Development of Functional Synaptic Connections in the Auditory System Visualized With Optical Recording: Afferent-Evoked Activity Is Present From Early Stages

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INTRODUCTION

Complex neural functions emerge from precisely organized neural circuits in the CNS. One of the fundamental questions for neuroscientists is how these complex patterns of connections are established in a precise manner during development. Concerning this issue, two major mechanisms have been proposed: one is an activity-independent process mediated by genetically determined molecular markers and the other is an activity-dependent process associated with sensory-evoked and spontaneous activity (Friauf and Lohmann 1999; Glover 2000; Goodman and Shatz 1993). Electrophysiological investigations of selected systems have suggested that in the early phases of neural circuit formation, sensory-evoked activity is not crucial, whereas molecular markers and spontaneous activity are important (Friauf and Lohmann 1999; Rubel and Fritzsch 2002; Shatz 1990, 1994). This notion is based partly on the observations that sensory-evoked activity is not detectable and/or that blockade of sensory information does not influence the degree of specificity arising during early phases of circuit formation. However, the emergence of physiological functions related to sensory-evoked activity has not been well examined because conventional electrophysiological techniques are often very difficult to use in the early embryonic nervous system because of the small size and fragility of immature neurons. Therefore it is important to reexamine the ontogenetic onset of physiological function in developing networks, using less-invasive techniques to monitor electrical activity.

Voltage-sensitive dye-imaging methods have made it possible to detect electrophysiological events in living systems that are inaccessible to microelectrodes. In addition, they have facilitated the simultaneous recording of electrical activity in large populations of neurons and have provided a powerful tool for monitoring the dynamic patterns of activity in the CNS (for reviews see Cohen and Salzberg 1978; Grinvald et al. 1988; Salzberg 1983). In our previous studies, we applied a voltage-sensitive dye-imaging technique to chicken and rat embryos and established its feasibility for recording electrical activity from the embryonic CNS (for reviews see Momose-Sato et al. 2001, 2002). In these studies, we assessed the onset and early development of action potential activity and postsynaptic function related to various cranial and spinal nerve inputs and outputs (Komuro et al. 1991; Miyakawa et al. 2004; Mochida et al. 2001; Momose-Sato et al. 1991, 1994, 2004; Sato et al. 1995, 1998, 1999).

The brain stem neural circuits related to the vestibulocochlear nerve (N. VIII) provide an excellent model to investigate the mechanisms of neural circuit formation because of their accessibility and well-described internuclear connectivity (Boord and Rasmussen 1963; Rubel and Fritzsch 2002; Ryugo and Parks 2003). In an earlier study on embryonic chicken brain stem slice preparations, we succeeded in recording optical responses from the area corresponding to Nucleus magnocellularis (NM), which were induced by electrical stimulation of the auditory afferents (Sato and Momose-Sato 2003). In these slice preparations, the pathways from NM to second- and third-order auditory nuclei were compromised, and we could not identify optical responses except in NM. In the present...
study, we have taken the next step in assessing the development of the auditory system by applying the optical recording technique to the intact medulla/brain stem preparation of the chicken embryo. Through a comprehensive survey of electrical activity in the intact preparation, we have been able to detect optical responses in several auditory nuclei including the higher-order nuclei and to trace the functional development of the auditory system from the earliest stages of synaptic connectivity.

Methods

Preparations

Intact medulla or brain stem preparations dissected from 5.5- to 8-day chicken embryos were used (n = 35). Experiments were carried out in accordance with the guidelines of Tokyo Medical and Dental University for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. Fertilized eggs of White Leghorn chickens (Saitama Experimental Animals Supply, Saitama, Japan) were incubated for 5.5 to 8 days in a forced-draft incubator (type P-008, Showa Incubator Laboratory, Urawa, Japan) at a temperature of 37°C and 60% humidity, and were turned once each hour. In the experiments reported here, the 5.5-day stage corresponded to Hamburger–Hamilton (H-H) stage 27, the 6-day stage to H-H stages 28–29, the 7-day stage to H-H stages 30–32, and the 8-day stage to H-H stages 33–34 (Hamburger and Hamilton 1951). The embryos were decapitated and brain stems, with the auditory branch of the eighth nerve (N. VIII) attached, were dissected from the embryos. This branch, termed pars cochlearis of N. vestibulocochlearis by Breazile and Yasuda (1979), carries afferents from the papilla basilaris and the macula lagenae with cell bodies residing in the cochlear/lagenar portion of ganglion VIII (D’Amico-Martel 1982; Kaiser and Manley 1996). It was identified on the basis of its position as the most medial (most posterior after dissection) of the three main branches of N. VIII, its large caliber relative to the other branches and its relationship to the cochlear duct (D’Amico-Martel 1982; Kaiser and Manley 1996). In the present study, we refer to this branch as N. VIIIc, and the remaining two vestibular branches of N. VIII as N. VIIIv. Although the subpopulation of afferents in N. VIIIc that project from the macula lagenae is believed to be vestibular (Kaiser and Manley 1996), in the results we describe below we saw no evidence of an activation of vestibular pathways, which we have studied extensively in a parallel study that will be published separately (JC Glover, H Mochida, Y Momose-Sato, and K Sato, unpublished observations).

The brain stems were placed in physiological saline (normal saline) that contained (in mM) NaCl, 138; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.5; glucose, 10; and Tris-HCl buffer (pH 7.3), 10, after which the meningeal tissues were carefully removed as much as possible (small excess (unbound) dye was washed away with dye-free physiological saline containing 0.2 mg/ml of the voltage-sensitive merocyanine–rhodanine dye, NK2761 (Hayashibara Biochemical Laboratories/Kankoh-Shikiso Kenkyusho, Okayama, Japan; Kamino et al. 1981; Momose-Sato et al. 1995; Salzberg et al. 1983), and the excess (unbound) dye was washed away with dye-free physiological saline before recording. This merocyanine–rhodanine dye has been shown to be particularly useful in embryonic nervous and cardiac tissues (Kamino 1991; Momose-Sato et al. 1995).

Electrical stimulation

The cut end of N. VIIIc was drawn into a suction electrode fabricated from TERUMO-hemotect tubing (VC-HO75P; Terumo, Tokyo, Japan), which had been hand-pulled to a fine tip (about 100 μm internal diameter) over a low-temperature flame. Positive (depolarizing) square-current pulses (30 μA/1 ms for 5.5- and 6-day preparations, 8 μA/5 ms for 7- and 8-day preparations), which evoked maximum responses, were applied to N. VIIIc at intervals of 10–15 min.

Glutamate receptor blocker experiments

Glutamatergic transmission was blocked by bath application of 200 μM APV [N-2-amino-5-phosphonovaleric acid, an N-methyl-D-aspartate (NMDA) receptor antagonist] and 5 μM CNQX (6-cyano-7-nitroquinolinic acid-2,3-dione, a non-NMDA–receptor antagonist). APV was from Sigma Chemical (St. Louis, MO) and CNQX was from Research Biochemicals International (Natick, MA).

Optical recording

The methods used for multiple-site optical recording of electrical activity in embryonic nervous systems are described in detail elsewhere (for reviews see Kamino 1991; Momose-Sato et al. 2001). In brief, bright-field illumination was provided by a 300-W tungsten–halogen lamp (Type JC-24V/300W, Kondo Philips, Tokyo, Japan) driven by a stable DC-power supply. The infrared light from the tungsten–halogen lamp was cut off by a heat filter, and incident light was collimated and rendered quasi-monochromatic with an interference filter with a transmission maximum at 703 ± 12 nm (half-width) (Asahi Spectra, Tokyo, Japan). The objectives (×10, 0.4 n.a. or ×4, 0.8 n.a.) and photographic eye piece (×2.5) projected a real image (magnification: ×25 or ×10) of the preparation onto a 34 × 34 element silicon photodiode matrix array mounted on a microscope stage. Changes in transmitted light intensity through the preparation were detected with the photodiode array and were recorded with a 1,020-site optical recording system constructed in our laboratory. Each pixel (element) of the array detected light transmitted by a square region (54 × 54 μm² using ×25 magnification; 116 × 116 μm² using ×10 magnification) of the preparation. This permitted us to make simultaneous spatiotemporal recordings of responses occurring in large areas and also to present the response waveforms at individual pixels. The time resolution of the system was about 1 ms (1,024 frames per 1,000 ms). The recordings were made in single sweeps at 10- to 15-min intervals except where noted. The optical recording was carried out at room temperature, 26–30°C. The preparations were continuously superfused with physiological saline, but this was stopped during recording to eliminate movement artifacts.

The fractional change in dye absorption ΔA/Δt is equal to −ΔI/Δt before staining − ΔI/Δt after staining, where A and I are light absorbance of the dye and light intensity transmitted through the preparation, respectively, and ΔA and ΔI are their changes (Ross et al. 1977). In most of our experiments, we had already stained the preparation before placing it in the recording chamber. This was done to maximize dye diffusion into the tissue, but it prevented us from measuring ΔI/Δt before staining and ΔI/Δt after staining from the same preparation. Therefore we compared ΔI/Δt before staining and ΔI/Δt after staining in a few preparations by measuring the light that reached the detectors before and after the preparation was stained for 20 min with 0.2 mg/ml NK2761 on the stage of the microscope. In that condition, regional variations in ΔI/Δt before staining and ΔI/Δt after staining were small (Momose-Sato and Sato 2006). Thus in the majority of experiments, in which we measured only ΔI/Δt after staining and ΔI/Δt, we expressed the optical signal as ΔI/Δt after staining.
assuming this to be linearly related to $-\Delta A/A$. Color-coded representations of optical signals to be used in a spatiotemporal activity map (Fig. 8) were constructed using the “NeuroPlex” program (RedShirtImaging, Fairfield, CT) and color-coding used to represent maximum signal amplitudes (Fig. 2A) was constructed using the “Transform” program (Fortner Research, Sterling, VA). The color code in the figures is linearly distributed between the minimum and maximum values of $\Delta I/I$ in each experiment.

RESULTS

Optical identification of the auditory nuclei in the medulla

In the avian auditory system, acoustic information is transmitted from hair cells of the basilar papilla to afferents coursing in the auditory branch of the eighth nerve (N. VIIIc). These auditory afferent fibers enter the medulla and bifurcate to terminate in Nucleus magnocellularis (NM) and Nucleus angularis (NA) (Boord and Rasmussen 1963; Ryugo and Parks 2003). NM and NA initiate two parallel pathways that use different anatomical relays and carry different types of information. Projections from NM terminate bilaterally in Nucleus laminaris (NL) (Boord 1968, 1969; Parks and Rubel 1975). All of these N. VIIIc-related auditory nuclei are located in the medulla. Thus we first surveyed N. VIIIc responses in the medullary region.

Figure 1 illustrates multiple-site optical recording of neural activity detected from an 8-day intact medulla, using a magnification of $\times 10$. The optical signals evoked by N. VIIIc stimulation were simultaneously recorded from 1,020 contiguous regions of the preparation using a 34-element photodiode array in a single sweep. In this figure, when a stimulating current ($8 \mu A/5$ ms) that gave the maximum response was applied to left N. VIIIc, optical responses were detected from two separated regions. One was distributedrostrocaudally on the stimulated side, as indicated by a light gray shadow, whereas the other was located at the level of the N. VIII root on the contralateral side, as indicated by a dark gray shadow.

The optical signals were eliminated at 630–640 nm, where the NK2761-dependent extrinsic absorption signal is absent (Momose-Sato et al. 1995), indicating that the detected optical signals are indeed dye-absorption changes related to the membrane potential and do not correspond to changes in light scattering related to mechanical or other factors. When we applied hyperpolarizing current pulses, no optical signal was observed within the medulla, although an electrotonic potential-related component was found within the ganglion.

Figure 2 shows another example of N. VIIIc responses obtained from an 8-day intact medulla. The response intensity in the response areas is color-coded in Fig. 2A, and the waveforms of optical signals detected from individual pixels within the four different response areas are shown in Fig. 2B (black traces): signals a–c were detected from the ipsilateral side and signal d was detected from the contralateral side. As can be seen in Fig. 2B, the waveforms of signals a and c were
tude of polarization and the density of polarized membrane in signal intensity at any given pixel is a function of the magni-

and Momose-Sato 2003). It is important to note that the optical

presynaptic terminals and the postsynaptic cell bodies (Sato 2003). Thus we performed optical recording in normal

Sato and Momose-Sato 2003), the slow signals detected in the present study in all four response areas a–d were eliminated in Ca\(^{2+}\)-free saline (not shown) and by application of the glutamate receptor antagonists APV and CNQX (Fig. 2B, red traces). This sug-
gests that they also reflect glutamatergic EPSPs and that the response areas therefore correspond to the sensory nuclei related to N. VIIIc.

The differences in the waveforms of signals a/c and b (Fig. 2B, black traces) suggested that ipsilateral responses could be divided into subgroups originating from different neuronal populations. To observe the ipsilateral response in more detail, we recorded optical signals at a higher magnification (×25). As can be seen in Fig. 3, we identified three ipsilateral response areas (Areas I, Iii, and III) based on differences in the optical signal waveforms. The contralateral response area (indicated with d in Fig. 2A) had a location symmetrical to Area IIi and was thus termed Area Iic. Based on previous anatomical studies (Breazile 1979; Cambronero and Puelles 2000; Cramer et al. 2000; Díaz et al. 2003; Kuenzel and Masson 1988; Marín and Puelles 1995), and consid-
ering that projections from NM to NL are bilateral, these areas evidently correspond to the following nuclei:

Area I  Nucleus magnocellularis (NM)
Area Iii/Iic  Nucleus laminaris (NL)
Area III  Nucleus angularis (NA)

In Figs. 1 and 2B, the optical responses in Area Iic (con-
tralateral-NL) showed apparent onset delays relative to those in Area I (NM) (30- to 55-ms delay, n = 5). Similar results were
obtained from the other tested preparations. These results are in
line with the notion that the contralateral response represented activity that was conducted from ipsilateral NM, although the delay was longer than might be expected for axonal conduction alone. Such a long delay was not obvious between the re-
sponses in Area I (NM) and Area Iii (ipsilateral-NL). One possible explanation for the unexpectedly long delay may be the immaturity of axons and synaptic connections in embryonic neural circuits (see DISCUSSION).

Development of synaptic function in the medullary nuclei

To study the development of functional synaptic transmission in NM, NA, and NL, we examined optical responses to N. VIIIc stimulation in the intact medulla at 5.5- to 7-day stages (Fig. 4). Glutamatergic synaptic transmission from N. VIIIc afferents is mediated by NMDA and non-NMDA receptors, and removal of Mg\(^{2+}\) from the extracellular solution has been shown to enhance postsynaptic responses (Sato and Momose-
Sato 2003). Thus we performed optical recording in normal and Mg\(^{2+}\)-free saline.

At the 5.5-day stage, although the fast spikelike signals corresponding to the presynaptic action potential were detected in the region of the afferent projection into the medulla, no slow signals were observed either in normal or in Mg\(^{2+}\)-free saline. At the 6-day stage, slow signals appeared, although their pattern varied from preparation to preparation (Fig. 4). In

similar and we could identify two optical signal components: a fast spikelike signal (indicated by arrows) and a delayed long-lasting slow signal. The waveforms of signals b and d were different from those of signals a and c. They were mainly composed of long-lasting slow signals and the signal peaks were delayed compared with those of signals a and c.

In our previous study using embryonic chicken brain stem slice preparations (Sato and Momose-Sato 2003), N. VIIIic stimulation elicited, in a region within the transverse plane corresponding to Nucleus magnocellularis, optical signals that were composed of a fast spikelike signal and a delayed long-lasting slow signal. In that study we showed that the slow signal represents a glutamatergic excitatory postsynaptic potential (EPSP) and that the fast signal corresponds to a sodium-dependent action potential, presumably arising both in the presynaptic terminals and the postsynaptic cell bodies (Sato and Momose-Sato 2003). It is important to note that the optical signal intensity at any given pixel is a function of the magni-
tude of polarization and the density of polarized membrane in that pixel (Obaid et al. 1985; Salzberg et al. 1977). In many cases the presynaptic action potential components cannot be seen even though a postsynaptic EPSP is seen. This is evi-
dently because the density of polarized membrane constituting the presynaptic terminals and the axon hillock is relatively low compared with the density of postsynaptic membrane polarized by the EPSP. As seen in our previous study (Sato and Momose-
Sato 2003), the slow signals detected in the present study in all four response areas a–d were eliminated in Ca\(^{2+}\)-free saline (not shown) and by application of the glutamate receptor antagonists APV and CNQX (Fig. 2B, red traces). This sug-
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some cases, slow signals were not detected in normal or Mg\textsuperscript{2+}-free saline (Fig. 5A), similarly to the 5.5-day preparations. In other cases, small slow signals were detected from Area I (NM) or Areas I (NM)/III (NA) in normal and/or Mg\textsuperscript{2+}-free saline (Fig. 4). In Areas IIi and IIC (NL), slow signals were not detected in normal saline, but were detected in one 6-day preparation in Mg\textsuperscript{2+}-free saline (Fig. 5B). These results suggest that functional synaptic transmission in the ipsilateral cochlear medullary relays appears at around the 6-day stage. At the 7-day stage, slow signals were detected from Area I (NM), Area III (NA), and Area IIi/IIC (bilateral NL) even in normal saline (Figs. 4 and 6). Although the optical signals at the 7-day stage were smaller than those in 8-day preparations (Fig. 6B, left traces), they were unequivocally identified in Mg\textsuperscript{2+}-free saline (Fig. 6B, right traces). These results suggest that functional synaptic transmission in the primary auditory nuclei (NM and NA) first appears at around 6 days, and that the internuclear connection from NM to bilateral NL is established by 6 to 7 days.

**Mapping of auditory nerve responses in the pons and cerebellum**

Next, to examine whether higher-order responses in the auditory pathways could be visualized, we performed optical recording using intact brain stem preparations that included the midbrain, pons, cerebellum, and medulla. Figure 7 shows an example of optical recordings detected from an 8-day preparation. In addition to Areas I, IIi, IIC, and III identified in the previous experiments, optical responses were seen in three additional areas (Areas IV, VI, and VC in Fig. 7A). Area IV was located in the contralateral cerebellum and Areas VI and VC were symmetrically distributed in the pons. Figure 7B shows enlarged traces of optical signals detected from six different areas. Between the signals in Areas I and III and signals in Areas IV, VC, and VI, there were significant delays, suggesting that the latter signals correspond to higher-order responses in the auditory pathways conducted by NM (Area I) and/or NA (Area III).

To visualize the spatiotemporal propagation pattern of the optical signals, we made time-sequenced color-coded maps (Fig. 8). Optical responses to N. VIIIc stimulation first appeared in Areas I and III (frames 5–20 ms). Within 50 ms after stimulation, optical responses were recognized in Areas IIC and VC on the contralateral side, as indicated by white arrowheads in the frame at 50 ms. Next, signals were detected in the contralateral cerebellum (Area IV) (frame at 60 ms) and finally they appeared in Area VI (frame at 110 ms). Differences in the onset latency presumably reflect conduction in the neural pathways linking each response area (see DISCUSSION).

To better chart the response details within each response area, we made contour line maps of slow signal amplitudes. The amplitude of the optical signal represents a weighted optical average of the potential change and membrane area imaged onto each detector (Obaid et al. 1985; Salzberg et al. 1977). Accordingly, the spatial distribution pattern of the slow signal reflects the spatial organization and differentiation of the functional synaptic connections. Figure 9 shows a typical example of the contour line map obtained from an 8-day preparation. In birds, it is known that two nuclei related to the auditory pathways are located in the pons. The first one is Nucleus lemnisci lateralis (NLL), which receives afferents from NL by the lateral lemniscus (Boord 1968; Ramón y Cajal...
1908; Stotler 1905). The second is Corpus trapezoidium (CTz), which receives third-order axons from NL and second-order axons from NM and NA (Boord 1968). Comparing anatomical investigations (Breazile 1979; Cambronero and Puelles 2000; Cramer et al. 2000; Kuenzel and Masson 1988; Marín and Puelles 1995), and considering that CTz is located ventromedially and extends from the medulla to the pons (Kuenzel and Masson 1988), we concluded that Areas Vi and Vc mainly correspond to ipsilateral- and contralateral-NLL, respectively, not to CTz. Concerning Area IV in the cerebellum, we could not relate it to any specific structure described in the adult bird (see DISCUSSION).

Development of auditory synaptic connectivity in the pons and cerebellum

We also examined the developmental sequence of functional synaptic connectivity in the higher-order auditory nuclei identified in the pons and cerebellum (Fig. 4). In normal saline, EPSP-related slow signals were detected from Area IV (contralateral cerebellum) and Vc (contralateral-NLL) from the 7-day stage. In Area Vi (ipsilateral-NLL), the slow signals were not identified until the 8-day stage, except for one 7-day preparation in which the signals were elicited in the Mg²⁺-free saline (Fig. 4, J2762). In the ipsilateral cerebellum, optical responses were not observed in any of the tested preparations.

DISCUSSION

Functional identification of auditory nuclei in the medulla and developmental profiles of synaptic connectivity

In this study, we identified three ipsilateral (Area I, III, and III) and one contralateral (Area Ic) response areas in the medulla, which evidently correspond to NM, ipsilateral-NL, NA, and contralateral-NL, respectively. Support for this identification comes from anatomical and developmental mapping studies. Ontogenetically, these nuclei originate from different ensembles of rhombomeres (r). Fate-mapping using the quail-
chick chimera technique has shown that NA, NL, and NM derive from rhombomeres 3 to 6 (r3–r6), r5–r6, and r6–r8, respectively (Cambronero and Puelles 2000; Marín and Puelles 1995), whereas fate-mapping using lipophilic dye injections has shown that NL arises mostly from r5 with a few cells arising from r6 and that NM arises from r5–r7 (Cramer et al. 2000). Both approaches demonstrate that NA, NL, and NM derive from sequential origins along the rostrocaudal axis. The distribution of optically identified NA, NL, and NM is consistent with this rostrocaudal alignment. In addition, three-dimensional reconstructions of these nuclei were previously made in the 11-day chicken embryo (Díaz et al. 2003), which are wholly consistent with the organization seen in our optical recordings.

By contrast to the auditory nuclei, vestibular neurons derive from a more extensive set of rhombomeres (r1–r7; Díaz et al. 1999) and occupy a longer span of the rostrocaudal axis in three-dimensional reconstructions (Díaz et al. 2003). Nevertheless, the vestibular nuclei overlap the areas containing the auditory nuclei in the plane of view we have used in our recordings. N. VIIIc also carries afferent fibers from the macula lagenae, a presumed gravistatic organ whose function has been only incompletely determined. These afferents terminate exclusively in the vestibular nuclei, Nucleus vestibularis lateralis, Nucleus vestibularis descendens, and Nucleus tangentialis (Kaiser and Manley 1996). In our previous study using brain stem slice preparations from the chicken embryo, we showed that stimulation of N. VIIIc using the parameters described here generated optical signals only in NM, not the vestibular nuclei, when viewed in the transverse plane (Sato and Momose-Sato 2003). Evidently, stimulation of the afferents from the macula lagenae at these stages is not sufficient to activate second-order neurons in the vestibular nuclei. By contrast, in the same study stimulation of the purely vestibular nerve branches (N. VIIIv) generated optical signals only in areas corresponding to the vestibular nuclei. Moreover, in a separate study in the whole brain stem preparation to be published elsewhere, stimulation of the rostral or caudal branches of N. VIIIv (pars vestibularis, R. rostralis, and R. caudalis of Barezile and Yasuda 1979) in intact preparations produced quite different response patterns from those shown in Figs. 1 and 2, consistent with the synaptic activation of vestibular nuclei and their motoneuron targets in the oculomotor, trochlear, and abducens nuclei and the cervical spinal cord (JC Glover, H Mochida, Y Momose-Sato, and K Sato, unpublished observations). Based on the above findings, the possibility that the optical responses we report here in the intact medulla are contaminated by a vestibular component mediated by afferents from the macula lagenae appears to be minimal.

Another issue to consider is that N. VIIIc contains not only afferent fibers but also inner ear efferents, which may have been stimulated antidromically. Although the efferent fibers invade the basilar papilla by 7 days (Fritzsch et al. 1993), we could not identify fast spikelike action potentials in the medial region of the medulla where the efferent neurons are located (Fritzsch et al. 1993; Simon and Lumsden 1993). The reason for this is not clear, but given the dependency of optical signal magnitude on the amount of polarized membrane, it might reflect a low density of active membrane per pixel in the area containing these neurons.

Given its role in the coincidence detection system that underlies sound localization, in mature birds NL receives inputs from ipsilateral and contralateral NM with little (<1 ms) delay; the projection distances of ipsilateral and contralateral...
NM axons within NL then provide differential delay lines for the detection of submillisecond interaural time differences (Carr and Konishi 1988). It is therefore striking that in the chicken embryo the response in the contralateral NL appears to be delayed by several tens of milliseconds compared with the response in ipsilateral NL. This difference may be exaggerated because it was difficult to pinpoint the onset of the response in ipsilateral NL with precision as a result of its proximity to activated NA and NM on the same side. Nevertheless, even a 10-ms difference would be difficult to reconcile on the basis of axon conduction because the distances involved would require a more than 300-fold greater conduction velocity in the ipsilateral compared with the contralateral NM axon. Although conduction velocities in chicken embryo axons can be very low (0.1–0.4 m/s; Sakai et al. 1991), a rough calculation of axon conduction velocity for the delay between NM and contralateral NL signals gives an exceptionally low value of 0.05 m/s. Thus it seems likely that the long delays observed in the developing auditory pathway arise in large part from synaptic immaturity, related, e.g., to low neurotransmitter release rates, longer diffusion distances, and lower densities of postsynaptic receptors. Such features might be expected to characterize transmitter release from arriving growth cones (Hume et al. 1983), transmission between still motile axonal and dendritic filopodia, or transmission at morphologically unspecialized pre- and postsynaptic contacts (Cohen-Cory 2002). Moreover, it cannot be ruled out that the dye used for optical recording, being lipid-soluble, might have effects that retard both conduction velocity and synaptic transmission relative to the situation in vivo.

In the medulla, Nucleus olivaris superior (SON) is known as another element of the auditory pathway (Conlee and Parks 1986; Takahashi and Konishi 1988). The outputs of NA and NL converge on SON and, in turn, the projections from SON neurons terminate in ipsilateral NA, NM, and NL, and contralateral SON (Burger et al. 2005; Conlee and Parks 1986; Monsivais et al. 2000). Srinivasan et al. (2004) used optical imaging in the postnatal rat brain stem slice and identified functional excitatory/inhibitory inputs to the superior olivary complex. In our optical recording, we could not identify a response area corresponding to SON. Because SON is located more ventrally than NM, NA, and NL (Kuenzel and Masson 1988), it is plausible that optical recording from the dorsal surface fails to detect the deeper SON responses or fails to discriminate them from overlying NM, NA, and NL responses. In Figs. 7 to 9, small signals were observed in a region medial to Area III, and it is possible that these might correspond to the responses in SON. Alternatively, the development of synaptic connections to SON may be delayed compared
FIG. 7. A: comprehensive optical recording of neural responses to N. VIIIc stimulation in an 8-day preparation. In addition to Areas I, III, IIc, and III in the medulla, optical responses were discerned in the bilateral pons (Areas VI and VC: indicated by green shadow) and contralateral cerebellum (Area IV: indicated by sky blue shadow). Colored-response areas were determined by eye, whereas the precise map is shown in Fig. 9. Optical recording was made from a region illustrated in the bottom left inset in Mg²⁺-free saline. B: enlarged traces of the optical signals detected from 6 different regions indicated by the numbers in A.
with other nuclei and thus not have been able to generate responses in our experiments.

Concerning projections from the cochleolagenar ganglion (CLG) to NM/NA and from NM to NL, morphological investigations have shown that 1) the final mitosis of CLG cells occurs between 4 and 7 days (D’Amico-Martel 1982); 2) some afferent axons enter the medulla while cell division is still occurring (Hemond and Morest 1991; Windle and Austin 1936) and, by 5 days, many auditory afferent axons penetrate the brain parenchyma (Book and Morest 1990; Knowlton 1967); 3) innervation of NM by auditory afferents occurs between 6 and 10 days (Jhaveri and Morest 1982a; Molea and Rubel 2003); 4) NM axons first reach ipsilateral-NL at 8 days (although they may contact migrating NL neurons before this), whereas their commissural branches reach contralateral-NL by 6 days (Young and Rubel 1986); 5) at 7.5–9 days, NM neurons have extensively ramifying dendritic processes, which undergo transformation to form by 17–18 days a mature synaptic structure, the end bulb of Held (Jhaveri and Morest 1982a,b), and 6) the NM neurons first become responsive to eighth nerve stimulation at 10 to 11 days (Jackson et al. 1982; Pettigrew et al. 1988).

The development of auditory pathway activation we document here has revealed that functional synaptic connections from the CLG to NM/NA arise at around 6 days and those from NM to bilateral NL are established by 6–7 days. This is substantially earlier than reported in previous functional studies (point 6 above), and is more in line with anatomical studies. However, even with respect to earlier anatomical studies the appearance of functional connections is earlier than might be expected. For example, in Mg^{2+}-free solution we could already detect responses in ipsilateral NL at 6 days, whereas Young and Rubel (1986) report that NM axons do not reach ipsilateral NL until 8 days, although they may contact newly generated NL neurons that are in the process of migrating to NL proper before this. These early signs of functional connections support the possibility that the responses detected as slow optical signals in the present study are mediated by immature synapses. The same notion has been suggested to explain the development of synaptic function in the embryonic rat trigeminal system (Momose-Sato et al. 2004). Similarly, Landmesser and Pilar (1972) demonstrated in the embryonic chick ciliary ganglion that synaptic transmission occurs before mature synaptic structures have been formed. That we
detect synaptic transmission at substantially earlier stages than in previous electrophysiological studies is probably explained by differences in the sensitivity and invasiveness of the recording methods.

**Functional identification of the auditory nuclei in the pons and developmental profiles of synaptic connectivity**

As shown in Figs. 7 and 9, we identified two response areas (Areas Vi and Vc) that appear to correspond to ipsi- and contralateral-NLL, respectively. In Fig. 8, responses in contralateral-NLL appeared at shorter latency than those in ipsilateral-NLL, at about the same time as those in contralateral-NL. The response in ipsilateral-NLL was also weaker than that in contralateral-NLL. These findings suggest that projections to ipsilateral-NLL do not derive directly from ipsilateral-NL, at least at the 8-day stage.

In the developmental profile of synaptic functions (Fig. 4), responses in ipsi- and contralateral-NLL were not induced at the 6-day stage even in Mg²⁺-free saline. This indicates that the development of auditory connections in NLL is slightly delayed compared with that in NM, NA, and NL. Differences in developmental profiles between Areas Vi and Vc also imply differences in functional synaptic development between ipsi- and contralateral-NLL. Nevertheless, the delay of slow signal appearance between Areas I–V was 1.5 days at most, suggesting that sensory information is transmitted to NLL soon after the initiation of synaptic function in NM and NA.

Because the responses in NLL were elicited by electrical stimulation applied to N. VIIIc, we cannot exclude the possibility that internuclear connections between NL and NLL are already established before the 7-day stage, but that propagation to NLL is not effective enough to elicit detectable EPSPs. We tried to examine functional connections between higher-order nuclei by applying direct stimulation to the medulla. However, this stimulation protocol elicited a large-scale depolarization wave that occluded the specific neuronal circuit activity (Momose-Sato et al. 2003) and we could not discriminate internuclear connections specific to the auditory pathway.

**Optical detection of a transient functional projection to the cerebellum**

In Figs. 7 to 9, slow signals were detected from the contralateral cerebellum (Area IV) in response to N. VIIIc stimulation. This was unexpected because, to our knowledge, no
nucleus related to the auditory pathway has been reported in the cerebellum of the adult chicken. It is unlikely that the cerebellar response is mediated by vestibular afferents running in N. VIIIc because vestibular afferents that project to the cerebellum do so only ipsilaterally. Another possibility is that the cerebellar response could represent crossed vestibulocerebellar projections activated by the macular afferents in N. VIIIc. However, in 5.5- to 8-day embryos, stimulation of the vestibular afferents in the rostral and caudal (vestibular) branches of N. VIII elicits no optical response in the cerebellum (JC Glover, H Mochida, Y Momose-Sato, and K Sato, unpublished observation).

Anatomical studies suggest, however, that there may be an auditory projection to the cerebellum at these stages. Young and Rubel (1986) described transient projections that arise from NM and course beyond NL anteriorly and dorsally toward the cerebellum. These projections are observed from 6 to 8 days. At 8–14 days, the NM axons projecting to NL increase severalfold in diameter and become dominated by their arborization in NL, whereas the axons extending beyond NL do not grow in diameter. By the 17-day stage, the projection to the cerebellum is barely visible or absent and it is not observed in posthatch chickens.

In the present study, the responses in the contralateral cerebellum were detected at 6–8 days, which is consistent with the morphological observations of Young and Rubel (1986). The physiological significance of this projection remains unknown. Although it is an interesting question when optical responses in the cerebellum disappear, we have not yet succeeded in recording optical signals in the cerebellum of embryos older than 8 days because of technical difficulties.

The potential role of sensory-evoked activity in the formation of auditory neural networks

In auditory network formation, it has been suggested that activity-independent molecular recognition combined with spontaneous activity are involved in target selection, the generation of the crude topography of auditory functional maps, and cell survival (Friauf and Lohmann 1999). In other words, it has been considered that most developmental events in auditory pathway formation take place independently of sensory-evoked activity. This concept is based on the observations that proliferation, early migration, and establishment of afferent and efferent topographic connections all occur before 10–11 days, when functional synaptic transmission from auditory afferents to NM is first thought to occur (Jackson et al. 1982; Pettigrew et al. 1988; reviewed in Rubel and Fritzsch 2002). On the other hand, more recent anatomical studies showed that auditory afferents reach NM starting at 6 days and contact target neurons in NM and NA in a tonotopic sequence over a period of a few days (Molea and Rubel 2003).

The results obtained in the present study reveal several new insights into the development of the auditory system of the chicken embryo. First, functional synaptic transmission in the primary auditory nuclei (NM and NA) appears around 6–7 days, much earlier than previously reported. Second, the higher-order nuclei (NL and NLL) are already responsive to sensory nerve stimulation within 1 day after the initiation of synaptic function in the primary auditory nuclei. Third, despite their early formation, functional neuronal networks in the auditory pathway exhibit a connectivity pattern similar to the adult pattern from the earliest stages. Similarly, the patterned tonotopic mapping of afferents onto NM and NA targets is present anatomically from the earliest stages of innervation by N. VIII afferents (Molea and Rubel 2003). These findings suggest that activity-independent mechanisms such as molecular recognition may play a major role in establishing the basic circuits, but also suggest that the role of sensory-evoked activity should be carefully reconsidered. The ability to elicit activity in this system by afferent stimulation from such early stages opens the possibility that sensory-evoked activity may influence the formation, consolidation, and maintenance of early established synapses, as well as the refinement of connections that may occur with continued development. Indeed, in future studies using the optical recording technique it should be possible to assess the development of tonotopic connections and the influence of sensory-evoked activity on their patterning.

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