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OPTICAL DETECTION OF POSTSYNAPTIC POTENTIALS EVOKED BY VAGAL STIMULATION IN THE EARLY EMBRYONIC CHICK BRAIN STEM SLICE

BY HITOSHI KOMURO*, TETSURO SAKAI, YOKO MOMOSE-SATO, AKIHIKO HIROTA AND KOHTARO KAMINO†

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

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SUMMARY

1. A voltage-sensitive dye and multiple-site optical recording of changes in membrane potential were used to reveal the postsynaptic potentials in the early embryonic chick brain stem slice preparation.

2. Vagus–brain stem preparations were isolated from 8-day-old chick embryos and then transverse slice preparations were prepared with both the right and left vagus nerve fibres intact. The slice preparations were stained with a voltage-sensitive merocyanine–rhodanine dye (NK2761).

3. Voltage-related optical (absorbance) changes evoked by vagus nerve stimulation with positive square current pulses using a suction electrode were recorded simultaneously from 127 contiguous loci in the preparation, using a 12×12-element photodiode array. Optical responses appeared in a limited area near the dorsal surface of the stimulated side.

4. When relatively large stimulating currents were applied, optical changes having two (or sometimes three) components were recorded. One component was the fast spike-like signal and another the delayed, long-lasting slow signal.

5. The size of the slow signal was decreased by continuous stimulation, reduced by low external calcium ion concentrations and eliminated in the presence of manganese or cadmium ions.

6. The slow signals were eliminated in the presence of kynurenic acid, and they were reduced by 2-APV (DL-2-amino-5-phosphono-valeric acid) and by CNQX (6-cyano-7-nitroquinoxaline-2,3-dione). We conclude that the slow signals correspond to excitatory postsynaptic potentials which are glutamate mediated.

INTRODUCTION

The ontogenetic approach to physiological events would be a useful strategy for analysing the complex and subtle functional organization/architecture of the central nervous system (CNS). However, for investigations of the early embryonic CNS,

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

† To whom all correspondence and reprint requests should be addressed.

MS 8639

conventional electrophysiological recording techniques, including microelectrodes and patch electrodes, are extremely difficult or impossible to employ, owing to the small size, fragility and topology of the immature or newly matured neurons. For this reason, investigations of physiology in the vertebrate CNS during the early phases of development have been hampered.

Optical techniques using voltage-sensitive dyes have made it possible to monitor electrophysiological events in living systems that are inaccessible to microelectrodes (Salzberg, Davila & Cohen, 1973; Cohen & Salzberg, 1978; Salzberg, 1983). Furthermore, optical recording methods have been developed into a powerful tool for recording electrical activity simultaneously from many sites in a living preparation (Salzberg, Grinvald, Cohen, Davila & Ross, 1977; Grinvald, Cohen, Lesher & Boyle, 1981; Komuro, Sakai, Hirota & Kamino, 1986; Salama, Lombardi & Elson, 1987; and for reviews see Salzberg, 1983; Cohen & Lesher, 1986; Grinvald, Frostig, Lieke & Hildesheim, 1988; Kamino, 1991). Applying these optical techniques, we have been able to monitor electrical activity from early embryonic hearts (e.g. Hirota, Kamino, Komuro, Sakai & Yada, 1985; Hirota, Kamino, Komuro & Sakai, 1987; and for reviews see Kamino, Hirota & Komuro, 1989*a*; Kamino, 1991) and embryonic nervous systems (Sakai, Hirota, Komuro, Fujii & Kamino, 1985; Kamino, Komuro, Sakai & Hirota, 1989*c*; Sakai, Komuro, Sato, Katoh, Sasaki & Kamino, 1989; Sakai, Komuro & Kamino, 1990), and we have overcome traditional obstacles to a functional approach to the embryonic heart and CNS during early development.

Using multiple-site optical recording methods, we have previously assessed the spatial pattern of neuronal response to vagal stimulation in the brain stem of early 7-day-old chick embryos (Kamino, Katoh, Komuro & Sato, 1989*b*; Kamino, Komuro, Sakai & Sato, 1990). Because, in these experiments, postsynaptic events were not detected, we have looked for postsynaptic potential-related optical signals in older embryos, and we have now been able to detect these responses in late 7- and 8-day-old embryonic brain stem slice preparations and study their physiological nature. This report is paired with the following paper (Momose-Sato, Sakai, Komuro, Hirota & Kamino, 1991) in which the development of vagal response patterns in the embryonic chick brain stem is described. Some of these results have been reported in a preliminary form (Komuro, Momose, Sakai, Hirota & Kamino, 1990).

METHODS

The methods that we have used for multiple-site optical recording of electrical activity in embryonic brain stem preparations have been described in detail elsewhere (Kamino *et al.* 1989*a*, *b*).

Preparation. In the present experiments, we used mainly embryonic chick brain stem slice preparations. Fertilized eggs of white Leghorn chickens were incubated for 4–9 days 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 brain stems, with vagus nerve fibres attached, were dissected from the embryos. The isolated brain stem preparation was attached to the silicone (KE 106LTV; Shinnetsu Chemical Co., Tokyo, Japan) bottom of a simple chamber by pinning it with tungsten wires. The preparation was kept in a bathing solution with 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. The solution was equilibrated with oxygen. The pia mater attached to the brain stem was carefully removed in the bathing solution under a dissecting microscope. Slices were then prepared, with the right and/or left vagus nerve fibres attached, by sectioning the embryonic brain stem transversely at the level of the root of the vagus nerve. The thickness of the slice was about 1 mm.

Dye staining. The isolated slice preparation was stained by incubating it for 15–25 min in a Ringer solution containing 0·1–0·2 mg/ml of the voltage-sensitive merocyanine–rhodanine dye NK2761 (Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan) (Kamino, Hirota & Fujii, 1981; Salzberg, Obaid, Senseman & Gainer, 1983; and Kamino *et al.* 1989*b*), and the excess (unbound) dye was washed away with dye-free Ringer solution before recording.

Electrical stimulation. For preparations in which the vagus nerve was stimulated, the cut 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 internal diameter) over a low-temperature flame.

Optical recording. Light from a 300 W tungsten–halogen lamp (Type JC-24 V/300 W, Kondo Sylvania Ltd, Tokyo, Japan) was collimated, rendered quasimonochromatic with a heat filter (32.5B-76, Olympus Optical Co., Tokyo, Japan) and an interference filter having a transmission maximum at 702 $\pm$ 13 nm (Type 1F-W, Vacuum Optics Co. of Japan, Tokyo), and focused on the preparation by means of a bright-field condenser with a numerical aperture (NA) matched to that of the microscope objective (S plan Apo, 0·4 NA). The objective and photographic eyepiece projected a real image of the preparation onto a 12 × 12-element silicon photodiode matrix array (MD-144-4PV; Centronic Ltd, Croydon) mounted on an Olympus Vanox microscope (Type AHB-L-1, Olympus Optical Co, Tokyo, Japan). The magnification of the image was usually 25 ×. Each pixel (element) of the array detected light transmitted by a square region (56 × 56 μm) of the preparation. 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 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). 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 from FORTRAN, under the RT-11 operating system (Version 5·0). The optical recording was carried out in a still chamber without continuous perfusion with Ringer solution, at room temperature (26–28 °C). The incident light was turned off except during the measuring period. Under these conditions, in 6- to 8-day-old embryonic brain stem preparations, the evoked optical signals can often be detected continuously for 60–150 min.

RESULTS

Voltage-sensitive dye signals from the 8-day-old embryonic brain stem slice

Figure 1 illustrates two examples of optical recordings of neuronal activity in an embryonic brain stem slice in response to vagus stimulation. The brain stem with vagus nerve fibres attached was dissected from an 8-day-old chick embryo and then a transverse slice preparation was made by sectioning at the level of the vagus nerve. The preparation was stained with a voltage-sensitive merocyanine–rhodanine dye (NK2761), and positive square current pulse stimuli, which depolarize the axonal membrane, were applied to the vagus nerve fibres with a suction electrode. The changes in the transmitted light intensity at 700 nm were detected simultaneously from 127 adjacent regions of the stained brain stem slice preparation using a 12 × 12-element photodiode array. The position of the photodiode array relative to the image of the preparation is illustrated at the top of the optical recordings. The recording of the left side was obtained using a 1·5 μA/7·0 ms positive square current pulse stimulus, and the recording of the right side with a 4·2 μA/7·0 ms pulse on the right vagus nerve.

As the strength of the stimulus current was increased, the signal size increased and the response area expanded (also see Figs 2 and 3). In Fig. 1, the optical signals evoked by a 4·2 μA/7·0 ms stimulus apparently consisted of two components: the spike-like fast signal and the succeeding long-duration slow signal. Enlargements of

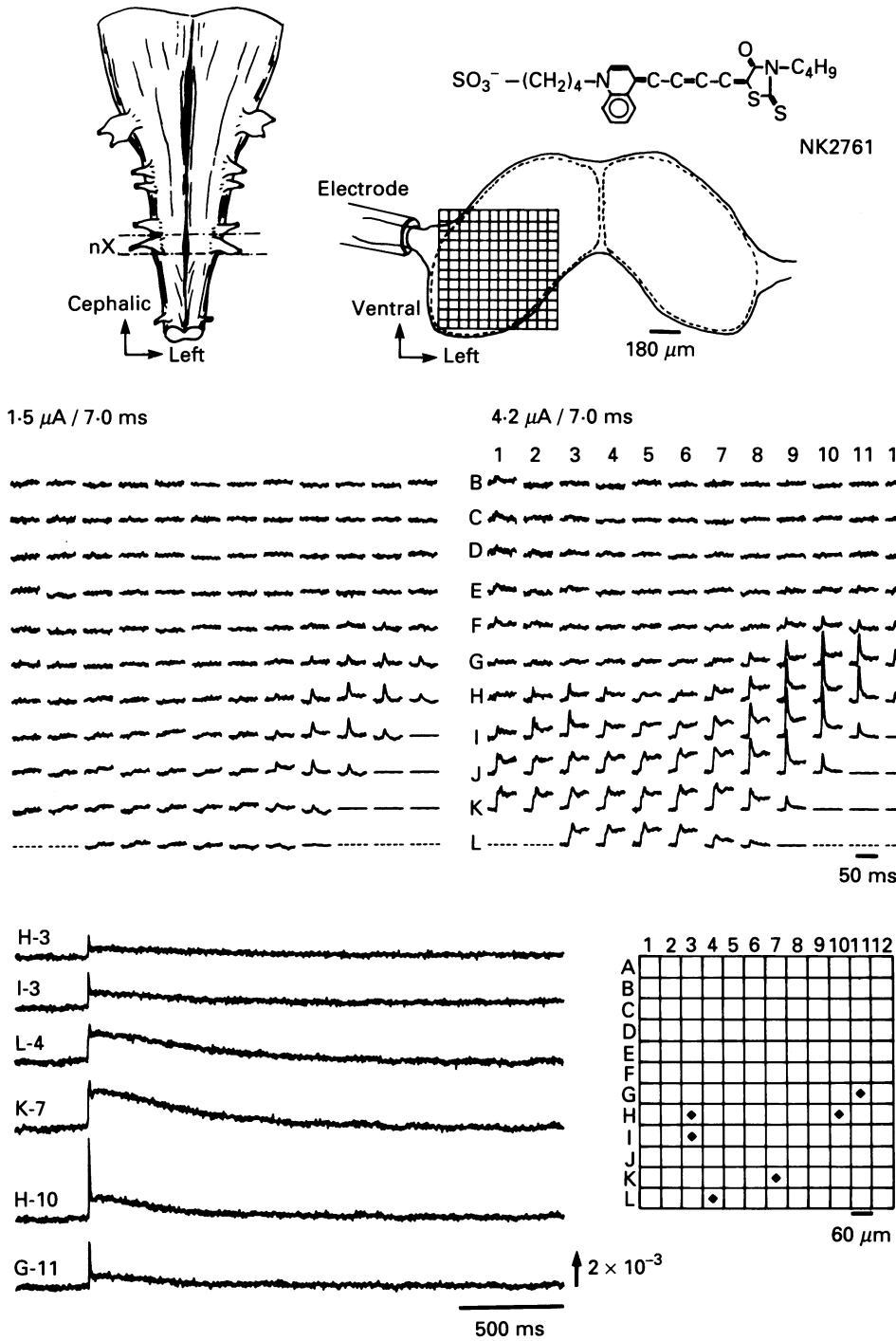

Fig. 1. For legend see facing page.

the signals detected from six different positions (H-3, I-3, L-4, K-7, H-10 and G-11) are shown on the bottom of Fig. 1. The action spectra of these two components were the same and both components were completely eliminated at 620–630 nm, the null wavelength for the NK2761 voltage-dependent optical change. No optical changes were detected from unstained preparations. These results indicate that both the fast signal and the slow signal are indeed dye-absorption changes related to changes in membrane potential and do not correspond to changes in light scattering related to mechanical or other factors.

Usually, when the duration of stimulating current was constant, the minimum strength of the current required to evoke the slow signal was slightly larger than that for the fast signal. One example is shown in Fig. 2. These traces were from one photodiode of the photodiode array. In this recording, the fast signal was detected using a 1·0 μA/7·0 ms current pulse stimulus, but the slow signal was only observed reliably using a 1·5 μA/7·0 ms stimulus. The sizes of both the fast and slow signals also depended on the duration of the stimulating current, and optimal combinations of strength and duration of the stimulating current could be determined. Examples of the combinations obtained from some representative preparations are summarized in Table 1. As these data suggest, chronaxy and rheobase were slightly different for the fast and slow components.

In addition, the area over which the slow signals were detected also depended on the strength of the stimulus current. Figure 3 illustrates the regional distributions of the fast and slow signals evoked by various current strengths (with a constant duration = 7·0 ms). In this preparation, a 0·8 μA/7·0 ms square current pulse evoked signals which appeared first in the root of the vagus nerve fibres and, subsequently, a fast signal was detected from only a small area (corresponding to one element) on the dorsal surface of the stimulated right side. The fast signal response area was enlarged as the current strengthened. Slow signals were first detected by two diodes positioned over the region of the right dorsal surface, and the slow signal response also spread gradually with increased stimulus strength. Eventually, the response areas of both the fast and slow signals reached a plateau size, with the areas of the fast and slow signals largely superimposed on the dorsal surface.

Fig. 1. Multiple-site optical recording of neural activity evoked by vagus stimulation in an embryonic chick brain stem slice preparation. The slice preparation was made by transverse sectioning of an 8-day-old embryonic brain stem at the level of the vagus nerve (nX, as illustrated in the upper left-hand corner). The preparation was stained with a merocyanine–rhodanine dye (NK2761; upper right). Positive square current pulses were applied to the right vagus nerve fibres by a suction electrode. The evoked optical signals were detected by the 12 × 12-element photodiode array positioned on the image of the right-side area of the brain stem. The relative position of the photodiode matrix array is illustrated in the upper right corner of the optical recording. The recordings were made with 1·5 μA/7·0 ms (for the left-hand recording) and 4·2 μA/7·0 ms (for the right-hand recording) square current stimuli. Wavelength 702 ± 13 nm. Enlargements of the optical signals obtained with 4·2 μA/7·0 ms stimulus current from six different positions (H-3, I-3, L-4, K-7, H-10 and G-11: indicated by ◆ on the grid corresponding to the photodiode matrix array shown on the right side) are shown in the bottom. The direction of the arrow to the right side of the signals indicates a decrease in transmitted light intensity (increase in absorption), and the length of the arrow represents the stated value of the fractional change(= the change in the light intensity divided by DC background intensity). The recordings in this and other figures were made in a single sweep.

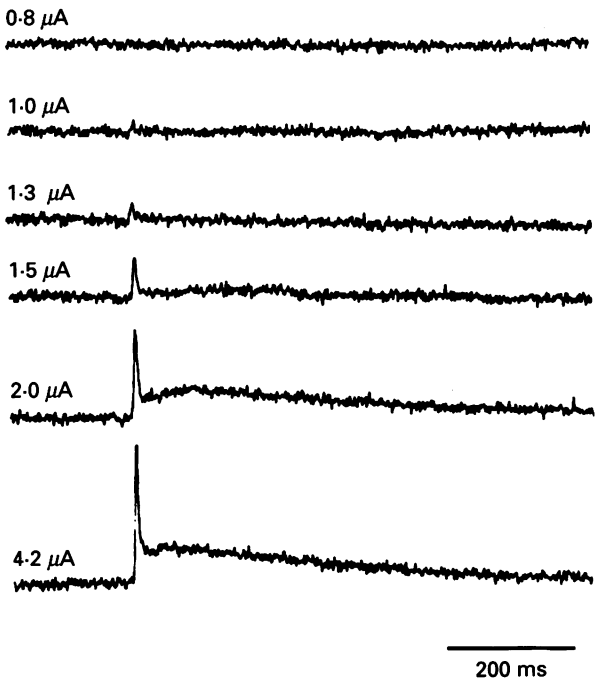

Fig. 2. Relationship between optical response and stimulus current strength in a slice preparation from an 8-day-old embryo. The signals were detected by one photodiode of the matrix array, with 0·8, 1·0, 1·3, 1·5, 2·0 and 4·2 μA currents (duration of 7·0 ms), and these recordings were made in a single sweep. Note that there is a difference in the threshold (the stimulus current strength which is just needed to produce an optical response) between the fast and slow signals.

TABLE 1. Combinations of the current strength and duration which are just needed to produce the optical responses

Preparation	Stimulating side	Duration (ms)	Strength (μA)	
			Fast signal	slow signal
J-686	Right	10·0	1·6	2·0
	Left	10·0	1·0	1·6
J-687	Right	7·0	1·0	1·5
	Left	7·0	0·8	1·1
J-681	Right	5·0	2·0	2·8
	Left	5·0	2·0	2·0
J-682	Right	5·0	1·5	2·0
	Left	5·0	1·5	1·8
J-680	Right	5·0	1·7	2·0
	Left	5·0	1·5	2·0
J-684	Right	3·0	2·7	3·6
	Left	3·0	2·4	2·5
J-685	Right	1·0	3·8	4·2
	Left	1·0	3·8	4·2
J-688	Right	0·5	12·0	12·0
	Left	0·5	8·0	8·0

These data were obtained from eight different slice preparations isolated from eight 8-day-old embryos.

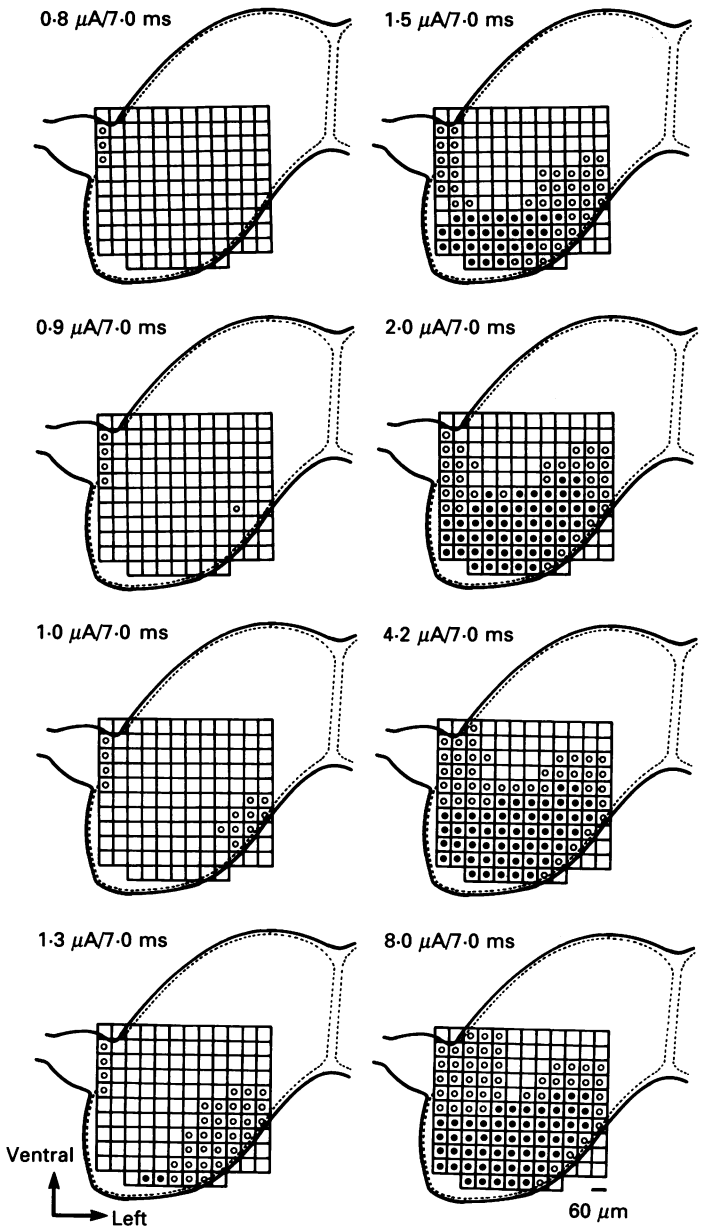

Fig. 3. Stimulus current strength-dependent changes in the area where the fast and slow signals were detected, in a slice preparation from an 8-day-old embryo. The right vagus nerve was stimulated by 0·8–8·0 μA square current pulses. ○ indicate the positions in which only the fast signal response appeared, and ● indicate the positions in which the slow signal response appeared with the fast signal. The duration of the stimulus current was 7·0 ms.

Properties of the slow signals

Because we suspected that the slow signal corresponded to a postsynaptic potential, we carried out several kinds of experiments designed to test this

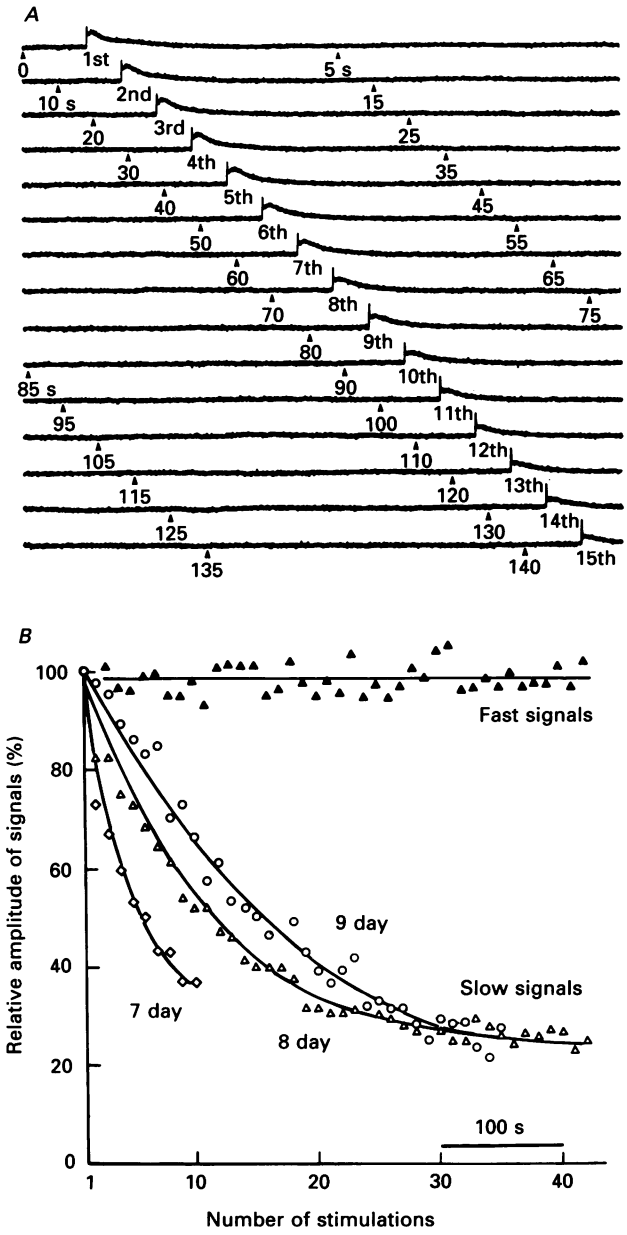

Fig. 4. *A*, illustration of decreasing slow signal size with continuous stimulation in an 8-day-old embryonic brain stem slice preparation. The square current pulses of 8·0 μA/5·0 ms and 0·1 Hz were applied to the right vagus nerve. A continuous recording is represented. In *B*, the relative amplitudes of the fast and slow signals are plotted against the number of stimuli. The data for the slow signal were obtained from late 7-day- (◇), 8-day- (△) and 9-day- (○) old preparations, and the data for the fast signal (▲) were obtained from an 8-day-old preparation. The abscissa indicates the number of stimuli together with time (in seconds).

hypothesis by comparing the properties of the slow signal with the physiological attributes of synaptic transmission.

Synaptic fatigue

Figure 4*A* shows the effects of continuous stimulation on the slow optical signals evoked in the right dorsal surface of an 8-day-old embryonic brain stem slice. When

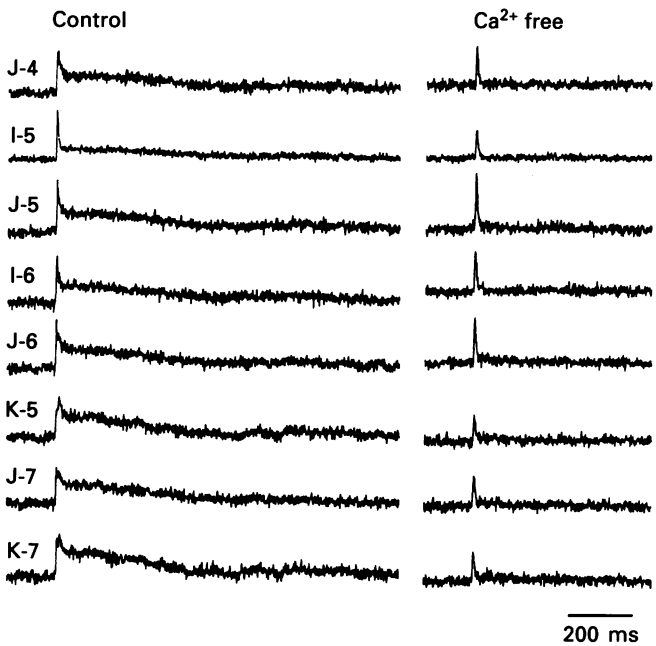

Fig. 5. Optical signals recorded in the control (left) and a Ca^{2+}-free bathing solution (right). In the Ca^{2+}-free Ringer solution, $CaCl_2$ was replaced by $MgCl_2$. The recording was made 15 min after the replacement of Ca^{2+} with Mg^{2+}. The signals from eight different positions (J-4, I-5, J-5, I-6, J-6, K-5, J-7 and K-7) are represented. An 8-day-old preparation was used. Note that the slow signals were eliminated in the Ca^{2+}-free bathing solution.

0·1 Hz continuous stimulation (8 μA/5·0 ms) was applied, the magnitude of the slow signal was gradually decreased, although the fast signal was not affected. In Fig. 4*B*, the normalized magnitudes of the fast and slow signals are plotted against the stimulus time. The fast signal size was nearly constant, but the slow signal size decreased exponentially. It is likely that this effect reflects synaptic fatigue and, if so, it argues that the slow signal is intimately related to a postsynaptic potential. Fatigue of this type was produced more rapidly in younger embryos than in older ones as shown in Fig. 4*B*.

Effects of external Ca^{2+}

Generally, lowering the extracellular calcium ion concentration reduces and ultimately blocks synaptic transmission (Dodge & Rahamimoff, 1967; Katz, 1969; also for a review see Augustine, Charlton & Smith, 1987). In the embryonic brain stem slice, the slow signal size decreased as the Ca^{2+} concentration in the Ringer solution was lowered. Figure 5 shows the results of a series of experiments designed

to examine the calcium dependence of the slow signal. The traces represent signals detected from eight different positions (J-4, I-5, J-5, I-6, J-6, K-5, J-7 and K-7) in the left dorsal surface of an 8-day-old embryo preparation. In each row, the optical signal on the left was recorded in control Ringer solution containing 1·8 mM-Ca^{2+},

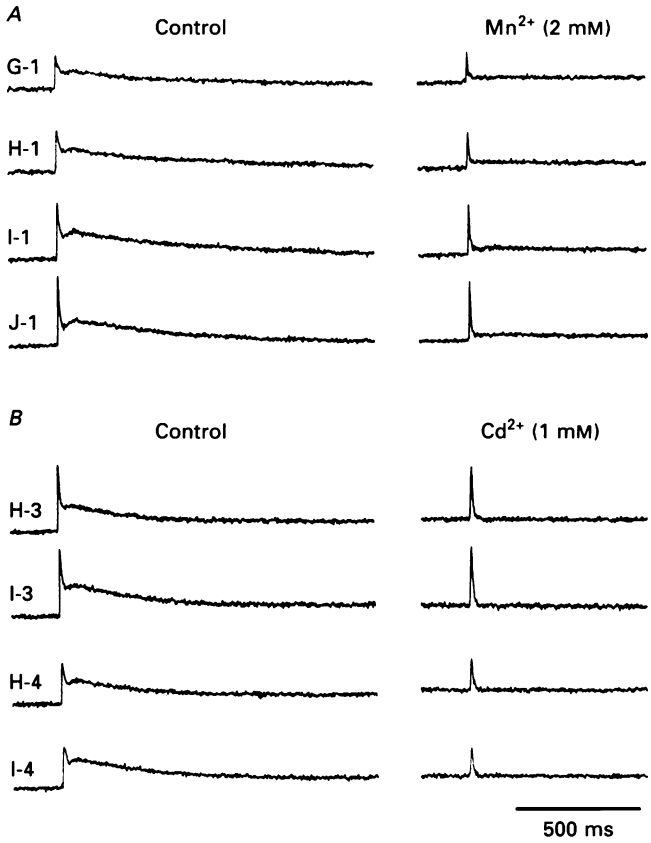

Fig. 6. Effects of manganese (in *A*) and cadmium (in *B*) ions on the evoked optical signals in an 8-day-old embryonic preparation. The records (shown in the right side) were obtained in the presence of manganese ions (2 mM) and in cadmium ions (1 mM). The signals were recorded 15 min after application of the ion tested. Signals recorded from four different positions (G-1, H-1, I-1 and J-1 in *A*; H-3, I-3, H-4 and I-4 in *B*) are illustrated. The data in *A* and *B* were obtained from two different preparations.

and the right-hand traces show the effect of Ca^{2+}-free (no added Ca^{2+}) Ringer solution. In the normal Ringer solution, both fast and slow signals were evoked by an 8 μA/5 ms stimulating current. When 1·8 mM-$CaCl_2$ was replaced by 1·8 mM-$MgCl_2$, the slow signals were entirely eliminated, while the fast signals were decreased only slightly. The effects of changes in external calcium concentration were always reversible.

Manganese and cadmium ions are also known to block synaptic transmission (Meiri & Rahamimoff, 1972; for review see Augustine *et al.* 1987). We examined the effects of Mn^{2+} and Cd^{2+} on the slow signals, and Fig. 6 shows the results after about

15 min in 2 mM-Mn^{2+} (right-hand traces in *A*) and after about 15 min in 1 mM-Cd^{2+} (right-hand traces in *B*). The signals were recorded simultaneously from four positions (G-1, H-1, I-1 and J-1 in *A*; H-3, I-3, H-4 and I-4 in *B*) in two 8-day-old embryonic brain stem slice preparations. In each case, the left-hand traces were obtained in normal Ringer solution. In a Ringer solution containing Mn^{2+} or Cd^{2+}, the slow signals were entirely eliminated. The fast signals were decreased only slightly. There was a partial recovery of the slow signals after the Mn^{2+} and Cd^{2+} were rinsed off.

The experimental results shown in Figs 5 and 6 are also consistent with the assumption that the slow signal corresponds to a postsynaptic potential. On the other hand, the reason for the small, but significant decrease in the sizes of the fast signals is unclear. One possible explanation that could account for this effect is that a small amount of the dye bound to the membrane was washed away by replacement of the external solution. In fact, when the stained preparation was washed with normal Ringer solution, a similar effect was found. Also, the possibility that the fast signal includes a small component reflecting a calcium contribution to an action potential (Llinás, Steinberg & Walton, 1981) has not been ruled out. In particular, if presynaptic terminal arborization contributes measurably to the total membrane area, a small calcium component to the fast signal is expected (Salzberg *et al.* 1983; Obaid, Orkand, Gainer & Salzberg, 1985). Dye bleaching was negligibly small during the course of this measurement.

Postsynaptic spike-like signal

Electrophysiologically, four separate events related to synaptic transmission are identified (Kandel, 1985): (1) the presynaptic action potential, (2) the Ca^{2+} current in the presynaptic neuron (nerve terminal), (3) the synaptic potential in the postsynaptic cell, and (4) the spike in the postsynaptic neuron. In the optical experiments reported here, a second spike-like signal was sometimes recorded in some preparations.

Figure 7 shows three traces of evoked optical signals composed of three components (first peak, second peak and slow change: indicated by 1, 2 and 3). These optical traces were obtained simultaneously from three different positions (I-9, G-10 and H-10) in an 8-day-old embryonic brain stem slice. The second peak depended on the strength of stimulating current and was entirely eliminated in Ca^{2+}-free Ringer solution and in the presence of Mn^{2+} or Cd^{2+}, while the first peak was not affected. We suppose that this second peak is the action potential in the postsynaptic cells. Similar signals can also be seen in the recording shown in Fig. 5 (positions K-5, J-7 and K-7).

Effect of blockers of chemical transmitters on the slow signals

From the viewpoint of the ontogenesis of synaptic function, it is of interest to know which neurotransmitters participate in the generation of the slow component of the optical signal. Accordingly, we have examined the effects of several blockers of synaptic transmission on the slow signals.

Figure 8 shows the effect of kynurenic acid, a blocker of glutamate-mediated excitatory synaptic transmission, on the evoked optical signals. The signals were

recorded simultaneously from 127 loci of an 8-day-old embryonic brain stem slice. The recordings on the left were obtained following stimulation of the right vagus nerve fibres, and those on the right followed stimulation of the left vagus nerve fibres. The position of the 12 × 12-element photodiode array with respect to the image of the

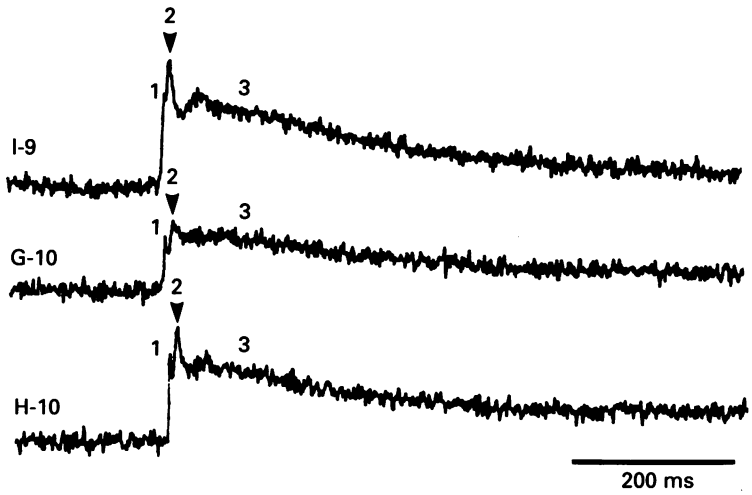

Fig. 7. Demonstration of the second spike-like optical response in an 8-day-old preparation. The recordings were obtained from three different positions (I-9, G-10 and H-10). In these signals, three components, first spike-like signal (indicated by 1, corresponding to the fast signal), second spike-like signal (indicated by 2) and slow signal (indicated by 3), are identifiable.

preparation is shown on the lower right. The two upper recordings show control experiments in normal Ringer solution and the two middle recordings were made in a Ringer solution containing 1·2 mM-kynurenic acid. Enlargements of the optical signal recorded from position J-7 (on the left side of the preparation), in the presence and absence of kynurenic acid, are shown on the lower left.

In the control recordings, the slow signals are clearly visible. These slow signals were abolished in the presence of kynurenic acid. There was some recovery of the signals after kynurenic acid was rinsed off. Not surprisingly, the fast component of the optical signals was insensitive to kynurenic acid. Kynurenic acid has been shown to inhibit excitatory synaptic transmission in the rat hippocampal slice and in isolated immature rat spinal cord (Ganong, Lanthorn & Cotman, 1983), and, recently, it has been shown that kynurenic acid is a blocker of glutamate-mediated excitatory synaptic transmission (e.g. Ganong & Cotman, 1986).

The glutamate receptor has been classified into four main subtypes, viz. NMDA (*N*-methyl-D-aspartate), quisqualate, kainate and 2-amino-4-phosphonobutyrate (APB) (Foster & Fagg, 1984). We also observed the effects of DL-2-amino-5-phosphono-valeric acid (2-APV: NMDA receptor antagonist) (Nelson, Pun & Westbrook, 1986) and of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX: non-NMDA receptor antagonist) (Yamada, Dubinsky & Rothman, 1989) on the postsynaptic slow signal. As shown in Fig. 9, 2-APV depressed the later phase of the slow signal, while CNQX reduced the initial phase of the slow signal. The slow signal was mostly abolished in the presence of both 2-APV and CNQX. These results suggest that the

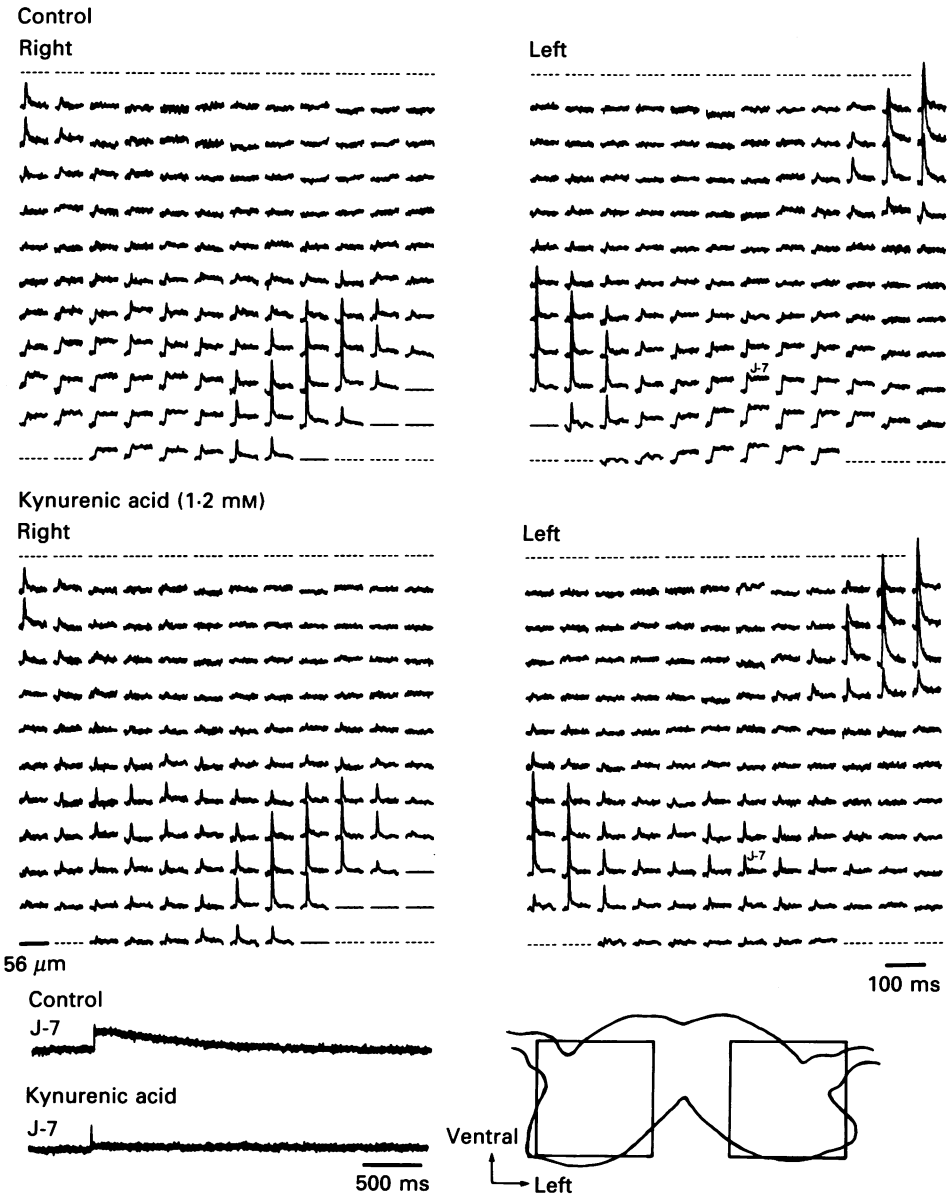

Fig. 8. Effects of kynurenic acid on the evoked optical signal. Signals were recorded simultaneously from 127 different positions in the right-side area (for the right vagus stimulation) and the left-side area (for the left vagus stimulation) of an 8-day-old preparation. The square current pulses of 6·0 μA/5 ms were applied to the right and left vagus nerves. The upper recordings are control, and the lower recordings were made in the presence of kynurenic acid. The recording was carried out 15 min after the addition of kynurenic acid (1·2 mM final concentration) to the bathing solution. Enlargements of the optical signals obtained from position J-7 (in the left side of the preparation) are shown at the bottom left and the relative position of the photodiode array on the image of the preparation is shown at the bottom right.

slow signals evoked by stimulation of the vagus nerve fibres represent a compound glutamate-mediated excitatory postsynaptic potential.

In order to examine the possibility that other chemical transmitters (such as adrenaline, acetylcholine, GABA and glycine) play significant roles in the synaptic

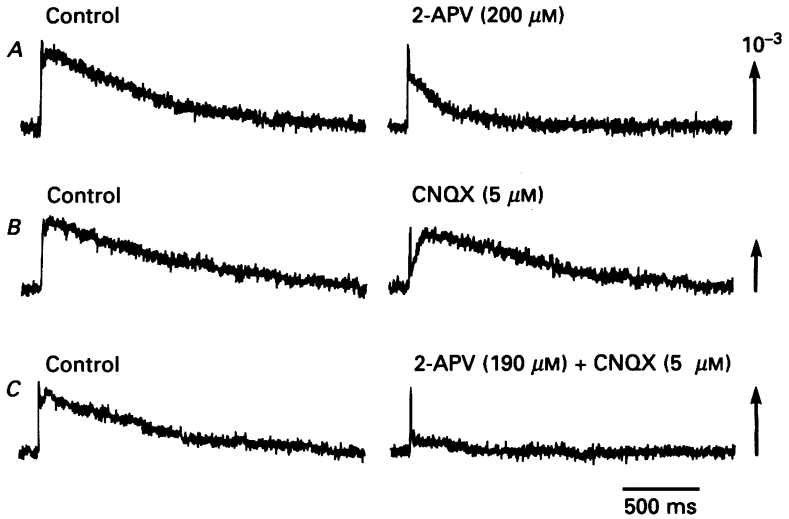

Fig. 9. The effects of 2-APV and CNQX on the postsynaptic slow signals in 8-day-old brain stem slices. In *A*, 2-APV (200 μM) was applied; in *B*, CNQX (5·0 μM) was applied; and in *C*, 2-APV (190 μM) and CNQX (5·0 μM) were applied together. The recordings were made about 15 min after application of the drugs. Recordings *A*, *B* and *C* were obtained from three different preparations.

physiology of the embryonic brain stem, we examined the effects of several other blockers (phentolamine (20 μM), propranolol (10 μM), *d*-tubocurarine (190 μM), atropine (10 μM), picrotoxin (100 μM) and strychnine (10 μM)). However, since we have not found significant effects of these blockers on the slow signals, it seems less likely that other transmitters are active in generating the slow signals.

DISCUSSION

The results presented here demonstrate postsynaptic potentials from the early embryonic vertebrate brain stem at times close to the origin of synaptic function, using an optical technique for multiple-site recording of electrical activity. In these experiments, optical signals consisting of, at least, two components were recorded. It seems important to first establish unequivocally the physiological event(s) that can account for each component of the recorded optical signal.

Concerning this problem, we must consider the following basic points:

(1) From histological evidence, it is likely that the vagal response area within the early embryonic brain stem includes many motoneurons and sensory nerve terminals and that glial cells might not be differentiated (Fujita, 1964). There is no detailed information concerning interneurons.

(2) The vagus nerve bundle contains both motor and sensory nerve fibres. Thus, in the present experiment, the applied stimulation was simultaneously orthodromic (for the sensory nerve fibres) and antidromic (for the motor nerve fibres). Unfortunately, at present at least, it is a formidable task to separate surgically the motor and sensory nerve fibres, because the early embryonic nerve fibres are very thin and fragile, and it is, therefore, technically impossible to stimulate separately the motor and sensory nerve fibres.

(3) In our optical recording system, each element of the photodiode array detects optical signals from many neurons and processes. Thus, the 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 these elements within the field viewed by one photodiode (Orbach, Cohen & Grinvald, 1985; Obaid *et al.* 1985; Kamino *et al.* 1989*a*).

In the brain stem preparation used in the present experiments, it is reasonable to assume that the fraction of the receptive field occupied by the nerve terminals of the sensory neurons is much smaller than that of the motoneurons. Therefore, although there is, as yet, no exact morphometry, we may suppose that the fast signal is mainly generated by the antidromic action potential in the motoneurons and that the component of the signal related to the orthodromic action potentials of the sensory nerve terminals is relatively small.

The results shown in Figs 4, 5 and 6 suggest that the slow signal reflects the postsynaptic potential. The duration of the slow signal was longer than 500 ms. This observation suggests that the postsynaptic potential in the early embryo is very slow, and this may be characteristic of embryonic synaptic transmission. Furthermore, the result shown in Fig. 4 shows that the embryonic synapse fatigues very readily. Landmesser & Pilar (1972) also reported rapid fatigue in the embryonic chick ciliary ganglion.

From these optical signals, we are not able directly to estimate the fractions of the motoneurons, sensory nerve terminals and postsynaptic neurons giving rise to the signals. A possible explanation for the optical signals is shown schematically in Fig. 10.

Because the optical receptive field of a single photodiode was $56 \times 56\ \mu m^2$ in the present experiments, we have not attempted to analyse the optical signals at the single-cell level, and the spatial resolution is not presently fine enough to allow a substantial fraction of the postsynaptic neurons to be identified. However, the results presented here demonstrate a substantial area of postsynaptic activity generated in the vagus nerve-related sensory nucleus during early phases of development. It should be pointed out that this sensory area is identified functionally in the accompanying report (Momose-Sato *et al.* 1991).

In the nucleus tractus solitarii of adult vertebrates, L-glutamate has been shown to be a chemical transmitter related to the baroreceptor reflex (Perrone, 1981; Talman, Granata & Reis, 1984). Thus, there is good reason to suggest that the postsynaptic response area detected in the present experiment corresponds to the vagus nerve-related sensory nucleus (nucleus tractus solitarii) and that in this nucleus, functional synaptic connections are first formed at the late 7- to early 8-day stage of development. As shown in the previous papers (Kamino *et al.* 1989*b*, 1990),

no synaptic potential-related optical signals were detected from early 7-day-old embryonic brain stem (also see the accompanying report: Momose-Sato *et al.* 1991). In a previous report (Kamino *et al.* 1989*b*), we have referred to the response area to vagus stimulation in the early embryonic brain stem as the vagus motor nucleus

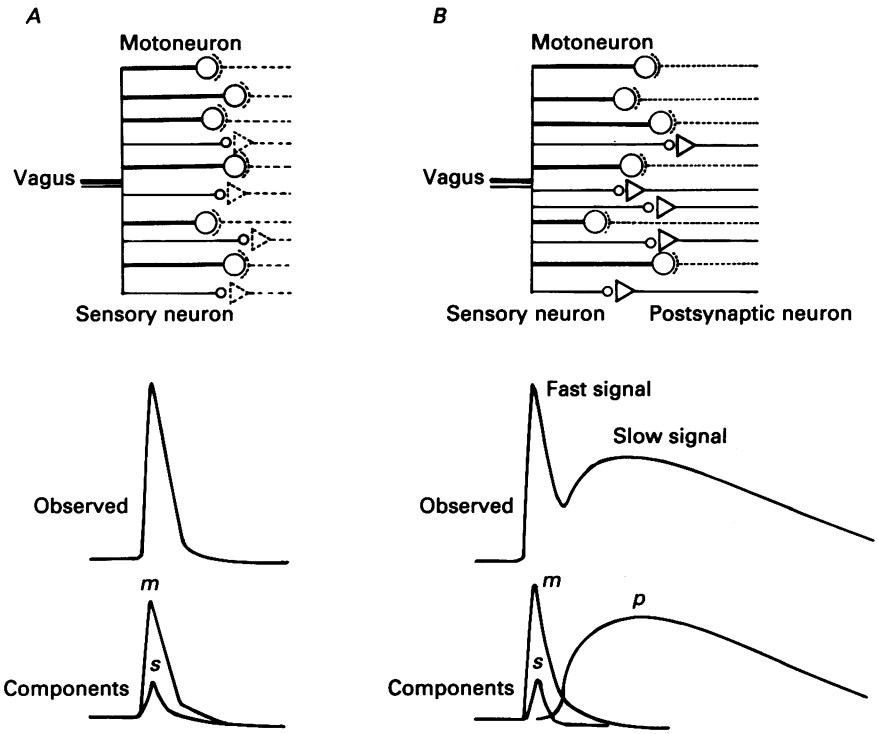

Fig. 10. Schematic representation of plausible origins of the different components of the optical signals evoked by vagus stimulation of the early embryonic chick brain stem. The upper panels show possible structures contributing to the optical signals recorded from the early embryonic brain stem related to the vagus nerve. *A* shows the case in which synaptic functions have not been generated; *B* shows the case in which sensory synaptic function is present. Large circles and thick lines indicate motoneurons (cell bodies and fibres); small circles and thin lines indicate sensory nerve terminals and fibres; triangles indicate postsynaptic cells; and semicircles indicate pre-motor nerve terminals. The middle and bottom panels display schematic drawings of the observed types of optical signals and their possible components for each case shown in *A* and *B*. In the bottom panels *m* is the component from motoneurons, *s* is the component from sensory neurons and *p* is the component from postsynaptic sensory neurons.

(nucleus dorsalis nervi vagi). In addition, the present data show that in the early embryonic brain stem, the areas of the sensory nerve nucleus and the dorsal motor nucleus of the vagus are partly superimposed. Here, the possibility of trans-synaptic pathways from the sensory nucleus to the motor nucleus is suggested. However, limitations in the present experimental technique prevent a temporal analysis of the onset of the slow component, because the first (fast) and second (slow) components of the optical signal cannot be separately recorded (see Fig. 10). A definitive conclusion requires further studies.

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REFERENCES

AUGUSTINE, G. J., CHARLTON, M. P. & SMITH, S. J. (1987). Calcium action in synaptic transmitter release. *Annual Review of Neuroscience* **10**, 633–693.

COHEN, L. B. & LESHER, S. (1986). Optical monitoring of membrane potential: methods of multisite optical measurement. In *Optical Methods in Cell Physiology*, ed. DE WEER, P. & SALZBERG, B. M., pp. 71–99. Wiley-Interscience, New York.

COHEN, L. B. & SALZBERG, B. M. (1978). Optical measurement of membrane potential. *Review of Physiology, Biochemistry and Pharmacology* **83**, 35–88.

DODGE, F.A. JR & RAHAMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *Journal of Physiology* **193**, 419–432.

FOSTER, A. C. & FAGG, G. E. (1984). Acidic amino acid binding sites in mammalian neuronal membranes: Their characteristics and relationship to synaptic receptors. *Brain Research Reviews* **7**, 103–164.

FUJITA, S. (1964). Analysis of neuron differentiation in the central nervous system by tritiated thymidine autoradiography. *Journal of Comparative Neurology* **122**, 311–327.

GANONG, A. H. & COTMAN, C. W. (1986). Kynurenic acid and quinolinic acid act at *N*-methyl-D-aspartate receptors in the rat hippocampus. *Journal of Pharmacology and Experimental Therapeutics* **236**, 293–299.

GANONG, A. H., LANTHORN, T. H. & COTMAN, C. W. (1983). Kynurenic acid inhibits synaptic and acidic amino acid-induced responses in the rat hippocampus and spinal cord. *Brain Research* **273**, 170–174.

GRINVALD, A., COHEN, L. B., LESHER, S. & BOYLE, M. B. (1981). Simultaneous optical monitoring of activity of many neurons in invertebrate ganglia using a 124-element photodiode array. *Journal of Neurophysiology* **45**, 829–840.

GRINVALD, A., FROSTIG, R. D., LIEKE, E. & HILDESHEIM, R. (1988). Optical imaging of neuronal activity. *Physiological Reviews* **68**, 1285–1366.

HIROTA, A., KAMINO, K., KOMURO, H. & SAKAI, T. (1987). Mapping of early development of electrical activity in the embryonic chick heart using multiple-site optical recording. *Journal of Physiology* **383**, 711–728.

HIROTA, A., KAMINO, K., KOMURO, H., SAKAI, T. & YADA, T. (1985). Early events in development of electrical activity and contraction in embryonic rat heart assessed by optical recording. *Journal of Physiology* **369**, 209–227.

KAMINO, K. (1991). Optical approaches to the ontogeny of electrical activity and related functional organization during early heart development. *Physiological Reviews* **71**, 53–91.

KAMINO, K., HIROTA, A. & FUJII, S. (1981). Localization of pacemaking activity in early embryonic heart monitored using a voltage-sensitive dye. *Nature* **290**, 595–597.

KAMINO, K., HIROTA, A. & KOMURO, H. (1989*a*). Optical indications of electrical activity and excitation–contraction coupling in the early embryonic heart. *Advances in Biophysics* **25**, 45–93.

KAMINO, K., KATOH, Y., KOMURO, H. & SATO, K. (1989*b*). Multiple-site optical monitoring of neural activity evoked by vagus nerve stimulation in the embryonic chick brain stem. *Journal of Physiology* **409**, 263–283.

KAMINO, K., KOMURO, H., SAKAI, T. & HIROTA, A. (1989*c*). Optical multiple-site real-time recording of neural activity in the CNS using voltage-sensitive dyes. (Abst.) *Neuroscience Research*, suppl. 9, S 21.

KAMINO, K., KOMURO, H., SAKAI, T. & SATO, K. (1990). Optical assessment of spatially ordered patterns of neural response to vagal stimulation in the early embryonic chick brain stem. *Neuroscience Research* **8**, 255–271.

KANDEL, E. R. (1985). Factors controlling transmitter release. In *Principles of Neural Science*, ed. KANDEL, E. R. & SCHWARTZ, J. H., pp. 120–131. Elsevier, New York.

KATZ, B. (1969). *The Release of Neural Transmitter Substances*. Liverpool University Press, Liverpool.

KOMURO, H., MOMOSE, Y., SAKAI, T., HIROTA, A. & KAMINO, K. (1990). Optical recording of synaptic potential in the embryonic chick brain stem slice preparation using a voltage-sensitive dye. (Abst.) *Neuroscience Research*, suppl. 11, S 33.

KOMURO, H., SAKAI, T., HIROTA, A. & KAMINO, K. (1986). Conduction pattern of excitation in the amphibian atrium assessed by multiple-site optical recording of action potentials. *Japanese Journal of Physiology* **36**, 123–137.

LANDMESSER, L. & PILAR, G. (1972). The onset and development of transmission in the chick ciliary ganglion. *Journal of Physiology* **222**, 691–713.

LLINÁS, R., STEINBERG, I. Z. & WALTON, K. (1981). Presynaptic calcium currents in squid giant synapse. *Biophysical Journal* **33**, 289–322.

MEIRI, U. & RAHAMIMOFF, R. (1972). Neuromuscular transmission: Inhibition by manganese ions. *Science* **176**, 308–309.

MOMOSE-SATO, Y., SAKAI, T., KOMURO, H., HIROTA, A. & KAMINO, K. (1991). Optical mapping of early development of the neural response pattern to vagal stimulation in embryonic chick brain stem. *Journal of Physiology* **442**, 649–668.

NELSON, P. G., PUN, R. Y. K. & WESTBROOK, G. L. (1986). Synaptic excitation in cultures of mouse spinal cord neurones: receptor pharmacology and behaviour of synaptic currents. *Journal of Physiology* **372**, 169–190.

OBAID, A. L., ORKAND, R. K., GAINER, H. & SALZBERG, B. M. (1985). Active calcium responses recorded optically from nerve terminals of the frog neurohypophysis. *Journal of General Physiology* **85**, 481–489.

ORBACH, H. S., COHEN, L. B. & GRINVALD, A. (1985). Optical mapping of electrical activity in rat somatosensory and visual cortex. *Journal of Neuroscience* **5**, 1886–1895.

PERRONE, M. H. (1981). Biochemical evidence that L-glutamate is a neurotransmitter of primary vagal afferent nerve fibers. *Brain Research* **230**, 283–293.

SAKAI, T., HIROTA, A., KOMURO, H., FUJII, S. & KAMINO, K. (1985). Optical recording of membrane potential responses from early embryonic chick ganglia using voltage-sensitive dyes. *Developmental Brain Research* **17**, 39–51.

SAKAI, T., KOMURO, H. & KAMINO, K. (1990). Optical monitoring of evoked electrical activity in the embryonic chick superior cervical ganglion using a voltage-sensitive dye. (Abst.) *Neuroscience Research*, suppl. 11, S 39.

SAKAI, T., KOMURO, H., SATO, K., KATOH, Y., SASAKI, H. & KAMINO, K. (1989). Developmental changes in conduction velocity of excitation in the embryonic chick cervical vagus nerve bundle assessed by multiple-site optical recording of action potential. (Abst.) *Neuroscience Research*, suppl. 9, S 55.

SALAMA, G., LOMBARDI, R. & ELSON, J. (1987). Maps of optical action potentials and NADH fluorescence in intact working hearts. *American Journal of Physiology* **252**, H384–394.

SALZBERG, B. M. (1983). Optical recording of electrical activity in neurons using molecular probes. In *Current Methods in Cellular Neurobiology*, vol. 3. *Electrophysiological Techniques*, ed. BARBER, J. L., pp. 139–187. John Wiley & Sons Inc., New York.

SALZBERG, B. M., DAVILA, H. V. & COHEN, L. B. (1973). Optical recording of impulses in individual neurones of an invertebrate central nervous system. *Nature* **246**, 508–509.

SALZBERG, B. M., GRINVALD, A., COHEN, L. B., DAVILA, H. V. & ROSS, W. N. (1977). Optical recording of neuronal activity in an invertebrate central nervous system: Simultaneous monitoring of several neurons. *Journal of Neurophysiology* **40**, 1281–1291.

SALZBERG, B. M., OBAID, A. L., SENSEMAN, D. M. & GAINER, H. (1983). Optical recording of action potentials from vertebrate nerve terminals using potentiometric probes provides evidence for sodium and calcium components. *Nature* **306**, 36–40.

TALMAN, W. T., GRANATA, A. R. & REIS, D. J. (1984). Glutamatergic mechanisms in the nucleus tractus solitarius in blood pressure control. *Federation Proceedings* **43**, 39–44.

YAMADA, K. A., DUBINSKY, J. M. & ROTHMAN, S. M. (1989). Quantitative physiological characterization of a quinoxalinedione non-NMDA receptor antagonist. *Journal of Neuroscience* **9**, 3230–3236.