OPTICAL MAPPING OF THE EARLY DEVELOPMENT OF THE RESPONSE PATTERN TO VAGAL STIMULATION IN EMBRYONIC CHICK BRAIN STEM

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SUMMARY

1. In both intact and slice preparations of vagus–brain stem isolated from 3- to 8-day-old chick embryos, the spatial pattern of neural responses to vagal stimulation and its development were assessed by means of multiple-site optical recording of electrical activity, using a voltage-sensitive merocyanine–rhodanine dye (NK2761) and a 12×12-element photodiode array.

2. The first neural responses, viz. fast optical signals (related to the action potential), were recorded in the 4-day-old brain stem preparation, and slow optical signals (related to excitatory postsynaptic potentials) were detected from late 7- and 8-day-old brain stem preparations.

3. The evoked optical signals appeared to be concentrated longitudinally in the central region of the stimulated side of the intact brain stem preparation and in a limited dorsal area in the slice preparation. The signal size gradually increased and the response area expanded as development proceeded.

4. Based on the above results, we have constructed developmental maps of the spatial patterns of the fast and slow optical responses. In the maps, the positions of the peak-size regions of the fast and slow signals were assessed and we have found that there were differences in the location of these areas for the fast *vs.* the slow signals in the late 7- and 8-day-old embryonic brain stem preparations.

5. In the maps for the late 7- and 8-day-old embryonic brain stems, the fast signal response area seems to correspond to the dorsal motor nucleus of the vagus nerve and the slow response area to the nucleus tractus solitarii.

INTRODUCTION

We have previously demonstrated the spatial response pattern to vagal stimulation in early 7-day-old embryonic chick brain stem preparations and some of its electrophysiological properties, using the technique of multiple-site optical

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recording of electrical activity (Kamino, Katoh, Komuro & Sato, 1989*b*; Kamino, Komuro, Sakai & Sato, 1990). In the preceding paper (Komuro, Sakai, Momose-Sato, Hirota & Kamino, 1991), we have reported optical recording of postsynaptic potentials in the 8-day-old embryonic brain stem slice preparations, and described some of the basic characteristics of the optical signal related to synaptic transmission.

These earlier experiments have led us to believe that the embryonic age of 7 and 8 days is a critical stage in the ontogeny of vagus-related synaptic function in the developing chick brain stem. However, morphological and/or electrophysiological approaches are not capable of completely resolving this problem. Thus, we have used optical recording methods to study the early development of neural responses to vagal stimulation in 3- to 8-day-old embryonic chick brain stem preparations. We present here early developmental maps of the spatial patterns of the neural responses, and we have uncovered the sequence of events in the generation of vagal response in the early embryonic chick brain stem. These experiments have been reported in preliminary form at the 67th Annual Meeting of The Physiological Society of Japan (Momose, Sakai, Komuro, Hirota & Kamino, 1990).

METHODS

Preparations. 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. Brain stems, with the vagus nerve fibres attached, were dissected from 3- to 8-day-old embryos, and slice preparations of about 1 mm thickness were made from the isolated brain stem at the level of the vagus nerve root. The slice preparation was attached to the silicone (KE 106; Shinnetsu Chemical Co., Tokyo, Japan) bottom of a simple chamber by pinning it with tungsten wires. The preparation was kept in an oxygen-equilibrated bathing solution having the following composition (in mM): NaCl, 138; KCl, 5·4; CaCl$_2$, 1·8; MgCl$_2$, 0·5; glucose, 10; and Tris-HCl buffer (pH 7·2), 10. In the present experiments, we used two intact preparations of 3-day-old, ten intact and one slice preparation of 4-day-old, sixteen intact and one slice preparation of 5-day-old, twenty-six intact and seven slice preparations of 6-day-old, fifty-five intact and twenty slice preparations of 7-day-old, and twenty-six intact and fifty-three slice preparations of 8-day-old embryonic brain stems.

Dye staining. The preparations were incubated for 15–25 min in a bathing solution to which was added 0·1–0·2 mg/ml of a merocyanine–rhodanine dye, NK2761 (Fujii, Hirota & Kamino, 1981; Salzberg, Obaid, Senseman & Gainer, 1983; Obaid, Orkand, Gainer & Salzberg, 1985; Komuro *et al.* 1991). The nature of this dye has been described previously in detail (Kamino, Hirota & Komuro, 1989*a*). After staining, the preparation was washed with several changes of normal bathing solution.

Electrical stimulation. For preparations in which the vagus nerve was stimulated, the end of the nerve was drawn into a suction electrode fabricated from TERUMO–haematocrit tubing (VC-HO75P; Terumo Co., Tokyo, Japan), which had been hand-pulled to a fine tip (about 100 μm in internal diameter) over a low-temperature flame. In the present experiments, current strengths which induced the maximum response in each preparation were used.

Optical measurement. The optical method that we have used in this experiment was identical to that described in the preceding report (Komuro *et al.* 1991). Light from a 24 V/300 W tungsten–halogen lamp was collimated, rendered quasimonochromatic with a heat filter and a 702 ± 13 nm interference filter, and focused on the surface of the preparation by means of a bright-field condenser with a numerical aperture (NA = 0·4) matched to that of the microscope objective. Light transmitted by the preparation was collected by a microscope objective (× 10) and a photographic eyepiece (× 2·5). The objective and eyepiece projected a real image of the preparation onto a 12 × 12-element silicon photodiode matrix array (MD-144-4PV; Centronic Ltd, Croydon) mounted in an Olympus Vanox microscope. Each pixel of the array detected light transmitted by a 56 μm square region of the preparation, discounting scattered light. The photocurrents generated

by each element of the photodiode array were separately converted to voltage, AC coupled (time constant 3 s) and amplified. All of the amplified outputs 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, USA). In this recording system, the sensitivity of the measurement is 10^{-4}, expressed as a fractional change in transmitted light intensity (change in transmitted intensity divided by the DC background intensity).

<div align="center">RESULTS</div>

<div align="center">*Embryonic initiation of vagal response in 3- and 4-day-old preparations*</div>

Intact preparations

Figure 1 shows optical recordings of the vagal response in the intact brain stem preparation dissected from a 4-day-old chick embryo. The preparation was stained in a Ringer solution containing 0·15 mg/ml merocyanine–rhodanine dye (NK2761) for 15 min, then washed with normal Ringer solution. Optical measurements were made with the preparation placed in the chamber ventral side up. Positive and negative current pulses (10 μA/5·0 ms, 1·0 Hz) were applied to the right vagus nerve fibres, and evoked optical signals were recorded from the right side of the brain stem.

Single-sweep optical recordings, using positive stimulation, revealed small, but significant, signals on the outputs of several photodiode elements positioned over the small central area in the stimulated side of the brain stem preparation, although the signal-to-noise ratios were very poor. In the recording shown in Fig. 1, eight trials were signal averaged in order to improve the signal-to-noise ratio. Here, when positive square current pulses were applied, action potential-related optical spike signals were clearly observed, and when negative current pulses were applied, extremely small optical changes related to the hyperpolarizing electrotonic potential were recorded by several photodiode elements positioned over the central part of the brain stem. At, and near, the root of the vagus nerve, large optical changes related to the electrotonic potential were recorded when either positive or negative current pulses were applied. Such an electrotonic potential-related change is unaffected by low external calcium concentration and insensitive to tetrodotoxin (see Kamino *et al.* 1989*b*). We also carried out similar experiments on 3-day-old embryonic brain stem preparations, and found that, although small electrotonic optical changes were recorded with positive and negative stimulation, no action potential-related optical signals could be observed.

Slice preparations

Figure 2 illustrates an optical recording of the vagus response in a slice preparation of a 4-day-old embryonic brain stem. The slice preparation was made at the level of the vagus nerve. The recordings in *A* were obtained by applying positive current pulses (3 μA/5·0 ms, 0·1 Hz) to the right (for the recording on the left) and left (for the recording on the right) vagus nerve fibres. The recordings in *B* were made using negative stimulation. These recordings were made by averaging four trials. The inset on the bottom illustrates the location of the image of the brain stem on the photodiode array and the relative position of the suction electrode.

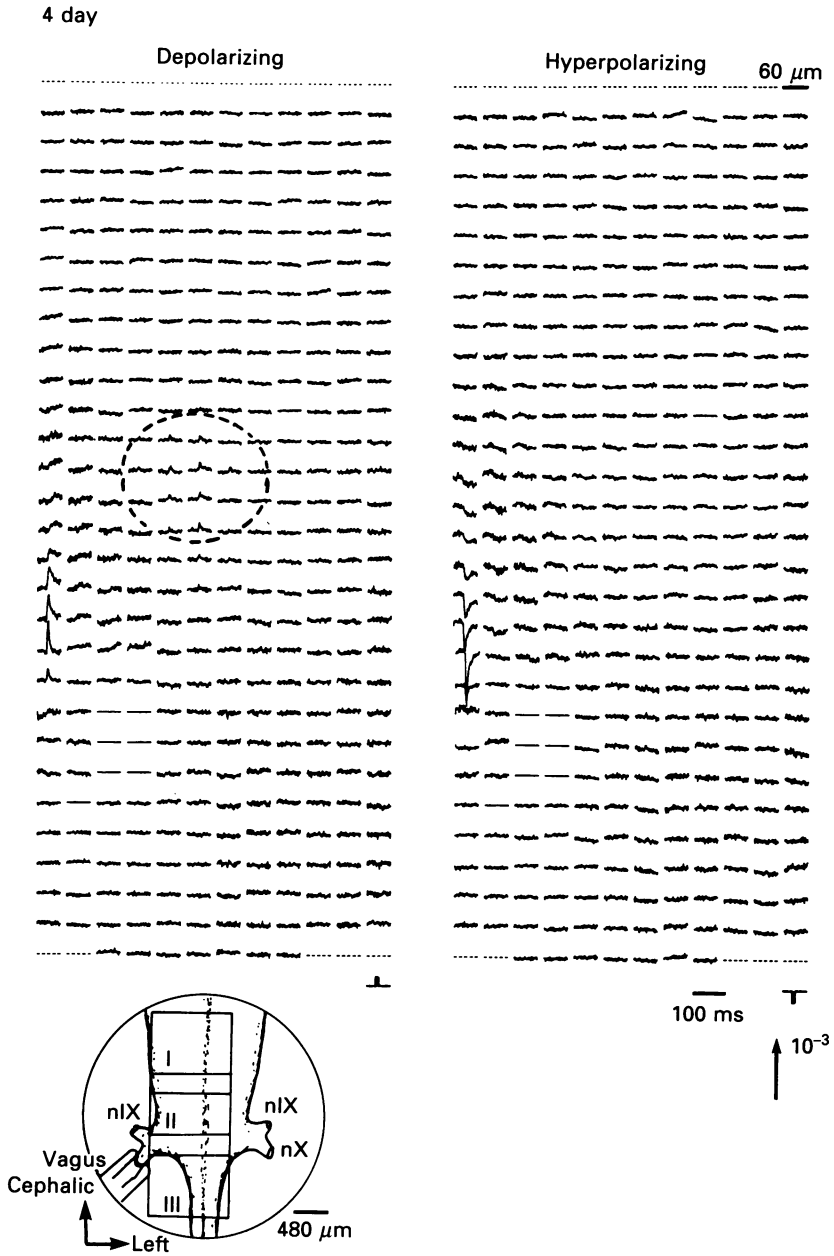In this preparation, when positive current pulses were applied, extremely small

4 day

Depolarizing

Hyperpolarizing   60 μm

100 ms

↑ 10⁻³

I

nIX   II   nIX

nX

Vagus

Cephalic   III

Left   480 μm

Fig. 1. For legend see p. 654.

4 day

A

Right

Left

Depolarizing

60 μm

B

Hyperpolarizing

100 ms

10⁻³

Vagus

Left nodose ganglion
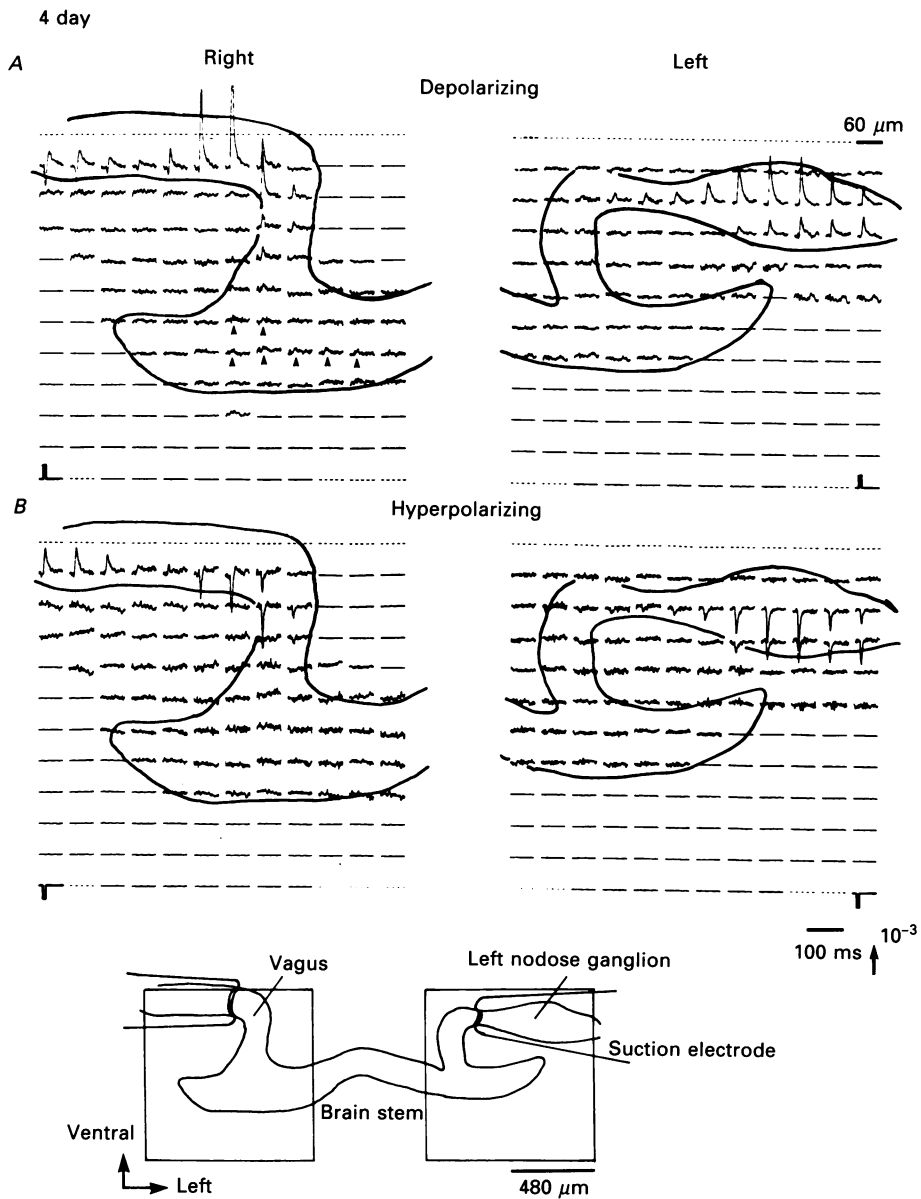
Suction electrode

Ventral

Brain stem

Left

480 μm

Fig. 2. For legend see p. 654.

but clearly recognizable signals were detected by several photodiodes (indicated by arrow-heads) positioned over the image of the right side of the brain stem. No signals were detected from the left side. Also, no significant optical changes were detected following negative current stimuli. Because the 4-day-old preparation is very delicate, we could not carry out more than one experiment with a single preparation. Nevertheless, this result indicates that the optical signals were related to the conducted action potentials. Since embryonic brain stem tissue less than 4 days old is extremely fragile, we were not able to make good slice preparations from 3-day-old embryos.

Response patterns in 5- to 6-day-old preparations

Intact preparations

Optical recordings obtained from intact and slice preparations of 5- to 8-day-old embryonic brain stems are shown in Fig. 3 (for intact preparations) and in Fig. 4 (for slice preparations). The response area and the size of the evoked optical signals usually depended on the strength of the stimulating current as demonstrated previously (Kamino *et al.* 1989*b*; Kamino *et al.* 1990). Therefore, the optical recordings shown in Figs 3 and 4 were made with stimulation parameters which produced the maximal response. These recordings were made by successive displacements of the photodiode array over the image of the brain stem, and all of the signals were recorded in single sweeps. Usually complete sets of such measurements were carried out within about 3 min.

Fig. 1. Multiple-site optical recording of neural responses to vagal stimulations in an intact preparation from a 4-day-old embryonic chick brain stem. Small signals were detected with positive pulses within a limited area (surrounded by a dashed line) in the brain stem. The preparation was stained with a merocyanine–rhodanine dye (NK2761: 0·15 mg/ml for 15 min). Positive (for the left recording) and negative (for the right recording) square current pulses (10 μA/5·0 ms, 1·0 Hz) were applied to the right vagus nerve fibres using a suction electrode. The figure was constructed with three 127-site simultaneous recordings using the 12 × 12-element photodiode array. In each recording, eight trials were averaged. The signals were detected from the ventral side of the brain stem, with a 702 ± 13 nm interference filter. The photodiode array was positioned over a 25 × magnified image of the brain stem, so that each trace represents signals detected by one photodiode from a 56 × 56 μm^2 area of the preparation. In this figure and Figs 2, 3 and 4, the traces are arranged so that their relative positions in the figures correspond to the relative positions of the area of the preparation imaged onto the detectors. The outputs of the individual detectors were divided by the resting level to compensate for the DC background intensity. The relative location of the photodiode array on the image of the brain stem is illustrated on the lower left. The direction of the arrow at the lower right corner of the optical recording indicates a decrease in transmission (an increase in absorption) and the length of the arrow represents the stated value of the fractional change.

Fig. 2. Optical recordings of vagal responses in a slice preparation made by transversely sectioning a 4-day-old embryonic brain stem. Positive (for *A*) and negative (for *B*) square current pulses (3·0 μA/5·0 ms, 0·1 Hz) were applied to the right and left vagus nerve fibres. When the positive pulses were applied to the right vagus nerve, small spike-like optical signals with small signal-to-noise ratios (indicated by arrow-heads) appeared in the brain stem. The inset on the bottom illustrates the location of the photodiode array and the suction electrode with respect to the preparation. These recordings were made by averaging four trials. Other experimental conditions and indications were the same as in Fig. 1.
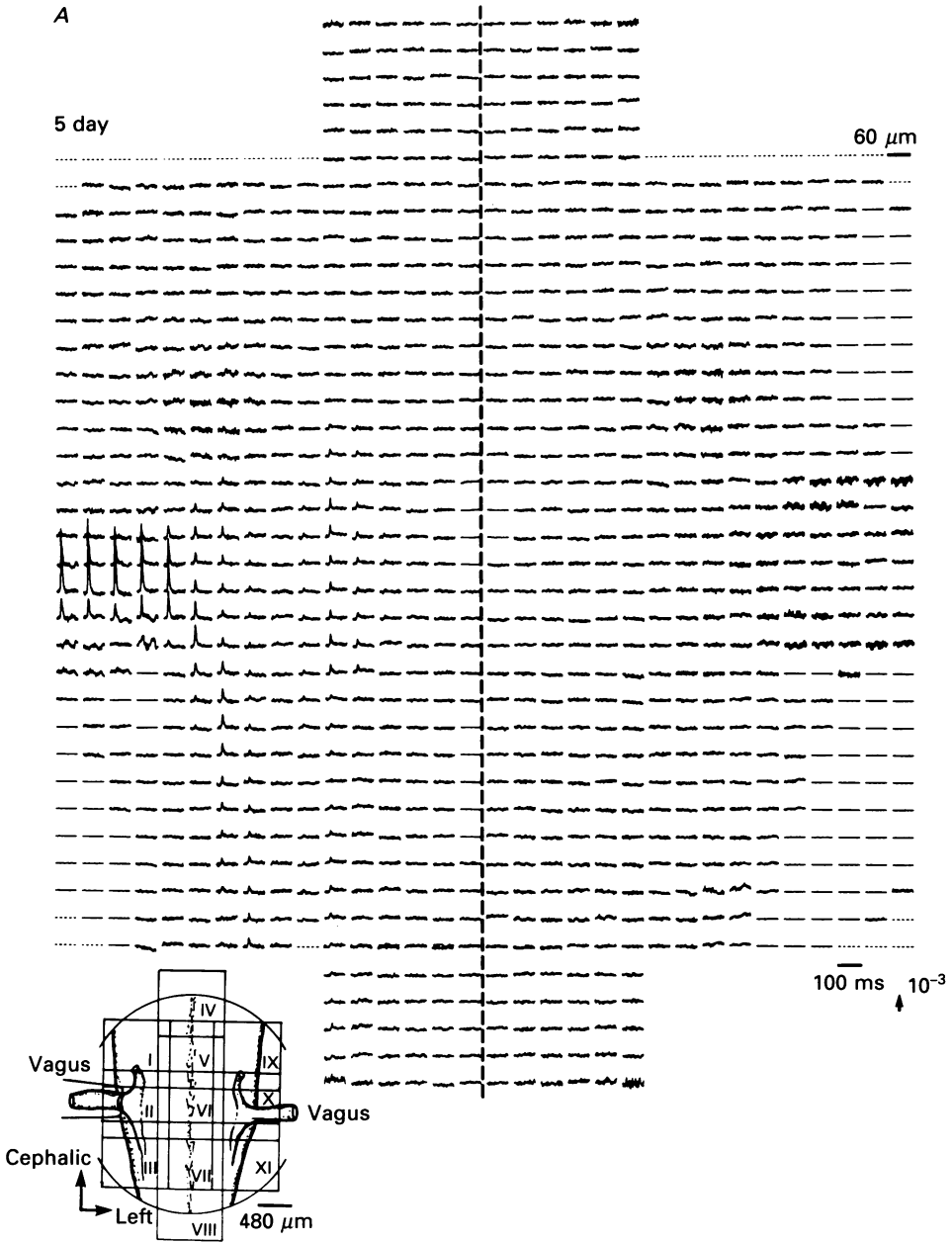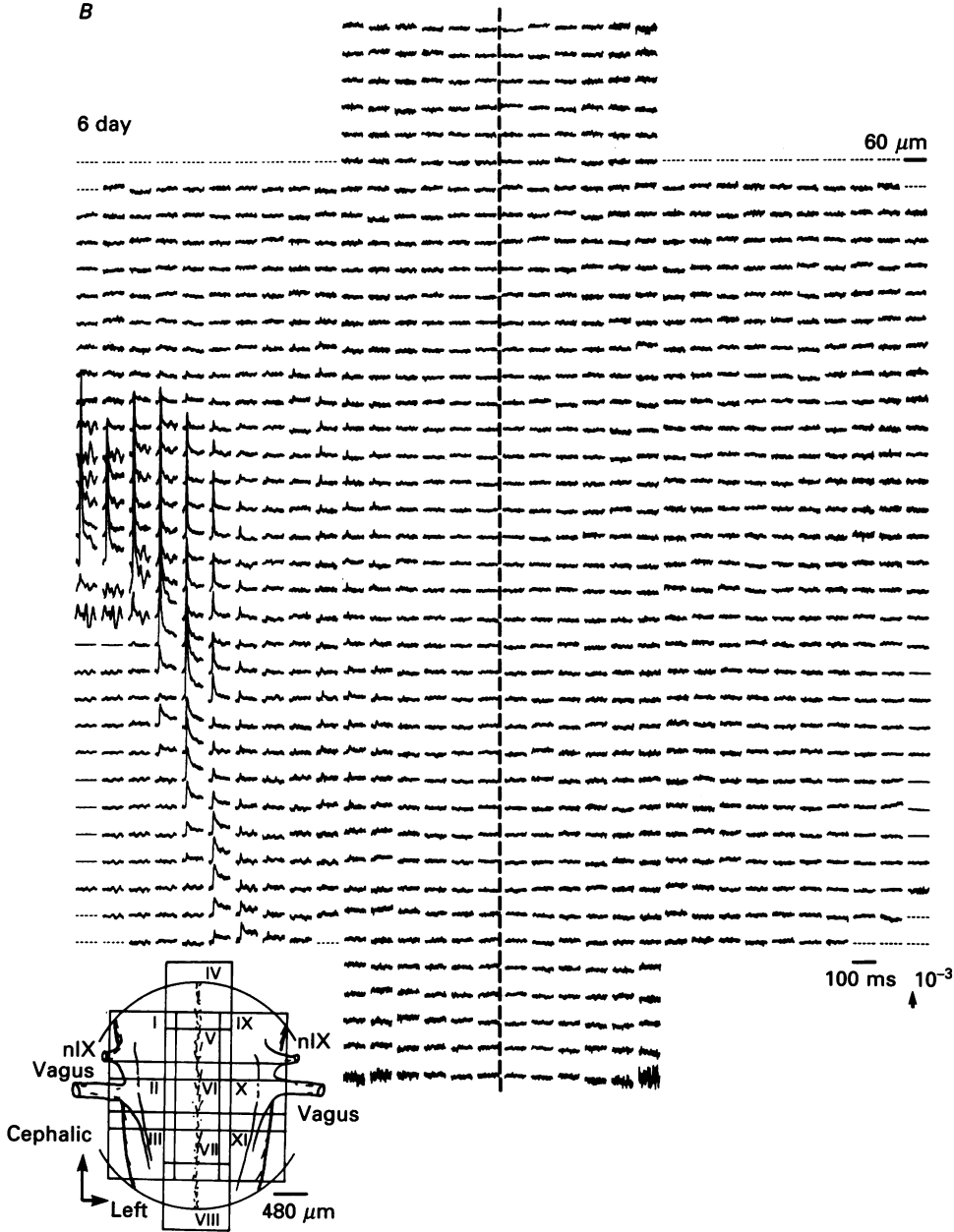
Fig. 3*A*. For legend see p. 659.

B

6 day

60 µm

100 ms 10⁻³
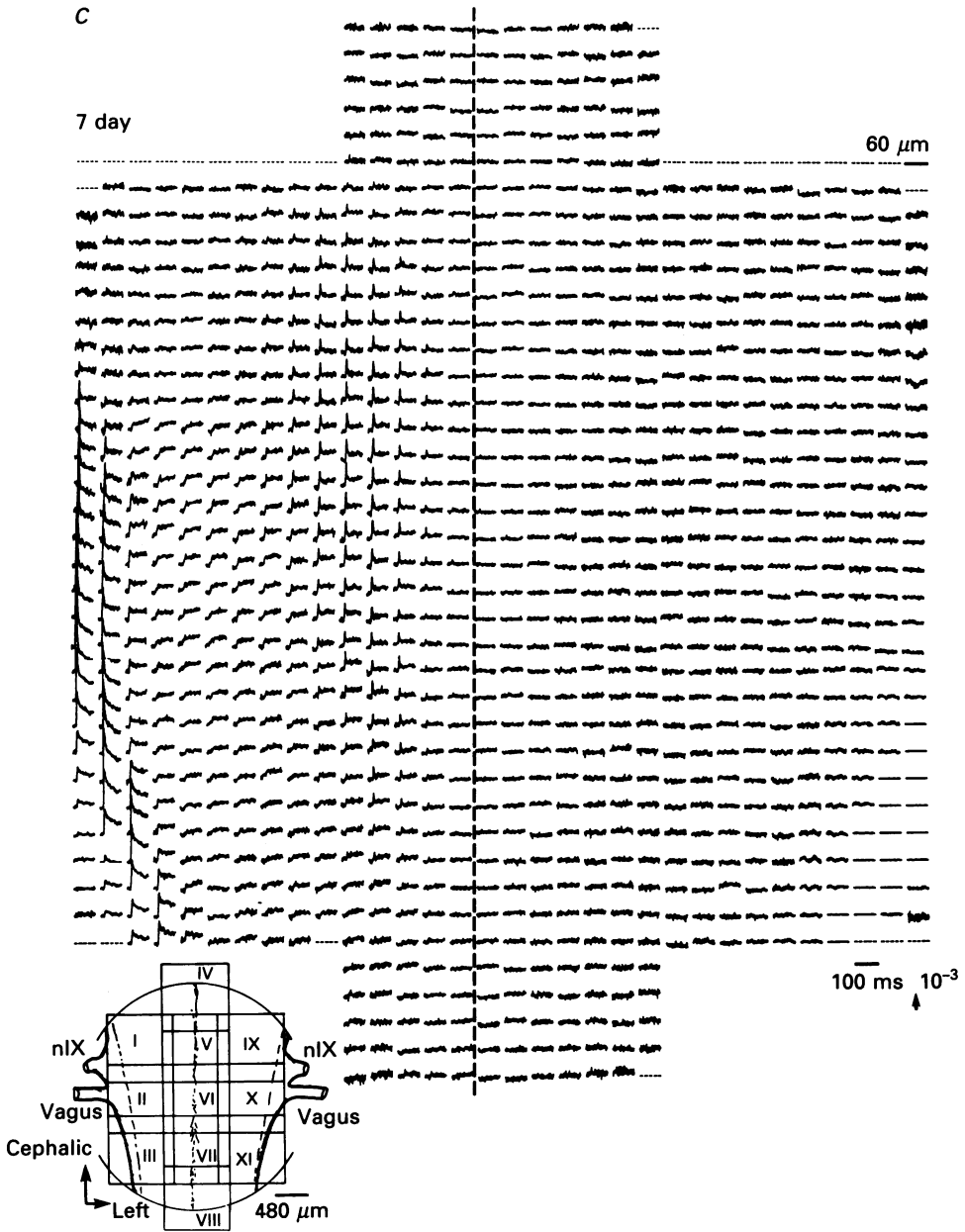
IV

I   IX

nIX   nIX
Vagus

II   X

Vagus

Cephalic   VIII   XI

Left   VIII   480 µm

Fig. 3*B*. For legend see p. 659.

C

7 day

60 µm

100 ms 10⁻³

nIX I IV V IX nIX

Vagus II VI X Vagus

Cephalic III VII XI

Left VIII 480 µm

Fig. 3*C*. For legend see p. 659.

D

8 day

60 μm

100 ms 10^{-3}

I

II VI   nIX

III VII   Left vagus

IV   VIII

V   480 μm
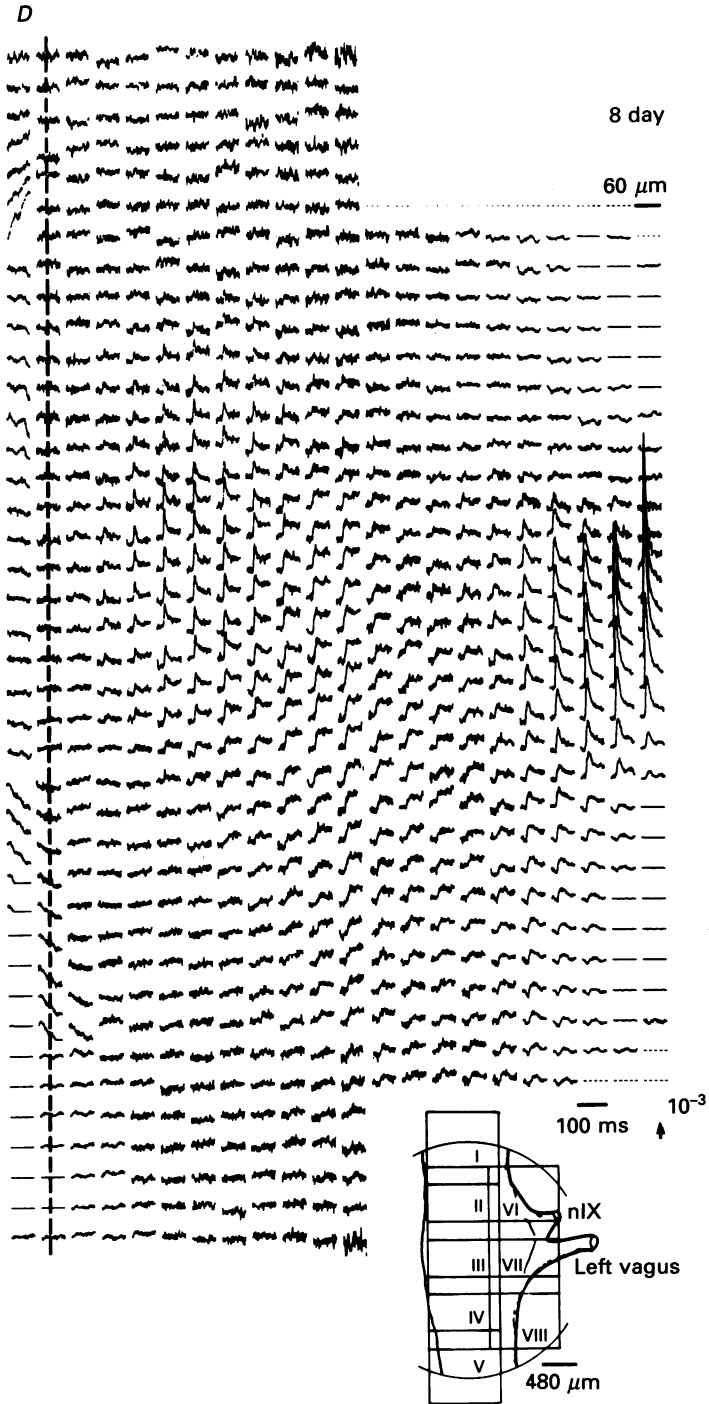
Fig. 3*D*. For legend see facing page.

Figure 3A shows the recording made using right vagus stimulation ($4\cdot0$ μA/$5\cdot0$ ms) in a 5-day-old intact brain stem preparation. In the recording made using left vagus stimulation ($4\cdot0$ μA/$5\cdot0$ ms) in the same preparation, a similar response pattern (on the left side) was obtained. Similarly, the recording shown in Fig. 3B was obtained using right vagus nerve stimulations ($5\cdot0$ μA/$5\cdot0$ ms) in a 6-day-old preparation. In the recording made using left vagus stimulation ($5\cdot0$ μA/$5\cdot0$ ms) in the same preparation, a similar response pattern (on the left side) was obtained.

As seen in these recordings, from 5- and 6-day-old preparations, both the signal size and response area increased dramatically, compared with the results shown in Fig. 1. In the central response area, the signal size, expressed as a fractional change in transmitted light intensity, increased to approximately 10^{-3} from a value of about 10^{-4} in the 4-day-old embryonic brain stem, and the response area expanded in an ellipsoidal shape along the longitudinal axis: the expansion in the caudal direction was larger than that in the cephalic direction. The lateral response area also extended to the caudal direction along the edge of the brain stem.

Slice preparations

Results obtained from slice preparations of the 5- and 6-day-old embryonic brain stems are shown in Fig. 4A (5 day) and 4B (6 day: top traces). In the 5- to 6-day-old embryonic brain stem, the cross-sectional area of the slice expanded and assumed a butterfly-like shape.

In Fig. 4A, the recording of the upper panel was obtained using right vagus stimulation ($5\cdot0$ μA/$5\cdot0$ ms) and the recording in the lower panel using left vagus stimulation ($5\cdot0$ μA/$5\cdot0$ ms). These recordings were made in single sweeps. In the 5-day-old preparation shown in Fig. 4A, the signals recorded from the right side were larger than those in the left side. In this preparation, when the stimulus was applied to the right vagus, the signals appeared to be distributed over much of the right side of the preparation. In the area near the mid-line of the brain stem, the signals were relatively large (fractional change $> 10^{-3}$), and in the lateral side of the brain stem they were smaller ($< 10^{-3}$). Although the signals were relatively small, a similar distribution of the signals was observed in the left area.

In the 6-day-old preparation shown in Fig. 4B (top traces), the signal size increased significantly, and the response area became localized on the dorsal side of the brain stem. This pattern seems to be established by the 6-day-old stage of development.

Fig. 3. Optical recordings of neural responses to vagal stimulation in 5- to 8-day-old intact embryonic brain stem preparations. Positive square current pulses were applied to the right vagus (for A, B and C), and to the left vagus (for D). The pulse conditions were: $4\cdot0$ μA/$5\cdot0$ ms for A, $5\cdot0$ μA/$5\cdot0$ ms for B, $7\cdot0$ μA/$5\cdot0$ ms for C and $8\cdot0$ μA/$5\cdot0$ ms for D. The recordings were made in a single sweep. Relative positions of the photodiode array with respect to the image of the preparation are illustrated on the lower left corners of A, B and C and on the lower right corner of D. The recordings were obtained from eleven (for A, B and C) and eight (for D) different contiguous areas by sliding the photodiode array over the image of the preparation. The dashed line corresponds to the mid-line of the brain stem. Since the dye did not diffuse easily into the tissue in the 8-day-old embryonic brain stem, the brain stem was separated by cutting along its mid-line, and then it was stained with the dye. Other experimental conditions and indications are the same as in Fig. 1.
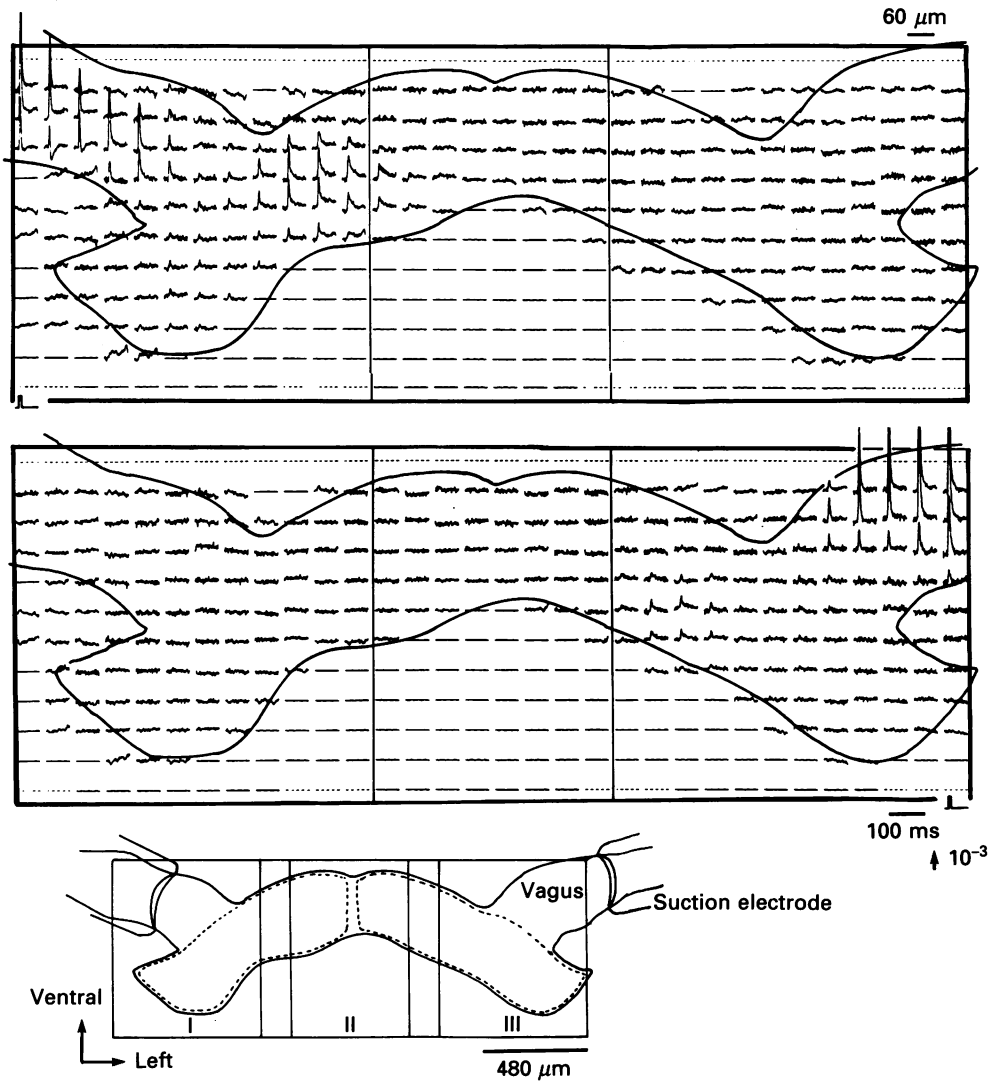
A 5 day

Fig. 4A. For legend see facing page.

Appearance of postsynaptic potential signals

Intact preparations

Figure 3C shows optical responses evoked by right vagus stimulation (7·0 μA/5·0 ms) from a late 7-day-old embryonic intact brain stem preparation. The signals observed in the central response area clearly show a slow signal. The size of this slow signal decreased as the calcium concentration in the Ringer solution was lowered, and the signal disappeared in the presence of manganese ions or cadmium
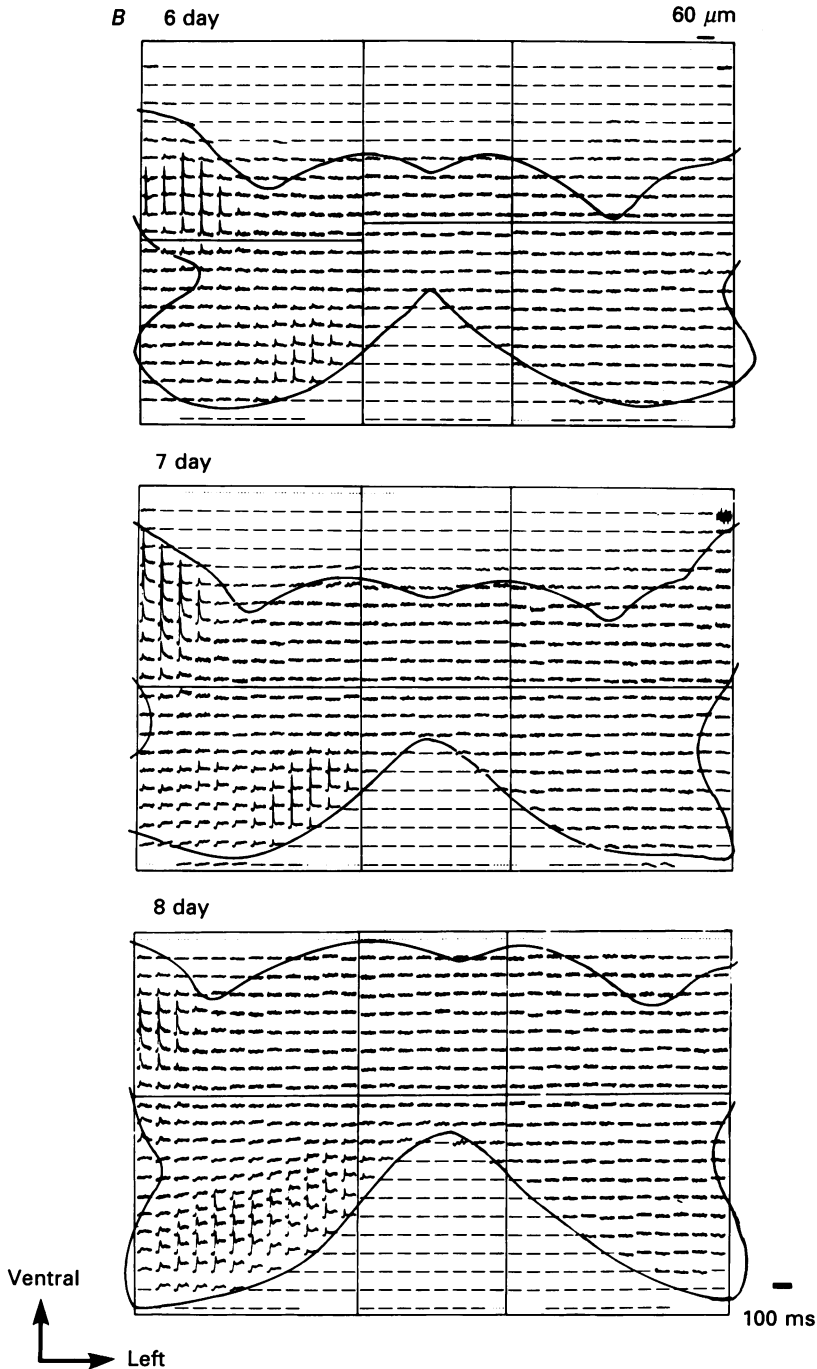
Fig. 4. Original recordings of optical signals evoked by vagus stimulation in 5- to 8-day-old embryonic brain stem slice preparations. Positive square current pulses (5·0 μA/5·0 ms for A; 6·0 μA/5·0 ms for top traces in B; 7·0 μA/5·0 ms for middle traces in B and 7·0 μA/5·0 ms for bottom traces in B) to the right (for the upper recordings in A and recordings in B) and the left (for the lower recordings in A) vagus nerve. A was constructed with three 127-site simultaneous recordings and B with six 127-site simultaneous recordings. The recordings were all made in single sweeps. Other experimental conditions and indications are the same as in Fig. 1.
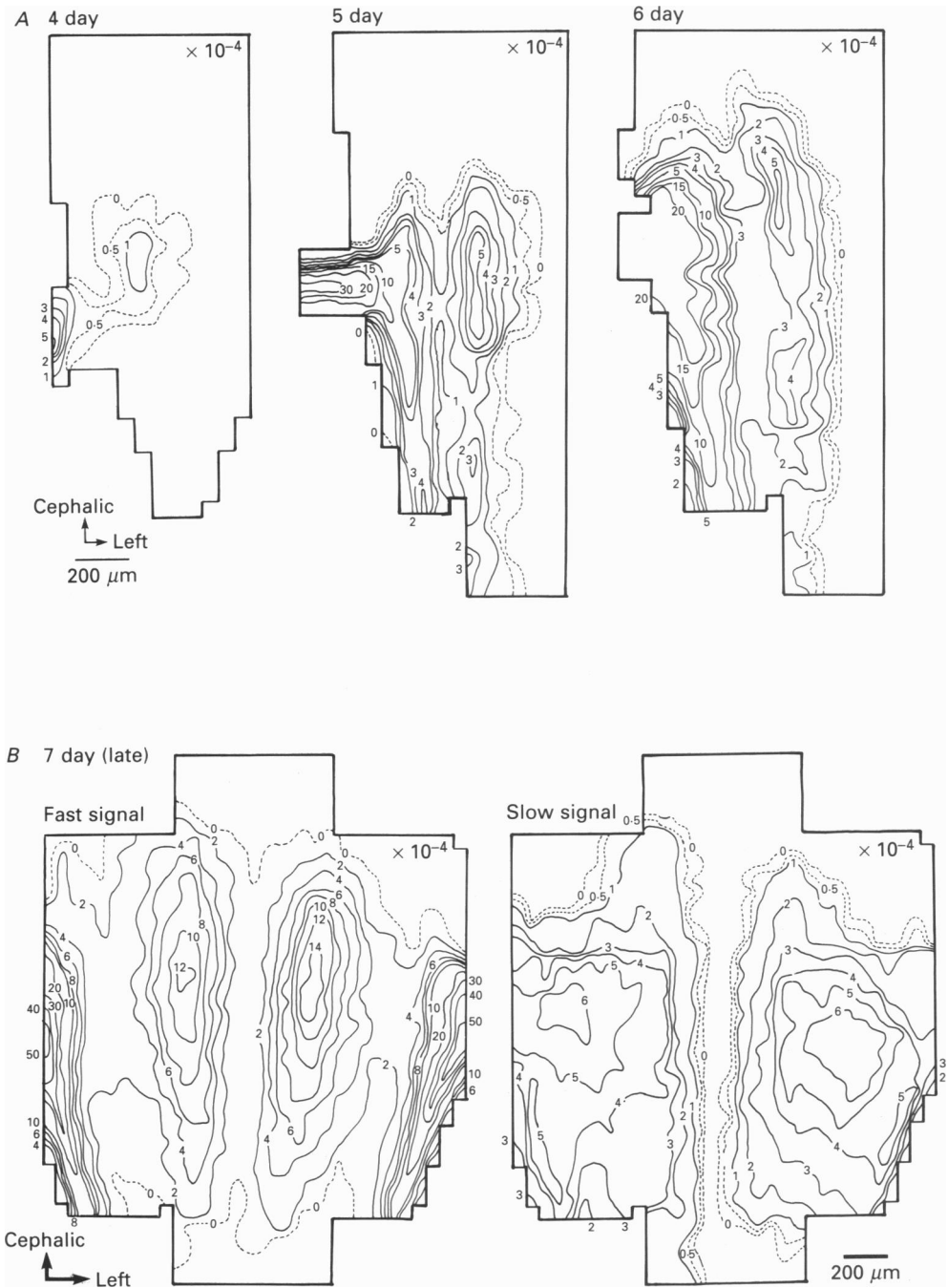
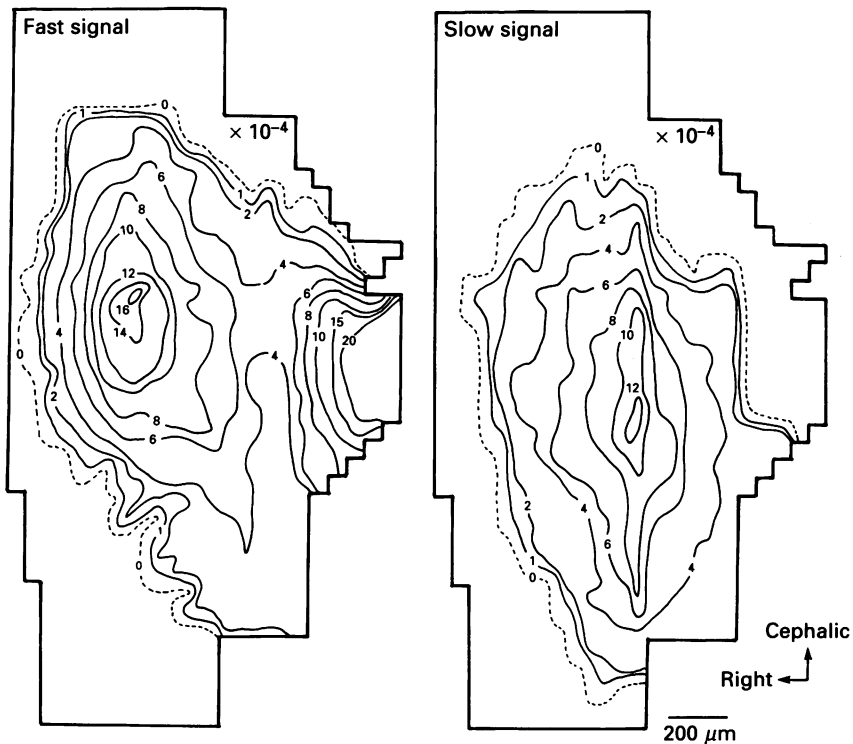Fig. 5A and B. For legend see facing page.

Fig. 5. Contour line maps of the size of the optical signals observed from 4- to 8-day-old intact embryonic brain stem preparations. Data used in these maps were obtained from the preparations shown in Figs 1 and 3. One contour interval represents an increment of 0.5×10^{-4}, 1.0×10^{-4} or 2.0×10^{-4} in the size of the optical signal: the numerals on the lines indicate the fractional changes multiplied by 10^4. A, the maps for the right half of the 4-, 5- and 6-day-old preparations; B, the maps of the fast signal (left side) and the slow signal (right side) in the late 7-day-old preparation (these maps were constructed using the data obtained by the right and left vagal stimulations); C, the maps of the fast signal (left side) and slow signal (right side) in the left half of the 8-day-old preparation.

ions (also see Komuro *et al.* 1991). In the lateral area near the electrode, optical changes related to electrotonic potentials were observed.

As shown in Fig. 3D, the slow signals were seen even more clearly in the 8-day-old embryonic brain stem. These signals were evoked by left vagus stimulation ($8.0\ \mu$A/5.0 ms), and appeared over a relatively wide area.

Slice preparations

Figure 4B (middle traces) shows the recording obtained using vagus stimulation ($7.0\ \mu$A/5.0 ms) from a late 7-day-old preparation. In this preparation, the slow component of the signal was clearly observed, and, by the 8-day-old stage (Fig. 4B, bottom traces), the slow component is prominent. These observations, made in slice preparations, are consistent with the results obtained from the intact preparations.

The results strongly suggest that, by late in the 7-day-old stage of embryonic

development of the chick brain stem, synaptic function is present in response to vagus stimulation.

Mapping

Generally, it is supposed that the size (represented as fractional change) of the optical signal is proportional to a weighted optical average of the potential change of the membrane area imaged onto each detector. Thus, we measured the size of the fast and slow signals shown in Figs 1, 2, 3 and 4, and we have drawn contour line maps of the signal size using an interpolation method.

Developmental maps of the 4- to 6-day-old intact preparations are illustrated in Fig. 5A. In the 4-day-old preparation, the response area was small and the peak size of the optical signal was 10^{-4}. In the 5-day-old preparation, the peak signal size was 5×10^{-4} and the response area expanded in an elliptical pattern. In the 6-day-old preparation, the response area expanded even further. These maps demonstrate that the area of peak signal size (in the central region) was positioned approximately at the level of the root of the vagus nerve, and that the contour lines demonstrate a layered pattern with the signal size decreasing peripherally.

Figure 5B shows two kinds of maps constructed from a late 7-day-old intact preparation: in the left panel the map of the fast signal is shown and in the right panel the map of the slow signal corresponding to the postsynaptic potential is shown. In the fast signal map, it is clear that, in comparison with the data mapped in Fig. 5A, the response area expands further, the signal size increases dramatically, and the right and left peak size areas are symmetrically positioned near the mid-line. In this preparation, the signal size and the peak size area on the right side were slightly smaller than those on the left side. In the slow signal map, the response area is shown largely to overlap the area of the fast signal. The peak size region of the slow signal is not superimposed on the peak size area of the fast signal: the peak area of the slow signal deviates in the lateral–caudal direction from that of the fast signal.

Figure 5C illustrates the maps of the 8-day-old preparation: the fast signal map is shown on the left side and the slow signal map on the right side. In this preparation, the features of the contour map are basically similar to those of the late 7-day-old preparation. The signal size is dramatically enhanced (the peak size is $1 \cdot 6 \times 10^{-3}$ for the fast signal and $1 \cdot 2 \times 10^{-3}$ for the slow signal), but the relative positions of the fast and slow signals are similar to those of the late 7-day-old preparation.

Figure 6 shows the maps of the 5- to 8-day-old slice preparations. In Fig. 6A it is very interesting to compare the maps of the 5-day-old and 6-day-old preparations. In the 5-day-old preparation, the response area occupies the greater part of the brain stem (on the stimulated side), and the peak-size area is positioned over the central part of the brain stem. In the 6-day-old preparation, the pattern of the contour line changes together with development of the brain stem: the peak-size area is now localized on the dorsal side of the brain stem. This position is maintained in the 7- and 8-day-old developmental stages, as can be seen in Fig. 6B and C.

In Fig. 6B and C, the relative positions of the peak-size area of the fast and slow signals are shown: in both the late 7- and 8-day-old preparations, the peak-size area of the slow signal is positioned somewhat lateral to that of the fast signal.
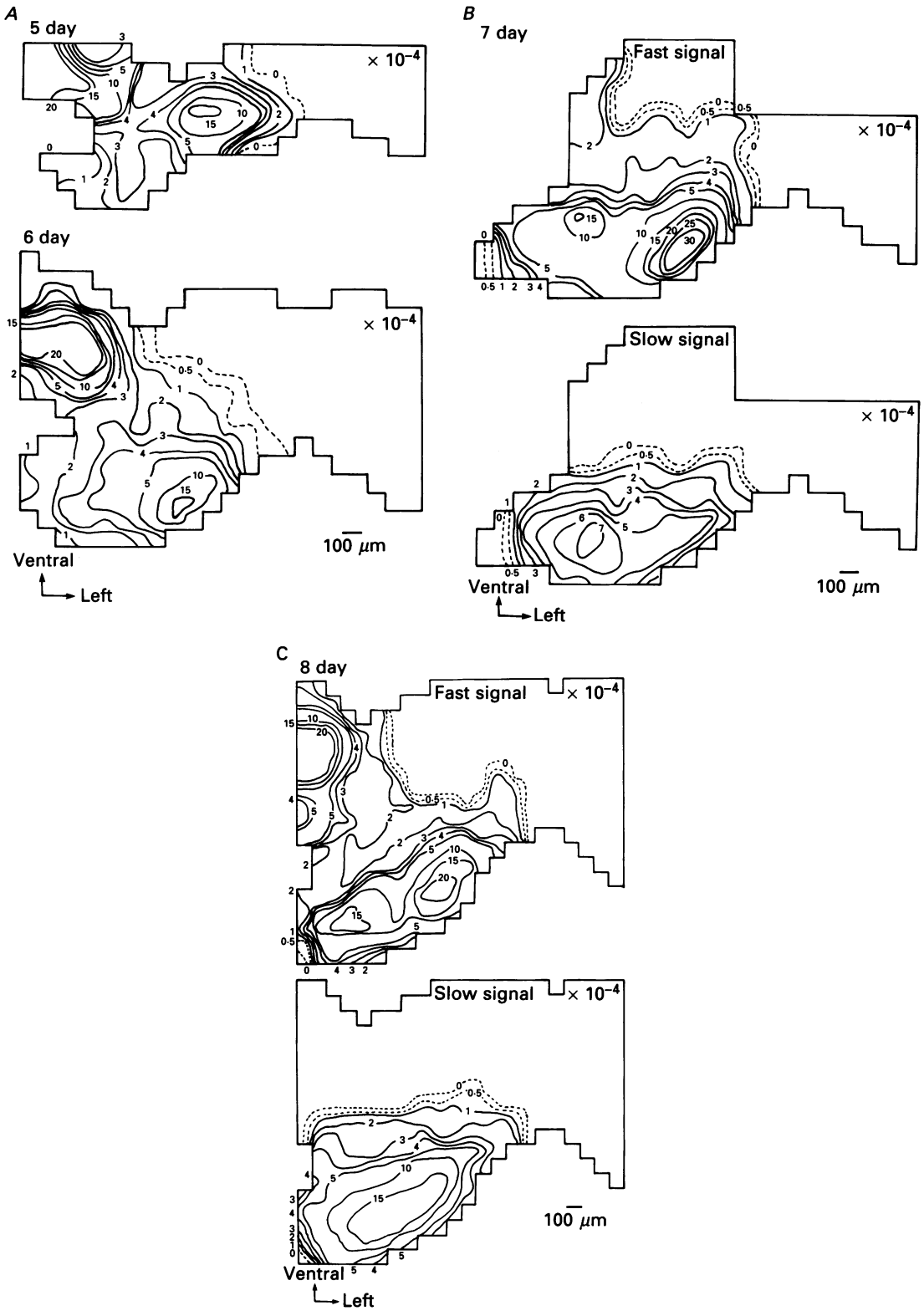
Fig. 6. Contour line maps of the size of the optical signals in 5- and 6-day (*A*) 7-day (*B*) and 8-day-old (*C*) slice preparations. In *B* and *C*, the upper maps are for the fast signal and lower maps for the slow signal. Data used in these maps were obtained from the preparations shown in Fig. 4. These maps show the right side of the brain stem. Other conditions are the same as in Fig. 5.

In the present study we have revealed the chronological sequence of the development of electrophysiological responses to vagus stimulation in the embryonic chick brain stem during early embryogenesis, using an optical technique. According to the pattern of the evoked optical (absorbance) signals in the early embryonic brain stem, we have classified two kinds of signal in response to vagus stimulation (Komuro *et al.* 1991): one is the fast signal related to the action potential and the other is the delayed slow signal related to the excitatory postsynaptic potential. We also suggest that the fast signal consists of two components related to antidromic action potentials in motoneurons and to orthodromic action potentials in sensory nerve terminals, and that the slow signal corresponds to postsynaptic potentials evoked in the postsynaptic neurons.

Because of limitations of the sensitivity of the measuring technique and of slice viability during the early stages of development, our results must be interpreted cautiously. However, the results obtained suggests that, in the response area in the embryonic brain stem related to vagus nerve stimulation, excitability of the motoneurons and/or the sensory nerve terminals is first generated no later than the 4-day stage of embryonic development. As can be seen in Figs 4*A* and 6*A* (upper), the initial vagal response appeared over the entire area of the slice preparation in the 5-day-old embryonic brain stem. This result indicates that the motoneurons and/or the sensory terminals of the vagus nerve initially distribute widely in the brain stem, and, subsequently, become concentrated near the dorsal surface by the 6-day stage of embryonic development. This distribution remains constant in the 7-day-old brain stem, suggesting that it is coupled with the growth of the brain stem itself. It would be interesting and important to separate the motoneuron activity and sensory nerve terminal activity. It would clearly be useful to stimulate an individual vagus nerve fibre, but, since the early embryonic nerve fibres are very fine and extremely fragile, the realization of this experiment would be forbiddingly difficult.

On the other hand, the slow signal is clearly related to the postsynaptic potential. Furthermore, as described in the preceding paper (Komuro *et al.* 1991), the postsynaptic potential is excitatory and glutamate mediated. Thus, we have concluded that synaptic function related to the vagus sensory neuron is generated initially in the brain stem of the 7-day-old embryo. On the other hand, there could be collateral activation of the antidromically driven motor fibres which may then innervate interneurons. However, since we have not found any evidence for cholinergic events in the present experiment, we feel that this possibility can reasonably be ruled out.

Although the fast signal and slow signal response areas for the late 7- and 8-day-old brain stem preparations are mostly superimposed, there are differences in the positions of the peak-size areas between the fast and slow signal responses. Accordingly, we suggest that the peak-size areas of the fast and slow signal responses correspond, respectively, to the centres of the dorsal motor nucleus of the vagus nerve and the nucleus tractus solitarii, and that their relative positions apparently correspond to those in the adult avian brain stem. It is known that, in birds, there is no nucleus ambiguus (Cohen & Schnall, 1970; Breazile, 1979). The second peak-size

area of the fast signal seen in Fig. 6*B* and *C* may reflect the area in which sensory nerve terminals are concentrated.

In the optical method, the linearity of the optical signal with changes in membrane potential has been established in other preparations (Cohen & Salzberg, 1978) and 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 the condition that the amount of dye bound to the membrane is uniform (Orbach, Cohen & Grinvald, 1985; Obaid *et al.* 1985; Kamino *et al.* 1989*a*; Kamino, 1991). Indeed, in the present experiments, the background intensities of transmitted light from the stained preparation were nearly constant from pixel to pixel and, in addition, the fractional sizes of the displayed signals were normalized by dividing by the slightly different background intensities. It is thus reasonable to interpret the amplitude of the slow signal as proportional to the number and/or activity of postsynaptic neurons imaged onto one photodiode. Therefore, we speculate that in the peak-size area of the slow signal, the level of synaptic activity is maximal, and that in the peak-size area of the fast signal, the number and/or activity of motoneurons is the largest. Roughly speaking, the number and/or activity of the synapses and the motoneurons decrease monotonically away from the peak, in a layered pattern.

As described above, the fast and slow signal response areas are superimposed for the most part. This evidence suggests that the dorsal motor nucleus of the vagus nerve and the nucleus tractus solitarii are not spatially separated but extensively overlapped in the early embryonic brain stem and that the motoneurons and sensory neurons are intermingled in the brain stem during early development.

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