Independent tonotopy and thalamocortical projection patterns in two adjacent parts of the classical primary auditory cortex in mice

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**HIGHLIGHTS**

- The classical AI is divided into two tonotopic regions, the AI and DM.
- In previous works, we did not determine the low frequency area in the DM.
- Here, we visualize the low frequency area in the DM using autofluorescence imaging.
- We also confirm that the AI and DM receive thalamic projections independently.
- The independency of the AI and DM is conclusive.

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**ABSTRACT**

Amid recent amendment of delineation of a mouse auditory cortical map, a caudal auditory field, originally defined as the primary auditory cortex (AI), was divided into the AI and dorsomedial field (DM), based on distinct high frequency areas. A low frequency area was not previously established in the DM because responses to low frequency tones were weak in this area. This may lead to the misconception that the DM is an atypical region that lacks a low frequency band. In the current study, we confirmed that the DM has a low frequency area that is completely independent from the AI. First, we conducted flavoprotein fluorescence imaging with improved signal to noise ratio and revealed the presence of two separated low frequency areas in the AI and DM. Next, we injected a retrograde neural tracer along the tonotopic axis of the AI or DM to reveal the thalamic origins in the ventral division of the medial geniculate body (MGv). We found that neurons projecting to low frequency areas of the AI and DM occupied different locations within the MGv and mutually independent topographic organizations consisting of thalamic neurons projecting to the AI or DM. These results indicate that the AI and DM have distinct low frequency areas with distinct thalamic projections from the MGv. Our findings re affirm that the AI and DM should be regarded as independent regions in the mouse auditory cortex.

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1. Introduction

In a traditional map of the mouse auditory cortex, the primary auditory cortex (AI) was delineated as a tonotopic region with a frequency area at the caudal end of the auditory cortex that shifted dorsoorostrally in response to high frequency tones (Fig. 1A) [1–4].

However, optical imaging that visualizes intrinsic fluorescence has revealed an additional tonotopic branch inside the classical AI that travels ventrorostrally (Fig. 1B) [5–8]. Therefore, the tonotopic gradients of the AI seem to be arranged in a V-shaped manner with one low frequency area and two high frequency areas (Fig. 1B). We have previously shown that the low frequency area and the ventral high frequency area have similar physiological, histological, and anatomical properties, while the dorsal high frequency area has distinct properties [6,9]. These findings strongly suggest that the classical AI is divided into two different parts: the AI, which includes

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the low frequency area and the ventral high frequency area: and the dorsal high frequency area, which should be considered separate from the AI. We call this area as the dorsomedial field (DM) (Fig. 1C). However, responses to a low frequency tone in the DM were weak, and we left distinct low frequency areas in the DM unrevealed in our previous studies using transcranial flavoprotein fluorescence imaging [1].

Here, we tried to identify the low frequency area within the DM using flavoprotein fluorescence imaging after craniotomy since the skull might obscure faint responses (Supplemental Fig. 1). To remove the effect of light scattering in the brain parenchyma, we deconvolved the original data using the Lucy–Richardson method. Furthermore, we investigated whether low frequency areas within the AI and DM had common or distinct thalamocortical projections. Our findings indicate that the AI and DM are independent regions with distinct tonotopic maps and thalamocortical projections (Fig. 1D).

2. Materials and methods

2.1. Animals

The experimental procedures in the present study were approved by the Committee for Animal Care at Niigata University. Data were obtained from 34 male C57BL/6 mice at 6–8 weeks of age (Charles River Japan, Kanagawa, Japan). The animals were housed in cages with ad libitum access to food pellets and water, and were kept on a 12-h light/dark cycle.

2.2. Flavoprotein fluorescence imaging

Responses in the right auditory cortex were observed using flavoprotein fluorescence imaging [10]. Cortical images were recorded at 9.1 Hz by a CCD camera system (AQUACOSMOS with ORCA-R2, Hamamatsu Photonics, Shizuoka, Japan) via an epifluorescence microscope (Ex, 450–490 nm; Em, 500–550 nm; M651 combined with MZ FL II, Leica Microsystems, Wetzlar, Germany). Mice were anesthetized with urethane (1.65 g/kg, i.p.; Wako, Osaka, Japan). The rectal temperature was maintained at ~37 °C. A craniotomy (~3 × 3 mm) was performed over the auditory cortex in the same way as described previously [6]. The auditory cortex was activated by sound waves using a LabVIEW program (National Instruments, Austin, TX) (Supplemental Fig. 2). Tone duration was 500 ms with a rise/fall time of 10 ms. Tones were amplitude modulated (20 Hz, 100% modulation) and set to ~60 dB SPL. Tones were presented to mice at intertrial intervals of 50 s, and images were averaged over 20 trials. Details of these procedures were described previously [6,11–13]. The area covered by one pixel was 20.4 × 20.4 μm. To remove the effect of light scattering in the brain parenchyma, Lucy–Richardson deconvolution with a 200-μm-wide Gaussian filter [5] was applied to original image data using a Matlab program (Mathworks, Natick, MA).

2.3. Histology

The neural tracer biotinylated dextran amine (BDA; molecular weight, 3000; Molecular Probes, Eugene, OR), was injected into cortical areas that were functionally identified by flavoprotein fluorescence imaging. BDA injection permitted visualization of projection neurons in the ventral division (MGv) of the medial geniculate body (MGB). A glass pipette (tip diameter 3–4 μm) filled with a 0.5% BDA solution in phosphate buffer and a platinum wire was introduced into the center of an auditory region ~500 μm below the surface. BDA was delivered iontophoretically by passing current pulses at 4 μA (7 s on, 7 s off) for 10 min. BDA was injected into a single site in each mouse. After seven days, mice were perfused transcardially with 4% paraformaldehyde and brains were removed. We prepared consecutive 40-μm-thick coronal cryosections including the MGB, and BDA was visualized by avidin-biotin interaction. Every fourth slice was evaluated and the slice with the largest number of BDA-positive neurons in the MGv was selected to obtain the coordinates for projection neurons. Since BDA was detected in a small area of the MGv, there were only few neurons in slices that were not selected [13]. The rostrocaudal distance between the selected slice and the bregma in the rostrocaudal view was determined by reference to the Paxinos brain atlas [14], which is the standard reference for navigation in the rodent MGB [15–17]. The distribution of neurons within a slice was measured with reference to the MGv center. The MGv boundary was determined by non-phosphorylated neurofilament (NNF) labeling in adjacent sections (Supplemental Fig. 3) [13]. For NNF labeling, a primary antibody of SM1-32 (1:2000, Covance Research Products, Berkeley, CA) and a secondary antibody of anti-mouse IgG (1:100; MBL, Nagoya, Japan) were used. Images were obtained using a DP80 color camera (Olympus, Tokyo, Japan) attached to a microscope (Eclipse Ni, Nikon, Tokyo, Japan). Injection, histological procedures, and analysis were performed as previously described [6,9,13].

2.4. Statistics

The Mann–Whitney U test or Wilcoxon signed-rank test was used to evaluate differences between unpaired or paired data from the two groups, respectively. These tests were conducted using SPSS (IBM, Armonk, NY).

3. Results

We observed response patterns to tonal presentation using flavoprotein fluorescence imaging. When a 5 kHz tone was presented to mice, responses in the AI were visualized (Fig. 2A, beige
and D'), and that was by and large in the opposite direction to neurons projecting to the AI. These data suggest that the AI and DM receive thalamic projections with a different topological fashion.

4. Discussion

Previous analysis using two-photon imaging has revealed that the ventrocaudal part in the DM contains neurons with a low best frequency (BF) [6]. However, we were not able to determine the presence of a low-frequency area in the DM using transcranial flavoprotein fluorescence imaging [6] because faint fluorescent signals were further weakened by light scatter in the skull and cortex. To address this, we removed the skull and deconvoluted the images to remove the effects of light scatter [7]. This permitted the visualization of separate activities in the DM and AI in response to low-frequency tones (Fig. 2), and functional visualization of regional boundaries between the two regions was possible. However, the regional boundary between the AI and DM is easily overlooked by functional mapping. Therefore, we performed additional anatomical analyses using tracers, and showed that thalamic neurons projecting to the AI or DM occupied rostrocaudally different locations in the MGv (Fig. 3). Furthermore, the topographic axis of neurons projecting to the AI and DM were completely independent in the MGv (Fig. 3). In particular, the ventrodorsal shift of topography represented by neurons projecting to the DM was newly found in the current study by analyzing neuronal distributions in coronal slices. Taken together, we concluded that the caudal auditory cortical regions should be classified according to Fig. 1D but not Fig. 1A–C.

A region delineated as the classical AI in the mouse is divided into two different tonotopic regions. Including these, at least five tonotopic regions have been identified using functional tools in/near the mouse auditory cortex [6,8,17]. Moreover, anatomical investigations have suggested that the mouse MGv includes at least four compartments, each with a distinct target in the auditory cortex [6,13,17]. Almost all of the compartments have a distinct topographic map, such that MGv neurons visualized by injecting tracers along a cortical tonotopic gradient are sequentially aligned. However, it is still unclear whether these topographic maps in MGv compartments represent tonotopic maps in the mouse. To answer this question, Hackett et al. tried to link tonotopy and topography in the mouse auditory thalamocortical pathway [18]. Comparing the location of thalamic projection neurons, a region annotated as the AI in the Hackett study is highly equivalent to the DM in the current study. Therefore, their results should confirm that topography in the rostral MGv compartment is consistent with tonotopy [18]. On the basis of recent anatomical refinement in the mouse thalamocortical auditory pathway, it is expected that all the topographic maps in thalamic compartments projecting to auditory cortical regions are very soon linked with tonotopy in the mouse.

It is important to compare auditory cortex maps between rodents for deeper understanding. In the rat which is another popular rodent model in neuroscience, vigorous physiological [19,20] and anatomical investigations [16] have revealed the presence of multiple frequency organization in the auditory cortex and multiple compartments in the MGv. There, differences in gene expression have been found between each pathway [21], and an electrophysiological study has been conducted to link several topographic maps with tonotopic maps in compartments of the rat MGv [22]. By comparing the spatial pattern of cortical multiple frequency organization, the DM, AI, and secondary auditory field (AI) in our mouse auditory cortex map may correspond to the AI, ventral auditory field (VAF), and suprachiasmatic auditory field (SRAF) in the rat auditory cortex map provided by Storace et al. [16,21], respectively. This assumption is partly validated by thalamocortical connection.
patterns; the positional relationship between the thalamic origins of the AI and VAF in the rat MGv [16] is quite similar to that of the DM and AI in the mouse (Fig. 3) [6]. When comparison of the thalamic origin of the mouse AI and rat SRAF is achieved, homology of the auditory cortex will be surely recognized between mice and rats, and a research platform to communicate scattered knowledge between two rodents will be established.
Precise brain mapping sometimes changes traditional regional delineation; therefore a consensus is necessary to be reached on how to set regional annotation. Especially, “AI” refers to the most cortical ventral region, and it is a sensitive problem which region is to be referred to as the AI. Here, we have tentatively named the ventral region as the AI and the dorsal region as the DM, because the ventral region originates from a low-frequency area of the classical AI [6]. However, the validity of this annotation cannot be affirmed at the current stage. Originally, the primary region indicates a region which receives thalamic inputs from the lemniscal thalamus, MGV. However, now that more than two frequency regions receive thalamic inputs from the lemniscal thalamus in the mouse [6,17] and rat [16,21], it may be difficult to annotate a particular region as the AI. Ultimately, regional annotation would be validated by collecting knowledge by comparing functional specialization and anatomical properties of each region across species. Again, since fine auditory cortical maps have recently been established in the mouse and rat, these studies will help to homologize regions and construct a common auditory cortical map in rodents.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2016.11.062.

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