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MINI-REVIEW

# Functiogenesis of the embryonic central nervous system revealed by optical recording with a voltage-sensitive dye

**Katsushige Sato**[1] · **Yoko Momose-Sato**[2]

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**Abstract** Clarification of the functiogenesis of the embryonic central nervous system (CNS) has long been problematic, because conventional electrophysiological techniques have several limitations. First, early embryonic neurons are small and fragile, and the application of microelectrodes is challenging. Second, the simultaneous monitoring of electrical activity from multiple sites is limited, and as a consequence, spatiotemporal response patterns of neural networks cannot be assessed. We have applied multiple-site optical recording with a voltage-sensitive dye to the embryonic CNS and paved a new way to analyze the functiogenesis of the CNS. In this review, we discuss key points of optical recording in the embryonic CNS and introduce recent progress in optical investigations on the embryonic CNS with special emphasis on the development of the chick olfactory system. The studies clearly demonstrate the usefulness of voltage-sensitive dye recording as a powerful tool for elucidating the functional organization of the vertebrate embryonic CNS.

**Keywords** Optical recording · Voltage-sensitive dye · Chick embryo · Development · Olfactory system · Oscillation

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✉ Katsushige Sato
katsu-satoh@komajo.ac.jp

[1] Department of Health and Nutrition Sciences, Komazawa Women's University Faculty of Human Health, 238 Sakahama, Inagi-shi, Tokyo 206-8511, Japan

[2] Department of Nutrition and Dietetics, College of Nutrition, Kanto Gakuin University, Yokohama 236-8501, Japan

## Introduction

Highly organized neural circuits in the central nervous system (CNS) give rise to the complex and sophisticated functions of the nervous system. During ontogenesis, when do the elements of the CNS, such as neurons and synapses, develop their structure and function? How are the neural circuits precisely formed within the CNS? Although these questions are fundamental issues in neuroscience, little is known about the "functiogenesis", which is a coined word to contrast with "morphogenesis" and means the emergence and development of excitability, action potentials, synaptic transmission, and network property [1, 2], despite large amounts of anatomical and molecular genetic information. This situation has partially resulted from methodological limitations of conventional electrophysiological means, with which it is difficult to record neural activities from small and fragile embryonic neurons.

Voltage-sensitive dye (VSD) recording enables us to monitor transmembrane voltage changes from excitable cells that are inaccessible by conventional electrophysiological techniques. Furthermore, the introduction of multi-channel photodiode arrays has provided powerful tools for monitoring the spatiotemporal dynamics of neural activity [for reviews see 3–6]. We have applied optical recording with VSDs to the embryonic nervous system and established its feasibility to analyze the dynamics of neural activity in the CNS [for reviews see 7–9].

In this review, we first discuss technical issues such as optical recording systems and VSDs that are suitable for monitoring small optical changes in the embryonic nervous system. Next, we summarize the application to date of VSDs to the embryonic nervous system. Finally, as an example of optical recording in the embryonic CNS, we introduce recent advances in optical studies on the

embryonic chick olfactory system [10, 11]. The functiogenesis of the embryonic brainstem and a widely spreading depolarization wave observed in the embryonic CNS are reviewed elsewhere [7, 12–15], and we will not describe these issues in this review.

## Technical considerations for VSD recording in the embryonic nervous system

For VSD recording in the embryonic nervous system, we need to take the special characteristics of embryos into consideration. First, the immaturity of the embryonic nervous system has some advantages in VSD recording. (1) The embryonic preparation has a loose cellular-interstitial structure, which allows the VSD to diffuse sufficiently from the surface to deeper tissues and stain neurons relatively well. (2) Incident light passes easily through the preparation because of the high translucency of the embryonic tissues. This enables us to detect optical signals from deeper regions in the preparation. (3) In absorption measurements, the high translucency also generates a large signal-to-noise ratio (S/N), since the S/N is proportional to the square root of the transmitted background light intensity, if the dominant noise source is shot noise [5, 6]. (4) The thinness and loose structure of the embryonic preparation reduce light scattering, which deteriorates the signals and decreases the spatial resolution of the optical measurements. (5) Although the optical signal detected by one detector is the sum of many signals from different cell populations, the shape of the optical signal is relatively simple in the embryonic nervous system because of its simpler structure and lower complexity of neural populations.

On the other hand, the fragility and immaturity of embryonic neurons also have some disadvantages. (1) It is difficult or impossible to directly compare the optical signal with the electrophysiological signal because conventional electrophysiological methods are hard to apply. (2) Postsynaptic potentials in the embryonic nervous system fatigue easily, and we need to detect the optical signals without signal averaging. (3) Embryonic postsynaptic potentials are usually long-lasting (duration $> 1$ s), and the waveforms of the optical signals are easily deteriorated by various noise sources.

In these situations, it is necessary to improve VSD recording to clearly monitor neural activity in the embryonic nervous system. Informative reviews concerning optical recording systems have been published [5, 6, 9, 16–19]. Here, we mention two important points: the optical recording system, especially the detector, and the VSD.

### Optical recording system

The recording systems are composed of optics, a detector, and a recording system with a computer (Fig. 1). In VSD recording, high sensitivity and high temporal resolution together with an adequate spatial resolution are needed to record small and millisecond-domain optical signals ($10^{-4}$–$10^{-2}$ as a fractional change: the change in light intensity divided by DC-background intensity). For monitoring embryonic neural activity, the following points should be considered: (1) the temporal resolution should be sufficient to detect the action potential. (2) Slow changes in the membrane potential, such as embryonic postsynaptic potentials (duration >1 s), should be detectable. (3) The S/N should be as large as possible to record neural activity without averaging because of rapid fatigue of the embryonic postsynaptic potential. (4) The dynamic range should match the fractional changes of the signals to be recorded.

In absorption measurements in in vitro embryonic preparations, the signals arise from a baseline of high-intensity transmitted light. As a detector, a photodiode array or a complementary metal oxide semiconductor (CMOS) sensor is desirable because other detectors such as a charge-coupled device (CCD) sensors would saturate at high light intensities. Compared with the CMOS sensor, the photodiode array enables us to record optical signals with a larger S/N, although the spatial resolution is lower. To detect optical signals with a similar S/N, the CMOS sensor needs signal averaging or spatial/temporal binning (Fig. 1). There is a possibility that the spatial/temporal binning deteriorates the shapes of optical signals by combining differing signals. In Fig. 1, a spike-like signal corresponding to an action potential may become smaller after the spatial/temporal binning. In our studies, we usually employ a home-made optical recording system with a $34 \times 34$ photodiode array [7, 20] and a 464-element photodiode system (NeuroPDA, RedShirtImaging, Fairfield, CT, USA).

### Voltage-sensitive dyes

Extensive research has led to marked progress in the development of VSDs for measuring rapid transmembrane voltage changes [for reviews see 21–23]. The VSDs are classified into absorption and fluorescence dyes. The high translucency of embryonic tissue gives a large S/N in absorption measurements, and thus, absorption, rather than fluorescence. VSDs have usually been employed in embryonic preparations [7, 9]. Figure 2 shows examples of optical recording of the depolarization wave [for a review see 14] observed in the embryonic chick CNS stained with a merocyanine-rhodanine absorption dye, NK2761, (Fig. 2a) and a styryl fluorescence dye, di-2-ANEPEQ,

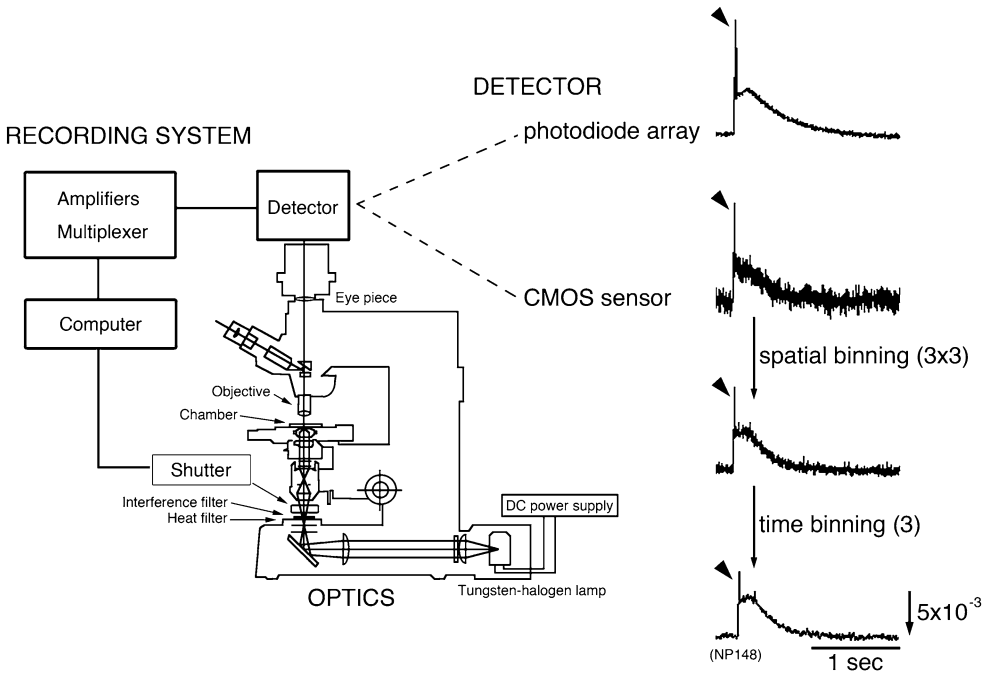


**Fig. 1** Schematic drawing of the optical recording system and optical signals induced by olfactory nerve (N.I) stimulation in a 12-day embryonic chick olfactory bulb. These signals were detected at the same region in one preparation using a 464-element photodiode array (NeuroPDA, RedShirtImaging, Fairfield, CT, USA), or a 128 × 128-element CMOS sensor (NeuroCMOS, RedShirtImaging, Fairfield, CT, USA) with a single sweep. One element of the photodiode array collects light from a round area (diameter = 46 μm), whereas that of the CMOS sensor collects light from a 12.5-$\mu m^2$ *square* region. The frame rate of the photodiode array is almost the same as that of the CMOS sensor (≈1 kHz). To increase the S/N, the optical signal detected using the CMOS sensor was processed with spatial binning (average of 3 × 3 elements) and time binning (average of three points). The *direction* of the *arrow* in the *lower right* indicates an increase in transmitted light intensity (a decrease in dye absorption), and the *length* of the *arrow* represents the stated value of the fractional change. *Arrowheads* indicate spike-like signals corresponding to action potentials

(Fig. 2b) [24]. Even with di-2-ANEPEQ, which performed the best among the fluorescence dyes screened, its S/N was inferior (approximately 20 %) to that of NK2761.

Absorption VSDs have also been favored because they are less phototoxic than fluorescence dyes and can be used in experiments requiring prolonged imaging [25–27]. In absorption VSDs, NK2761 (produced by Hayashibara Biochemical Laboratories Inc./Kankoh-Shikiso Kenkyusho, Okayama, Japan) [28] is the most commonly used dye in embryonic hearts [for reviews see 29, 30] and nervous systems [31, 32, for reviews see 7, 9]. Large optical signals have also been obtained using NK2776, NK3224, and NK3225, with no serious pharmacological or phototoxic effects on the evoked action potential [33].

On the other hand, fluorescence VSDs may be useful for detecting optical signals in preparations of later developmental stages or postnatal/posthatched animals because of their lower translucency. Among the fluorescence VSDs, di-2-ANEPEQ had the largest S/N in the embryonic chick brain, while (1) its photobleaching and detachment of dye bound to the cell membrane were faster (Fig. 3) and (2) the recovery of neural responses after staining was slower than those of di-4-ANEPPS and di-3-ANEPPDHQ (Fig. 4) [24]. Di-4-ANEPPS and di-3-ANEPPDHQ showed large S/Ns (Fig. 4a), together with less photobleaching (Fig. 3). These styryl dyes also exhibited large S/N in cardiac myocyte preparations [34]. However, these dyes also required a relatively long time for the recovery of neural activity after staining (Fig. 4). Furthermore, the background fluorescence intensity changed unstably with time (Fig. 3), the mechanism of which is unknown. Using fluorescence VSDs in the embryonic nervous system, we need to pay attention to the adequacy of the recovery time.

## Application of VSDs to the embryonic nervous system

Since the first application of VSDs to the embryonic nervous system [31, 32], extensive investigations have been performed in the brainstem, forebrain, spinal cord, and peripheral nervous system of chick embryos as well as rat and mouse fetuses. Table 1 summarizes VSD analysis of the functiogenesis of the embryonic nervous system. Some of these investigations were discussed previously in detail [7, 8, 9, 12, 13, 25, 35].

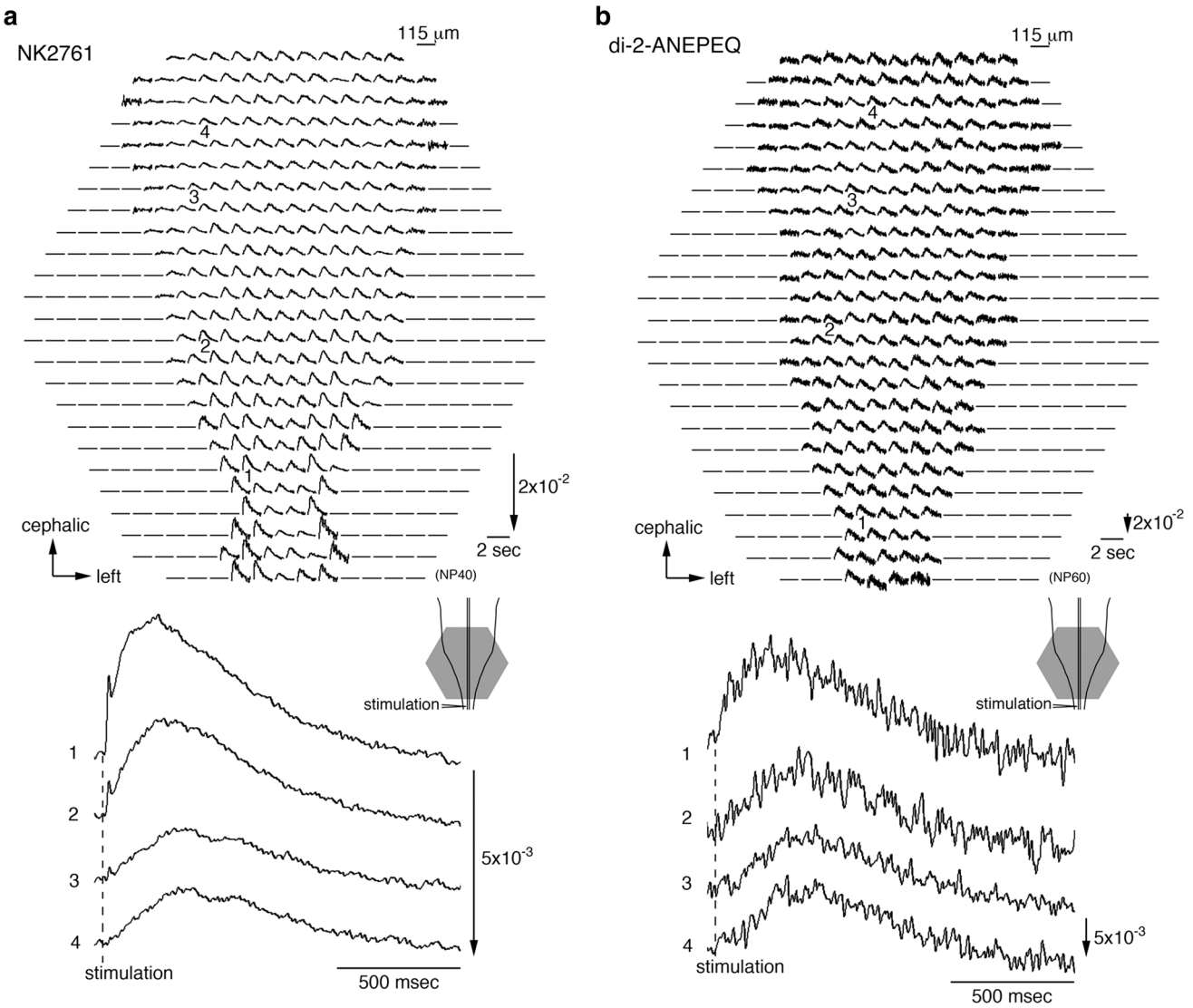


**Fig. 2** Multiple-site optical recording of neural responses to upper spinal cord stimulation in 7-day embryonic chick brainstem-spinal cord preparations. The preparation was stained with a merocyanine-rhodanine absorption dye, NK2761 (**a**), or a styryl fluorescence dye, di-2-ANEPEQ (**b**). The optical signals evoked by electrical stimulation (200 μA/1 ms) were recorded simultaneously from 464 contiguous regions of the preparation with a magnification of ×4 (an objective) ×1.67 (an eyepiece). Electrical stimulation elicited a propagating depolarization wave, widely propagating correlated activity, in the spinal cord and brainstem. Illustrations of the preparations are shown in the lower right, in which the detected areas are marked with *gray hexagons*. The recording was made in a single sweep. For each recording, enlarged traces of the optical signals detected from four regions indicated by *1–4* are shown in the *lower panels*. Modified from Ref. [24]

## Functiogenesis of the chick olfactory system

As an example of the application of VSD recording to the embryonic nervous system, we show investigations of the functiogenesis of the chick olfactory pathway. The chick embryo is easy to handle for ontogenetic analysis. In addition, the chick olfactory nerve (N.I) is long, and we can selectively stimulate the N.I with a suction electrode. Furthermore, re-evaluation of the basic structure of the avian brain has been facilitated in comparison with the mammalian brain by virtue of elucidation of the chick genome [95, 96]. This allows us to gain a deeper understanding of vertebrate brain function through the study of birds.

### Optical detection of neural responses in the olfactory bulb (OB) and forebrain

In a 9-day embryonic N.I-OB-forebrain preparation, N.I stimulation elicited neural responses in the OB and forebrain (Fig. 5a). In the OB, the optical response was composed of a fast spike-like signal, corresponding to a

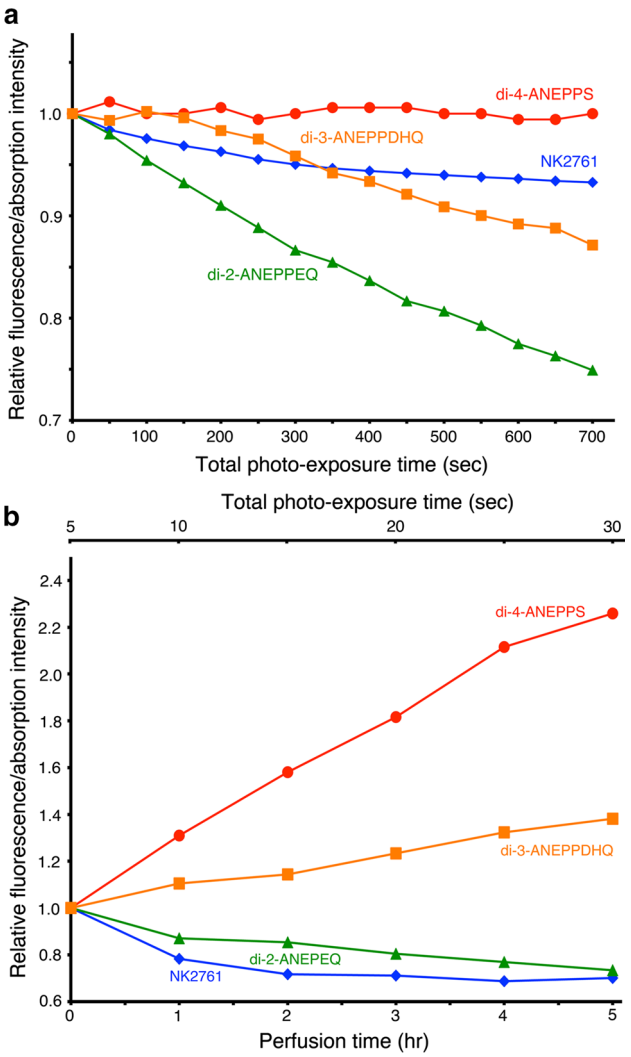


**Fig. 3** **a** Effects of illumination on the fluorescence/absorption background intensity of the dye. The ordinate represents the relative fluorescence/absorption intensity (DC-background light intensity at each time divided by that at time 0), and the abscissa is the time of continuous illumination. **b** Effects of perfusion on the fluorescence/absorption background intensity of the dye. The incident light was turned off except during the measuring period. The ordinate represents the relative fluorescence/absorption intensity (DC-background light intensity at each time divided by that at time 0). The lower abscissa is the time after staining (perfusion rate: 1 ml/min), and the upper abscissa is the total illumination time. In each figure, the results of an absorption dye (NK2761) and three fluorescence dyes (di-4-ANEPPS, di-3-ANEPPDHQ, and di-2-ANEPEQ) are shown. Modified from Ref. [24]

sodium-dependent action potential, and a long-lasting slow signal, corresponding to an excitatory postsynaptic potential (EPSP) [10] (Fig. 5b). Concerning the EPSP-related slow signal, the following features were commonly observed in the OB [10]. (1) Synaptic transmission was mediated by glutamate, and *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors were functionally expressed. (2) The initial phase of the EPSP was attributed to non-NMDA receptors, while the later phase was mediated by NMDA receptors. (3) The EPSP has a duration in the order of seconds. (4) The EPSP readily fatigued. These characteristics were also seen in the embryonic brainstem [for reviews see 7, 8, 9], and may be the basic principles of synaptic transmission in the embryonic CNS.

In the forebrain of a 9-day embryo, the slow signal was observed in a restricted area, which corresponds to the higher-ordered nucleus receiving inputs from the N.I (Fig. 5a) [10]. In pigeons [97–99], efferents of the OB project (1) to the medial septal nucleus and to more dorsal parts of the medial telencephalon through a medial olfactory tract (MOT), (2) to the piriform cortex through a lateral olfactory tract (LOT), and (3) to the olfactory tubercle and rostral part of the lobus parolfactorius (medial striatum: [100]) through an intermediate olfactory tract (IOT). In our recording, we could not determine the anatomical region for the response area in the forebrain because there are some differences between chicks and pigeons and also between embryos and adults.

## Development of functional synaptic connections in the OB and forebrain

At an 8-day embryonic stage, N.I stimulation elicited neural responses in the OB but not in the forebrain in normal physiological solution (Fig. 5c). The removal of $Mg^{2+}$ from the bathing solution, which activated the NMDA receptor function, enhanced the slow signal in the OB. In the forebrain, the removal of $Mg^{2+}$ elicited a small slow signal, indicating that the synaptic function latently emerged in the forebrain at this stage. The ontogenetic investigation (Fig. 5d, e) revealed that: (1) functional synaptic transmission in normal physiological solution appears at around the 6 ~ 7-day embryonic stage in the OB and at around the 9-day embryonic stage in the forebrain, (2) NMDA receptor function is latently expressed earlier than the non-NMDA receptor function at the 6-day embryonic stage in the OB and the 8-day embryonic stage in the forebrain, and that (3) functional synaptic transmission is gradually strengthened during development.

## Oscillations in the embryonic OB

Since its discovery by Adrian in the hedgehog [101], it has been known that odorant molecules induce oscillations, i.e., stereotyped sinusoidal neural activity, in the OB of various species [102]. In the embryonic chick OB, oscillation was also optically detected in addition to fast and slow signals (Fig. 6).

The oscillation had some unique characteristics [11]. (1) The oscillation was distributed in the distal half of the OB near the N.I. (2) The onset was around the 9-day embryonic

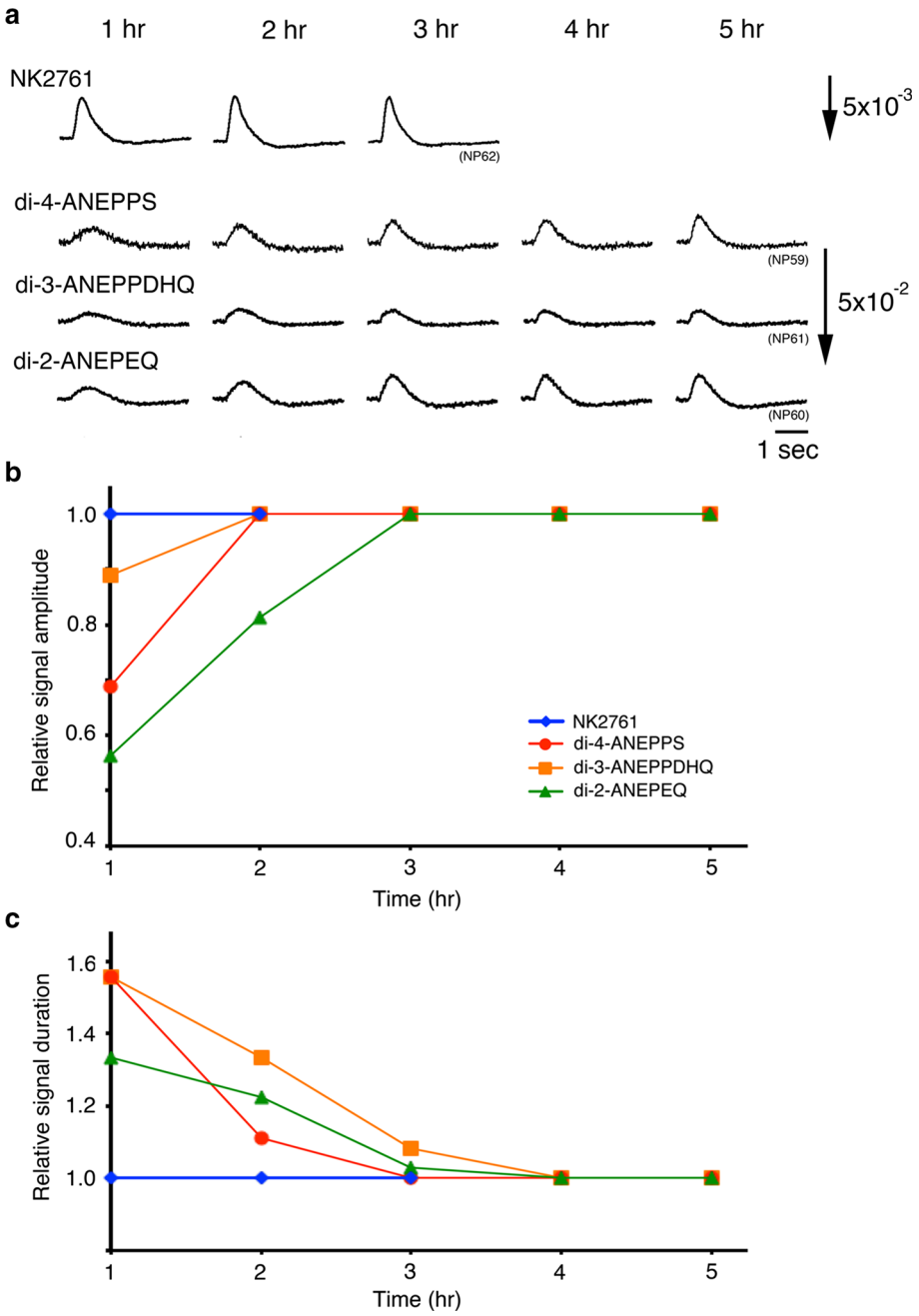


**Fig. 4** Time-dependent changes in optical signals after staining. The preparations were stained with an absorption dye and three fluorescence dyes. **a** Optical signals related to the depolarization wave detected with a magnification of ×10 ×1.67 in single sweeps at 1, 2, 3, 4, and 5 h after staining. **b**, **c** Amplitudes (**b**) and durations (**c**) of the optical signal normalized to the values at 1 h after staining are plotted against time. The signal duration was measured at 50 % of the maximum signal amplitude. Modified from Ref. [24]

stage. (3) The incidence increased with development. (4) The duration and mean frequency showed preparation-to-preparation variations even at the same developmental stage. (5) The duration became longer as development proceeded, while the relative area did not change during the embryonic 9- to 12-day stages. (6) The mean frequency became higher with development, while it remained in the range of the theta oscillation at all stages. These results indicate that the oscillation in the chick OB dynamically changes during embryogenesis. Such changes may continue beyond the 12-day embryonic stage, suggesting that the neural network responsible for the oscillation is still not fully differentiated for at least 12 days.

## Developmental sequence of neural function in the chick olfactory system

Figure 7 summarizes the functional development of the chick olfactory system. In the OB, functional synaptic transmission appears at around the 6 ∼ 7-day embryonic stage. Previous morphological investigations demonstrated the developmental sequence of olfactory structure

**Table 1** Application of voltage-sensitive dyes to the embryonic nervous system

| | |
|---|---|
| Chick olfactory system (I) | Sato et al. [10, 11] |
| Chick visual system (II) | Miyakawa et al. [36] |
| Chick oculomotor, trochlear, and abducens nuclei (III, IV, VI) | Glover et al. [37]* |
| Chick trigeminal nucleus (V) | Momose-Sato and Sato [38] |
| | Sakai et al. [31] |
| | Sato et al. [39] |
| Chick vestibulo-cochlear nucleus (VIII) | Asako et al. [40] |
| | Glover et al. [37]* |
| | Momose-Sato et al. [41] |
| | Sato and Momose-Sato [42] |
| Chick glossopharyngeal nucleus (IX) | Momose-Sato et al. [43] |
| | Sato and Momose-Sato [44, 45] |
| | Sato et al. [46–48] |
| Chick vagal nucleus (X) (including pharmacological studies on NTS neurons) | Kamino et al. [32, 49] |
| | Komuro et al. [50] |
| | Momose-Sato and Sato [51] |
| | Momose-Sato et al. [43, 52–57] |
| | Sato and Momose-Sato [44] |
| | Sato et al. [48, 58–62] |
| Chick spinal cord | Arai et al. [63, 64] |
| | Mochida et al. [65] |
| Rat trigeminal nucleus (V) | Momose-Sato et al. [66, 67] |
| Rat facial nucleus (VII) | Momose-Sato et al. [68] |
| Rat glossopharyngeal nucleus (IX) | Momose-Sato et al. [69] |
| Rat vagal nucleus (X) | Momose-Sato et al. [69–71] |
| | Sato et al. [72, 73] |
| Rat spinal cord | Demir et al. [74] |
| Rat respiratory center | Onimaru and Homma [75] |
| Mouse vagal nucleus (X) | Momose-Sato and Sato [76] |
| Mouse respiratory center | Ikeda et al. [77] |
| | Onimaru et al. [78] |
| Peripheral nervous system | Momose-Sato et al. [79, 80] |
| | Sakai et al. [31, 81] |
| Correlated wave activity | |
| Chick | Arai et al. [64] |
| | Komuro et al. [82] |
| | Mochida et al. [83, 84] |
| | Momose-Sato and Sato [85] |
| | Momose-Sato et al. [86–89] |
| Rat | Momose-Sato et al. [67, 68] |
| | Ren et al. [90] |
| Mouse | Momose-Sato et al. [91, 92] |
| Dye screening and improvements in optical recording systems | Hirota et al. [20] |
| | Momose-Sato et al. [33] |
| | Mullah et al. [24] |
| | Tsau et al. [93] |
| | Wenner et al. [94] |

Roman numerals in parentheses indicate the number of cranial nerves, which were stimulated to induce the response, except for the oculomotor, trochlear, and abducens nuclei (III, IV, and VI), which were identified in the vestibulo-ocular reflex pathway by stimulation of the vestibular nerve

The application of voltage-sensitive dyes in postnatal/posthatched animals is a major branch of optical studies in developmental neuroscience. However, this issue is beyond the scope of this review and not mentioned here. Modified from Ref. [9]

"*" shows that the results are presented in abstract form

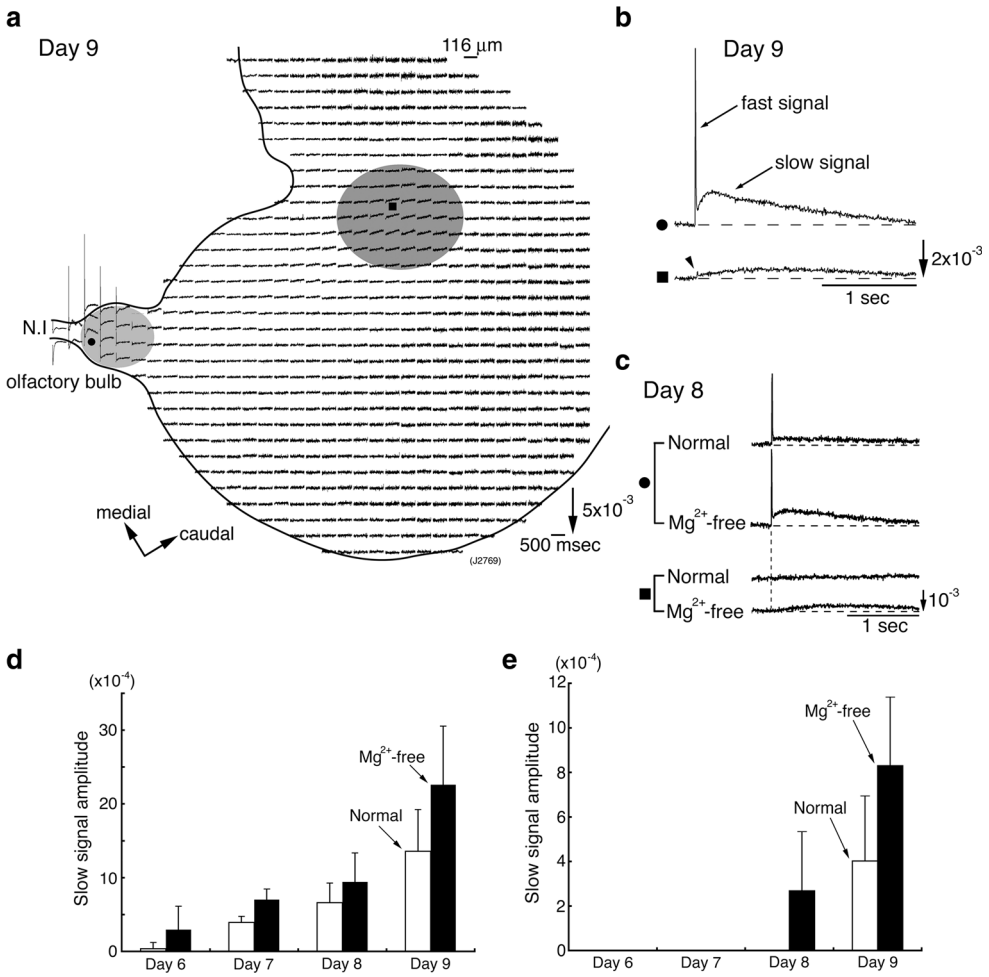


**Fig. 5** **a** Multiple-site optical recording of neural responses to N.I stimulation in a 9-day embryonic N.I-OB-forebrain preparation. In this recording, two response areas were identified: in the OB (shadowed in *light gray*), and in the forebrain (shadowed in *dark gray*). **b** Enlarged traces of the optical signals detected from the OB (*upper trace* marked by a *black circle* in **a**) and the forebrain (*lower trace* marked by a *black square* in **a**). In the *lower trace*, a small spike-like signal corresponding to the presynaptic action potential was identified (marked by an *arrowhead*). **c** Appearance of the slow signal in the forebrain of an 8-day chick embryo upon the removal of $Mg^{2+}$ from the bathing solution. In the forebrain (marked by a *black square*), no slow signal was observed in normal solution, whereas a small slow signal was evoked in $Mg^{2+}$-free solution. **d**, **e** Comparison of the slow signal amplitude in the OBs (**d**) and the forebrains (**e**) at 6- to 9-day embryonic stages. The slow signal amplitudes in normal (*white bars*) and $Mg^{2+}$-free saline (*black bars*) are presented as the mean + SD in the fractional change. Modified from Ref. [10]

formation in chicken embryos [95, 103–108]: (1) the nasal placode forms at the 2-day embryonic stage; (2) the differentiation of nasal pits, the formation of the OB, and growth of the N.I towards the brain occur by the 4-day embryonic stage; (3) the olfactory tuberculum and piriform cortex appear at the 5-day embryonic stage; (4) the olfactory epithelium is observed within the nasal capsule at the 6-day embryonic stage; and (5) the axons of olfactory neurons reach the OB by the 7-day embryonic stage.

Compared with morphogenesis, functional synaptic connections in the OB are considered to be formed at almost the same time as the N.I fibers project into the OB. This result raises the possibility that the responses detected as slow signals are not mediated by mature synapses. Using VSD recording combined with electron microscopic observations, we have shown that synaptic transmission occurs before mature synaptic structures have been formed in the embryonic rat trigeminal system [66]. Similar phenomena were observed in embryonic chick ciliary ganglia [109] and developing neuromuscular junctions in culture obtained from Xenopus embryos [110]. The precise communication mechanisms in these immature synapses are not yet known. Functional communication at immature synapses can be mediated by transmitter release from arriving growth cones [111], chemical transmission between motile axonal and dendritic filopodia, and transmission at morphologically unspecialized pre- and postsynaptic contacts [112].

Optical responses to N.I stimulation in the forebrain were detected from around the 9-day embryonic stage. This means

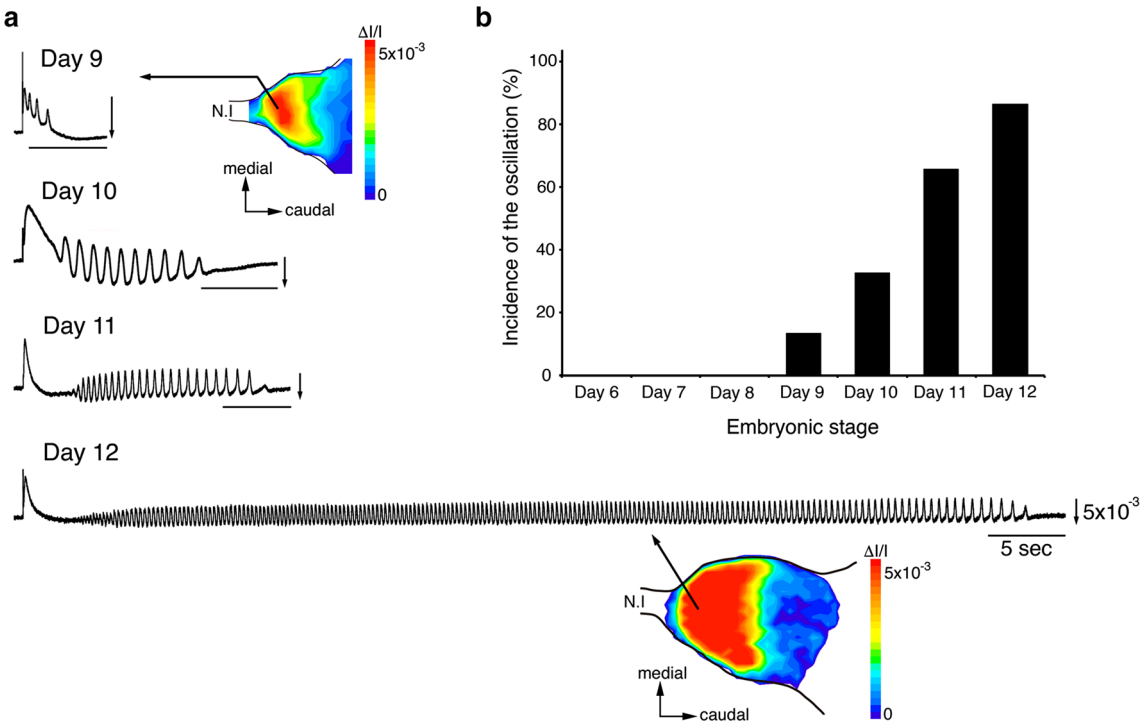


**Fig. 6** **a** Oscillations evoked by N.I stimulation in 9 ~ 12-day embryonic chick OBs. *Color-coded maps* of the oscillation in 9- and 12-day preparations are also shown. **b** Comparison of the incidence of the oscillation in the OB at 6- to 12-day embryonic stages. Modified from Ref. [11]

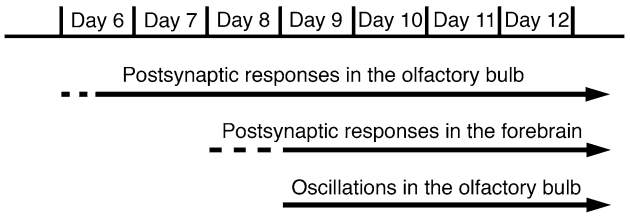


**Fig. 7** Functiogenesis of neural activity in the chick olfactory system

that functional neural circuits from the first- to higher-ordered nucleus are generated by the 9-day embryonic stage. In the chick brainstem sensory nuclei related to the N.VIII [41] and the N.X [62], synaptic transmission between the first- and higher-order nuclei is established by the 7-day embryonic stage. These profiles suggest that neural circuits from the first- to higher-ordered nuclei related to the cranial nerves have the ability to transfer sensory information from the early stages of development, irrespective of being in the brainstem or forebrain.

The appearance of the oscillation was ontogenetically later than the emergence of functional synaptic transmission in the OB. It has been reported that the theta oscillation is supported by low-frequency burst firing of external tufted cells in the glomerular layer [113]. If this is the case in the chick embryo, our results suggest that intra-bulbar neural circuits responsible for the oscillation mature later than synapses between the N.I and mitral/tufted cells.

The role of the oscillation during functiogenesis is unclear. The oscillation in the embryonic chick OB showed a gradual increase in duration with development, and the prolonged oscillation possibly causes a sustained intracellular $Ca^{2+}$ concentration. This might be beneficial for the development of the olfactory system, because the elevated intracellular $Ca^{2+}$ concentration plays an essential role in the development of the CNS, such as neural growth, survival, and differentiation [114–116].

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that there are no conflicts of interest.

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