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DEVELOPMENT OF VAGAL AFFERENT PROJECTIONS CIRCUMFLEX TO THE OBEX IN THE EMBRYONIC CHICK BRAINSTEM VISUALIZED WITH VOLTAGE-SENSITIVE DYE RECORDING

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Abstract—Using voltage-sensitive dye recording, we surveyed neural responses related to the vagus nerve in the embryonic chick brainstem. In our previous studies, we identified four vagus nerve–related response areas in the brainstem. On the stimulated side, they included (1) the nucleus of the tractus solitarius (NTS: the primary sensory nucleus) and (2) the dorsal motor nucleus of the vagus nerve (DMNV), whereas on the contralateral side, they corresponded to (3) the parabrachial nucleus (PBN: the second/higher-ordered nucleus) and (4) the medullary non-NTS region. In the present study, in addition to these areas, we identified another response area circumflex to the obex. The intensity of the optical signal in the response area was much smaller than that in the NTS/DMNV, and the spatio-temporal pattern could be discerned after signal averaging. The conduction rate to the response area was slower than that to the other four areas. Ontogenetically, the response area was distributed on the stimulated side at the 6-day embryonic stage, and it spread into the contralateral side in 7- and 8-day embryonic stages. These distribution patterns were consistent with projection patterns of vagal afferent fibers stained with a fluorescent tracer, suggesting that the response area included a primary sensory nucleus. In comparison with the functional development of the other four response areas, we traced the functional organization of vagus nerve–related nuclei in the embryonic brainstem.

Key words: voltage-sensitive dye recording, chick embryo, vagus nerve, brainstem, area postrema, development.

In the CNS, the brainstem is a pivotal region to integrate various sensory information from somatic/visceral organs and to produce motor reflexes. In the brainstem, the autonomic nervous system has extensive networks that are indispensable for regulating visceral functions, including cardiovascular responses, respiratory activity and gastric function (Spyer, 1982; Dampney, 1994; Saper, 1995; Travagli et al., 2006). In order to understand the integration of the visceral information in the brainstem, one of the fundamental questions is how and when the neural circuits are established in the brainstem. Although many anatomical and molecular genetic investigations have been made so far on brainstem neural circuits (for reviews see Friauf and Lohmann, 1999; Glover, 2000; Rubel and Fritzsch, 2002), electrophysiological studies of neural network organization have been limited because of the technical difficulty of monitoring neural activity from small and fragile embryonic neurons (for reviews see Fortin et al., 2000; Borday et al., 2004, 2005).

Voltage-sensitive dye recording has made it possible to monitor transmembrane voltage changes from living cells that are inaccessible to conventional electrophysiological means. Furthermore, the introduction of multi-element photodiode arrays has provided powerful tools for monitoring the spatio-temporal patterns of neural activity from a variety of invertebrate and vertebrate central nervous systems (for reviews see Cohen and Salzberg, 1978; Salzberg, 1983; Grinvald et al., 1988; Ebner and Chen, 1995; Baker et al., 2005).

In our previous studies, we established the usefulness of the voltage-sensitive dye recording for monitoring electrical activity from the embryonic nervous systems (for reviews see Momose-Sato et al., 2001, 2002), and we investigated the development of neural functions related to various cranial nerves, including the glossopharyngeal and vagus nerve.

First, we examined spatio-temporal patterns of neural activity evoked by glossopharyngeal/vagal stimulation in embryonic brainstems, and obtained three-dimensional profiles of neural responses in the visceral motor and sensory nuclei (Komuro et al., 1991; Momose-Sato et al., 1991, 1994, 1999; Sato et al., 1995, 1998, 2002). In the sensory nucleus of the vagus and glossopharyngeal nerves (nucleus of the tractus solitarius: NTS), (1) the optical signals were composed of fast and slow components, which corresponded to the action potential and the glutamatergic excitatory postsynaptic potential (EPSP), respectively; (2) the slow signal easily fatigued with repetitive stimuli, and the initial and later phases of the slow signal were mainly attributable to non-NMDA (*N*-methyl-D-aspartate) and NMDA receptors, respectively; (3) in normal Ringer's solution, the postsynaptic responses were first expressed in the NTS at embryonic day 7 in the chick embryo and embryonic day 15 in the rat embryo.

Second, we surveyed optical responses from a wider region of the chick brainstem, and identified the second/

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Abbreviations: DiI, 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate; DMNV, the dorsal motor nucleus of the vagus nerve; EPSP, excitatory postsynaptic potential; NA, nucleus ambiguus; NMDA, *N*-methyl-D-aspartate; NTS, the nucleus of the tractus solitarius; PBN, the parabrachial nucleus.

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higher-ordered nucleus, possibly corresponding to the parabrachial nucleus (PBN), at the pons/rostral medulla on the contralateral side (Sato et al., 2004). Furthermore, we found a primary vagal projection to the contralateral non-NTS region in the embryonic chick brainstem (Momose-Sato and Sato, 2005).

During these studies, we noticed that very small optical responses were induced by vagal nerve stimulation in the area circumflex to the obex. In the present study, we examined the development of the small vagal responses in the embryonic chick brainstem with voltage-sensitive dye recording and optical signal averaging.

EXPERIMENTAL PROCEDURES

Preparations

Experiments were carried out in accordance with the guidelines of the US National Institutes of Health and 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, and all of the experiments were done in Tokyo Medical and Dental University. Fertilized eggs of White Leghorn chickens (Saitama Experimental Animals Supply Co. Ltd., Saitama, Japan) were incubated for 6–8 days in a forced-draft incubator (type P-008, Showa Incubator Laboratory, Urawa, Japan) at 37 °C and 60% humidity, and were turned once each hour. In the present experiments, the day on which incubation was started was termed day 0: day 6 corresponds to the Hamburger-Hamilton stages (Hamburger and Hamilton, 1951) 28–29, day 7 to 30–32, and day 8 to 33–34. The embryos were decapitated, and intact brainstem preparations with the vagus nerve fibers attached were dissected from the embryos. For slice preparations, 1200–1300 μm thick slices were made from the isolated brainstem at the level of the vagus nerve root. The meningeal tissue was carefully removed in a bathing solution that 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 100% oxygen. Each preparation was stained by incubating it for 20 min in a solution containing 0.2 mg/ml of the 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), and the excess (unbound) dye was washed away with a dye-free solution before recording. After staining with the dye, the preparation was attached to the silicone (KE 106LTV; Shin-etsu Chemical Co., Tokyo, Japan) bottom of a simple chamber with the ventral side up for the intact preparation or the caudal side up for the slice preparation.

Electrical stimulation

The cut end of the vagus nerve was drawn into a glass microsuction electrode (about 100 μm internal diameter). Positive depolarizing square current pulses at 7–8 μA/5 ms, which evoked maximum responses, were applied to the right vagus nerve. For single sweep recordings, a single stimulus was applied to the nerve. When the signals were averaged, 16–50 stimuli were delivered at 1 Hz.

Optical recording

The optical recording system which we used was similar to that previously described (Momose-Sato et al., 2001). In brief, light from a 300 W tungsten-halogen lamp (Type JC-24V/300W, Kondo Philips Ltd., Tokyo, Japan) was collimated, rendered quasi-monochromatic with a heat filter and an interference filter with a transmission maximum at 703±15 nm (Asahi Spectra Co., Tokyo, Japan), and focused onto the preparation. An objective (Plan Apo, 10×, 0.45 numerical aperture) and a photographic eyepiece projected a real image of the preparation (magnification 25×) onto a multi-element silicon photodiode matrix array mounted on an upright microscope. We used a 128-site optical recording system with a 12×12-element silicon photodiode array which was constructed in our laboratory (for reviews see Kamino, 1991; Momose-Sato et al., 2001). The time resolution of the system was 1 ms at 1000 frames/s. The recordings were usually made with a time interval of 10–15 min. To see the small fast component of the optical signal, 16–50 recordings were averaged off-line. Optical measurements were carried out in a still chamber without continuous perfusion with the bathing solution at 26–30 °C. For short time recording of optical signals evoked by vagal nerve stimulation, there was no difference in the signal intensity between with and without continuous perfusion and between the bathing solution at 26–30 °C and at 35–37 °C.

The fractional change in dye absorption $\Delta A/A_r$ is equal to $-\Delta I/(I_b-I_a)$, where I is the light intensity transmitted through the preparation and I_b, I_a are the intensity before and after staining, respectively (Ross et al., 1977). When we recorded vagal responses, we stained the preparation before pinning it to the chamber to allow the dye to diffuse into the tissue. Thus, the measurement of I_b and I_a from the same position was technically difficult. We compared I_b and I_a by measuring the light that reached the detectors before and after the preparation was stained for 20 min with 0.2 mg/ml NK2761 on the stage of the microscope (Momose-Sato and Sato, 2006). Under this condition, I_a/I_b averaged 64% in the 8-day-old intact medulla with only a small variation between regions ($n=3$ preparations). In slice preparations, although the central part of the slice was less intensely stained than the peripheral region, I_a/I_b was relatively constant within the vagal response area (dorsal region): in 8-day-old slices ($n=3$), I_a/I_b averaged 42% in the dorsomedial region and 40% in the dorsolateral region. Since regional variations in I_a/I_b in the vagal response area were small, we measured I_a and ΔI, and expressed the optical signal as $\Delta I/I_a$.

DiI labeling

The DiI labeling method that we used was essentially similar to that described by Godement et al. (1987). Brainstems with the vagus nerve attached were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. A small crystal of the fluorescent neuronal tracer, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA), was placed in the cut ends of the vagus nerve. Preparations in which DiI had been placed were stored in 4% paraformaldehyde for 2–8 weeks at room temperature. The brainstem dissected free from surrounding tissue was embedded in 3% gelatin, and was sectioned in the horizontal (coronal) plane at 50 μm on a Vibratome (microslicer DTK-2000, Dosaka EM, Kyoto, Japan). Wet-mounted sections were examined with an epifluorescence microscope (FLUOPHOT, Nikon Co., Tokyo, Japan) equipped with a rhodamine filter set at excitation 520–550 nm and emission >570 nm using a 575 nm dichroic mirror.

RESULTS

Optical signals induced by vagal nerve stimulation in the medulla

Fig. 1A shows an example of multiple-site optical recordings of neural activity induced by vagal nerve stimulation in a 7-day-old embryonic chick brainstem intact preparation. The thickness (light-path from the dorsal surface to the