Optical Approaches to Functional Organization of Glossopharyngeal and Vagal Motor Nuclei in the Embryonic Chick Hindbrain

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Optical approaches to functional organization of glossopharyngeal and vagal motor nuclei in the embryonic chick hindbrain. J Neurophysiol 88: 383–393, 2002; 10.1152/jn.00664.2001. We investigated the functional organization of the glossopharyngeal and vagal motor nuclei during embryogenesis using multiple-site optical recording with a fast voltage-sensitive dye. Intact brain stem preparations with glossopharyngeal and vagus nerves were dissected from 4- to 8-day-old chick embryos. Electrical responses evoked by glossopharyngeal/vagus nerve stimulation were optically recorded from many loci of the stained preparations. In 4- to 6-day-old preparations, action potential-related fast spikelike signals were detected from the nucleus of the glossopharyngeal nerve and the dorsal motor nucleus of the vagus nerve. Contour line maps of the signal amplitude showed multiple-peak patterns, suggesting that the neurons and/or their activity were not uniformly distributed within the nuclei at early developmental stages. As development proceeded from 4 to 6 days, the peaks fused with each other and the number of peaks decreased gradually. In most 7- and 8-day-old preparations, only a single peak was identified in the nuclei, and the distribution of the signal amplitude formed a layered pattern surrounding the peak-signal area. These results suggest that functional organization of the motor nuclei in the embryonic hindbrain changes dynamically with development, resulting in a rearrangement of functional nuclear cores from multiple-peaks to a single peak.

INTRODUCTION

In the study of functional organization and architecture of the complex CNS, it is a useful strategy to ontogenetically approach the emergence of spatiotemporal patterns of electrical activity in neural circuits. During the early stage of ontogenesis in the CNS, it is well documented that segmentation along the anteroposterior axis plays an important role in pattern formation within the neural epithelium. In the neural epithelium, transient periodic swellings, neuromeres, have long been recognized, and, especially in the brain stem, rhombomeres are thought to be key structures forming the cranial nerve nuclei (Keynes and Lumsden 1990; Lumsden 1990; Lumsden and Keynes 1989). Moreover, recent progress in molecular biology has made it clear that the Hox genes form a network of transcription factors implicated in the regulation of axial patterning in vertebrate development (Keynes and Krumlauf 1994; Wilkinson 1993). However, electrophysiological experiments on the early embryonic CNS are often difficult or impossible because of the small size and fragility of the young embryonic cells.

Optical techniques that use 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 and also facilitate the simultaneous recording of electrical activity from multiple sites in living systems such as the CNS (Cohen and Salzberg 1978; Grinvald et al.1988; Kamino 1990; Salzberg 1983; Salzberg et al. 1977). We applied this optical technique to the embryonic rat and chick nervous systems and established the feasibility of using optical techniques to record electrical activity in brain stems (for a review, see Momose-Sato et al. 2001), spinal cords (Arai et al. 1999; Mochida et al. 2001), and peripheral ganglia (Momose-Sato et al. 1991a, 1999) isolated from developing chick and rat embryos.

In our previous reports, we demonstrated the ontogenesis of neural excitability and synaptic function in the embryonic chick vagal nuclei (Komuro et al. 1991; Momose-Sato et al. 1991b, 1994). Furthermore, we succeeded in three-dimensional recording of neural activity from the glossopharyngeal nerve-related nuclei in the middle stage of embryonic development (Sato et al. 1995). In a subsequent study that targeted the early process of glossopharyngeal nuclear organization, we noticed that the functional architecture of the motor nucleus changed dynamically.

We demonstrate here how brain stem motor nuclei are organized during early embryogenesis, focusing on the early stages of functional organization of the glossopharyngeal and vagal motor nuclei in the chick embryo. We recorded voltage-sensitive dye signals evoked by glossopharyngeal and vagal nerve stimulation in 4- to 8-day-old brain stem preparations and pursued developmental changes in the spatial distribution patterns of the optical signals. Preliminary results have been presented in abstract form (Sato et al. 2001).

METHODS

Preparations

Intact preparations dissected from 4- to 8-day-old embryonic chick brain stems were used. Experiments were carried out in accordance

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with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Fertilized eggs of white Leghorn chickens (Saitama Experimental Animals Supply, Saitama, Japan) were incubated for 4–8 days in a forced-draft incubator (type P-03; Showa Incubator, Urawa, Japan) at 37°C and 60% humidity and were turned once each hour. The brain stems, with glossopharyngeal and vagus nerve bundles attached, were dissected from the embryos. The pia mater attached to the brain stem was carefully removed in a bathing solution. The isolated brain stem preparation was attached to the silicone (KE 106L; TV; Shin-etsu Chemical, Tokyo, Japan) bottom of a simple chamber with the ventral side up. The preparation was kept in a bathing solution with the following composition (in mM): 138 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>, 10 glucose, and 10 Tris · HCl buffer (pH 7.3). The solution was equilibrated with oxygen.

Voltage-sensitive dye staining

Each isolated intact preparation was stained by incubating it for 20 min in a Ringer solution containing 0.2 mg/ml of the voltage-sensitive merocyanine-rhodanine dye NK2761 (Hayashibara Biochemical Laboratory/Kankoh Shikiso Kenkyusho, Okayama, Japan; Kamino et al. 1981, 1989; Salzberg et al. 1983; Momose-Sato et al. 1995), and the excess (unbound) dye was washed away with dye-free Ringer solution before recording was performed. This merocyanine-rhodanine dye has been established to be particularly useful in embryonic nervous and cardiac tissues (Kamino 1990, 1991; Momose-Sato et al. 1995). Furthermore, it has been confirmed that the immature cellular and interstitial structure of the early embryonic brain stem preparations allows the dye to diffuse readily from the surface to the interior regions (Sato et al. 1995).

Electrical stimulation

For preparations in which the glossopharyngeal/vagus nerves were stimulated, the cut end of the nerve was drawn into a microsuction electrode fabricated from TERUMO-hematocrit tubing (VC-HO75P; TERUMO, Tokyo, Japan) that had been hand-pulled to a fine tip (∼50–100 μm internal diameter) over a low-temperature flame. Positive (depolarizing) square current pulses (10 μA/1 ms for 4-day-old preparations and 8 μA/5 ms for 5- to 8-day-old preparations), which evoked maximum responses, were applied to the right glossopharyngeal/vagus nerves.

Optical recording

The optical responses to glossopharyngeal nerve stimulation

Figure 1 illustrates two examples of multiple-site optical recordings of neural activity induced by glossopharyngeal nerve stimulation in 4-day- (Fig. 1A) and 7-day- (Fig. 1B) old embryonic chick brain stem-intact preparations. The thickness (light path from the dorsal surface to the ventral surface) of these preparations was 300–1000 μm, and they were translucent. Thus we could detect neural voltage responses as changes in transmitted light intensity. The evoked optical signals were recorded simultaneously from contiguous regions of the preparation with a 12 × 12 (Fig. 1A) or 34 × 34 (Fig. 1B) element photodiode array.

In Fig. 1A, simultaneous 128-site optical recordings were made in four areas of the preparation by moving the photodiode array over the image of the preparation, and the recordings were reconstructed from 16 trials. When a stimulating current (10 μA/1 ms), which gave the maximum response, was applied to the right glossopharyngeal nerve, two response areas that were spatially separated were discriminated on the stimulated side of the preparation: one was located cephalic to the level of the glossopharyngeal nerve root (area 1), and the other was located at the level of the glossopharyngeal/vagus nerve roots (area 2). In both areas, fast spikelike optical signals were recorded. The action spectra of the fast spikelike signals were similar to those of NK2761-dependent extrinsic absorption signals, and the signals 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 indicated that the fast spikelike signals were indeed dye-absorption changes related to the membrane potential and did not correspond to changes in light scattering related to mechanical
or other factors. When we applied hyperpolarizing current pulses, optical signals were observed only in an area near the root of the glossopharyngeal nerve, which is outside both area 1 and area 2 (data not shown). These remaining optical signals were considered to be electrotonic potential-related optical signals. Compared with previous reports (Breazile 1979; Sato et al. 1995), areas 1 and 2 were considered to correspond to the nucleus of the glossopharyngeal nerve (Nuc IX; motor nucleus) and the nucleus of the tractus solitarius (NTS; sensory nucleus/tractus solitarius; TS; sensory tract), respectively. The enlarged traces in Fig. 1A show the spikelike optical signals in area 1 (indicated by an asterisk at left). When we compared the amplitude of the optical signals from the top to the bottom of the row, several peaks and valleys were recognized.

Figure 1B illustrates part of a simultaneous 1,020-site optical recording in a 7-day-old chick brain stem-intact preparation. The signals are shown in a single sweep. When a stimulating current (8 μA/5 ms) was applied to the right glossopharyngeal nerve, two response areas (areas 1 and 2) were also identified. In this recording, only the fast spikelike signals were recorded from area 1, whereas in area 2, slow components were recorded together with the fast signals. The action spectra of the slow components were the same as those of the fast spikelike optical signals (data not shown). This result indicates that the slow signals are also dye-absorption changes related to the membrane potential. As shown in our previous report (Sato et al. 1995), the slow signals were glutamatergic excitatory postsynaptic potential (EPSP)-related optical signals. Figure 1B, right, shows enlarged traces of optical signals from a row indicated by an asterisk at left. In these enlarged traces, only one peak of the optical signal amplitude was recognized in area 1. This pattern is different from that seen in the 4-day preparation shown in Fig. 1A.

To examine how the distribution pattern of the optical signals changes during development, we detected evoked optical signals in the motor nucleus in other developmental stages. Figure 2 illustrates optical signals evoked by glossopharyngeal nerve stimulation in 5-day and 6-day preparations. Optical signals evoked in the motor nucleus (hatched areas) are shown. In these preparations, the peak of the signal amplitude was not...
single, but the pattern was less complex than that in the 4-day preparation.

Optical responses to vagus nerve stimulation

To examine whether the multiple-peak patterns of the signal amplitude are recognized in other motor nuclei, we investigated functional development of the dorsal motor nucleus of the vagus nerve (DMNV).

Figure 3 illustrates multiple-site optical recordings of neural activity induced by vagus nerve stimulation in 4-day (Fig. 3A) and 7-day (Fig. 3B) preparations. These preparations were the same as those shown in Fig. 1. In the 4-day preparation (Fig. 3A), when a stimulating current (10 μA/1 ms) was applied to the right vagus nerve bundle, these optical signals were detected from the same preparations shown in Fig. 1. Other experimental conditions were the same as those in Fig. 1.

FIG. 3. Multiple-site optical recordings of neural responses to vagus nerve stimulation in 4-day (A) and 7-day (B) preparations. Optical signals were evoked by applying a brief positive square current pulse (10 μA/1 ms, A; 8 μA/5 ms, B) to the right vagus nerve bundle. These optical signals were detected from the same preparations shown in Fig. 1. Other experimental conditions were the same as those in Fig. 1.

FIG. 2. Multiple-site optical recordings of neural responses to glossopharyngeal nerve stimulation in 5-day and 6-day preparations. Hatched areas, optical signals evoked in each scheme of the preparation, corresponding to the nucleus of the glossopharyngeal nerve (Nuc IX).
the right vagus nerve, fast spikelike optical signals were recorded on the stimulated side. Compared with previous reports (Breazile 1979; Komuro et al. 1991; Momose-Sato et al. 1991b), the fast spikelike optical signals recorded from the medial region were considered to be the antidromic action potential in the motoneurons (DMNV), and those located laterally were the orthodromic action potential in the fiber and terminal of the sensory nerve (TS/NTS). Figure 3A, right, shows enlarged traces of the spikelike optical signals in the DMNV, which are indicated by an asterisk at left. As is the case in Fig. 1A, several peaks and valleys were recognized in the amplitudes of the fast spikelike signals.

Figure 3B illustrates part of a simultaneous 1,020-site optical recording in a 7-day preparation. When a stimulating current pulse (8 μA/5 ms) was applied to the right vagus nerve, two signals, viz., fast spikelike signals and slow long-lasting signals, were identified. As we described previously (Komuro et al. 1991), the slow signals were glutamatergic EPSP-related optical signals in the NTS. Figure 3B, right, shows enlarged traces of the optical signals recorded from the row indicated by an asterisk at left. The distribution patterns of the fast spikelike signal amplitude were unimodal and were different from those seen in the 4-day preparation.

Figure 4 illustrates optical signals evoked by vagal stimulation in 5-day and 6-day preparations. As is the case of the Nuc IX, optical signals evoked in the DMNV are shown. In these preparations, multiple peaks and valleys of the signal amplitude were less clear than in the 4-day preparation but not as simple as in the 7-day preparation.

**Contour line maps of the optical signals**

To trace developmental changes in the spatial distribution pattern of the motoneuronal action potentials, we measured the amplitude of the fast spikelike signals and constructed contour line maps as shown in Fig. 5. The maps of the glossopharyngeal nerve (Nuc IX) and vagus nerve (Nuc X) were obtained from the same preparation. For the 7-day preparation, the distribution pattern of the EPSP-related slow signals evoked in the NTS is also shown. These maps revealed the following characteristics of the optical responses in the Nuc IX, DMNV, and NTS: 1) in the 4- to 6-day preparations, the optical signals were distributed in multiple-peak patterns; 2) the distribution patterns became simpler as development proceeded; in the 7-day preparation, only one signal amplitude peak was identified in the motor nuclei, and the distribution of the signal amplitude formed a layered pattern surrounding the single peak; 3) the size of the optical signals increased as development proceeded; 4) the area of the Nuc IX did not overlap with that of the DMNV in early developmental stages; and 5) the distribution pattern of the slow optical signals in the NTS was simple; only one peak was recognized from the initial stage of slow signal expression (7 day).

It is possible that the distribution pattern and number of peaks depend on either a magnification of optics or signal-to-noise ratio. Figure 6A illustrates optical signals evoked by glossopharyngeal nerve stimulation in a 4-day preparation. The signals were recorded with ×25 and ×50 magnifications and were averaged 20 times. In both recordings, two signal peaks were identified independent of the magnification. Figure 6B shows optical signals evoked by glossopharyngeal nerve stimulation in a 5-day preparation; these signals were obtained with ×25 (a) and ×50 (b) magnifications. In b, a single trial recording (left) and an averaged (4 times) recording (right) are also compared. In all of these recordings, two apparent peaks were identified, and the response pattern was almost the same for all. The distribution pattern of the optical signals was also unchanged with off-line low-pass filtering (data not shown). Similar results were obtained with the optical signals induced by vagus nerve stimulation.

**Peak locations of the glossopharyngeal and vagal responses**

Figure 7 illustrates peak locations of the fast spikelike optical signals and their developmental changes. Red areas are the peak locations for glossopharyngeal nerve stimulation and blue areas are those for vagus nerve stimulation. This figure shows more clearly that the number of peaks decreased gradually with development to a single peak in the 7-day-old embryonic stage. In addition, this figure demonstrated that 1) the peak locations of the optical signals in the Nuc IX were different from those in the DMNV and that they did not overlap each other during development; 2) the number of peaks in the DMNV was higher than that in the Nuc IX in the 4- to 6-day-old preparations; 3) the peak locations were positioned with an alignment on a single rostrocaudal axis; and 4) the final
In Table 1, preparation-to-preparation differences in the number of signal peaks are summarized for the 4- to 8-day-old embryonic stages. In the 7-day preparations, the evoked optical signals in the Nuc IX and DMNV exhibited one peak in most preparations, whereas in some preparations, they presented two peaks. In all of the 8-day preparations, only one peak was identified.

Comparison with morphology

To compare the spatial distribution patterns of the optical signals with morphological structures, we investigated motoneuronal distributions in the Nuc IX and DMNV. Figure 8 shows photographic views of the Nuc IX and DMNV obtained from 4-day and 5-day preparations in which the glossopharyngeal and vagus nerves were labeled with a carbocyanine dye, DiI. In these

core in the 7-day-old stage was located in the center of the nucleus.

In Table 1, preparation-to-preparation differences in the number of signal peaks are summarized for the 4- to 8-day-old embryonic stages. In the 7-day preparations, the evoked optical signals in the Nuc IX and DMNV exhibited one peak in most preparations, whereas in some preparations, they presented two peaks. In all of the 8-day preparations, only one peak was identified.
photographs, the cell density in the motor nuclei was not uniform. However, we could not find any significant structural bases that corresponded to the peaks of the optical signal amplitude.

DISCUSSION

In this study, we focused on the motor nuclei of the glossopharyngeal and vagus nerves and provided interesting baseline data on how the brain stem motor nuclei are organized functionally during early embryogenesis. Concerning the vagal nuclei, we previously reported the basic profiles of nuclear development/organization (Momose-Sato et al. 1991b). Thus in the following sections, we first discuss the early process of functional organization of the glossopharyngeal nuclei. We then discuss the spatial distribution pattern of the optical signal and its developmental change, providing a basic principle of functional organization for the embryonic brain stem nuclei.

Onset of neural excitability and synaptic function in the glossopharyngeal nuclei

In embryonic brain stem preparations younger than the 7-day stage, only spikelike signals were evoked by glosso-
pharyngeal nerve stimulation. These signals correspond to antidromic action potentials evoked in the motoneurons and orthodromic action potentials evoked in the terminals of the sensory nerves. In the present experiments, we detected action potential-related optical signals induced by glossopharyngeal nerve stimulation from 4-day preparations. Thus excitability of the motoneurons and/or the sensory nerve terminals is generated no later than the 4-day stage of embryonic development.

As shown in Figs. 1 and 2, the EPSP-related slow signal evoked by glossopharyngeal nerve stimulation was first detected in the 7-day-old embryonic stage. In our previous reports (Momose-Sato et al. 1991b, 1994), we showed that vagus nerve stimulation also induced an EPSP-related slow signal...
from 7-day preparations in normal Ringer solution. These results imply that the onset timing of functional synaptic expression in the chick NTS is the same for the glossopharyngeal and vagus nerves.

How do the motor nuclei develop functionally?

Before we consider the results of functional development of the glossopharyngeal and vagal motor nuclei, we need to be reminded of the basic features of optical recording. In the optical technique for monitoring membrane potential changes, the linearity of the optical signal with changes in membrane potential has been established in other preparations (Cohen and Salzberg 1978). It has been assumed that the fractional signal size is proportional to the magnitude of the membrane potential changes in each cell and process and to the number and membrane area of activated neural elements within the field detected optically by one photodiode under conditions in which the amount of the dye bound to the membrane is uniform (Kamino 1991; Kamino et al. 1989; Obaid et al. 1985; Orbach et al. 1985). According to these assumptions, the spatial distribution of the optical signals reflects the spatial pattern of neural activity as a function of the membrane area of the activated cells and the magnitude of the membrane potential change. Thus the peak optical signal amplitude corresponds to

As shown in Fig. 5, the contour line maps of the optical signal amplitude represented multiple-peak patterns in the motor nuclei.
tor nuclei of 4- to 6-day preparations, and the number of peaks decreased as development proceeded. In the 7-day preparations, only one peak was recognized, and the distribution of the signal amplitude formed a layered pattern. These findings suggest that the neurons and/or their activity do not distribute uniformly within the motor nuclei at early developmental stages and that such arrangements change dynamically during development. In the morphological views shown in Fig. 8, we could not identify any significant structural basis corresponding to the peaks of the optical signal amplitude. This result demonstrates the usefulness of optical mapping to analyze the functional organization and/or architecture of the CNS structures, which is not necessarily coincident with the morphological construction identified with histological techniques.

In the earlier stages of development in chick embryos, it is known that repeated segmental structures, rhombomeres, exist in the hindbrain. Rhombomere formation begins at Hamburger and Hamilton (Hamburger and Hamilton 1951) stage 9 (1.5 day), and by stage 12 (2 day) the process is complete (Vaage 1969). These rhombomeres are transiently-expressed structures in the hindbrain, and by stage 24 (4 day), the rhombomeres become invisible again. Lumsden and Keynes (1989) showed that the rhombomeres is a key structure, forming branchial and visceral motor nuclei morphologically, and extensive studies have shown that many of the neurons and most of the basic structures of the hindbrain are formed in interaction with the rhombomere (Fortin et al. 1994; Gilland and Baker 1993; Wake 1993). In the present experiments, we could not obtain successful recordings from brain stem preparations younger than the 4-day stage, so we analyzed optical signals in 4- to 8-day-old embryos. In these stages, the rhombomeres had already disappeared or were rudimentary (at 4 days), and we could not directly compare the locations of the optical signal peaks with the rhombomeres. Nonetheless, the present results extracted some interesting profiles concerning the formation of the Nuc IX and DMNV.

First, Lumsden and Keynes (1989) demonstrated that the Nuc IX is formed in the sixth and seventh rhombomeres and that the DMNV is formed in the seventh and eighth rhombomeres. In other words, the Nuc IX and DMNV overlap in the seventh rhombomere at early developmental stages. In the present study, as shown in Fig. 5, the response area to glossopharyngeal nerve stimulation did not overlap with that of the vagus nerve stimulation, even at the 4-day-old embryonic stage. This result indicates that the Nuc IX is functionally separated from the DMNV before the rhombomere rudiment disappears completely.

Second, as shown in Fig. 7 and Table 1, the number of optical signal peaks was >2 in the 4-day preparations. As described above, the Nuc IX and DMNV are generated within two adjacent rhombomeres. The present result suggests the possibility that there are functional subsegments in each rhombomere at early developmental stages.

Finally, considering that the developmental pattern was almost the same for the glossopharyngeal and vagal nuclei, a general scheme of the functional organization of the brain...
stem motor nuclei is proposed. 1) At the initial stage of functional organization, multiple cores, in which neural activities are higher than in the surroundings, are generated in the brain stem motor nuclei. 2) As the developmental stage proceeds, functional rearrangement occurs in the motor nucleus; the cores fuse with each other and the number of cores decreases. 3) At the matured stage, neurons are functionally arranged in a hierarchical pattern surrounding the central core. Considering that cell death occurs at later stages in these motor nuclei (McConnell and Sechrist 1980; Wright 1981), it seems likely that these processes are related to neuronal generation/differentiation rather than neuronal death/degeneration. The distribution pattern of the EPSP-related slow signal in the NTS was simple from the onset of synaptic function, as shown in Fig. 5. This suggests that the developmental sequence of functional organization is different between motor and sensory nuclei.

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