Optical mapping reveals developmental dynamics of Mg$^{2+}$/APV-sensitive components of glossopharyngeal glutamatergic EPSPs in the embryonic chick NTS

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ABSTRACT

To examine whether there are any differences in functional organization between the glossopharyngeal nerve (N. IX)- and vagus nerve (N. X)-projecting areas in the nucleus of the tractus solitarius (NTS), we performed optical recording of neural responses evoked by N. IX stimulation in 5- to 9-day old embryonic chick brainstem preparations, and compared the results with those in our previous studies concerning the N. X-related NTS. First, we investigated $\text{DL}$-2-amino-5-phosphonovaleric acid (APV)-/Mg$^{2+}$-sensitivity of the glutamatergic excitatory postsynaptic potentials (EPSPs) in the N. IX-related NTS. In 7- to 9-day old preparations, we found regional differences in the degree of both the APV-induced reduction and Mg$^{2+}$ free-induced enhancement of the EPSPs. We constructed developmental maps of spatial patterns of the APV- and Mg$^{2+}$-sensitive components, and showed that functional expression of the N-methyl-$D$-aspartate (NMDA) receptor dynamically changed during development. Second, we studied initial expression of synaptic functions in the N. IX-related NTS. In 6-day old preparations, although action potentials alone were usually detected in normal Ringer’s solution, small EPSPs were elicited in a Mg$^{2+}$-free solution. This result suggests that the NMDA receptor-mediated synaptic function is latently generated in the N. IX-related NTS at the 6-day old embryonic stage, and that external Mg$^{2+}$ regulates the onset of synaptic functions. Developmental patterns of APV-/Mg$^{2+}$-sensitivity and the stage of initial expression of the glossopharyngeal EPSP were similar to those of the N. X, suggesting that the developmental sequence of the synaptic function in the NTS is the same for the N. IX- and N. X-related NTS.
INTRODUCTION

The nucleus of the tractus solitarius (NTS) is a sensory nucleus that has unique features in the central nervous system (CNS). First, the NTS is the first relay nucleus to receive visceral information from different kinds of peripheral organs, such as cardiovascular, pulmonary and gustatory organs. Second, the NTS is the nucleus which receives projections from different cranial nerves, such as the facial (N. VII), glossopharyngeal (N. IX) and vagus (N. X) nerves. These nerves carry different information from the peripheries, which is integrated within the NTS. For example, in the cardiovascular reflexes, information from the carotid body and aortic arch is conducted via the N. IX and N. X, respectively, and then transferred to several regions in the brainstem and higher CNS (for reviews see Spyer, 1982; Kumada et al., 1990; Andresen and Kunze, 1994; Dampney, 1994; Saper, 1995). Therefore, the investigation of the NTS function is of great importance; not only to reveal how visceral information is integrated in the brainstem, but also to elucidate, as one of the fundamental models, how the CNS processes sensory information from the peripheries.

Although many anatomical and physiological studies have been done in adult animals, the ontogenetic approach to the physiological functions of the NTS has been hampered because of the small size and fragility of embryonic NTS neurons. We employed an optical recording technique with voltage-sensitive dyes (for reviews see Cohen and Salzberg, 1978; Salzberg, 1983; Grinvald et al., 1988; Wu et al., 1998), and proved that the optical technique is a useful tool for analyzing the embryogenetic expression of neural functions in the CNS (for reviews see Kamino, 1990; Momose-Sato et al., 2001, 2002).
In our previous investigations, we examined the spatio-temporal patterns of neural activity evoked by glossopharyngeal/vagal stimulation in embryonic brainstems. We demonstrated three-dimensional profiles of the glossopharyngeal/vagal response areas corresponding to the NTS (sensory nucleus), the dorsal motor nucleus of the vagus nerve (DMNV: motor nucleus) and the nucleus of the glossopharyngeal nerve (Nucl IX: motor nucleus) in the chick and rat embryos (Komuro et al., 1991; Momose-Sato et al, 1991, 1994, 1999; Sato et al., 1995, 1998, 2002a, 2002b, 2004). In these studies, we proved the following characteristics of the embryonic chick NTS. (1) In both the N. IX- and N. X-related NTS, optical signals were composed of fast and slow signals, and the fast signal corresponded mainly to the presynaptic action potential and the slow signal to the glutamatergic excitatory postsynaptic potential (EPSP) (Komuro et al., 1991; Momose-Sato, 1994; Sato et al., 1995). (2) In both the N. IX- and N. X-related NTS, the glutamatergic EPSPs consisted of non-NMDA (N-methyl-D-aspartate) and NMDA receptor components (Komuro et al., 1991; Momose-Sato et al., 1994). (3) In the N. X-related NTS, the glutamatergic EPSP was expressed at 7 day of incubation in normal Ringer’s solution. However, synaptic function mediated by NMDA receptors was already generated latently at the 6-day old embryonic stage, and the onset of synaptic function was regulated by a Mg²⁺ block on the NMDA receptors (Momose-Sato et al., 1994). (4) In the N. X-related NTS, the DL-2-amino-5-phosphonovaleric acid (APV)- and Mg²⁺-sensitivity of the vagal glutamatergic EPSPs changed during development (Momose-Sato et al., 1994).

In the ontogenetic approaches to elucidate a manner of sensory information
processing in the NTS, it is important to clarify whether there are any differences in
developmental organization of synaptic functions between the N. IX and N. X. In the
present study, we addressed two questions, i.e., (1) whether the dynamic changes in the
APV-/Mg$^{2+}$-sensitivity of the glutamertagic EPSPs are also observed in the N. IX-related
NTS and (2) whether the synaptic function in the N. IX-related NTS emerges at the same
time in the N. X-related NTS. From the results obtained, we extracted principles of the
developmental expression of the NTS function.
MATERIALS AND METHODS

Preparations: Brainstem slice preparations dissected from 5- to 9-day old embryonic (E5-E9) chicks were used (n=36). Experiments were carried out in accordance with the Tokyo Medical and Dental University guidelines 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., Saitama, Japan) were incubated for five to nine days in a forced-draft incubator (type P-008, Showa Incubator Lab., Urawa, Japan) at a temperature of 37 °C and 60% humidity, and were turned once each hour. In the present experiment, E5 corresponded to the Hamburger-Hamilton stages (H-H stages: Hamburger and Hamilton, 1951) 27, E6 to stages 28-29, E7 to stages 30-32, E8 to stages 33-34, and E9 to stage 35. The embryos were decapitated, and brainstems, with the glossopharyngeal nerve fiber attached, were dissected from the embryos. Slice preparations of about 1500 μm thickness were made from the isolated brainstem at the level of the glossopharyngeal nerve root. The pia mater was carefully removed in the bathing solution. After staining with the dye (see below), the preparation was attached to the silicone (KE 106LTV; Shin-etsu Chemical Co., Tokyo, Japan) bottom of a simple chamber with the spinal cord side up. The bathing solution contained (in mM) NaCl, 138; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.5; glucose, 10; and Tris-HCl buffer (pH 7.3), 10. The solution was equilibrated with oxygen.

Voltage-sensitive dye staining: Each preparation was stained by incubating it for 20 min in Ringer’s 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; Momose-Sato et al., 1995), and the excess (unbound) dye was washed away with dye-free Ringer’s solution before recording. This merocyanine-rhodanine dye has been shown to be particularly useful in embryonic nervous and cardiac tissues (Kamino, 1991; Momose-Sato et al., 1995).

Electrical stimulation: The cut end of the glossopharyngeal nerve was drawn into a micro-suction electrode fabricated from a haematocrit tube (VC-HO75P; TERUMO Co., Tokyo, Japan), which had been hand-pulled to a fine tip (about 100 µm internal diameter) over a low-temperature flame. Positive (depolarizing) square current pulses (8 µA/5 msec), which evoked maximum responses, were applied to the glossopharyngeal nerve at intervals of 10-15 min.

Optical recording: 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 on the preparation. An objective (S Plan Apo, x10, 0.4 NA (numerical aperture)) and a photographic eyepiece projected a real image of the preparation (magnification 25x) onto a multi-element silicon photodiode matrix array mounted on an Olympus Vanox microscope (Type AHB-L-1, Olympus Optical Co., Tokyo, Japan). The focal plane was set on different depths with moving a microscope stage. To detect the largest optical responses from the N. IX-related NTS, we set the focal plane at the level of the glossopharyngeal nerve root (Fig. 1; also see Sato et al., 1995). In the present
experiments, we used the 128ch optical recording system using a 12 x 12-element silicon photodiode array (MD-144-4PV; Centronic Ltd., Croydon, UK), which was constructed in our laboratory (for reviews, see Kamino, 1991; Momose-Sato et al., 2001). Each pixel (element) of the array detected light transmitted by a square region (56 x 56 μm$^2$ using x25 magnification) of the preparation. The output of each detector in the diode array was passed to an amplifier (time constant of AC-coupling $\approx$ 3 sec) via a current-to-voltage converter. The amplified outputs from 127 elements of the detector were first recorded simultaneously on a 128-channel recording system (RP-890 series, NF Electronic Instruments, Yokohama, Japan), and then were passed to a computer. The time resolution of this system was 1 msec. The time interval between each recording was 10-15 min, and incident light was turned off except during the measuring period. In this condition, little or no signal fatigue was observed, and the degree of variability between successive recordings in terms of amplitude and duration of the signals was small. The recordings were made in a single sweep. The optical measurement was carried out in a still chamber without continuous perfusion with Ringer’s solution at room temperature, 26-30 °C. The recorded signals were presented as the fractional change $\Delta I/I$ (the change in the light intensity divided by DC background intensity).
RESULTS

Optical detection of neural activity in the N. IX-related brainstem nuclei

To show a typical response pattern of neural activity in the N. IX-related brainstem nuclei, we illustrate color-coded representations of multiple-site optical sectioning recordings of neural activity induced by N. IX stimulation in an 8-day old preparation (Fig. 1), which was recorded in a similar way as described previously (Sato et al., 2002a). The focus was set to two different depths, viz., Focus 1 corresponding to 500µm cephalic to the level of the glossopharyngeal nerve root and Focus 2 corresponding to the level of the glossopharyngeal nerve root. With a stimulating current applied to the right glossopharyngeal nerve, action potentials (fast signals) were most clearly recorded from the dorso-medial region in Focus 1, whereas glutamate-mediated excitatory postsynaptic potentials (EPSPs: slow signals) were detected mainly from the dorso-lateral region in Focus 2. These regions correspond to the nucleus of the glossopharyngeal nerve (Nucl IX; motor nucleus) and the nucleus of the tractus solitarius (NTS; sensory nucleus), respectively (Breazile, 1979; Sato et al., 1995).

In our previous studies, we did similar experiments in other focal planes and in every developmental stage tested (5- to 9-day old embryos). Although the signals were not completely separated in the two focal planes, the response in the Nucl IX was most clearly detected from Focus 1, whereas that in the NTS was largest at the level of Focus 2 (Sato et al., 1995, 2002a). In the present study, we recorded slow signals on Focus 2, and examined dynamic changes in the N. IX-related synaptic function in the NTS.
Developmental changes in APV-sensitivity of the slow optical signal

Figure 2 shows optical signals induced by glossopharyngeal nerve stimulation and the suppressive effects of \( \text{DL-2-amino-5-phosphonovaleric acid (APV)} \) on the optical signals. In Fig. 2A, the left recording shows control signals recorded in normal Ringer's solution and the right one shows signals recorded in an APV-containing solution in an 8-day old preparation. The later phase of the slow signals was markedly reduced when APV (200 \( \mu \text{M} \)) was added to the bathing solution (also see Fig. 2B), while the initial phase of the slow signals was reduced by an application of 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX; 5\( \mu \text{M} \)) (Fig. 2C). These results imply that the later phase of postsynaptic potentials in the NTS is mediated by N-methyl-\( \text{D} \)-aspartate (NMDA) receptors, while the initial phase is mediated by non-NMDA receptors. In Fig. 2B, we show enlarged traces of optical signals detected from 7- to 9-day old preparations. The amplitude of the slow signal gradually increased with development. In the presence of APV, the later phase of the slow signal was reduced in every developmental stage.

As can be seen in the original recordings shown in Fig. 2A, there were regional differences in the amplitude of the slow signals in normal and APV-containing solutions. With the optical technique for monitoring membrane potential changes, the linearity of the optical signal with changes in membrane potential has been established (Cohen and Salzberg, 1978). It has also been assumed that the fractional signal size is proportional to the magnitude of the membrane potential changes in each cell and process, and to the number and membrane area of activated neural elements within the field detected optically by one
photodiode under conditions where the amount of dye bound to the membrane is uniform (Obaid et al., 1985; Orbach et al., 1985; Kamino et al., 1989). To reveal the spatial distribution pattern of the glossopharyngeal responses and the APV-sensitivity, we measured the areas under the slow signal curves (signal-area) obtained in normal and APV-containing solutions, and then constructed maps of the contour lines of the signal-areas. The maps shown in Fig. 3A were constructed using the recordings from 7- to 9-day old preparations. The top row shows the maps of the control signals in each preparation, the second row was made with data obtained from the APV-containing solution, and the third row with the differences ($\Delta S = [\text{the slow signal area in normal solution}] - [\text{the area estimated in the APV-containing solution}]$). The value of $\Delta S$ reached a saturation level with the APV concentration used in the present study.

The sizes of the slow signal areas were regionally distributed in a layered pattern surrounding the site in which the signal-area exhibited the maximal size (peak-area site). Under the control condition, the peak size ($S_{\text{max}}$) increased from 4 (arbitrary units) to 7 (arbitrary units) as development proceeded from the 7-day to 9-day embryonic stage (also see Table 1). There were differences in the regional distribution pattern among the three developmental stages: the position of the peak-area site in the 9-day old preparation was translocated to the medial direction compared to those in the 7- and 8-day old preparations. Similar patterns were observed in all the tested preparations ($n=4$).

In these maps, the relative positions of the peak-area site in the control (top row) and in the APV-containing solution (second row) coincided with each other in the 7- to 9-day
The distribution pattern of $\Delta S$ (third row) was also very similar to that of the control signal area with the relative position of the peak-area site of $\Delta S$ corresponding to that of the control signal area. The size of $\Delta S$ gradually increased with development, suggesting that the NMDA-receptor-mediated component of the glutamatergic excitatory postsynaptic function gradually increases in the NTS during development (also see Table 1).

To examine the relative sensitivity to APV, we estimated the ratios of the APV-induced reduction area ($\Delta S$) to the control area ($S$), and made distribution maps of the ratios ($\Delta S/S$). Fig. 3B shows the distribution of the ratio for the signal component reduced by APV. The data were obtained from the maps shown in Fig. 3A. In these maps, the values of the ratio ($\Delta S/S$) are indicated by the relative size of the solid circles: the ratios were divided into five sizes. There were differences in the regional distribution pattern of the ratio ($\Delta S/S$) between these preparations. In the 7-day old preparation, the ratio was almost homogenous in the NTS; in the 8-day old preparation, the ratio was larger in the medial region, and in the 9-day old preparation, the ratio increased over the whole response area with the distribution pattern being similar to that in the 8-day old preparation (also see Table 1). These features are considered to represent the regional distribution of APV-sensitivity and of the ratio of functionally expressed NMDA-receptors to total glutamatergic receptor function.
Developmental changes in Mg$^{2+}$-sensitivity of the slow optical signal

It is well known that the NMDA receptor function is blocked by extracellular Mg$^{2+}$ ($\text{Mg}^{2+}_o$) (Mayer et al., 1984; for a review see Collingridge and Lester, 1989). Fig. 4 shows the effects of Mg$^{2+}_o$ removal on the optical signals evoked by glossopharyngeal nerve stimulation. In Fig. 4A, the left recording shows control signals recorded in normal Ringer's solution and the right one shows signals recorded in a Mg$^{2+}$-free solution in an 8-day old preparation. Enlarged traces of the optical signals detected from 7- to 9-day old preparations are shown in Fig. 4B. The slow signal was markedly enhanced in the Mg$^{2+}$-free solution in every developmental stage. This effect was blocked by APV (200 µM; data not shown), suggesting that the enhancive effect of Mg$^{2+}_o$ removal is mediated by the NMDA receptor.

As can be seen in the original recordings shown in Fig. 4A, there were regional differences in the amplitude of the slow signals in normal and Mg$^{2+}$-free bathing solutions. We evaluated the areas under the slow signal curves (signal-area), and constructed contour line maps of the signal-areas. The maps shown in Fig. 5A were constructed using the recordings from 7- to 9-day old preparations. The top row illustrates the maps of the control signals, the second row was made with the data obtained in the Mg$^{2+}$-free solution, and the third row with the differences ($\Delta S = \text{[the slow signal area estimated in the Mg}^{2+}\text{-free solution]} - \text{[the area in normal solution]}$). In Fig. 5A, the patterns of the three contour line maps (control, Mg$^{2+}$-free and difference) were similar in each developmental stage. At embryonic 7 and 8 days, the peak-area sites were positioned in the lateral region, while at
embryonic 9 day, they were located in the medial region.

In order to examine the regional differences in the relative sensitivity of the slow signal to external Mg$^{2+}$, we estimated the ratio of $\Delta S$ to the control area (S). In Fig. 5B, the values of the ratio ($\Delta S/S$) are represented by the relative sizes of solid circles: the size was divided into five grades. The data were obtained from the three preparations shown in Fig. 5A. In these maps, it was shown that the relative sensitivity to external Mg$^{2+}$ was greatest in the 8-day old preparation (also see Table 2), and that the signals in the ventral region of the response area were relatively more affected by external Mg$^{2+}$ than in the dorsal region. These results show that the relative sensitivity to Mg$^{2+}$ does not increase in a simple fashion but dynamically changes during development.

The initial appearance of Mg$^{2+}$-sensitivity in the N. IX-related NTS

At the 6-day old embryonic stage, glossopharyngeal nerve stimulation evoked no or very small postsynaptic responses in the NTS (n=4). We examined whether the removal of Mg$^{2+}$ induced expression of postsynaptic responses which were not significant in normal Ringer's solution.

Figure 6A shows two examples of optical recordings obtained with glossopharyngeal nerve stimulation in 6-day old preparations. In these preparations, glossopharyngeal nerve stimulation evoked only fast spike-like signals in normal Ringer's solution: the slow signal was not significant ($<1 \times 10^{-4}$) (left traces). However, when Mg$^{2+}$ was removed from the extracellular solution, significant slow signals were elicited (right
traces) in the region corresponding to the NTS (indicated by yellow in the upper drawings). The slow signals induced in the Mg$^{2+}$-free solution were blocked by APV (200 µM), suggesting that they are attributable to NMDA receptors.

In Fig. 6B, we show two other examples of optical recordings obtained from 6-day old preparations. In these preparations, very small slow signals were detected even in normal Ringer’s solution (left traces) in the region indicated in red in the upper drawings. In the Mg$^{2+}$-free solution, these signals were markedly enhanced (right traces), and additional slow signals were detected from the surrounding region (indicated in yellow in the upper drawings). In 5-day old preparations, slow signals were not observed in either normal Ringer’s or Mg$^{2+}$-free solution (n=3). Taken together, the results suggest that although there is animal-to-animal variation, the 6-day old embryonic stage is the critical stage at which synaptic function is generated in the N. IX-related NTS.
DISCUSSION

In the present experiments, using an optical recording technique with a voltage-sensitive dye, we examined developmental changes in regional distributions of the APV- and Mg$^{2+}$-sensitive components of the EPSPs evoked by glossopharyngeal nerve stimulation in the embryonic chick brainstem. The glossopharyngeal nerve of the embryo is very thin and fragile, and the present study seems to be the first report to have succeeded in examining developmental dynamics of the N. IX-related synaptic function in the embryonic brainstem. The results demonstrated (1) developmental changes in NMDA and non-NMDA receptors, (2) developmental dynamics of Mg$^{2+}$-sensitivity of the NMDA receptors, and (3) the profile of initial expression of synaptic function in the N. IX-related NTS. We discuss these issues in comparison with data obtained for the N. X-related NTS (Momose-Sato et al., 1994), and consider a principle of the developmental expression of the NTS function.

Developmental changes in APV-/Mg$^{2+}$-sensitivity of the glutamatergic EPSPs in the NTS

The glutamate receptor is conventionally divided into the NMDA receptor and non-NMDA receptor, and APV is considered to be a specific antagonist to the NMDA receptor (Davies et al., 1981; for reviews see Watkins and Evans, 1981; Collingridge and Lester, 1989; MacDonald and Nowak, 1990). In the present study, we used APV as a pharmacological tool to separate the NMDA and non-NMDA receptor components of the EPSP-related slow optical signal. In Fig. 3, it seems reasonable to consider that the signal-
area diminished at the saturation level of APV (Difference ($\Delta S$), the third row in Fig. 3A) corresponds to the NMDA receptor component, and that the signal-area unaffected by APV (the second row in Fig. 3A) corresponds to the non-NMDA-receptor component. As shown in Fig. 4, the slow signal induced by N. IX stimulation was enhanced by Mg\(^{2+}\) removal, and this effect was blocked by APV. Therefore, in Fig. 5, it is considered that the signal-area enhanced by Mg\(^{2+}\) removal (Difference ($\Delta S$), the third row in Fig. 5A) reflects the distribution pattern of Mg\(^{2+}\)-sensitivity of the NMDA receptor.

In Figs. 3 and 5, we extracted the following characteristics of the N. IX-related synaptic function in the embryonic chick NTS. (1) The total areas of the glutamatergic EPSP gradually expanded, and the NMDA and non-NMDA receptor components also increased as development proceeded from the 7- to 9-day embryonic stage (Fig. 3A and Table 1). (2) The ratio of the NMDA-receptor function to total glutamatergic receptor function slightly increased with development, and the distribution patterns of the ratio changed from a homogenous pattern to a medially-shifted pattern (Fig. 3B and Table 1). (3) The peak-area site of Mg\(^{2+}\)-sensitivity (Fig. 5A) moved medially with development, and this was consistent with the developmental changes in the NMDA-receptor component (Fig. 3A). (4) The ratio of the Mg\(^{2+}\)-sensitivity increased at the 8-day embryonic stage, and decreased again at the 9-day embryonic stage (Fig. 5B and Table 2).

In our previous study (Momose-Sato et al., 1994), we examined developmental changes in the slow signals evoked by vagus nerve stimulation, and reported similar characteristics of the APV- and Mg\(^{2+}\)-sensitivity of the glutamatergic EPSPs in the N. X
related-NTS. These results indicate that the developmental sequence of the glutamatergic receptor function in the NTS is the same for the N. IX- and N. X-related NTS.

Concerning Mg\(^{2+}\)-sensitivity of the glutamatergic EPSPs, there seem to be some causes in its developmental change: one is that the total fraction of the NMDA-receptor decreases at the 9-day old embryonic stage, and another is that the Mg\(^{2+}\)-sensitivity of the NMDA receptor changes with development. Considering that the inhibitory effect of Mg\(^{2+}\) on the NMDA receptor is voltage-dependent (Mayer et al., 1984; Nowak et al., 1984), it is also possible that changes in resting membrane potential may play a role in the developmental changes in the Mg\(^{2+}\) sensitivity of the NMDA receptor. During early development, it is known that changes in excitatory amino acid receptors, particularly NMDA receptors, occur in the central nervous system, and a transient increase in the expression of glutamate, NMDA, and AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors has been demonstrated in different brain areas (Baudry et al., 1981; Tremblay et al., 1988; Insel et al., 1990; Miller et al., 1990). In addition, it has been reported that the Mg\(^{2+}\)-sensitivity of NMDA receptors changes during development, and it has been suggested that changes in Mg\(^{2+}\) regulation of the NMDA receptor may play a role in its development in the central nervous system (Bowe and Nadler, 1990; Morrisett et al., 1990). The developmental change in the expression and the sensitivity to Mg\(^{2+}\) of the NMDA receptor may be related to the early development of synaptic transfer efficacy within the NTS.
The initial expression of synaptic function in the NTS

As shown in Fig. 6, in normal Ringer's solution containing 0.5 mM Mg$^{2+}$, only fast spike-like signals were usually observed in the 6-day old preparations, whereas small slow signals were elicited by Mg$^{2+}$ removal. In the embryonic chick N. X-related NTS, it has been shown that synaptic function mediated by NMDA receptors is latently generated at the 6-day old stage, and that the onset of synaptic function is regulated by a Mg$^{2+}$ block on the NMDA receptors (Momose-Sato et al., 1994). These data indicate that, irrespective of projecting nerves, viz., N. IX and N. X, synaptic function mediated by the NMDA receptor is generated in the NTS as early as the 6-day old embryonic stage, and is suppressed by external Mg$^{2+}$.

In an anatomical investigation (Hiscock and Straznicky, 1986), it has been reported that neurons in the distal glossopharyngeal and vagal ganglia are generated between the 2nd and 5th days of incubation, and those in the proximal ganglia are produced between the 4th and 7th days. The results obtained with optical recording suggest that functional synapses of the glossopharyngeal and vagal nerves have already been generated by the 6-day old embryonic stage, at which the neuronal generation in the proximal ganglia has not yet been completed.

The NTS has a number of unique anatomical and phenotypical features that contribute to its pivotal role in neuronal regulation and integration of autonomic functions. In adults, it is considered that the NTS is not a simple "relay" nucleus, but rather that it performs complex integration of information from multiple synaptic inputs from the
periphery and central origins (Paton and Kasparov, 2000). In the present study, we demonstrated that the initial expression and principal characteristics of synaptic function are similar between the N. IX and N. X-related NTS. These results suggest that the peripheral information conducted by the N. IX and N. X may be simultaneously processed and integrated in the NTS from the beginning of nuclear organization.

One question in ontogenetic investigations of sensory information processing is whether there is any common feature in developmental organization of synaptic functions between different nerves that project to the same nucleus. The present study demonstrated that this was the case with the N. IX and N. X responses in the embryonic chick NTS. In Fig. 7, we summarize the sequence of the postsynaptic function in the chick NTS, which was common irrespective of the projecting nerves, viz., the N. IX and N. X.
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FIGURE LEGENDS

Figure 1. Color-coded representations of multiple-site/sectioning recordings of neural activity evoked by right glossopharyngeal nerve (N. IX) stimulation in an 8-day old preparation. The evoked signals were recorded from multiple sites of a slice preparation at two different focal planes. Focus 2 corresponds to the level of the N. IX root, and Focus 1 is 500 µm cephalic to Focus 2. Glossopharyngeal nerve stimulation induced optical responses in two different areas; the nucleus of the glossopharyngeal nerve (Nucl IX) and the nucleus of the tractus solitarius (NTS). The waveforms obtained from each area are shown in the right row. The direction of the arrow on the lower right corner indicates an increase in transmitted light and the length of the arrow represents the stated value of the fractional change.

Figure 2. A. Multiple-site optical recordings of neural activity from an 8-day old preparation. The electrical activity was evoked by a depolarizing square current pulse (8 µA/5 msec) applied to the left glossopharyngeal nerve using a micro-suction electrode. This stimulating condition was adequate for eliciting the maximum responses. The left recording was obtained in normal Ringer's solution and the right one was obtained in an APV (200 µM)-containing solution. In this figure and Fig. 4A, the traces are arranged so that their relative positions in the figures correspond to the relative positions of the sites of the preparation imaged onto the detector. The recordings were made in a single sweep. B. Enlargements of optical signals from 7- to 9-day old preparations in normal (left row) and
APV (200 µM)-containing (right row) solutions. C. Enlarged traces from an 8-day old preparation in normal (left row) and CNQX (5 µM)-containing (right row) solutions.

**Figure 3.** A. Contour line maps of the time-integrated area of the slow optical signals in 7- to 9-day old preparations. The top row shows the control; the second row is for the signals in the APV (200 µM)-containing solution; and the third row is the difference ($\Delta S$) = [the control area] - [the area in the APV-containing solution]. The numerals on the contour lines represent the areas in arbitrary units. B. Regional distributions of the fraction of the slow optical signal area reduced by APV to the control area. The fractions are displayed by black circles, which were ranked into five classes according to the size of the ratio (%). Data were obtained from the same preparations shown in A.

**Figure 4.** A. Multiple-site optical recordings of neural activity from an 8-day old preparation in normal Ringer's solution (left traces) and in a Mg$^{2+}$-free solution (right traces). The optical signals were evoked by a depolarizing square current of 8 µA/5 msec. B. Enlargements of optical signals from 7- to 9-day old preparations in normal (left row) and Mg$^{2+}$-free (right row) solutions.

**Figure 5.** A. Contour line maps of the time-integrated area of the slow optical signals in 7- to 9-day old preparations. The top row shows the control; the second row is for the signals in the Mg$^{2+}$-free solution; and the third row is the difference ($\Delta S$) = [the area in the Mg$^{2+}$-free
solution] - [the control area]. The numerals on the contour lines represent the areas in arbitrary units. B. Regional distributions of the fraction of the slow optical signal area enhanced by Mg\(^{2+}\) removal to the control area. The fractions are displayed by black circles, which were ranked into five classes according to the size of the ratio (%). Data were obtained from the same preparations shown in A.

**Figure 6.** A. Examples of the areas in which slow optical signals appeared after Mg\(^{2+}\) removal in 6-day old preparations. The positions indicated in yellow are the sites at which slow signals were elicited in the Mg\(^{2+}\)-free solution. Enlargements of optical signals from three sites of a 6-day old preparation in normal and Mg\(^{2+}\)-free solutions are illustrated in the lower panel. Note that very small slow signals were elicited in the Mg\(^{2+}\)-free solution. B. Two other examples of the areas in which slow optical signals were detected in 6-day old preparations. The positions indicated in red are the sites at which small slow signals were detected in normal Ringer's solution; the positions indicated in yellow are the sites at which slow signals were elicited in the Mg\(^{2+}\)-free solution. In the lower panel, enlargements of optical signals detected in normal and Mg\(^{2+}\)-free solutions are illustrated.

**Figure 7.** Summary of the sequence of events in the embryonic emergence of the neural responses in the chick NTS. The N. IX- and N. X-related NTS are illustrated in yellow green and green, respectively. The Mg\(^{2+}\)-sensitivity of the glutamatergic EPSPs (glu-EPSPs) is displayed by red circles. In this figure, data obtained in the present study are
summarized in combination with the results in our previous studies (Momose-Sato et al, 1994; Sato et al., 1995).
Table 1. Parameters associated with the maximum area of optical signals under the normal and APV conditions

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Control ($S_{max}$)</th>
<th>Difference ($\Delta S_{max}$)</th>
<th>$\Delta S_{max} / S_{max}$ (%)</th>
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<td>J1280</td>
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<td>42</td>
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| 8 day       |                      |                               |                                 |
| J1039       | 7.6                  | 5.9                           | 78                              |
| J1041       | 9.7                  | 8.3                           | 86                              |
| J1042       | 6.3                  | 4.7                           | 75                              |
| J1163L      | 6.4                  | 3.9                           | 61                              |
| J1163R      | 9.0                  | 6.9                           | 77                              |

| 9 day       |                      |                               |                                 |
| J1251L      | 9.8                  | 7.7                           | 79                              |
| J1251R      | 8.2                  | 7.0                           | 85                              |
| J1252       | 5.8                  | 4.7                           | 81                              |
| J1281       | 7.8                  | 6.1                           | 78                              |

$63\pm14$  
$75\pm9$  
$81\pm3$
Table 2. Parameters associated with the maximum area of optical signals under the normal and Mg\textsuperscript{2+}-free conditions

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<th>Difference ((\Delta S_{\text{max}}))</th>
<th>(\Delta S_{\text{max}} / S_{\text{max}} (%))</th>
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Fig. 1
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7