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Single-Neuron Labeling in the Cat Auditory Nerve

Abstract. *Single auditory nerve fibers in the cat were labeled intracellularly with horseradish peroxidase. The sample of fibers was selected to represent different response types over a wide range of characteristic frequencies. All 56 labeled neurons were found to be radial fibers innervating inner hair cells, suggesting that none of the single-unit data reported to date has been from the outer hair cell innervation. Differences in rates of spontaneous discharge and thresholds to tones among these labeled neurons were closely correlated with morphological differences in the caliber and location of their unmyelinated terminals on the body of the inner hair cell.*

The auditory nerve bundle contains primary neurons connecting cochlear sensory cells with the cells of the cochlear nucleus. The classic morphological studies of cochlear innervation (*1*) suggested that there are at least two fundamentally different types of afferent fibers in the auditory nerve: radial fibers innervating inner hair cells (IHC's) and outer spiral fibers innervating the outer hair cells. Recent morphological studies suggest that radial fibers can be further divided into three distinct types on the basis of caliber, mitochondrial content, and synaptic morphology of the peripheral terminals (*2*) and that radial fibers outnumber outer spiral fibers by 20 to 1 (*3*).

In the years since the first measurements of spike discharges from auditory nerve fibers (*4*), a number of single-unit classification schemes have been devised (*5*, *6*), each suggesting possible correlations with the morphological data available at the time. In the work reported here the validity of such schemes was tested by intracellular labeling of functionally identified neurons with standard horseradish peroxidase (HRP) techniques (*7*).

Injections of HRP were made iontophoretically through beveled micropipettes filled with a 10 percent solution of Sigma type VI HRP in 0.05*M* tris buffer (*p*H 7.3) containing 0.15*M* KCl. The electrodes yielded resting potentials in the auditory nerve as high as −60 mV and passed currents of 4 to 5 nA (50-msec pulses, 50 percent duty cycle, electrode positive). Injection of HRP was initiated only if the unit's resting potential was more than −30 mV and was terminated when the potential became smaller than −15 mV. An injected unit was visible as a labeled fiber only if the product of nanoamperes and minutes of injection exceeded 11 (*8*). For each single unit selected, a tuning curve (tone threshold as a function of stimulus frequency) and a measure of spontaneous discharge rate (SR) were obtained prior to HRP iontophoresis. For almost all units selected, additional tuning curves (typically three or four) and measures of SR (typically one or two) were obtained after the injection was terminated.

Roughly 30 hours after the last injection, the cochlea was fixed (*9*), reacted in toto by perfusion of solutions containing cobalt chloride and diaminobenzidine (*10*) through the oval and round windows, and embedded in Epon. The organ of Corti was then dissected out and mounted on glass slides so that all the sensory cells could be examined by light microscopy with an oil-immersion, phase-contrast objective lens at ×2600 (*9*).

In each experiment the aim was to label four or five fibers sufficiently separated along the length of the cochlea to allow unambiguous correlation between physiology and histology. The overall sample (from 14 cats) included fibers with characteristic frequencies (CF's) evenly distributed from 0.16 to 36 kHz. Within each CF region, units were selected to represent the full spectrum of SR's. The SR ranges from 0 to over 100 spikes per second and is strictly correlat-

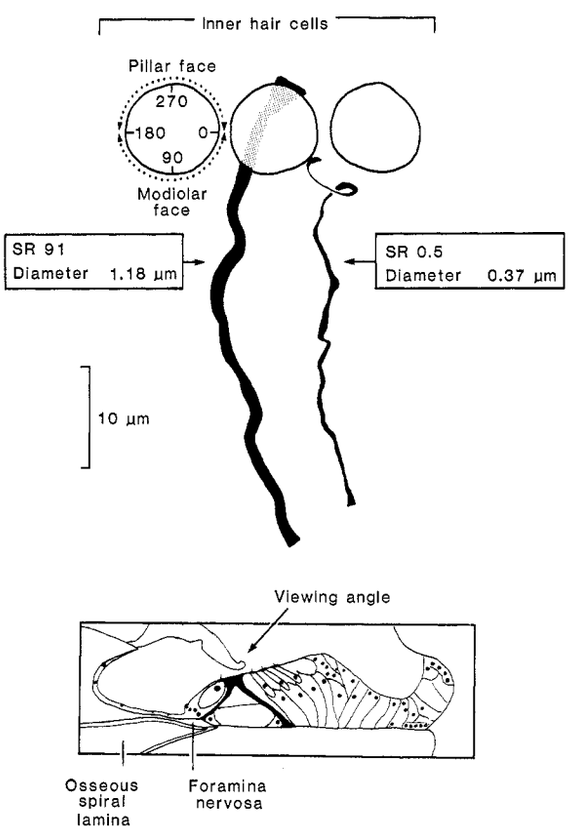

Fig. 1. Tracings of three IHC's and two radial fiber terminals as they appear when viewed from the endolymphatic surface of the organ of Corti. Outer hair cells would be toward the top of the figure. The conventions for dividing the hair cell circumference (to quantify radial fiber position) are shown on the IHC at the left. The two fibers did not innervate the same IHC, although both were from the same cochlea.

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ed with sensitivity. Among the units in one CF region, low-SR units (< 0.5 spike per second) are 20 to 80 dB less sensitive than high-SR units (>17.5 spikes per second), which, in turn, are roughly 10 dB more sensitive than medium-SR units (*6*).

All 56 neurons labeled were found to be radial fibers innervating IHC's only. All except two were unbranched, contacting a single IHC with a single terminal swelling. Tracings of the peripheral terminals of typical low- and high-SR units are shown in Fig. 1. As suggested by these tracings, and as illustrated more systematically in Fig. 2a, the average diameter of the radial fiber terminals is highly correlated with SR. Although there is some overlap in diameter across SR classes, high-SR units typically had larger diameters than low- or medium-SR units, and the four fibers with the smallest diameters were all low-SR units.

There is also a spatial separation of radial fiber terminals according to SR (Fig. 1). Each IHC is roughly barrel-shaped (circular in cross section) and is innervated by 20 to 25 radial fibers (*2, 3*). The terminal swellings of radial fibers, where virtually all the afferent synapses are located, can be found at all points around the hair cell circumference at any cross-sectional level below the nucleus. However, the terminal swellings contacting the side of the IHC facing the pillar or outer hair cells are exclusively those of high-SR units, while all the low- and medium-SR units contact the side of the IHC facing the modiolus (Fig. 2b).

The present data strongly suggest that the activity of the outer hair cell innervation has never been sampled in any of the single-unit recordings from the auditory nerve. Low-SR units constitute the only single-unit subclass that has ever been demonstrated to satisfy the prerequisites for an outer spiral fiber correlate (a response type constituting 5 to 10 percent of the total unit sample in all CF regions from any normal animal) (*6*). The data clearly show that these units innervate the IHC's. The apparent inability to record from outer spiral fibers is consistent with the recent suggestion that their central axons are unmyelinated and less than 0.5 μm in diameter (*11*). The central axons of all radial fiber types have diameters between 3 and 5 μm (*2*).

These findings also corroborate the suggestion that fibers of different calibers innervate different sides of the IHC's and that the thick fibers innervating the pillar face all have high SR's while the thin fibers on the modiolar face have low or medium SR's (*2*). It has been suggested that a morphological differentiation between low- and medium-SR units could be made on the basis of synaptic ultrastructure (*2*). The ultrastructural data indicate that virtually every radial fiber forms only one synaptic complex with a hair cell. Thin-fiber and thick-fiber synapses appear similar in most respects (for example, the number of presynaptic vesicles seems roughly the same). However, some of the thinnest fibers show a larger and more complex region of synaptic membrane specialization and a longer synaptic bar. The suggestion that the presence or absence of this synaptic complexity is correlated with the distinction between low- and medium-SR units (*2*) can now be directly tested.

Electron microscopic study of the IHC area (*2*) has also shown that the thin fibers innervating the modiolar faces contain very few mitochondria compared with the thick fibers innervating the pillar faces. It is not clear whether these differences in caliber and mitochondrial content cause the observed differences in SR or whether they merely reflect differences in resting spike activity arising because of (i) structural or chemical differences at sites such as the synapse or (ii) differences in the electrical environments on the two sides of the IHC.

The strict correlation between SR and threshold to tones has recently been corroborated by single-unit data from the auditory nerves of the Mongolian gerbil and the chinchilla (*12*). The presence of the same phenomenon in a number of different species indicates that this type of SR classification has functional significance of some generality, as does the consistent correlation between unit response and SR in a wide variety of stimulus conditions. It may be that low-, medium-, and high-SR units play fundamentally different roles in auditory perception. If so, the central projections of these three neuronal types may differ. Such a suggestion is directly testable by intracellular HRP techniques.

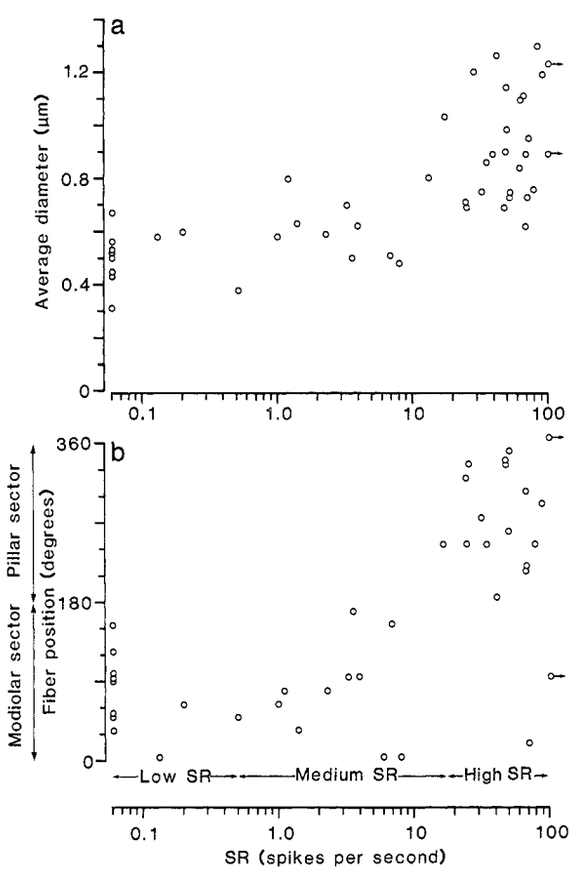

Fig. 2. (a) Relation between SR and fiber diameter. Fiber diameter is determined by tracing (with a drawing tube at an overall magnification of ×2600) the unmyelinated terminal portion of each labeled fiber from the foramen nervosum (Fig. 1) to the terminal swelling. The fiber's contour is then digitized with a Talos graphics tablet interfaced to a PDP-11 computer, and the average diameter is determined by dividing the area by the length. SR determination is based on a 15-second sample. Units showing no spikes per 15 seconds are included with those showing one spike per 15 seconds (SR = 0.06). Units with SR's greater than 100 are placed at 100 and marked with arrows. Only units with CF's above 15 kHz are included. (b) Relation between SR and location of terminal swelling on the hair cell circumference.

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Interaction of Convulsive Ligands with Benzodiazepine Receptors

Abstract. *The γ-aminobutyric acid (GABA)–benzodiazepine receptor complex, which is composed of distinct proteins embedded in the neuronal plasma membrane, is important for several effects of benzodiazepines, including protection afforded against convulsions. During structural modification of ethyl β-carboline-3-carboxylate an agent was discovered which has high affinity for brain benzodiazepine receptors but which is a potent convulsant. Also in contrast to benzodiazepines, this type of benzodiazepine receptor ligand favors benzodiazepine receptors in the non-GABA–stimulated conformation, which may explain the convulsive properties.*

Specific binding sites for benzodiazepines have been demonstrated in the central nervous system of higher vertebrates, including man (*1*). Evidence suggests that these binding sites are receptors for the benzodiazepine type of minor tranquilizers (*2*).

It was initially thought that only pharmacologically active benzodiazepines, and a few benzodiazepine-like agents, interacted with high affinity with these receptors (*2*). However, agents such as ethyl β-carboline-3-carboxylate (β-CCE) (*3*) and Ro 15-1788 (*4*) have been discovered which have a high affinity for benzodiazepine receptors but which lack the anticonvulsant and anticonflict effects of benzodiazepines (*4–6*). These agents can block or reverse benzodiazepines from eliciting their usual effects (*4–6*), indicating that their interaction with benzodiazepine receptors is functional and novel. In testing structural modifications of β-CCE we observed that methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) (Fig. 1) is a potent convulsant in mice and rats. Our findings suggest that DMCM produces convulsions by interacting with benzodiazepine receptors in a particular way. It appears that, by affecting the γ-aminobutyric acid (GABA)–benzodiazepine receptor complex (*7*), DMCM may reduce GABA-mediated neurotransmission.

To determine the nature of its convulsive properties we administered DMCM (1 to 300 mg/kg intraperitoneally) to mice (Fig. 1) or rats (5 to 10 mg/kg intraperitoneally). Clonic-tonic convulsions followed within 3 to 5 minutes. The convulsions were similar in quality to those produced by pentylenetetrazol; a short period of clonic fore- and hindlimb convulsions was followed by tonic extensor spasms. At DMCM doses above 15 mg/kg, all mice died within 10 to 15 minutes from respiratory depression. Less frequent and briefer convulsions were induced by methyl β-carboline-3-carboxylate (β-CCM) at 5 to 100 mg/kg intraperitoneally. The convulsive effects of DMCM and β-CCM occurred at doses at which these agents are bound to benzodiazepine receptors in living mice (Fig. 1). Similar effects were observed in rats (data not shown). As expected, convulsions induced by DMCM (15 mg/kg intraperitoneally) in mice were inhibited by benzodiazepines and barbiturates [mean effective dose (ED_{50}) for lorazepam, 0.5 mg/kg intraperitoneally; for diazepam, 9 mg/kg intraperitoneally; and for phenobarbital, 20 mg/kg orally]. The ability of Ro 15-1788 (ED_{50}, 7 mg/kg orally) and β-CCE (ED_{50}, 70 mg/kg, intraperitoneally, administered 35 minutes before DMCM) to inhibit convulsions induced by DMCM was surprising. Ro 15-1788 has been described as a pure benzodiazepine antagonist (*4*), but, to our knowledge, it has not been shown to antagonize convulsive agents. β-CCE not only fails to antagonize convulsions induced by pentylenetetrazol, picrotoxin, bicuculline, and strychnine, but actually has some proconvulsant activity—that is, it enhances the effect of several convulsive treatments (*5*). Both β-CCE and Ro 15-1788 can occupy benzodiazepine recep-

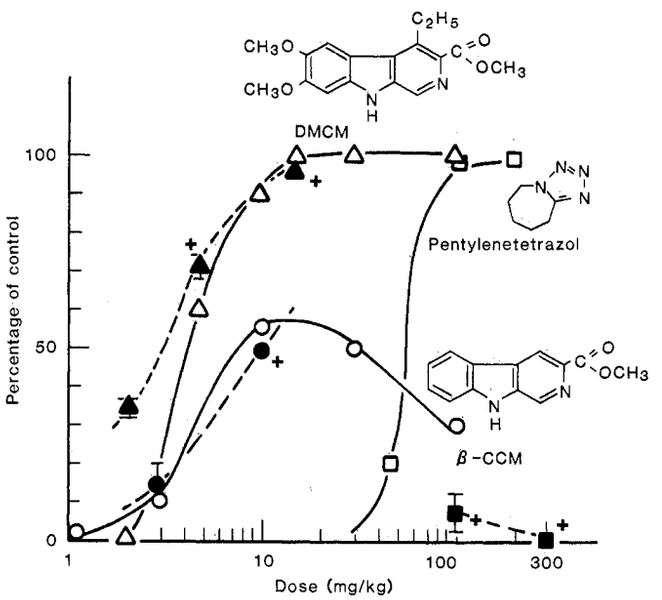

Fig. 1. Dose-related induction of clonic convulsions in male mice by DMCM (△), β-CCM (○) given intraperitoneally, and pentylenetetrazol (□) given subcutaneously. Relation to benzodiazepine receptor occupancy in vivo (filled symbols) as measured by inhibition of specific binding of [^{3}H]flunitrazepam mainly according to Chang and Snyder (*20*). DMCM and β-CCM were administered 15 minutes before decapitation, and pentylenetetrazol was administered 30 minutes before. Since binding of [^{3}H]flunitrazepam to receptors could not be determined in convulsing animals, we pretreated some animals (+) with phenobarbital (200 mg/kg orally, 45 minutes before decapitation). This dose of phenobarbital sodium did not by itself affect specific [^{3}H]flunitrazepam binding (data not shown). Vertical bars denote standard error of the mean (S.E.M.) values which extended outside the symbol.

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