Optical Imaging of Large-Scale Correlated Wave Activity in the Developing Rat CNS

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INTRODUCTION

The vertebrate CNS consists of a large number of neurons that are synthetically interconnected in a precise manner. Electrophoretically encoded information within the CNS is conducted through specific neuronal circuits, which enable the expression of proper behavior. In the developing CNS, immature synaptic networks generate correlated neuronal activity in a wide range (for reviews, see Ben-Ari 2001; Champagnat and Fortin 1997; Feller 1999; O’Donovan 1999; Roerig and Feller 2000). Although the roles of this activity during ontogenesis remain unresolved, one traditional view is that it contributes to the activity-dependent developmental organization of neuronal circuits with specific functions (Goodman and Shatz 1993; Hanse et al. 1997; Katz and Shatz 1996; Sur and Leamley 2001). For example, in the developing mammalian cortex, the synchronized activity of neurons in locally correlated areas, termed neuronal domains, has been suggested to lead to a columnar unit of interconnected neurons in the adult brain (Yuste et al. 1992). In slice preparations including the retina and lateral geniculate nucleus, the bursting activity of retinal ganglion cells is transmitted to the lateral geniculate nucleus, suggesting a role for retinal inputs in the regulation of visual pathway formation (Mooney et al. 1996).

A recent finding of a novel form of correlated wave activity in the embryonic chick CNS suggested another possibility concerning the role of the correlated activity during embryogenesis. This activity was recorded using a multiple-site optical recording technique with a fast voltage-sensitive dye as widely spreading depolarizing optical signals and was termed the depolarization wave (Momose-Sato et al. 2001b). A novel phenomenon of the depolarization wave is that it travels over a wide region of the CNS including the medulla, spinal cord, pons, cerebellum, midbrain, and telencephalon (Mochida et al. 2001; Momose-Sato et al. 2001b, 2003a). Another outstanding feature is that it is nonspecifically triggered by various types of cranial and spinal sensory inputs as well as endogenous spontaneous activity (Mochida et al. 2001; Momose-Sato et al. 2003b). These depolarization wave profiles suggest that it may not serve as a simple regulator of specific neuronal circuit formation but might play a more global role in CNS development (Momose-Sato et al. 2003b).

An important question is whether correlated activity similar to the depolarization wave is generated in other species, especially in mammals. The aim of the present study was to examine whether widely propagating wave activity can be observed in the embryonic rat CNS and if so, then to examine the characteristics of the wave activity including spatiotemporal patterns and pharmacological natures for comparison with the chick depolarization wave. Preliminary results have appeared in abstract form (Momose-Sato and Sato 2004; Sato et al. 2004).

METHODS

Preparations

The experiments were carried out in accordance with the guidelines of Tokyo Medical and Dental University for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. Wistar rats at 15–17 days gestation (E15–E17; Saitama Experimental Animals Supply, Saitama, Japan) were used. Females were caged with males in the evening and were checked for
sperm positivity the next morning, and this day was termed E0. Pregnant rats were anesthetized with ether, and their fetuses were then surgically removed. To investigate the spatial distribution pattern of evoked waves of activity, three types of semi-intact preparations were used: Type-A, brain stem preparations that included the medulla, cervical spinal cord, pons, cerebellum with or without the midbrain (Fig. 1A); Type-B, whole brain preparations that included the diencephalon and telencephalon in addition to Type-A preparation; Type-C, spinal cord preparations that included the medulla and spinal cord. Most experiments were carried out using E16 brain stem (Type-A) preparations. The number of tested preparations and the incidence of wave activity are summarized in Table 1. The preparations were kept in a bathing solution of (in mM) 149 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 10 glucose, and 10 Tris-HCl buffer (pH 7.4), and the solution was equilibrated with oxygen. In some experiments, we used artificial cerebrospinal fluid (ACSF) that contained (in mM) 124 NaCl, 5 KCl, 1 MgSO₄, 2.5 CaCl₂, 1.25 NaH₂PO₄, 22 NaHCO₃, and 10 glucose, equilibrated with a mixture of 95% O₂-5% CO₂ (pH 7.4) and obtained similar results. The pia mater was carefully removed in the bathing solution under a dissection microscope, and the preparation was next stained by incubating it for 10–20 min in the solution containing 0.2–0.4 mg/ml of the voltage-sensitive merocyanine-rhodanine dye NK2761 (Hayashibara Biochemical Laboratories/Kankoh-Shikiso Kenkyusho, Okayama, Japan) (Momose-Sato et al. 1995). After staining, the preparation was attached to the silicone bottom of a recording chamber with the ventral or dorsal side facing up by pinning it down with tungsten wires. The preparation was continuously perfused with the bathing solution at 2–3 ml/min at 26–30°C. Wave activity was evoked by applying square current pulses to the upper cervical cord 2–3 mm caudal to the obex using a concentric bipolar tungsten electrode at 200 μA/1 ms or to the cranial nerves with a glass micro-suction electrode at 8 μA/5 ms.

Materials

For the pharmacological experiments, DL-2-amino-5-phosphonovaleric acid (APV), strychnine, bicuculline, picrotoxin, tetrodotoxin, octanol, and 18β-glycyrrhetinic acid were acquired from Sigma

FIG. 1. Optical recording of wave activity in the embryonic rat brain. A: schematic drawing of a brain stem preparation without the midbrain. The brain was cut at the caudal midbrain, and the brain stem-cervical spinal cord (shadowed) was stained with the voltage-sensitive merocyanine-rhodanine dye NK2761. After staining, the preparation was flattened by bilaterally reflecting the cerebellum and was fixed with tungsten wires to the recording chamber with the ventral side face-up. Stimuli (200 μA/1 ms) was applied to the upper cervical cord 2 mm caudal to the obex via a concentric bipolar electrode (stim), and simultaneous 1,020-site optical recordings were made from a region indicated by the square. G. VIII, vestibulo-cochlear ganglion. B: multiple-site optical recording of membrane potential changes evoked by direct stimulation of the upper cervical cord in an E16 brain stem preparation. The optical signals are arranged according to the positions of the photodiode array elements, and the signals outside the preparation are omitted for clarity. Each element (pixel) of the photodiode array detected light transmitted by a square region of 116 x 116 μm² of the preparation. The arrow pointing to the lower right of the recording indicates an increase in transmitted light intensity (decrease in absorption), and the length of the arrow represents the stated value of the fractional change Δ/I, the change in the light intensity divided by the DC background intensity. The upward direction of the signal corresponds to membrane depolarization. In this and other figures, the recordings were obtained in single sweeps. C: wavelength dependence of the optical signal. The optical signal detected at 700 nm was not observed at 630 nm, where the membrane potential-related absorption change in the dye is absent. D: simultaneous recordings of the optical response and electrical signal. The electrical signal was recorded from the vagus nerve root with a suction electrode, and the optical signal was detected from the region corresponding to the vagal motor nucleus.

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The incidence of wave activity elicited by direct stimulation of the upper cervical cord and stimulation applied to the cranial nerves. The upper values show the number of preparations in which the wave activity was observed in normal solution, and the lower values show the number of trials with wave activity in the wave-positive preparations, which was evaluated after the wave was restored following dissection. Values in parentheses are in percentages. For cranial nerve stimulation, the percentage of success in inducing waves was assessed for the first stimulation of a series of repetitive stimuli. N. V1, ophthalmic branch of the trigeminal nerve; N. V2, maxillary branch of the trigeminal nerve; N. V3, mandibular branch of the trigeminal nerve; N. X, vagus nerve. For details on the preparations, see METHODS.

TABLE 2. Comparison of the spatial extent of wave activity

<table>
<thead>
<tr>
<th>Trigger Source</th>
<th>Lumbar Spinal Cord</th>
<th>Cervical Spinal Cord</th>
<th>Medulla</th>
<th>Pons</th>
<th>Cerebellum</th>
<th>Midbrain</th>
<th>Diencephalon</th>
<th>Telencephalon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct stimulation</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+ (n = 5) or − (n = 16)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>N. V2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>N. V3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>N. X</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+ (n = 1) or − (n = 2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The spatial extent of wave activity elicited by direct stimulation of the upper cervical cord and stimulation applied to the cranial nerves. The second to ninth columns show the appearance of the wave activity in each area. Data were obtained from E16 preparations shown in Table 1. For each stimulation, similar results were obtained from every preparation except the cerebellum for which the incidence of the wave is indicated with the number of preparations (n). For direct stimulation, data obtained from three types of preparations were pooled.

TABLE 3. Comparison of the half-width duration of the wave-related optical signal

<table>
<thead>
<tr>
<th>Trigger Source</th>
<th>Rostral Medulla, s</th>
<th>Pons, s</th>
<th>Cerebellum, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct stimulation</td>
<td>1.2 ± 0.2 (29)</td>
<td>1.2 ± 0.2 (32)</td>
<td>0.8 ± 0.2 (5)</td>
</tr>
<tr>
<td>N. V2</td>
<td>1.2 ± 0.3 (13)</td>
<td>1.1 ± 0.1 (6)</td>
<td>1.0 ± 0.1 (3)</td>
</tr>
<tr>
<td>N. X</td>
<td>1.2 ± 0.1 (7)</td>
<td>1.2 ± 0.1 (9)</td>
<td>1.0 ± 0.1 (3)</td>
</tr>
</tbody>
</table>

The half-width duration of the wave-related optical signals elicited by direct stimulation of the upper cervical cord and stimulation applied to the cranial nerves. Optical signals with the largest amplitude were identified in the rostral medulla, pons and cerebellum, and the duration at the 1/2 amplitude was measured (mean ± SD). Data were obtained from E16 preparations. In parentheses, are the number of waves.
ShirtImaging LLC, Fairfield, CT), and that for the maximum signal amplitude was performed using “Transform” software (Fortner Research LLC, Sterling, VA). The color code for each figure was linearly distributed between the minimum and maximum values of ΔI/II.

**Ca**<sup>2+</sup> fluorescence imaging

For the detection of changes in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]), we used the Ca<sup>2+</sup> indicator Ca green-1 AM (Molecular Probes, Eugene, OR). The preparation was stained by incubating it for 60 min in HEPES-buffered (HEPES-NaOH, pH 7.4, 10 mM) Ringer solution containing 20 μg/ml Ca green-1 AM with 0.5% dimethyl-sulfoxide (DMSO) and 0.005% cremophor. It was then perfused with dye-free Ringer solution for 60 min before recording. The preparation chamber was mounted on an inverted microscope (Axiovert 135TV, Carl Zeiss, Göttingen, Germany), and a 75-W Xenon lamp provided epi-illumination of the field. The excitation light was passed through a heat filter and a band-pass filter (450–490 nm) to the preparation, and the emitted light was then passed through a dichroic mirror (510

**FIG. 2.** Spatiotemporal pattern of wave activity. A: pseudocolor spatio-temporal images of the optical signals shown in Fig. 1B. The frame interval was 30 ms, and stimulation was applied during the first frame. B: spatial distribution patterns of the wave-related optical signals. Color-coded (left) and contour line (right) maps represent the maximum optical signal amplitude at each element within two seconds after stimulation. For the contour line map, 4 peak areas were discriminated, indicated with white asterisks in the left map. C: enlarged traces of the optical signals detected from 4 peak areas identified in B. Red: cerebellum, green: medial pons, blue: lateral pons, purple: rostral medulla. Note that there were delays in signal onset between the regions. D: an example of biphasic optical signals recorded from the medial pons. E: an isochrone map of the wave-related optical signals shown in A. Each solid line shows the wave front at intervals of 20 ms, and a dotted line show the wave front 30 ms after stimulation. Wave propagation was not radial, and the appearance of signals in the medial pons and cerebellum was delayed compared with that of the lateral pons.
TABLE 4.  Comparison of the onset latency of the wave-related optical signal

<table>
<thead>
<tr>
<th>Trigger Source</th>
<th>Nucleus, ms</th>
<th>Rostral Medulla, ms</th>
<th>Pons, ms</th>
<th>Cerebellum, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct stimulation</td>
<td></td>
<td>9–24</td>
<td>22–47</td>
<td>98–134</td>
</tr>
<tr>
<td>N. X</td>
<td>5–15</td>
<td>541–2299</td>
<td>610–2472</td>
<td></td>
</tr>
</tbody>
</table>

The delay of wave-related optical signals elicited by direct stimulation of the upper cervical cord and stimulation applied to the cranial nerves. The onset latency of the optical signal was identified in the peak signal area located in the rostral medulla, pons, and cerebellum. For cranial nerve stimulation, the onset latency of the excitatory postsynaptic potential was also identified in the center of each sensory nucleus. The upper values show the onset latency of the signal in each area. The lower values show the differences in the onset latency between two regions, together with the conduction velocity. Data were obtained from E16 preparations. Values in parentheses are in millimeters/second.

![Diagram](http://example.com/diagram.png)

**FIG. 3.** Rostrocaudal extent of wave propagation. The propagation of wave activity was examined in a whole brain (A) and intact spinal cord (B) dissected from E16 and E17 embryos, respectively. For the whole brain preparation (A), the dorsal midline of the tectum and cerebellum was cut, and the preparation was flattened by bilaterally reflecting the cerebrum, tectum and cerebellum. Stimuli were applied to the upper cervical cord. Simultaneous 1,020-site optical recordings were made in four areas of each preparation by moving the photodiode array over the image of the preparation: the displayed images were then reconstructed by combining individual recordings. The frame interval was 60 ms for A and 30 ms for B. Lower insets: color-coded maximum signal amplitudes (left) and waveforms (right) of the optical signals. *Inset A:* the amplitude scale at the top of the trace is different from that of the others. *Inset B:* “pin” indicates tungsten wires which were to fix the preparation. LS, lumbosacral spinal cord.
nm) and a band-pass filter (515–565 nm). The objective (Plan NEOFLUAR, ×5, 0.15 NA) projected a real image of the preparation (magnification: ×5) onto a cooled CCD camera system (PXL 3700, Photometrics, Tucson, AZ) mounted on the microscope, where the camera head contained a half-masked 512 × 512 pixel CCD chip operated in the frame-transfer mode and the system had a 12-bit dynamic range. Groups of 2 × 2 pixels were binned together, and images were recorded with a frame interval of 500 ms.

Electrical recording

Wave activity was electrically monitored by recording the population activity of vagal motoneurons with a suction electrode applied to the cut end of the vagus nerve root. Signals were amplified with filters set at 0.08 Hz and 1 kHz and digitally recorded at 4 kHz with an A/D converter (MacLab/8S, ADInstruments, Castle Hill, Australia).

FIG. 3. (continued)
Immunohistochemistry

Ten fetal (E16) rats were used. Their brains and spinal cords were removed and immersed in cold (4°C) fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), for 2–3 h, and the tissues were then cryoprotected in 10 mM PB containing 30% sucrose. They were finally directly embedded in Tissue-Tek/O.C.T. (Sakura Finetechnical, Tokyo, Japan) and rapidly frozen in liquid nitrogen. Tissues from the liver of adult rats (n = 2) were used as a positive control. Horizontal sections of the flattened brains and spinal cords were cut at a thickness of 6–8 μm using a cryostat, air-dried, and processed following the labeled streptavidin-biotin (LSAB) method.

FIG. 4. Wave activity evoked by trigeminal nerve stimulation. A: pseudocolor images of membrane potential changes evoked by stimulation of the maxillary branch of the trigeminal nerve (N. V2). The preparation was dissected from an E16 embryo with the right trigeminal nerve intact and recordings were made in the region indicated by the square in C. Top: maxillary nerve stimulation with a glass micro-suction electrode (8 μA/5 ms) evoked optical signals in a restricted region that corresponded to the trigeminal nuclear complex (Nc. V). Bottom: stimulation of the nerve with the same intensity elicited spreading wave activity after the nuclear response. The frame interval was 40 ms. B: enlarged traces of the wave-related optical signals induced by maxillary nerve stimulation. The signals were detected from the trigeminal nuclear complex (position 1), rostral medulla (positions 2 and 3), and lateral pons (position 4) shown in C. C: spatial distribution pattern of wave-related optical signals induced by maxillary nerve stimulation. The meningial tissue surrounding the trigeminal ganglion was not removed to keep the trigeminal nerve root intact, and thus optical signals were not detected from this region because of insufficient staining.
using a Dako LSAB2 kit (DakoCyto, Kyoto, Japan). The sections were washed three times for 10 min each in 10 mM phosphate-buffered saline (PBS, pH 7.4), and then endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 min at room temperature. After washing in PBS, the sections were blocked for 30 min in 10% normal goat serum (Vector Laboratories, Burlingame, CA), followed by incubation overnight at 4°C with the primary antibodies against connexins. In the rat embryo, connexins26, -32, -43, -45 are expressed in the cortex and/or midbrain floor (Leung et al. 2002; Nadarajah et al. 1997), although no study has been carried out on the lower brain stem. Thus we examined the immunoreactivity of these connexins in the brain stem region, in which the optical signals were most prominent. We used 1/200 diluted rabbit polyclonal anti-mouse connexin26 antibody (Chemicon International, Temecula, CA), 1/100 diluted mouse monoclonal anti-connexin32 antibody (Zymed Laboratories, South San Francisco, CA), 1/100 diluted rabbit anti-connexin43 antibody (Zymed Laboratories), and 1/100 diluted rabbit polyclonal anti-connexin45 antibody (Chemicon International). The sections were then washed again three times in PBS, incubated with streptavidin conjugated to horseradish peroxidase (DakoCyto) for 10 min at room temperature, and reacted for 10 min in 50 mM Tris-HCl buffer (pH 7.4) containing 0.02% 3,3′-diaminobenzidine (DAB) and 0.005% hydrogen peroxide. Control sections were treated using the same immunohistochemical protocol except for incubation with primary antibodies. No immunostaining was seen in the control sections. After counterstaining with hematoxylin, the sections were dehydrated in a graded alcohol series, cleared with xylene, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). Photomicrographs were then taken using a microscope (Nikon ECLIPSE E600, Tokyo) equipped with a Nikon DCM digital camera system at a resolution of 3,600 × 2,880 pixels. The captured images were processed using Adobe Photoshop software (Adobe Systems) and were printed at a resolution of 300 dpi.

**RESULTS**

**Widely propagating depolarizing wave activity in the embryonic rat CNS**

In the present study, we first examined whether correlated wave activity was evoked by direct stimulation of the upper cervical cord, which had been shown in the chick embryo to induce depolarization waves (Momose-Sato et al. 2003a). Figure 1B shows an example of the optical recordings obtained from an E16 brain stem preparation. When we applied stimuli to the upper cervical cord, depolarizing optical signals were evoked throughout the medulla, pons, and cerebellar primordium. Optical signals were not detected at 630 nm (Fig. 1C), where the membrane potential-related absorption change of NK2761 is absent (Momose-Sato et al. 1995), indicating that the obtained signals were voltage-dependent absorption changes in the dye and not intrinsic signals or mechanical
artifacts. Similar results were obtained from 24 brain stem preparations dissected from E15 to E16 embryos, although the responses in the cerebellum were significant \((\Delta I/I \geq 2.0 \times 10^{-4})\) only in five E16 preparations (Tables 1 and 2).

In Fig. 1D, an enlargement of the optical signal is shown together with an electrical signal which was recorded from the vagus nerve root with a suction electrode. The time course of the optical signal was coupled to the DC potential change in the electrical signal, suggesting that the depolarization of cranial motoneurons was included in the wave activity. The wave-related optical signal exhibited a smooth waveform with a long duration in the order of seconds (also see Table 3), and fast spike-like discharges were not clearly identified. The magnitude of the optical signal represents the weighted optical
average of the potential change and the active membrane area imaged onto each detector (Kamin et al. 1989; Obaid et al. 1985; Orbach et al. 1985). Thus if the action potentials were asynchronous between neighboring neurons or originated from a small active membrane area, it is possible that they were not observed as clear spike-like signals.

The pseudocolored images of the wave activity shown in Fig. 2A indicate that the optical signals spread like a wave with a unique spatial distribution pattern that was symmetrical with respect to the midline of the preparation. Contour line maps of the signal amplitudes (Fig. 2B) allowed us to identify several peak areas, which were located in the rostral medulla, lateral pons, medial pons, and caudal cerebellum as indicated by white asterisks on the left map. In each peak area, the optical signals exhibited a similar monophasic waveform lasting a few seconds (Fig. 2C, Table 3). In the medial pons, biphasic optical signals were sometimes recorded (Fig. 2D), suggesting that neural populations with different onsets of optical responses might have been dorsoventrally overlapped (Momose-Sato et al. 2003b).

As seen in Fig. 2C, there were delays in the signal onset between regions. To calculate the propagation velocity, we measured the delay time of each signal. An isochrone map of the wave front (Fig. 2E) showed that wave propagation was not radial, and the appearance of signals in the medial pons and cerebellum was usually delayed compared with that of the lateral pons (also see Table 4). When we assumed that the wave evoked in the spinal cord traveled along the caudorostral axis to the lateral pons and was then horizontally propagated to the cerebellum, the conduction velocity was 34–158 mm/s (mean ± SD = 93.8 ± 27.6 mm/s; n = 41 events for 7 preparations) for the former and 6–9 mm/s (7.3 ± 1.3 mm/s; n = 8 events for 2 preparations) for the latter pathway. It was not clear why the conduction velocity was different between the regions. This may reflect differences in neural networks or differences in developmental maturity between the regions.

In the present study, the preparations were stained with a bath-applied voltage-sensitive dye, so it is possible that cell populations other than neurons contributed to the optical signals. The conduction velocities calculated in the preceding text were faster than the speed of the Ca$^{2+}$ waves passing through astrocytes and radial glia (Dani et al. 1992; Weissman et al. 2004), suggesting that the wave in the present study is different from previously reported glial waves. We tested the oxonol dye RH482 (NK3630), which is relatively insensitive to glial cell membrane potentials in the skate cerebellum (Konnerth et al. 1987), and observed similar wave activity, although this does not exclude the possibility of a glial cell contribution to the optical signal because the cell specificity of RH482 has not been proven in the embryonic rat brain.

It was interesting to determine to what extent the waves were propagated within the embryonic brain. Figure 3 shows the propagation of the optical signals in the whole brain (Fig. 3A) and intact spinal cord (Fig. 3B) dissected from E16 to E17 embryos. When we applied stimuli to the upper cervical cord, optical signals were propagated rostrally to the medulla, pons, and midbrain (Fig. 3A). Small signals were also observed in the thalamus and part of the cerebrum.

**FIG. 6.** Wave activity vs. localized postsynaptic response. A: multiple-site optical recordings of neural responses evoked by N. V2 stimulation in an E16 preparation. Left: optical signals recorded within 1 s after stimulation. Right: expanded time base of the early phase (125 ms) of the left recording. The signals were obtained from a square region (right inset). The dotted vertical lines indicate the midline of the preparation. Contralateral responses with a short latency were identified in the region surrounded by the dotted circle. Right inset: maximum amplitudes of the wave-related optical signals are shown by a color-coded representation. G. V: trigeminal ganglion. B: enlarged traces of the optical signals detected from the trigeminal nuclear complex (red asterisk), the contralateral short-latency response area (green asterisk), and the region with wave activity alone (blue asterisk). The signals were obtained from the region indicated on the right of recording A. Right: amplified, expanded time base of the left recordings. Note that there was a small delay between the signals in the trigeminal nuclear complex (red asterisk: a part of the right trace is truncated) and the contralateral response area (green asterisk). C: left: distributions of the optical signals related to the excitatory postsynaptic potential (EPSP) in the Ne. V (black), contralateral short-latency responses (dark gray) and wave activity (light gray). Middle: contour line maps of the contralateral short-latency responses (red lines) and wave activity (blue lines) in the region in the square on the left map. The red and blue lines exhibited different patterns. Right: optical signals detected from 2 regions marked on the middle contour line map. Top: contralateral short-latency response (arrowhead) was detected with a small wave-related signal (asterisk). Bottom: no short-latency response was identified despite a large wave-related signal. D: relationship between the wave activity and contralateral short-latency response. The amplitudes of the contralateral response were plotted against those of the wave-related optical signal. Data were obtained from the contour line map shown in C. Each dot corresponds to a signal detected by one photodiode.
In a caudal direction, the wave was propagated to the lumbo-sacral region (Fig. 3B). These results show that the depolarizing wave activity is widely distributed in the embryonic CNS (Table 2).

Wave activity triggered by cranial sensory input

Next, we examined whether the wave activity shown in Figs. 1 and 2 was elicited by other natural triggers such as synaptic inputs from the sensory nerves. Figures 4 and 5 show that this was the case. In Fig. 4A, two patterns of optical responses obtained by stimulation of the maxillary branch of the trigeminal nerve (N. V2) are shown. In the upper images, trigeminal nerve stimulation evoked pre- and postsynaptic responses in a restricted region corresponding to the trigeminal nuclear complex (Momose-Sato et al. 2004), whereas in the lower images trigeminal nerve stimulation at the same intensity elicited the wide propagation of optical signals following the nuclear response. The former pattern was often observed shortly after dissection of the preparation or after repetitive stimuli, whereas the latter pattern was usually observed after the preparation was fully recovered. In Fig. 4B, enlargements of the wave-related optical signals detected from four regions shown in Fig. 4C are presented. Similar results were obtained via maxillary nerve stimulation from 9 of 10 preparations dissected from E15 to E16 embryos (Table 1).

Wave activity similar to that shown in Fig. 4 was also elicited through activation of the mandibular nerve (N. V3) and vagus nerve (N. X; Fig. 5, Table 1). When we applied stimuli to the ophthalmic nerve (N. V1), wave activity was not evoked in normal solution. This might be because the excitatory postsynaptic potential (EPSP) related to the ophthalmic nerve is extremely small (Momose-Sato et al. 2004). When we stimulated the ophthalmic nerve in the Mg2+/H11001-free solution, in which an N-methyl-D-aspartate (NMDA) receptor-mediated component of the EPSP was potentiated (Momose-Sato et al. 2004), the wave appeared in every preparation (Table 1).

In Tables 2–4, we compared the spatiotemporal characteristics of the waves evoked by upper cervical cord stimulation and cranial nerve stimulation. The spatial extent of the wave activity (Table 2) and the time course of the wave-related optical signal (Table 3) were similar in every preparation although the onset latency of the signal (Table 4) showed some differences between stimulations: the onset latency was more variable with cranial nerve stimulation than with direct stimulation of the cervical cord. A comparison of the onset latency of the signal between different regions (Table 4, bottom) showed that the delay was most variable between the cranial sensory nucleus and the medulla, and that propagation of the wave to the pons and cerebellum was not largely different between stimulations.

Similarities in the characteristics of the wave-related optical signals between different nerves suggests that although each nerve stimulation first activated a specific set of neural structures within the sensory nucleus, there was a nonspecific spread of depolarization from the nucleus that led to global activation of the brain. Another possibility is that the net effects of secondary and tertiary synaptic links led to widespread depolarization with each nerve stimulation. Figure 6 suggests that the spatiotemporal characteristics of the wave activity were different from those of the second/higher-ordered synaptic responses evoked by cranial nerve stimulation as seen by the multiple-site optical recordings of maxillary nerve responses detected in the E16 medulla. In Fig. 6A, left (1-s recording), optical signals related to the EPSP were identified in the lateral region corresponding to the trigeminal nuclear

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**FIG. 8.** Effects of chemical synaptic antagonists on the wave activity. The effects of Cd2+ (10 µM) (A), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 µM), dl-2-amino-5-phosphonovaleric acid (APV, 200 µM) (B), atropine (10 µM), d-tubocurarine (dTc, 100 µM) (C), strychnine (20 µM), and bicuculline (200 µM) (D) are shown with color-coded representations of the maximum signal amplitude (top) and enlarged traces of the optical signals detected from the lateral pons (top traces) and rostral medulla (bottom traces). The blockers were applied for 10 min. Data were obtained from E16 brain stem preparations with stimulation of the upper cervical cord.
complex as shown by the red asterisk in the right short-time recording, and wave-related optical signals were observed over the entire medulla. The expanded time base of the same recording (Fig. 6A, right: 125-ms recording) revealed that there was another population of neural responses with a short latency on the contralateral side as indicated by the dotted circle. Comparison of the onset latency of the signals from three different regions, the trigeminal nuclear complex (red asterisk), the contralateral short-latency response area (green asterisk), and the region with wave activity alone (blue asterisk; Fig. 6B) showed that there was a small delay of 10–20 ms between the first-ordered EPSP in the trigeminal nuclear complex (red asterisk) and the contralateral response (green asterisk). The onset latency of the wave-related optical signal (blue asterisk) was long and highly variable as indicated in Table 4. Contour line maps of the contralateral responses (Fig. 6C, red lines) and wave activity (Fig. 6C, blue lines) exhibited different patterns with no correlation between the amplitudes of the two responses (Fig. 6D, correlation coefficient = 0.333); in one position (Fig. 6C, top right trace) the contralateral response (arrowhead) was detected with the small wave-related optical signal (asterisk), whereas in another (Fig. 6C, bottom right trace) no contralateral response was identified despite the large wave-related optical signal. Furthermore, wave activity was detected from preparations in which contralateral responses were not clearly identified.

From the characteristics described in the preceding text, we believe that the contralateral short-latency responses reflected second/higher-ordered synaptic responses in the trigeminal network for which the wave activity was neither temporally or spatially correlated.

$\text{Ca}^{2+}$ waves associated with the depolarizing wave activity

One question we asked was whether the widely propagating depolarizing wave activity detected with a voltage-sensitive dye could afford information limited to the electrogenic interaction of cells or provide biochemical signaling such as via $\text{Ca}^{2+}$ through the network. We thus examined whether the spreading depolarization was accompanied by a change in the intracellular $\text{Ca}^{2+}$ concentration ([$\text{Ca}^{2+}$]$_i$) using a $\text{Ca}^{2+}$-imaging technique and the fluorescent $\text{Ca}^{2+}$-indicator Ca green-1 AM.

FIG. 9. Effects of chemical synaptic antagonists and gap junction blockers on the wave activity. A: effects of chemical synaptic antagonists are presented as signal amplitudes normalized against the control signal (mean ± SD). The number of preparations tested is indicated by $n$. For each preparation, 5–10 signals surrounding the peak signal areas in the lateral pons (right) and medulla (left) were analyzed. The concentration of picrotoxin was 200 μM, and that of the other blockers was the same as shown in Fig. 8. nd: not detected, the amplitude of the optical signals in the presence of the blocker was <1.5 × 10^{-4}. B: normalized signal amplitudes of the wave activity with combined application of the blockers. The data were obtained from 10 positions in the medulla (●) and 10 positions in the lateral pons (○) of an E16 preparation, and the blockers were applied during the period indicated by the horizontal lines. In the presence of a mixture of glutamatergic, cholinergic, glycinergic, and GABAergic blockers, the signals were eliminated. Atr, atropine; Str, strychnine; Bic, bicuculline; PTX, picrotoxin. C: normalized amplitudes of the wave-related optical signals with a mixed application of blockers. The optical signals were eliminated in the pons in all tested preparations ($n = 7$ using bicuculline and $n = 2$ using picrotoxin). In the medulla, the optical signals were eliminated in 5 of 8 preparations using bicuculline and 3 of 3 preparations using picrotoxin. D: effects of gap junction blockers on the wave activity. Normalized signal amplitudes of the wave activity with the application of octanol (1 mM: 10–20 min application) and 18β-glycyrrhetinic acid (GA; 100 μM: 60 min application) are presented.
Figure 7 shows an example of Ca$^{2+}$-imaging obtained via stimulation of the N. V₂. When we stimulated the nerve, Ca$^{2+}$ responses were first observed in the trigeminal nuclear complex (1st frame), followed by an elevation in \([\text{Ca}^{2+}]_i\) over a wide region of the medulla, spinal cord and pons. Ca$^{2+}$ responses exhibited a multiple-peak pattern with peak locations in the rostral medulla, lateral and medial regions of the pons, and spinal cord just caudal to the obex. This spatial distribution pattern was similar to that of the spreading depolarizing optical signals (Fig. 2). These results suggest that the wave activity identified in the present experiment provides not only electrical synchrony but also biochemical signals through the network distributed over a wide region of the CNS.

**Neural network mechanisms underlying the wave activity**

We next performed pharmacological examinations to study neural network mechanisms underlying the propagation of the wave. Experiments were performed using E16 brain stem preparations with triggering wave activity by upper cervical cord stimulation.

The wave activity was eliminated by Cd$^{2+}$ (10 μM; \(n = 7\) preparations; Figs. 8A and 9A), Ca$^{2+}$-free solution (\(n = 2\); data not shown) and tetrodotoxin (TTX; 1 μM; \(n = 2\); data not shown), suggesting that activity in chemical synaptic networks was involved. To assess which neurotransmitters mediate the wave activity, we applied antagonists to neurotransmitter receptors. The wave activity was markedly inhibited by the application of glutamate receptor antagonists. In the presence of the \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)/kainite receptor antagonist CNQX (5 μM; \(n = 4\)), the wave-related optical signals were significantly reduced in amplitude (Figs. 8B and 9A). The wave activity was also inhibited when the nicotinic acetylcholine receptor antagonist \(d\)-tubocurarine (dTc; 100 μM; \(n = 4\)) was applied, although the muscarinic acetylcholine receptor antagonist atropine (10 μM; \(n = 4\)) did not significantly affect it (Figs. 8C and 9A).

In the developing CNS, the inhibitory neurotransmitters GABA and glycine exert excitatory effects by inducing membrane depolarization (Cherubini et al. 1991; Ben-Ari 2002). In embryonic rat brain stem preparations, application of the gly-
neurotransmitters including glutamate, acetylcholine, glycine, and GABA.

We previously reported that the depolarization wave in the chick embryo is blocked by gap junction blockers such as octanol, 18β-glycyrrhetinic acid and carbenoxolone, in addition to chemical synaptic antagonists (Momose-Sato et al. 2003a). To compare the pharmacological characteristics of the wave activity with those of the chick depolarization wave, we examined the effects of these blockers. When we applied octanol to the bathing solution (1 mM; n = 4), the wave-related optical signals were reversibly suppressed within 10–20 min (Fig. 9D). Similar results were obtained with 18β-glycyrrhetinic acid (100 µM; n = 4), although the degree of suppression was relatively mild (Fig. 9D).

### Connexin immunohistochemistry

A gap junction is formed by two hemichannels (connexons), each of which is a hexamer composed of connexin (Cx) protein (Evans and Martin 2002). We performed an experiment involving connexin immunohistochemistry in the embryonic rat brain (Fig. 10).

In sections of E16 rat brain stems and spinal cords, connexin26-immunoreactive (IR) spots (Fig. 10A, 1–4) and connexin32-IR spots (Fig. 10, B–D) were identified. The IR spots were mostly localized on the surface of the cell bodies. A similar dot-like staining pattern was also confirmed in sections of the liver of adult rats (data not shown). No immunoreactivity was detected in negative control sections as shown in Fig. 10E. Connexin-IR spots were widely distributed in the brain stem and were dense in the regions corresponding to the peak areas of the optical signals; the caudal cerebellum (Fig. 10A, 1, and B), lateral pons (Fig. 10A, 2, and C), and rostral medulla (Fig. 10A, 3 and D). We also examined the immunohistochemistry of connexins 43 and 45. However, we could not identify clear immunoreactivity of these connexins in the E16 rat brain stem.

### DISCUSSION

In the present study, we examined the large-scale depolarizing wave activity that occurs in the embryonic rat CNS, which is propagated over a wide region of the brain-spinal cord across anatomical boundaries. Wave-related optical signals were observed in the medulla, pons, midbrain, and spinal cord, and also detected in the cerebellum and forebrain.

In the mammalian CNS, using electrophysiological methods or Ca\(^{2+}\)-imaging techniques, it was found that some activities travel widely over distances of a few millimeters or more (Garaschuk et al. 2000; Hanson and Landmesser 2003; Leinekugel et al. 1998; Nakayama et al. 1999; Peinado 2000; Ren and Greer 2003). However, these activities were essentially described within specific structures that become part of functional ensembles such as the cerebral cortex, limbic system, and medulla-spinal cord. The wave activity optically identified in the present study showed wide propagation of membrane depolarization and [Ca\(^{2+}\)]\(_i\) elevation, which has not been previously described for the mammalian CNS. The only comparable phenomenon noted in past studies is the depolarization wave in the chick embryo, which was also recorded using the optical recording technique with voltage-sensitive
dyes (Mochida et al. 2001; Momose-Sato et al. 2001b, 2003a,b). Between the wave activity in the rat embryo and the depolarization wave in the chick embryo, there are many similarities (summarized in Table 5). These include the extent of wave propagation, although responses in the cerebellum were smaller in the rat embryo, the waveform of the optical signal, the conduction velocity, the trigger sources, association with an increase in \([\text{Ca}^{2+}]\), and sensitivity to multiple blockers of chemical neurotransmitters and gap junctions. These similarities suggest that the large-scale depolarization wave is globally generated in different species. In preliminary experiments, we also detected similar depolarizing wave activity in mouse embryos.

As shown in Fig. 1D, the wave activity was coupled to the electrical discharges of cranial motoneurons. In the E15–E17 rat embryos and the mouse embryos at corresponding stages, correlated neuronal activity occurs as spontaneous electrical discharges of cranial and spinal motoneurons (Abadie et al. 2000; Hanson and Landmesser 2003; Ren and Greer 2003). In the rat spinal cord, spontaneous electrical activity travels throughout the cord during these developmental stages (Nakayama et al. 1999; Ren and Greer 2003). It is important to clarify the relationship between the optically identified depolarization wave and the electrically recorded spontaneous activities. Although we preliminarily observed spontaneous optical signals that spread like a wave, detailed analyses could not be performed in the present study because of technical problems associated with our optical recording system. Resolving this issue will be a major challenge in future.

We examined network mechanisms responsible for wave propagation via pharmacological manipulation. Propagation of the wave-related optical signals was dependent on multiple neurotransmitters such as glutamate, acetylcholine, GABA, and glycine and was most sensitive to the NMDA and GABA_\text{A} receptor antagonists. In the mouse spinal cord between E15 and E17, it has been suggested that functional inhibitory connections mediated by glycine are in the process of developing (Whelan 2003). In the E16 rat brain stem preparation, the effects of GABA and glycine receptor antagonists on the wave activity were inhibitory in every case.

In addition to chemical synaptic antagonists, wave activity was inhibited by gap junction blockers such as octanol and 18β-glycyrrhetinic acid. Recently, several investigations reported using octanol and glycyrrhetic acids (18α-glycyrrhetinic acid, 18β-glycyrrhetic acid, and carbenoxolone) that gap junction function is necessary in addition to chemical synaptic transmission in mediating correlated neuronal activity in the embryonic retina (Catsicas et al. 1998; Wong et al. 1998) and spinal cord (Hanson and Landmesser 2003; Milner and Landmesser 1999). In the embryonic mouse spinal cord, gap junctions are necessary for the propagation of local episodes of activity throughout the cord but not for the local episode itself evoked by antidromic motoneuron activation (Hanson and Landmesser 2003). Another study on the neonatal mouse retina showed that spatially restricted retinal waves are triggered by local, synaptically mediated depolarization and that widely propagating waves induced by L-type \([\text{Ca}^{2+}]\)-channel activation depend on gap junction function (Singer et al. 2001). These studies suggest that the synergistic activities of gap junctions and chemical synapses might be necessary for the long-distance propagation of correlated activity in the developing CNS.

Lipophilic compounds such as octanol and heptanol have been widely used as gap junction inhibitors (Spray and Bennett 1985). Glycyrrhetic acid and its derivatives such as carbenoxolone have been developed as more specific inhibitors of gap junctions (Davidson and Baumgarten 1988). However, nonspecific effects of these reagents have also been suggested (Deutsch et al. 1995; Rouach et al. 2003; Todorovic and Lingle 1998; Vessey et al. 2004), and thus it is of concern that clear interpretations of results gained using these blockers are not forthcoming. Although our morphological observation of connexin immunoreactivity suggests that gap junctional communication systems are generated in the embryonic rat brain, the hypothesis that these systems contribute to the propagation of wave activity will require careful confirmation in future.

In our immunohistochemical study, we observed significant immunoreactivity of connexins26 and 32 in the E16 rat brain stem. These connexins are expressed in several types of neural cells in the brain. In the postnatal and adult rodent brain, expression of the connexin26-protein was previously reported in leptomeningeal cells, ependymal cells, astrocytes, and neurons (Dermietzel et al. 1989; Nadarajah et al. 1997; Nagy et al. 2001; Solomon et al. 2001). Some neurons and oligodendrocytes express connexin32 in the postnatal and adult rat brain (Dermietzel et al. 1989; Kunzelmann et al. 1997; Nadarajah et al. 1996). Unfortunately, we could not identify specific cell-type connexin immunoreactivity in the present study. The expression of connexins is developmentally regulated in the rat brain. In the midbrain floor, connexins26 and 32 are highly expressed during the embryonic period with peaks at E14 and E16, respectively. Thereafter they significantly decrease toward birth (Leung et al. 2002). Throughout the entire cortex, connexin26 expression also occurs during the embryonic stages (Nadarajah et al. 1997). However, in the rat striatum and cerebral cortex, connexin32 shows no distinct activity throughout the prenatal and neonatal periods but gradually increases during the postnatal stages (Dermietzel et al. 1989; Nadarajah et al. 1997). Our results showed for the first time the dense distribution of immunoreactive spots of both connexins at the levels of the pons and medulla in E16 rat embryos. The punctate labeling pattern of connexins26 and 32 showed that the immunoreactive spots were concentrated on the plasma membrane as plaques, suggesting that these connexins organize fully functional gap junction plaques (Yeager et al. 1998).

What might be the functional significance of the wave activity in the embryonic rat CNS? The wave activity identified in the present study provides evidence of the existence of two kinds of information that travel through the network, one being electrical signals characterized by membrane depolarization and the other being biochemical signals accompanied by \([\text{Ca}^{2+}]\) elevation. It was previously suggested that transient elevation in \([\text{Ca}^{2+}]\) is important in some aspects of growth and differentiation including proliferation (LoTurco et al. 1995), migration (Komuro and Rakic 1996), neuronal differentiation, and the expression of transmitter phenotypes (Gu and Spitzer 1997), axonal pathfinding (Kater et al. 1988), and dendritic growth and patterning (Wong and Ghosh 2002). In the brain stem and spinal cord in which the wave activity was most prominent in the present study, neurogenesis occurs in most regions by E15, although in several brain stem nuclei such as the cochlear nucleus, cell production continues beyond E16.
referring the day of sperm positivity as E0 (Altman and Bayer 1982, 1984). Thus the role of the wave activity is not limited to the control of early neuronal proliferation/differentiation and probably involves the regulation of later development such as synaptogenesis. Although activity-dependent processes are required for the proper formation of neuronal circuitry (Goodman and Shatz 1993; Hanse et al. 1997; Katz and Shatz 1996; Sur and Leamney 2001), the profile of large-scale, nonspecific wave activity propagation across anatomical boundaries suggests that this activity may not serve as a simple regulator of specific neuronal circuit formation either.

One possible role of the wave activity is that it transmits electrical and biochemical signals that occur in any restricted region of the nervous system throughout the CNS via a widely distributed network. This would allow neurons/neural precursor cells involved in the network to be activated at a higher rate than expected for local, poorly developed specific neuronal circuits and then facilitate the processes responsible for neural development via its nurturing effects.

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