Optical Detection of Developmental Origin of Synaptic Function in the Embryonic Chick Vestibulocochlear Nuclei

Katsushige Sato and Yoko Momose-Sato
Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Graduate School and Faculty of Medicine, Tokyo 113-8519, Japan

Submitted 26 December 2002; accepted in final form 10 February 2003

Sato, Katsushige and Yoko Momose-Sato. Optical detection of developmental origin of synaptic function in the embryonic chick vestibulocochlear nuclei. J Neurophysiol 89: 3215–3224, 2003; 10.1152/jn.01169.2002. Functional organization of the brain stem vestibulocochlear nuclei during embryogenesis was investigated using a multiple-site optical recording technique with a fast voltage-sensitive dye. Brain stem slices with the cochlear and/or vestibular nerves attached were dissected from 6- to 8-day-old (E6–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 ventrolaterally. At E6, cochlear nerve stimulation evoked only the fast spike-like signals in normal Ringer solution. However, when we removed Mg2+ from the extracellular solution, significant slow signals were elicited in the E6 preparation. The present results demonstrated that in the chick vestibulocochlear nuclei, functional synapses are already generated by the E7 embryonic stage and that postsynaptic activity related to N-methyl-D-aspartate 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 vestibulocochlear ganglion neurons link hair cells and brain stem nuclei, and transmit acoustic and balance information from the inner ear to the 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 brain stem 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 1995; Lippe and Rubel 1985; Sanes et al. 1989). 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 cellular communications in the developing CNS (Boord 1968; Parks and Rubel 1975; Rubel and Parks 1975; Rubel et al. 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 multielement 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 (Cohen and Salzberg 1978; Grinvald et al. 1988; Kamino 1990; Salzberg 1983; Salzberg et al. 1977).

In our previous studies, we established the feasibility of optical techniques to record electrical activity from the brain stem, 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 spatiotemporal 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 vestibulocochlear 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 brain stems and found that the developmental...
origin of postsynaptic function is much earlier than has been reported previously (Asako et al. 1999; Jackson et al. 1982; Kuba et al. 2002a,b; Lippe 1994; Pettigrew et al. 1988). Preliminary results have been presented in abstract form (Sato et al. 2002a).

METH ODS

Preparations

Brain stem 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 Institutes 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, Saitama, Japan) were incubated for 6 to 8 days in a forced-draft incubator (type P-008, Showa Incubator, 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 brain stems, with the vestibular and cochlear nerve fibers attached, were dissected from the embryos. Slice preparations of ~1,500 μm thickness were made from the isolated brain stem at the level of the facial/vestibulocochlear nerve root (Fig. 1A). The pia mater was carefully removed in the bathing solution. After staining with the dye (see following text), the preparation was attached to the preparation. An objective [S Plan Apo, Kondo Philips, Tokyo, Japan) was collimated, rendered quasi-monomode with a heat filter and an interference filter with a transmission maximum at 703 ± 15 nm (Asahi Spectra, Tokyo) and focused on the preparation. An objective [S Plan Apo, ×10, 0.4 NA (numerical aperture)] and a photographic eyepiece projected a real image of the preparation (magnification: ×25) onto a multi-element silicon photodiode matrix array mounted on an Olympus Vanox microscope (Type AHB-L-1, Olympus Optical, 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 that were constructed in this laboratory (for reviews, see Kamino 1991; Momose-Sato et al. 2001). One was a 1,020-site optical recording system with a 34 × 34-element silicon photodiode array (Hamamatsu Photonics, 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 × 54 μm² using ×25 magnification) of the preparation. The outputs from 1,020 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 × 12-element silicon photodiode array (MD-144–4PV; Centronic, Croydon, UK). In this system, each pixel of the array detected light from a square region (56 × 56 μm² using ×25 magnification) of the preparation. The output of each detector in the photodiode array was passed to an amplifier (AC coupling = 3 s) 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, Tokyo, Japan) and were then passed to a computer. The time resolution of these systems was ~1 ms (1,024 frames per 1,000 ms in the 1,020-site recording system and 1,000 frames per 1,000 ms 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

Voltage-sensitive dye staining

Each preparation was stained by incubating it for 20 min in Ringer solution containing 0.2 mg/ml of a voltage-sensitive merocyanine-rhodamine dye, NK2761 (Hayashibara Biochemical Laboratories/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 solution before recording. This merocyanine-rhodamine 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. 1998).

Electrical stimulation

The cut end of the cochlear or one branch of the vestibular nerve (Breazile and Yasuda 1979) nerve was drawn into a micro-suction electrode fabricated from TERRUO-hematocrit tubing (VC-HOT5P; Terumo, Tokyo, Japan), which had been hand-pulled to a fine tip (~100 μm ID) over a low-temperature flame. Positive (depolarizing) square current pulses (8 μA/5 ms), 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, 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, Tokyo) and focused on the preparation. An objective [S Plan Apo, ×10, 0.4 NA (numerical aperture)] and a photographic eyepiece projected a real image of the preparation (magnification: ×25) onto a multi-element silicon photodiode matrix array mounted on an Olympus Vanox microscope (Type AHB-L-1, Olympus Optical, 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 that were constructed in this laboratory (for reviews, see Kamino 1991; Momose-Sato et al. 2001). One was a 1,020-site optical recording system with a 34 × 34-element silicon photodiode array (Hamamatsu Photonics, 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 × 54 μm² using ×25 magnification) of the preparation. The outputs from 1,020 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 × 12-element silicon photodiode array (MD-144–4PV; Centronic, Croydon, UK). In this system, each pixel of the array detected light from a square region (56 × 56 μm² using ×25 magnification) of the preparation. The output of each detector in the photodiode array was passed to an amplifier (AC coupling = 3 s) 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, Tokyo, Japan) and were then passed to a computer. The time resolution of these systems was ~1 ms (1,024 frames per 1,000 ms in the 1,020-site recording system and 1,000 frames per 1,000 ms 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 solution at room temperature, 26–30°C. The recorded signals were presented as the fractional change $\Delta/I$ (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). The color code in the figure is linearly distributed between the minimum and maximum values of $\Delta/I$.

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 brain stem. The thickness (light-path from the cephalic surface to the caudal surface) of the preparations was ~1,500 $\mu$m, 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 1,020 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 $\mu$A/5 ms), which gave the maximum response, was applied to the left cochlear nerve, optical responses were detected from the dorsolateral region on the stimulated side of the preparation (top). 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 (bottom).

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 (top), the fast spike-like signals were more prominent and the slow signals were larger than those of the vestibular nerve response (bottom).

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 brain stem, although an electrotonic potential-related component was found within the ganglion (data not shown).

FIG. 2. Multiple-site optical recordings of neural responses to cochlear (top) or vestibular (bottom) nerve stimulation in an E7 (A) and E8 (B) chick brain stem slice. The optical signals were evoked by applying a brief positive square current pulse (8 $\mu$A/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.
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 brain stems 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\(^{2+}\)/H\(_{11001}\)-free Ringer solution and Cd\(^{2+}\)/H\(_{11001}\)-containing solution 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\(^{2+}\)/H\(_{11001}\)-free and Cd\(^{2+}\)/H\(_{11001}\) traces, there appeared to be a small downward deflection. However, the amplitude of the deflection was negligibly small (<1.0 × 10\(^{-3}\)), and thus we did not analyze them in the present study. Figure 4B shows the effects of repetitive stimuli on the slow optical signal. When we applied repetitive stimuli (0.1 Hz), the amplitude of the slow optical signals decreased gradually, whereas 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, N-methyl-D-aspartate (NMDA) receptor antagonist] and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 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 \(\mu\)M) or CNQX (5 \(\mu\)M) partly reduced the slow signal (Fig. 5B). The initial phase of the slow signal was much more sensitive to CNQX, whereas 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 \(\mu\)M), 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.

Spatiotemporal distribution patterns

From experimental results presented in the preceding text, 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.

FIG. 4. A: effects of a Ca\(^{2+}\)/H\(_{11001}\)-free bathing solution and Ca\(^{2+}\)/H\(_{11001}\)-containing solution on the evoked optical signals in response to cochlear nerve stimulation. Data were obtained from an E8 preparation. Top: control; middle and bottom: obtained in the Ca\(^{2+}\)/H\(_{11001}\)-free bathing solution and the Ca\(^{2+}\) (1 mM)-containing solution, respectively. B: decreasing slow signal amplitude with repetitive stimuli in an E8 brain stem preparation. Square current pulses of 8 \(\mu\)A/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.
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 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 solution (Fig. 8A), cochlear nerve stimulation evoked only the fast spike-like signals in the dorsolateral region: the slow component was not significant (<1.0 × 10⁻⁴). However, when Mg²⁺ was removed from the bathing solution (Fig. 8B), distinct slow signals were elicited in the dorsal region. The slow signals induced in the Mg²⁺-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 brain stem 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 brain stem**

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 brain stem. The vestibulocochlear nerve contains sensory (afferent) and (efferent) nerve fibers, and the stimulation applied to the vestibulocochlear nerve was simultaneously orthodromic for the sensory nerve fibers and antidromic for the efferent nerve fibers. Unfortunately, it was impossible to separate the sensory and efferent nerve fibers surgically because the early embryonic nerve fibers are very thin and fragile.

From the results that the slow signal was blocked by Ca²⁺-free or Cd²⁺-containing solutions, and considering that the efferent neurons are located bilaterally in the medial region (Fritzsch et al. 1993; Simon and Lumsden 1993), it is reasonable to interpret that the observed optical responses originate from the sensory nucleus and that 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 contributes 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 ms). In the present study, we applied adequate stimulation (8 μA/5 ms) (Kamino et al. 1990; Sato et al. 1993) to the cochlear and vestibular nerves individually with a suction electrode. We verified that no electrotonic

---

**FIG. 6.** A: contour line maps of the amplitude of the evoked optical signals in response to left cochlear (top) and vestibular (bottom) nerve stimulation obtained from an E8 preparation. Left: the fast signals; right: the slow signals. The numerals on the contour lines indicate the fractional change multiplied by 10⁴. The dotted lines represent the signal size of 1.0 × 10⁻⁴. B: comparison of the response areas of the cochlear (top) and vestibular (bottom) nerves in different preparations. The relative location of the area in which the optical signals >1 × 10⁻⁴ were evoked is illustrated in each drawing. *, positions of the amplitude peak.
response was contained in the detected signal by confirming that application of hyperpolarizing current pulses evoked no optical signal within the brain stem.

In birds, the afferent fibers of the cochlear nerve terminate in topographic fashion on two brain stem nuclei, Nucleus angularis (NA) and Nucleus magnocellularis cochlearis (NM) (Breazile 1979). In the development of the brain stem, 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 (Combronero and Puelles 2000; Martín and Puelles 1995). 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 vestibulocochlear nerve (the 8th cranial nerve) root and the glossopharyngeal nerve (the 9th 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 brain stem, 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 (Breazile and Yasuda 1979), and this might be the cause of the small responses. Although the brain stem 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 dor-
soventral/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 (Glover 2000; Glover and Petursdottir 1991; Peusner and Morest 1977).

Characteristics of the slow signals

The EPSP-related slow optical signal recorded in the present experiment was very slow (≈1-s 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 vestibulocochlear nucleus fatigues rapidly. We have reported similar observations in other brain stem nuclei (Komuro et al. 1991; Sato et al. 1995, 1998, 1999), spinal cord (Arai et al. 1999) and sympathetic ganglion (Momo-se-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, whereas 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 brain stem nuclei.

Developmental origin of functional synapse formation

With cochlear nerve stimulation, the EPSP-related slow signal was 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 solution, was evoked in the Mg\(^{2+}\)-free Ringer solution. Previously, we have shown that, in the nucleus of the tractus solitarius (NTS) of the chick and rat embryos, synaptic func-
tion 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 to 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 brain stem parenchyma by E5 (Hemond and Morest 1991; Windle and Austin 1936) 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 brain stem cochlear center have already been generated at E6–E7, when production of sensory neurons has 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.

We express gratitude to Dr. Kohtarou Kamino for helpful discussion throughout the course of our work. This research was supported by grants from the Monbuk-Kagakusho of Japan [Priority Areas (C) Advanced Brain Science Project] and research funds from Uehara Memorial Life Science Foundation, Inamori Foundation, Brain Science Foundation, Shimadzu Science Foundation, and Nissan Science Foundation.

**REFERENCES**


