

USING guinea-pig isolated whole brain preparation *in vitro*, synaptic responses to electrical stimulation of auditory nerves were examined in intracellularly recorded and stained neurons of posteroventral and dorsal divisions of the cochlear nucleus. Stimulation of the contralateral auditory nerve evoked exclusively IPSPs in 70% of neurons, with amplitude of 2.3 ± 1.2 mV. Neurons of all major cell types were inhibited from the contralateral side. In the majority of responding cells (78%) IPSPs were induced at latencies of 3–9 ms suggesting di- and trisynaptic connections from contralateral auditory afferents or, respectively, mono- and disynaptic connections from the contralateral cochlear nucleus. Few cells responded with long-latency IPSPs (13.5–23 ms), indicating involvement of polysynaptic pathways. These data demonstrate the existence of functional, direct and indirect inhibitory connections between the cochlear nuclei. *NeuroReport* 10:1913–1917 © 1999 Lippincott Williams & Wilkins.

Key words: Cochlear nucleus; EPSP; Intracellular recording and staining; IPSP; Isolated whole brain; Synaptic potentials

Inhibitory synaptic interactions between cochlear nuclei: evidence from an *in vitro* whole brain study

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Introduction

Interactions between the cochlear nuclei (CN) might represent the first level of processing of auditory information relevant for binaural hearing. Anatomical studies have demonstrated the existence of direct commissural projections between the CN [1–8]. Since a substantial proportion of these projections appeared to be glycinergic [3,8–10], the crossed pathways from one CN to the other were proposed to be inhibitory. Extracellularly recorded CN units, however, exhibited both inhibitory and excitatory responses to acoustic stimulation of the contralateral ear [11–16]. The question arises, therefore, as to the nature and precise pattern of synaptic processes underlying the influence of the contralateral auditory input on CN neurons, and to the identity of the target cells in the CN. Previous investigations using traditional approaches could not address these issues. In the present study, using the *in vitro*, isolated whole brain (IWB) preparation of the guinea-pig, we intracellularly recorded and stained individual CN neurons in which the effects of electrical stimulation of ipsilateral and contralateral

auditory nerves (AN) were tested. Thus, the IWB preparation allowed us to characterize functional synaptic inputs to identified CN cells from auditory afferents on both sides. While the detailed account of responses triggered by the ipsilateral AN stimulation will be presented elsewhere, this report is mainly dealing with the effects of the contralateral AN nerve stimulation on CN neurons. A part of these data has already been presented in an abstract form [17].

Materials and Methods

The methods used to isolate the brain and maintain it *in vitro* have been previously described in detail [18] and were similar to those originally employed by Mühlethaler *et al.* [19]. Briefly, guinea-pigs (body weight 150–300 g) were deeply anesthetized with a lethal dose of pentobarbital and perfused through the heart with cold (10–12°C) Ringer's solution for 5–6 min. The animal was then decapitated and the brain dissected from the skull with special care to avoid damaging the arterial network on the ventral surface of the brain. The isolated brain was trans-

ferred to the perfusion/recording chamber maintained at 12–13°C, submerged in saline, and fixed in place in a ventral side up position. One of the vertebral arteries was cannulated with a fine tube connected to the perfusion system and the other vertebral artery was ligated. Major leaks of perfusate from the brain arterial system were eliminated by ligating the arteries cut during the dissection (e.g., carotid and hypophyseal arteries). Finally, the brain was gradually (0.2°C/min) warmed to 29°C and the perfusion rate progressively increased from an initial 2–2.5 ml/min to 4.5–5 ml/min. The perfusion solution had the following composition (in mM): NaCl 126; NaHCO₃ 26; MgSO₄ 1.3; KH₂PO₄ 1.2; KCl 3; CaCl₂ 2.4; glucose 15; dextran (mol. wt 70 000, Macrodex, Sweden) 1.5%. It was continuously gassed with carbogene (95% O₂/5% CO₂).

Auditory nerves were stimulated with bipolar stainless steel electrodes (interpolated distance 0.5–1 mm) inserted in the cut edge of nerve stumps. Threshold stimulation intensities could be as low as 20–30 µA, whereas supramaximal intensities, at which all responses reached their maximal amplitudes, did not usually exceed 300–400 µA. These values were much smaller than those (1–1.2 mA) resulting in direct activation of CN neurons by current spread. Intracellular recordings from CN neurons were made by glass micropipettes filled with a solution of 1–3% neurobiotin (or biocytine) in 2 M K-acetate (resistance 120–200 MΩ). Neurons were stained by passing positive currents of 0.5–1.5 nA through the intracellular microelectrode for 5–26 min in a 200 ms on/off duty cycle. Conventional electrophysiological equipment was used for recording and storage of signals. Averaged quantitative data are expressed as mean ± s.d.

At the end of each experiment the brain was perfused with 4% paraformaldehyde. Injected cells were revealed on transverse brain sections (thickness 100 or 50 µm) using standard ABC histochemistry [20].

Results

The effects of the contralateral AN stimulation were tested in 33 cells located in the posteroventral (PVCN) and dorsal (DCN) divisions of the CN. The mean resting membrane potential of neurons was 62.8 ± 3.8 mV (range –52 to –70 mV). In response to ipsilateral AN stimulation, most of these cells (25 of 33) exhibited EPSP–IPSP sequences, seven cells exhibited only EPSPs, and one neuron displayed only an IPSP (see examples in Fig. 1A, Fig. 2A, top traces). The mean latency of EPSPs and the EPSP component of mixed responses was 1.33 ± 0.21 ms (range 1–1.9 ms; *n* = 30) suggesting

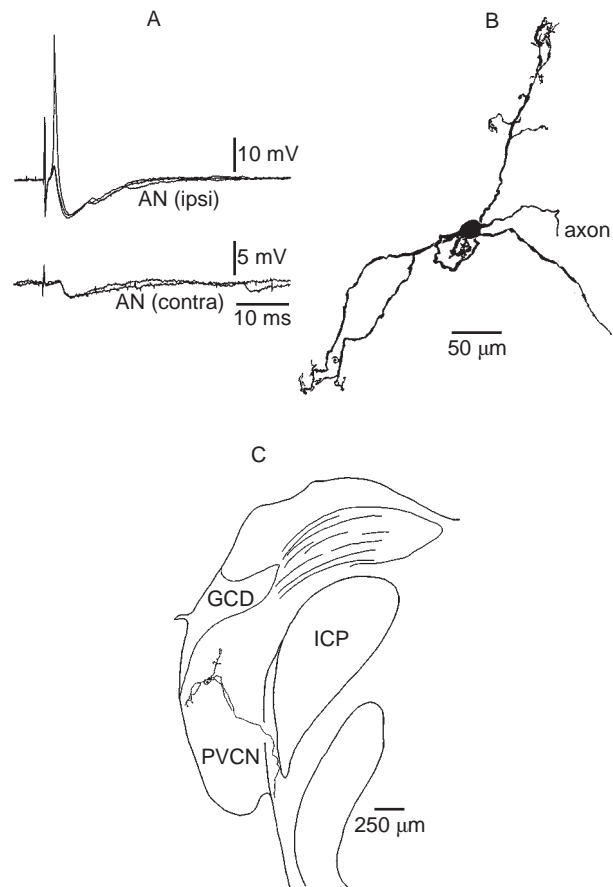


FIG. 1. Physiological and morphological characteristics of a stellate neuron in PVCN. (A) Intracellular recordings at a resting membrane potential of –61 mV. Stimulation of the ipsilateral AN evoked an EPSP–IPSP sequence and, at higher stimulation intensity, produced a spike superimposed on the EPSP (upper traces, AN (ipsi)). Contralateral AN stimulation induced only an IPSP (lower traces, AN (contra)). (B) High magnification reconstruction of the neuron whose location is shown on a transverse section of the CN (C). Note that the axon of the neuron is directed towards the ventral acoustic stria. Abbreviations: GCD, granule cell domain; ICP, inferior cerebellar peduncle.

monosynaptic activation of the majority of neurons from ipsilateral auditory afferents. Most of IPSP components of mixed responses and the pure IPSP had latencies of 3.09 ± 0.6 ms (range 2–4.2 ms; *n* = 23) compatible with disynaptic delays.

Recorded neurons belonged to the major cell types described in the PVCN and DCN [21]. Among 33 neurons, 15 were identified as stellate cells, six as octopus cells, three as giant cells, two as pyramidal cells, and one as a vertical cell. The remaining six cells were either not recovered (five neurons) or could not be assigned to defined cell categories (one neuron). Stimulation of the contralateral AN induced synaptic responses in 23 of 33 tested neurons (70%), whereas 10 cells were not affected. The only observed responses were hyperpolarizing post-synaptic potentials identified as IPSPs (see below). We did not find any evidence of

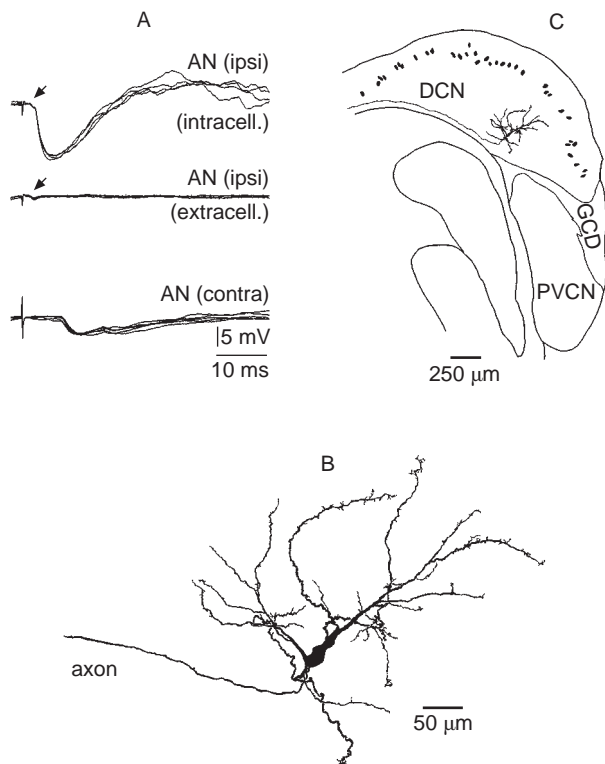


FIG. 2. Physiological and morphological characteristics of a giant neuron in DCN. (A) Intracellular recordings at a resting membrane potential of -62 mV. Stimulation of the ipsilateral AN evoked a large IPSP (AN (ipsi), intracell.). These records are accompanied by extracellular records (AN (ipsi), extracell.) to show that the small negative deflection at the beginning of the response (arrows) was due to the extracellular field potential. Contralateral AN stimulation induced a longer latency and smaller IPSP (lower traces, AN (contra)). (B) High magnification reconstruction of the neuron whose location is shown on a transverse section of the CN (C). Note that the cell axon is leaving the CN via the dorsal acoustic stria. Abbreviation: GCD, granule cell domain.

excitation induced from the contralateral side. Figure 1 shows an example of a stellate cell in the PVCN with its electrophysiological characteristics. Single shock stimulation of the contralateral AN induced an IPSP at a latency of 3.3 ms with approximate amplitude of 2.5 mV (Fig. 1A, lower traces). Another typical example of our recordings is illustrated in Fig. 2 for a giant cell, recorded and stained in the deep layer of the DCN. Activation of the contralateral AN evoked an IPSP with a latency of 8 ms and an amplitude of 4 mV (Fig. 2A, bottom traces). The observed hyperpolarizing responses (including those evoked from the ipsilateral side) were identified as IPSPs since they could be reduced in amplitude and, sometimes, even reversed by hyperpolarization of the cell membrane with currents passed through the intracellular electrodes (not shown).

Stimulation of the contralateral AN induced IPSPs in all types of neurons found in the present study. The distribution of IPSPs among cells was as follows: IPSPs were observed in 10 of 15 stellate

cells (67%), four of six octopus cells (67%), three of three giant cells (100%), one of one vertical cell (100%), one of two pyramidal cells (50%) and four of six unrecovered or unidentified cells (67%). The amplitudes of recorded IPSPs were relatively small. They ranged from 0.6 to 5 mV, and had a mean value of 2.3 ± 1.2 mV ($n = 23$).

The latencies of IPSPs evoked by contralateral AN stimulation were distributed in a wide range from 3 to 23 ms, with an average of 8.8 ± 5.8 ms ($n = 23$). The distributions of IPSP latencies for different types of CN neurons and for all recorded cells are presented in Fig. 3. The large majority of cells (18 of 23; 78%) responded with latencies ranging from 3 to 9 ms, whereas five neurons exhibited IPSPs with latencies of 13.5 ms or longer. Our previous studies of central vestibular networks, using the same preparation, demonstrated that the latencies of well established disynaptic and trisynaptic events partially overlapped and were in ranges of 2.1–4.4 and 4–7.7 ms, respectively [18]. In those experiments, the distances between the stimulation and recording sites were usually several millimeters shorter than in the present study. If we assume that some additional time would be required for action potentials to travel the longer pathways in the present experiments, then the observed IPSP latencies within the range 3–9 ms are compatible with di- and trisynaptic delays. Altogether, these results suggest that stimulation of the contralateral AN evoked mainly di- and trisynaptic IPSPs in the CN neurons. Since all primary auditory afferents terminate in the CN, it implies that di- and trisynaptic IPSPs would be respectively due to mono- and disynaptic inhibitory influences from the contralateral CN.

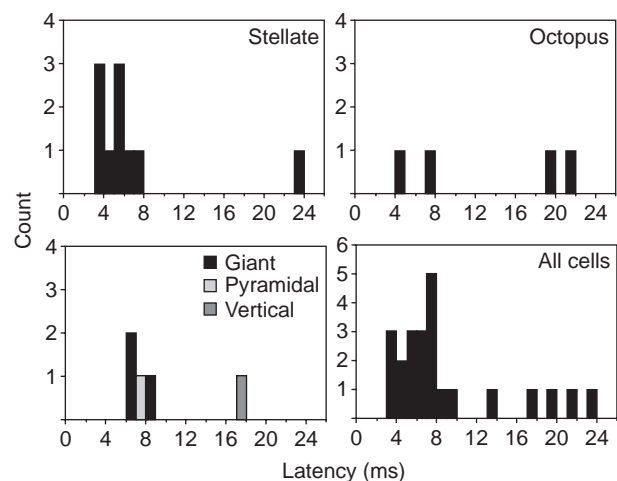


FIG. 3. Histograms of latency distributions of IPSPs produced by contralateral AN stimulation. Plots are for different types of CN neurons, and for all cells pooled together. Note that due to small sample size the latencies for giant, pyramidal and vertical cells were plotted together.

Discussion

The present study demonstrates the existence of functional inhibitory synaptic connections between the two CN. The cells of all major categories in the PVCN and DCN receive mono- and disynaptic inhibitory inputs from the contralateral CN. The monosynaptic inhibitory interactions between the two CN are consistent with morphological data showing direct commissural projections connecting two CN [1–8] and a predominant glycinergic nature of these projections [3,8–10]. A recent study in the guinea-pig has demonstrated boutons of anterogradely labelled commissural fibers, originating from the contralateral CN, in close apposition to neurons of all major cell types throughout the CN [6]. These observations are consistent with our results showing commissural inhibitory responses in most categories of cells of the PVCN and DCN.

In addition to direct monosynaptic inhibitory projections, our data suggest the existence of disynaptic connections between the two CN. Such connections might operate via superior olivary complexes (SOC) and/or through an additional synaptic relay formed between commissural axons and local inhibitory neurons within the CN itself. Both explanations are plausible. First, the possibility of disynaptic inhibitory connections between the two CN via SOC is supported by existence of well-known projections from the CN to SOC bilaterally and from the latter ones to the CN of the opposite side [22]. Furthermore, a large proportion of the projections from SOC on both sides to the CN was found to be glycinergic [9]. Second, anterogradely labelled commissural axons originating from the contralateral CN seem to contact not only principal cells in the CN, but also small cells including those in granule cell domain [6]. Such connections could serve as a neuronal substrate for disynaptic inhibitory interactions between the CN if we postulate that these small neurons (presumably inhibitory ones) could be excited by some excitatory commissural axons and exert inhibitory actions on neighbouring cells in the CN. Indeed, only a part of the population of commissural cells was shown to be glycinergic [3,8], leaving open the possibility that some of commissural axons are excitatory.

In a few CN cells, stimulation of the contralateral AN induced very long-latency IPSPs (13.5–23 ms), indicating their polysynaptic origin. These IPSPs could be mediated through additional synaptic relays at the brain stem level and/or at the level of higher auditory centers (such as the midbrain or cortex).

In the present study, stimulation of the contralateral AN evoked exclusively IPSPs in CN cells.

However, extracellular recordings from the CN have demonstrated both inhibition [11–14,16] and excitation [15,16] of neuronal activity associated with contralateral acoustic stimulation. The facilitation of neuronal activity observed in previous studies could be related to the complex nature and relatively long duration of sound stimuli, compared with single shock electrical stimulation of the AN. Indeed, the excitatory responses usually had much longer latencies [15] than the inhibitory ones [14]. Therefore, it is likely that these excitatory effects were mediated indirectly, through higher auditory centers, and reflected a long-lasting multisynaptic activity triggered by sounds. The discrepancy between our findings and previously observed short-latency excitation of CN neurons [15] could have the following explanation. As proposed above, the disynaptic inhibitory interactions between the CN could be partially mediated via small inhibitory neurons located within the CN and excited by some of commissural axons. Moreover, stable and lasting intracellular recordings are possible mainly from relatively large neurons, whereas extracellular recordings are possible from smaller units. Altogether then, it is possible that excitatory responses in the extracellular recordings were obtained from small neurons, which were not sampled in the present investigation. Alternative explanations would include species differences (guinea-pig in the present study; chinchilla and cat in previous ones) and local variations in recording sites in different studies.

In our study, recordings were restricted to the PVCN and DCN. Therefore the effects of the contralateral AN stimulation on bushy neurons, located mainly in the anteroventral division of the CN, were not assessed. Further experiments are needed to obtain this information in order to establish a more complete picture of synaptic interactions between the two CN.

Conclusion

Intracellular recordings from identified CN neurons in the IWB preparation of the guinea-pig allowed us to characterize, for the first time, the synaptic responses underlying the commissural interactions between the two CN. Stimulation of the contralateral AN induced mainly di- and trisynaptic IPSPs in various CN neurons, in turn implying mono- and disynaptic inhibitory connections between the two CN. A few neurons exhibited long-latency IPSPs mediated most likely through many synaptic relays. Our data indicate that the auditory pathways including distant projections and local circuits are preserved and remain functional in the IWB preparation. These advantages of IWB preparation,

as compared to brain slices, emphasize a significant research potential of the method for studies of the auditory system.

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ACKNOWLEDGEMENTS: This work was supported by grants and financial contributions from Swiss National Science Foundation (No.31-045731.95), CNRS (France), NIH/NIDCD (DC00232 and DC00979), Novartis Research Foundation and the Embassy of France in Switzerland. The experiments were conducted at the LNRS (Head: Dr P.-P. Vidal), CNRS (Paris). The authors would like to thank V. Moret and C. Roulin for their excellent technical assistance.

**Received 21 April 1999;
accepted 27 April 1999**