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Primary Vagal Projection to the Contralateral Non-NTS Region in the Embryonic Chick Brainstem Revealed by Optical Recording

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Abstract. Using multiple-site optical recording with the voltage-sensitive dye, NK2761, we found that vagus nerve stimulation in the embryonic chick brainstem elicits postsynaptic responses in an undefined region on the contralateral side. The characteristics of the contralateral optical signals suggested that they correspond to the monosynaptic response that is related to the vagal afferent fibers. The location of the contralateral response was different from the vagal motor nucleus (the dorsal motor nucleus of the vagus nerve) and sensory nucleus (the nucleus of the tractus solitarius), and other brainstem nuclei that receive primary vagal projection. These results show that the vagus nerve innervates and makes functional synaptic connections in a previously unreported region of the brainstem, and suggest that sensory information processing mediated by the vagus nerve is more complex than expected.

Key words: Optical recording — Voltage-sensitive dye — Vagus nerve — Sensory nucleus — Nucleus of the tractus solitarius — Synapse

Introduction

Understanding the structural configuration of the central nervous system (CNS) is one of the most important challenges in neuroscience. However, what is fascinating about the CNS is not just its structure but its functional architecture, i.e., the spatial pattern of neuronal activity and its temporal dynamics based on the morphological arrangement of active neurons. Although electrophysiology is the most direct method for monitoring electrical signals within neurons, its application to the study of neuronal network organization is limited because of the difficulty in simultaneous multiple-site recording. Optical recording techniques involving voltage-sensitive dyes have made it possible to monitor transmembrane voltage changes from multiple regions (Salzberg et al., 1977), and are powerful tools for studying the characteristics of membrane properties and spatio-temporal patterns of neuronal network activity (for reviews *see* Cohen and Salzberg, 1978; Salzberg 1983; Grinvald et al., 1988; Ebner and Chen, 1995; Baker et al., 2005).

We have been studying the functional organization of the CNS using an ontogenetic approach and by applying multiple-site optical recording techniques (for reviews *see* Kamino, 1990; Momose-Sato, Sato & Kamino, 2001). One of our targets is the brainstem, which is the pivotal region involved in the integration of multiple sensory/motor processes and cardiovascular/respiratory functions. In the course of our study on the brainstem cranial nerve nuclei, we found that stimulation of the vagus nerve in the chick embryo elicits monosynaptic-like responses in an undefined contralateral region in addition to the ipsilateral nucleus of the tractus solitarius (NTS), which is the first relay nucleus of the visceral nerves. In the present study, we report on this hitherto unknown projection of the vagus nerve by analyzing the characteristics and spatial distributions of the optical signals.

Materials and Methods

Preparations

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. Fertilized eggs of White Leghorn chickens (Saitama Experimental Animals Supply Co. Ltd.,

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Saitama, Japan) were incubated for 6–11 days in a forced-draft incubator (type P-008, Showa Incubator Lab., Urawa, Japan) at 37°C and 60% humidity, and were turned once each hour. The embryos were decapitated, and intact brainstem preparations with the vagus nerve fibers attached were dissected from the embryos. Slice preparations of 1,200–1,300 μm thickness were made from the isolated brainstem at the level of the vagus nerve root. The pia mater was carefully removed in a bathing solution which contained (in mM) NaCl, 138; KC1, 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. 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, Hirota and Fujii, 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 micro-suction electrode (about 100 μm internal diameter). Positive depolarizing square current pulses at 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 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 NA (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 two optical recording systems, which were constructed in our laboratory (for reviews *see* Kamino, 1991; Momose-Sato et al., 2001). One was a 1,020-site optical recording system with a 34 × 34-element silicon photodiode array (for details *see* Hirota et al., 1995), and the other was a 144-site optical recording system with a 12 × 12-element silicon photodiode array. The time resolution of these systems was 1 ms at 1,024 frames per second for the 1,020-site recording system and 1,000 frames per second for the 144-site recording system. The recordings were usually made in single sweeps with a time interval of 10–15 min. To see the fast component of the contralateral 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.

The fractional change in dye absorption $\Delta A/A_r$ is equal to $-\Delta I/(I_{\text{before staining}} - I_{\text{after staining}})$, where I is the light intensity transmitted through the preparation (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 $I_{\text{before staining}}$ and $I_{\text{after staining}}$ from the same position was technically difficult. We compared $I_{\text{before staining}}$ and $I_{\text{after staining}}$ 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. Under this condition, $I_{\text{after staining}} / I_{\text{before staining}}$ 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, $I_{\text{after staining}} / I_{\text{before staining}}$ was relatively constant within the vagal response area (dorsal region): in 8-day old slices ($n = 3$), $I_{\text{after staining}} / I_{\text{before staining}}$ averaged 42% in the dorsomedial region and 40% in the dorsolateral region. Since regional variations in $I_{\text{after staining}} / I_{\text{before staining}}$ in the vagal response area were small, we measured $I_{\text{after staining}}$ and ΔI, and expressed the optical signal as $\Delta I/I_{\text{after staining}}$.

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 phosphat 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) (Cohen et al., 1974), was placed in the cut ends of the vagus nerve. Preparations with DiI placements were stored in 4% paraformaldehyde for 2–4 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.

Abbreviations

APV: $_{DL}$-2-amino-5-phosphonovaleric acid
CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione
CNS: central nervous system
DiI: 1,1′ -dioctadecyl-3,3,3′ ,3′ -tetramethylindocarbocyanine perchlorate
DMNV: dorsal motor nucleus of the vagus nerve
GABA: γ-aminobutyric acid
NMDA: N-methyl-$_D$-aspartate
NTS: nucleus of the tractus solitarius
PBN: parabrachial nucleus

Results

Detection of Contralateral Signals with Vagal Stimulation

Figure 1 illustrates an example of multiple-site optical recordings of neural activity induced by vagus nerve stimulation. The slice preparation was dissected from an 8-day old chick embryo (Stage 34) at the level of the vagus nerve root, and a simultaneous 1,020-site optical recording was obtained in a single sweep. When the right vagus nerve was stimulated with a current that gave the maximum response, optical signals were differentiated in the dorsal region on the stimulated (ipsilateral: shadowed with light gray) and contralateral (shadowed with dark gray) sides. The distributions of the optical signals are presented with a contour line map of the signal amplitudes in Fig. 2*A*.

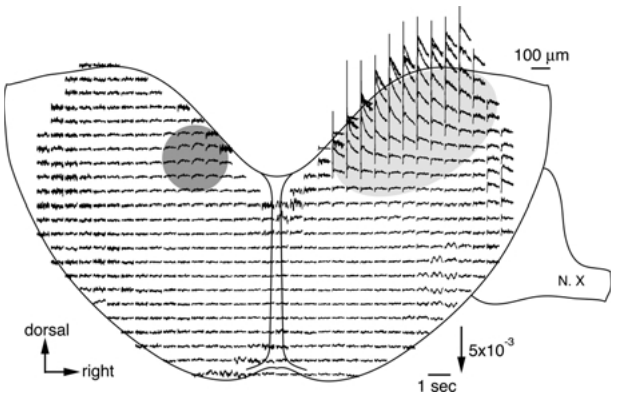

Fig. 1. An example of multiple-site optical recordings of neural responses to vagal stimulation in an 8-day old chick brainstem slice. The preparation was stained with the merocyanine-rhodamine dye, NK2761, and optical signals were evoked by applying a brief square current pulse to the right vagus nerve (*N. X*) with a microsuction electrode. The evoked optical signals were detected from the caudal side of the preparation in a single sweep. The direction of the arrow on the lower right side indicates an increase in transmitted light intensity (a decrease in absorption) and the length of the arrow represents the stated value of the fractional change. In this recording, two response areas were observed; one was located on the stimulated side (shadowed in light gray), and the other was on the contralateral side (shadowed in dark gray). The former area includes the nucleus of the tractus solitarius (NTS), the lateral region, and the dorsal motor nucleus of the vagus nerve (DMNV), the medial region.

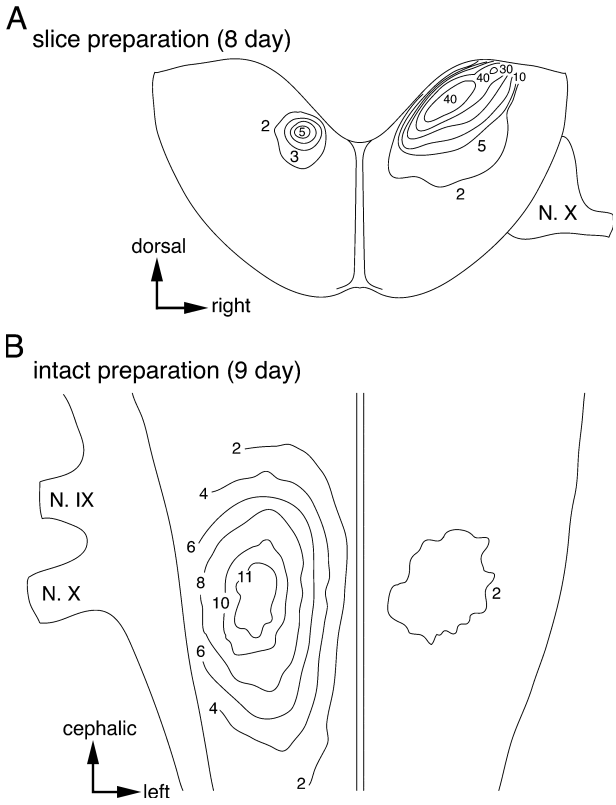

Fig. 2. Contour-line maps of the amplitude of the slow optical signal evoked by vagus nerve stimulation in an 8-day old slice (*A*) and 9-day old intact (*B*) preparation. Stimulation was applied to the right vagus nerve (*N. X*) (right side in *A* and left side in *B*), and the maximum amplitude of the slow component was measured. The preparation shown in *A* was the same as that in Fig. 1. The numerals on the contour lines indicate the fractional change multiplied by 10^{-4}. *N. IX*, glossopharyngeal nerve.

The ipsilateral response area corresponds to the nucleus of the tractus solitarius (NTS: lateral region) and the dorsal motor nucleus of the vagus nerve (DMNV: medial region) as previously reported (Komuro et al., 1991; Momose-Sato et al., 1991; for a review *see* Momose-Sato et al., 2001). The contralateral response has not been described so far. In a previous study, we reported that polysynaptic responses in the higher-order nucleus of the vagal pathway, possibly the parabrachial nucleus (PBN), appear on the contralateral side with stimulation of the vagus nerve in the chick embryo (Sato, Miyakawa & Momose-Sato, 2004). However, the present contralateral response area did not seem to be the parabrachial nucleus since the former was located at the level of the vagus nerve root (Fig. 2B), while the latter was located at a more cephalic level (Sato et al., 2004) and was not included in the present slice preparation.

There are several possible factors that might have caused our failure to detect the contralateral response in previous studies. One is the small amplitude and lability of the contralateral optical signals (also *see* Discussion). The contralateral signal decayed quickly with repetitive stimuli, and became indistinguishable from noise after only two or three stimuli were delivered at 0.1 Hz in 8-day old preparations. When the records were carefully examined, the contralateral response was detected from the 7- to 8-day old embryonic stages in slice preparations and from the 8- to 9-day old embryonic stages in intact preparations (Table 1: contralateral slow signal).

The Contralateral Signal Reflects a Monosynaptic Response

Figure 3 shows enlargements of the optical signals detected from the ipsilateral NTS (*left traces*) and contralateral response area (*right traces*) at different stages of embryonic development. The signals in the NTS consisted of fast spike-like and slow signals, which correspond to the sodium-dependent action potential and postsynaptic response, respectively (Komuro et al., 1991; Momose-Sato et al., 1994). The contralateral signals exhibited a slow waveform, and a spike-like signal was not clearly identified in younger preparations. As development proceeded, the slow component became larger, and the initial upstroke became clearer, as indicated with arrowheads in the 10- and 11-day recordings.

Table 1. Amplitudes of the optical signals

Preparation	Embryonic day	Contralateral slow signal ($\times 10^{-4}$)	Contralateral fast signal ($\times 10^{-5}$)	Ipsilateral slow signal ($\times 10^{-4}$)	Ipsilateral fast signal ($\times 10^{-4}$)
Slice	6	nd ($n = 2$)	3.4 ± 2.0 ($n = 2$)	nd ($n = 38$) or 1.8 ± 0.4 ($n = 2$)	31.5 ± 7.8 ($n = 40$)
	7	nd ($n = 4$) or 2.0 ($n = 1$)	3.4 ± 0.2 ($n = 3$)	7.5 ± 2.8 ($n = 50$)	48.1 ± 15.0 ($n = 50$)
	8	nd ($n = 1$) or 4.1 ± 2.0 ($n = 13$)	5.5 ± 3.9 ($n = 5$)	24.1 ± 6.5 ($n = 50$)	58.9 ± 15.7 ($n = 50$)
	9	4.8 ± 1.7 ($n = 20$)	7.5 ± 3.1 ($n = 9$)	28.4 ± 5.7 ($n = 40$)	45.6 ± 15.2 ($n = 31$)
	10	5.7 ± 2.4 ($n = 3$)	11.0 ± 7.8 ($n = 2$)	26.7 ± 3.2 ($n = 3$)	44.6 ± 31.2 ($n = 3$)
	11	7.0 ($n = 1$)		37.2 ($n = 1$)	
Intact	6	nd ($n = 2$)	nd ($n = 2$)		
	7	nd ($n = 3$)	3.7 ± 0.3 ($n = 3$)		
	8	nd ($n = 4$) or 2.2 ($n = 1$)	4.5 ± 1.3 ($n = 2$)		
	9	2.3 ± 0.4 ($n = 2$)	5.0 ($n = 1$)		

Maximum amplitudes of the optical signals ($\Delta I/I$: mean ± SD) detected from the contralateral side of the preparation at the level of the vagus nerve root. For slice preparations, maximum amplitudes of the optical signals detected from the ipsilateral side are also shown for comparison. Data was obtained with single-sweep recordings except for the contralateral fast signal for which 16–50 trials were averaged, nd: $\Delta I/I < 2.0 \times 10^{-5}$ for the contralateral fast signal and $\Delta I/I < 1.5 \times 10^{-4}$ for the other signals, *n*: the number of tested preparations.

Fig. 3. Enlarged traces of the optical signals detected from the ipsilateral NTS (*left traces*) and the contralateral response area (*right traces*). The arrowheads indicate possible fast spike-like signals in the contralateral response.

The slow time course of the contralateral signal suggested that it reflects postsynaptic events as does the slow component of the ipsilateral NTS signal. Synaptic transmission in the embryonic chick vagal pathway is mediated by glutamate (Komuro et al., 1991; Momose-Sato et al., 1994; Sato et al., 2004). Thus, we examined the effects of glutamate receptor antagonists on the contralateral signal. Figure 4 shows the effects of kynurenic acid (1.2 mM) on the ipsi- and contralateral signals detected from a 9-day old intact preparation. The contralateral signal and the slow component of the ipsilateral signal were mostly suppressed by kynurenic acid. The normalized amplitude of the largest contralateral signal was calculated to be 2.0 ± 4.5% (mean ± SD, $n = 5$ preparations) in the presence of kynurenic acid. The contralateral signal was also reduced by the N-methyl-$_D$-aspartate (NMDA) receptor antagonist $_{DL}$-2-amino-5-phosphonovaleric acid (APV) at 200 μM (12.4 ± 14.4% of the control, $n = 4$) and the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) at 5 μM (35.7 ± 16.9%, $n = 3$). Taken together with the observation that the contralateral signal decayed rapidly with repetitive stimuli, the results suggest that the contralateral slow signal reflects the postsynaptic event mediated by glutamate.

The next question was whether the contralateral response area corresponds to the first relay nucleus of the vagus nerve, as does the NTS, or to a higher-order nucleus, such as the PBN. Comparison of the onset latency of the signals in the ipsilateral NTS and the contralateral response area detected from an 11-day old preparation (Fig. 5*A*) showed that there was no significant delay between the two optical signals. This observation favors the idea that the contralateral response area corresponds to the first relay nucleus of the vagus nerve.

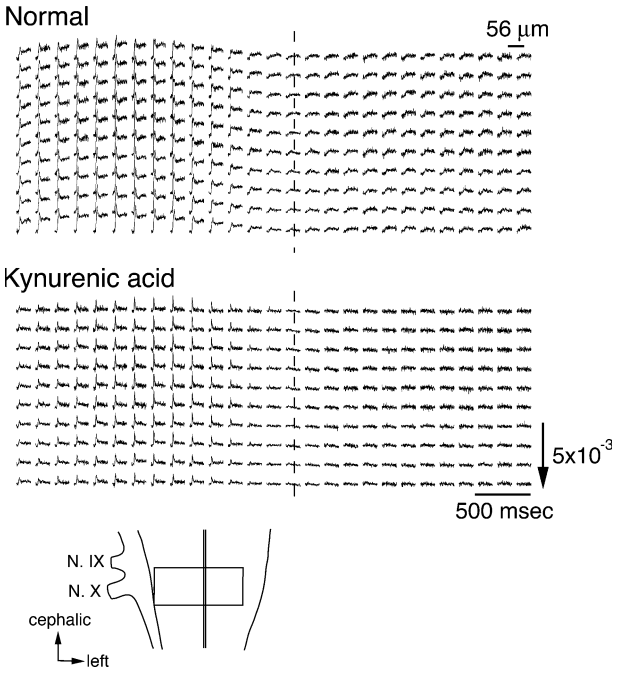

Fig. 4. The effects of kynurenic acid (1.2 mM) on optical signals detected from a 9-day old intact preparation. The recordings were obtained from a region surrounded by a square in the lower inset. The dotted line indicates the midline of the preparation.

To support this hypothesis, we examined whether fast signals corresponding to the presynaptic action potential occurred on the contralateral side in young embryos after averaging the optical signals. Figure 5*B* shows the early phase of the contralateral signal recorded in a single sweep (*top trace*) and with 16 times averaging (*middle trace*) together with the ipsilateral NTS signal (*bottom trace*) in a 9-day old slice preparation. In this preparation, fast spike-like signals were not visible on the contralateral side in the single-sweep recording (Fig. 5*B*, *top trace*), similar to the 8- and 9-day recordings shown in Fig. 3. After averaging, however, the fast signal became clearly apparent (Fig. 5*B*, *middle trace*). Similar results were obtained from other preparations at different developmental stages (Table 1: contralateral fast signal).

Between the averaged contralateral fast signal and the signal detected from the NTS, there was no significant delay for the signal onsets (Fig. 5*B*, *middle* and *bottom traces*). When signal averaging was performed in the presence of kynurenic acid, contralateral fast signals were still observed (*data not shown*). These results suggest that the contralateral fast signal reflects the direct response of the vagus nerve fibers, possibly corresponding to the presynaptic action potential, with which the slow postsynaptic response is associated.

In Fig. 6 are shown contour line maps of the averaged fast optical signal. On the contralateral side, the fast signal was concentrated in the dorsomedial

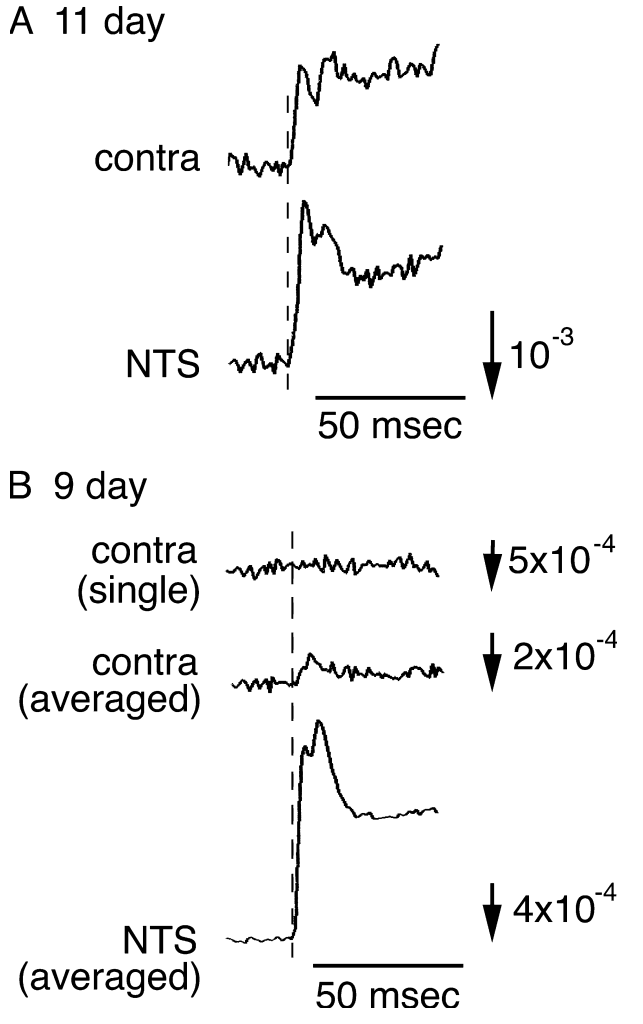

Fig. 5. Optical signals on an enlarged time scale showing the initial phase of the signal. (*A*) Optical signals recorded simultaneously from the contralateral response area (*upper trace*) and the ipsilateral NTS (*lower trace*). Data was obtained from an 11-day old slice preparation. In this preparation, fast spike-like signals were detected in a single sweep from the contralateral response area and the ipsilateral NTS. Note that there was no significant delay in signal onset between the two signals. (*B*) Optical signals detected from the contralateral response area with a single sweep (*top trace*) and after averaging 16 trials (*middle trace*) together with the ipsilateral NTS signal obtained after averaging 16 trials (*bottom trace*). Note that a small fast signal, which had no significant delay compared to the NTS signal, appeared on the contralateral side after averaging.

region, similar to the slow signal identified with single-sweep recordings (Fig. 2A). Figure 6 also suggests that the contralaterally-projecting fibers cross the midline on the dorsal side. When we cut the ventral half of the midline, contralateral responses were still observed, while the signals disappeared from the contralateral side when a complete incision was made along the midline (Table 2).

To identify morphologically the vagal projection to the contralateral side, we traced the vagus nerve

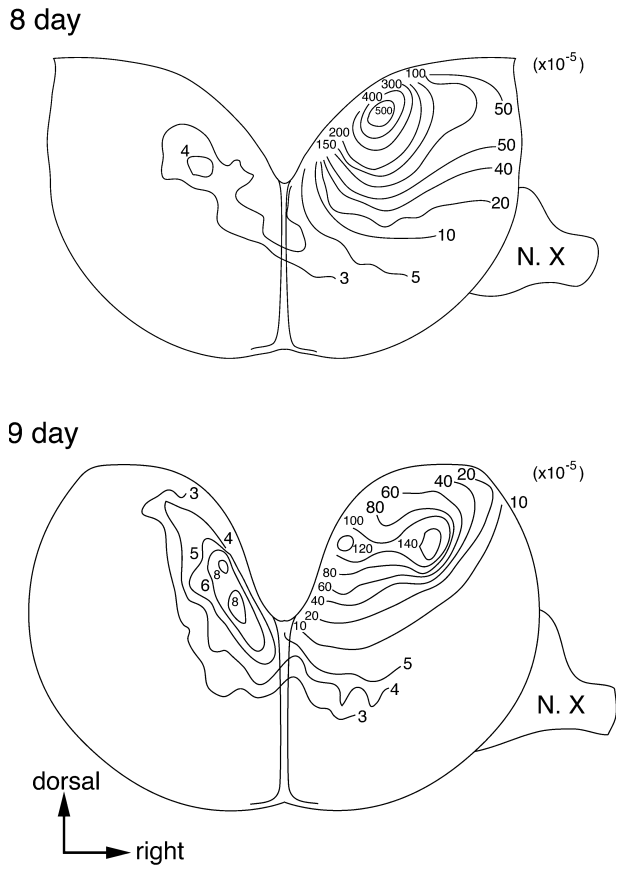

Fig. 6. Contour-line maps of the amplitude of the fast spike-like signal evoked by vagus nerve (*N. X*) stimulation. Data was obtained from an 8-day (*upper*) and 9-day (*lower*) slice preparation after averaging the optical signals following 50 trials. The numerals on the contour lines indicate the fractional change multiplied by 10^{-5}.

fibers by labeling it with the carbocyanine dye 1,1′-dioctadecyl 1-3,3,3′,3′- tetramethylindocarbocyanine perchlorate (Dil). Figure 7 is a photographic view of a horizontal (coronal) section of the 7-day old intact preparation at the level of the vagus nerve root, showing that the labeled thin fibers cross the midline and project to the contralateral side.

Relative Location of the Contralateral Response Area

To compare the relative location of the contralateral response area with that of the vagal sensory and motor nuclei, we mapped in Fig. 8 the peak signal area, i.e., the area surrounded by the curve at 70% of the largest signal amplitude, of the contralateral slow signal (*black*), ipsilateral slow signal (*light gray*) and ipsilateral fast signal (*dark gray*). The latter two areas reflect the center of the functionally-identified NTS (sensory nucleus) and DMNV (motor nucleus), respectively (Momose-Sato et al., 1991). In all preparations the ipsilateral and contralateral responses were asymmetrical with respect to the midline: the contralateral response area was slightly deviated ventromedially. This implies that the contralateral response area does not correspond to the NTS or DMNV on the contralateral side, and suggests that the vagus nerve projects to a region outside the vagal nuclei.

Table 2. The effects of midline cutting

	Preparation reference	Contralateral slow signal	Contralateral fast signal
Ventral side cut	J1216R	+	+
	J1216L	+	+
	J1217L	?	+
	J1218R	+	+
	J1218L	+	+
	J1219L	−	+
All cut	J1223R	−	−
	J1223L	−	−

The effects of midline cutting on the optical signals detected from the contralateral side. A 9-day old slice preparation was cut along the midline on the ventral side (ventral side cut) or ventro-dorsally (all cut). Contralateral slow and fast signals were positive when the signal amplitude was $\geq 1.5 \times 10^{-4}$ with the single sweep recording and $\geq 2.0 \times 10^{-5}$ with 50 trials of averaging, respectively. Note that the contralateral signals were not abolished with ventral side cutting.

Discussion

In previous studies using multiple-site optical recording, several response areas were identified in the embryonic chick brainstem with vagal stimulation. These included the dorsal motor nucleus of the vagus nerve (DMNV), the nucleus of the tractus solitarius (NTS) and a higher-order nucleus, possibly corresponding to the parabrachial nucleus (PBN) (Kamino et al., 1989; Komuro et al., 1991; Momose-Sato et al., 1991,1994; Sato et al., 2002, 2004). The first two areas were identified on the ipsilateral side at the level of the vagus nerve root, and the last one was found on the contralateral side at the level of the pons. The medullary contralateral response area identified in the present study does not seem to correspond to any of these nuclei, since the location and characteristics of the optical signal were different from those reported earlier.

In previous studies, we could not identify the medullary contralateral response although the area was included in the measured region. Several factors could have caused our failure to detect the contralateral response. First, the signals are small and decay easily, so that measurements have to be made with extreme care. Since our primary targets in earlier studies were the vagal nuclei, special attention was not paid to morphologically undefined regions. In the

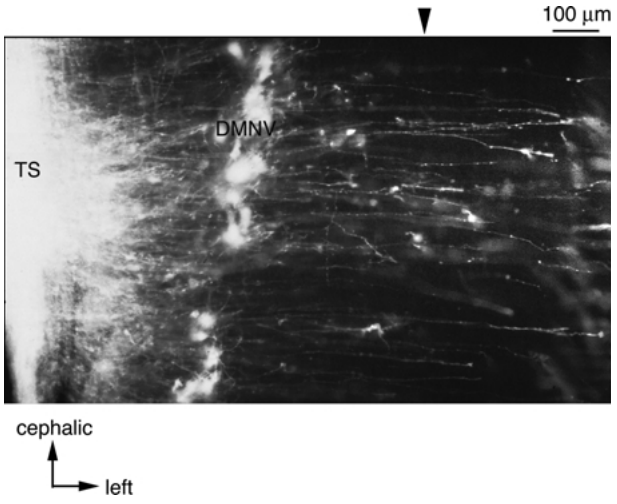

Fig. 7. Morphological view of the vagal pathway. The photograph was obtained from a horizontal (coronal) section of a 7-day old intact preparation at the level of the vagus nerve root. The carbocyanine dye, DiI, was applied to the right vagus nerve (left side of the figure). *TS*, tractus solitarius; *DMNV*, dorsal motor nucleus of the vagus nerve. The arrowhead indicates the midline of the preparation.

study where we surveyed vagal responses from a wide region of the brainstem (Sato et al., 2004), we used intact preparations younger than 9 days, in which the medullary contralateral response was usually insignificant in single-sweep recordings (Table 1). Second, the contralateral response consisted mainly of the slow component, especially in younger embryos. This resulted in signal ambiguity when there was a large movement artifact having a slow time course. Third, the contralateral response was positive only when the preparation was completely healthy. Improvements in the skill of dissection might have resulted in successful detection of the contralateral response.

The characteristics of the contralateral slow signal suggest that it reflects a monosynaptic response, although it remains possible that polysynaptic potentials contribute as well. In morphological investigations on adults, the central terminals of primary vagal afferents are found mainly in the NTS (Saper, 1995; Berthoud & Neuhuber, 2000). Smaller numbers of projections have also been reported in the DMNV, area postrema and trigeminal nuclear complex (Berthoud & Neuhuber, 2000). The location of the contralateral response was different from that of the NTS and DMNV (Fig. 8), and was also inconsistent with the location of the area postrema and trigeminal nuclear complex (Kuenzel & Masson, 1988, Sato et al., 1999).

At present, structural bases for the contralateral response have not been clarified. Possible candidates include the nucleus intercalatus, which lies between the vagus and hypoglossal nerve nuclei (Breazile, 1979; Kuenzel & Masson, 1988). In birds, the nucleus

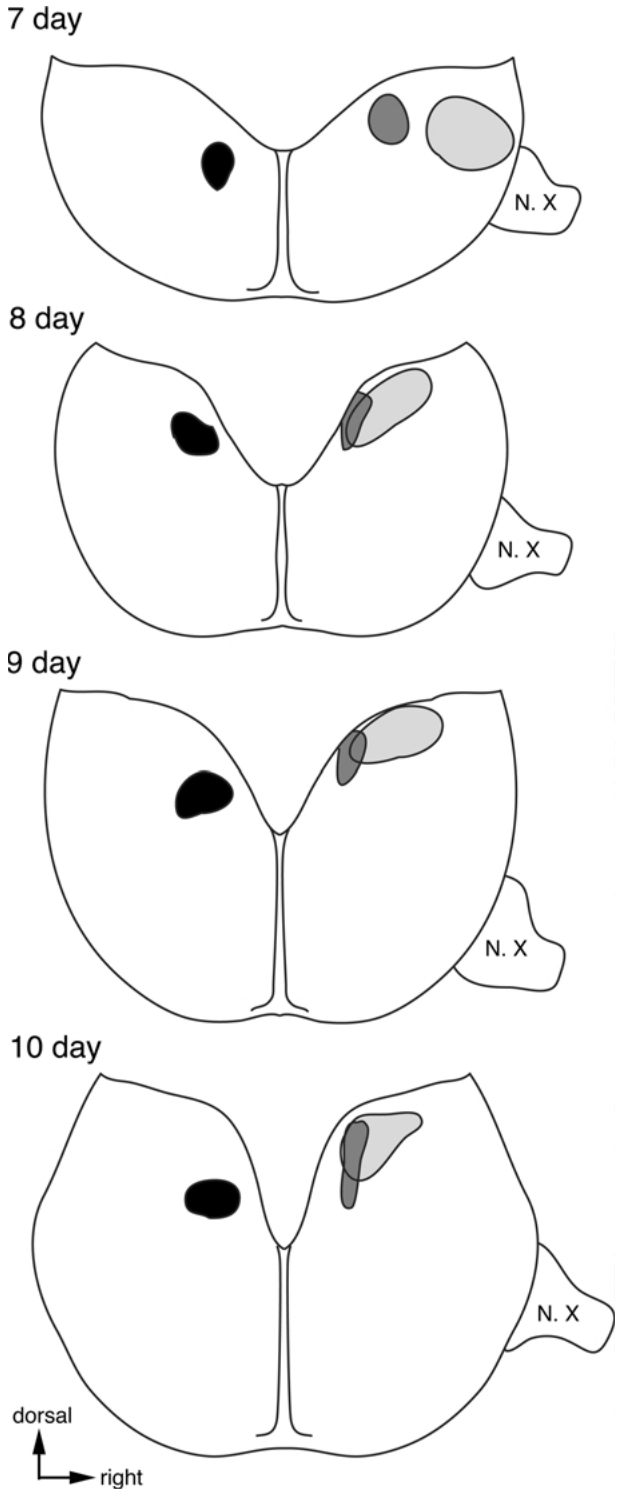

Fig. 8. Location of the peak areas of the contralateral slow signal (*black*), ipsilateral slow signal (*light gray*) and ipsilateral fast signal (*dark gray*). The areas surrounded by the curve at 70% of the largest signal amplitude are shown. The light and dark gray areas correspond to the centers of the functionally-identified NTS and DMNV, respectively.

intercalatus and the DMNV are present as separate entities, although the nucleus intercalatus has often been referred to as the dorsomedial nuclear group of

the vagus nerve (Kuenzel & Masson, 1988). It is possible that the contralateral response reflects innervation of the vagus nerve to the contralateral nucleus intercalatus, although such a projection has not been reported. Alternatively, it might be that the contralateral response reflects synaptic events in a nucleus that has not been anatomically defined. Another possibility is that growing afferent fibers, which finally project to the contralateral NTS, make synaptic and/or non-synaptic contacts with cells surrounding their pathway. In the maturing brain, neurotransmitters like GABA (γ-aminobutyric acid) and glutamate are released from migrating neurons and growth cones as a diffusible factor that acts before synapse formation (Nguyen et al., 2001; Demarque et al., 2002; Represa & Ben-Ari, 2005). However, our observation that the location of the contralateral response area did not change during the 7- to 10-day old embryonic stages (Fig. 8) does not support this hypothesis. Finally, it is possible that the contralateral response originates from a transient afferent projection that later disappears. Transient projections at particular developmental stages have been reported in the embryonic chick visual (Thanos and Bonhoeffer, 1984; O'Leary & Thanos, 1985) and auditory (Young & Rubel, 1986) systems, and a similar phenomenon might be present in the visceral system.

Whatever the structure underlying the contralateral response, the point that must be emphasized is that functional synaptic connections were identified in a region where morphological evidence for such connections has not been reported. This suggests that the sensory information processing mediated by the vagus nerve is more complex than previously known. In an earlier study on the embryonic rat trigeminal system, we reported that postsynaptic responses are detectable in the trigeminal nuclear complex before the appearance of the morphological structure of conventional synapses (Momose-Sato et al., 2004). These results suggest that the functional approach using optical recording with potentiometric dyes is useful and frequently exhibits distinct advantages over purely morphological investigations in the study of CNS organization.

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