Optical Mapping of Neural Network Activity in Chick Spinal Cord at an Intermediate Stage of Embryonic Development

YOSHIYASU ARAI, YOKO MOMOSE-SATO, KATSUSHIGE SATO, AND KOHTARO KAMINO
Department of Physiology, Tokyo Medical and Dental University School of Medicine, Tokyo 113-8519, Japan

Arai, Yoshiyasu, Yoko Momose-Sato, Katsushige Sato, and Kohtaro Kamino. Optical mapping of neural network activity in chick spinal cord at an intermediate stage of embryonic development. J. Neurophysiol. 81: 1889–1902, 1999. We have applied multiple-site optical recording of transmembrane potential changes to recording of neuronal pathway/network activity from embryonic chick spinal cord slice preparations. Spinal cord preparations were dissected from 8-day-old chick embryos at Hamburger-Hamilton stage 33, and transverse slice preparations were prepared with the 13th cervical spinal nerve or with the 2nd or 5th lumbosacral spinal nerve intact. The slice preparations were stained with a voltage-sensitive merocyanine-rhodamine dye (NK2761). Transmembrane voltage-related optical (dye-absorbance) changes evoked by spinal nerve stimulation with positive square-current pulses using a suction electrode were recorded simultaneously from many loci in the preparation, using a 128- or 1,020-element photodiode array. Optical responses were detected from dorsal and ventral regions corresponding to the posterior (dorsal) and anterior (ventral) gray horns. The optical signals were composed of two components, fast spike-like and slow signals. In the dorsal region, the fast spike-like signal was identified as the presynaptic action potential in the sensory nerve and the slow signal as the postsynaptic potential. In the ventral region, the fast spike-like signal reflects the antidromic action potential in motoneurons, and the slow signal is related to the postsynaptic potential evoked in the motoneuron. In preparations in which the ventral root was cut microsurgically, the antidromic action potential-related optical signals were eliminated. The areas of the maximal amplitude of the evoked signals in the dorsal and ventral regions were located near the dorsal root entry zone and the ventral root outlet zone, respectively. Quasiconcentric contourline maps were obtained in the dorsal and ventral regions, suggesting the functional arrangement of the dorsal and ventral synaptic connections. Synaptic fatigue induced by repetitive stimuli in the ventral synapses was more rapid than in the dorsal synapses. The distribution patterns of the signals were essentially similar among C13, LS2, and LS5 preparations, suggesting that there is no difference in the spatiotemporal pattern of the neural responses along the rostrocaudal axis of the spinal cord at this developmental stage. In the ventral root-cut preparations, comparing the delay times between the ventral slow optical signals, we have been able to demonstrate that neural network-related synaptic connections are generated functionally in the embryonic spinal cord at Hamburger-Hamilton stage 33.

INTRODUCTION

A basic question in neurophysiology concerns the emergence of patterns of electrical activity in neuronal circuits/networks at the population level in addition to the single cell level. However, for answering this question, conventional electrophysiological recording methods, including microelectrodes and patch electrodes, have practical and theoretical limitations: conventional microelectrode recording from younger embryonic neurons is often difficult because of their small size and fragility; further, the use of microelectrodes usually cannot provide simultaneous recordings from multiple sites in in vivo or in vitro CNSs.

Optical techniques using fast voltage-sensitive dyes have made it possible to monitor electrical activities in small and/or fragile cells that are difficult or impossible to access by traditional electrophysiological means and further facilitate the simultaneous recording of electrical activity from multiple sites in living systems such as central nervous and cardiac systems (e.g., for reviews, see Cohen and Salzberg 1978; Grinvald et al. 1988; Kamino 1990; Salzberg 1983, 1989). A particularly important application of this technique using photodiodes involves the analysis of the components of pre- and postsynaptic potentials based on the analysis of the waveform of the signals (Grinvald et al. 1982; Kamino 1991; Kamino et al. 1989a; Momose-Sato et al. 1994).

Our previous studies have established the feasibility of optical methods for recording electrical activity in brain stem preparations isolated from early developing embryos: using such optical techniques, we have investigated the early development of action potential and synaptic potential activities evoked by vagal or glossopharyngeal stimulation in embryonic chick and rat brain stems (Kamino et al. 1989b, 1990; Momose-Sato et al. 1991, 1994; Sato et al. 1995, 1998).

The optical recording technique often is used efficiently for monitoring the neuronal network activity in invertebrate ganglia (Nakashima et al. 1992; Salzberg et al. 1977; Tsau et al. 1994), cultured neural circuits (Parsons et al. 1989), and the submucous plexus of the guinea pig ileum (Obaid et al. 1996). However, in the CNSs of vertebrates, optical recording techniques have been used mainly for the qualitative mapping of the spatial spread of neural activity and have seemed inadequate for analyzing neural circuit activity. Thus we have tried to examine the feasibility of using optical recording techniques to monitor neural circuit activity. For this experiment, we manipulated embryonic chick spinal cord slice preparations, which are treated as quasi-two-dimensional structures, in which electrical activity in the relatively primitive neural circuits/networks underlying the spinal reflex can be detected at the neural population level.

In particular, our interest in the present work concerns the optical representation of the spatiotemporal patterning of electrical activity that may occur in the neural circuit/network as a result of synaptic coupling between cells in the embryonic...
spinal cord. A great deal of electrophysiological investigations have been devoted to the functional development of synaptic transmission in the embryonic spinal cord. However, these analyses using electrophysiological methods have been limited to recordings from single neurons or recordings of compound action potentials from ventral roots. Although examination of the spatial distribution pattern of synaptic responses would provide important information about the organization of synaptic networks, such approaches rarely have been found in the spinal cord. Thus we have introduced a multiple-site optical recording technique to the present experiment to examine the spatial distribution of synaptic responses after spinal nerve stimulation.

Lee and coworkers (1988) have reported that the morphological and functional bases for a monosynaptic connection between afferents and motoneurons are present by Hamburger-Hamilton stage 32 in the embryonic chick spinal cord and that polysynaptic connections between femorotibialis afferents and motoneurons in the chick form earlier in development than do the monosynaptic connections. However, intracellular recordings from motoneurons have not been attempted earlier than at the Hamburger-Hamilton stages 32–33 (O’Donovan 1987). From this evidence, we have considered that around Hamburger-Hamilton stage 32–33 a critical stage for the morphological formation of the neural circuit/network underlying the spinal reflex and that this stage also provides a landmark in the search for the early phases of functional organization of the neural circuit. Consequently, in the present work, as a first step, we focused our efforts on the spinal cord isolated from 8-day-old chick embryos, corresponding to Hamburger-Hamilton stage 33. Ho and O’Donovan (1993) also have reported that the spinal rhythmogenic capacity was not uniformly distributed along the rostrocaudal axis of the spinal cord. Therefore, it is interesting to examine whether there is a cephalo-caudal gradient of development of the spinal cord function. Thus we have selected the 13th cervical and 2nd and 5th lumbar spinal nerve segments, which are the intumescence parts for the cervical, lumbar and sacral spinal cords, respectively. We have been able to optically monitor the synaptic activity suggesting the formation of the neural circuit/network in the preparations. Preliminary reports of this work and related experiments have already appeared (Arai et al. 1997a,b).

**Methods**

**Preparations**

In the present experiments, we used embryonic chick spinal cord slice preparations. Fertilized eggs of white Leghorn chickens were incubated for 8 days (Hamburger-Hamilton stage 33) (Hamburger and Hamilton 1951) in a forced-draft incubator (type P-03, Showa Incubator Laboratory, Urawa, Japan) at a temperature of 37°C and 60% humidity and were turned once each hour. The spinal cords, with spinal nerve fibers attached, were dissected from the embryos. The isolated spinal cord preparation was attached to the silicone (KE 106LTV; Shin-etsu Chemical, Tokyo) bottom of a simple chamber by pinning it with tungsten wires. The preparation was kept in an ice-cold bathing solution (Ringer solution) with the following composition (in mM): 138 NaCl, 5.4 KCl, 1.8 CaCl2, 0.5 MgCl2, 10 glucose, and 10 Tris-HCl buffer (pH 7.2). The solution was equilibrated with oxygen. The pia mater attached to the spinal cord was removed carefully in the bathing solution under a dissecting microscope. Slices were prepared, with the right and/or left spinal nerve fibers attached, by sectioning the embryonic spinal cord transversely at the level of the roots of the 13th cervical spinal nerve and the 2nd and 5th lumbar spinal nerves. The thickness of the slice was ~1 mm.

**Voltage-sensitive dye staining**

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

**Electrical stimulation**

For preparations in which the spinal nerve was stimulated, the cut end of the nerve was drawn into a microsuction electrode fabricated from hematocrit tubing (VC-H075P, Terumo, Tokyo), which had been hand-pulled to a fine tip (~100 µm ID) over a low-temperature flame. Positive (depolarizing) square-current pulses (8.0 µA/5.0 ms), which evoked maximum responses, were applied to the right or left spinal nerve.

**Optical recording**

Light from a 300 W tungsten-halogen lamp (Type JC-24V/300W, Kondo Philips, Tokyo) was collimated, rendered quasimonochromatic with a heat filter (32.5B-76, Olympus Optical, Tokyo) and an interference filter having a transmission maximum at 703 ± 15 (half width) nm (Asahi Spectra, 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, ×10, 0.4 NA). The objective and photographic eyepiece (×2.5 or ×3.3) projected a real image of the preparation (magnification ×25 or ×33) onto a multielement silicon photodiode matrix array mounted on an Olympus Vanox microscope (Type AHB-L-1, Olympus Optical). In the present experiments, we used two optical recording systems. One is a recently constructed (Hirota et al. 1995) 1,020-site optical recording system with a 34 × 34-element silicon photodiode array (Hamamatsu Photonics, Hamamatsu, Japan). In this system, each pixel (element) of the array detects light transmitted by a square region (41 × 41 µm2 using ×33 magnification) of the preparation. The outputs from the 1,020 elements were fed into amplifiers via current-to-voltage converters and then passed to 32 sets of 32-channel analog multiplexers. Each output from the 32-channel multiplexers was fed into a subranging type AD converter system with a resolution of 18 bit and was sent to a computer (LSI-11/73 system, Digital Equipment, Tewksbury, MA). Another recording system is a 128-channel multiple-site optical recording system using a 12 × 12-element silicon photodiode array (MD-144–4PV; Centronic, Croydon, UK). This system has been described often in detail elsewhere (Komuro et al. 1991; Momose-Sato et al. 1991, 1994; also for reviews, see Kamino 1990, 1991). In this system, each pixel of the array detected light from a square region (56 × 56 µm2 using ×25 magnification) of the preparation. The output of each detector in the diode array was passed to an amplifier (AC coupling = 3 s) via a current-to-voltage converter. The amplified outputs from 127 elements of the detector first were 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). The 128-channel data recording system is com-
posed of a main processor (RP-891), eight I/O processors (RP-893), a 64K word wave-memory (RP-892) and a videotape recorder. The program for the computer was written in the assembly language (Macro-11) called from FORTRAN, under the RT-11 operating system (version 5.0). The time resolution of these systems was 1 ms. The recordings were made in a single sweep, and no off-line filtering was used (also see Fig. 1 legend for additional details). The optical measurement was carried out in a still chamber without continuous perfusion with Ringer solution at room temperature, 26–30°C. The incident light was turned off except during the measuring period. Under these conditions, the evoked optical signals often can be detected for 60–150 min.

**DiI labeling**

The 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeling method that we have used was essentially similar to that described by Godement et al. (1987). Embryos used for DiI-labeling studies were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. A small crystal of the fluorescent neuronal tracer DiI (Molecular Probes, Eugene, OR) was placed in the spinal nerve. Spinal cord slice preparations with DiI placements were stored in 4% paraformaldehyde for 2–4 wk at room temperature. The spinal cord dissected free from surrounding tissue was embedded in 3% gelatin and was sectioned in the transverse plane at a thickness of 50 μm on a vibratome (microslicer DTK-2000, Dosaka EM, Kyoto, Japan). Wet-mounted sections were examined with an epifluorescence microscope (FLUOPHOT, Nikon, Tokyo) equipped with a Rhodamine filter set (excitation 520–550 nm, emission >570 nm).

**RESULTS**

**Response areas**

We first searched for the areas in which optical responses occurred in isolated spinal cord slice preparations. For this...
search, we used a 1,020-element photodiode array recording system (Hirota et al. 1995). An example of multiple-site optical recordings of the neuronal response is illustrated in Fig. 1. The optical signals were elicited by a brief square-current pulse (8 μA/5 ms), which gave the maximal response, applied to the mixed spinal nerve fibers with a microsuction electrode in a spinal cord slice preparation stained with a merocyanine-rhodamine dye (NK2761). The slice was made by sectioning an embryonic spinal cord at the level of the fifth lumbar spinal nerve (LS5) at Hamburger-Hamilton stage 33 (8-day-old embryonic age). Such a preparation is termed “E8/LS5-preparation” in this report. The evoked optical signals were recorded simultaneously from multiple contiguous loci of the preparation. The relative position of the 1,020-element photodiode array on the image of the preparation is illustrated in Fig. 1, bottom. The photodiode array covered the entire image of the preparation. The array was positioned over a ×3 magnified image of the preparation, so that each photodiode detected the signals from a square region 41 μm on a side. Knowing the action spectrum of the merocyanine-rhodamine dye (e.g., Momose-Sato et al. 1995), we made the recordings using incident light at 700 nm; no averaging was required.

In Fig. 1, the areas in which the optical signals were evoked were identified easily: the optical signals appeared to spread from the dorsal to ventral areas on the side of the preparation that was stimulated with no optical signal detected contralaterally. Essentially, similar response patterns were obtained from E8/C13 and E8/LS2 preparations (n = 25) dissected at the level of the 13th cervical spinal nerve (C13) and the 2nd and 5th lumbar sacral spinal nerves (LS2 and LS5). Optical signals were recorded using a 12 × 12-element photodiode array in a single sweep. Other experimental conditions were the same as in Fig. 1.

Characterizations of optical signals

Figure 2 shows optical signals that were recorded from E8/C13, E8/LS2, and E8/LS5 preparations. Recordings were made in normal Ringer solutions (left) and in a Ca²⁺-free Ringer solution (right). For each preparation, the stimulating current was applied to the 13th cervical spinal nerve (C13) and the 2nd and 5th lumbar sacral spinal nerves (LS2 and LS5). Optical signals were recorded using a 12 × 12-element photodiode array in a single sweep. Other experimental conditions were the same as in Fig. 1.
projected an image of the stimulated side of the preparation. The relative positions of the array on the images of the preparation are illustrated on the left.

In the recordings, apparently, some differences in the waveform of the optical signals were observed between the dorsal and ventral regions. To see the waveform in more detail, the enlarged traces of several signals extracted from the recordings in Fig. 2 are shown in Fig. 3. In both the dorsal and ventral regions, the optical signals evoked in normal Ringer solution consisted of two components, the first, fast and spike-like, and the second, slow. From similar observations in embryonic brain stem preparations (e.g., Komuro et al. 1991; Momose-Sato et al. 1991, 1994), we first assumed that the slow signal represents a postsynaptic potential. Because lowering the extracellular calcium ion concentration reduces and ultimately blocks synaptic transmission (Dodge and Rahamimoff 1967; Katz 1969; also for a review, see Augustine et al. 1987), we examined the effects of external Ca\(^{2+}\) concentrations on the optical signals.

The slow signal amplitude decreased as the Ca\(^{2+}\) concentration in the Ringer solution was lowered. In the Ca\(^{2+}\)-free Ringer solution, the slow signals were reduced dramatically in both the dorsal and ventral regions as shown in Figs. 2 and 3. The effects of changes in the external Ca\(^{2+}\) concentration were always reversible. The slow signals also were blocked in the presence of Mn\(^{2+}\) (1 mM) or Cd\(^{2+}\) (1 mM) in the solution (data not shown). On the other hand, in the Ca\(^{2+}\)-free solution, the fast signals were seen clearly in both the dorsal and ventral regions. The fast signals were blocked completely by applying tetrodotoxin (TTX: 1.0–2.0 \(\mu\text{M}\); data not shown), but they were not blocked either in the Ca\(^{2+}\)-free solution or by Cd\(^{2+}\) or Mn\(^{2+}\). These results are consistent with the assumption that the slow signal corresponds to a postsynaptic potential.

In the recordings shown in Figs. 2 and 3 (also in Fig. 1), differences in the waveform of the slow optical signals were observed between the dorsal and ventral regions. The rate of rise of the upstroke of the slow signals detected from the dorsal region was rapid so that the dorsal slow signal seemed to be closely linked with the fast signal. On the contrary, the rate of rise in the slow signals in the ventral region was slower than that of the slow signal in the dorsal region.

From anatomic and physiological considerations, viz. that sensory nerve fibers enter the cord dorsally and motor nerve fibers leave the cord ventrally and considering that in the present experiment the stimulation was applied simultaneously to the sensory (dorsal root) and motor nerve (ventral root)
fibers, the dorsal fast signal was referred to as the presynaptic afferent action potential, whereas the ventral fast signal was referred to as the antidromic action potential. This assignment will be taken up in DISCUSSION.

**Ventral root-cut preparations**

In the present experiments using the Hamburger-Hamilton stage 33 embryonic spinal cord slice preparations, it is very difficult for us to separate surgically the dorsal and ventral root nerve fibers without damage, although Lee et al. (1988) succeeded to separate them. It is thus technically impossible to stimulate separately and individually the sensory and motor nerve fibers with a microsuction electrode. Accordingly, to circumvent this experimental difficulty and to eliminate antidromic action potentials evoked in the motoneurons, we used preparations in which the ventral root was cut microsurgically. (Such a preparation was termed a “cut preparation.”)

Figure 4 shows the multiple-site optical recordings of neural responses made in E8/C13- and E8/LS5-cut preparations. In every preparation, the part corresponding to the ventral root was cut microsurgically as indicated in the inset. For the LS2 preparation, it has been difficult to perform the microsurgery.

In the dorsal region, the waveform of the evoked optical signals obtained in normal Ringer solution was very similar to that of the signals recorded from the noncut preparation (Fig. 2). Whereas in the ventral region, as expected, the fast signals were eliminated, and only the slow optical signals were detected. This result demonstrates that in the noncut preparations (Figs. 1–3), the ventral fast signals correspond to the antidromic action potential in motoneurons via the ventral root. In Fig. 4, no signal reflecting the presynaptic potential was identified in the ventral region (see DISCUSSION). In the Ca\(^{2+}\)-free solution, in both the dorsal and ventral regions, the slow optical signals were completely blocked (data not shown).

**Contour-line mapping of optical signals**

As may be seen in the original recordings shown in Fig. 2, there are regional differences in the amplitude of the evoked optical signals. To quantitate the regional differences, we measured the amplitudes of the fast spike-like and slow signals, and we constructed contour-line maps of the amplitudes of the signals. Figure 5A illustrates the maps that were constructed with the data obtained from the C13-, LS2-, and LS5-noncut preparations shown in Fig. 2. For each preparation, the maps of the fast and slow signals recorded in normal Ringer solution (top and middle) and the maps of the fast signals in a Ca\(^{2+}\)-free Ringer solution (bottom) are shown. From these maps, we can extract the following features; the signal areas appear to be concentrated near the dorsal root entry in the dorsal gray horn (for the signals in the dorsal region) and near the ventral root outlet in the ventral gray horn (for the signals in the ventral region); in normal Ringer solution, the areas of the fast and slow signals mostly are superimposed in the dorsal and ventral regions; and in each map, the areas of the fast signals recorded in normal Ringer solution are the same as the areas of the signals recorded in the Ca\(^{2+}\)-free Ringer solution. In addition, Fig. 5B shows the maps that were constructed with the data obtained from the C13- and LS5-cut preparations shown in Fig. 4. In these maps, the ventral fast signals were eliminated.

Figure 6 gives the preparation-to-preparation variations in the relative locations of the areas of the larger amplitude signals.
Comparison with morphology

Figure 8 shows the dorsal and ventral pathways of the spinal nerve fibers. This photographic view was obtained from an E8/LS5 preparation in which the spinal nerve was labeled with a carbocyanine dye, DiI. In this photograph, distributions of the motoneurons and the afferent nerve fibers are seen clearly. In Fig. 8, it appears that the motoneuron dendrites expand into the intermediate zone and the white matter. In comparison with the optical responses with this morphological profile, it seems
likely that the small spike-like signals detected from the intermediate region of the noncut preparation correspond to firing activity evoked in the motoneuron dendrites. In addition, this morphological view shows that the afferent nerve fibers/terminals are labeled in the dorsal white matter. This profile is also consistent with the result that the presynaptic action potential-related optical signals were detected from the region corresponding to the dorsal white matter.

**Delay of the slow signals**

We compared the delay times of the slow signals in the ventral regions. In Fig. 9, A1 and B1, the traces of the optical signals taken from the simultaneous multiple-site recording in C13 and LS5 preparations, in which the ventral roots were surgically severed, are arranged vertically in a row. In these series of the traces, the delays of the onset of the slow signals were assessed using the onset of the dorsal signals as the zero-point reference for the timing. According to the delay times, the ventral slow signals were classified into two groups: those without a delay and the group of the signals with a delay. Within the accuracy of our present measurements at least, it has been difficult to classify the signals into three or more groups, and the delay times were estimated to be \( \sim 43 \) ms for the C13 preparation in Fig. 9A1 and 34 ms for the LS5 preparation in Fig. 9B1. Statistically, we obtained 36.3 \( \pm 2.7 \) ms \((n = 3)\) for the LS5 preparations.

In addition, Fig. 9, A2 and B2, shows the regional distributions of the slow signals with (●) and without (▲) the delay as well as the dorsal signals (○). As can be seen in these figures, the regions of the slow signals with and without the delay appear to be separated: the signals with the delay distributed on the ventral horn and the signals without the delay distributed on the intermediate region.

**Synaptic fatigue**

According to the assumption that the slow signals correspond to postsynaptic potentials, we performed repetitive stimulation experiments. The results obtained from an LS5 preparation are shown in Fig. 10. When 0.1-, 0.2-, or 1.0-Hz repetitive stimulation of 8 \( \mu \)A/5 ms was applied to the spinal nerve fibers, the amplitude of the slow signals gradually decreased, whereas the repetitive stimuli were without significant effects on the fast signals. In Fig. 10A, the normalized amplitudes of the dorsal fast signals and the slow signals detected...
from the dorsal, intermediate, and ventral regions are plotted against the stimulus number with time. The fast signal amplitudes were nearly constant, but slow signal amplitudes decreased gradually; the rate of decreasing in the slow signal amplitudes was dependent on the frequency of the applied stimuli, and the order of the rate was $1.0 > 0.2 > 0.1$ Hz. In Fig. 10B, to compare the time course directly, the slow signal amplitudes are plotted against the time in the same scale. From commonly observed properties of central synapses, it seems reasonable to suppose that this decrease in the size of the slow signal was due to synaptic fatigue. In addition, these graphs indicate that the synaptic fatigue induced by repetitive stimuli was most rapid in the ventral horn, and most slow in the dorsal horn, suggesting differences in synaptic behaviors between these regions.

**Spatiotemporal activity mapping**

To examine the patterns of spread of the neural responses more clearly, we made “pseudocolor imaging maps.” Figure 11 illustrates two examples of the imaging maps constructed from C13- and LS5-cut preparations in normal Ringer solution. In such pseudocolor imaging-displays, it is often difficult and/or impossible to identify the pre- and postsynaptic signals. Thus the imaging maps are compared with the waveforms of the optical signals obtained simultaneously from the corresponding preparations.

In these maps, we can read the time sequence of the neural response as follows. For the C13 preparation (Fig. 11A), the **dorsal fast-signal area** expands during 6~10 ms after the application of the stimulation $\rightarrow$ reaches its maximum at 10 ms $\rightarrow$ reduces gradually during 10~15 ms; the **dorsal slow-signal area** expands during 15~25 ms $\rightarrow$ reaches its maximum at 60 ms $\rightarrow$ reduces gradually after 60 ms; and the **ventral slow-signal area** appears at 30 ms $\rightarrow$ expands during 100~250 ms $\rightarrow$ reaches its maximum $\rightarrow$ reduces gradually after 250 ms.

In these maps, the signals having fractional intensity changes $>1.5 \times 10^{-4}$ are displayed so that the early phase of the signals in the intermediate region is not shown. Nonetheless, these maps reflect dynamically the spatiotemporal patterns of the neural circuit activity.

**DISCUSSION**

In this article, we have demonstrated that multiple-site optical recording techniques can be used successfully to monitor the presence of synaptic connections in E8 embryonic chick. We have demonstrated the spatiotemporal pattern of neural responses within the spinal cord and shown for the first time the presence of synaptic connections between dorsal horn neurons and primary afferent fibers at this stage of development. The development of connections between afferents and motoneurons has been well studied previously by Lee et al. (1988) and Davis et al. (1989). Therefore, the primary novelty of the present work is not establishing the development of this pathway but rather in showing the spatial/regional distribution of synaptic responses in the transverse $x$-$y$ plane of the spinal cord at three different levels at an intermediate stage of embryonic development.

The linear relationship between the voltage-sensitive dye optical signal and changes in intracellularly recorded membrane potential has been established (e.g., for reviews, see Cohen and Salzberg 1978; Grinvald et al. 1988; Kamino et al. 1989a; Salzberg 1983). It further is assumed that, when the multiple-site optical recording method using a multielement photodiode matrix array (Cohen and Lesher 1986; Grinvald et al. 1981; Hirota et al. 1995; Kamino et al. 1989b; Salzberg et al. 1983) is applied to vertebrate central nervous systems, one photodiode sums the activities from a population of neurons together with processes. Accordingly, the fractional signal amplitude is proportional to the voltage changes and the fraction of the active area within the receptive field of one photodiode. These characteristics of the optical recording technique are considered first as a basis to discuss the experimental results in the present study.
In the present experiments, electrical stimuli were applied to mixed spinal nerve fibers. For this protocol, we are reminded further of the following general basic features.

1) The mixed spinal nerve contains both afferent sensory and efferent motor nerve fibers. Thus electrical stimulation applied to the mixed spinal nerve was simultaneously orthodromic for the sensory nerve fibers and antidromic for the motor nerve fibers. Unfortunately, at present at least, it is very difficult to stimulate the dorsal and ventral roots separately because the embryonic nerve fibers are very fragile and thin.

2) Anatomically, it is likely that the response areas contain many sensory nerve terminals together with postsynaptic nerve cells (possibly interneurons) in the dorsal gray horn and many motoneurons and possibly interneurons in the ventral gray horn.

In the noncut preparation, the detected optical signals were classified into four categories, viz., fast spike-like signals in the dorsal gray horn (dorsal fast signal), slow signals in the dorsal gray horn (dorsal slow signal), fast spike-like signals in the ventral gray horn (ventral fast signal), and slow signals in the ventral horn (ventral slow signal). Based on the results obtained from the experiment using the ventral root-cut preparations, it is now apparent that the ventral fast signals are antidromic action potentials evoked in the motoneurons. Further, from evidence that the slow signals were detected from both the dorsal and ventral regions, it is suggested that different kinds of synaptic connections are formed in the dorsal and ventral regions. Here we assume that the dorsal slow signals contain the components related to the spinal reflex pathway and the ascending tract pathway.

The ventral slow signals were classified into two groups according to the difference in the delay time (Fig. 9): slow signals with delay and those without delay. It has been shown previously that direct monosynaptic connections form between lumbosacral muscle afferents and motoneurons by stage 32: electrical recordings from the ventral roots at this stage reveal the presence of short-latency ventral root potential (Lee et al. 1988). These potential have an onset latency from a stimulus applied to a muscle nerve of ~7 to 8 ms, and they peak within

FIG. 9. Comparisons of the delay time among the slow signals. Ventral slow signals were classified according to the delay time. Onset of the dorsal fast signal is regarded as the 0-point reference for the timing of the ventral slow signals. Thin vertical lines are placed at the onset of the dorsal fast signals and ventral slow signals and S indicates the time of the application of the stimulating current. Signals in A1 were from a C13-cut preparation, and those in B1 were from an LS5-cut preparation. In A2 and B2, the positions in which the ventral slow signals without the delay (●) and with the delay (Œ) and the dorsal signals (E) are marked.
15–20 ms of the stimulus. In the present experiments, the earliest detectable signals in the ventral part of the cord, where motoneurons are located, were ~30 ms after the occurrence of the orthodromic spike in the dorsal horn (ventral slow signals with delay). This result suggests that these signals reflect polysynaptic pathways activated by the stimulus and that the monosynaptic potentials are not detected in the somatic region.

Electrical stimulation of spinal nerves can trigger regenerative network activity in the spinal cord that results in much larger membrane potential changes than afferent-evoked monosynaptic or disynaptic responses. Considering that the delay time in the present experiment is relatively large, it is possible that some of the detected signals reflect the regenerative activation of spinal networks.

For the ventral slow signals without delay, one possible explanation is that they reflect postsynaptic potentials in the interneurons located in the intermediate zone. Another possibility is that they are related to synaptic connections on the motoneuron dendrites because the response area is overlapped spatially with the dendritic region identified morphologically (Fig. 8).

An additional question remains: in the ventral region of the cut preparations, the expected signal corresponding to the presynaptic action potential was not detected. As a first approximation, the magnitude of the optical signal (fractional change) is proportional to the amplitude of the membrane potential changes in each cell and process and to the number of these elements within the receptive field of one photodiode (Kamino et al. 1989a; Obaid et al. 1985; Orbach et al. 1985; Salzberg 1983). On the basis of this, we suggest that the amplitude of the membrane potential changes times the fraction of the active membrane area of the presynaptic terminals that are viewed by one photodiode is too small to be detected optically. In the cut preparations, there was no firing activity due to activation of motoneurons by sensory input. There are two possible explanations for this result. The first possibility is

---

**FIG. 10.** Graphic representation of decreasing of slow signal amplitudes with repetitive stimuli. Square-current pulses of 8 μA/5 ms with a frequency of 0.1, 0.2, or 1.0 Hz were applied to the mixed spinal nerves. Data were obtained from 3 dorsal fast, 3 dorsal slow, 3 intermediate slow (ventral slow without delay), and 3 ventral slow (ventral slow with delay) signals in an E8/LS5 preparation. Different symbols correspond to different positions. A: amplitudes of the fast and slow signals are plotted against the stimulation number. B: plots in A are transformed into the plots of the amplitude vs. time (s) in the same scale.
FIG. 11. Spatiotemporal imaging maps of the neuronal responses in C13 (A) and LS5 (B)-cut preparations. Images are compared with a dorsal signal and 2 ventral slow signals obtained from each preparation. Of the 2 ventral slow signals, 1 is the signal without the delay (m) and the other is the signal with the delay (v). For each preparation, the dorsal signal and the ventral slow signals with and without the delay were recorded from the positions indicated by D, m, and v (left), respectively. S is placed at the time of the application of the stimulating current. Pseudocolor imagings were constructed from the optical recording signals of which amplitudes were $>1.5 \times 10^{-4}$ in the fractional change using an interpolation method of Transform (Fortner Research LLC, Starling, VA).
that the slow optical signal reflects the subthreshold change in membrane potential in the postsynaptic cells. The second possibility is that firing activities in the motoneurons are small and incomplete, so that they are fused and are not observed as a spike-like signal.

It is likely that the regional distributions of the amplitudes of the fast and slow optical signals reflect approximately the number times the voltage activity of the neurons/nerve terminals and the functional synaptic connections, respectively. On the above assumption, the results shown in Fig. 7 suggest that the neural activity and/or the number of neurons at the level of LS2 are smaller than those at C13 and LS5. In the dorsal and ventral regions, the functional synaptic connections appear to be distributed in a quasicircular pattern around the areas of maximal activity located near the dorsal root entry zone (for the sensory nerve) and near the ventral root outlet zone (for the motoneuron) (Fig. 5). The spatial distribution pattern of the signals was essentially similar among C13, LS2, and LS5 preparations. This result suggests that there is no rostral-to-caudal gradient of neural responsiveness in the spinal cord at this developmental stage.

The slow signals decreased gradually with repetitive stimulation of a low frequency, such as 0.1 Hz. Similar observations have been reported in the embryonic chick and rat brain stem preparations (Komuro et al. 1991; Sato et al. 1995, 1998) and the embryonic chick spinal cord (Lee and O’Donovan 1991), suggesting that such a rapid fatigue is typical of early embryonic synapses. In the embryonic synapses, a decrease in small quantal contents (the small amount of transmitter per synaptic vesicle) may be implicated in such a rapid fatigue. Between the dorsal and ventral synaptic connections, the difference in synaptic fatigue appears to indicate the regional difference in the degree of the functional synaptic connection.

In the present work, some problems remain to be resolved, although they are outside the scope of this report. First, because we examined exclusively the preparations at Hamburger-Hamilton stage 33, we have not traced the development of the neural circuit activity and, second, the information obtained is limited to the levels of the C13, LS2, and LS5 spinal nerves. Further, we have not characterized the neural transmitters. These problems will be taken up in future reports of our continuing investigations.

We thank L. Cohen and B. Salzberg for reading the manuscript and useful comments and Drs. Norio Kudo (University of Tsukuba) and Tetsuro Sakai for discussion. In addition, we thank journal reviewers especially for helpful and useful suggestions and comments concerning the manuscript. We also are pleased to acknowledge the helpful cooperation of A. Hirota in the construction of the optical recording system and E. Bandai for cooperation in the preparation of the manuscript. Y. Arai is grateful to Drs. Kohtaro Furuya and Kenichi Shinomiya for kind permission to work in the present department.

We are pleased to acknowledge the helpful cooperation of A. Hirota in the construction of the optical recording system and E. Bandai for cooperation in the preparation of the manuscript. Y. Arai is grateful to Drs. Kohtaro Furuya and Kenichi Shinomiya for kind permission to work in the present department.

This work was supported by grants from the Mombusho of Japan, Ichiro Kanehara Foundation, and Konica Imaging Science Foundation.

Address for reprint requests: K. Kamino, Dept. of Physiology, Tokyo Medical and Dental University School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan.

Received 19 February 1998; accepted in final form 3 December 1998.

REFERENCES


