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RELATION BETWEEN INTRINSIC CONNECTIONS AND ISOFREQUENCY CONTOURS IN THE INFERIOR COLLICULUS OF THE BIG BROWN BAT, *Eptesicus fuscus*

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Abstract—Information processing in the inferior colliculus depends on interactions between ascending pathways and intrinsic circuitry, both of which exist within a functional tonotopic organization. To determine how local projections of neurons in the inferior colliculus are related to tonotopy, we placed a small iontophoretic injection of biodextran amine at a physiologically characterized location in the inferior colliculus. We then used electrophysiological recording to place a grid of small deposits of Chicago Sky Blue throughout the same frequency range to specify an isofrequency contour. Using three-dimensional computer reconstructions, we analyzed patterns of transport relative to the physiologically determined isofrequency contour to quantify the extent of the intrinsic connection lamina in all three dimensions. We also performed a quantitative analysis of the numbers of cells in different regions relative to the biodextran amine injection. Biodextran amine-labeled fibers were mainly located dorsomedial to the injection site, confined within the isofrequency contour, but biodextran amine-labeled cells were mainly located ventrolateral to the injection site. When we counted numbers of labeled cells classified by morphological type, we found that both elongate and multipolar cells were labeled within the isofrequency contour. Because the dendrites of multipolar cells typically extend outside the isofrequency lamina, it is likely that they receive input from other isofrequency contours and relay it to more dorsomedial portions of their specific isofrequency contour, along with the frequency-specific projections of the elongate cells. Within a given isofrequency contour, there is a consistent organization in which intrinsic connections ascend from the ventrolateral portion to more dorsomedial points along the contour, forming a cascaded system of intrinsic feedforward connections that seem ideally suited to provide the delay lines necessary to produce several forms of selectivity for temporal patterns in inferior colliculus neurons.

Key words: laminae, biodextran amine, electrophysiology, delay lines, multipolar cells, elongate cells.

The inferior colliculus (IC) is a major center for integration of auditory information. Some of the main sources of input to the IC include ascending projections from a variety of lower brainstem auditory nuclei, crossed projections from the contralateral IC, descending inputs from the auditory cortex, and intrinsic projections within the IC itself (Casseday et al., 2002). These projections all terminate within a structure that has a single clear tonotopic organization, as seen by electrophysiological recording, and a system of fibrodendritic laminae, as seen in anatomical studies. The two main hypotheses to be tested in this study were: 1) that the intrinsic projections would be confined within an electrophysiologically defined isofrequency lamina and, 2) that they would originate in a specific cell type.

The tonotopic organization of the IC and its laminar structure has been the focus of much research over the years. Separate studies using electrophysiology and anatomical mapping techniques such as connectional tracing, 2-deoxyglucose labeling, and c-fos mapping all show that the basic tonotopic organization is oriented in the same plane as the main fibrodendritic laminae (e.g. monkey, Fitzpatrick, 1975; ferret, Moore et al., 1983; cat, Serviere et al., 1984; bat, Pollak and Casseday, 1989; Casseday and Covey, 1992; guinea-pig, Malmierca et al., 1995, rat, Saldana and Merchan, 1992; Saldana et al., 1996; see Caird et al., 1991 and Ehret, 1997, for reviews). Moreover, the intrinsic projections appear to follow the same general orientation as the fibrodendritic laminae. However, the question remains of how the tonotopic map, as determined electrophysiologically, is correlated with the anatomically determined fibrodendritic laminae, especially with regard to the intrinsic connections. Therefore, we combined tracer techniques with electrophysiology in the IC of the big brown bat to determine the relationship of intrinsic connections to tonotopy and anatomy within the IC.

The morphology of neurons in the IC has previously been described using methods of Golgi-impregnation, Nissl staining, and horseradish peroxidase (HRP) labeling (Fitzpatrick, 1975; Malmierca et al., 1993; Oliver et al., 1991; Oliver and Morest, 1984; Zook and Casseday, 1982; Zook et al., 1985). Intracellular HRP labeling has further defined cell morphology (Peruzzi et al., 2000; Poon et al., 1990). Two general classes of neurons have been described, elongate (disk-shaped) cells and multipolar (stellate) cells. To determine the extent to which the two cell types contribute to the intrinsic projections and to examine their relation to the overall laminar shape and tonotopy, we categorized labeled cell types within the fibrodendritic laminae and quantified their distribution patterns within the IC.

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Abbreviations: BDA, biodextran amine; CSB, Chicago Sky Blue; HRP, horseradish peroxidase; IC, inferior colliculus; MW, molecular weight; PBS, phosphate-buffered saline; SAM, sinusoidal amplitude modulation; SFM, sinusoidal frequency modulations; TDT, Tucker Davis Technologies.

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EXPERIMENTAL PROCEDURES

Surgical procedures

Nineteen big brown bats (*Eptesicus fuscus*), bred in captivity in the laboratory, were used in this study. To prepare a bat for electrophysiological recording, a small stainless steel post was attached to the skull. The bat was anesthetized with isoflurane (inhalation, 2–4% at 1 l/min, Abbot Laboratories, North Chicago, IL, USA) and given a general analgesic (buprenorphine hydrochloride, 0.025 mg/kg, Reckitt Benckiser Pharmaceuticals, VA, USA). The head was immobilized by placing the animal in a molded bite bar with a modified isoflurane mask for continuous anesthesia. The bite bar was attached to manipulators that allowed the head to be placed in a standard position. The hair overlying the scalp was removed and the skin scrubbed with surgical scrub. Local analgesic (bupivacaine, 0.6 mg/kg, Abbot Laboratories) was administered before making a midline incision in the scalp. The temporal muscles were reflected, and the skull was cleaned of any remaining tissue. The post was glued to the skull overlying the dorsal surface of the cortex using cyanoacrylate gel adhesive and a liquid acrylic hardener. A chlorided silver wire was placed under the temporal musculature to serve as a reference electrode. Recording began 1–4 days after surgery. Before recording, each bat was given a s.c. injection of a neuroleptic (19.1 mg/kg fentanyl citrate/droperidol mixture, Abbott Laboratories). Bats were then placed in a foam-lined body restraint that was suspended in a flexible sling supported by springs within a stereotaxic frame (ASI Instruments, Warren, MI, USA) mounted atop a floating vibration table (TMC, Inc., Peabody, MA, USA), and the head post was clamped in a customized holder mounted on a stereotaxic micromanipulator (David Kopf Instruments, Tujunga, CA, USA). A small opening was made in the skull and in the dura overlying the IC for insertion of the recording electrode. Each bat was used in 1–4 recording sessions lasting ~6 h/day. Experiments were terminated if the bat showed any sign of discomfort. Between sessions, the wound was covered with Gel foam and coated with Neosporin. Bats were housed in individual cages in a temperature- and humidity-controlled environment and were given *ad libitum* access to food and water. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee and were carried out in accordance with guidelines published by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were employed to minimize the use of animals and their suffering.

Acoustic stimuli

The apparatus and procedures for delivering acoustic stimuli have been previously described by Faure et al. (2003). Sound stimuli were generated and controlled by Tucker Davis Technologies (TDT) System 2 hardware (Alachua, FL, USA) and custom designed software. All stimuli were presented monaurally contralateral to the recording IC from a Brüel & Kjaer (Norcross, GA, USA) type 4135 1/4-inch condenser microphone modified for use as a loudspeaker with a circuit to correct for nonlinearities in the transfer function (Frederiksen, 1977). Sound was presented as pure tones of short durations (3–7 ms) and downward frequency sweeps with a depth of 10 kHz and durations between 10 and 20 ms. A range of search frequencies covering the main part of the audible range for *Eptesicus* was presented as the electrode was advanced dorsoventrally through the IC (Casseday and Covey, 1992; Poon et al., 1990).

Electrophysiology

Sound-evoked responses were recorded during experiments in which we made injections of biodextran amine (BDA) and Chicago Sky Blue (CSB). Neural responses were recorded with glass micropipettes filled with one of the following solutions: 5–10% BDA (3000 MW and 10,000 MW, Molecular Probes, Inc., Eugene, OR, in 0.9% sterile saline), or a 2.5% solution of CSB (Sigma Aldrich, St. Louis, MO, USA) in 0.5 M sodium acetate. The tip diameters of the electrodes ranged from ~1–10 μm with impedances that varied from 10–25 MΩ. Electrodes were visually aimed at the IC, which in bats extends to the dorsal surface of the brain and is visible through the skull. Electrodes were advanced with a stepping hydraulic micropositioner (David Kopf Instruments model 650). Action potentials were recorded with a Neuroprobe amplifier (A-M Systems model 1600, Sequim, WA, USA), the 10× output of which was further amplified, bandpass-filtered (TDT PC1; filter cutoff frequency, 700 Hz and 3 kHz), and passed through a spike discriminator (TDT SD1). Spikes were monitored audiovisually to determine best frequency at the recording site. When a single unit or group of units was isolated, we conducted routine tests to determine whether it responded best to pure tones, noise, or frequency sweeps. If the unit(s) responded to pure tones, the best frequency and threshold were determined. If the strongest response was to sweeps, we determined the best sweep depth, response frequency range, and threshold.

Injection of tracers

Once a specific frequency band was identified physiologically, an iontophoretic injection of BDA was placed (4 μA positive pulsed current for 5 min) at that location. A combination of two types of BDA (3000 MW and 10,000 MW) was used in order to achieve maximum transport in both anterograde and retrograde directions. Injections were placed in different isofrequency contours in different animals, and were variously placed in dorsomedial, central, and ventrolateral locations within the contour.

After a 4 to 8 day survival period we electrophysiologically mapped the isofrequency contour containing the BDA injection in all dimensions and placed a grid of CSB deposits at sites where neurons were tuned to the same frequency as those at the BDA injection (4 μA negative pulsed current for 5 min). CSB injections were placed approximately 200 μm apart, except to avoid obvious arterioles on the surface of the brain. The target frequency for all of the CSB injections in a given animal was the same as the frequency to which neurons at the BDA injection site responded. For injections made early on in the project, the injection of the tracer or dye was made as soon as the target frequency was reached. In later experiments, the determination of where to place the injection was made by noting the depth at which a target frequency was first encountered and the depth at which it was last encountered, then backtracking to the halfway point to make the injection at the center of the isofrequency contour. The total number of CSB injections placed in each animal varied between eight and 17. The mapping procedure was performed in nine animals.

Histology

Once the CSB injections were completed, animals were given a lethal dose of sodium pentobarbital (Abbott Laboratories) and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline solution (PBS) overnight, and the next day 30 or 40 μm sections were cut on a freezing microtome and placed into 0.1 M PBS for histology. Tissue was soaked in 0.3% Triton X-100 in 0.1 M PBS for 30 min followed by three PBS rinses. Tissue was then soaked in an avidin/biotin solution (ABC, Vector Laboratories, Burlingame, CA, USA) for 1.5 h followed by one rinse in 0.1 M PBS and two rinses in 0.1 M phosphate buffer. To visualize the BDA injections, sections were reacted with a 0.05% 3,3 diaminobenzidine tetrahydrochloride solution (Sigma, with 0.003% hydrogen peroxide in 0.1 M phosphate buffer). Sections were mounted onto chrome-alum slides, air-dried, dehydrated in ethanol and xylene, and then coverslipped for subsequent microscopic analysis.