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Multimodal cortical sensory pathways revealed by sequential transcranial electrical stimulation in mice

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ABSTRACT

We investigated polysynaptic cortical pathways linking primary to multimodal sensory association areas in mice using transcranial flavoprotein imaging combined with sequential application of transcranial electrical stimulation (TES). Stimulation of primary visual cortex (V1) elicited activity in lateral and medial areas of secondary visual cortices (V2), which were reciprocally connected. Stimulation of V2 areas elicited activity in area 2. Similarly, corticocortical pathways from primary somatosensory cortex (S1) through the corresponding secondary somatosensory areas (S2) to area 2 were observed. Auditory pathways from primary auditory area (A1) through peripheral region (area 22) to area 2 and from anterior auditory field to area 2 were also found. Stimulation in area 2 elicited activity in part of parietal association cortex (PtA), which was reciprocally connected with area 2, and in some areas near the midline including retrosplenial cortex (RSA). A cortical pathway from RSA through anterior cingulate cortex (aCC) to frontal areas was also visualized. These results indicate that area 2, surrounded by visual, somatosensory and auditory cortices, may receive inputs from all three primary sensory areas, and may send outputs through the parietal association cortex to frontal areas, suggesting that area 2 may have an important role in multimodal sensory integration in mice.

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1. Introduction

Multimodal sensory processing is performed in cortical circuits that integrate sensory information of different modalities and send the integrated information to higher areas (Olson and Colby, 2013). However, the cortical pathways responsible are not well understood, particularly in mice models that are suitable for investigating the molecular mechanisms underlying multimodal sensory processing (Carvell and Simons, 1987; Hofstetter and Ehret, 1992; Wang et al., 2012). Previously, we reported that the auditory cortex is vital to audio-visual integration in discrimination learning in rats (Ono et al., 2006). The auditory cortex was also reported to be functionally connected with surrounding cortical areas in rat cerebral slices (Hishida et al., 2003, 2007). However, in slice preparation studies it is not possible to trace entire cortical pathways underlying audio-visual integration, as many circuits are severed during the slicing procedure. As such, analyses of functional cortical pathways in the whole living brain are required for further understanding of multimodal sensory integration.

Flavoprotein fluorescence imaging *in vivo* has enabled analysis of cortical activity elicited by sensory stimuli such as somatosensory (Kitaura et al., 2010; Komagata et al., 2011; Shibuki et al., 2003, 2006), visual (Tohmi et al., 2006, 2009) and auditory stimuli (Kubota et al., 2008; Ohshima et al., 2010; Takahashi et al., 2006). This method is especially useful for imaging mouse cortical activity as neural activity can be observed transcranially with almost no damage to the cortex (Shibuki et al., 2007). However, activity in higher sensory areas is only minimally elicited by simple sensory stimuli. To visualize the polysynaptic activity in higher areas propagated from primary areas, we previously used transcranial electrical stimulation (TES) of primary areas (Hishida et al., 2011). In TES, the stimulus currents flow from an electrode through the shaved skull to the cortex, bypassing the space occupied with

Abbreviations: A1, primary auditory cortex; A2, secondary auditory cortex; AAF, anterior auditory field; AC, auditory cortex; aCC, anterior cingulate cortex; aV2, anterior part of V2; FP, forepaw area in S1; HP, hindpaw area in S1; lV2, lateral part of V2; MM, mediomedial area; mV2, medial part of V2; PtA, parietal association cortex; PMBSF, posteromedial barrel subfield in S1; RSA, retrosplenial agranular cortex; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; SW, small whiskers of anterior snout area in S1; TES, transcranial electrical stimulation; V1, primary visual cortex; V2, secondary visual cortex.

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cerebrospinal fluid. TES mainly activates the supragranular layers, which are responsible for corticocortical connections (Amaral and Strick, 2013). Using a combination of TES and flavoprotein fluorescence imaging in mice, we have previously visualized the reciprocal corticocortical connection between the lateral and medial parts of the secondary visual cortices (Hishida et al., 2011) and the corticocortical connections between both hemispheres (Yamashita et al., 2012).

In the present study, we used the combination of TES and flavoprotein fluorescence imaging to trace comprehensive polysynaptic cortical pathways underlying multimodal sensory integration over the whole cerebral cortex of mice. We applied TES sequentially from each primary cortex to higher areas. This sequential TES procedure enabled us to determine a broad overview of the cortical pathways, in which neural activity originating from the visual, somatosensory and auditory systems converge onto a particular multimodal area and then propagate further to higher cortices.

2. Materials and methods

The Ethics Committee of Niigata University approved the experimental protocols used in the present study. Male C57BL/6 strain mice (8–10 weeks old) were used in the experiments.

2.1. Surgical procedures

Mice were anesthetized with urethane (1.65 g/kg, i.p.). Rectal temperature was maintained at 37.5 °C throughout the recordings using a silicon rubber heater. Surgical procedures were conducted under sterile conditions. After subcutaneous injection of the local anesthetic, bupivacaine (AstraZeneca, Osaka, Japan), the disinfected skin covering the skull was incised, and part of the left temporal muscle was removed to expose the dorsal left cortex. A metal piece was attached to the right side of the skull with dental resin, and the head was fixed in position by screwing the metal piece into a manipulator. For TES, the left side of the skull was shaved with the blade of a dental drill as described previously (Fig. 1A; Hishida et al., 2011). The surface of the skull was covered with liquid paraffin to prevent drying and to keep the skull transparent. Operations were completed within 45 min. An additional dose of urethane (0.2 g/kg, s.c.) was administered when necessary. At the end of the experiments, mice were euthanized with an overdose of pentobarbital (i.p.).

2.2. Imaging in anesthetized mice

Imaging experiments started approximately 1 h after administering the anesthetic. The animal was set on a stage slanted at an angle of 45° (Fig. 1B). The dorsolateral view of the left hemisphere was observed with an epifluorescence microscope (MZ FLIII; Leica) with a 75 W xenon light source. A cooled CCD camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan) attached to the microscope was used to take cortical images (128 × 168 pixels) of endogenous green fluorescence (λ = 500–550 nm) under blue light excitation (λ = 450–490 nm) at a rate of 7 frames/s. Spatial averaging in 5 × 5 pixel areas was used to improve image quality. Twenty sets of images were captured at 20–60 s intervals, and the averaged data were normalized by dividing fluorescence intensity changes in each pixel (ΔF) by a basal value (F_0), obtained by averaging three images taken immediately prior to stimulation. A pseudocolor scale was used to depict relative changes in fluorescence ($\Delta F/F_0$). For TES, we used a sewing needle with a slightly blunt tip (diameter of approximately 100 μm) as the stimulus electrode. Electrical stimulation (500 μA, 10 Hz, 10 pulses) was applied as previously reported (Hishida et al., 2011). The ground electrode was attached to the temporal muscle.

In our previous study, a cortical map was made, which was used as a template for approximate positioning of a stimulus electrode and the identification of activated areas (Hishida et al., 2011). We made the template as follows. The primary sensory cortices were identified on the basis of the cortical activity elicited by natural stimuli. The other areas were mapped with respect to the location of the primary sensory areas. Visual areas of the left hemisphere were evoked by stimulation of the right eye using a light emitting diode (LED) light which was positioned between 0° and 90° in the horizontal plane. The LED light caused strong cortical activities in V1 and weak activities in lateral part of V2 (lV2). V1 was mapped with the comparisons of the strongly elicited sites with the previously published cortical maps (Caviness, 1975; Franklin and Paxinos, 2008; Wang and Burkhalter, 2007). V2 was mapped with respect to the location of the V1 based on a published map of the mouse visual cortex (Wang and Burkhalter, 2007). Somatosensory areas of the left hemisphere were evoked by vibratory stimuli to the right whiskers and electrical stimuli to the right forepaw and hindpaw. These stimuli evoked strong cortical activities in some areas in left S1 (PMBSF, SW, FP, and HP). Weak activities also often appeared in an area immediately ventral to S1, which presumably corresponds to S2. Based on these results and published results (Caviness, 1975; McIlvain et al., 2003; Hunt et al., 2006; Franklin and Paxinos, 2008), we mapped S1 and S2. Part of the boundary between S1 and S2 was verified based on the area-specific patterns of myeloarchitecture, which were observed in fresh cortical slices containing the boundary (data not shown, Hishida et al., 2003). Auditory areas were elicited by sound stimuli of 5 or 10 kHz. AAF, A1 and A2 were identified as the localized activities (Stiebler et al., 1997; Takahashi et al., 2006). Area 22 was mapped as a belt-like region which surrounded AAF, A1 (Stiebler et al., 1997) and did not overlap lV2 and S2. After the cortical mappings of visual, somatosensory and auditory areas as described above, we found a region which was surrounded by the sensory areas of 3 different modalities and did not belong to any of these areas. This region was identified as area 2. The site in S2 responding to whisker stimuli was not included, the area 2 we mapped was slightly small on the ventral side compared with the previous description (Caviness, 1975). Several regions such as frontal areas were left unlabeled in the template, because we could not confidently identify the regions which are far away from the clearly-observed primary areas. But in these unlabeled regions we could identify the sites very near the mapped areal boundaries or the anatomical landmarks such as bregma or midline.

In each TES experiment, we first mapped some cortical areas based on the activity elicited by sensory stimuli: visual areas (V1 and lV2), somatosensory areas (PMBSF, FP, HP and part of S2 responding to whisker stimuli) and auditory areas (AAF, A1 and A2). With respect to the location of these areas, the other areas were mapped using the template mentioned above. Individual differences in the location of the primary areas were minimal, so almost the same template was used for most experiments. Where differences occurred, area mapping required a slight adjustment, made with reference to the location of the primary areas.

The propagation of neural activity from each stimulation point to the cortical area of interest was assessed using the activity propagation index (API), calculated as follows:

$$\mathrm{API}\,(\%) = (\mathrm{RESPONSE\ AREA}/\mathrm{CORTICAL\ AREA}) \times 100,$$

where CORTICAL AREA represents the square measurement of a cortical area of interest, expressed in pixels, and RESPONSE AREA represents the number of pixels in the cortical area of interest, in which $\Delta F/F_0$ was larger than 1/6 of the maximal response amplitude measured 1 s after stimulus onset.

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