Optical Detection of Developmental Origin of Synaptic Function in the Embryonic Chick Vestibulo-Cochlear Nuclei

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ABSTRACT

Functional organization of the brainstem vestibulo-cochlear nuclei during embryogenesis was investigated using a multiple-site optical recording technique with a fast voltage-sensitive dye. Brainstem slices with the cochlear and/or vestibular nerves attached were dissected from 6- to 8-day old (E6 to E8) chick embryos. Electrical responses evoked by cochlear or vestibular nerve stimulation were optically recorded simultaneously from many loci of the preparations. In E7 and E8 preparations, we identified two components of the optical response with cochlear or vestibular nerve stimulation; one was a fast spike-like signal related to the action potential, and the other was a slow signal related to the glutamate-mediated excitatory postsynaptic potential. The location of the cochlear nerve response area was mainly located on the dorsolateral region, while that of the vestibular nerve was deviated ventro-medially. At E6, cochlear nerve stimulation evoked only the fast spike-like signals in normal Ringer’s solution. However, when we removed Mg$^{2+}$ from the extracellular solution, significant slow signals were elicited in the E6 preparation.

The present results demonstrated that in the chick vestibulo-cochlear nuclei functional synapses are already generated by the E7 embryonic stage, and that postsynaptic activity related to NMDA receptors emerges latently, at least in the cochlear nerve-related nucleus, at the E6 embryonic stage. This chronological sequence of the emergence of postsynaptic function is different from that reported previously (E10-E11), suggesting that the developmental origin of sensory information transfer in the auditory pathway is much earlier than has been anticipated.
INTRODUCTION

The vestibulo-cochlear ganglion neurons link hair cells and brainstem nuclei, and transmit acoustic and balance information from the inner ear to the central nervous system (CNS). In the chick hindbrain, the cochlear ganglion cells innervate two cochlear nuclei (Nucleus magnocellularis cochlearis and Nucleus angularis; Breazile, 1979), and form the second-order synapses of the auditory pathway with the brainstem neurons. In these nuclei, it has been shown that a precise pattern of topographic connections is produced from the early stage of development (Levi-Montalcini, 1949; Lippe and Rubel, 1985; Sanes et al., 1989; Lippe, 1995). With this property, the embryonic chick auditory pathway has been one of the excellent models for developmental studies of neuronal connectivity/networks, and has been widely studied not only by neuroscientists focusing on acoustic information processing, but also by researchers interested in cell-cell communications in the developing CNS (Boord, 1968; Parks and Rubel, 1975; Rubel and Parks, 1975, 1976; for reviews see Friauf and Lohmann, 1999; Rubel and Fritzsch, 2002). However, most studies so far have been made in the anatomical/biochemical fields, and physiological investigations are rare. This situation is a result of the methodological limitation in which conventional electrophysiological techniques are difficult or impossible to employ, because of the small size and fragility of the young embryonic neurons.

Optical recording techniques using fast voltage-sensitive dyes have made it possible to monitor electrical activities in small cells that are difficult or impossible to access by traditional electrophysiological means. The introduction of a multi-element photodiode array has also facilitated the simultaneous recording of electrical activity and provided a powerful technique for monitoring the dynamic patterns of neural responses in living systems such as CNSs (Salzberg et al., 1977; Cohen and Salzberg, 1978; Salzberg, 1983; Grinvald et al., 1988; Kamino, 1990).

In our previous studies, we established the feasibility of optical techniques to record electrical activity from the brainstem, spinal cord and peripheral nervous systems isolated from developing chick and rat embryos (for reviews see Momose-Sato et al., 2001, 2003; Sato et al., 2003). We unraveled for the first time the onset and early development of action potential activity and postsynaptic function in the trigeminal, glossopharyngeal and vagal nuclei (Kamino et al., 1989; Komuro et al., 1991; Momose-Sato et al., 1991, 1994; Sato et al., 1995, 1998, 1999, 2002b, c). Throughout these investigations, we examined spatio-temporal patterns of neural responses, and succeeded in identifying the functional architecture of motor and sensory nuclei at early developmental stages.

In the present study, we applied the optical recording method to the embryonic chick vestibulo-cochlear nuclei, and traced their development and functional organization during early embryogenesis. We monitored optical responses evoked by cochlear/vestibular nerve stimulation in 6- to 8-day old (E6-E8) chick brainstems, and found that the developmental origin of postsynaptic function is much earlier than has been reported previously (Jackson et al., 1982; Pettigrew et al., 1988; Lippe, 1994; Asako et al., 1999;
Kuba et al., 2002a, 2002b). Preliminary results have been presented in abstract form (Sato et al., 2002a).
MATERIALS AND METHODS

Preparations: Brainstem slice preparations dissected from 6- to 8-day old embryonic (E6-E8) chicks were used (n=36). Experiments were carried out in accordance with the National Institute of Health guidelines 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 Co. Ltd., Saitama, Japan) were incubated for six to eight days in a forced-draft incubator (type P-008, Showa Incubator Lab., Urawa, Japan) at a temperature of 37 °C and 60% humidity, and were turned once each hour. In the present experiment, E6 corresponded to the Hamburger-Hamilton stages (H-H stages: Hamburger and Hamilton, 1951) 28-29, E7 to stages 30-32, and E8 to stages 33-34. The embryos were decapitated, and brainstems, with the vestibular and cochlear nerve fibers attached, were dissected from the embryos. Slice preparations of about 1500 µm thickness were made from the isolated brainstem at the level of the facial/vestibulo-cochlear nerve root (Fig. 1A). The pia mater was carefully removed in the bathing solution. After staining with the dye (see below), the preparation was attached to the silicone (KE 106LTV; Shin-etsu Chemical Co., Tokyo, Japan) bottom of a simple chamber with the spinal cord side up. The bathing solution contained (in mM) NaCl, 138; KCl, 5.4; CaCl$_2$, 1.8; MgCl$_2$, 0.5; glucose, 10; and Tris-HCl buffer (pH 7.3), 10. In the Mg$^{2+}$-free experiments, MgCl$_2$ was replaced with CaCl$_2$. The solution was equilibrated with oxygen.

Voltage-sensitive dye staining: Each preparation was stained by incubating it for 20 min in Ringer’s solution containing 0.2 mg/ml of a voltage-sensitive merocyanine-rhodanine dye, NK2761 (Hayashibara Biochemical Laboratories Inc./Kankoh-Shikiso Kenkyusho, Okayama, Japan: Kamino et al., 1981; Momose-Sato et al., 1995), and the excess (unbound) dye was washed away with dye-free Ringer’s solution 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). Further, it has been confirmed that the immature cellular-interstitial structure of early embryonic brain preparations allows the dye to diffuse readily from the surface to the interior regions (Sato et al., 1995).

Electrical stimulation: The cut end of the cochlear or one branch of the vestibular nerve (R. caudalis; Breazile and Yasuda, 1979) nerve was drawn into a micro-suction electrode fabricated from TERUMO-haematocrit tubing (VC-HO75P; TERUMO Co., 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 (8 µA/5 msec), which evoked maximum responses, were applied to the cranial nerve at intervals of 10-15 min.

Optical recording: Light from a 300 W tungsten-halogen lamp (Type JC-24V/300W, Kondo Philips Ltd., Tokyo, Japan) was collimated, rendered quasi-monochromatic with a heat filter and an interference filter with a transmission maximum at 703 ± 15 nm (Asahi
Spectra Co., Tokyo, Japan), and focused on the preparation. An objective (S Plan Apo, x10, 0.4 NA (numerical aperture)) and a photographic eyepiece projected a real image of the preparation (magnification 25x) onto a multi-element silicon photodiode matrix array mounted on an Olympus Vanox microscope (Type AHB-L-1, Olympus Optical Co., Tokyo, Japan). The focal plane was set on the caudal surface of the slice preparation. In the present experiments, we used two optical recording systems which were constructed in this laboratory (for reviews see Kamino, 1991; Momose-Sato et al., 2001). One was a 1020-site optical recording system with a 34 x 34-element silicon photodiode array (Hamamatsu Photonics Co., Hamamatsu, Japan) (Fig. 1B; for details see Hirota et al., 1995). In this system, each pixel (element) of the array detected light transmitted by a square region (54 x 54 µm² using x25 magnification) of the preparation. The outputs from 1020 elements were fed into amplifiers via current-to-voltage converters, and then passed to 32 sets of 32-channel analog multiplexers. Each output from the 32-channel multiplexers was fed into a subranging type AD converter system with a resolution of 18 bits, and was then sent to a computer. The other recording system was a 144-site optical recording system using a 12 x 12-element silicon photodiode array (MD-144-4PV; Centronic Ltd., Croydon, UK). In this system, each pixel of the array detected light from a square region (56 x 56 µm² using x25 magnification) of the preparation. The output of each detector in the diode array was passed to an amplifier (AC coupling = 3 sec) via a current-to-voltage converter. The amplified outputs from 144 elements of the detector were digitized by 16-bit AD converter units (PS-2032GP, TEAC corporation, Tokyo, Japan), and were then passed to a computer. The time resolution of these systems was ≈1 msec (1024 frames per 1000 msec in the 1020-site recording system and 1000 frames per 1000 msec in the 144-site recording system). The time interval between each recording was 10-15 min, and incident light was turned off except during the measuring period. In this condition, little or no signal fatigue was observed, and the degree of variability between successive recordings in terms of amplitude and duration of the signals was small. The recordings were made in single sweeps. The optical measurement was carried out in a still chamber without continuous perfusion with Ringer’s solution at room temperature, 26-30 °C.

The recorded signals were presented as the fractional change ΔI/I (the change in the light intensity divided by DC background intensity). Color-coded representation for a spatio-temporal activity map (Fig. 7) was constructed using “NeuroPlex” (RedShirtImaging LLC, Fairfield, CT, USA). The color code in the figure is linearly distributed between the minimum and maximum values of ΔI/I.
RESULTS

Optical responses to cochlear/vestibular nerve stimulation

Figure 2 illustrates four examples of multiple-site optical recordings of neural activity detected from an E7 (A) and E8 (B) chick brainstem. The thickness (light-path from the cephalic surface to the caudal surface) of the preparations was approximately 1500 µm, and they were translucent. Thus, we could detect neural voltage responses as changes in transmitted light intensity. The optical signals evoked by cochlear or vestibular nerve stimulation were recorded simultaneously from 1020 contiguous regions of the preparation using a 34 x 34 element photodiode array, in a single sweep.

In Fig. 2, when a stimulating current (8 µA/5 ms), which gave the maximum response, was applied to the left cochlear nerve (right side of the figure), optical responses were detected from the dorso-lateral region on the stimulated side of the preparation (upper recordings). When the vestibular nerve was stimulated, optical responses were also induced in the ipsilateral dorsal region, but the signals were usually smaller than those of the cochlear nerve stimulation (lower recordings).

Enlarged traces of the optical signals evoked by cochlear or vestibular nerve stimulation are presented in Fig. 3. In these recordings, we identified two components of the optical signals, viz., a fast spike-like signal and a delayed long-lasting slow signal. In the cochlear nerve recording (upper traces), the fast spike-like signals were more prominent, and the slow signals were larger, than those of the vestibular nerve response (lower traces).

The action spectra of the two components were similar, and both components were eliminated at 620-630 nm where the NK2761-dependent extrinsic absorption signal is absent (Momose-Sato et al., 1995; data not shown). This result indicates that both the fast and slow 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 brainstem, although an electrotonic potential-related component was found within the ganglion (data not shown).

Characteristics of the slow signals

In the previous studies, we reported that similar two-component signals were evoked by trigeminal, vagal or glossopharyngeal nerve stimulation in early embryonic brainstems, and that the fast component corresponded to the action potential and the slow component to the glutamate-mediated excitatory postsynaptic potential (EPSP) (Komuro et al., 1991; Sato et al., 1995, 1999). In the following experiments, we examined characteristics of the slow signal.

Figure 4A shows the effects of Ca\(^{2+}\)-free Ringer’s solution and Cd\(^{2+}\) on the slow optical signals evoked by cochlear nerve stimulation. The slow signals were reduced or eliminated by lowering the external Ca\(^{2+}\) concentration, and by addition of Cd\(^{2+}\) (1 mM) to the bathing solution. In the Ca\(^{2+}\)-free and Cd\(^{2+}\) traces, there appeared to be a small
downward deflection. However, the amplitude of the deflection was negligibly small (<1.0 x 10^-4), and thus we did not analyze them in the present study. Fig. 4B shows the effects of repetitive stimuli on the slow optical signal. When we applied repetitive stimuli (0.1Hz), the amplitude of the slow optical signals decreased gradually, while the fast signal changed insignificantly. This decline seems to reflect synaptic fatigue, and it argues that the slow signal is intimately related to the postsynaptic potential. The rate of decreasing in the slow signal amplitude was dependent on the frequency of the applied stimuli, and more rapid fatigue was observed when we used 1.0 Hz repetitive stimuli. These characteristics were also observed in the slow signals evoked by vestibular nerve stimulation (data not shown).

Figure 5 shows the effects of glutamate receptor antagonists, kynurenic acid, APV (DL-2-amino-5-phosphonovaleric acid, an NMDA receptor antagonist) and CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione, a non-NMDA receptor antagonist). When we applied kynurenic acid (1.2 mM), the slow signal was eliminated completely (Fig. 5A). On the other hand, application of APV (200 µM) or CNQX (5 µM) partly reduced the slow signal (Fig. 5B). The initial phase of the slow signal was much more sensitive to CNQX, while the later phase was reduced by APV. When APV and CNQX were added together, the slow signal was eliminated completely (Fig. 5B bottom). When we applied tetrodotoxin (TTX: 20 µM), the fast spike-like signal was eliminated together with the slow signal (data not shown). Similar results were obtained in all tested preparations (n=3). These results imply that the slow signal represents the glutamate-mediated EPSP, and that the fast signal corresponds to the sodium-dependent action potential. These results also suggest that the initial phase of the slow signal was mainly attributable to non-NMDA receptors, and that the later phase was mediated by NMDA receptors.

Spatio-temporal distribution patterns

From experimental results presented above, it is reasonable to interpret the detected optical signals as reflecting neural responses in the cochlear and vestibular sensory nuclei. To reveal functional organization/architecture of the cochlear/vestibular nuclei, we examined spatio-temporal distribution patterns of the optical signals.

First, we measured the amplitudes of the fast and slow signals, and constructed contour line maps of the signal amplitudes using an interpolation method (Hirota et al., 1987). Fig. 6A illustrates typical examples of maps constructed with the recordings obtained from an E8 preparation, in response to left cochlear or vestibular nerve stimulation. In cochlear nerve stimulation (upper panels), the distribution of the contour lines represented a layered pattern, with the signal size decreasing peripherally. In vestibular nerve stimulation (lower panels), the layered pattern was also observed in the E8 preparation. Similar maps were also obtained from E7 preparations, whereas the maximum amplitude of the slow signal increased from E7 to E8. Generally, it is understood that the size (represented as fractional change) of an extrinsic optical signal is proportional to a weighted average of the potential change of the membrane area imaged onto each detector (Kamino et al., 1989, Obaid et al.,
Thus, the change in the slow signal amplitude is assumed to be correlated with a developmental change in functional synaptic connections within the sensory nucleus (also see Discussion).

Figure 6B shows preparation-to-preparation differences in the optical response areas in E8 preparations. In these drawings, the response areas of the cochlear (upper maps) and vestibular (lower maps) nerves largely overlapped with each other, although the areas of the cochlear nerve were deviated dorso-laterally compared with those of the vestibular nerve. Positions of the amplitude peak (asterisks), which corresponds to the center of each nucleus, were also different between the cochlear and vestibular responses. Between the E7 and E8 preparations, there was no distinct difference in the distribution pattern (data not shown).

To compare the dynamic pattern and time course of the neural responses, we made spatiotemporal activity maps. Fig. 7 illustrates the images of the optical responses recorded from an E7 (upper panels) and E8 (lower panels) preparation. In both the preparations, the neural response first appeared in the brainstem nucleus 4 msec after stimulation (the third frame); it expanded gradually, and reached its maximum spatial extent by the frame recorded at 18 msec (the tenth frame). Similar results were obtained in other E7 and E8 preparations (n=10). In these imaging maps, no consistent difference in the time course of neural responses was observed between the cochlear and vestibular nerves.

Development of synaptic function in the cochlear nuclei

It is an interesting question in what stages functional synaptic transmission is expressed within the cochlear and vestibular nuclei. With cochlear nerve stimulation, the EPSP-related slow optical signals were recorded from E7, and no slow component was detected from E6 preparations in normal Ringer’s solution.

Figure 8 shows an example of optical recordings in response to cochlear nerve stimulation in an E6 preparation. Enlarged traces of the optical signals indicated by asterisks are also presented in Fig. 9. In normal Ringer’s solution (Fig. 8A), cochlear nerve stimulation evoked only the fast spike-like signals in the dorso-lateral region: the slow component was not significant (< 1.0 x 10^-4). However, when Mg\textsuperscript{2+} was removed from the bathing solution (Fig. 8B), distinct slow signals were elicited in the dorsal region. The slow signals induced in the Mg\textsuperscript{2+}-free solution were blocked in the presence of APV (200 µM) (data not shown), suggesting that they are attributable to NMDA receptors (see Discussion). These results suggest that, in the sensory nucleus of the cochlear nerve, synaptic function is latently generated as early as E6.

Concerning the vestibular nerve, we could not analyze postsynaptic function in E6 preparations, because the vestibular nerve was so fine and fragile at this stage that we could not make a good preparation with the vestibular nerve intact.
DISCUSSION

In the present experiment, we focused on the brainstem nuclei related to the cochlear and vestibular nerves, and provided evidence that postsynaptic activity is already expressed at the E7 embryonic stage. In addition, we demonstrated that at least in the cochlear nucleus, synaptic function is latently generated as early as E6. These stages are much earlier than those reported previously (E10-E11). We will discuss the present results in relation to functional development of the acoustic neuronal pathway during early embryogenesis.

Cochlear nerve-related nucleus in the brainstem

As shown in Figs. 2 and 3, cochlear nerve stimulation induced fast spike-like signals followed by slow signals in the dorsolateral region of the brainstem. The cochlear nerve bundle contains sensory (afferent) and motor (efferent) nerve fibers, and the stimulation applied to the cochlear nerve was simultaneously orthodromic for the sensory nerve fibers and antidromic for the motor nerve fibers. Unfortunately, it was impossible to separate the sensory and motor nerve fibers surgically, because the early embryonic nerve fibers are very thin and fragile.

From the results that the slow signal was blocked by Ca\(^{2+}\)-free or Cd\(^{2+}\)-containing solutions, and considering that the cochlear motor neurons are located bilaterally in the medial region (Fritzsch et al., 1993; Simon and Lumsden, 1993), it is reasonable to interpret that (1) the observed optical responses originate from the sensory nucleus of the cochlear nerve and that (2) the fast signal corresponds to the orthodromic action potential evoked in the sensory nerve fibers/terminals and the slow signal to the postsynaptic potential evoked in the postsynaptic neurons. It might be possible that some fraction of the postsynaptic firing component also contribute to the fast optical signal.

Previously, Asako et al. (1999) recorded optical signals with fast and slow components from E8 chick embryos in response to stimulation of the cochlear nerve with a tungsten microelectrode. However, there remained a possibility that the detected signals contain electrotonic responses because, as the authors noted in their discussion, the stimulation intensity used was very strong (3 mA/5 msec). In the present study, we applied adequate stimulation (8 µA/5 msec) (Kamino et al., 1990; Sato et al., 1993) to the cochlear and vestibular nerves individually with a suction electrode. We verified that no electrotonic response was contained in the detected signal by confirming that application of hyperpolarizing current pulses evoked no optical signal within the brainstem.

In birds, the afferent fibers of the cochlear nerve terminate in topographic fashion upon two brainstem nuclei, *Nucleus angularis* (NA) and *Nucleus magnocellularis cochlearis* (NM) (Breazile, 1979). In the development of the brainstem, rhombomeres (r) are thought to be key structures forming the cranial nerve nuclei (Lumsden and Keynes, 1989). Morphological studies using quail-chick chimeras showed that the NA and NM derive from rhombomeres 3 to 6 (r3-r6; mainly r3 and r4) and from r6 to r8 (mainly r7 and r8),
respectively (Marín and Puelles, 1995; Cambronero and Puelles, 2000). These studies also showed that at H-H stages 35-36, the NA is mainly localized at the level of the cerebellar peduncle, and that the NM is situated at the level of the cochlear nerve root.

In the present experiments, the focal plane of the optical recording was set on the caudal surface of the slice preparation, which corresponded to the midpoint between the vestibulo-cochlear nerve (the eighth cranial nerve) root and the glossopharyngeal nerve (the ninth cranial nerve) root. Therefore, it seems reasonable to consider that the recorded signals originate mainly from the NM, and that the contribution of the deeply located NA is relatively small. In the following discussion, we refer the optical response area in response to cochlear nerve stimulation as the cochlear nerve-related sensory nucleus.

**Comparison with vestibular nerve-related nucleus**

In the present study, we also detected optical signals evoked by vestibular nerve stimulation. The vestibular nerve stimulation also produced the fast spike-like signal followed by the slow signal in the dorsal region of the brainstem, but the responses were usually smaller than those of the cochlear nerve. In the present experiments, we stimulated only one branch of the vestibular nerve (*R. caudalis*; Breazile and Yasuda, 1979), and this might be the cause of the small responses. Although the brainstem center of the vestibular nerve is anatomically divided into several nuclei (Breazile, 1979) or groups (Glover, 2000), we could not identify each in the present study.

As shown in Fig. 6, the response area of the cochlear nerve largely overlapped with that of the vestibular nerve in a dorsomedial/mediolateral (X-Y) plane of the slice. This result suggests the possibility that these nuclei are not separated functionally at early developmental stages. The vestibular nerve-area was somewhat deviated ventrally to the cochlear nerve-area, which is consistent with the morphological observations in older embryos (Peusner and Morest, 1977; Glover and Petursdottir, 1991; Glover, 2000).

**Characteristics of the slow signals**

The EPSP-related slow optical signal recorded in the present experiment was very slow (=1 sec duration; Fig. 4A). Such slow signals were also detected from a preparation stained with an oxonol dye (RH482) (unpublished observations), which is relatively insensitive to glial cell membrane potential changes (Konnerth et al., 1987). Thus, we conclude that the slow optical signals were neuronal in origin, and interpret the long duration of the slow signal as attributable to slow synaptic transmission in the embryonic preparation.

The amplitude of the slow optical signal decreased with repetitive stimuli at 0.1 Hz (Fig. 4B), indicating that the embryonic synapse in the vestibulo-cochlear nucleus fatigues rapidly. We have reported similar observations in other brainstem nuclei (Komuro et al., 1991; Sato et al., 1995, 1998, 1999), spinal cord (Arai et al., 1999) and sympathetic ganglion (Momose-Sato et al., 1999) of the chick and rat embryos. These results suggest that such a
rapid fatigue is a typical characteristic of early embryonic synapses. As shown in Fig. 5, the slow optical signals were eliminated by application of kynurenic acid or APV/CNQX. We therefore interpret the slow signal as reflecting the glutamate-mediated (glutamatergic) EPSP. Furthermore, it was shown that both NMDA and non-NMDA receptors were related to synaptic function, and that the initial phase of the EPSP was mainly attributable to non-NMDA receptors, while the later phase was mediated by NMDA receptors. Similar results were also obtained from other preparations (Komuro et al., 1991; Sato et al., 1995, 1998, 1999), suggesting that glutamate-mediated synaptic transmission appears globally in the early embryonic chick and rat brainstem nuclei.

Developmental origin of functional synapse formation

With cochlear nerve stimulation, the EPSP-related slow signal was first detected from E7, suggesting that synaptic function in the chick acoustic system is already generated at this developmental stage. As shown in Figs. 8 and 9, in the E6 preparation, the slow signal, which was not detectable in normal Ringer’s solution, was evoked in the Mg$^{2+}$-free Ringer’s solution. Previously, we have shown that, in the nucleus of the tractus solitarius (NTS) of the chick and rat embryos, synaptic function mediated by NMDA receptors is latently generated one day before the expression of the EPSP, and that the onset of synaptic function is regulated by a Mg$^{2+}$ block on the NMDA receptors (Momose-Sato et al., 1994; Sato et al., 1998). The present results also suggest that postsynaptic function related to NMDA receptors emerges, in latent form, within the cochlear nerve-related nucleus at the E6 embryonic stage.

This chronological sequence of the emergence of postsynaptic function is different from the previous observations that NM neurons were responsive to eighth-nerve stimulation from E10-E11 (Jackson et al., 1982; Pettigrew et al., 1988). As Jackson et al. (1982) pointed out, this discrepancy may be due to differences in sensitivity of the measurement systems.

In the present study, we could not determine the developmental origin of functional synaptic formation in the vestibular nucleus because of technical limitations. The result that vestibular nerve stimulation elicited the EPSP-related slow signal in the E7 preparation suggests that synaptic function in the chick vestibular nucleus is also generated at least by the E7 embryonic stage. In morphological studies, formation of the vestibular center is anticipated to be one day earlier than that of the cochlear center (Rubel and Fritzsch, 2002). Functional development of the vestibular nucleus might also be earlier than that of the cochlear nucleus: further investigation will uncover this interesting question.

Role of electrical activity in neural circuit formation

In the chick cochlear ganglion, neurons of the neural crest origin are produced between E4 and E7 (D’Amico-Martel, 1982). Axons of the cochlear neurons penetrate the brainstem parenchyma by E5 (Windle and Austin, 1936; Hemond and Morest, 1991), and
project to the cochlear nuclei by E10 (Jhaveri and Morest, 1982a, b). Based on the previous reports that E10-E11 was the first age at which postsynaptic action potentials in the NM could be evoked by eighth-nerve stimulation (Jackson et al., 1982; Pettigrew et al., 1988), it is suggested that most developmental events in the auditory pathway formation take place independently of excitatory afferent activity: it has been recognized that proliferation, early migration and establishment of afferent and efferent topographic connections all occur before functional afferent synapses are formed (Rubel and Fritzsch, 2002). However, as shown in the present study, functional synaptic connections in the brainstem cochlear center have already been generated at E6-E7, when production of sensory neurons have not been completed. This result suggests the possibility that the activity-dependent process might be present at early developmental stages and have some influences on neural circuit formation of the auditory pathway.
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REFERENCES
Hirota, A., Kamino, K., Komuro, H., and Sakai, T.  Mapping of early development of


Levi-Montalcini, R. The development of the acoustico-vestibular centers in the chick embryo in the absence of the afferent root fibers and of descending fiber tracts. *J. Comp.*


Pettigrew, A. G., Ansselin, A. D. and Bramley, J. R. Development of functional innervation in the second and third order auditory nuclei of the chick. *Development* 104,


Sato, K., Momose-Sato, Y., Sakai, T., Hirota, A., and Kamino, K.  Responses to glossopharyngeal stimulus in the early embryonic chick brainstem: Spatiotemporal


Windle, W. F. and Austin, M. F.  Neurofibrillar development in the central nervous system of chick embryo up to 5 days’ incubation.  *J. Comp. Neurol.* 63, 431-463, 1936.
FIGURE LEGENDS

**Figure 1.** A. An example of the relative position of the image of the brainstem slice preparation dissected from an E8 chick embryo and the 1020-element photodiode array grids. B. A schematic drawing of the simultaneous 1020-site optical recording.

**Figure 2.** Multiple-site optical recordings of neural responses to cochlear (upper recordings) or vestibular (lower recordings) nerve stimulation in an E7 (A) and E8 (B) chick brainstem slice. The optical signals were evoked by applying a brief positive square current pulse (8 µA/5 ms) to the left cochlear (N. C) or vestibular (N. V) nerve with microsuction electrodes. The signals were detected in a single sweep. The direction of the arrow on the lower right of the figure indicates an increase in transmitted light intensity (a decrease in dye absorption), and the length of the arrow represents the stated value of the fractional change. Signals indicated by asterisks are enlarged in Fig. 3.

**Figure 3.** Enlarged traces of the evoked optical signals induced by cochlear (upper traces) and vestibular (lower traces) nerve stimulation in the E7 (A) and E8 (B) preparations shown in Fig. 2.

**Figure 4.** A. Effects of a Ca$^{2+}$-free bathing solution and Cd$^{2+}$-containing solution on the evoked optical signals in response to cochlear nerve stimulation. Data were obtained from an E8 preparation. The upper trace was control, and the middle and lower traces were obtained in the Ca$^{2+}$-free bathing solution and the Cd$^{2+}$ (1 mM)-containing solution, respectively. B. Decreasing slow signal amplitude with repetitive stimuli in an E8 brainstem preparation. Square current pulses of 8 µA/5 ms and 0.1 Hz were applied to the cochlear nerve. The relative amplitudes of the fast and slow signals are plotted against time in seconds.

**Figure 5.** A. Effects of kynurenic acid (1.2 mM) on the evoked optical signals in response to cochlear (upper traces) and vestibular (lower traces) nerve stimulation. Data were obtained from an E8 preparation. B. Effects of APV and CNQX on the evoked optical signals in response to cochlear nerve stimulation. Data were obtained from an E8 preparation in normal Ringer’s solution (upper trace), APV (200 µM)-containing solution (the second trace), CNQX (5 µM)-containing solution (the third trace), and APV (190 µM)/CNQX (5 µM)-containing solution (bottom trace).

**Figure 6.** A. Contour line maps of the amplitude of the evoked optical signals in response to left cochlear (upper panels) and vestibular (lower panels) nerve stimulation obtained from an E8 preparation. In each paired drawing, the left map is for the fast signals, and the right map for the slow signals. The numerals on the contour lines indicate the fractional change multiplied by 10$^4$. The dotted lines represent the signal size of 1.0 x 10$^{-4}$. B.
Comparison of the response areas of the cochlear (upper panels) and vestibular (lower panels) nerves in preparations. The relative location of the area in which the optical signals larger than $1 \times 10^{-4}$ were evoked is illustrated in each drawing. The positions of the amplitude peaks are indicated by asterisks.

**Figure 7.** Spatiotemporal activity mapping. The time lapse imaging representations of the neural response detected from an E7 and E8 preparation are shown. The frame interval was 2 msec. Red in the color bar corresponds to $5 \times 10^{-4}$.

**Figure 8.** Appearance of the slow signal in the Mg$^{2+}$-free bathing solution. The signals were obtained from an E6 preparation in response to cochlear nerve stimulation. In normal Ringer’s solution (A), no slow signal was observed. In the Mg$^{2+}$-free solution (B), slow signals were evoked in the dorsal region. Signals indicated by asterisks are enlarged in Fig. 9. The recordings were obtained from the region indicated by a square in the lower inset using the 144-element photodiode array.

**Figure 9.** Enlarged traces of the optical signals evoked by cochlear nerve stimulation in the E6 preparation shown in Fig. 8. Upper traces were recorded in normal Ringer’s solution, and lower traces were obtained in the Mg$^{2+}$-free solution.
A 7 day
N. cochlearis stimulation

B 8 day
N. cochlearis stimulation

N. vestibularis stimulation

stimulation

10^3

100 msec

Fig. 3
Fig. 4
Fig. 5

A
N. cochlearis stimulation
control
KYN
stimulation
N. vestibularis stimulation
control
KYN
stimulation
100 msec

B
control
APV
CNOX
APV +
CNOX
stimulation
100 msec
6 day

A: Control

56 μm

B: Mg²⁺ free

500 msec

(j2288)

ventral

left

N. VIII

Fig. 8