MINIREVIEW

Optical Studies of Early Developing Cardiac and
Neural Activities Using Voltage-Sensitive Dyes

Kohtaro KAMINO

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

The ontogenetic approach to generation of physiological events during natural development could be a useful and powerful strategy to study heart and central nervous systems: it would allow us to analyze progressively the complicated functional organization and architecture of both cardiac and nervous systems in a manner reminiscent of the expansion of a complex function in a power series. However, the experimental analysis of early embryonic cardiac and nervous systems is technically difficult because the cells are extremely inaccessible: microelectrode examination of heart cells and neurons, which provides the most direct test of their electrophysiology, is often difficult because of the small size of the cells. For this reason, electrophysiological studies of very early embryonic hearts and nervous systems have been hampered.

In such a situation, optical methods for monitoring electrical activity using voltage-sensitive dyes have been introduced, and it has been possible to make recordings from embryonic hearts and neurons at very early stages of their development. Recently, the progress of the optical studies on the early embryonic heart activity has been extensively reviewed [31, 33]. In this article, some results obtained with optical recordings of electrical activity in the early embryonic heart and nervous systems are briefly sketched.

A. OPTICAL METHODS FOR MONITORING CELLULAR
ELECTRICAL ACTIVITY

Optical methods for monitoring cellular electrical events offer two principal advantages over more conventional electrophysiological techniques. One is that it is possible to make optical recording from very small cells and subcellular organelles which are inaccessible to microelectrode impalement [4, 10, 53, 55, 56], and the other is that multiple regions of a preparation can be monitored simultaneously and provide spatially-resolved mapping of electrical activity [17, 18, 31, 39, 44, 54]. Accordingly, early embryonic heart cells and neurons are particularly suitable subjects for the optical method.

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1. Voltage-sensitive dyes

Optical methods for monitoring action potential activity are based on the observation that optical properties such as absorption, fluorescence and/or birefringence of giant axons of squid (*Loligo pealei*) stained with certain dyes change with changes in the membrane potential [4].

Over 1,500 dyes have been tested for their responses to changes in membrane potential in the squid giant axon, and fluorescence, absorption and/or birefringence signals were found with about half of these [5, 21, 47]. Of the dyes that exhibit an absorption response, several merocyanine-rhodanine dyes have a relatively high sensitivity to rapid changes in membrane potential. Subsequently, many additional voltage-sensitive dyes have been synthesized [19, 41].

In voltage-clamp experiments using squid giant axons, absorption changes in voltage-sensitive dyes are often linearly related to the membrane potential in the physiological range of resting potential ± 100 mV [47]. This property is very useful for optical measurement.

In earlier experiments on embryonic hearts, monitoring electrical activity as an absorbance signal was accomplished using a merocyanine-oxazolone dye (NK2367) and merocyanine-rhodanine dyes (Dye XVII [13, 14] and Dye XXIII [15]). Subsequently, in light of the fact that early embryonic heart cells are primitive and may be very sensitive to small changes in the environment, we designed an improved merocyanine-rhodanine dye, NK2761 [15, 32]. This dye was synthesized by Nippon Kankoh-Shikiso Kenkyusho Co. Ltd., Okayama, Japan. It is an analogue of Dye XVII [47] and XXIII [21, 54], in which the alkyl chain (ethyl) attached to the rhodanine nucleus is replaced by a butyl [15]. This dye has several advantages in that (1) for staining the preparation, a lower concentration (0.05–0.1 mg/ml) is used; (2) neither the pharmacologic effects nor the photodynamic damage are serious; (3) dye bleaching time is relatively long; and (4) the optical response follows the voltage-change with a time constant of less than 2.0 μ s.

In addition, NK2761 is sufficiently soluble in physiological Ringer's solution. However if the butyl is replaced by a longer alkyl chain such as a hexyl, heptyl, or octyl, the dye is more hydrophobic and not so easily soluble in water.

The size of the voltage-related absorption change (fractional change = the change in intensity divided by DC background intensity) closely depends on the wavelength of the incident light. The action spectra in the tissues stained with NK2761 are usually biphasic with a decrease in absorption from 520 to 600 nm, and an increase from 630 to 750 nm. The absorption change disappears at 610–620 nm and the change exhibits its largest value at 700 nm [15].

2. Optical recording system

In the original experiments for simultaneous optical monitoring of electrical activity from several neurons in an invertebrate central nervous system, photodiodes glued directly to light guides were employed [20, 54]. Subsequently, a similar method was applied to the early embryonic heart [15, 29, 51]. Light from the image of the

embryonic heart was conducted to individual photodiodes, using fiber optics as the light guides. The maximum number of photodiodes in this method was 16, so that the number of regions from which electrical activity could be simultaneously recorded was 16.

Multiple-site optical methods were advanced with introduction of a 10×10 - or 12×12 -element photodiode array in Cohen's and Cohen-school researchers' laboratories [17, 27, 48, 56]. This array allowed a large increase in the number of sites which could be monitored. The review by COHEN and LESHER [3] emphasizes the basic experimental method and the measuring system.

The multiple-site optical recording system that we currently use consists of four main parts: optics, detection system, multiple-channel recording system, and computer (Fig. 1). The preparation chamber is placed on the stage of a microscope mounted on a vibration-isolation table. Bright field illumination is provided by a tungsten-halogen lamp driven by a stable DC power supply. Incident light is collimated, passed through a heat filter, rendered quasi-monochromatic with an interference filter, and focused onto the preparation by means of an aplanatic/achromatic condenser. When merocyanine-rhodanine dyes are used, a 700 ± 11 (or 702 ± 13) nm interference filter is employed.

An objective (S plan or S plan Apo) and a photographic eyepiece form a magnified real image of the preparation, at the image plane. The transmitted light intensity at the image plane of the objective and photographic eyepiece are detected using 100- or 144-element of a 10×10 or 12×12 square matrix array of silicon photodiodes. Thus, spatial resolution is limited only by microscope optics and noise considerations. Recently, using a water-immersion- $\times 40$ objective together with a $\times 6.7$ photographic eyepiece, the spatial resolution has been enhanced to $2.7 \times 2.7 \mu\text{m}^2$ (H. Komuro, Y. Momose, T. Sakai, A. Hirota, and K. Kamino, unpublished results).

The outputs of the detectors in the diode array are fed to 144 amplifiers via 144 current-to-voltage converters. The amplified outputs from 128-elements of the diode array are first recorded simultaneously on a 128-channel data recording system, and then are fed into a computer. The programs are originally written in assembly language (Macro-11) called by Fortran under the RT-11 operating system (version 5.0). In other laboratories, instead of the multi-channel recording system, a multiplexer(s) and an A-D converter(s) are used [3, 18, 40, 49, 56]. In the experiments on the heart, relatively long continuous recordings are often required for analyzing rhythm generation, so that the multi-channel recording system is preferred.

B. OPTICAL MONITORING OF SPONTANEOUS ACTIVITY FROM EARLY EMBRYONIC HEARTS

In the early cardiogenesis of the chick embryo, heart primordia are brought together at the midline and fuse with each other progressively caudally [6, 7, 42, 46]: this process results in the formation of the primitive tubular heart which begins

A

m=3 and n=2, Dye XVII

m=4 and n=3, Dye XXIII

m=4 and n=4, NK 2761

B

beating spontaneously [16, 45, 46].

On the basis of the results obtained using conventional electrophysiological measurements, MANASEK [43], in "Handbook of Physiology" published in 1979, tentatively concluded that the onset of electrical activity occurs at stage 10 of Hamburger-Hamilton (corresponding to the 10-somite stage and to about 30 h of incubation). VAN MIEROP [61] has been the only person to record a spontaneous action potential from an 8-somite embryonic chick heart using an ultra-microelectrode. In addition, it has been commonly accepted that the first beating of the embryonic heart begins at the 10-somite stages of development. Accordingly, we first attempted to monitor spontaneous electrical activity optically in the 8- to 9-somite chick embryonic hearts, and in 1978, although the signal-to-noise ratio was very poor, we succeeded for the first time in detecting spontaneous optical signals from a 9-somite embryonic heart stained with a merocyanine-oxazolone dye (NK2367 [11, 22]).

1. *Optical signals from the early embryonic heart stained with voltage-sensitive dyes*

When the merocyanine-oxazolone dyes were used, the signals obtained at a wavelength of 675 nm were eliminated by illumination with white light [12, 22]. Furthermore, the wavelength dependence of the signals was examined in more detail with a merocyanine-rhodanine dye (Dye XVII). In the 8- and early 9-somite embryonic hearts stained with this dye, the signal size depended sensitively on the wavelength of the incident light: the action spectrum of the dye signal was triphasic, with an increase in transmittance from 525 to 600 nm, a decrease from 625 to 720 nm, and an increase again at 750 nm. The signal was largest at 700 nm and was completely eliminated at 625 nm, where the action spectrum of the absorption signal exhibited by this dye has a null, and no optical signal was detected from the unstained heart.

A similar spectral pattern is also obtained with another merocyanine-rhodanine dye (NK2761) [15], and it is the same as the action spectrum of the action potential-related optical signal. These spectral characteristics demonstrate that the signals are due to absorbance changes associated with spontaneous action potentials in the stained embryonic heart.

On the other hand, in embryonic hearts at the middle and later periods of the 9-somite stage, optical signals composed of two components are often detected. The 1st-component varies with the wavelength of incident light, and has the same action spectrum as the optical signal related to the action potential; the 2nd-component, however, is detected at 620 nm and under illumination with white

Fig. 1. Chemical structure of merocyanine-rhodanine dyes (A), and schematic diagram of the multiple-site optical recording system that is currently used for monitoring changes in transmitted light from the stained preparation (B). Light from a tungsten-halogen lamp was passed through a heat filter and an interference filter. A 12×12 -element photodiode array was placed in the plane where the objective and eyepiece form the image of the preparation.

light. Thus, the 1st-component is the absorbance signal due to the action potential, and the 2nd-component is the light scattering change related to the contraction of the cardiac muscle [12, 26, 28, 33].

From these results, it is concluded (1) that in the embryonic chick heart, the first contraction begins at the middle period of the 9-somite stage, and (2) that spontaneous electrical activity is generated prior to the initiation of contraction.

2. *Onset of spontaneous electrical activity in the pre-fused cardiac primordium*

Although we suggested earlier that in the embryonic chick heart the spontaneous action potential would be generated initially during the middle period of the 7-somite stage of development, corresponding to the initiation of fusion of the paired right and left cardiac primordia [12–15], additional experiments indicated that this result was not reliable. In experiments using a multiple-site optical recording method with a 100-element photodiode array, we have now been able to detect spontaneous electrical activity in the pre-fused cardiac primordia at the 6- and early 7-somite stages.

The size of the signals detected from the pre-fused cardiac primordium was very small, and was estimated to represent fractional changes of less than 5×10^{-4} . The signal-to-noise ratio was smaller than 1.5–2.0. Nevertheless, the signals appeared rhythmically and were nearly synchronized among several different positions. In addition, the signals from the preparation stained with NK2761 disappeared when a 610 ± 9 nm interference filter was used. These temporal and optical characteristics of the signals have refined our identification: we have concluded that, although the signal-to-noise ratio was very small, these signals are indeed absorption changes related to spontaneous action potentials and do not correspond either to noise or mechanical artifacts [25].

The signal sizes in the early 7-somite pre-fused cardiac primordia seem to be somewhat larger than those observed at the 6-somite stage. However, as in the 6-somite embryos, the action potential signals were always detected from only one side of the primordia.

The combination of voltage-sensitive dyes and a multi-element photodiode matrix array has made monitoring of the electrical activity simultaneously over the entire extent of the paired right and left cardiac primordia feasible, and the signal-to-noise ratio has also been improved using this photodiode array. Under these conditions, we found spontaneous electrical activity, for the first time, in the pre-fused chick cardiac primordia at the 6- and early 7-somite stages. Thus, our findings strongly suggest that the conventional idea concerning the onset of electrical activity of the embryonic chick heart needs to be re-considered (see Fig. 8 in [43]).

A remarkable finding is that spontaneous action potential-related signals occur rhythmically in the pre-fused cardiac primordium. This strongly suggests that the genesis of excitability, spontaneity, and rhythmicity appears to be coupled in the cardiac primordium.

The size of the optical signals obtained from the pre-fused cardiac primordium

is very small and often indistinct, and the electrical activity appeared in a limited narrow area. This result suggests that a few cells with very slight electrical activity are differentiated in a limited area in the right or left cardiac primordium before fusion has begun.

3. *Early development of electrical activity*

Figure 2 shows multiple-site optical recordings of spontaneous action potentials from different contiguous regions of a 9-somite embryonic pre-contractile chick heart, stained with the merocyanine-rhodanine dye (NK2761). Optical signals were recorded from 100 elements of a 10×10 -element photodiode matrix array. As development proceeds from the 6-somite to the 7-somite stage, the size of the optical action signals gradually increases, the area in which the signals occur spreads widely over the heart, and the size of the optical signals is different in different areas (also see Figs. 1–5 of [25]).

The regional differences of the optical signal and their spatial patterns are shown in more detail in contour-line maps of optical action signals in the 6- to 9-somite embryonic pre-contractile hearts (see Fig. 7 in [25]).

The signal size (fractional change) should be proportional to the magnitude of the action potential generated per unit area in each active cell, to the number of electrically active cells in the field detected by one element, and to the amount of the dye bound to the membrane. Here, we shall assume that the amount of the dye bound to the membrane is regionally homogeneous and, consequently, that regional differences in signal size reflect regional variations in the number of the electrically active cells and/or in the magnitude of electrical activity of each cell.

Accordingly, such multiple-site optical recordings and contour maps include the following feature: (1) the electrical activity is generated and developed independently and in parallel in the right and left regions originating from each cardiac primordium at the 6-somite stage; (2) both the electrical activity and the active area increase gradually through the 6- and 7-somite stages and increase rapidly through the 8- and 9-somite stages; (3) during the middle 7- to early 8-somite stages, two peaks of electrical activity coexist in the right and left cono-ventricular regions; (4) subsequently, at the 9-somite stage, several peaks of electrical activity appear in the right and left sides of the ventricular region and (5) development of electrical activity near the fusion line in the midline of the heart is relatively poor.

Experiments similar to those on the embryonic chick heart were carried out on the embryonic rat heart [27]. Spontaneous optical signals were first recorded from a limited central part of the 3-somite embryonic rat heart (for morphology see [16]), and their sizes were very small. These optical signals are closely similar to those recorded from chick embryonic heart. However, we have not been able to obtain signals from the 2-somite embryonic rat heart stained with voltage-sensitive dyes. It appears, therefore, that the first spontaneous action potential activity in embryonic rat heart occurs at the 3-somite stage of development, prior to the beginning of contraction.

Fig. 2. Simultaneous optical recording of spontaneous electrical activity from 91 contiguous regions of a 9-somite embryonic pre-contractile heart, using a 12×12 -element photodiode matrix array. The preparation was stained with a merocyanine-rhodanine dye (NK2761). The photodiode array was positioned over a magnified image of the heart. Thus, each trace represents signals detected by one photodiode from a $42 \times 42 \mu\text{m}^2$ area of the heart. The outputs of the individual detectors have been divided by the resting light level (DC-background intensity). Early embryonic preparations are usually very thin, so that the effect of scattering from the preparation is not serious. The measurement was carried out at $36.6\text{--}37.5^\circ\text{C}$. A $702 \pm 13 \text{ nm}$ interference filter was used (modified from Fig. 1 in [36], with permission).

4. *Ionic properties*

In embryonic chick hearts incubated for over 2 days, various ionic and/or membrane properties related to electrophysiological events have been analyzed. For example, effects of tetrodotoxin [30, 57], potassium flux and permeability [1, 59], and voltage-activated K-channels [2], have been mentioned.

It has been established that in the embryonic chick heart the mechanisms that underlie action potential generation develop during the second day of incubation and for the next few days are markedly different from those that characterize adult hearts [58, 60]. Based on this conception, the ionic properties of the action potential in the early embryonic heart were examined optically [50]. In the 7- to 9-somite embryonic pre-contractile hearts, when the external Ca^{2+} is partially replaced by Mg^{2+} , the frequency of the spontaneous action potentials decreases strikingly, and the signal size decreases. Similar effects are caused by reducing external free Ca^{2+} concentration by adding GEDTA (or EGTA) (glycoletherdiaminetetraacetate or ethyleneglycol-bis-(α -amino-ether) N,N'-tetraacetic acid) to the bathing solution, and by the addition of Mn^{2+} . Furthermore, from the slope of the dose (Ca^{2+} concentration)-response (signal size and frequency) curve, there is a difference in the degree of responsiveness to low external Ca^{2+} concentration among the stages of development.

Conversely, as the external Ca^{2+} concentration is increased, both the size and frequency of the spontaneous action potentials increase gradually up to a saturation level. In particular, in embryonic hearts at the 7-somite stage or the early period of the 8-somite stage, the optical action signals are often very small in the normal bathing solution, but the size of signals are increased dramatically when the external Ca^{2+} concentration is elevated. At the same time, the spontaneous action potentials are independent of external Na^{+} and insensitive to tetrodotoxin (TTX).

On the basis of these observations, it is tentatively concluded that spontaneous action potentials in the early embryonic heart do not depend on a component due to a fast Na^{+} current, and that they are best characterized as Ca^{2+} -action potentials, generated by the slow inward Ca^{2+} current which is responsible for the plateau phase (phase 2) in the adult. Indeed, the rate of rise of the optical action signal is small in the 6- to 9-somite embryonic hearts: the initial rapid upstroke (phase 0), which results from fast Na^{+} current is absent in the action signals from the embryonic pre-contractile heart.

5. *Rhythm generation*

As described above, in the pre-fused cardiac primordium at the 6-somite stage, initial spontaneous action potentials occur rhythmically. This evidence really shows that "rhythmicity" is generated simultaneously with "spontaneous excitability" in the pre-fused cardiac primordium [25].

However, the spontaneous action potential-related optical signals which are detected from the pre-fused cardiac primordium or the primitive embryonic heart

at the 6- to 7-somite stages are very low in frequency, and the action signals do not necessarily recur with a completely regular rhythm [14, 25]. The frequency of the spontaneous action signals gradually increases, as development proceeds from 6-somite to 9-somite stage, and the rhythm is often completely organized by the early period of the 9-somite stage (see Fig. 1 in [14]). The time-interval histograms of spontaneous optical signals obtained from four embryonic hearts at the 7- to early 9-somite stages were compared (see Fig. 5 in [14]).

Cooling causes a marked decrease in the frequency of occurrence of the spontaneous action potential. This slowing is not associated with a disordering of the rhythmicity of the action potential: however, as the temperature continues to decline, the rhythm becomes distorted and arrhythmic action potential signals are often observed [23].

The rhythmicity of the spontaneous action signals is also strongly affected by the external Ca^{2+} concentration. Reducing the external Ca^{2+} concentration disturbs the rhythm of the spontaneous action potentials in the embryonic precontractile heart. Comparing the time interval histograms (peak-to-peak) obtained from a 9-somite heart in normal bathing solution and in low Ca^{2+} -concentration, the histogram in the low Ca^{2+} concentration was remarkably broad: the mean interval shifts from 1.17 ± 0.06 s in the normal condition to 2.92 ± 1.29 s in $1/2 \text{ Ca}^{2+}$ concentration (0.9 mM) (see Fig. 4 in [50]).

In the dose-response curves, the frequency decreases with a reduction in the external Ca^{2+} concentration in each heart at the 7- to 9-somite stage of development, and the slopes of the dose-response curves, reflecting the resistance to low external Ca^{2+} concentration, decrease as development proceeds [50].

In summary, "rhythmicity" is generated in the cardiac primordia at the 6-somite stage. The rhythmicity in the embryonic pre-contractile primitive heart is easily disturbed by lowering the temperature. This feature strongly suggests that the dissipative structure is related to the subcellular and/or molecular mechanism(s) of the generation and early development of rhythmicity during the early phases of cardiogenesis. In addition, it is likely that the origin of "rhythmicity" is intimately related to external Ca^{2+} .

6. *Spreading of spontaneous action potential*

As can be seen in Fig. 2, the optical signals that reflect spontaneous action potentials are nearly synchronized among the different regions. Nevertheless, short delays between the feet of the signals are evident. These delays reflect the conduction-time of excitation. In recordings at higher sweep speed, short delays between the signals are clearly observed, and we can see that the action potential first appears in position J-8 (indicated by an asterisk). Thus, it is likely that, in this heart, the pacemaking area was located at this position, and the excitation spread over the entire region of the heart from this pacemaking area.

In a plot of the delays in the optical signals against the radial straight line distance from the center of the pacemaking area, a simple linear relationship is

often obtained. This observation suggests that excitation propagates radially and uniformly over the entire region of the heart from the pacemaking area. From the slope of the straight line, the conduction velocity can be readily calculated. In the 9-somite embryonic chick heart, it is around 1.5 mm/s.

The radial propagation of excitation at a uniform rate seems to be common to early embryonic hearts of the rat [27] and chick [29, 35, 36, 38]. Thus, we tentatively suggest that neither preferential conduction pathway(s) nor special conduction system(s) are differentiated in the earliest phases of cardiogenesis in vertebrates. The propagation of action potentials in the early embryonic heart would be favored by a low-resistance pathway for current flow between the cell interiors [9]. One possible mechanism is that gap-junctions are the sites of intercellular communication [8]. This idea is supported by the finding that the conduction velocity decreases when intracellular Ca^{2+} concentrations are elevated in the presence of a Ca^{2+} ionophore [38].

Applying the multiple-site optical recording method, we also found that the pacemaking area is located in various regions of the heart, at the 7- to 8-somite stages, and that as development proceeds to the 9-somite stage, it is located in the left atrial primordium and subsequently shifts to the sinus primordial region at the 11-somite stage [32].

Furthermore, from graphical analysis of the data obtained from simultaneous multiple-site optical recording, it has been shown that the pacemaking area was basically circular in shape in the later 8- to 9-somite embryonic hearts. The size of the pacemaking area was estimated to be 1,200–3,000 μm^2 . We suggest that the pacemaking area is composed of 60–150 cells, and that the pacemaking area remains at a relatively constant size throughout the 7- to 9-somite stages. Thus, it was proposed that a population of pacemaking cells, rather than a single cell, serves as a rhythm generator in the embryonic chick heart [35, 36]. In connection with the functional organization of the pacemaking area, regional gradients of rhythmicity were examined using congenital and experimental double-hearted embryos [15, 24, 62].

C. OPTICAL RECORDINGS OF NEURAL RESPONSES IN EARLY EMBRYONIC CENTRAL NERVOUS SYSTEMS

Although a complete understanding of the functional organization/architecture of the central nervous system requires a detailed knowledge of early developmental process of the generation of electrophysiological events in the embryonic nervous system, the small size and fragility of the cells have made direct recording of electrical activity difficult or impossible. Therefore, in contrast with studies of morphogenesis, studies of the origin and organization of “physiological functions” in the nervous system during the early phases of development made little progress. Thus, the prospect of using optical techniques to record electrical events from the early embryonic neurons intrigued us.

1. *Embryonic trigeminal ganglion*

The largest and the most clearly defined of the ganglia present in 4-day-old chick embryos is the trigeminal ganglion of the fifth cranial nerve. It lies ventro-laterally, opposite the most anterior neuromere of the myelencephalon. From its cells sensory nerve fibers grow mesiad into the brain and distad to the facial and oral regions. In 4-day-old chick embryos the beginning of the ophthalmic division of the fifth nerve extends from the ganglion toward the eye, and the beginning of the mandibulo-maxillary division is growing toward the angle of the mouth [46].

In the early experiments we tried to monitor electrical activity optically in the embryonic chick trigeminal ganglion [52]. Changes in absorbance of voltage-sensitive merocyanine-rhodanine dyes were used to monitor electrical responses in the trigeminal ganglion of 4- to 10-day-old developing chick embryos. The electrical responses were recorded simultaneously from many positions in the ganglion. Stimulation of the afferent nerve fibers (the ophthalmic division of cranial nerve V) with a suction electrode led to changes in light absorption of the stained ganglia. With both depolarizing and hyperpolarizing pulses, the change was largest at 700 nm and was eliminated at a wavelength of 620 nm where the voltage-dependent absorption change of the dyes disappears. In 4- to 10-day-old embryonic ganglia, two types of optical membrane potential responses, "non-conducted" and "conducted" responses, were identified. The non-conducted response varied with the intensity of the stimulus and behaved as electrotonic spread. Furthermore, this non-conducted response exhibited an "initial upstroke response" followed by the steady-state plateau evoked by larger depolarizing pulses. The conducted responses were blocked by tetrodotoxin (TTX) or by high external potassium concentration. The incidence of the conducted responses increased as development proceeded from the 5th to the 10th day of age. Thus, the TTX-sensitive action potential activity is probably generated initially in the trigeminal ganglion during the 5- to 10-day stage of development. These data represent the first demonstration of membrane potential responses in intact early embryonic nervous systems, and these studies demonstrate the utility of voltage-sensitive dyes for the analysis of the organizing process of embryonic neuronal functions during these early stages of development.

2. *Early embryonic brain stem*

The investigations on the embryonic origin and early development of cardiac function guided our interest to the early embryonic brain stem in connection with the functional organization/architecture of the cardiovascular central nervous system.

Thus, we have attempted to apply optical methods for monitoring electrical activity to the embryonic chick brain stem. As a first step, we have assessed the spatial pattern of neural response to vagal stimulation in the brain stem of the 7-day old chick embryo, and we have begun to study the functional organization of the vagal nucleus in the embryonic brain stem [34, 37]. In these experiments,

we verified that the multiple-site optical recording method is also effective for the analysis of functional organization in the embryonic central nervous system.

Figure 3 shows an example of original recordings of optical signals obtained from an embryonic brain stem in response to suction electrode stimulation of the vagus nerves. The vagus nerve-brain stem preparation was isolated from a 7-day-old chick embryo, and stained for 15 min with a 0.2 mg/ml solution of the voltage-sensitive merocyanine-rhodanine dye (NK2761). The preparation was 500–700 μm thick and translucent. We therefore detected optical responses as changes in transmitted light intensity. These changes were detected simultaneously from 127 adjacent regions of the stained brain stem preparation using a 12×12 -element photodiode array. A 702 ± 13 nm interference filter was used to select the wavelength. The simultaneous 127-site optical recordings were made at eleven successive times by moving the photodiode array over eleven positions on the image of the brain stem. As sketched on the lower left corner in Fig. 3, the 12×12 -element photodiode array was moved in the order X $\rightarrow$ II $\rightarrow$ I $\rightarrow$ IX $\rightarrow$ VI $\rightarrow$ V $\rightarrow$ III $\rightarrow$ XI $\rightarrow$ VII $\rightarrow$ IV $\rightarrow$ VIII. When we changed the position of the photodiode array, the incident light was off, except during the measuring period; dye bleaching was small. When we returned to position II, essentially similar results were obtained. The right vagus nerve fibers were stimulated with depolarizing pulses (5.0 μA and 5 ms). Eight trials were averaged.

As can be seen in Fig. 3, the evoked optical responses appeared to be concentrated in a limited central region and in a lateral region, both on the stimulated side of the brain stem, and they did not spread to the contralateral side. The DC background intensities measured by individual photodetectors were somewhat different, so that the signals were normalized to obtain fractional changes (= the change in transmitted intensity divided by the DC background intensity) by a computer program which compensated for the DC background intensity. The fractional changes of these optical signals were 10^{-4} to 10^{-3} .

In the recording shown in Fig. 3, we are able to see only the two-dimensional distribution pattern of the optical signals; from such recordings it is not simple to obtain information about the spread in depth of the electrical responses. To determine the depth distribution of the optical responses, similar experiments were carried out on transverse slices of brain stem. In the experiments using the slice preparations, the optical signals were obtained by stimulation with depolarizing pulses delivered to the right and left vagus nerve fibers with a suction electrode. The evoked optical signals were detected by several photodiodes positioned over the image of the dorsal area on the stimulated side of the brain stem. The results indicate that neurons responding to stimulation of the vagus nerve fibers are concentrated in a circumscribed region of the dorsal areas on the right and left sides of the brain stem.

These optical action potentials evoked by brief depolarizing stimuli were conducted slowly and were blocked completely by tetrodotoxin. With relatively long-duration depolarizing and hyperpolarizing stimulations, electrotonic responses

were recorded. Furthermore, when $30\ \mu\text{A}/20\ \text{ms}$ hyperpolarizing pulse stimulations were applied, anode-break excitation signals were detected, and these signals were also blocked by tetrodotoxin. On the basis of the data obtained from these experiments, we constructed a three-dimensional feature of the electrical response area.

Furthermore, we found that these response areas changed in an orderly fashion according to changes in the strength and in the duration of the stimulating current: the response area expanded as the strength or the duration of the stimulating current was increased. The size of the evoked optical signals also depended on the strength and on the duration of the stimulating current. We also measured critical (threshold) values of the strength and duration of the stimulating current to produce the optical responses, and we found that there was a regionally ordered distribution of the critical values in the brain stem.

Eventually, we hope to obtain more detailed four-dimensional maps of the development of the spatial and temporal organization/architecture of neural function within the brain stem in connection with cardiovascular control central nervous system.

SUMMARY

Using optical methods for monitoring cellular electrical activity based on voltage-sensitive dyes, we have overcome several obstacles to the study of electrical function in the embryonic heart and central nervous system during early development. We have been able to monitor, for the first time, spontaneous electrical activity in the pre-fused cardiac primordia in early chick embryos at the 6- and early 7-somite stages of development and to follow the early development of electrical

Fig. 3. Multiple-site optical recording display of neural responses to stimulation of vagus nerve fibers in a 7-day-old embryonic chick brain stem preparation. The preparation was stained with a merocyanine-rhodanine dye (NK2761). The figure was constructed with eleven 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 $702 \pm 13\ \text{nm}$ interference filter. Each group of signals surrounded with an outline corresponds to one recording with the photodiode array. The traces represent signals detected by one photodiode from a $56 \times 56\ \mu\text{m}^2$ area of the brain stem. The traces are arranged so that their relative positions correspond to the relative positions of the area of the preparation imaged onto the detectors. The outputs of the individual detectors have been normalized by dividing by the DC background intensity. The direction of the arrow at the lower right corner of the optical recordings indicates a decrease in transmission (increase in absorption) and the length of the arrow represents the stated value of the fractional change. The recording was obtained with $5.0\ \mu\text{A}/5\ \text{ms}$ depolarizing current applied to the right vagus nerve fibers with a suction electrode. The relative location of the photodiode array on the image of the brain stem is illustrated on the lower left (from Fig. 1 in [34], with permission).

activity in the pre-contractile heart at the 7- to 9-somite stages. In addition, we have monitored neural responses in the early embryonic chick brain stem by optical means, and determined the spatial pattern of the response.

Key words: optical recording, voltage-sensitive dyes, embryonic heart, embryonic ganglion, embryonic brain stem.

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