Stimulus-dependent changes in optical responses of the dorsal cochlear nucleus using voltage-sensitive dye

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Licari FG, Shkoukani M, Kaltenbach JA. Stimulus-dependent changes in optical responses of the dorsal cochlear nucleus using voltage-sensitive dye. J Neurophysiol 106: 421–436, 2011. First published May 4, 2011; doi:10.1152/jn.00982.2010.—Optical imaging with voltage-sensitive dye was used to examine the spatiotemporal dynamics of stimulus-driven activity on the surface of the dorsal cochlear nucleus (DCN). Stimulation with tones at low to moderate levels produced localized regions of activation that were most commonly elongated rostrocaudally. The size of these activation areas expanded with increases in sound level, while their centers shifted from the lateral direction to the medial direction with increases in stimulus frequency. In contrast to the tonotopic patterns of activation evoked by tones, electrical stimulation of the DCN surface resulted in bands of activation that were elongated along the medial-lateral axis; response latencies increased with distance along these bands from the point of stimulation. Shifting the site of electrical stimulation from the rostral direction to the caudal direction induced corresponding shifts in the rostrocaudal location of the activation band; moving the electrode tip to subsurface depths resulted in loss of the elongated band. Transecting the DCN along the rostrocaudal axis midway between its medial and lateral extremities blocked propagation of the response to the half of the DCN distal to but not proximal to the stimulating electrode. The results suggest that the two modes of stimulation activated two distinct populations of neurons, one involving primarily tonotopically organized cells and the other crossing these tonotopic zones and likely representing the activation of parallel fibers. These results reveal a number of new features in the spatial patterns of tone-elicted activation that are not readily predicted by responses recorded electrophysiologically.

optical imaging; JPW-1114; tonotopic organization; parallel fibers

NEURONS IN THE AUDITORY CENTERS of the brain are organized functionally around the principle of tonotopy, according to which sounds of differing frequency are represented by the place of activation across the neuronal population. Historically, this organization has been demonstrated by mapping the frequency tuning properties of neurons or small groups of neurons along a coordinate grid that spans the thickness or width of a given auditory center. The best known examples have come from recordings of the auditory cortex (Harrison et al. 2000; Kim et al. 2006; Nelken et al. 2008; Song et al. 2006), although tonotopic maps for subcortical auditory centers have also been well described (Snyder et al. 2000; Malmierca et al. 2008; Kaltenbach and Lazor 1991; Spirou et al. 1993; Wenstrup 1999). While electrophysiologically defined maps using microelectrodes have provided some details of this organization, the distribution pattern and time course of activity across the tonotopic array evoked by even the simplest stimuli are difficult to characterize from electrophysiological data and have rarely been reported.

There has been increasing interest in the use of optical imaging technology to examine the spatial and temporal patterns of stimulus-driven activity in sensory systems (Zecevic et al. 2003; Grinvald et al. 1999; Chemnia and Chavane 2010). Some progress in using optical imaging to map auditory responses has been made by imaging intrinsic signals (Versnel et al. 2002; Harrison et al. 1998; Harel et al. 2000; Kalatsky et al. 2005 Ojima et al. 2005; Tsytserav and Tanaka 2002). These include signals generated by changes in the metabolic or vascular properties of tissue, changes in cell volume, or changes in the oxidative state of intrinsic molecules. By nature, these signals are extremely weak and follow slow time courses. Consequently, the images of auditory responses obtained by this method have generally required time scales on the order of several hundred milliseconds or seconds of stimulation and typically many minutes of response averaging before an interpretable result is obtained. Furthermore, these signals are only an indirect reflection of neuronal activity. For these reasons, investigations of auditory responses that use optical imaging technology increasingly rely on the use of voltage-sensitive dyes (VSDs) (Nishimura et al. 2006, 2007; Hosokawa et al. 2004; Horikawa et al. 2001). These dyes are potentiometric, acting as molecular transducers, converting neural potential changes of neurons and neighboring glial cells to changes in the optical signal level (Cohen and Salzberg 1978). The strength of these signal changes is a function of the dye’s peak sensitivity to incident light, which normally occurs over a restricted band of wavelengths, but the additional changes in membrane potential caused by neuronal activation produce subtle shifts in the dye’s fluorescent emission spectrum, which can now be detected with submillisecond time resolution.

Our laboratory has been researching the use of the watersoluble dye, JPW-1114, for imaging stimulus-evoked activity on the surface of the dorsal cochlear nucleus (DCN). In many species, the DCN is ideally suited for surface imaging owing to its near-horizontal orientation on the brain stem surface and the fact that electrophysiological studies have revealed a wide representation of the audible frequency spectrum that is spread over its surface (Kaltenbach and Lazor 1991; Meleca et al. 1997). In addition, microelectrode recordings have demonstrated surface potentials that are robust. For example, the signal-to-noise ratios of multiunit voltages evoked by acoustic stimuli are typically more than five times higher than those of the voltages of background spontaneous activity (Kaltenbach and McCaslin 1996; Kaltenbach and Afman 2000). Since

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VSD-dependent signals increase with the size of the voltage changes, optical responses of the DCN have proven to be particularly strong and amenable to systematic study with variations in parameters of stimulation.

On the other hand, optically recorded signals based on VSD do not necessarily have exclusively neuronal origins, and the neural components may differ from those represented in single and multiunit recordings. Unlike single and multiunit recordings, which reflect primarily spiking activity of neuronal somata, VSD signals can have multiple sources, including neurons, glia, and vascular and extravascular fluid compartments. Moreover, the neuronal contributions to VSD signals often reflect activity originating from neuronal dendrites rather than their somata, so significant differences are sometimes found between the responses of neural tissue recorded optically and those recorded electrophysiologically. In other cases, or even at the same time in the same preparation, close correspondences have been found between VSD-generated signals and averaged spiking activity recorded electrophysiologically, but the extent to which these different types of signals predict each other depends not only on the dye but also on the properties of underlying cellular elements, all of which vary with each specific preparation.

Previously, we were able to show good agreement between the location of the peaks (or so-called epicenters) of the optically defined activation areas in the DCN and the characteristic frequencies (CFs) of neuron clusters defined electrophysiologically based on recordings on the DCN surface (Kaltenbach and Zhang 2004). In the present study, we performed a more systematic study of tone-elicited and electrically evoked responses of the DCN using VSD-based optical imaging technology. This investigation characterized how the spatial and temporal patterns of these responses were affected by changes in stimulus frequency, intensity, and duration. We also examined the patterns of activation evoked by stimulation of the surface of the DCN molecular layer using surface electrodes. The results provide a means of differentiating activity patterns in two major components of DCN circuitry. The results also show how activation throughout the neural population is distributed spatially across the tonotopic range and how this activation changes over time throughout the course of steady-state and dynamically changing stimulation. Finally, we were able to show significant similarities and differences between optically and electrophysiologically recorded responses to sound in the DCN.

METHODS

Animal subjects and surgical procedures. Male Syrian golden hamsters, 70–90 days upon arrival, were obtained from Charles River Laboratories. The care and use of animals for this study were approved by the Institutional Animal Care and Use Committee in accordance with federal, state, and local regulations. Animals were anesthetized intramuscularly with a combination of ketamine (117 mg/ml) and xylazine (18 mg/kg). The depth of anesthesia was achieved when the toe withdrawal reflex was no longer observed and breathing became stable. The animal was placed inside a sound attenuation booth (Industrial Acoustics). A tracheotomy was performed, and the animal was transferred to a platform on which the head was positioned and held firmly using a head brace. An occipital craniotomy was performed, and the cerebellum was partially aspirated along with associated portions of the choroid plexus to reveal the DCN and surrounding brain stem. Special care was taken to avoid damage to local blood vessels on the floor of the IVth ventricle that could affect the functional integrity of the DCN.

Throughout the surgical procedure and during recordings, the temperature was continuously monitored by a rectal probe and maintained at 37°C by automatic adjustment of a warming pad (TCAT-2DF, Physitemp) placed beneath the animal. The heart rate was monitored using a cardiotachometer (CT1000, CWM Industries), and the breathing rate was visually monitored to ensure that the proper level of anesthesia was maintained. To ensure stability of anesthesia, supplements of ketamine-xylazine were given intramuscularly either when heart rate increased above 240 beats/min or breathing rate exceeded 23 breaths/min; typically, one of these signs became apparent every 30–45 min.

Dye application. The styryl VSP JPW-1114, also known as di-2-ANEPEQ (Invitrogen), was prepared in a solution of 500 μl artificial cerebrospinal fluid at a pH of 7.4 and stored at 2–6°C. Before application, the dye was slowly warmed to physiological temperature. The DCN and neighboring regions of the brain stem were flooded with ~0.1 ml of the dye solution. Fresh solution was applied every 20 min over a period of 60–90 min until adequate staining was achieved.

Optics. For dye excitation, either a 120-W metal halide or halogen light source (Xelite 120, Exfo, Moritex) was used. The DCN was illuminated from the light source using a liquid light guide attached to a stereomicroscope (Leica MZ16F) modified for epifluorescence imaging using triplebeam technology. This microscope was equipped with a Plan ×0.8 LWD M series objective with a working distance of 50–130 mm and a numerical aperture of 0.71–0.113, depending on zoom position. The excitation beam passed through a 530 (~10)-nm filter while the emitted fluorescence was passed through a ~600–nm long-pass filter (Leica FluolIII). These optics enabled us to view the surface of the DCN, which spans roughly 1.5 mm along the medio-lateral axis and 1.0 mm along the rostrocaudal axis. The maximal thickness of the DCN across this range is ~0.4 mm.

Acoustic stimulation. Sound stimuli were generated by a National Instruments signal generation unit (PCle-6251) or a programmable function generator (Hewlett Packard 3325A). The produced signals were amplified and applied to a Beyer Dynamic (DT48) speaker. At the beginning of each experiment, the intensities of acoustic stimuli were calibrated using a B&K ¼-in. microphone inserted into a conical tube that was used to focus the sound down to the animal’s concha. Customized MATLAB software was used to adjust the voltage input to the speaker to allow stimulation of the ear at the desired sound levels.

To quantify how responses to sound were affected by changes in stimulus conditions, some of the stimulus parameters was varied while holding the other two constant. After measurement of the response to the first stimulus condition, the parameter being tested was changed, and the measurement was performed again. This process was repeated until a set of responses was obtained over a range of stimulus conditions. Generally, the effects of varying only one stimulus parameter were examined in a given animal. Variations in sound intensity were carried out with a 10-kHz tone with intensities varied between 6 and 96 dB or 6 and 120 dB sound pressure level (SPL); durations were held constant at values of 250, 500, or 1,500 ms. Variations in stimulus duration were studied with a 10-kHz tone (84 dB SPL) with durations varying between 25 and 3,000 ms. This level was selected to ensure that the responses would be robust and would require less averaging to define the spatial boundaries of the response. Responses to sounds of varying frequency were examined by imaging the DCN during a succession of tones increased in frequency from 3 to 32 kHz, while holding intensity (84 dB SPL) and duration (200–250 ms) constant. Most experiments were carried out in the left DCN, although in a few cases the right DCN was studied for comparison.

Electrophysiological recordings. The methods used for recording tuning properties and CFs as well as for mapping activity profiles along the medial-lateral axis were the same as those described previously (Kaltenbach and Lazor 1991; Kaltenbach and Afman 2000).
Electrical stimulation. Electrical stimuli were applied to the surface of the DCN using glass micropipette electrodes with impedances ranging between 1.2 and 1.6 MΩ. The temporal parameters of the electrical stimuli were shaped using MiCAM data-acquisition software, and the pulse train was applied to an electrical stimulus isolator (SIU91A, Cygnus Technologies). This device was used to control the current level, which was varied between 75 and 225 μA, comparable to the range used in a previous study in which current stimuli were applied to the DCN surface in vivo (Davis et al. 1996). A typical pulse train used for this study was 50 pulses, each with a duration of 100 μs followed by an interpulse interval of 2 ms. This stimulus condition was found to be effective in evoking sustained responses that could be imaged throughout the stimulus period with minimal loss of signal. The total acquisition time for this set of experiments was 1 s (0.5-ms frame time, 2,048 frames), and between 15 and 32 acquisitions were averaged.

Image acquisition. A CMOS camera equipped with a 100 × 100 photodiode array (Miacam 01 and Miacam Ultima, Shimadzu, Tokyo, Japan) was mounted at the top of the microscope column, far above the DCN surface, and was used for imaging. Image sequences (i.e., movies) were collected using acquisition software custom designed for the MiCAM camera. In each stimulus cycle, image collection was programmed to begin 200 ms after opening of a light shutter for passage of the fluorescent light used to excite the VSD. This delay was necessary to avoid light and sound artifacts during the shutter opening period. The temporal resolution of the image sequence was determined by the frame time, which was varied between 0.2 and 16.0 ms. Image sequences most commonly consisted of between 512 and 2,048 frames and lasted between 1.5 and 17 s. The image sequence for each stimulus cycle consisted of a 200-ms prestimulus period followed by a period of stimulation and a poststimulus recovery period. The durations of the stimulus and poststimulus periods were varied depending on the experiment. Responses to tones were often apparent after a single stimulus presentation, but it was common to average responses to repetitive stimuli to reduce noise. Captured images were downloaded to an image processor that contained circuits for noise reduction and further signal amplification. Images were background corrected by subtracting an averaged image of the DCN taken in the absence of an acoustic stimulus from each frame in an image sequence obtained in the presence of a stimulus. The resulting postsubtraction image sequences were stored on a hard drive for offline analysis.

Image processing. Saved image sequences were analyzed offline using MiCAM image-analysis software (BV-analyzer) and customized software developed in MATLAB. Image sequences were normalized to correct for regional variations in background fluorescence (F0) by computing ΔF/F signals. Optical signal strengths were color encoded for each pixel in an image frame. The activation areas were sometimes smoothed by averaging images with 3 × 3 Gaussian spatial and lowpass filters. In some cases, the sequences were also temporally averaged using a four-frame sliding window filter. However, in cases where the images were smoothed, the comparisons were also made with unsmoothed (raw) images to ensure that the smoothing process did not distort the activation areas or introduce exaggerated hot spots of activation that were not consistent with the spatial and temporal features of the raw response patterns.

Histology. After optical recording sessions, selected animals were used for histological analysis to determine the depth and distribution of stain with VSD. These animals were perfused transcardially with 4% paraformaldehyde while under anesthesia. The fixed brain in each animal was removed, placed overnight in the same fixative, and then transferred to a 30% sucrose solution. After 24 h, the brain was sectioned at a thickness of 38 μm in transverse planes that spanned the full rostrocaudal extent of the cochlear nucleus. The stained sections were mounted on slides (Plus Gold), allowed to dry, and then examined with fluorescent illumination using an Olympus BX61 microscope. Excitation and emission wavelengths were similar to those used to perform optical imaging experiments. Images of sections that included the ipsilateral and contralateral DCN, posteroventral cochlear nucleus (PVCN), and anteroventral cochlear nucleus (AVCN) were obtained at magnifications from ×125 to ×20. Selected sections were then counterstained with thionin to highlight the principal cell laminae of the DCN and cytoarchitectural boundaries between the cochlear nucleus subdivisions.

RESULTS

General effects of steady-state tonal stimulation. Tonal stimulation evoked vigorous optical responses in the DCN that could be readily imaged with JPW-1114. When using frame times of >6 ms, clearly defined responses were evoked by single stimulus presentations without the need for averaging. With intermediate frame times (i.e., 1–2 ms) averaging of responses across 4–8 stimulus repetitions was necessary to clearly distinguish the response from surrounding background fluorescence. The shortest frame times (0.2–0.7 ms) required between 8 and 32 repetitions to define a response. The shapes of responding areas varied depending on stimulus parameters, but for low to moderate level stimuli (36–64 dB SPL), they typically assumed an oval shape whose axis of elongation was oriented approximately parallel to the rostrocaudal axis. An example of an activation area evoked by a moderate level 4-kHz tone is shown in Fig. 1A. Note that the activation area did not reach the rostral and caudal extremities of the DCN. This was expected since a previous electrophysiological study has shown very weak responses to sound in these areas (Kaltenbach and Lazor 1991). This may reflect a decline in density of responsive cells toward the rostral and caudal borders due to thinning of cell layers and/or downward curvature of the cell-sparse molecular layer. Within the activation area, there was a gradient of signal strengths, indicating that the level of excitation was variable. Low-pass and spatial filtering converted the map of activated pixels to a color-coded relief map (Fig. 1B andSupplemental Material, movie 1), with the peak response represented by red and baseline by green. This peak area is flanked by several different color bands representing decreasing levels of excitation. Figure 1C shows the plane in which the activation area was cut to view the level of activation as a function of distance along the medial-lateral axis. The resulting profile of activation is shown in Fig. 1D. The location of the maximal response or epicenter occurred at 20% of the distance from the lateral edge to the medial border of the DCN. Figure 1E shows the time course of the response at the epicenter, revealing how the optical signal intensity, corresponding to levels of excitation, varied before, during, and after the stimulus. We were unable to distinguish inhibition of activity below the background level (indicated by absence of dark purple) at the end of stimulation or in the spatial distribution of the response.

Depth of dye penetration. JPW-1114 emitted strong enough background fluorescence to be visible in brain sections examined under fluorescent illumination. Sections cut in the plane of the cochlear nuclei postmortem after an optical recording session revealed an intense fluorescent signal that was concentrated in the left DCN with little or no fluorescence observed in the DCN contralateral to the optical recordings (Fig. 2A). The dye signal was present throughout all layers of the DCN,

1 Supplemental Material for this article is available at the Journal of Neurophysiology website.
although staining was less intense in the molecular layer than in the ependyma and fusiform cell layer (100 and 200 μm below the DCN surface; Fig. 2, B–D). Within the fusiform cell layer, somata of large neurons (fusiform cells) were often stained at a lower intensity than the space between these cells (arrows in Fig. 2C). Staining was also higher in the outer half of the deep layer than the inner half bordering on the dorsal acoustic stria (Fig. 2, C and D). The maximum response levels are shown by the red bars, representing the response epicenter (pixels 15–18). E: time course of the optical response at the epicenter defined in D. A more complete record of the responses in A and B is shown in movie 1.
area of activation, as shown in Fig. 3A for the left DCN of a single animal. Here, an expansion of the activation area with stimulus level involved a radial spread of the area’s borders in all directions. At higher levels of stimulation, there was a tendency for the spread toward the medial direction to dominate over the spread in other directions. Thus, whereas at low to moderate levels of stimulation (<84 dB SPL) the patches of activation were elongated rostrocaudally and remained circumscribed on approximately the same locus, at high levels (>84 dB SPL) the activation areas were elongated medial laterally. The relationship between the area of activation and the stimulus level is shown quantitatively in Fig. 3A for animals in the square shown in the 3rd from the rostral-most section in A. Note the dense staining in the ipsilateral DCN and absence of stain in the PVCN. C: enlargement of the area shown in the rectangle in B. Large arrows point to large neurons, likely fusiform cells. Ep, ependyma; ML, molecular layer; FCL, fusiform cell layer; DL, deep layer; DAS, dorsal acoustic stria. D: optical intensity profile for JPW-1114 staining as a function of depth below the DCN surface along an axis radial to the DCN laminae (dashed line in C). The section shows the highest intensity of staining in the ependyma and fusiform cell layer.

Increasing the stimulus level also caused increases in the response amplitude. This effect is shown in Fig. 3, C–E. Figure 3C shows the topographic profiles of the responses to a 1,500-ms tone (10 kHz) over a stimulus range of 48–96 dB SPL. Note that the spread of the activation area toward the medial direction did not greatly shift the location of the peak or epicenter of the activation area (up arrow below abscissa) except at the highest level of stimulation (96 dB SPL), where a second peak of slightly higher amplitude occurred in the medial portion of the profile (down arrow). This pattern could have resulted from some degree of damage or fatigue at the peripheral receptor level, thus weakening the response at the original epicenter of the activation area. Figure 3D shows the increase in response strength during a series of tone bursts of similar frequency (10 kHz) that were serially increased from 6 to 120 dB SPL over a period of 25 s. The dynamic range of the response in this case was 72 dB, with increases in signal strength occurring between threshold (36 dB SPL) and saturation (108 dB SPL). This value is similar to the mean dynamic range of 70 dB of the optical responses averaged across animals (n = 3). The change in latency of the onset of the response as a function of stimulus level, based on time course plots similar to those in Fig. 3E, is shown in Fig. 3F. A tendency toward shorter response latencies was observed with increases in stimulus level; latencies decreased from a mean value of 85 ms at 48 dB SPL to a minimum of between 10 and 18 ms at 72 dB SPL and above.

Effect of stimulus duration changes. Examples of time- and duration-dependent effects of acoustic stimulation are shown in Fig. 4. For the relatively long-duration stimulus shown (1,000 ms), the growth of the response was biphasic, consisting of a steep rise in the response magnitude up to the 250-ms time point during stimulation, but this was followed by a more gradual increase until the response reached its maximal strength at stimulus offset. Regardless of stimulus duration, the response amplitude typically increased throughout the stimulus interval, reaching a peak near the end of the stimulus period (Fig. 4A). The increase in response amplitude was parallelized by an increase in the area of activation (top row of images). A low-amplitude response appears as a small circumscribed spot in the lateral region of the DCN shortly after stimulus onset.
The effects of stimulus duration changes were studied by holding stimulus frequency and intensity constant (10 kHz, 84 dB SPL) and measuring responses to tonal stimuli varied in duration from 5 to 3,000 ms (Fig. 4). The shortest stimulus eliciting detectable responses was 15 ms. Increases in tone duration beyond this 15-ms threshold caused corresponding increases in the duration of the response. Both the response amplitude and area activated by the stimulus also increased with stimulus duration (Fig. 4, B and C), although the more gradual second phase of the biphasic response observed for long-duration stimuli (1,000–3,000 ms) was absent from the response to shorter-duration stimuli (15–250 ms). The increase in the area of the response with stimulus duration resembled that observed with increases in stimulus intensity (Fig. 3A) except that the expansion did not show as much preferential spread toward the medial direction. Both increases in amplitude and area of activation were observed up to at least 1–2 s of stimulation and were usually robust. In the example shown in Fig. 4D, the increase in area was asymptotic above 2,000 ms.

After the tone offset, the response recovered toward baseline (Fig. 4B). The time to recovery increased with duration, such that for tones lasting <250 ms, recovery was almost complete within 200 ms after the tone offset; for longer-duration tones, recovery was more gradual and sometimes required several seconds before approaching baseline (data not shown). However, in the latter cases, the time required to recover to baseline levels could be shortened by lowering the light level of the excitation beam.

**Effects of frequency changes.** We characterized responses to 50 tone bursts swept in logarithmically spaced intervals from 3 to 32 kHz over periods of up to 32 s/image sequence. Both fast and slow rates of frequency change (2–10 frequencies/s) were tested, and the unaveraged responses to single sweeps as well as the averaged response to repetitive sweeps were examined. Typical examples of the frequency-dependent shift in the activation areas are shown in Fig. 5. The images in Fig. 5A were generated by averaging responses of the left DCN to a
A series of tones swept four times from low to high frequency at a rate of 3 frequencies/s. The activation area can be seen to shift systematically from the lateral to the medial direction in the series as the frequency was increased from 6 to 20 kHz (from the topmost to the bottommost image). Below 6 kHz, no responses could be detected, and above 20 kHz, the responses were usually too weak and too restricted in spatial extent to detect further changes in position with further increases in frequency. The weakening of signal strength at the frequency extremities may have resulted from attenuation of fluorescence due to the curvature of the DCN away from the horizontal plane in the medial (high frequency) and lateral (low frequency) regions (see Fig. 2D). The spatial profiles of activation (images in Fig. 5B), which were obtained by slicing through the three-dimensional projections of the activation areas (as shown in Fig. 1C), more precisely show the loci of the peak response (epicenter) for each frequency of stimulation. The images in Fig. 5C and movie 2 show a similar series for the right DCN of a different animal. This series is similar to that for the left DCN except that the direction of the shift in the response is a mirror image of that for the left DCN shown in Fig. 5A. This bilateral symmetry is further shown in Fig. 5, D and E, which compares the tonotopic gradients derived from the topographic profiles from the left and right DCNs, respectively.

Examining responses to tone sweeps also revealed substantial frequency-dependent changes in the size and shape of the activation areas. In the example shown in movie 2, the band of activation expanded from 7.1 to 7.8 kHz, contracted from 8.2 to 10.3 kHz, and then divided into two subareas at 10.8 kHz before rejoining at 12.5 kHz. In the example shown in movie 3, the response band at most frequencies was rostrocaudally elongated, but the band changed in its width and curvature as the tone was swept across the frequency range. It was initially...
suspected that such changes were artifacts of brain pulsations; however, further analysis failed to show a synchronization of any feature of these changes with the rate of pulsation. Moreover, as shown in the example in Fig. 6 and movie 3, responses to frequency sweeps repeated a few minutes apart showed much the same pattern. Although the precise borders of the activation areas varied, their general shapes were similar.

Tests of a possible hemodynamic origin of signals. Since changes in fluorescence can occur as a result of hemodynamic changes (e.g., changes in blood flow and changes in oxygenation state of hemoglobin), the possibility was considered that a significant component of the optical responses observed in this study may have been of intrinsic origin, independent of the VSD. To address this possibility, we first conducted an experiment in which the blue VSD RH-1691 was substituted for JPW-1114. RH-1691 has an excitation wavelength outside the absorption spectrum of hemoglobin, thus greatly decreasing the contribution of hemodynamic signals. We initially tested RH-1691 at a concentration of 1.0 mg/ml. This concentration caused noticeable phototoxic injury to the DCN, resulting in a loss of responsiveness to sound. However, responsiveness was clearly apparent when the concentration of RH-1691 was decreased to 0.5 mg/ml. At this concentration, we tested the responses of the DCN to three different frequencies of stimulation. The results are shown in Fig. 7A. As can be seen, the overall sizes, shapes, and locations of the activation areas obtained with RH-1691 for these stimulus frequencies did not differ appreciably from those shown in Fig. 5 for JPW-1114 and those obtained with the blue dye RH-1691. A more complete record of the responses in C is shown in movie 2.

We sought additional verification that our optical responses were dependent on dye transduction by examining responses to sound at different excitation wavelengths. If the observed responses were related to the VSD, then they should show changes in signal level that correspond to the excitation spectrum of the dye. The response should also be absent in...
unstained tissue. The spectrum of excitation for related styryl dyes are biphasic, showing a peak response in the 500- to 600-nm region, a zero crossing at 450–500 nm, and a change of sign below 450 nm (Xu and Loew 2003). We compared optical responses to tonal stimuli at two different excitation wavelengths, one at 535 nm and the other at 365. A comparison of the DCN response to tonal stimulation using filters for these two wavelengths is shown in Fig. 7B. A strong positive response was elicited with the 535-nm excitation filter, but a negative response was obtained using the 365-nm filter. Similar results were observed in two other animals in which this experiment was performed and further support the interpretation that the measured responses are VSD related and not of intrinsic origin.

A final test of a possible intrinsic origin of the signals contributing to our optical responses involved comparison of images of the DCN responding to sound before and after staining with JPW-1114. If the recorded optical signals have a significant intrinsic component, that component should be evident in images of the DCN in the absence of stain. As shown in Fig. 7C, no optical responses were observed in tissue that was not stained with JPW-1114. This indicates that any intrinsic signals that were present in the DCN during its response to sound were below the threshold of detection and thus did not contribute to the optical signals recorded with this dye.

Electrical stimulation of the molecular layer. The imaging of parallel fiber activation was attempted by examining the JPW-1114-stained DCN during local electrical stimulation of the DCN surface. Since parallel fibers run parallel to the DCN across isofrequency bands, it was expected that stimulation of these fibers would manifest as a band of activation orthogonal to those evoked by low to moderate level tones. To test this hypothesis, trains of 100-μs current pulses (50 pulses/train, 2-ms interpulse intervals) were delivered at levels of 75–225 μA through the tip of a micropipette placed in contact with the DCN surface. Stimulation with current above 150 μA produced widespread activation of the DCN as well as surrounding regions well beyond the DCN borders and extending onto the floor of the fourth ventricle, particularly in the region between the right and left DCNs. The current delivery site was very localized in these cases, and the areas responding with increased optical signal far exceeded the range of the intended target. Reducing the
Fig. 7. Tests of a possible contribution of intrinsic signals to the optical responses of the DCN. A: images of the DCN surface responding to tones of different frequencies after staining with the blue dye RH-1691, which is excited at wavelengths remote from the peak of absorption of hemoglobin. These yielded a similar tonotopic gradient as those obtained with JPW-1114 (see Fig. 5F). Tone durations were 500 ms. B: change in optical response polarity of the DCN to a 10-kHz tone using JPW-1114 when the excitation wavelength was shifted from 535 nm (left) to 365 nm (right). The change in sign of the response is consistent with the biphasic action spectrum of styryl dyes. C: comparison of responses of the DCN to a 10-kHz tone (1,500 ms) before (left) and after (right) staining with JPW-1114. Only subtle fluctuations in baseline activity, likely resulting from random variations in hemodynamic, volumetric, or vascular signals, were observed before and during the period of stimulation in the absence of dye. Intrinsic signals generated by these sources thus contributed negligibly to the optical response to stimulation observed after staining. Bars under graphs show periods of stimulation.

current level to 150 μA resulted in an increased optical signal that was confined to a narrow beam that extended across the medial-lateral length of the DCN, as shown in Fig. 8A and movie 4; optical responses outside the beam were very weak to nonexistent (see the time course plot below the images in Fig. 8B), such that the width of the beam between its peak amplitude and half its peak amplitude was 100–150 μm. Similar beams of activation were observed with other placements of the electrode tip along the rostrocaudal axis. The locations of the activation bands were highly dependent on the locations of the electrode tip along the rostrocaudal axis. The widths of the activation bands were highly dependent on the locations of the electrode tip along the rostrocaudal axis, although the beams shifted from the rostral (Fig. 8B) to the caudal borders of the DCN (Fig. 8D) with corresponding shifts in the site of stimulation. Throughout the duration of stimulation the beam varied in its continuity, sometimes breaking up into a string of islands in some frames and decreasing in its amplitude with distance from the stimulus electrode (compare Fig. 8, B with C). No bands of activation were observed with current pulses delivered to sites caudal to the DCN where there are no known auditory neurons. These features are consistent with the activation of bundles of parallel fibers in the molecular layer. This interpretation is further supported by the observation that conduction of optical responses along the beam could be blocked by transecting the molecular layer of the DCN along the rostrocaudal axis midway between the medial and lateral extremities of the DCN, so as to cut the projection of the parallel fibers (compare Fig. 8, E with F). Furthermore, the latency of the response increased with distance away from the stimulating electrode. At distances proximal to the stimulus site, minimum response latencies were 5 ms, but this increased with distance away from the stimulus electrode. Conduction velocities were in the range of 0.05–0.1 m/s. Moving the electrode to subsurface levels of the DCN resulted in loss of the beam-like appearance of the optical response and sometimes was replaced by a more circumscribed response similar to that observed in response to tonal stimulation.
Fig. 8. Activation patterns evoked by electrical stimuli applied to the DCN surface. A: a train of 50 pulses (100 μs/pulse, 2-ms interpulse intervals, 150 μA) delivered to the DCN at the point marked “X” produced an elongated band of activation that extended along the medial-lateral axis of the DCN. This pattern is consistent with the axis of projections of parallel fibers and contrasts with the tonotopic patterns of activation evoked by tonal stimulation (see Fig. 5). B–D: effect of changes in stimulus electrode location on response band location along the rostrocaudal axis. Moving the electrode caudally or rostrally resulted in corresponding shifts in the activation band along the rostrocaudal axis. The graphs show the time courses of activation at the XYZ locations indicated in the frames above. E and F: comparison of activation bands resulting from electrical stimulation in an intact DCN with those observed after a shallow cut was made in the DCN surface along the rostrocaudal axis at the level indicated by the upwardly pointing arrow in the bottom right image; the dashed line in the bottom left frame shows the corresponding axis in the intact DCN. Graphs above the images show the time course of the optical response at locations indicated by the smaller arrows. After the DCN was sectioned, the activation band extended only up to but not beyond the point where the DCN was cut. Scale bars in B–D = 200 μm.
Comparison with electrophysiological recordings. Comparisons of data obtained by optical imaging with those obtained by electrophysiological recordings are shown in Fig. 9. The spatial profile of optically defined activation seen in cross section compares very closely with the activity profile of multiunit responses to the same sound (Fig. 9A). Both show a peak at the same locus with sharp rolloffs toward the medial and lateral directions. The tonotopic gradients obtained by optical imaging also compared closely with the gradient based on mapping of CFs at multiple sites along the medial-lateral axis (Fig. 9B). Thus, in terms of tonotopicity, the optically defined responses retain the same basic characteristics as those yielded by electrophysiological recordings. An interesting contrast was observed in the comparison of the optically and electrophysiologically based input-output functions (Fig. 9C). The rate-intensity functions derived from recordings of multiunit responses to a 10-kHz tone had narrower dynamic ranges than those based on imaging. The dynamic range of units recorded from the 10-kHz locus were generally in the range of 40–50 dB, whereas those based on imaging averaged 70 dB. Thus, while the tonotopic specificity of the optical and electrophysiological responses are similar, it is clear that important differences also exist.

DISCUSSION

The results present, for the first time, the spatial patterns of activation evoked in the DCN by simple acoustic and electrical stimuli. They further show how these spatial patterns change temporally throughout stimulation and how they are affected by changes in stimulus parameters. Although the details of these responses were found to be sensitive to changes in incident light levels and changes in sampling rate (frame time), the general features of these responses showed a high degree of consistency across experiments. We now discuss what these changes reveal about the functional organization of the DCN, compare them with properties that have been characterized here and in previous studies using electrophysiological methods, and consider what they suggest about the likely underlying generators.

Differentiation of synaptic circuit components of the DCN by VSD imaging. Our results demonstrate a clear dichotomy between activation patterns evoked by acoustic stimuli and those evoked by electrical stimulation of the DCN surface. Over most of the range of intensities and durations tested, tonal stimulation elicited well-defined responses whose locations varied with the tone’s frequency. At low levels of stimulation (≤60 dB SPL), the area of activation was approximately oval or round, and when oval, the axis of elongation was most often oriented rostrocaudally. With increases in intensity up to ~84 dB SPL, this area expanded radially, often assuming a more rounded configuration, but as long as the frequency was not changed, the area remained approximately centered on the same locus (Fig. 3); elongation along the medial-lateral axis was not observed in the low to medium intensity range. When the frequency was increased while holding intensity constant, the locus of activation shifted dramatically from the lateral to the medial direction (Fig. 5). Both the rostrocaudal orientation of the activation area and the medial shift of the area’s center point with increases in frequency are consistent with the tonotopic gradient for the DCN inferred from electrophysiological data presented in Fig. 9A and B. The rate-intensity functions derived from recordings of multiunit responses to a 10-kHz tone had narrower dynamic ranges than those based on imaging. The dynamic range of units recorded from the 10-kHz locus were generally in the range of 40–50 dB, whereas those based on imaging averaged 70 dB. Thus, while the tonotopic specificity of the optical and electrophysiological responses are similar, it is clear that important differences also exist.

Fig. 9. Comparison of measures of DCN responses to tones based on optical and electrophysiological recordings. A: activity profiles plotting the magnitude of the response to a 10-kHz tone (84 dB SPL, 1,000 ms) along the medial-lateral axis at a location halfway between the rostral and caudal margins of the DCN. Both data sets are from the same animal. The optical response represents the average of eight stimulus repetitions at the time point of the maximal response. The electrophysiological data are based on measures of multiunit discharge rate with a 100-mV trigger level. Both sets of measures were normalized to the maximum response. B: tonotopic gradients defined optically, based on locations of response epicenters using 84 dB SPL tones (250-ms duration), and electrophysiologically, based on the distribution of characteristic frequencies. The numbers on the abscissa represent the stimulus frequency for imaged data and characteristic frequency for the electrophysiological data. These two data sets were from different individuals. C: growth of the optically (n = 2) and electrophysiologically (n = 5) characterized response amplitudes with increases in stimulus level. Each point represents the mean response to a 250-ms tone repeated eight times. The electrophysiological data were obtained within minutes after optical imaging.
logical recordings, although our results show for the first time how this activity is spatially spread across this gradient as well as within isofrequency bands.

In contrast, electrical stimulation of discrete points on the DCN surface elicited bands of activation that were narrow and extended lengthwise along the medial-lateral axis, orthogonal to the tonotopic areas evoked by tones. The rostrocaudal position of this activation band moved caudally when the tip of the stimulus electrode was moved toward the caudal border of the DCN. A different result was obtained when the stimulating electrode was moved to subsurface positions or when the electrode was placed 50–100 μm caudal to the DCN border. When current pulses were delivered at depths of 100 μm or more below the surface, the activation area became more tonotopic, resembling the shape and pattern of activation resulting from tonal stimulation. Stimulation of sites outside the DCN did not produce bands of activation. Taken together, these results suggest that stimulation of the DCN surface activated beams of parallel fibers while tonal stimuli and electrical stimuli delivered at subsurface levels activated cell populations whose input is tonotopic.

Nonuniformity of the tonotopic map. An intriguing aspect of our results was the changes in the size and shape of the activation areas that were observed over time during a stimulus presentation as well as in tests in which the frequency of the tonal stimulus was varied while holding intensity and duration constant. In the former case, activation started as a small area that expanded throughout the stimulus period, reaching its maximum size at the beginning of the stimulus offset (Fig. 4A). Although the elicited bands of activation tended to be elongated along the rostrocaudal axis, the specific shape of the band varied considerably with changes in frequency. Contractions, expansions, and/or bending of the bands or subregions of the bands were commonly encountered during the course of a frequency sweep. In some cases, a specific subregion of the band pinched off from the main band or condensed into one or two small islands of activation as the tone swept through a specific frequency range but then merged or recombined at higher frequencies. Some of these nonuniformities in the shape and size of the activation areas might reflect instabilities in the preparation due to the presence of tissue movements such as cardiac or respiratory pulsations or incompletely damped vibrations. However, such nonuniformities were commonly observed throughout the frequency sweep even when only low-level pulsations or vibrational noise was present in the record.

Moreover, in those cases wherein pulsatation or vibrational artifacts were present, the timing of the expansions and contractions were neither synchronized with nor dependent on any particular phase of the pulsation or vibrational noise. Finally, when the sweep was repeated the general shape of the activation area remained consistent, demonstrating reproducibility across stimulus trials.

We interpret these changes in the size and shape of activation areas with frequency as a reflection of irregularities and distortions of the tonotopic organization of the DCN. This interpretation is consistent with a number of observations that have been noted previously based on electrophysiological mapping studies in the hamster DCN. Kaltenbach and Lazor (1991) showed that isofrequency contour lines have irregular, wavy trajectories, but the shape of those trajectories are variable from one CF region to another. This could account for the general changes in the contours of the activation area observed in the present study. In electrophysiologically defined maps, CFs sometimes appear displaced from the locations expected from their neighboring CFs. Such “ectopic CFs” could account for the islands of activity that were sometimes observed to pinch off from the main band of activation. Electrophysiologically defined maps also reveal that certain frequencies appear as CF plateaus and discontinuities in the tonotopic gradient, particularly when CFs are mapped along a single row of recording sites that parallels the tonotopic axis (Meleca et al. 1997). The nonuniform nature of the frequency bands observed during a frequency sweep are thus in line with observations from electrophysiologically defined frequency maps of the DCN. Similar discontinuities and plateaus have recently been shown to exist in the tonotopic gradient of the inferior colliculus (Malmeier et al. 2008). The results thus suggest that the tonotopic organization of primary auditory centers has a more complex microstructure than commonly assumed.

Intrinsic signals are not major components of the optical signals. A possibility that must be considered is that our optical signals might have represented intrinsic responses of nonneuronal origin rather than shifts in the emission spectrum of the VSD caused by changes in neural membrane potentials. Intrinsic responses have been well characterized in numerous studies in other systems and include changes in the physical properties of the tissue (e.g., blood flow, blood volume, and cell volume) as well as changes in the oxidative state of hemoglobin (Grinvald et al. 1999; Devor et al. 2003, 2005) and other endogenous fluorophores (e.g., flavoproteins, serotonin, and tryptophan) that are involved in cell signaling and metabolic pathways (Lillard and Yeung 1997; Williams et al. 1999; Chance et al. 1962; Reinert et al. 2007; Dunbar et al. 2004). Changes in blood volume or blood flow in the DCN as the main factor underlying our optical responses seems dubious for several reasons. First, previous studies have shown that blood flow changes have onset response latencies on the order of 700–800 ms (Shoham et al. 1999; Grinvald et al. 1999), and measures of blood flow changes in the DCN during tonal stimulation are consistent with these findings (Quirk et al. 1996). In contrast, the optical responses in the present study had onset response latencies ranging between 10 and 85 ms. A hemodynamic origin of our optical responses is also doubtful in view of the similarity between the spatial patterns, time courses, and dependence on stimulus parameters obtained with JPW-1114 and those obtained with the blue dye RH-1691, whose excitation spectrum falls outside the peaks of the absorption spectrum of hemoglobin (Shoham et al. 1999; Lippert et al. 2007). Moreover, a clear reversal of response polarity occurred when the excitation wavelength was shifted from 535 to 365 nm (Fig. 7B); this property is consistent with the wavelengths at which positive and negative components of the action spectrum occur for styryl dyes (Xu and Loew 2003). And, finally, no responses to sound were evident when the DCN was imaged in the absence of dye staining. Thus, any intrinsic signals that may have been present were below the threshold of detectability at the gain setting used to image responses with dye.

Comparison between VSD-based properties and electrophysiological properties. Some of the optically defined stimulus-driven properties of DCN cell populations in the present

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The activation of DCN parallel fiber bundles in vivo can be compared with patterns of parallel fiber activation evoked in the DCN, based on studies conducted in vitro (Manis 1989; Manis and Molitor 1996; Molitor and Manis 1997). In these studies, compound action potentials were evoked in the molecular layer of the DCN by current pulses applied to the ependymal surface using a microwire electrode. The induced potentials propagated most strongly along the strial axis of the DCN, and these weakened sharply in amplitude along an axis orthogonal to the strial axis. In contrast, both single and multiunit recordings show minimum latencies of 3 ms (Joris and Smith 1998; Kaltenbach 1984; Godfrey et al. 1975; Starr 1965), rise times of a few milliseconds, and recovery rates that are nearly immediate (Godfrey et al. 1975). Finally, the optical responses did not reveal inhibitory effects, such as lateral inhibition, that have been defined electrophysiologically. The inhibitory components of the responses to tones are likely to have been smeared out by the summed responses of the many neurons contributing to the optical response.

Some of the differences just discussed may reflect contrast sources from which the measured signals arise. Whereas single and multiunit recordings are dominated by spiking activity originating from neuronal somata, VSD-based optical signals are often dominated by subthreshold postsynaptic potentials arising from dendritic processes (Ebner and Godfrey 1995; Grinvald et al. 1999), with additional contributions from extracellular K\(^+\) and potentials arising from glial activation (Manis and Freeman 1988; Konnerth et al. 1987, 1988). Most of the latter signal types have relatively low-frequency spectral content (<100 Hz), unlike spiking activity, which contains higher-frequency spectral components, from a few 100 Hz to 10 kHz. During single and multiunit recordings, the low-frequency potentials arising from dendrites and glia are usually removed by filtering. Such signals are uncovered by extending the filtering into the low-frequency range. Indeed, better agreement between optically and electrophysiologically recorded responses have sometimes been observed when the time courses of VSD-based signals recorded in vivo are compared with those of local field potentials (LFPs), which are dominated by low-frequency spectral components. For example, in the barrel cortex, the time course of optical responses to whisker deflection has been found to be almost identical to the time course of LFPs (Kim et al. 1989; Elias et al. 1993; Berger et al. 2007). One might infer from these considerations that the responses recorded in the DCN using VSD may not represent the same neural elements as those represented in single unit recordings but instead may represent predominantly dendritic responses or responses of glia. If this is correct, then optical imaging of the DCN using JPW-1114 has revealed the spatiotemporal patterns of predominantly nonspiking activity. In some ways, these patterns show close correspondence with those of spiking activity, but at the same time, important differences also exist that could reveal fundamentally important differences in the way these elements respond to sound.

A new model system for in vivo detection of parallel fiber activation. The activation of DCN parallel fiber bundles in vivo can be compared with patterns of parallel fiber activation evoked in the DCN, based on studies conducted in vitro (Manis 1989; Manis and Molitor 1996; Molitor and Manis 1997). In these studies, compound action potentials were evoked in the molecular layer of the DCN by current pulses applied to the ependymal surface using a microwire electrode. The induced potentials propagated most strongly along the strial axis of the DCN, and these weakened sharply in amplitude along an axis orthogonal to the strial axis. The beam width to half-maximal amplitude was reported to be 200 \(\mu\)m, while conduction velocities averaged 0.3 m/s. The optically evoked responses of parallel fibers observed in the present study were similar to those observed in vitro in that they extended along the medial-lateral axis of the DCN, which corresponds to the strial orientation of beams in the brain slice. Additionally, the width of the activated beam (100–150 \(\mu\)m) in the present study was not markedly different from the beam width of 200 \(\mu\)m reported by Manis (1989). A notable difference in our results was the slower conduction velocity of parallel fibers, which averaged between 0.05 and 0.1 m/s, markedly slower than that observed in vitro (0.3 m/s). Several factors could account for this difference. First, it is possible that the shorter latency response is of too small a magnitude to be detected optically owing to the weaker signal-to-noise ratio inherent in optical imaging. Higher noise due to background fluorescence substantially raises the baseline level of the optical signal and could mask the early phase of the optical response to stimuli. This disadvantage might be overcome by using dyes with higher signal-to-noise ratios. A second factor that could contribute to the more sluggish optical responses to parallel fiber activation is that the underlying signals may originate from glia and dendrites, whose subthreshold responses have slower time courses. Since parallel fibers exert synaptic effects on both cartwheel cells and fusiform cells (Wall et al. 1996; Manis and Molitor 1996; Davis et al. 1996; Fuijino and Oertel 2003; Zhao et al. 2009; Tzounopoulos et al. 2004), some component of the optical response may have originated from dendrites of these two cell types as well as their neighboring glial cells (astrocytes). A third possibility is that a slower conduction velocity could be in part related to the use of anesthetic in vivo. This possibility is raised by a recent report showing that various anesthetics, including ketamine-xylazine, have a slowing effect on conduction velocities of peripheral nerves (Oh et al. 2010). Despite these differences, our results demonstrate the ability to resolve the spatial and temporal patterns of parallel fiber activity with a high degree of spatial and temporal detail. This preparation may provide a useful model system for investigating the functional role of this pathway and its influence on abnormal patterns of activity that develop in the DCN in certain pathological states.
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DISCLOSURES

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