-unit activity was seen only during spontaneous or stimulus-elicited NM sweeps. All units in Acc ABD were extremely sensitive to corneal and periorbital stimulation. Small air puffs or slight movements of hairs in the orbital region elicited vigorous driving. No qualitative evidence of habituation of this trigeminal input was seen.

All cells but two fired just at or before the onset of the conditioned or unconditioned NM sweep (Fig. 4). The other two fired during the behavioral response but after its onset. Our population of single neurons was too small to get a solid estimate of the population variance in cellular onset firing times to NM extension. The particular single cells illustrated in Figure 4 do not necessarily reflect the average amount of time that Acc ABD neuron firing preceded the behavioral response. Many cells probably fired earlier. As an example, the Acc ABD multiple-unit activity illustrated on two individual trials in Figure 5 preceded conditioned eyeball retraction by at least 25 msec on each trial. This comparison illustrates the range of variation in onset time which must be present in the cell population. The cell illustrated in *panel II* of Figure 4 was not typical of our population but is included because it showed an interesting, short-latency auditory response to the auditory CS presentation as well as a motor response which was well correlated with NM extension. The unit fired either one or two spikes at CS onset, thus the two short-latency peaks in the unit histogram. Unfortunately, the rabbit from which it was recorded did not show behavioral conditioning within the 90 trials this cell was held.

Microstimulation within Acc ABD elicited NM sweeps at currents as low as 10 μA (0.1-msec pulses, 200 Hz, 250- to 500-msec pulse trains). All single neurons were histologically located within the Acc ABD nucleus or in the reticular formation immediately adjacent to it by electrode track reconstruction.

Acc ABD Lesions. We first mapped the abducens field potential region. We then located the Acc ABD region slightly anterior, lateral, and ventral to it. After mapping Acc ABD, we placed an electrolytic lesion in its center by passing 0.5 to 1.5 mA of direct current through the recording electrode (with electrode negative) for 30 sec. We checked the VIth nerve field potential before and after the lesion. If the field potential was not eliminated, we moved the electrode tip

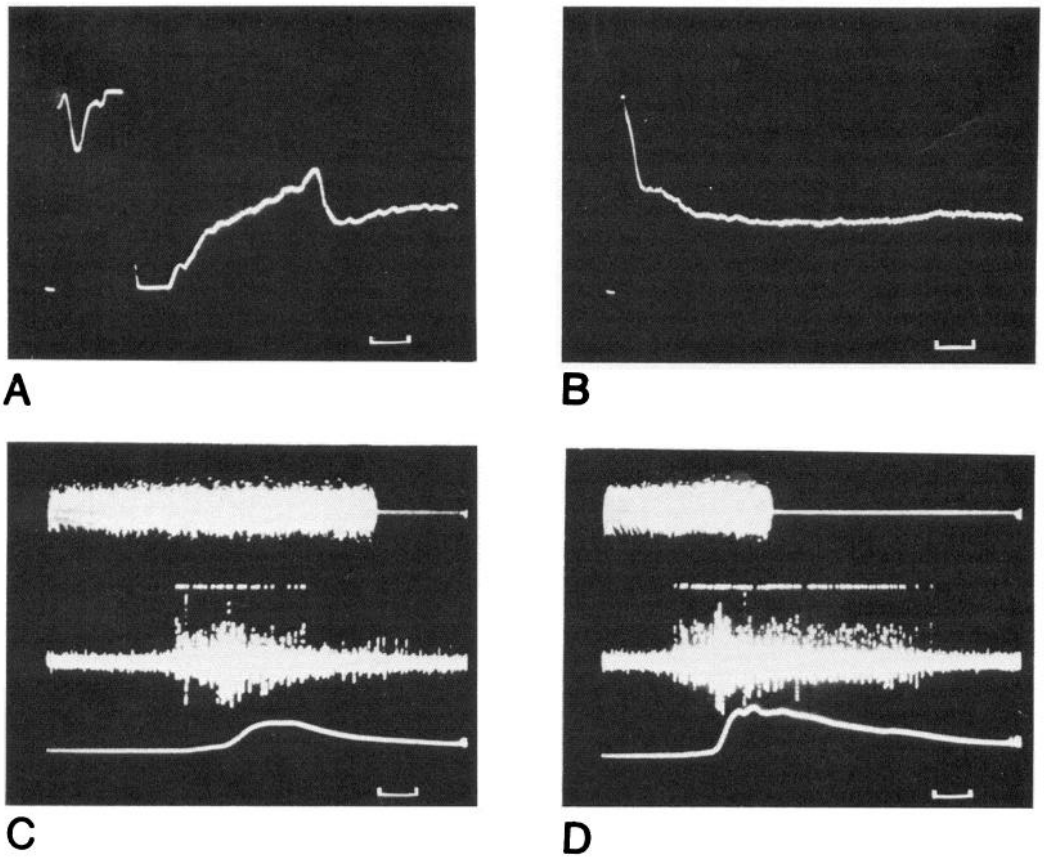


Figure 5. Field potentials and multiple units recorded from an Acc ABD lesion site. *A*, Field potential recorded from a microelectrode in response to VIth nerve stimulation. Stereotaxic location of microelectrode and latency of the evoked field potential indicated that the microelectrode was in the Acc ABD nucleus. This was confirmed histologically. *Calibration*, 0.5 msec. *B*, Field potential at the same location as in *A*, recorded following an electrolytic lesion (1 MA, 30 sec). The early peak is a stimulus artifact. The lesion has completely disrupted the field potential, indicating destruction of the Acc ABD nucleus. *Calibration*, 0.5 msec. *C*, Multiple units during a CS-alone trial consisting of a 400-msec burst of white noise presented alone in a well trained rabbit. *Top trace*: White noise onset and offset. *Middle trace*: Acc ABD multiple unit activity. Note the lack of spontaneous background activity. *Bottom trace*: Eyeball retraction. Note the onset of unit activity which precedes the onset of conditioned eyeball retraction. *Calibration*, 50 msec. *D*, Multiple-unit response during a conditioning trial in which CS (white noise) was paired at a 250-msec latency with a 5 psi puff of compressed nitrogen to the periorbital region. *Top, middle*, and *bottom traces*: same as in *C*. Note correlation of multiple-unit activity with conditioned and unconditioned eyeball retraction. *Calibration*, 100 msec. Note that sweep speeds in *C* and *D* are different.

slightly and made another lesion. Figure 5 shows an example of evoked potential and multiple-unit data gathered during a lesion experiment. The saturated Acc ABD field potential and its absence after the lesion are shown in Figure 5, *A* and *B*. Multiple-unit activity, recorded at the lesion site before it was made, correlated with the conditioned and unconditioned NM response as shown in Figure 5, *C* and *D*. Note that in Figure 5*C*, just the tone CS was presented, so that the multiple units correlate with only the conditioned NM response. In the case illustrated, a complete Acc ABD electrolytic lesion was made.

Electrolytic lesions which destroyed all or most of Acc ABD immediately reduced the size of conditioned and unconditioned eye retractions (or NM extensions) in 8 of 10 cases. However, the responses returned to, or exceeded, prelesion amplitudes within 3 days in 5 cases (Fig. 6*A*). In the 3 cases with the largest postlesion reduction, the responses never returned totally to their prelesion sizes (Fig. 6*B*). Even in these cases, a substantial postlesion eye retraction/NM extension response remained.

These data suggested to us that our hypothesis that Acc ABD was the major controller of eye retraction was not adequate. As will be discussed below, two previous reports in the literature (Lorente de No, 1933; Berthier and Moore, 1980) had indicated that the extraocular muscles, other than retractor bulbi, contracted during eye retraction/NM extension. In order to get a more accurate estimate of their contribution to normal eye retraction, we detached the four rectus and two oblique muscles from the eyeball on one side in three rabbits. The intact eye served as the control. We found that the eye retraction response was reduced after extraocular muscle detachment, especially at higher shock levels (Fig. 7). At lower shock levels, asymptotic eye retraction was equal in the eyes with and without the extraocular muscles. In these cases the eye retraction response rise time was slower, even though the asymptotic response was the same (Fig. 7). These data indicate that retractor bulbi is assisted by the recti and oblique muscles in eye retraction even at low shock levels. As the stimulus intensity was increased, asymptotic retractor bulbi contraction was apparently reached and the contribution of the remaining extraocular muscles became more obvious. An additional, qualitative behavioral observation we made was that the eye appeared much less responsive to light puffs of air and corneal taps after extraocular muscle detachment.

The point of the extraocular muscle section experiments was to test the possibility that the recti and oblique muscles could possibly have mediated the eye retraction response after Acc ABD lesion. The results clearly indicated that they could have. Therefore, in two final experiments, we first detached the extraocular muscles and then lesioned the Acc ABD nucleus.

In the first of these experiments, the Acc ABD lesion was complete but the lateral rectus and superior oblique muscles were not completely detached. The response to periorbital shock, evident 1 month after the lesion, was lateral rotation of the eye followed by a slight

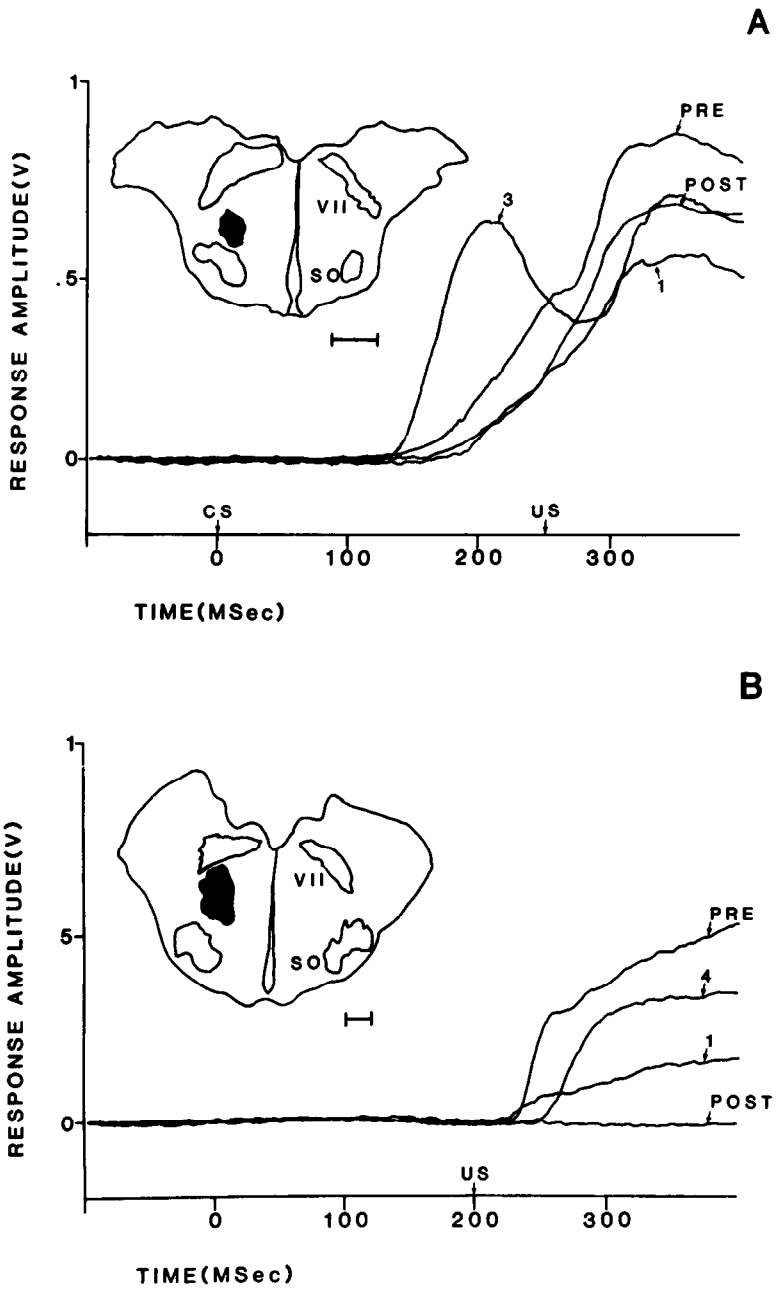


Figure 6. Acc ABD lesion effects on eyeball retraction response. *A*, A lesion largely confined to Acc ABD caused an immediate reduction in conditioned and unconditioned response amplitude (*Pre* versus *Post*). The postlesion effect on the conditioned response had disappeared by 3 days after the lesion, although in this case the unconditioned response remained reduced. *B*, A large lesion caused a large postlesion unconditioned response reduction which was still reduced 4 days after the lesion. The CS in *A* was white noise; the US in *A* and *B* was periorbital air puff. The eyeball retraction responses are 15 (*A*) and 10 (*B*) trial averages. The *insets* show outline drawings of sections through Acc ABD with largest lesion. *VII*, facial nerve; *SO*, superior olivary complex.

eye retraction. The eye retraction which remained was attributable to the remaining extraocular muscle attachments. In the final experiment, the Acc ABD lesion caused a virtually complete and permanent elimination of eye retraction to periorbital shock after the Acc ABD lesion (Fig. 8*C*). HRP was injected into the retractor bulbi before sacrificing this rabbit. Abducens motor neurons were heavily labeled, but there were no labeled cells in the lesion area, which covered the normal location of Acc ABD (Fig. 8*B*). Note that a slight amount of retraction remained after the lesion. As will be discussed below, this is attributable to the small population of abducens and oculomotor motor neurons which send their axons to the retractor bulbi muscle.

Discussion

The series of experiments we have done demonstrate two aspects of the motoneuronal system controlling eye retraction/NM extension in the rabbit. First, anatomical tracing showed that the Acc ABD nucleus is the primary source of the axons which innervate the retractor bulbi muscle in the rabbit. Physiological studies showed that Acc ABD neurons fired in close correlation with eye retraction and are very responsive to stimulation of the cornea and periorbital region. When Acc ABD was lesioned after detachment of the extraocular muscles other than retractor bulbi, eye retraction was permanently eliminated. Thus, the major role of Acc ABD motor neurons to retractor bulbi muscle in control of eye retraction in the rabbit was firmly established. Second, our lesion studies showed that the extraocular muscles other than retractor bulbi normally play an important role in eye retractions and may mediate relatively normal eye retractions when the Acc ABD nucleus has been lesioned.

Our anatomical tracing experiments demonstrated quite convincingly that Acc ABD is the primary source of axons to the retractor bulbi in the rabbit. Thus, innervation of the rabbit retractor bulbi is similar to that in the cat (Grant et al., 1979; Hutson et al., 1979; Spencer et al., 1980). Cegavske and his colleagues (1984) also concluded that Acc ABD was the major source of retractor bulbi axons in the rabbit. Gray et al. (1981), however, concluded that abducens controlled both lateral rectus and retractor bulbi in the rabbit. In addition to the neurons in Acc ABD which sent axons to retractor bulbi, they reported that 72% of abducens neurons did so.

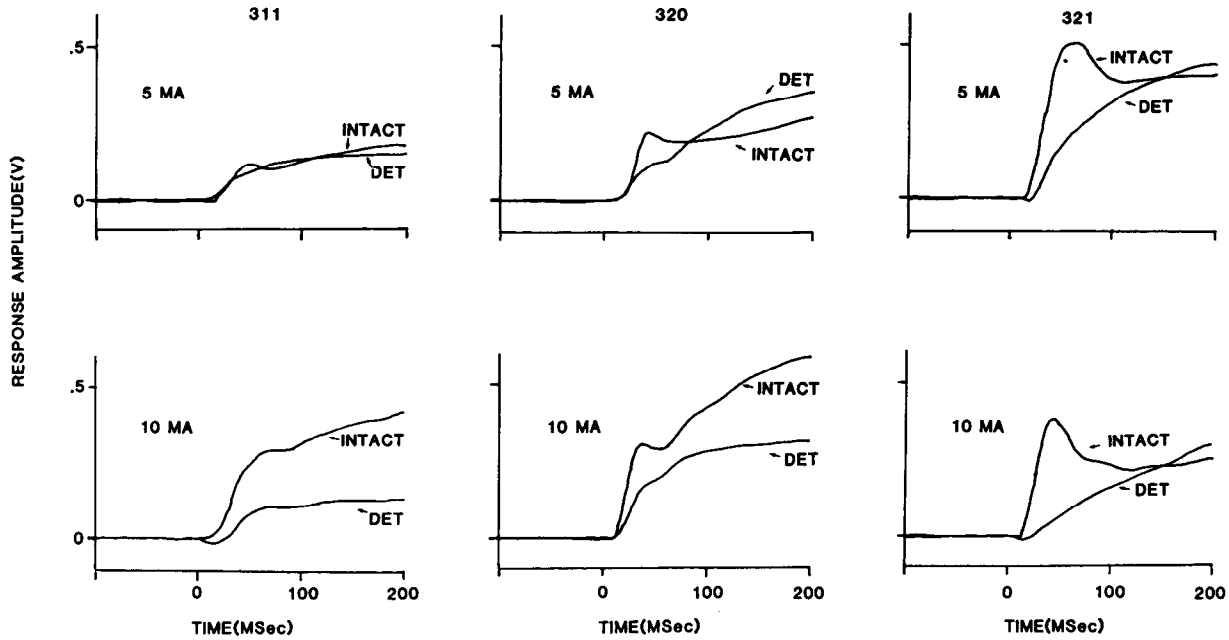


Figure 7. Extraocular muscle detachment effect on eye retraction. Average responses are shown for three rabbits (*311*, *320*, and *321*) to 5- and 10-mA periorbital shock trains. The extraocular muscles other than retractor bulbi were detached from one orbit (*DET*); the other eye served as the unoperated control (*INTACT*). Detachment caused a clear reduction at the higher shock level. At the lower shock level, the effect was primarily to slow the rise time of the retraction; the asymptotic response was not affected.

We did find a small number of abducens neurons which were labeled in our anatomical control experiments (Fig. 2C), but in nowhere near the numbers they reported. We should note that our anatomical control experiments, i.e., cautery of the abducens nerve branch to lateral rectus followed by HRP injection into retractor bulbi, showed consistent results in three replications; few ABD neurons were labeled. In addition, our lesion data are consistent with our anatomical tracing experiments. If a large proportion of abducens neurons sent their axons to retractor bulbi, detachment of the extraocular muscles other than retractor bulbi followed by Acc ABD lesions would be thought to leave a substantial remaining eye retraction. This did not occur (Fig. 8). Finally, our anatomical tracing data are consistent with those of other groups who have considered the location of retractor bulbi motor neurons in the rabbit (Cegavske et al., 1985) and cat (Spencer et al., 1980).

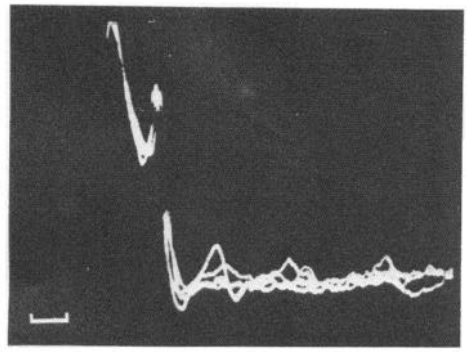
Our physiological observation that single neurons in and around Acc ABD were correlated in essentially a one-to-one fashion with eye retraction/NM extension was expected and confirmed our anatomical tracing experiments. The significant corneal and periorbital trigeminal input was also expected. This is the type of stimulus which elicits eye retractions and eye blinks as a defensive reflex in the rabbit. A disynaptic orbital trigeminal input has been demonstrated in the cat with intracellular recording (Baker et al., 1980; Grant and Horcholle-Bossavit, 1983). Intracellular injections of identified Acc ABD motor neurons with HRP in the cat have demonstrated that the dendritic field of these large motor neurons actually extends laterally into the adjacent trigeminal nucleus (Grant et al., 1979; Baker et al., 1980). Durand et al. (1983) have demonstrated (in the cat) that trigeminal neurons responsive to corneal input and filled with HRP by intracellular injection have axonal terminations in the Acc ABD nucleus. Berthier and Moore (1983) also demonstrated a trigeminal input onto Acc ABD neurons in the anesthetized rabbit.

We reported the one cell illustrated in *panel II* of Figure 4 because it illustrates the possibility that tone-puff associations may occur at the level of the Acc ABD output motor neurons, or in the reticular formation directly adjacent to it. This extremely well isolated cell showed one or two spikes just at tone onset on every CS presentation in addition to a response, less time locked, associated with the NM extension. It is possible that the later response was also an auditory response to the white noise component of the corneal air puff. If so, it was extremely well correlated with the NM response. Even if both components were auditory, this cell (in such a case, an interneuron) illustrates the fact that the auditory CS information is gaining close access to the Acc ABD output neurons. Since Acc ABD motor neurons have direct trigeminal input, tone-puff association may be occurring in that brainstem region at, or very close to, the output motor neurons.

Our lesion data are perhaps the most surprising finding of our series of studies. Taken as a whole, they make a coherent picture and explain what were some apparent inconsistencies in the literature.

First, it is clear that our initial hypothesis that Acc ABD was the principal output motor neuron pool for conditioned and unconditioned eye retractions was inadequate. This hypothesis was based on two considerations: (*1*) the retractor bulbi muscle is uniquely placed in the orbit for retraction, and (*2*) our anatomical and physiological studies showed that Acc ABD was the source of retractor bulbi motor neurons and that these neurons were very responsive to trigeminal input. In repeated experiments, we failed to permanently affect eye retraction/NM extension with total Acc ABD lesions (Fig. 4*A*). Even in those experiments in which we found a large response reduction immediately after the Acc ABD lesion, the response had largely returned within 3 or 4 days (Fig. 4*B*). It should be noted that the largest response reductions were seen in those cases in which the lesion encroached significantly upon the reticular formation surrounding Acc ABD. This reticular formation area may well contain premotor neurons important for control of abducens (Weiss and Disterhoft, 1985) and/or Acc ABD motor neurons.

Second, our initial Acc ABD lesion results caused us to reconsider several previous studies. First, Lorente de No reported more than 50 years ago, in quite a different context, that corneal stimulation caused activation of the retractor bulbi as well as the other six extraocular muscles in rabbit. Cegavske et al. (1976, 1979) had reported that abducens multiple units were highly correlated with conditioned and unconditioned NM extension in the rabbit. Later, Harrison et al. (1978) reported the same phenomenon for oculomotor nucleus multiple units. Finally, Berthier and Moore (1980) had reported that the extraocular muscles can mediate an NM extension



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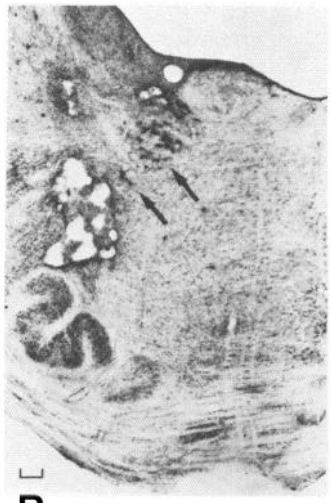

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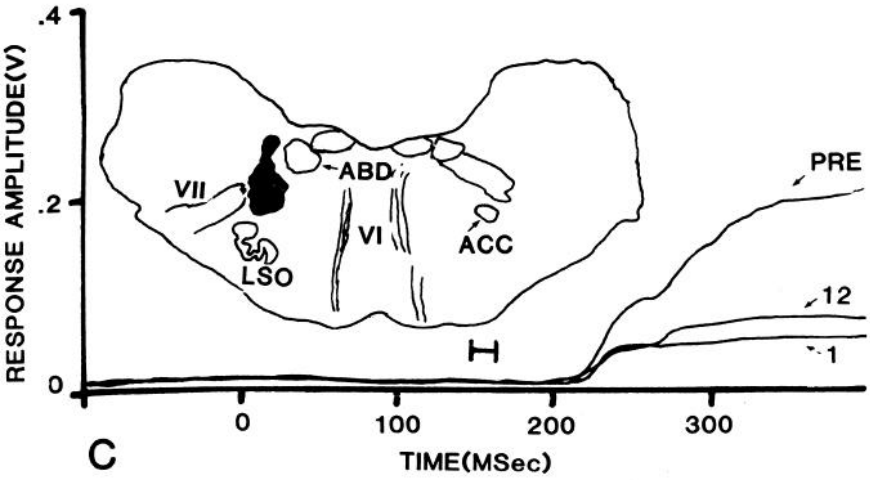

Figure 8. Acc ABD lesion plus extraocular muscle detachment effects on eye retraction response. *A*, Field potential and antidromically activated Acc ABD neuron at lesion location. Five traces are overlaid. *Calibration*, 0.5 msec. *B*, Brightfield photomicrograph through the center of the Acc ABD lesion. An outline drawing of this section is seen in *C*. *Arrows* point to abducens motor neurons retrogradely labeled after HRP injected into the orbit prior to sacrifice was taken up by lateral rectus motorneurons. No Acc ABD motor neurons were labeled. *Calibration*, 0.2 mm. *C*, Outline drawing of the section shown in *B*. Eye retraction responses to periorbital shock (which began at 200 msec) are shown before (*PRE*), the day after (*1*), and 12 days after (*12*) the Acc ABD lesion. All measurements were taken after extraocular muscle section, explaining why the *PRE* retraction is reduced from what would be expected in the intact situation (see Fig. 7). *VI*, *VI*th nerve; *VII*, facial nerve; *ABD*, abducens; *ACC*, accessory abducens; *LSO*, lateral superior olive. *Calibration*, 0.5 mm.

which was reduced 50% after VIth nerve section in naive rabbits. Paradoxically, they found no effect of extraocular muscle section on NM extension. These studies, in light of our Acc ABD lesion result, suggested that the recti and oblique muscles plus the retractor bulbi acted in a coordinated fashion to produce eye retraction.

Our final series of extraocular muscle detachment and lesion studies supported our revised hypothesis nicely. Detachment of the extraocular muscles was shown to have an effect on the rise time of the eye retraction response at low stimulus levels and on the asymptotic response at higher shock levels (Fig. 7). Thus, the extraocular muscles clearly do contribute to the eye retraction response. When retractor bulbi contraction was largely eliminated by Acc ABD lesions, the remaining extraocular muscles were very quickly able to produce responses of approximately the same amplitudes as before the lesion. When the Acc ABD lesion was combined with detachment of the extraocular muscles, the eye retraction response was essentially eliminated (Fig. 8). That small amount of eye retraction which remained after this combined treatment may be attributable to the small population of abducens and oculomotor nucleus motor neurons which send axons to the retractor bulbi (Spencer et al., 1980; Cegavske et al., 1984). These neurons were intact and the retractor bulbi muscle itself was unharmed; thus, some residual retraction remained.

Marek et al. (1984) reported that sectioning Acc ABD axons, without cutting the extraocular muscles, was adequate to almost eliminate NM extension elicited by air puff in the rabbit. In addition, they found that removal of the extraocular muscles, leaving retractor bulbi intact, did not affect NM extension amplitude elicited by air puff or shock. Their experiments were done with knife cuts within the brainstem which sectioned Acc ABD axons before they joined the VIth cranial nerve. It is possible that this manipulation severed the trigeminal afferents to other extraocular motor neuron pools or damaged important premotor areas to the other extraocular motor neurons (Weiss and Disterhoft, 1984) and caused the phenomena they observed.

Our lesion data can be easily fit with the important study of Cegavske et al. (1976), which pointed to the role of axons running in nerve VI for eye retraction/NM extension. Stimulation of nerve VI caused contraction of retractor bulbi along with lateral rectus and caused a coordinated eye retraction response. Stimulation of the oculomotor nerve or the trochlear nerve alone caused some movement of the eyeball; but because all of the extraocular muscles were not contracting in unison, no eye retraction resulted. Since the effect of nerve VI stimulation was so dramatic, it was quite reasonably emphasized in their report. Our data indicate that all of the extraocular muscles contract together to cause eye retraction. Within this context, the further observation of Cegavske et al. (1976, 1979) that abducens multiple units showed firing highly correlated to NM extension is quite reasonable. Lateral rectus (as well as the other extraocular muscles) contracts along with retractor bulbi to produce the response.

We draw two major conclusions from our experiments. First, the Acc ABD nucleus is the primary source of axons to retractor bulbi in the rabbit. Second, there are two important functional groups of

motor neurons which control eye retraction in the rabbit. Acc ABD forms one group. Abducens, oculomotor nucleus, and trochlear nucleus, acting in coordination, form another group. Either group is sufficient but not necessary for eye retraction to occur. We have not systematically tested the proposition, but it seems quite reasonable to suppose that neurons in abducens, oculomotor, and trochlear nuclei fire almost synchronously to assist in producing a coordinated eye retraction. Thus, we refer to these three nuclei as a functional group in regard to eye retraction.

Eye blink, controlled by the facial nucleus, is also coordinated with eye retraction/NM extension as components of one global defensive response which is conditioned during training (McCormick et al., 1982). Thus, a common premotor center must exist, at some point in the conditioned reflex arc, to control activity in the five nuclear regions controlling eye retraction/NM extension and eye blink. This center either is efferent from, or intimately involves, the deep cerebellar nuclei where lesions affect the conditioned but not the unconditioned reflex (McCormick and Thompson, 1984).

Our data show that even an apparently "simple" Pavlovian conditioned response like eye retraction/NM extension in the rabbit is relatively complex at the level of final output motor neuron pools. Also, we have not considered other adjustments of the skeletal musculature, heart rate, respiration, etc., which must be occurring simultaneously during conditioning. The central circuits which comprise the reflex arc may be even more complex or, alternatively, may have central nodes which control activity in several peripheral loci simultaneously. Our experiments suggest that a systematic physiological, anatomical, and behavioral approach can hope to make progress in unraveling the circuit elements, at several levels of the neuraxis, which must underly even this simple conditioned reflex arc.

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