Optical Assessment of Spatial Patterning of Strength-Duration Relationship for Vagal Responses in the Early Embryonic Chick Brainstem

Katsushige SATO, Yoko MOMOSE-SATO, Tetsuro SAKAI,
Akihiko HIROTA, Hitoshi KOMURO*,
and Kohtaro KAMINO

*Department of Physiology, Tokyo Medical and Dental University
School of Medicine, Bunkyo-ku, Tokyo, 113 Japan*

Abstract The spatial patterning of strength-duration relationship for neural responses to vagus nerve stimulation in the early embryonic chick brainstem preparation was assessed by means of an optical method for monitoring neural activity using a voltage-sensitive dye. Intact brainstem, and brainstem slice preparations, together with the attached vagus nerve, were dissected from 6- to 8-day (d)-old chick embryos. They were stained with a voltage-sensitive merocyanine-rhodanine dye (NK2761). Application of depolarizing square current pulses to the vagus nerve fibers using a micro-suction electrode evoked voltage-related optical (absorption) responses in the brainstem. The optical responses were recorded simultaneously from 127 contiguous sites in both the intact and slice preparations, using a 12×12 -element photodiode array. The amplitude of the evoked signals and the extent of the response area depended on the strength and duration of the stimulating current pulse. Thus, we determined the threshold current strength needed to evoke the fast spike-like optical signal, corresponding to the action potential, in response to vagal stimulation at a given duration, and we obtained strength-duration curves for 100-200 contiguous regions in the preparation. The strength-duration curves could be apparently fitted by the empirical formula of Weiss. From these curves we obtained the rheobasic currents as a possible indication of neural responsiveness to peripheral vagus stimulation, and we constructed the map of the spatial distribution of the rheobasic currents. The rheobasic current is smallest in the central region of the response area, and it is distributed in a layered pattern of increasing value. In accordance with such a pattern, the minimum rheobasic current decreased and the response area expanded developmentally. The results suggest that the

Received on March 19, 1993; Accepted on June 10, 1993

*Present address: Section of Neurobiology, Yale University School of Medicine, New Haven, CT 06510, USA

ordered patterns of spatial distributions of the size of the axon, the specific membrane resistance, sodium channel activity, and other electrophysiological factors underlie the initial process of functional organization of the dorsal motor nucleus within the brainstem during early embryonic development.

Key words: embryonic brainstem, vagal responsiveness, optical recording, strength-duration relationship.

Understanding of brain functions would be enhanced by a more detailed analysis of electrical events in the early embryonic central nervous system. This problem has been studied through a variety of conventional electrophysiological approaches (e.g. see Ref. [1]). However, because early embryonic neural cells and processes are very small and fragile, it is technically very difficult or impossible to impale them with microelectrodes. For this reason, in contrast to morphological investigations, electrophysiological studies on the early embryonic central nervous system have been hampered.

Optical methods for monitoring of cellular electrical events have made it possible to measure electrical activity in small cells, and to record electrical activity simultaneously from many sites of a living preparation [2–8].

In the previous works, we have established the feasibility of using optical techniques to record neural activity simultaneously from multiple sites in early embryonic nervous systems [9–11], and we have investigated the early development of spatial and temporal patterns of the response to vagal stimulation in the early embryonic chick brainstem [12–15].

Subsequently, we have previously found that in the experiments using 7-d-old embryonic brainstems, the neural responses to vagal stimulation depend closely upon two factors such as the strength and duration of the stimulating square current pulse, and we have demonstrated that the regional distribution of the threshold of the strength and duration of the stimulating current exhibited a layered pattern [13]. Accordingly, we have recalled the strength-duration relationship as a protocol to examine electrophysiological parameters possibly related to neural responsiveness to the vagal stimulation and its early development in the embryonic brainstem.

In this report, in order to assess early development of the spatial patterning of the vagal responsiveness in the 6- to 8-d-old embryonic chick brainstem, in which the electrical responses are initially generated [15], we have assessed the strength-duration relationship and constructed maps of spatial distribution of the rheobasic current, using the multiple-site optical recording technique. We have discussed the results focusing on early embryonic development of functional organization/architecture of the dorsal motor nucleus. Some of the results reported here have appeared in a preliminary form [16].

MATERIALS AND METHODS

Preparations. In the present study, we have used 6-, 7-, and 8-d-old embryonic chick brainstems. Fertilized eggs of chicks (white Leghorn) were incubated in a forced draft incubator (Type P-03, Showa Incubator Lab., Urawa, Japan) at a temperature of 37°C and 60% humidity, and were turned once each hour. The brainstems with vagus nerve fibers were dissected from the embryos. The pia mater attached to the brainstem was carefully removed. The intact vagus-brainstem preparations were attached dorsally to the silicone (KE 106; Shin-etsu Chemical Co., Tokyo, Japan) bottom of a simple chamber by pinning it with tungsten wires. Slice preparations about 1 mm thick were cut transversely from the isolated brainstems at the level of the vagus nerve root, and were pinned to the chamber with the spinal cord side up. The preparations were kept in a bathing solution having the following composition (in mM): NaCl, 138; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.5; glucose, 10; and Tris HCl buffer (pH 7.2), 10. The solution was equilibrated with oxygen.

Voltage-sensitive dye staining. The isolated preparations were incubated for 20 min in a bathing solution to which was added 0.2 mg/ml of a merocyanine-rhodanine dye (NK2761) [7, 17–19], which was synthesized by Nippon Kankoh Shikiso Kenkyusho Co., Okayama, Japan (the nature of this dye has been described previously: see Ref. [7]). After staining, the preparation was washed with several changes of normal bathing solution.

Electrical stimulation. The cut end of the nerve was drawn into a micro-suction electrode fabricated from TERUMO-hematocrit tubing (VC-HO 75 P; TERUMO Co., Tokyo, Japan), which had been hand-pulled to a fine tip (about 100 μ m in caliber) over a low temperature flame.

Optical recording. The optical recording system is basically similar to that described by Cohen and Leshner [2], with slight modifications [7, 20]. The preparation chamber was mounted on the stage of an Olympus Vanox microscope (Type AHB-L-1). Bright field illumination was provided by a 300 W tungsten-halogen lamp (Type JC-24V-300 W, Kondo Sylvania Ltd., Tokyo, Japan) driven by a stable dc-power supply (Model PAD 35–20L, 0–35 V 20 A, Kikusui Electronic Corp., Kawasaki, Japan). Incident light was made quasimonochromatic by a 703 $\pm$ 15 nm interference filter (Asahi Spectra Co., Tokyo, Japan) placed between the light source and the preparation. A microscope objective (S plan Apo, 0.40 n.a.) and a photographic eyepiece formed a magnified real image of the preparation at the image plane. Magnification was usually 25 $\times$. The transmitted light intensity at the image plane was monitored using a 12 $\times$ 12 square array of silicon photodiodes (MD-144-4PV; Centronic Ltd., Croydon, UK). The image of the preparation was focused onto the photodiode array. The output of each detector in the diode array was passed to an amplifier via a current to voltage converter. The amplified outputs from 127 elements of the detector and the stimulus current were first recorded simultaneously on a 128-channel recording system (RP-890 series, NF

Electronic Instruments, Yokohama, Japan), and then were passed to a computer (LSI-11/73 system, Digital Equipment Co., Tewksbury, MA). The 128-channel data recording system is composed of a main processor (RP-891), eight I/O processors (RP-893), a 64 K word wave-memory (RP-892) and a videotape recorder. The program for the computer was written in assembly language (Macro-11) called by Fortran, under the RT-11 operating system (Version 5.0). All experiments were carried out at room temperature, 26 to 28°C.

RESULTS

Optical recordings of vagal responses to various stimulations

First experiments were carried out on intact brainstem preparations. The vagus nerve-brainstems were dissected from 6-, 7-, and 8-d-old chick embryos. The isolated brainstem preparations were stained with a voltage-sensitive merocyanine-rhodanine dye (NK2761). The electrical responses in the brainstem preparation were evoked by applying square current pulses, with various combinations of strength and duration, to the attached vagus nerve fibers using a micro-suction electrode. The evoked electrical responses in the brainstem were recorded as extrinsic optical (absorption) signals of which the fractional change is linearly related to the membrane potential in the physiological range of resting potential ± 100 mV [3].

Figure 1 shows three examples of optical recordings of the evoked neural response in the brainstem preparation from a 7-d-old embryo. The optical signals were recorded by applying three different combinations of the strength and dura-

Fig. 1. Multiple-site optical recording of electrical responses evoked by three combinations of strength and duration of positive (depolarizing) square current pulses applied to the left vagus nerve (N. X) in a 7-d-old embryonic chick brainstem. The intact brainstem preparation, to which the vagus nerve fibers were attached, was stained with a merocyanine-rhodanine dye (NK2761). The preparation was fixed to the silicone bottom of the chamber with the dorsal side down. The figure was constructed using two 127-site simultaneous recordings from two areas on the left side (stimulated side) of the brainstem, obtained with a 12×12 -element photodiode array. In each recording, 4 trials were averaged. A 703 ± 15 nm interference filter was used. The photodiode was positioned over a $25 \times$ image of the preparation, so that each signal was detected from a $56 \times 56 \mu\text{m}^2$ area of the preparation. The relative position of each element of the diode array is illustrated on the lower left. The recordings were obtained with $7.35 \mu\text{A}/1.0$ ms, $1.30 \mu\text{A}/15$ ms, and $1.25 \mu\text{A}/30$ ms positive square current pulses. The direction of the arrow in the lower right corner of the recording indicates a decrease in transmission (increase in absorption), and the length of the arrow represents the stated value of the fractional change of the transmitted light intensity.

tion of the stimulating square current pulses, $7.35\mu\text{A}/1.0\text{ ms}$, $1.30\mu\text{A}/15\text{ ms}$, and $1.25\mu\text{A}/30\text{ ms}$, to the left vagus nerve fibers. The simultaneous optical recordings from 127 adjacent loci were made in 2 areas in the preparation by moving a 12×12 -element photodiode over the image of the left side of the brainstem.

Clearly, the size of the optical signal and the extent of the response area varied, depending upon the combination of strength and duration of the stimulating current. In general, when the vagus nerve bundle was stimulated by a micro-suction electrode, the size of the evoked optical signal increased with increase in the strength and/or duration of the stimulating current. Also the response area expanded with increase in the strength and/or duration of the stimulating current. These results suggest that the relation between the strength and the duration of the stimulus current pulse is a factor in determining the response pattern, in the brainstem, to vagal stimulation. Next, we examined the strength-duration relationship for the vagal response in 6-, 7-, and 8-d-old embryonic brainstem preparations.

Strength-duration curves

In order to construct strength-duration relation curves for the brainstem response to vagal stimulation, we determined the threshold current strength needed to produce an optical signal in response to the vagal stimulation, varying the strength of the test stimulus at several fixed values of the pulse duration, at all positions of the optical recording. In the present measurements, the minimum visible size of the signals was considered to be a signal-to-noise ratio (S/N) ≈ 2 . These minimum signals were completely eliminated at 620–630 nm, the null wavelength for the NK2761 voltage-dependent optical change [7]. From this spectral characteristic, these minimum signals were indeed absorption changes related to the action potential, and were not artifact(s).

In 4- to 6-d-old embryonic brainstems, only fast spike-like signals related to action potential have been detected, and in 7- and 8-d-old embryonic brainstems under suitable combinations of strength and duration of the stimulating current, the optical signals comprised two components: a fast spike-like signal, and a slow long-duration signal related to the postsynaptic potential [14, 15]. The threshold (critical strength) of the stimulation current needed to evoke the slow signal is somewhat higher than that for the fast spike-like signal, and the slow signal shows rapid synaptic fatigue in response to a train of stimuli [14]. Thus, in the experiments on 7- and 8-d-old embryonic brainstems, we tested the threshold current strength only for the fast signals.

Figure 2 shows three examples of the relation between the duration of a square pulse and the current strength that just elicited the optical response in the brainstem. These data were obtained from three positions in the 7-d-old embryonic brainstem shown in Fig. 1. The strength-duration curves were all basically hyperbolic. Nevertheless, these curves were not identical and there were clear regional differences. Next, in order to examine more quantitatively the relationship between strength (i) and duration (t), we plotted their product ($i\cdot t$) against t (Fig.

Fig. 2. Strength-duration curves for responses to vagal stimulation of a 7-d-old embryonic intact brainstem. The curves were made with the data obtained from 3 different positions, A-11, F-1, and D-10. The minimum strength of the stimulus current which evoked the signal was plotted against the duration. In the present measurements, the minimum visible size of the signals was considered to be at a signal-to-noise ratio ≈ 2 .

Fig. 3. A: Plots of stimulus charge ($i \cdot t$) against duration (t), where i is the minimum strength of the stimulus current and t the duration shown in Fig. 2. B: Plots of i against $1/t$. The data were the same as those shown in Figs. 2 and 3A.

3A). Here, $i \cdot t$ was linearly related to t , and the strength-duration curves were well fitted with the formula:

$$i \cdot t = at + b, \quad (1)$$

where a and b are constants. In Fig. 3A, the intercept on the y -axis gives the value of b , and the intercept on the x -axis gives $-b/a$. The value of a can be estimated from the slope of the straight line.

Furthermore, to examine the accuracy, we also plotted i against $1/t$, and we obtained the linear relationship shown in Fig. 3B. Here, the intercept on the y -axis gives the value of a , and the intercept on the x -axis gives $-a/b$. Table 1 compares the values of a , b , and b/a obtained from the $i \cdot t$ vs. t plot and from the i vs. $1/t$ plot. From this comparison, it appears that similar values were obtained from both plots, and that they are reasonable data. In addition, from the linear relationship in both these plots, we concluded that the strength-duration curves shown in Fig. 2 apparently obeyed the classical empirical formula of Weiss [21], with a corresponding to the rheobase, and b/a to the chronaxie.

a-values. Figure 4 shows distributions of a -values obtained from three intact preparations from 6-, 7-, and 8-d-old embryos. The a -values scattered in the wide range, from 0.2 to 0.9 μA , and in every tested preparation, the distribution showed a multimodal structure. Such features approximately characterize the distribution of the a -value. Furthermore, it is shown that the histogram of the a -value shifted to the left side and the minimum a -value tended to decrease as development proceeded from 6- to 8-d-old (also see next section and DISCUSSION).

b-values. In comparison with the a -values, the distributions of the b -values were relatively small in all tested preparations. One example of the results is shown in Fig. 5. The histogram in this figure also appears to have several peaks in the small range of the b -value.

Table 1. Comparison of the parameters obtained from the i -($1/t$) and $i \cdot t$ - t plots.

Recording	Position	Plot	a (μA)	b ($\mu\text{A} \cdot \text{ms}$)	b/a (ms)
Trial 1	B-10	i -($1/t$)	0.36	7.2	20.1
		$i \cdot t$ - t	0.36	6.9	19.4
	G-16	i -($1/t$)	0.34	6.6	19.3
		$i \cdot t$ - t	0.36	6.8	18.9
Trial 2	F-1	i -($1/t$)	0.39	6.6	16.8
		$i \cdot t$ - t	0.44	6.3	14.3
	L-1	i -($1/t$)	0.62	6.1	9.9
		$i \cdot t$ - t	0.63	6.3	10.0
	B-6	i -($1/t$)	0.66	5.6	8.5
		$i \cdot t$ - t	0.67	5.7	8.4

Fig. 4. Histograms of the α -value obtained from a 6-d-old, a 7-d-old, and an 8-d-old embryonic brainstem intact preparation. The ordinate is the number of the positions at which the signals were detected. The bin width is $0.01 \mu\text{A}$.

Fig. 5. Histogram of b -value obtained from a 7-d-old embryonic brainstem intact preparation. The bin width is $0.1 \mu\text{A} \cdot \text{ms}$.

Fig. 6. Plots of the b/a -value against the a -value. Data were obtained from the $i-t$ plots at 200 different positions of a 7-d-old intact embryonic brainstem preparation.

Relationship between a and b/a

In Fig. 6, a -values corresponding to rheobase are plotted against b/a -values corresponding to chronaxie. The data were obtained from many different positions in a 7-d-old embryonic brainstem. In contrast with the conventional behavior observed in a single cell or a single nerve fiber (e.g. Refs. [22, 23]), the changes in the b/a -values (chronaxie) were not parallel with the changes in the a -values (rheobase): the b/a -values decreased with increasing a -values. However, considering that the range of changes in the b -values is smaller than in the a -values (Figs. 4 and 5), mathematically, b/a should decrease with increases in a . In addition, in this graph, there appears to be several groups of the relationship between the a -value and b/a -value. For convenience, in the present experiment, the a -value has been used as an index of the relative degree of the responsiveness to the vagal stimulation in the embryonic brainstem (see DISCUSSION).

Regional distribution of a -values

We assume that, in the present experiment, the a -value reflects the threshold of stimulating current at infinite duration (∞) to evoke a neural response to vagal stimulation, and that the a -value reflects the responsiveness of neurons within the particular response area in the brainstem. Accordingly, we examined regional differences in the a -value.

Mapping. We have evaluated the a -values for the evoked optical signals, in all positions where the signals were detected, in preparations from 6-, 7-, and 8-d-old embryos. We constructed maps of the regional distribution of the a -values; these

are shown in Fig. 10A. The a -values evaluated at each locus of the brainstems have been represented by eight colors. The regional distribution patterns in 6-, 7-, and 8-d-old preparations were basically similar: the minimum a -value appeared in the central region of the response area, and the a -value tended to increase toward the periphery. This characteristic was observed in all the tested preparations ($n=18$). Nevertheless, the area of brainstem in which the a -value was smaller than $0.2\mu\text{A}$ expanded gradually as embryonic age proceeded from 6 to 8 days. In the 6-d-old preparation, the areas exhibiting the minimum a -value were dispersed throughout the central region of the response area. Furthermore, the multimodal distribution of the a -value corresponds roughly to the maps.

Slice preparations

The spatial resolution of the optical recording is somewhat limited for three-dimensional preparations. In the experiments on intact preparations, information about the z -axis distribution of the responses is lacking. Therefore, to overcome this limitation, we have also carried out a series of experiments on transversely sectioned slice preparations from the brainstems ($n=12$). We prepared slice preparations attached to the vagus nerve fibers by transversely sectioning brainstems from 6- to 8-d-old embryos using the same method described previously [12]. The thickness of the slices was about $1,000\mu\text{m}$.

We recorded optical signals from 127 contiguous loci in the preparation using a 12×12 -element photodiode array: the optical signals were evoked by applying stimuli with various combinations of strength and duration to the vagus nerve fibers. The amplitude of the optical signal and the extent of the response area varied with the strength and duration of the stimulating current.

Figure 7 shows three examples of optical signals from the slice preparation from a 7-d-old embryonic brainstem, evoked by three different combinations of strength and duration of the stimulating square current pulse, $2.85\mu\text{A}/3\text{ms}$, $1.40\mu\text{A}/8\text{ms}$, and $1.05\mu\text{A}/15\text{ms}$. The inset on the lower left illustrates the location of the image of the slice preparation with respect to the photodiode array. The evoked optical signals appeared to be concentrated in the dorsal region of the stimulating side, and both the size and the response area varied with the combination of the strength and duration of the stimulating current.

Strength-duration relationship. Figure 8 shows the strength-duration curves obtained from three different areas (A-2, K-10, and E-7) in a slice preparation from the 7-d-old embryonic brainstem shown in Fig. 7. Again, spatial variations in the strength-duration curve were revealed.

In the slice preparations, as in the intact preparations, we made the $i\cdot t$ vs. t plot and the i vs. $1/t$ plot, and we obtained linear relationships between $i\cdot t$ and t , and between i and $1/t$. In these two plots, we have again evaluated a -, b -, and b/a -values. The distributions of a -values (corresponding to rheobase), obtained from 6-, 7-, and 8-d-old slice preparations are shown in Fig. 9. Distribution patterns similar to those observed in the intact preparations were obtained.

Fig. 7. Multiple-site optical recordings of the electrical responses evoked by three conditions of the strength and duration of the stimulus square current pulse. The signals were detected simultaneously from 127 different contiguous loci in a 7-d-old embryonic brainstem slice preparation. The stimulus currents of 2.85 $\mu\text{A}/3\text{ ms}$, 1.40 $\mu\text{A}/8\text{ ms}$, and 1.05 $\mu\text{A}/15\text{ ms}$ were applied to the left vagus nerve fibers. In each recording, 4 trials were averaged. The relative position of the photodiode array on the image of the preparation is illustrated on the lower left.

Fig. 8. Strength-duration curves for optical responses in the slice preparation prepared from a 7-d-old embryonic brainstem. Stimulus square current pulses were applied to the left vagus nerve. The data were obtained from 3 different positions in the preparation.

Fig. 9. Histograms of the a -value obtained from a 6-d-old, a 7-d-old, and an 8-d-old embryonic brainstem slice preparation. The ordinate is the number of the positions at which the signals were detected. The bin width is $0.01 \mu A$.

Fig. 10. Spatial pattern of the distribution of the α -value in intact (A) and slice (B) preparations of a 6-d-old, a 7-d-old, and an 8-d-old embryonic brainstem. The values of α are classified into 8 colors, as shown on the right side of the maps. The grid corresponds to the elements of the photodiode array, and its relative positions on the image of the preparations are illustrated at the bottom of each map.

Mapping. We also constructed maps of the regional distribution of the a -value in 6-, 7-, and 8-d-old slice preparations (Fig. 10B). In every preparation, the area of the minimum a -value was located in the medial and dorsal region. Although the response area gradually expanded as embryonic age proceeded from 6- to 8-d-old, the regional distribution pattern of the a -value was similar at every embryonic age, and the relative position of the minimum a -value region was the same in every preparation.

DISCUSSION

Our purpose in the present work has been to know initial development of the spatial distribution pattern of responsiveness of the neural population to peripheral vagus stimulation in the early embryonic chick brainstem. Using an optical technique for recording neural activity simultaneously from multiple sites in a preparation, we have studied the spatial patterning of the strength-duration behavior for neural response to peripheral vagus stimulation in the 6- to 8-d-old embryonic brainstem in which electrical responses are initially detected [15].

The strength-duration relationship is a rather old-fashioned concept in electrophysiology [21, 23–27], and it has been used to look for an optimal stimulating current in the central nervous system (e.g. Refs. [28, 29]), and to assess contractile responsiveness of the skeletal muscle [30–32]. The strength-duration relationships are often fitted by the classic and simple equation of Weiss [21], and it may be expected that two parameters, rheobase and chronaxie, reflect the relative excitability of cells and tissues.

In the present experiments, the data were well fitted by the Weiss's equation. In this relationship, it was noticed that, in our data obtained from multiple-site optical measurement of the brainstem response to peripheral vagus stimulation, the change in the b/a -value (corresponding to chronaxie) behaved opposite to the change in the a -value (corresponding to rheobase). The b/a - and a -values were inversely related in different regions of the brainstem, as shown in Fig. 6. This result shows that the two parameters, a and b/a , did not change in the same fashion. As shown in Figs. 4 and 9, the a -value varied regionally over a wide range. Whereas the rate of the changes in b , in the Weiss's equation (Eq. (1)), was relatively small (Fig. 5). This event reflects that the rate of the changes in the effective threshold stimulus charge defined as $b = (i - a) \cdot t$ is relatively smaller than the rate of the changes in the rheobasic current (a). From this simple consideration, it is explained that the direction of the changes in the chronaxie was opposite to the direction of the changes in the rheobase.

According to Bostock [33], the rheobasic current primarily depends on the input conductance, whereas the strength-duration time constant (equivalent to chronaxie) depends on the electrotonic time constant and the rate of sodium activation. The input conductance is, in turn, related to myelination, the size of the axons, and specific membrane resistance. The rate of sodium activation is associat-

ed with sodium channel activity. On the other hand, empirically, the chronaxie is determined subordinately to a determination of the rheobasic current, so that the chronaxie should be also dependent on the input conductance. These standpoints would complicate the interpretation of the changes in the chronaxie. For this reason, as a parameter of neural responsiveness, we chose the rheobase rather than the chronaxie.

In optical recording of electrical activity, generally, the signal size is proportional to the membrane area of the activated cells and the magnitude of the membrane potential change in each activated cell within the receptive field of one photodiode [2, 7, 8]. In the present experiments, each element of the photodiode array monitored optical signals from many neurons and processes. The vagus nerve bundle contains both sensory and motor nerve fibers. Thus, vagus nerve stimulation was orthodromic for the sensory neurons, and antidromic for motoneurons. As discussed previously, each element of the photodiode array (pixel) will detect optical signals from both sensory neurons and motoneurons in a given region of the brainstem preparation [14]. When a stimulus current with greater strength and/or longer duration is applied to 7- and 8-d-old embryonic preparations, a slow optical signal related to postsynaptic potentials is detected together with a fast spike-like optical signal. However, in the present experiments, because stimulus currents of lower strength and/or shorter duration were used, we detected only the fast spike-like optical signal that mainly reflects antidromic action potentials in motoneurons [14, 15].

When the stimulating current strength was reduced, at a given duration, a decrease in the size of the evoked optical signal was accompanied by a decrease in the response area. The critical strength of the stimulating current was defined as the current strength which was critically needed to produce a minimum response signal(s) which was significantly distinguished at a signal-to-noise ratio $\simeq 2$. Thus, one may assume, as a first approximation, that the signals evoked by the minimum stimuli originate from the most responsive neurons, which are at the vicinity of the lowest thresholds, within the field detected optically by one photodiode. It is thus plausible that the observed rheobasic current (*a*-value) is a function of the lowest threshold and the number of the neurons at the vicinity of the lowest threshold, within the field detected optically by one photodiode. The *a*-value decreases (increases) with a decline (increase) of the lowest threshold and with an increase (decrease) in the number of the neurons at the vicinity of the lowest threshold. We assume that differences in the size of action potentials of the neurons are small.

As demonstrated in a previous report [11], in earlier stages of development, the embryonic cervical vagus nerve bundle contains many nerve fibers with various diameters, and they are all unmyelinated. Therefore, for the present experimental data, a possible explanation is (1) that the regional differences in the *a*-value (rheobasic current) reflect regional differences in the size and the specific membrane resistance of the axons, and the number of the most responsive neurons which are at the vicinity of the lowest thresholds, and (2) that the developmental decrease

in the rheobasic current reflects a developmental increase in the size and the specific membrane resistance of the axons, and the number of the most responsive neurons. It is considered that the threshold membrane potential should be related to voltage-dependent activation and inactivation of sodium channels. Thus, regional differences and developmental changes in the rheobase must reflect regional differences and developmental changes in the specific characteristics of voltage-dependent activation and inactivation of sodium channels and in the density of the sodium channels formed in the neural membrane.

From the multimodal distributions in the histograms shown in Figs. 4 and 9, it seems that, in 6- to 8-d-old embryonic brainstems, the neurons connected with the vagus nerve fibers may be classified into several groups according to the a -values (rheobasic currents), and that the pattern of the groups changes developmentally. In addition, several groups can be recognized in the graph shown in Fig. 6. Here, we suggest that these features correspond to several classes of axons and/or cell bodies in the response area, and that they reflect the gradation in the a -value in the maps shown in Fig. 10.

Previously, we have identified the fast signal-response area and the slow signal-response area in embryonic brainstem preparations at several stages, and we have constructed their maps [15]. We have suggested that the fast signal-response area corresponds to the dorsal motor nucleus of the vagus nerve and the slow signal-response area corresponds to the nucleus of the solitary tract [15]. Comparison of the regional distribution maps of the a -value with the maps of the fast and slow signal-response areas indicates that the area corresponding to the minimum a -value coincides with the area of the fast signal-response in which the optical signal is largest.

Finally, it is concluded that the dorsal motor nucleus is initially composed of a neural population having higher responsiveness which is generated at the earliest stage, and, subsequently, surrounding this neural population as a central core, the dorsal motor nucleus is gradually organized with a spatial gradient of responsiveness to peripheral antidromic vagal stimulation during early brainstem development. The responsiveness in the central core also developmentally increases and expands. We also speculate that this emergence is closely related to the development of the size of the axons, specific membrane resistance, and sodium channel activity.

We are most grateful to Drs. Larry Cohen and Brian Salzberg for their critical reading of the first draft of the manuscript and pertinent comments. We also thank the journal reviewers for helpful suggestions concerning the manuscript. This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by the grants from the Fukuda Foundation and the Mitsubishi Foundation.

REFERENCES

1. Patterson PH and Purves D: Readings in Developmental Neurobiology, Cold Spring Harbor Lab, Cold Spring Harbor, NY, pp 1-700, 1982
2. Cohen LB and Leshner S: Optical monitoring of membrane potential: Methods of multisite optical measurement. *In: Optical Methods in Cell Physiology*, ed. De Weer P and Salzberg BM, Wiley-Interscience, New York, pp 71-99, 1986
3. Cohen LB and Salzberg BM: Optical measurement of membrane potential. *Rev Physiol Biochem Pharmacol* **83**: 35-88, 1978
4. Grinvald A, Frostig RD, Lieke E, and Hildesheim R: Optical imaging of neuronal activity. *Physiol Rev* **68**: 1285-1366, 1988
5. Kamino K: Optical studies of early developing cardiac and neural activities using voltage-sensitive dyes. *Jpn J Physiol* **40**: 443-461, 1990
6. Kamino K: Optical approaches to ontogeny of electrical activity and related functional organization during early heart development. *Physiol Rev* **71**: 53-91, 1991
7. Kamino K, Hirota A, and Komuro H: Optical indications of electrical activity and excitation-contraction coupling in the early embryonic heart. *Adv Biophys* **24**: 45-93, 1989
8. Salzberg BM: Optical recording of electrical activity in neurons using molecular probes. *In: Current Methods in Cellular Neurobiology*, Vol. 3. Electrophysiological Techniques, ed. Barker JL and McKelby JF, John Wiley & Sons, New York, pp 139-187, 1983
9. Momose-Sato Y, Komuro H, Sakai T, Hirota A, and Kamino K: Optical monitoring of cholinergic postsynaptic potential in the embryonic chick ciliary ganglion using a voltage-sensitive dye. *Biomed Res* **12** (Suppl 2): 139-140, 1991
10. Sakai T, Hirota A, Komuro H, Fujii S, and Kamino K: Optical recording of membrane potential responses from early embryonic chick ganglia using voltage-sensitive dyes. *Dev Brain Res* **17**: 39-51, 1985
11. Sakai T, Komuro H, Katoh Y, Sasaki H, Momose-Sato Y, and Kamino K: Optical determination of impulse conduction velocity during development of embryonic chick cervical vagus nerve bundles. *J Physiol (Lond)* **439**: 361-381, 1991
12. Kamino K, Katoh Y, Komuro H, and Sato K: Multiple-site optical monitoring of neural activity evoked by vagus nerve stimulation in the embryonic chick brain stem. *J Physiol (Lond)* **409**: 263-283, 1989
13. Kamino K, Komuro H, Sakai T, and Sato K: Optical assessment of spatially ordered patterns of neural response to vagal stimulation in the early embryonic chick brainstem. *Neurosci Res* **8**: 255-271, 1990
14. Komuro H, Sakai T, Momose-Sato Y, Hirota A, and Kamino K: Optical detection of postsynaptic potentials evoked by vagal stimulation in the early embryonic chick brain stem slice. *J Physiol (Lond)* **442**: 631-648, 1991
15. Momose-Sato Y, Sakai T, Komuro H, Hirota A, and Kamino K: Optical mapping of the early development of the response pattern to vagal stimulation in embryonic chick brain stem. *J Physiol (Lond)* **442**: 649-668, 1991
16. Sakai T, Momose-Sato Y, Komuro H, Sato K, Hirota A, and Kamino K: Optical mapping of spatial responsiveness to vagal stimulation in the early developing chick brain stem. *Jpn J Physiol* **41**(Suppl): S261, 1991 (abstract)

17. Fujii S, Hirota A, and Kamino K: Action potential synchrony in embryonic pre-contractile chick heart: Optical monitoring with potentiometric dyes. *J Physiol (Lond)* **319**: 529–541, 1981
18. Kamino K, Hirota A, and Fujii S: Localization of pacemaking activity in early embryonic heart monitored using a voltage-sensitive dye. *Nature* **290**: 595–597, 1981
19. Salzberg BM, Obaid AL, Senseman DM, and Gainer H: Optical recording of action potentials from vertebrate nerve terminals using potentiometric probes provides evidence for sodium and calcium components. *Nature* **306**: 36–40, 1983
20. Hirota A, Kamino K, Komuro H, Sakai T, and Yada T: Early events in development of electrical activity and contraction in embryonic rat heart assessed by optical recording. *J Physiol (Lond)* **369**: 209–227, 1985
21. Weiss G: Role de la quantite d'electricite dans l'excitation des nerfs. *Compt Rends Soc Biol Paris* **53**: 440–441, 1901 (cited from Monnier [26])
22. Noble D and Stein RB: The threshold conditions for initiation of action potentials by excitable cells. *J Physiol (Lond)* **187**: 129–162, 1966
23. Ranck JB Jr: Extracellular stimulation. *In: Electrical Stimulation Research Techniques*, ed. Patterson MM and Kesmer RP, Academic Press, New York, pp 1–36, 1981
24. Davson H: *A Textbook of General Physiology*, 4th ed, J & A Churchill, London, Vol 2, pp 1041–1126, 1970
25. Lucas K: The excitable substances of amphibian muscle. *J Physiol (Lond)* **36**: 113–135, 1907
26. Monnier M: *Function of the Nervous System*, Elsevier Science Publishing, New York, pp 59–65, 1968
27. Woodbury JW: Action potential: Properties of excitable membranes. *In: Physiology and Biophysics*, 19th ed, ed. Ruch TC and Patton HD, WB Saunders Company, Philadelphia and London, pp 26–72, 1965
28. Shinoda Y, Arnold AP, and Asanuma H: Spinal branching of corticospinal axons in the cat. *Exp Brain Res* **26**: 215–234, 1976
29. Shinoda Y, Ghez C, and Arnold AP: Spinal branching of rubrospinal axons in the cat. *Exp Brain Res* **30**: 203–218, 1977
30. Adrian RH, Chandler WK, and Hodgkin AL: The kinetics of mechanical activation in frog muscle. *J Physiol (Lond)* **204**: 207–230, 1969
31. Costantin LL: Contractile activation in frog skeletal muscle. *J Gen Physiol* **63**: 657–674, 1974
32. Gilly WF and Hui CS: Mechanical activation in slow and twitch skeletal muscle fibres of the frog. *J Physiol (Lond)* **301**: 137–156, 1980
33. Bostock H: The strength-duration relationship for excitation of myelinated nerve: Computed dependence on membrane parameters. *J Physiol (Lond)* **341**: 59–74, 1983