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Research paper

Specific distribution of non-phosphorylated neurofilaments characterizing each subfield in the mouse auditory cortex

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HIGHLIGHTS

- Each auditory subfield of mice connected to the contralateral counterparts.
- We compared physiological and anatomical features of six auditory subfields.
- Each auditory subfield has the specific immunostaining pattern for NNF.
- Functionally-identified auditory subfields were morphologically supported.

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ABSTRACT

Recent imaging studies revealed the presence of functional subfields in the mouse auditory cortex. However, little is known regarding the morphological basis underlying the functional differentiation. Distribution of particular molecules is the key information that may be applicable for identifying auditory subfields in the post-mortem brain. Immunoreactive patterns using SMI-32 monoclonal antibody against non-phosphorylated neurofilament (NNF) have already been used to identify or parcellate various brain regions in various animals. In the present study, we investigated whether distribution of NNF is a reliable marker for identifying functional subfields in the mouse auditory cortex, and found that each auditory subfield has region-specific cellular and laminar patterns of immunoreactivity for NNF.

1. Introduction

The mouse auditory cortex is composed of at least six subfields including the primary auditory field (AI), the anterior auditory field (AAF), the dorsomedial field (DM), the secondary auditory field (AII), the dorsoposterior field (DP) and the dorsoanterior field (DA) [17,35]. Although these subfields can be visualized reproducibly using flavoprotein fluorescence imaging [18,19,39,40], the morphological properties underlying the functional map remain to be elucidated. It is partly because it is impossible to identify a particular auditory subfield on the tissue sections based on a reliable marker.

SMI-32 is a monoclonal antibody, which recognizes a non-phosphorylated epitope of neurofilament proteins (NNF) [36]. SMI-32 labels specifically the dendrites, axons and perikarya of a subset of neocortical pyramidal neurons [22,30,36,44]. Recently, growing evidence has shown that differences in the immunohistochemical pattern of NNF can be successfully used for the identification of fine cortical areas in different mammalian species, *e.g.* in rodents [1,3,4], monotremes [13], felines [24,29,41] and primates [2,6,26]. Therefore, NNF can be a candidate for a histological marker to identify auditory subfields in mice. NNF has already been applied for the identification of subfields in the auditory cortex in rats [32] and Mongolian gerbils [4]. However, fine distribution patterns of NNF-immunoreactivity in the mouse auditory cortex remain unknown, although the mouse begins to be widely used in the auditory research.

In the present study, we investigated NNF immunoreactive patterns in the auditory subfields, which have been delineated by flavoprotein fluorescence imaging [40]. We injected a neural tracer, biotinylated dextran amine (BDA), into the physiologically-defined auditory subfields to set the histological landmark on the contralateral auditory subfields through the callosal fibers, and then performed NNF immunohistochemistry to examine the NNF distribution patterns.

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2. Materials and methods

2.1. Mice

Twenty-eight male C57BL/6N mice of 5–7 weeks old (Charles River Japan,Tokyo, Japan) were used for experiments. The experimental protocols used in this study were approved by the Animal Committee of Niigata University.

2.2. Flavoprotein fluorescence imaging

Precise auditory cortical subfields in the right auditory cortex were identified by flavoprotein fluorescence imaging prior to BDA-injection [23,31,37]. Mice were anesthetized with urethane (1.65 g/kg, i.p.). The rectal temperature was kept at 37.0 °C. The craniotomy was performed over the right auditory cortex. Cortical images were recorded by a CCD camera system (AQUACOSMOS/Ratio with ORCA-R2 camera, Hamamatsu Photonics, Hamamatsu, Japan) via an epifluorescence microscope (Ex, 500–550 nm; Em, 450–490 nm; M651 combined with MZ FL II, Leica Microsystems, Wetzlar, Germany). Details are described in the Supplemental methods and our previous works [23,31,37].

2.3. Sound stimuli

Sound waves were made with LabVIEW programs (National Instruments, Austin, TX, USA). To identify the places of AAF, AI, DM and AII, 5 kHz or 20 kHz AM tones (20 Hz, 100% modulation) were used. To identify the place of DA and DP, FM differential imaging method was used [18]. Details are described in the Supplemental methods and our previous works [18,39].

2.4. Confirmation of callosal connectivity between bilateral auditory subfields

We used callosal connections to histologically mark a particular subfield by injection of BDA into the contralateral subfield. To confirm the presence of callosal connections with the accuracy at the subfield level, AAV vector including the cDNA of GCaMP3, a fluorescent Ca^{2+} indicator, (AV-1-PV1627, Penn Vector Core, Philadelphia, PA, USA) was injected into a functionally-identified subfield of the right AI to label the axonal branches reaching the contralateral auditory cortex [38,40]. A glass capillary (tip diameter: 20–30 μm) filled with AAV solution (500 μl) was introduced to a depth of 500 μm from the surface, and slowly pressure-injected for 30 min. After injection, the hole in the skull was covered with 2% agarose (Type 1-B, Sigma–Aldrich, MO, USA), and the skin was sutured. Mice were recovered from anesthesia in their home cages. Two weeks after the injection, the contralateral auditory cortex in the left hemisphere was observed to detect fluorescence derived from GCaMP3 positive axon terminals (excitation wavelength, 470–490 nm; emission wavelength, 500–550 nm).

2.5. Neural tracer injection

Injection of the neural tracer, BDA, was performed as described previously [18,19,39,40] and detailed methods are described in the Supplemental methods.

2.6. Tissue preparation, visualization of tracers and immunohistochemistry for NNF

Tissue preparation, visualization of tracers and immunohistochemistry for NNF was performed as described previously [18,19,39,40] and detailed methods are described in the Supplemental methods. In all animals, 8–10 sections of auditory cortex per animal was used for analysis.

2.7. Measuring the location of BDA injection sites and BDA-labeled axon terminals

The precise locations of the right BDA injection sites and the left sites, where BDA-labeled axon terminals were accumulated, were measured as follows. In antero-posterior axis, the distance from bregma was judged by coronal brain views in reference to those in the published mouse brain atlas [10]. In dorso-ventral axis, the distance from the dorsal edge of the rhinal fissure (red dots) was measured as shown in Fig. 1C. These values were measured using cellSense application (Olympus)(Fig. 1H, I). All the data in the text were presented as mean ± SEM.

3. Results

3.1. Callosal connectivity between bilateral auditory subfields

We had to observe NNF immunolabeling patterns on the post-mortem brain sections. Therefore, prior to NNF immunohistochemistry, BDA was injected to mark the functionally-identified subfields of living mice (Fig. 1A–C). However, the injected site exhibited high background staining with BDA, that prevents observation of fine NNF staining patterns (sFig. 1A), probably because endogenous mouse IgGs leaked from blood vessels in the BDA injected site produced the high background staining (sFig. 1B). To avoid this technical problem, we investigated NNF staining patterns in the auditory cortex contralateral to the injected site. It is possible because an individual auditory subfield has strong projections to its counterpart, as reported in cats [8,20,34], monkeys [7,9], Mongolian gerbils [4], squirrels [25] and rats [33]. First, we confirmed that an auditory subfield has dense callosal connections to the contralateral counterpart by injecting AAV-GCaMP3 into AI. Since GCaMP3 is anterogradely transported [40], we observed GCaMP3-positive axon terminals in the contralateral counterpart two weeks after AAV injection (Fig. 1D, E). We further verified the existence of callosal connections between the auditory subfields using BDA, which works as an anterograde neural tracer. In all twenty eight mice sections examined, the ipsilateral BDA-stained area was successfully found within a cylindrical cortical area with a diameter of approximately 350 ± 50 μm (Fig. 1F). We measured the precise dorsal deviation of the injected sites from the dorsal edge of the rhinal fissure and posterior deviation from the bregma as internal references. The injected sites were 1002 ± 38 μm dorsal and 3080 ± 44 μm posterior in AI, 1197 ± 68 μm and 2407 ± 53 μm in AAF, 1787 ± 80 μm and 2820 ± 140 μm in DM, 580 ± 20 μm and 3080 ± 43 μm in AII, 1951 ± 26 μm and 3244 ± 90 μm in DP, 1602 ± 123 μm and 2780 ± 90 μm in DA, respectively (Fig. 1H, I). In all twenty eight mice sections examined, the accumulation of BDA-labeled axon terminals was found at the contralateral sites with homotopic locations (Fig. 1F, G, sFig. 2). Dorsal and posterior deviations of the accumulation site were 1087 ± 29 μm dorsal and 3120 ± 60 μm posterior in AI, 1234 ± 30 μm and 2407 ± 53 μm in AAF, 1825 ± 40 μm and 2868 ± 100 μm in DM, 540 ± 40 μm and 3176 ± 44 μm in AII, 1970 ± 79 μm and 3320 ± 139 μm in DP, 1634 ± 167 μm and 2712 ± 66 μm in DA, respectively (Fig. 1H, I). Based on the precise symmetrical locations of the injected and transported sites, we evaluated NNF-staining patterns in the auditory subfields contralateral to the injected sites.