Optical Analysis of Depolarization Waves in the Embryonic Brain: A Dual Network of Gap Junctions and Chemical Synapses

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Optical analysis of depolarization waves in the embryonic brain: a dual network of gap junctions and chemical synapses. J Neurophysiol 89: 600–614, 2003; 10.1152/jn.00337.2002. Correlated neuronal activity plays a fundamental role in the development of the CNS. Using a multiple-site optical recording technique with a voltage-sensitive dye, we previously described a novel type of depolarization wave that was evoked by cranial or spinal nerve stimulation and spread widely over the whole brain region in the chick embryo. We have now investigated developmental expression and neuronal network mechanisms of this depolarization wave by applying direct stimulation to the brain stem or upper cervical cord of E5–E11 embryos, which elicited wave activity similar to that evoked by nerve stimulation. Spatial distribution patterns of the depolarization wave changed dynamically with development, and this change appeared to be related to the regional differences in neuronal differentiation. The depolarization wave was completely eliminated by application of either gap junction blockers or an N-methyl-D-aspartate (NMDA)-receptor antagonist, indicating that functions of both gap junctions and NMDA receptors are indispensable for wave propagation. A possible interpretation of the results is that dual networks of gap junctions and chemical synaptic coupling mediate large-scale depolarization waves in the developing chick CNS.

INTRODUCTION

Correlated neuronal activity plays a fundamental role in the development of the CNS. Activation of a particular neuronal population is recognized to be essential for shaping aspects of circuit development. These include axonal path finding (Catalano and Shatz 1998; Dantzker and Callaway 1998), neuronal domain formation (Yuste et al. 1995), and circuit refinement/remodeling (Goodman and Shatz 1993; Katz and Shatz 1996). Correlated activity at later developmental stages involves the synchronous activity of neurons of a particular network through their synaptic interactions (O’Donovan 1999). In contrast, activity produced by the early nervous system, before the formation of synaptic networks, is largely mediated by gap junctions (Katz 1993). Characterization of the various modes of circuit activation and their spatiotemporal patterns now seems essential for an understanding of the full extent of the role of correlated activity.

Most of our knowledge about the functional organization of neuronal networks is based on the analysis of firing patterns of individual neurons or neuronal populations using electrophysiological techniques. Although this approach enables assessment of event-related variations in discharge rates, it does not provide information about the extent and spatiotemporal patterns of neuronal activity. Optical recording techniques using Ca2+ indicators or fast voltage-sensitive dyes have made it possible to monitor neuronal activity from many sites and provided a useful and powerful method for analyzing the spatiotemporal patterns of network activity (Grinvald et al. 1988; Salzberg 1983; Tsien 1989; Wu et al. 1998). Ca2+-sensitive dyes have very large signals and provide both an indirect reflection of the electrical activity and a direct measurement of \([\text{Ca}^{2+}]_i\) changes associated with second-messenger systems (Tsien 1989). On the other hand, voltage-sensitive dyes have better temporal resolution and allow direct monitoring of electrical events in cells (Grinvald et al. 1988; Salzberg 1983; Wu et al. 1998). In addition, voltage-sensitive dyes have advantages in terms of detecting cell activity with small \([\text{Ca}^{2+}]_i\) changes and monitoring subthreshold events or inhibitory potentials.

Applying an optical recording method with a fast voltage-sensitive dye, we investigated development and functional organization of the embryonic CNS (for a review, see Momose-Sato et al. 2001a). Throughout the experiments, we found a novel type of depolarization wave that was evoked by vagal stimulation and spread widely from the brain stem to the whole brain region in the chick embryo (Momose-Sato et al. 2001b). This depolarization wave was triggered by glutamate-mediated excitatory postsynaptic potentials (EPSPs) in the nucleus of the tractus solitarius (NTS), and its propagation was dependent on functions of gap junctions. Subsequently, we found that a similar depolarization wave was evoked by dorsal root stimulation in an embryonic chick spinal cord–whole brain preparation (Mochida et al. 2001a). The novelty of these waves was that they traveled widely over almost all of the regions of
the CNS, including the brain stem, cerebellum, spinal cord, and telencephalon.

In the studies mentioned in the preceding text, we could not identify the full profile of wave expression/development because vagal or spinal nerve stimulation does not evoke the EPSP, which triggers the wave, at early embryonic stages (Mochida et al. 2001b; Momose-Sato et al. 1991, 1994). In addition, although the depolarization wave was eliminated by an N-methyl-D-aspartate (NMDA) receptor antagonist (Momose-Sato et al. 2001b), it was not clear whether the result was via the effects on wave initiation (i.e., the effects on the glutamate-mediated EPSP) or wave propagation. To overcome these problems, in the present experiments, we used another experimental approach that allowed us to separately analyze the mechanism of wave propagation without considering triggering synaptic inputs. We applied direct stimulation to an experimental approach that allowed us to separately analyze glutamate-mediated EPSP) or wave propagation. To overcome via the effects on wave initiation (i.e., the effects on the mose-Sato et al. 2001b), it was not clear whether the result was

Buffer (pH 7.3). In the Mg$_2^+$-free experiments, MgCl$_2$ was removed from the bathing solution or replaced with CaCl$_2$. The solution was kept in Ringer solution with the following composition (in mM): 138 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 10 glucose, and 10 Tris–HCl buffer (pH 7.3). In the Mg$^{2+}$-free experiments, MgCl$_2$ was removed from the bathing solution or replaced with CaCl$_2$. The solution was equilibrated with oxygen. The pia mater was carefully removed in the bathing solution under a dissecting microscope. After staining with the dye (see following text), the preparation was attached to the silicone (KE 106LT; Shin-etsu Chemical, Tokyo, Japan) bottom of a simple chamber with the ventral side up by pinning it with tungsten wires. The preparation was continuously perfused with Ringer solution at a rate of 1.5 ml/min at room temperature, 26–30°C, except during the measuring period.

Voltage-sensitive dye staining

The preparation was stained by incubating it for 20 min in a Ringer solution containing 0.2 mg/ml of a voltage-sensitive merocyanine-rhodanine dye, NK2761 (Hayashibara Biochemical Laboratories/ Kankoh-Shikiso Kenkyusho, Okayama, Japan) (Kamino et al. 1981; Momose-Sato et al. 1995), and the excess (unbound) dye was washed away with a dye-free Ringer solution before recording. This merocyanine-rhodanine dye has been shown to be particularly useful in embryonic nervous and cardiac tissues (Kamino 1990, 1991; Momose-Sato et al. 1995).

Extracellular electrical recording

Extracellular field potentials were recorded with a glass microelectrode (0.6–0.9 MΩ resistance) filled with 2M-NaCl and placed in the region of the pons. The signals were amplified with filters set at 0.08 Hz and 1 kHz and digitally recorded at 2 kHz with an A/D converter (PowerLab/8sp, ADInstruments, Castle Hill, Australia).

Optical recording

The preparation chamber was mounted on the stage of an Olympus Vanox microscope (Type AHB-L-1). Bright-field illumination was provided by a 300-W tungsten-halogen lamp (Type JC-24V-300W, Kondo-Philips, Tokyo, Japan) driven by a stable DC-power supply. Incident light was made quasimonochromatic by an interference filter [703 ± 15 (half width) nm; Asahi Spectra, Tokyo, Japan] placed between the light source and the preparation. A microscope objective (×4, S plan Apo, 0.16 n.a.) and a photographic eyepiece (×2,5) formed a magnified (×10) real image of the preparation at the image plane. The transmitted light intensity at the image plane was detected using a multi-element silicon photodiode matrix array. In the present experiments, we used a 1,020-site optical recording system with a 34 × 34-element silicon photodiode array (Hamamatsu Photonics, Hamamatsu, Japan), which was constructed in this laboratory (Hirata et al. 1995). The outputs from 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 multiplexers was fed into a subranging type A/D converter system with a resolution of 18 bits and sent to a computer. The time resolution of the system was ~1 ms (1,024 frames/s). The recordings were made in single sweeps at 10-min intervals. The incident light was turned off except during the measuring period. In these conditions, little or no deterioration of the optical response was observed for a few hours in terms of the amplitude and duration of the signals.

The recorded signals were presented as the fractional change ΔI/I (the change in the light intensity divided by DC background intensity). Color-coded representation for a spatiotemporal activity map (Figs. 3 and 7) was constructed using “Neuroplex” (RedShirtImaging LLC, Fairfield, CT), and that for the maximum signal amplitude (Fig. 8) was made using “Transform” (Fortner Research LLC, Sterling, VA). The color code in each figure is linearly distributed between the minimum and maximum values of ΔI/I.

Materials

The sources of chemicals used for pharmacological experiments were as follows: DL-2-amino-5-phosphonovaleric acid (APV), picrotoxin, bicuculline, strychnine, propranolol, 18β-glycyrrhetinic acid, glycyrrhizic acid, and carbeneoxolone were from Sigma Chemical (St Louis, MO); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was from Research Biochemicals International (Natic, MA); d-tubocurarine (d-Tc), atropine, and tetrodotoxin (TTX) were from Wako Pure Chemical Industries (Osaka, Japan); phenoxylbenzamine was from Tokyo Kasei (Tokyo, Japan); 2-hydroxysalofen was from Toecis Cookson (Bristol, UK).

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Evoked depolarization wave is known to be elicited more frequently in a Mg\(^{2+}\)-free solution than in normal Ringer solution (Momose-Sato et al. 2001b). Thus we first examined whether wave-like activity was elicited by direct stimulation of the brain stem in a Mg\(^{2+}\)-free solution.

Figure 1B shows an example of multiple-site optical recordings obtained from an E8 (stage 34) preparation. When we applied the stimulation to the region near the nucleus of the tractus solitarius (NTS), optical signals with long durations were elicited over the entire region of the preparation. These signals exhibited the wavelength dependence characteristic of NK2761 (data not shown), indicating that they were voltage-dependent absorption changes in the dye, corresponding to depolarization of the membrane potential.

To reveal the relative timing of the optical response, enlarged traces of the optical signals, indicated by asterisks shown in Fig. 1B, are presented in Fig. 2. These overlapping signals showed that there were differences in the delay time of signal onsets between the regions. The appearance of the optical signal in the lateral pons (the root of the cerebellum) preceded that in the medial pons and cerebellum. The spatio-temporal propagation image of the optical signal (Fig. 3A) showed clearly that the depolarizing response spread like a wave, but its propagation was not radial. The propagation pattern of the bipolar-evoked optical signals (Fig. 3A), as well as the waveform and spatial distribution pattern (Fig. 1B), was similar to that of the vagus nerve-evoked depolarization wave (Momose-Sato et al. 2001b: Figs. 14–16).

We reported previously that dorsal root stimulation in the embryonic chick spinal cord induced a depolarization wave that propagated to the brain stem and exhibited distribution patterns similar to that evoked by vagal stimulation (Mochida et al. 2001a). Direct stimulation applied to the upper cervical spinal cord (2 mm caudal to the obex) elicited optical signals which spread through the entire region of the brain stem (Fig. 3B; for raw signals also see Figs. 4, 5, and 10). In this case, the propagation pattern was also nonradial, suggesting that the neuronal network mediating wave propagation is not homogeneous. Although the early phase of signal propagation was not identical, the final distribution patterns shown in Figs. 3, A and B, were almost the same. These results suggest that globally distributed neuronal networks, including brain stem and spinal neurons, are responsible for the depolarization wave.

A depolarization wave similar to that shown in Fig. 3 was detected from every E7–E8 preparation in the Mg\(^{2+}\)-free solution (n = 26). The delay time of signal onsets, however, varied with different preparations. For example, in E8 preparations, when the stimulation was applied to a region near the

![Diagram of neural responses evoked by brain stem stimulation](image)

**FIG. 1.** Multiple-site optical recording of neural responses evoked by brain stem stimulation. A: an example of the relative position of the image of the brain stem preparation (E7) and the 1,020-element photodiode array grids. N. V, trigeminal nerve; N. VIII, cochlear/vestibular nerve; N. IX, glossopharyngeal nerve; N. X/NG, vagus nerve and nodose ganglion. B: simultaneous multiple-site optical recording of neural responses evoked by direct stimulation (200 \(\mu\)A/1 ms) applied with a concentric bipolar electrode to the region near the nucleus of the tractus solitarius (stim.). The preparation was dissected from an E8 (stage 34) embryo. In this and other figures, the recordings were obtained in single sweeps. The signals outside the preparation are omitted. The direction of the arrow (bottom left) indicates an increase in transmitted light intensity (decrease in absorption), and the length of the arrow represents the stated value of the fractional change \(\Delta I/I\) (the change in the light intensity divided by DC background intensity).

**RESULTS**

Depolarization waves evoked by stimulation of the brain stem or cervical spinal cord

As reported previously, the depolarization wave evoked by vagus nerve stimulation propagated to the whole brain region including the brain stem, spinal cord, diencephalon, and cerebrum peduncle (Momose-Sato et al. 2001b). In the present experiments, the optical recordings were made in the brain stem/cerebellum region, as illustrated in Fig. 1A. The preparation was stained with a voltage-sensitive merocyanine-rhodamine dye (NK2761), and the optical responses were recorded using a 1,020-element photodiode array. The vagus nerve-evoked depolarization wave is known to be elicited more frequently in a Mg\(^{2+}\)-free solution than in normal Ringer solution (Momose-Sato et al. 2001b). Thus we first examined whether wave-like activity was elicited by direct stimulation of the brain stem in a Mg\(^{2+}\)-free solution.

Figure 1B shows an example of multiple-site optical recordings obtained from an E8 (stage 34) preparation. When we applied the stimulation to the region near the nucleus of the tractus solitarius (NTS), optical signals with long durations were elicited over the entire region of the preparation. These signals exhibited the wavelength dependence characteristic of NK2761 (data not shown), indicating that they were voltage-dependent absorption changes in the dye, corresponding to depolarization of the membrane potential.

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A depolarization wave similar to that shown in Fig. 3 was detected from every E7–E8 preparation in the Mg\(^{2+}\)-free solution (n = 26). The delay time of signal onsets, however, varied with different preparations. For example, in E8 preparations, when the stimulation was applied to a region near the

![Diagram of neural responses evoked by brain stem stimulation](image)

**FIG. 2.** Relative time course of the optical signals. Enlarged optical signal traces were overlapped to show the relative timing of the optical response. The traces were obtained from the regions indicated by asterisks shown in Fig. 1B.
NTS, the signal latency ranged from 20 to 80 ms in the lateral pons and from 150 to 300 ms in the cerebellum. When the stimulation was applied to the upper cervical cord, variations were relatively small, and the signal delay was 20–35 ms in the lateral pons and 90–130 ms in the cerebellum. Because the signal propagation was not radial and the propagation pathway was unknown, it was difficult to precisely determine the conduction velocity of the depolarization wave. When we assumed that the wave evoked by spinal cord stimulation traveled linearly along the caudorostral axis to the lateral pons and then horizontally to the cerebellum, the conduction velocity was calculated to be 170–250 mm/s in the former, and 8–12 mm/s in the latter, pathway.

In normal Ringer solution, the wave-like activity was also recorded from E7 to E8 preparations, but the frequency was lower than in the Mg\(^{2+}\)-free solution (21 of 27 preparations). In addition, amplitudes of the evoked signals were often smaller (see DISCUSSION). An example of optical recordings obtained in normal Ringer solution is shown in Fig. 4, left. The characteristics of the wave-related optical signals recorded in normal and Mg\(^{2+}\)-free solutions were basically the same. In the following experiments, we examined the profiles of the depolarization wave mainly in the Mg\(^{2+}\)-free solution.

**Developmental changes in the distribution pattern of the depolarization wave**

To investigate developmental expression of the depolarization wave, we examined optical responses to direct stimulation in stage 26 (E5) to stage 37 (E11) preparations. Figure 5 shows examples of optical recordings obtained from stage 27 (E5), stage 29 (E6), stage 31 (E7), stage 34 (E8), and stage 35 (E9) chick embryos. The depolarization wave was evoked by stimulation of the upper cervical spinal cord (E5-E8) or the region near the NTS (E9). In Fig. 6, enlarged traces of the optical signals detected from the regions indicated in Fig. 5 (*) are...
FIG. 4. Depolarization waves in normal and Mg²⁺-free solutions. Direct stimulation was applied to the upper cervical spinal cord of an E8 (stage 33) preparation. The recordings were made in normal (left) and Mg²⁺-free (right) Ringer solutions.

FIG. 5. Developmental changes in the depolarization wave. Simultaneous multiple-site optical recording of bipolar-evoked neural responses obtained from E5 to E9 preparations. Stimulation was applied to the upper cervical spinal cord (E5 to E8) or brain stem (E9).
presented. Although the relative timing of signal appearance was basically the same, the spatial extents of the optical signals were completely different between the stages. In the stage 27 preparation, the optical signals were restricted to the lower brain stem and did not appear in the pons. At stage 29, the response area expanded rostrally, and at stage 31 the activity invaded the cerebellar anlage. The optical responses were most prominent in the stage 34 (E8) preparation, showing maximum spatial extents with large signal amplitudes.

In Figs. 7 and 8 we compared spatial distribution patterns of the depolarization wave in different stages. The images in Fig. 7 represent dynamic propagation patterns of the optical signal, and the maps in Fig. 8 show locations of peak response areas. From these maps, we found that the amplitude of the signals exhibited multiple peaks that were symmetrical with respect to the midline of the preparations; in younger embryos, the distribution pattern was relatively simple becoming more complex as development proceeded; and in the E9 preparation, the responses in the middle region of the brain stem were lacking, although the signals in the cerebellar region were larger than those in the E8 preparation.

According to the distribution pattern observed in the E8 embryo, we determined six regions of signal-peak areas as indicated on the color image of the E8 preparation (Fig. 8). Area 1 is the midregion of the pons, and area 2 is the region just lateral to area 1. Area 3 is positioned in the medial midbrain rostral to area 2, and area 4 is located at the root of the cerebellum. Areas 5 and 6 correspond to caudal and rostral regions of the cerebellum. Although the optical signals with long durations were observed in the lower brain stem in every preparation, we did not evaluate them in the following developmental analysis because they were close to the stimulated site.

To investigate the relationship between embryonic age and regional distribution patterns of the depolarization wave, we examined the incidence of wave activity for each area. Table 1 summarizes the results obtained from stage 26 (E5) to stage 37 (E11) chick embryos. In this table, −, shows that no significant optical signal was detected (ΔH/Δt < 2 × 10⁻⁵); ± means that there were signals but no regional peak, and + indicates that a distinct regional peak of signal amplitude was identified at each location. The data show clearly that there was an age-dependent region specificity of depolarization wave expression. In younger embryos, the wave was restricted to the medial region of the brain stem (areas 1 and 2). As development proceeded, the area expanded to the rostral and lateral regions (areas 3 and 4) and then to the cerebellum (areas 5 and 6). Between the brain stem stimulation (BS) and spinal cord stimulation (SC) groups, no difference was observed.

To quantitatively analyze the developmental changes in wave activity, we measured the values of peak signal amplitudes in each area and plotted them against embryonic age (Fig. 9). The results showed that the wave-related optical response had its maximum amplitude at particular developmental stages. For example, the activity in the midregion of the brain stem (area 1) reached its maximum at stage 29 and remained almost constant until stage 34. Whereas, the root of the cerebellum (area 4) exhibited the largest signals during stages 31–35.

The linearity of the optical signal with changes in membrane potential has been well established: it is thought that the amplitude of the optical signal represents a weighted optical average of the potential change and the active membrane area imaged onto each detector (Kamino et al. 1989; Obaid et al. 1985; Orbach et al. 1985). Considering that the signal-peak areas distributed symmetrically, it is conceivable that they correspond to the brain stem nuclei, in which the activity and/or cellular membrane area are thought to be largest. The spatial distribution pattern of the depolarization wave and its developmental change may reflect the organization and functional differentiation of neural populations involved in the depolarization wave during embryogenesis (see Discussion).

**Neural network mechanisms responsible for the depolarization wave**

It is of interest to know whether the depolarization wave is mediated by chemical synapses or gap junctions. Thus we examined the effects of several blockers on gap junctions and synaptic transmission.

Figure 10A shows the effects of a gap junction blocker, 18β-glycyrrhetinic acid, on the depolarization wave. The preparation was dissected from an E8 (stage 34) embryo, and the depolarization wave was induced by spinal cord stimulation. When we applied 18β-glycyrrhetinic acid (100 μM) to the Mg²⁺-free bathing solution, the depolarization wave was slowly reduced and mostly eliminated after 40 min (Fig. 10A, right). Enlarged traces of the optical signals obtained from areas 2 (medial pons), 4 (lateral pons), and 5 (cerebellum) are presented in Fig. 11. The effect of 18β-glycyrrhetinic acid was
reversible, and the depolarization wave was fully recovered 45–60 min after removal of the blocker from the bathing solution (Fig. 10B, left). Similar results were obtained with another gap junction blocker, carbenoxolone (100 μM). Glycyrrhizic acid (100 μM), an inactive derivative of 18β-glycyrrhetinic acid (Davidson et al. 1986), did not affect the depolarization wave.

Figure 10B represents another experiment made on the same preparation shown in Fig. 10A, using an NMDA-type glutamate receptor antagonist, APV. Surprisingly, the depolarization wave was also abolished completely in the presence of APV (200 μM) (Fig. 10B, right; also see Fig. 11). Similar results were obtained with application of Cd^{2+} (10 μM; Table 2). When we applied tetrodotoxin (TTX: 1 μM) to the bathing solution, the depolarization wave was completely eliminated (data not shown). Wave activities observed in normal Ringer solution and in E7 preparations were also abolished when these blockers were applied to the bathing solution.

These results suggest that functions of NMDA receptors, in addition to gap junctions, are necessary for wave propagation. The TTX sensitivity of the depolarization wave also suggests that action potentials play a fundamental role in wave propagation.

To check the possibility that small currents that may not cause a large voltage change exist in the presence of the blockers, we made extracellular field potential measurements. Figure 12 shows examples of the recordings obtained from a nonstained preparation under a stimulation condition that evoked the depolarization wave. The signals were recorded from the dorsolateral region of the pons. When we applied 18β-glycyrrhetinic acid (middle) or APV (bottom), the signal was completely eliminated. The effects of the blockers were reversible, and the signal was recovered after removal of the blockers from the bathing solution. In the present experiment, in addition to the negative field potential changes, the signals with a positive direction, and with a long duration similar to that shown in Fig. 12, were often recorded from a wide region of the pons. These signals were also eliminated in the presence of 18β-glycyrrhetinic acid and APV. These results confirm that the wave-related ac-

FIG. 7. Spatiotemporal activity maps of the depolarization wave. The images were constructed from the recordings shown in Fig. 5. The frame interval was 20 ms. Red color of the scale bar corresponds to $19 \times 10^{-4}$ (E6), $22 \times 10^{-4}$ (E7 and E8), and $25 \times 10^{-4}$ (E9) in the fractional change.

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tivity was completely blocked by the gap junction blocker and the NMDA receptor antagonist.

In Figs. 10A and 11, we noted that a small signal component remained in the lower brain stem and the lateral pons (area 4) in the presence of a gap junction blocker. This component was reduced in amplitude by APV (Figs. 10B and 11) and Cd²⁺ (data not shown), suggesting that it reflects the synaptic component mediated by NMDA receptors.

To check the specificity of the gap junction blocker, we examined the effects of the drug on vagus nerve- and dorsal-root-evoked EPSPs. In the presence of 18β-glycyrrhetinic acid, the vagal EPSP within the NTS was not significantly affected, although the spinal polysynaptic EPSP was reduced in amplitude (data not shown). These results, together with the uneffectiveness of glycyrrhizic acid, seem to provide a good argument for blocker specificity, although the possibility of unspecific drug effects cannot be excluded.

We tested the effects of other blockers of chemical transmission, such as CNQX (5 μM), d-Tc (100 μM), atropine (10 μM), phenoxybenzamine (10 μM), propranolol (10 μM), picrotoxin (200 μM), bicuculline (200 μM), 2-hydroxysaclofen (200 μM), and strychnine (20 μM), in E7–E8 preparations. Significant effects (consistent reduction in signal amplitudes of >20% of the control signal) were observed with CNQX (a non-NMDA receptor antagonist), d-Tc (a nicotinic acetylcholine receptor antagonist), picrotoxin and bicuculline [γ-aminobutyric acid (GABA)-A receptor antagonists]. Figure 13 presents typical examples of the optical signals obtained from E8 preparations. Unlike APV, although these blockers reduced the amplitude and slowed the onset of wave-related optical signals, they did not abolish wave propagation. To examine whether there was a regional specificity of the effects of the blockers, we measured changes in the signal amplitudes in areas 1–6 (Table 2). Except for APV and Cd²⁺, which showed complete reduction of the signal in every location, the effects of the blockers were variable depending on the preparations, and no significant regional specificity was observed.

DISCUSSION

In the present study, we have unraveled the characteristics of a recently discovered depolarization wave in the embryonic brain, including its developmental profile, spatiotemporal patterns and mechanisms underlying its propagation. The depolarization wave was mediated by multiple types of receptor functions as well as gap junctions. These findings suggest that dual networks of gap junctions and chemical synapses mediate the depolarization wave.

Development of the depolarization wave

The developmental study in the present experiment showed that, at the level of thepons, the wave activity was first expressed at E6 in the medial region, followed by a later appearance in the lateral region and the cerebellum at the E7–E8 developmental stages. In E5 embryos, the optical signals were only detected from the lower brain stem. In the chick embryo, the first differentiated neurons in the medulla (the reticular neurons) become postmitotic about 24 h after incubation (McConnell and Sechrist 1980). Motor and sensory neu-
rons in the cranial nuclei appear subsequently (McConnell and Sechrist 1980), and most of the brain stem nuclei are differentiated morphologically by the E9 developmental stage (Tan and Le Douarin 1991). While, differentiation of the cerebellum is relatively late: production of the cerebellar neurons occurs after the third day of incubation (Fujita 1964), and migration of the progenitor cells from the rhombic lip, a proliferative region, to the developing cerebellum begins at E6 (Hanaway 1967). Production of neurons in the external granule or germinal layer, another proliferative zone, continues until about E18 with the formation of postmitotic granule cells beginning at about E8 (Quesada and Genis-Galvez 1983). The later appearance of the depolarization wave in the cerebellum seems to reflect a chronological sequence of neuronal differentiation. An important question is whether the glial component is involved in the depolarization wave. Generally, glial differ-

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The first and second columns give the embryonic day and Hamburger-Hamilton (H-H) stage, respectively. The third column gives the preparation references: BS and SC indicate that the stimulation was applied to the brain stem and cervical spinal cord, respectively. The fourth to ninth columns show the appearance of the depolarization wave in areas 1–6. –, no significant optical signal was detected (∆M1 < 2 × 10⁻⁴); ±, there were signals but no regional peak; and *, a distinct regional peak of signal amplitude was identified in each area. In stage 37 preparations, area 6 was not identified because there were ≥2 signal-peak areas in the rostral cerebellum (*). **, the corresponding area was not included in the preparation.
Differentiation occurs later in development after neuronal differentiation and migration are largely complete. In the chick embryo, a mature form of neuroglia is not observed before E8 (Fujita 1965; Linser and Perkins 1987; Petralia and Peusner 1990; Thomas et al. 2000), although glial progenitor cells and radial glia-like structures appear earlier (Striedter and Beydler 1997; Thomas et al. 2000). In a previous study (Momose-Sato et al. 2001b), we tested another voltage-sensitive dye, RH482, which is known to be relatively insensitive to glial cell membrane potential changes (Konnerth et al. 1987), and obtained signals similar to those recorded with NK2761. Because the affinity of the dye to glial progenitor cells or radial glia is not known, our data did not allow us to identify the relative contribution of the glial components. Nevertheless, considering the result obtained with RH482 together with the morphological observation of glial differentiation, it seems likely that at least adult-type astrocytes and oligodendrocytes are not the main contributor to the depolarization wave detected from preparations earlier than E8.

Disappearance of the depolarization wave at later developmental stages observed in the medial region of the brain stem requires some consideration. The time of wave disappearance corresponded to the stage when the adult pattern of cytoarchitecture is established in most of the brain stem nuclei (Tan and Le Douarin 1991). The simplest interpretation of this result is that the depolarization wave is only expressed during particular developmental periods, and the ability of neurons to produce the wave becomes lost as development proceeds. Another possibility that should be considered in optical recording is that the medial portion of the brain stem was not stained well with the dye at later developmental stages because of an increase in the preparation thickness. Although we cannot exclude the second possibility, several lines of evidence suggest that disappearance of the depolarization wave cannot be attributable simply to the staining artifact. First, although the midregion of the brain stem (area 1) was thinner than other regions of the brain stem (e.g., areas 2 and 4), the signals there diminished earliest. Second, the thickness of the preparation was almost the same between the lower brain stem and the pons (1,300–1,600 µm at E9 and ~2,000 µm at E11), while the optical responses were recorded from the former, but not the latter, at E10–E11. Third, in some preparations at E9, the distribution pattern of the optical signal changed during the measurements from a laterally restricted pattern (like the recording shown in Fig. 5) to an overall expressed pattern (like a recording from the E8 preparation), suggesting the possibility that the medial brain stem neurons were stained with the dye, but in a relatively inactive state, by unknown mechanisms. Further experiments using slice preparations at various levels of the brain stem may help to address this issue.

Neural network mechanisms responsible for the depolarization wave

An interesting finding in the present study was that the depolarization wave was completely abolished in the presence of either gap junction blockers or APV. These results indicate that both gap junctions and NMDA receptors are indispensable for wave propagation and a functional loss of either of the two results in a blockade of the depolarization wave. Because TTX also blocked the depolarization wave, it seems likely that active conduction of action potentials is involved in the mechanism of wave propagation. The other blockers of chemical transmission, such as CNQX, d-Tc, picrotoxin and bicuculline, showed small effects on the depolarization wave, suggesting that non-NMDA receptors, nicotinic acetylcholine receptors and GABA_A receptors play modulatory, rather than essential, roles in propagation of the depolarization wave.
In the chick embryo, synaptic transmission mediated by NMDA and non-NMDA types of glutamate receptors are already generated at E7–E8 in several brain stem nuclei (Komuro et al. 1991; Momose-Sato et al. 1994; Sato et al. 1995, 1999). Functional GABA receptors are also expressed in E7–E8 brain stems (Momose-Sato et al. 1997): at these stages GABA depolarizes the cell membrane, suggesting its excitatory actions on developing neurons (Momose-Sato et al. 1998). Although functional evidence is not yet available, immunohistochemical studies have shown that nicotinic acetylcholine receptors appear in some brain stem nuclei around at E6 (Torraño et al. 2000). The results obtained in the present experiments suggest that these receptors are widely distributed in the brain stem/cerebellum at early embryonic stages, and play a role in neuronal synchronization.

Coordinated neuronal network activity produced in the early developing CNS is mediated by gap junctions or chemical synapses (Katz 1993; O’Donovan 1999; Roerig and Feller 2000). One hypothesis is as follows: before the formation of synaptic networks, the activity comprises groups of cells coupled by gap junctions, and once chemical synaptic networks form, another type of activity driven by synaptic interaction emerges (O’Donovan 1999). For example, cortical neurons are coupled via gap junctions during early embryogenesis, but this coupling disappears gradually by the end of chemical synaptogenesis (Peinado et al. 1993; Roerig et al. 1996).

The present experimental results suggest that large-scale coactivation of neurons in the early embryonic chick CNS is mediated by coordination of gap junctions and chemical synapses, especially NMDA-type glutamate receptors. It is also possible that APV acts by blocking the actions of extracellular glutamate rather than synaptically released glutamate. If glutamate is present in the extracellular space, blocking its depolarizing action depresses neuronal excitability, possibly below that necessary for wave propagation.

Concerning the mechanisms underlying the interactions of gap junctions and NMDA receptors, one hypothesis is that electrical inputs via gap junctions and NMDA receptors synergistically work in parallel to activate a neuronal network to the suprathreshold level necessary for wave propagation. Another possibility is that NMDA receptors affect gap junctional communication by modulating electrotonic coupling (Pereda and Faber 1996) or changing the transfer function of gap junction-permeable signaling molecules, such as inositol 1,4,5-trisphosphate (InsP3), cyclic adenosine monophosphate (cAMP), and Ca2+ (Sáez et al. 1989; Meyer 1991; Sanderson et al. 1994), via intracellular events linked to Ca2+ permeates through NMDA receptor-channels (Pumain et al. 1987).

The depolarization wave was elicited more frequently in a Mg2+-free solution than in normal Ringer solution. Furthermore, amplitudes of the wave-related optical signals were often larger in the Mg2+-free solution. These results suggest that NMDA receptor functions are partly suppressed by Mg2+ in normal Ringer solution (Mayer et al. 1984; Nowak et al. 1984). Alternatively, it might be possible that low Mg2+ increased the excitability of neurons by shifting the voltage sensitivity of voltage-sensitive channels (Frankenhaeuser and Hodgkin...
Applied to the Mg$_2^+$-free solution. It might be possible that Mg$_2^+$ serves as a regulator of the depolarization wave activity, it seems reasonable to assume that the dual junction blockers in normal Ringer solution also eliminated the wave-related optical signals detected from areas 1–6. The concentrations of the blockers were as follows: 2-amino-5-phosphonovaleric acid (APV, 200 μM), 6-cyano-7-nitroquinazoline-2,3-dione (CNQX, 5 μM), d-tubocurarine (d-Tc; 100 μM), picrotoxin (200 μM), and Cd$_2^+$ (10 μM). The blockers were applied to the Mg$_2^+$-free solution.

### Physiological features of the depolarization wave

Correlated neuronal activity has been observed in a wide variety of circuits in immature nervous systems, including the spinal cord (Milner and Landmesser 1999; O’Donovan et al. 1994; Wenner and O’Donovan 2001), cortex (Garaschuk et al. 2000; Kandler and Katz 1998; Peinado 2000; Yuste et al. 1992, 1995), hippocampus (Avignone and Cherubini 1999; Ben-Ari et al. 1989; Garaschuk et al. 1998; Leinekugel et al. 1997), and retina (Feller et al. 1997; Meister et al. 1991) (for reviews, see O’Donovan 1999; Roerig and Feller 2000). Highly correlated spontaneous activity has also been reported in the embryonic chick hindbrain (Fortin et al. 1994, 1995, 1999). The mechanisms underlying these activities are different and include communication through multiple networks of chemical synapses, as well as gap junctions, in the spinal cord (Chub and O’Donovan 1998; Milner and Landmesser 1999) and retina (Catsicas et al. 1998; Feller et al. 1996; Wong et al. 1998), gap junctional (Kandler and Katz 1998; Yuste et al. 1992) or chemical synaptic (Garaschuk et al. 2000; Peinado 1999) coupling in the cortex, the depolarizing action of GABA$_A$ receptors in the hippocampus (Garaschuk et al. 1998; Leinekugel et al. 1997).

### Table 2. Changes in the optical signal amplitude by the blockers

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<td>d-Tc</td>
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The first column gives the embryonic day, the second column gives the blockers, the third to eighth columns show the effects of the blockers on the wave-related optical signals in areas 1–6. Values in each column indicate the percent reduction in the peak signal amplitude in each area (mean ± SD; n = 3–4). —, no regional peak of signal amplitude was identified. The concentrations of the blockers were as follows: 2-amino-5-phosphonovaleric acid (APV, 200 μM), 6-cyano-7-nitroquinazoline-2,3-dione (CNQX, 5 μM), d-tubocurarine (d-Tc; 100 μM), picrotoxin (200 μM), and Cd$_2^+$ (10 μM). The blockers were applied to the Mg$_2^+$-free solution.

### FIG. 11. Effects of gap junction and NMDA receptor blockers on the depolarization wave

Enlarged traces of the optical signals detected from areas 2 (medial pons), 4 (lateral pons), and 5 (cerebellum). Thick lines correspond to the control signals and the signals in the presence of APV. Thin lines correspond to the signals in the presence of 18β-glycyrrhetinic acid. The signals were obtained from the recordings shown in Fig. 10. 1957) or by enhancing synaptic release of neurotransmitters (Katz and Miledi 1969). Because application of APV or gap junction blockers in normal Ringer solution also eliminated the wave activity, it seems reasonable to assume that the dual junction blockers in normal Ringer solution also eliminated the wave-related optical signals detected from areas 1–6. The concentrations of the blockers were as follows: 2-amino-5-phosphonovaleric acid (APV, 200 μM), 6-cyano-7-nitroquinazoline-2,3-dione (CNQX, 5 μM), d-tubocurarine (d-Tc; 100 μM), picrotoxin (200 μM), and Cd$_2^+$ (10 μM). The blockers were applied to the Mg$_2^+$-free solution.

### FIG. 12. Effects of gap junction and NMDA receptor blockers on the field potential change

The field potential change was recorded extracellularly from the dorsolateral region of the pons. The signals were obtained from a non-stained E8 (stage 34) preparation. Stimulation was applied to the upper cervical spinal cord. Other experimental conditions were the same as those in Fig. 10.
made 10 min after application of the drug to the Mg\(^{2+}\) were made in a Mg\(^{2+}\) medial region of the pons (corresponding to area 2). The control recordings of the depolarization wave detected from E8 (stage 34) preparations. Stimulation was by tubocurarine (d-Tc, 10 \(\mu\)M; A), \(d\)-tubocurarine (d-Tc, 10 \(\mu\)M; B), and picrotoxin (200 \(\mu\)M; C) on the depolarization wave detected from E8 (stage 34) preparations. Stimulation was applied to the upper cervical spinal cord. The signals were obtained from the medial region of the pons (corresponding to area 2). The control recordings were made in a Mg\(^{2+}\)-free solution, and the recordings with the blocker were made 10 min after application of the drug to the Mg\(^{2+}\)-free solution.

FIG. 13. Effects of synaptic transmission blockers on the depolarization wave. Effects of 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 5 \(\mu\)M; A), \(d\)-tubocurarine (d-Tc, 10 \(\mu\)M; B), and picrotoxin (200 \(\mu\)M; C) on the depolarization wave detected from E8 (stage 34) preparations. Stimulation was applied to the upper cervical spinal cord. The signals were obtained from the medial region of the pons (corresponding to area 2). The control recordings were made in a Mg\(^{2+}\)-free solution, and the recordings with the blocker were made 10 min after application of the drug to the Mg\(^{2+}\)-free solution.

al. 1997), and the inhibitory action of GABA\(_A\) receptors together with glutamatergic excitation in the hindbrain (Fortin et al. 1999).

Concerning the roles of the correlated activity in the developing CNS, one traditional view is that activity-dependent processes are operating and contribute to developmental organization of neuronal circuits or neuronal domains with specific functions (Goodman and Shatz 1993; Katz and Shatz 1996; Yuste et al. 1995). The depolarization wave reported here was not restricted to a specific region and showed a wide range of wave propagation (also see Momose-Sato et al. 2001b). In addition, we observed preliminarily that the depolarization wave was triggered by multiple types of peripheral inputs, such as general/special somatic and visceral nerve afferents, and spontaneous activity (Momose-Sato et al. 2003). These profiles suggest the possibility that the depolarization wave may not serve as a simple regulator of specific neuronal circuit formation but plays more global roles in CNS development. The depolarization wave studied in the present experiment was only expressed at particular stages of embryonic development. This profile also suggests that the depolarization wave may not simply reflect the network activity of an immature respiratory rhythm generator that is thought to be located within the hindbrain.

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