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# MAINTENANCE OF THE LARGE-SCALE DEPOLARIZATION WAVE IN THE EMBRYONIC CHICK BRAIN AGAINST DEPRIVATION OF THE RHYTHM GENERATOR

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**Abstract—Widely correlated spontaneous activity in the developing nervous system is transiently expressed and is considered to play a fundamental role in neural circuit formation. The depolarization wave, which spreads over a long distance along the neuraxis, maximally extending to the lumbosacral cord and forebrain, is an example of this spontaneous activity. Although the depolarization wave is typically initiated in the spinal cord in intact preparations, spontaneous discharges have also been detected in the isolated brainstem. Although this suggests that the brainstem has the ability to generate spontaneous activity, but is paced by a caudal rhythm generator of higher excitability, a number of questions remains. Does brainstem activity simply appear as a passive consequence, or does any active change occur in the brainstem network to compensate for this activity? If the latter is the case, does this compensation occur equally at different developmental stages? Where is the new rhythm generator in the isolated brainstem? To answer these questions, we optically analyzed spatio-temporal patterns of activity detected from the chick brainstem before and after transection at the obex. The results revealed that the depolarization wave was homeostatically maintained, which was characterized by an increase in excitability and/or the number of neurons recruited to the wave. The wave was more easily maintained in younger embryos. Furthermore, we demonstrated that the ability of brainstem neurons to perform such an active compensation was not lost even at the stage when the depolarization wave was no longer observed in the intact brainstem.** 

**Key words: optical recording, voltage-sensitive dye, depolarization wave, brainstem, development.**

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*Abbreviations:* AC, alternating current; DC, direct current; SEM, standard error of the mean.

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## INTRODUCTION

Spontaneous activity in the developing nervous system is transiently expressed during a particular period, and is considered to play a fundamental role in neural circuit formation (Feller, 1999; Ben-Ari, 2001; Moody and Bosma, 2005; Blankenship and Feller, 2010). An example of early spontaneous activity is the wave spreading over a long distance along the neuraxis, maximally extending to the lumbosacral cord and forebrain, which has been referred to as the depolarization wave (Momose-Sato et al., 2001b, 2007, 2012a; for a review Momose-Sato and Sato, 2013).

Optical imaging analyses of the depolarization wave revealed that the origin of the activity is typically located in the upper cervical cord at the early stage and lumbosacral cord at the later stage, with a mixed pattern being observed during the middle period (Momose-Sato et al., 2007, 2009, 2012a). Although the depolarization wave is rarely initiated in the region rostral to the obex in intact preparations, spontaneous discharges similar to those associated with the depolarization wave have been detected in isolated brainstems and medulla slices (Fortin et al., 1995, 1999; Abadie et al., 2000; Thoby-Brisson et al., 2005). This finding suggests that the brainstem has the ability to generate spontaneous activity, but is paced by a caudal rhythm generator of higher excitability. Although this hypothesis is reasonable, a number of questions remains. For example, does brainstem activity simply appear as a result of the deprivation of dominant activity, or does any active change occur in the brainstem network to compensate for this activity? If the latter is the case, does active compensation occur equally at different developmental stages? Where is the new rhythm generator(s) in the isolated brainstem? A comparison of activity before and after isolation of the brainstem is required to answer these questions, especially the first and second ones. In the present study, we analyzed the spatio-temporal patterns of spontaneous activity detected in the chick brainstem before and after transection at the obex using an optical recording technique with a voltage-sensitive dye.

## EXPERIMENTAL PROCEDURES

### Preparations

Experiments were carried out in accordance with the Japan Society for the Promotion of Science guidelines

for the care and use of laboratory animals with the approval of the Ethics Committee of Kanto Gakuin University. All efforts were made to minimize the number of animals used and their suffering. The fertilized eggs of White Leghorn chickens (Saitama Experimental Animals Supply Co. Ltd., Saitama, Japan) were incubated for 5–9 days (stage 26 to stage 35) in a forced-draft incubator (type P-008, Showa Incubator Lab., Urawa, Japan) at 38 °C and 60% humidity, and were turned once each hour. The embryo was decapitated, and a brainstem-whole spinal cord preparation with the vagus nerve attached was dissected. The second type of preparation, an isolated brainstem, was made by transecting the brainstem-spinal cord preparation at the level of the obex. Transection was performed using a hand-held blade. The thickness of the brainstem along the ventrodorsal axis was about 1000 μm at day 8 of incubation. The early embryonic brain had a histologically loose structure with immature neurons and undifferentiated connective tissue and was relatively resistant to anoxia. Therefore, a brain-whole spinal cord preparation was available *in vitro*, which made it possible to investigate the functional organization of neural networks in the intact brain-spinal cord. Preparations were maintained in a bathing solution, which contained (in mM) NaCl, 138; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.5; glucose, 10; and Tris–HCl buffer (pH 7.3), 10. The solution was equilibrated with oxygen.

### Staining with a voltage-sensitive dye

For optical recording, the meningial tissue surrounding the brain was carefully removed, and the preparation was stained by incubating it for 10–20 min in a solution containing 0.2 mg/ml of a voltage-sensitive merocyanine-rhodanine dye, NK2761 (Hayashibara Biochemical Laboratories Inc./Kankoh-Shikiso Kenkyusho, Okayama, Japan: Kamino et al., 1981; Salzberg et al., 1983; Momose-Sato et al., 1995). The immature cellular-interstitial structure of the embryonic tissue allowed the dye to diffuse well from the surface and into deeper regions. After the staining, the preparation was attached to the silicone bottom of a recording chamber with the ventral side facing up by pinning it with tungsten wires. The preparation was continuously superfused with the bathing solution at 2–3 ml/min at 28 ± 1 °C.

### Electrical recording of spontaneous cranial nerve discharges

Spontaneous activity was electrically monitored to check the feasibility of stained preparations and to set the timing of illumination for optical recording. Population activity of vagal motoneurons was recorded with a glass micro-suction electrode applied to the cut end of the vagus nerve's root. Previous studies have shown that spontaneous activity in the embryonic brainstem can be recorded from every cranial motor nerve with a high correlation (Greer et al., 1992; Fortin et al., 1995; Abadie et al., 2000). The vagus nerve was selected to monitor electrical activity in the present study since it is laterally attached to the medulla, and thus, did not disturb the optical recording made from the ventral surface of the preparation. Electrical signals were amplified with filters set at 0.08 Hz and 1 kHz and were digitally recorded at 4 kHz with an analog-to-digital converter (MacLab/8S, ADInstruments, Castle Hill, Australia) or were fed into one channel of the analog-to-digital converter of the 1020-site optical recording system.

### Optical recording using a voltage-sensitive dye

Bright-field illumination was provided by a 300-W tungsten-halogen lamp (Type JC-24V/300W, Kondo Philips Ltd., Tokyo, Japan) driven by a stable dc-power supply, and incident light was collimated and rendered quasi-monochromatic using an interference filter with a transmission maximum at 699 ± 13 nm (half-width) (Asahi Spectra Co., Tokyo, Japan). The objective (Plan Apo, ×2: 0.1 NA and × 4: 0.2 NA) and photographic eyepiece lenses (×2.5) projected a real image of the preparation onto a 34 × 34-element silicon photodiode matrix array mounted on a microscope. The focus was set to the ventral surface, but the optical signals seemed to include activity from every depth, since it had been shown that the dye diffused readily from the surface into the interior region of the embryonic brainstem (Sato et al., 1995) and that neuronal responses in the dorsally-located cranial nerve nucleus can be detected from the ventral surface (Momose-Sato et al., 1991). Changes in the transmitted light intensity through the preparation were detected with the photodiode array and were recorded with a 1020-site optical recording system that was constructed in our laboratory. This system is based on one described previously (Hirota et al., 1995; Momose-Sato et al., 2001a) with modifications to its analog-to-digital converter unit. Each pixel (element) of the photodiode array detected light transmitted by a square region ($116 \times 116\ \mu m^2$ using ×10 magnification) of the preparation. The outputs from the 1020 elements of the photodiode array were fed into individual current-to-voltage converters followed by individual pre-amplifiers. The AC (alternating current) component of each signal was further amplified (time constant of AC coupling = 3 s), passed though an low-pass filter (time constant = 470 μs), digitized with a 16-bit dynamic range, and sampled at 1024 Hz. This system makes it possible to detect optical signals without the drift in the DC (direct current) baseline that is associated with dye bleaching. Continuous sampling and storage of data at a resolution of 1 ms is possible over 30 min, but to minimize phototoxic damage, the illumination was limited to as much as possible and an optical recording was made only when the electrically-monitored spontaneous activity appeared. All the recordings were made in single sweeps without averaging.

### Data analysis

The fractional change in dye absorption $\Delta A/A_r$ is equal to $-\Delta I/(I_{before\ staining} - I_{after\ staining})$, where I is the light intensity transmitted through the preparation (Ross et al., 1977). We had already stained the preparation