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Initial localization of the acoustic conditioned stimulus projection system to the cerebellum essential for classical eyelid conditioning

(anatomical tracing/conditioned stimulus pathway/electrophysiological recording/lesions/pontine nuclei)

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ABSTRACT **Previous experiments have demonstrated a sufficient and necessary involvement of mossy fibers in projecting conditioned stimulus information to the cerebellum during classical eyelid conditioning in rabbits. Presented here are electrophysiological, anatomical, and lesion data that suggest that cells within the lateral pontine nuclear region may be essentially involved in projecting information concerning the occurrence of acoustic conditioned stimuli to the cerebellum during classical conditioning.**

A number of theories concerning cerebellar function have postulated a role for the cerebellum in motor learning (1–5). A common theme is that cerebellar plasticity is thought to be due to the conjunctive activation of mossy and climbing fibers on a common element, the Purkinje cell, whose axons project ventrally to cerebellar and brainstem nuclei. In our laboratory, a number of lesion, recording, and stimulation studies have demonstrated that the cerebellum is critically involved in the acquisition and retention of classically conditioned skeletal muscle responses (6–14). From these data we have proposed that neuronal plasticity involved in acquisition and retention of conditioned responses (CRs) is localized to regions of the cerebellum where the conditioned stimulus (CS) and the unconditioned stimulus (US) converge, and that the CS is projected to the cerebellum along mossy fibers while the US is projected to the cerebellum along climbing fibers (15).

We have demonstrated the essential involvement of climbing fibers in projecting the US to the cerebellum during classical conditioning. First, lesions placed in rostromedial portions of the dorsal accessory olive (DAO) prevent acquisition in naive rabbits and cause behavioral extinction with continued training in rabbits given lesions after acquisition training (16, 17). Second, stimulation of the DAO–climbing fiber system, which produces a variety of behaviors (e.g., eyeblink, head turn, limb flexion) can serve as an effective US for classical conditioning (18–20). When a tone CS and DAO-stimulation US trials are paired, rabbits develop CRs that are identical to the behavior elicited by DAO stimulation. Together, these data indicate that the DAO forms a portion of the neural circuit that is responsible for projecting the US to neural areas involved in classical conditioning.

We also have demonstrated the essential involvement of mossy fibers in projecting the CS to the cerebellum during classical conditioning. First, direct stimulation of mossy fibers or their cells of origin (the pontine nuclei) is an adequate CS for classical eyelid conditioning when paired with an air-puff or DAO-stimulation US (21–23). Second, bilateral lesions of the middle cerebellar peduncle (MCP) prevent acquisition and abolish eyeblink CRs when a tone, light, tactile, or mossy fiber stimulation CS is paired with an air-puff US (24, 25). These data provide strong evidence that many, if not all, CSs commonly used in classical conditioning are projected to the cerebellum by way of mossy fibers in the MCP. Although these data demonstrate that mossy fibers constitute a portion of the essential CS circuitry for classical conditioning, we have yet to determine the exact origin of CS mossy fibers activated by peripheral CSs. We focus here on the acoustic CS pathway; data are presented from electrophysiological (26), anatomical (27), and lesion–behavioral (28) studies that have identified portions of the dorsolateral and lateral pontine nuclei (DLPN and LPN, respectively) as critical sites for relaying information to the cerebellum concerning occurrence of acoustic CSs in classical conditioning.

MATERIALS AND METHODS

Acute Electrophysiological Recording. Twelve male albino rabbits were anesthetized with halothane, the skull above the right pontine nuclei was removed, and the entire extent of the right pontine nuclei and an area 2 mm caudal to the nuclei was mapped for auditory evoked field potentials. Mapping consisted of systematically lowering an insulated, stainless steel recording electrode (50-μm exposed tip) in 1 mm increments to obtain recordings from the entire right pontine nuclei, ventral portions of the right ventral nucleus of the lateral lemniscus (NVLL), and rostral areas of the right superior olive (SO) and trapezoid nucleus (NTB). Three clicks (65 decibels sound pressure level) were presented at each recording site via a speaker located 10 cm from the right ear, and auditory-evoked field potentials were bandpass filtered at 100–1000 Hz, digitized, and stored for further analysis. At the end of the recording session, electrolytic marking lesions were placed in the pontine nuclei, the animals were perfused, and the brains were prepared for standard histological verification of recording sites (22).

Anatomical Tracing. Twelve rabbits were anesthetized with Rompun/ketamine and the right LPN was stereotaxically injected over a 10-min period with 0.2 μl of the fluorescent tracer fast blue (5%). The injecting needle was withdrawn after 10 min and the animals were returned to their cages for 8 days to allow for transport of the dye. Two additional rabbits received control injections in the NVLL. Each rabbit then was given an overdose of sodium pentobarbital, perfused via the aorta with saline followed by 3% paraformaldehyde/0.2 M sodium phosphate buffer, pH 7.4,

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Abbreviations: CR, conditioned response; CS, conditioned stimulus; DAO, dorsal accessory olive; DCN, dorsal cochlear nucleus; DLPN, dorsolateral pontine nucleus; IC, inferior colliculus; LPN, lateral pontine nucleus; MCP, middle cerebellar peduncle; NTB, nucleus of the trapezoid body; NVLL, ventral nucleus of the lateral lemniscus; PMPN, paramedian pontine nucleus; SO, superior olive; US, unconditioned stimulus; VCN, ventral cochlear nucleus.

and finished with a wash of 10% sucrose in the buffered paraformaldehyde. The brains then were stored in 30% sucrose in phosphate buffer for 24 hr, sectioned at 40 μm, dried, and coverslipped using a low-fluorescing mounting medium (DPX, BDH Chemicals). The brain sections then were examined microscopically for fluorescence, using ultraviolet filters.

Lesions of the Pontine Nuclei. Fourteen rabbits were anesthetized with halothane and bilaterally implanted with insulated stainless steel lesion electrodes (200- to 400-μm exposed tips) in the region of the DLPN and LPN. In addition, 5 rabbits were implanted with a lesion electrode in rostral areas of the paramedian pontine nucleus (PMPN). Since subsequent lesions of the PMPN failed to affect conditioned responding, data from these rabbits are not presented here. Electrodes were guided into position stereotaxically and by recording field potentials evoked by presentations of a click. Once implanted, the electrodes and a headstage designed to hold a potentiometer to monitor movements of the left nictitating membrane and an air-puff nozzle were cemented onto the skull. After a 1-wk recovery period, the rabbits were adapted in a conditioning chamber and daily classical eyelid conditioning sessions were begun. Details of the training procedures have been published previously (17, 22). In brief, the animals were trained to a conditioning criterion by pairing a 350-msec tone CS with a coterminating 100-msec air-puff US, then overtrained with an additional session. The rabbits were next trained to criterion, then overtrained by pairing a light CS with an air-puff US. Immediately after the last light training session, the rabbits were anesthetized lightly with halothane and lesions were delivered through the chronically implanted electrodes by passing 1.0–1.5 mA of dc current for 20–90 sec. Paired training was then reinstated 24 hr after the lesions. The rabbits then were given one tone session, one light session, three to seven additional tone sessions, and three additional light sessions. After the last postlesion session, all rabbits were overdosed and perfused, and the brains were prepared for standard histological examination of lesion sites.

RESULTS

Electrophysiology. Mapping the pontine nuclear region for auditory-evoked field potentials revealed three areas that were responsive to the click stimulus: a medial area of the PMPN, an area in the rostral DLPN, and an area confined to dorsolateral portions of the LPN. The PMPN potentials were isolated within rostral portions of the nucleus, while LPN potentials were found in caudal areas of the nucleus. When the neural recordings were bandpass filtered at 500–5000 Hz, clear multiunit activity evoked by the click could be discerned, thus suggesting that the field potentials were recorded from cells within the pontine nuclei and not from fibers projecting through the pons from auditory structures. For comparison, auditory-related potentials were recorded from the NVLL, SO, and NTB. Onset latencies and amplitudes of the potentials differed. Potentials recorded in the NVLL (4- to 5-msec onset), SO (2.5–3.5 msec), NTB (3–5 msec), and LPN (3–5 msec) were relatively large, while DLPN (5–7 msec) and PMPN (3–5 msec) potentials were somewhat smaller. The PMPN potentials could not be reliably elicited from all animals. Fig. 1 shows examples of auditory-evoked field potentials recorded in the DLPN, LPN, PMPN, and NVLL. These recording data suggest three potential regions of the pontine nuclei (the DLPN, LPN, and PMPN) that may relay information about acoustic stimuli to the cerebellum during classical conditioning.

Anatomical Tracing. Injections of fast blue into the lateral region of the caudal LPN, a pontine area in which auditory-related field potentials were recorded (see above), resulted in marked labeling in the contralateral ventral cochlear nucleus (VCN). Less labeling was found in the contralateral dorsal cochlear nucleus (DCN), ipsilateral SO, and ipsilateral inferior colliculus (IC). Control injections placed in the NVLL produced labeling in the contralateral DCN and less in the VCN. Fig. 2 depicts the LPN injection site and the resulting labeling in the VCN and DCN. These data indicate that direct projections may exist from the VCN and DCN to the contralateral LPN, a pathway that could account for the short-latency auditory-related potentials recorded in the caudal LPN.

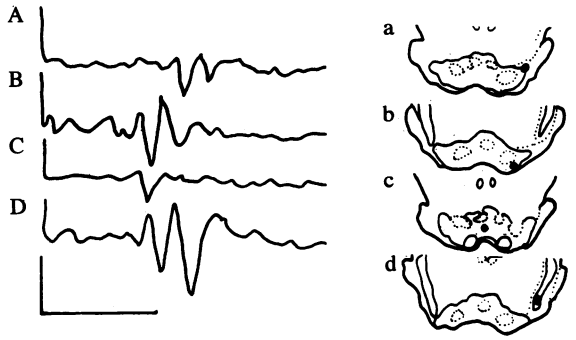

FIG. 1. Digitized click-evoked field potentials recorded in the DLPN (*A*), LPN (*B*), PMPN (*C*), and NVLL (*D*). The respective recording sites, as identified by marking lesions, are depicted in *a*, *b*, *c*, and *d*. The neural recordings were bandpass filtered at 100–1000 Hz. The vertical bar represents 3 mV and the horizontal bar represents 5 msec.

Pontine Lesions. All rabbits reached conditioning criterion on the second day of acquisition when trained with the tone CS and on the second or third day of acquisition when trained with the light CS (see Figs. 3–5). Lesions placed in lateral regions of the pontine nuclei, however, altered performance of the CR. On the basis of postlesion performance of CRs, the rabbits could be placed in one of three groups: (*i*) group AB (n = 4), rabbits that demonstrated abolition of CRs with presentations of the tone CS (less than 10% of prelesion responding) but maintained prelesion responding rates to the light CS (Fig. 3); (*ii*) group RA (n = 4), rabbits that demonstrated reacquisition with the tone CS (less than 30% prelesion responding on the first day of postlesion training)

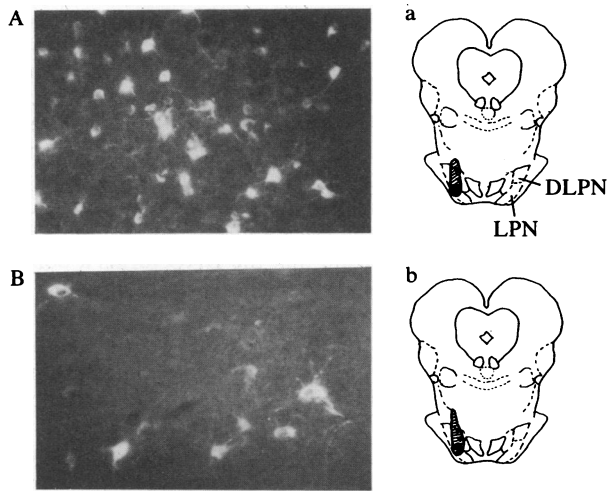

FIG. 2. Histofluorescence results and injection sites of fast blue in two rabbits. Photomicrographs (×80) of labeled cells within the contralateral VCN (*A*) and contralateral DCN (*B*) are shown with their respective LPN injection sites (black dots shown in *a* and *b*). The hatched areas surrounding the injection sites depict the maximal extent of spread of the dye.

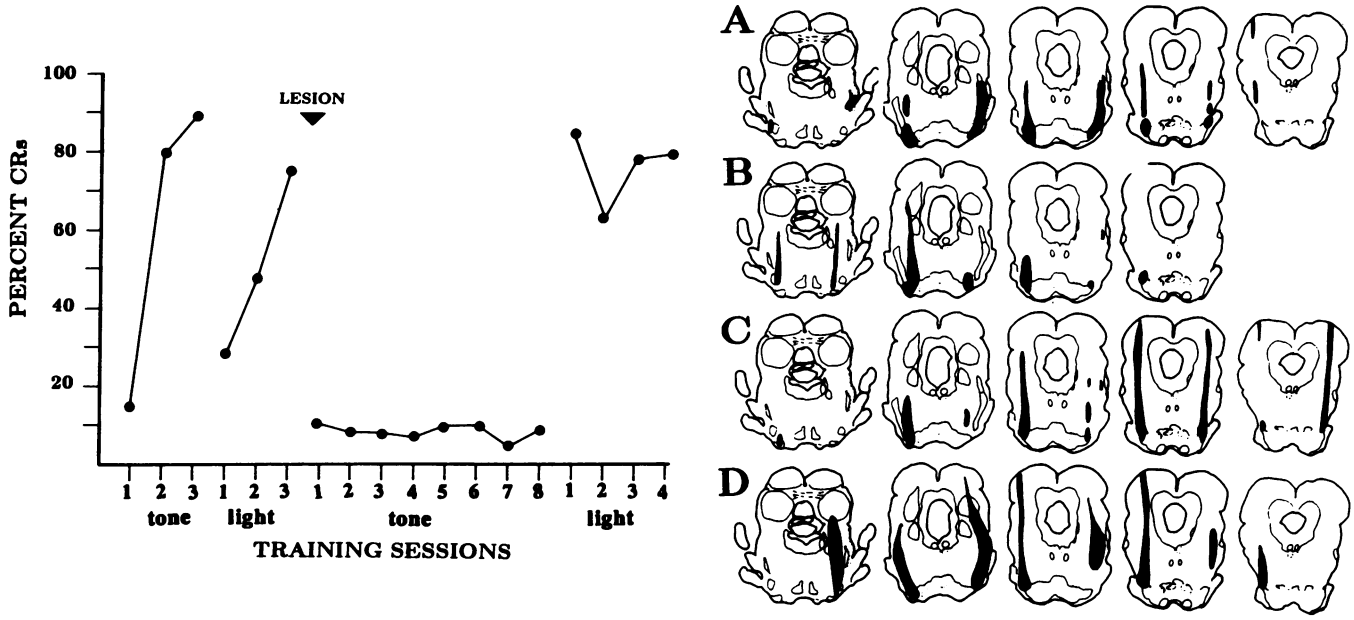

FIG. 3. Percent CRs and drawings of lesion damage in four rabbits (A–D). These rabbits were given three tone sessions and three light sessions (108 trials per session) before bilateral pontine lesions were induced. The lesions caused abolition of tone CRs but did not affect response to the light CS. [The drawings show coronal sections of the brainstem including caudal (left) to rostral (right) aspects of the pontine nuclei.]

but maintained prelesion responding rates to the light CS (Fig. 4); and (*iii*) group NE ($n = 6$), rabbits that demonstrated no postlesion deficits with presentations of tone or light CSs (Fig. 5).

Histological examination of the lesion sites revealed different patterns of destruction in the three groups of rabbits. All AB animals (Fig. 3, animals A–D) had large portions of the left DLPN and dorsal portions of the left caudal LPN removed. In addition, other areas with lesions could be identified. Animal A received damage to the right DLPN, dorsal portions of the right caudal LPN, and massive damage to the right lateral lemniscal system (tract and nuclei). Animal B had right DLPN damage, some damage in dorsal areas of the right LPN, and very slight damage to ventral aspects of the left NVLL. Animal C sustained damage in rostral areas of the right DLPN but no damage in the right LPN. Animal D received no damage to the right DLPN or LPN but had massive bilateral damage in the lateral lemniscal system (including an area immediately dorsal to caudal portions of the left and right pontine nuclei). The following general conclusion can be drawn from these data: tone CRs can be selectively abolished when caudal aspects of the LPN in conjunction with the DLPN receive lesions or when partial lesions of the DLPN and LPN are given in conjunction with large bilateral lesions of the lateral lemniscal system.

Two patterns of lesion damage were observed in rabbits

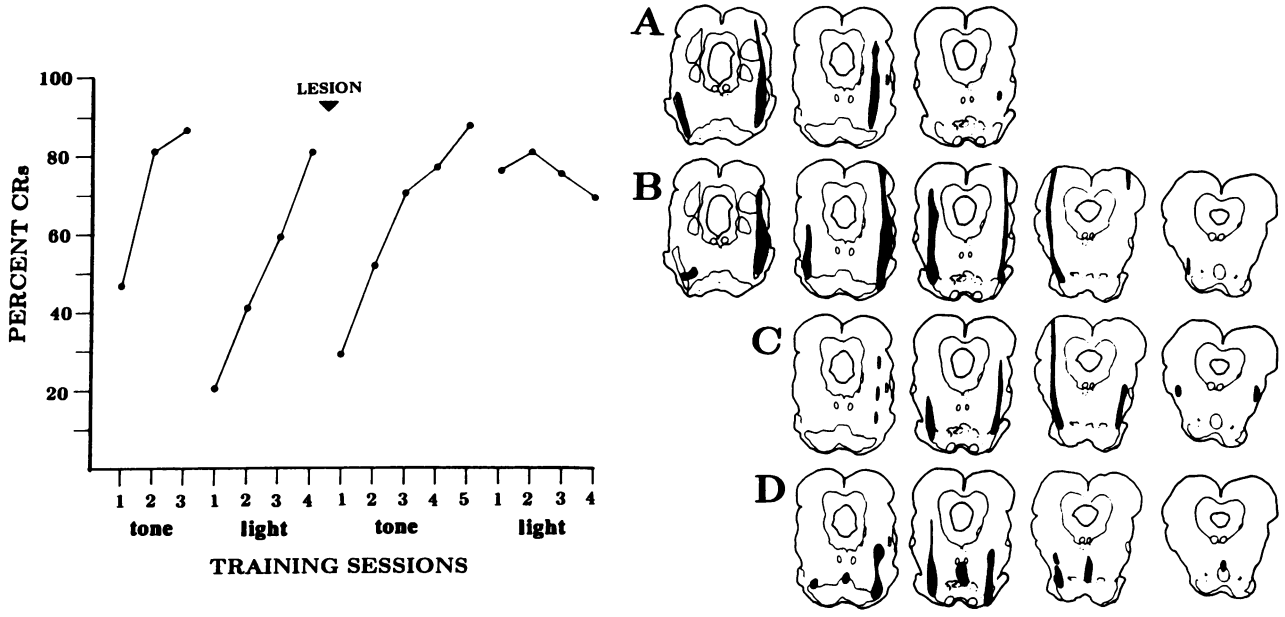

FIG. 4. Percent CRs and drawings of lesion damage in four rabbits (A–D). These rabbits were given three tone sessions and four light sessions (108 trials per session) before bilateral pontine lesions were induced. The lesions caused loss of tone CRs, followed by reacquisition, but did not affect response to the light CS. [The drawings show coronal sections of the brainstem including caudal (left) to rostral (right) aspects of the pontine nuclei.]

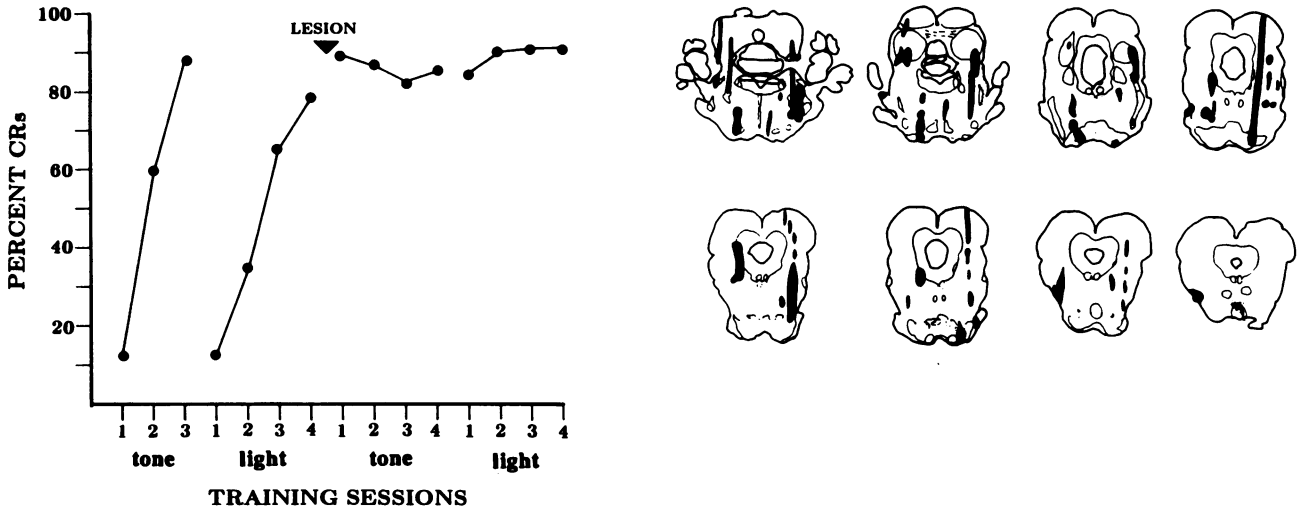

FIG. 5. Percent CRs and composite drawings of lesion damage in six rabbits (*Right*). These rabbits were given three tone sessions and four light sessions (108 trials per session) before lesions were induced. No postlesion CR deficits were observed in these animals. [The drawings show coronal sections of the brainstem from an area caudal to the pontine nuclei (upper left) to an area rostral to the pontine nuclei (lower right).]

that demonstrated reacquisition of tone CRs (Fig. 4, animals A–D): either extensive bilateral damage of the tract and nuclei of the lateral lemniscus (animals A and B) or bilateral damage to rostral portions of the DLPN (animals C and D). None of the RA animals received damage to caudal aspects of the LPN or DLPN. These results suggest that lesions confined to either the lateral lemniscal system or rostral DLPN can cause a temporary deficit in conditioned responding to a tone CS that can be overcome with additional paired presentations of the tone CS and air-puff US.

Fig. 5 shows composite drawings of lesion damage in six rabbits that exhibited no CR deficits after the lesions. No damage involving the DLPN or LPN and only slight damage to the lateral lemniscal system was observed in these rabbits. Other areas with lesions included small regions of the IC and superior colliculus, a portion of the left SO, portions of the right trigeminal complex, medial areas of the pontine nuclei, parts of the left and right pyramidal tract, small regions of the right MCP, and large areas of the reticular formation above the pontine nuclei. These data indicate that lesions placed in areas of the brainstem surrounding, but not invading, lateral regions of the pontine nuclei and the lateral lemniscal system do not affect performance of CRs established with tone or light CSs.

DISCUSSION

The observation of acoustic-related responses in the DLPN is in agreement with a number of previous anatomical and recording studies, which have demonstrated that rostral portions of the DLPN receive auditory projections from the IC and auditory cortex (29–33). In the present study, acoustic-related potentials with 5- to 7-msec onset latencies were recorded from areas of the DLPN. The latencies of these potentials suggest that they may have been recorded from pontine cells that receive projections from the IC. However, potentials recorded more ventral and caudal than the DLPN sites (as determined by marking lesions placed in the sites after recording) were observed in a small area of the LPN. These potentials had a shorter onset latency (3–5 msec), which suggests that the LPN cells were not responding to input from only the IC, but rather were receiving direct projections from lower auditory nuclei. Our anatomical findings when retrograde transport of fast blue was studied suggest strong, direct connections from the contralateral VCN to the caudal LPN, with weaker connections from the contralateral DCN, ipsilateral SO, and ipsilateral IC. Furthermore, the onset latencies of the LPN potentials were generally consistent with response latencies recorded in auditory nuclei that receive direct input from the cochlear nuclei (e.g., the SO). Together, the electrophysiological and anatomical data argue that information concerning occurrence of acoustic stimuli is projected to lateral regions of the pontine nuclei.

Extensive bilateral damage of the DLPN and LPN, as well as extensive damage of the left DLPN and LPN with some damage of the right lateral lemniscal system dorsal to the pontine nuclei, was effective in selectively abolishing CRs to a tone (but not a light) CS. Abolition of the CR after bilateral lesions of the DLPN and LPN is consistent with the idea that these pontine regions are essential relays to the cerebellum for acoustic CSs. A possible explanation for CR abolition after combined destruction of the left DLPN and LPN and right lateral lemniscal system is that the right lesions, placed immediately dorsal to the pontine nuclei, may have interrupted auditory input to the right lateral pontine nuclear region. In either case, selective abolition of the tone CR appears to have been caused by the elimination of necessary input to the cerebellum that arises from the lateral pontine nuclear region. It should be noted that the lesions could have interrupted fibers of passage that project from auditory structures to the cerebellum without synapsing in the pontine nuclei [e.g., projections from the VCN to the vermis (34)]. Our recording and anatomical tracing data, however, indicate that the lateral pontine regions receive direct auditory input from a variety of auditory structures, whereas previous data suggest that the cerebellar hemisphere and interpositus nucleus do not (34, 35). It seems likely that acoustic CS information is relayed through the pontine nuclei before reaching the cerebellum.

Animals with damage confined to either the lateral lemniscal system or the DLPN showed only temporary deficits in conditioned responding to the tone CS and eventually relearned the CR with additional postlesion training. Since the caudal LPN was not damaged in this group it seems likely that auditory input, relayed to the cerebellum from the cochlear nuclei by way of the LPN, was sufficient to eventually reinstate conditioned responding. However, the fact the reacquisition was observed in these animals indicates that acoustic CSs are also projected to the cerebellum by way of relays in areas of the DLPN, LPN, or both that receive input from portions of the lateral lemniscal system that had been

damaged. In a previous study (36, 37), bilateral injections of a local anesthetic (lidocaine) into regions of the lateral lemniscus dorsal to the caudal aspects of the pontine nuclei produced a temporary selective abolition of tone CRs. Subsequent injections of ibotenic acid into the same regions failed to permanently abolish the learned response. These data argue that fibers contained in or near the lateral lemniscus are involved in projecting information concerning occurrence of acoustic CSs, a result that is consistent with the possibility that damaging portions of the lateral lemniscal system interrupts auditory inputs to the DLPN, LPN, or both, thus producing a transient effect on conditioned responding, namely, loss of the tone CR followed by reacquisition.

In summary, the present data suggest that lateral regions of the pontine nuclei are essential relays for projecting acoustic (but not visual) CS information to the cerebellum during classical conditioning. The data also suggest that the lateral pontine regions receive at least two sources of auditory input: direct projections from the VCN and DCN and input from other auditory structures (e.g., the IC) that project along fibers located in or near regions of the lateral lemniscal system that received lesions in the present study. The present results also suggest that critical acoustic CS information is projected to the cerebellum along parallel pathways that converge in rather wide regions of the lateral pontine nuclei. It is likely that the lateral pontine nuclei, in turn, distribute mossy fiber input to several regions of cerebellar cortex, nuclei, or both that are involved in establishing and maintaining the neuronal plasticity that underlies classical conditioning of skeletal muscle responses. In principle, this parallel arrangement of acoustic CS inputs is similar to the parallel visual pathways that transmit CS information during classical heart-rate conditioning in pigeons (38) as well as parallel pathways implicated in transmitting visual CS information during classical eyelid conditioning in rabbits (39).

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