OPTICAL CHARACTERIZATION OF A NOVEL GABA RESPONSE IN EARLY EMBRYONIC CHICK BRAINSTEM

Y. MOMOSE-SATO,* K. SATO, A. HIROTA, T. SAKAI, X.-S. YANG, and K. KAMINO

Department of Physiology, Tokyo Medical and Dental University School of Medicine, Bunkyo-ku, Tokyo 113, Japan

Abstract. To examine the functional expression of embryonic GABA receptors, the inhibitory effects of GABA (GABA responses) on the excitatory postsynaptic potentials evoked by vagal stimulation in seven- to ten-day-old embryonic chick brainstem slice preparations. A multiple-site optical recording technique was used, with a multielement photodiode array system and a fast voltage-sensitive microfluorochrome dye (5(6)FAM). First, in the GABA response, three components were pharmacologically identified: component 1, related to GABA\textsubscript{A} receptors; component 2, related to GABA\textsubscript{B} receptors; and component 3, which is insensitive to GABA\textsubscript{A} and GABA\textsubscript{B} antagonists, but is stimulated by both GABA\textsubscript{A} and GABA\textsubscript{B} analogues. Subsequently, the embryogenesis and early development of the three components were investigated, and early developmental maps of regional distribution patterns of these components were constructed. Components 1 and 2 have already emerged in the seven-day-old embryonic brainstem preparation; component 2 appeared in the eight-day-old preparations. No component related to GABA\textsubscript{A} receptors was observed in the seven- to ten-day-old embryonic stages.

From the pharmacological properties of component 3, we suggest that it is related to a new subtype, the GABA\textsubscript{B} receptor.

Key words: GABA responses, embryonic brainstem, nucleus tractus solitarius, optical recording, spatiotemporal patterns.

GABA provides inhibitory modulation of excitatory postsynaptic potentials (EPSPs),\textsuperscript{1, 2, 3, 22-25} and classically, two classes of GABA receptors, namely GABA\textsubscript{A} and GABA\textsubscript{B} receptors, have been identified.\textsuperscript{1, 2, 3, 22-25} GABA acts postsynaptically at GABA\textsubscript{A} receptors, which are ligand-gated Cl\textsuperscript{-} channels.\textsuperscript{26} GABA\textsubscript{B} receptors, which activate K\textsuperscript{+} and Ca\textsuperscript{2+} channels by coupling to GTP-binding regulatory protein (G protein).\textsuperscript{11, 12, 27-29} The GABA\textsubscript{A} receptors are blocked by the competitive antagonist, bicuculline, and the non-competitive blocker, picrotoxin.\textsuperscript{26} The GABA\textsubscript{B} receptors are specifically blocked by 3-hydroxyxazolidine \textsuperscript{22, 23} in addition, picrotoxin-sensitive but bicuculline-insensitive GABA\textsubscript{B} receptors have been described\textsuperscript{30} (for a review, see Ref. 3). Recently, possible novel classes of GABA receptors have also been reported.\textsuperscript{12, 26, 27, 29} although it is suggested that they are similar to the GABA\textsubscript{A} receptors.

In a previous short note,\textsuperscript{9, 10} we have provided preliminary evidence for a novel type of GABA response, which is insensitive to antagonists of both GABA\textsubscript{A} and GABA\textsubscript{B} receptors, but is stimulated by agonists of both GABA\textsubscript{A} and GABA\textsubscript{B} receptors. These results were found for vagal glutamatergic EPSPs in seven- to eight-day-old embryonic chick brainstem slices, using an optical technique employing a fast voltage-sensitive dye.

Experimental analysis of the early embryonic nervous system is difficult because the cells are relatively inaccessible. Microelectrode and patch-clamp examinations of neurons, which provide the most direct test of their electrophysiological behavior including single channel currents, are often technically difficult or impossible because of the small size and fluidity of the cells.

In these cases, optical methods using voltage-sensitive dyes for monitoring cellular electrical events offer two principal advantages over conventional electrophysiological techniques. One is that it is possible to make optical recordings from very small cells which are inaccessible to microelectrode implantation and/or patch-clamp application\textsuperscript{11, 12, 26} and the other is that multiple sites of a preparation can be monitored simultaneously.\textsuperscript{11, 12, 26} Thus, the optical recording using voltage-sensitive dyes has provided a unique tool for measuring neural electrical activity, including postsynaptic potentials, in early developing embryonic nervous systems.\textsuperscript{13}

In the present study, using such a multiple-site optical recording technique, more detailed analyses...
Fig. 1. Developing patterns of multiple-site optical recording of neural responses in the 7-day to 10-day-old embryonic chick brainstem preparation. The neural responses were evoked by back-rheobase currents (0.5-2.5 mA) applied to the right vagus nerve. The recordings were made in normal Ringer's solution (left panel) and in a 50 μM GABA-containing Ringer's solution (right panel). The evoked optical signals were detected with a 783 ± 15 nm interference filter, and in a single sweep. The photodiode array was positioned over the image (×25) of the right/dorsal area of the preparation. The relative positions of the photodiode array on the array were indicated in the left side of each recording. The direction of the arrow in the right side of the figure indicates a decrease in transmitted light intensity (increase in absorption), and the length of the arrow represents the relative value of the fractional change.
were carried out on GABA responses in the early embryonic brainstem. Three components were pharmacologically identified and their embryogenesis and early development specified.

EXPERIMENTAL PROCEDURES

The methods used for multi-site optical recording of electrical activity in embryonic brainstem preparations have been described in detail elsewhere.19,20

Preparation

In the present experiments embryonic chick brainstem slice preparations were used. Fertilized eggs of white Leghorn chickens were incubated for seven to 10 days in a forced-draft incubator (Type P-03, Sanyo Incubator Lab., Osaka, Japan) at a temperature of 37°C and 65% humidity, and were turned once each hour. The brainstem, with vagus nerve fibers attached, were dissected from the embryos. The isolated brainstem preparation was attached to the silicone (KE 1067TV, Shin-etsu Chemical Co., Tokyo, Japan) bottom of a simple chamber by passing it with tungsten wires. The preparation was kept in a bathing solution with the following composition (in mM): NaCl 138, KCl 5.4, CaCl2 1.8, MgCl2 0.5, glucose 10, and Tris-HCl buffer (pH 7.2), 10. The solution was equilibrated with oxygen. The pinwheel attached to the brainstem was carefully removed in the bathing solution under a dissecting microscope. Slices were then prepared, with the right and/or left vagus nerve fibers attached, by sectioning the embryonic brainstem transversely at the level of the root of the vagus nerve. The thickness of the slice was about 1 mm.

Dye staining

The isolated slice preparation was stained by incubating it for 15–25 min in a Ringer's solution containing 0.1–0.2 μM of the voltage-sensitive nernstian-electrode dye NK-261 (Nippon Kaneko Shikitsu Kagyo Co., Osaka, Japan), and the excess (unbound) dye was washed away with dye-free Ringer's solution before recording.

Materials

The sources of chemicals used for pharmacological experiments were as follows: GABA, picROTOXIN, naphenol, and aprotinin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; bicinchoninic acid and MBO were from Research Biochemicals Inc., Natick, MA, U.S.A.; diacetin was from Tokyo Kasei Co., Tokyo, Japan; 2-hydroxyacetoaldehyde, phaeophytin and 6-aminonicotinic acid (CAAC) were from Toxins Co., Ltd., U.K.; and CGB3334 was a gift from Chua-Grey (Basel, Switzerland).

Electrical stimulation

For preparations in which the vagus nerve was stimulated, the cut end of the nerve was drawn into a micropipette electrode made from TERUMO-hemostatic tubing (VC-H075; TERUMO Co., Tokyo, Japan) which had been hand-pulled to a fine tip (about 100 μm internal diameter) over a low-temperature flame.

Optical recording

Light from a 300 W tungsten-halogen lamp (Type JU-24V/300 W, Kondo Philips, Tokyo, Japan) was collimated, reduced quasi-monochromatic with a heat filter (325–56, Olympus Optical Co., Tokyo, Japan), and an interference filter with a transmission maximum at 700 ± 15 nm (Asahi Spectra Co., Tokyo, Japan), and focused on the preparation by means of a bright-field condenser with a numerical aperture (NA) matched to that of the microscope objective (Plan 100, x10, 0.4 NA). The objective and photographic eyepiece (x2.5) projected a real image of the preparation onto a 12 × 12-channel silicon photodiode matrix array (MD-4444P, Central, Co., Ltd., UK) mounted on an Olympus Vanox microscope (Type AIB-L-1, Olympus Optical Co., Tokyo, Japan). The magnification of the image was x23. Each pixel (element) of the array detected light transmitted by a square region (50 × 50 μm) of the preparation. The output of each detector in the diode array was passed to an amplifier (time constant of AC-coupling=10) via a current-to-voltage converter. The amplified output 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 (LSI-11/73 system, Digital Equipment Co., Woburn, MA, U.S.A.). The 128-channel data recording system is composed of a main processor (RP-511), eight I/O processors (RP-893, 64 k word core-memory (RP-932) and a videotape recorder. The program for the computer...
RESULTS

GABA responses

Multiple-site optical recordings of neural activities in response to vagal stimulations in seven- to 10-day-old embryonic chick brainstem slice preparations are illustrated in Fig. 1. The optical signals were evoked by positive (depolarizing) square current pulses (8.0 μA/5.0 ms) applied to the right vagus nerve with a microelectrode electrode, in a normal Ringer's solution (left panel) and in a Ringer's solution containing GABA (30 μM) (right panel). The subcell corresponds to the photodiode and these are shown in the inset on the left.

The evoked optical signals consisted of two components: a fast spike-like signal and a delayed long-duration slow signal. It has already been shown often in our previous reports,21,31,32 that the slow signals reflect EPSPs which are mediated by glutamate, and arise within the nucleus of the tracts solitarius (NTS). Further, the initial phase of the slow signal is sensitive to 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and the later phase to N-methyl-D-aspartate (NMDA) receptors, and that the latter phase is mediated by NMDA receptors.21,32 We have not been able to detect slow signals from six-day-old embryonic preparations in normal Ringer's solution, so that, in the present experiments, we used preparations that were more than seven days old.

The slow optical signals were reversibly reduced in the presence of GABA. In these experiments, GABA was applied to the bathing solution for each
fig. 5. plots of the area (arybitrary units) against the corresponding signal amplitude (in fractional change). The signal area was calculated as the time-integral of the slow signal. The data were obtained from an eight-day-old embryonic preparation.

Preparation (the final concentration 50 μM). The signals were recorded 5–20 min after application of GABA. There was no change in baseline signal over the period of GABA exposure and desensitization of the GABA effects was not observed. As shown in Fig. 2, the slow optical signals were fully recovered after GABA was rinsed off. In the recordings shown in Fig. 1, there were differences in the GABA responses, according to the embryonic age. When 50 μM GABA was applied, in the seven- and eight-day-old preparations, the slow signals were completely suppressed, whereas, in the nine- and 10-day-old preparations, the slow signals partly remained.

To examine the dose dependence of the inhibitory effect of GABA in detail, we used enlargements of the signals from experiments similar to those in Fig. 1 (Fig. 3). The traces were obtained from the recordings in the eight- and nine-day-old preparations. These traces demonstrate that the slow signal decreased with the concentration of the bath-applied GABA.

The curves in Fig. 4 show the dose-response relation constructed by plotting the peak amplitude of the slow signal as a function of the concentrations of GABA in the bathing solution. The data for the plots were obtained from eight- and nine-day-old preparations. The shapes of the curves for these preparations were basically similar. However, in the nine-day-old preparation, the curve appeared to be shifted toward the right; the slow signals were completely suppressed by 500 μM GABA in the bathing solution for eight-day-old preparations, and the critical concentration to suppress completely the slow signals in the nine-day-old preparation was larger than 100 μM.

The correlation between the signal amplitude and the signal area is shown in Fig. 5, indicating that the signal amplitude changes in parallel with the signal area. The signal amplitude has been routinely used to assess the GABA response.

fig. 6. expansions of the fast spike-like optical signal characteristic recorded from two positions (1-5 and D-7) of an eight-day-old embryonic preparation. S1 and S2 indicate the timings of the first spike-like signals and the second spike-like signals, respectively. The second spike-like signals (S2) were eliminated in 50 μM/500 μM GABA-containing bathing solutions.

The fast signal. In Fig. 3, the fast signals also appear to be reduced by higher concentrations of GABA. To study the details of the GABA response on the fast signal, we examined expanded traces of the fast part of the signals. The traces of the signals recorded from two sites of an eight-day-old preparation are represented in Fig. 6. The expanded time-base makes it clear that the fast signals viewed at a slower speed (shown in Fig. 3) contain two signals separated temporally by a very small interval (4.5–7.0 ms). The two signals are labeled S1 and S2 in Fig. 6.

Thus, the amplitude of the fast spike-like signals which were apparently observed as a simple signal in Fig. 3 is the sum of the first spike-like signal (S1) and the second spike-like signal (S2). The second spike-like signals correspond to action potentials firing in postsynaptic cells, and the first spike-like signals contain antidromic action potentials in motor neurons and presynaptic action potentials in sensory nerve terminals, as reported previously.2 The later signals (S2) were reduced or eliminated in the presence of GABA, while GABA was ineffective in blocking the earliest signals (S1). Therefore, it seems reasonable to attribute the reduction of the fast spike-like signals observed in Fig. 3 to a reduction in the size of the later signal (S2).

Effects of GABA antagonists on the GABA response.

The effects of various GABA antagonists on the GABA responses in multiple site optical recordings made in a nine-day-old embryonic brainstem slice preparation are illustrated in Fig. 7. The recordings were obtained in normal Ringer's solution, and in a
Fig. 7. Optical signals recorded in normal Ringer's solution and in a Ringer's solution containing GABA (50 μM), GABA (50 μM) and bicuculline (200 μM), GABA (50 μM) and picrotoxin (100 μM), or GABA (50 μM), picrotoxin (100 μM) and 2-hydroxyxanthene (200 μM). These concentrations were maximally effective. The signals were evoked by right vagal stimulations in a nine-day-old preparation.

Ringer's solution containing a non-competitive GABA_A antagonist (picrotoxin) or a competitive GABA_A antagonist (bicuculline). In addition, a recording was made in the presence of picrotoxin together with a GABA_A antagonist (2-hydroxyxanthen). In Fig. 7, the effects of these antagonists were compared at their maximally effective concentrations. The GABA responses (inhibitory effects on the slow optical signals) were slightly reduced by these GABA antagonists. The dose-response curves for these antagonists are shown in Fig. 8. In a Ringer's solution containing GABA antagonists alone, the slow optical signals were the same as those in normal Ringer's solution (Fig. 9).

Figure 10 illustrates the expanded traces of the optical signals chosen from two positions (G-9 and J-5) of the recording from the nine-day-old preparation shown in Fig. 7. Most of the slow signal was reduced with 50 μM CABA, although a small fraction remained, in the presence of bicuculline.
Responses to GABA agonists

The responses to agonists of GABA_A receptors (muscimol) and to GABA_B receptors (baclofen) were examined. Both muscimol and baclofen reduced the slow optical signal. The responses were dependent on the concentrations of the agonists. The dose-response curves for baclofen and muscimol are shown in Fig. 13, together with CACA.

The effects of the antagonists of GABA_A and GABA_B receptors on the muscimol and baclofen responses are shown in Fig. 14. Although the muscimol response was partly blocked by picrotoxin, most of the response was insensitive to picrotoxin. There was no significant difference in the effects between bicuculline (200 μM) and picrotoxin (200 μM), suggesting that the typical GABA_C response was undetectable or negligibly small. Similarly, the baclofen response contained a fraction which was insensitive to an antagonist of GABA_B receptors (2-hydroxydiallofen). These picrotoxin- and 2-hydroxydiallofen-insensitive fractions were considered to be equivalent to fraction D in Fig. 12.

Regional distribution maps

On the basis of GABA antagonist experiments on the GABA response, evaluations were made of fraction A which was sensitive to bicuculline, fraction B which was sensitive to 2-hydroxydiallofen and insensitive to picrotoxin, fraction C which was sensitive to picrotoxin and insensitive to bicuculline, and fraction D which was insensitive to 2-hydroxydiallofen and to picrotoxin. Using these data, we constructed maps of the regional distributions of fractions A, B, C, and D. The pattern of regional distribution of total GABA responses, and fractions A, B, and C can be seen in Fig. 15. In these maps, the amplitude fractions are represented by gray levels.

From these maps, we have been able to conclude the following: (i) the GABA response area expands with development and the region of the peak-response is localized to the dorsal region in every preparation; (ii) the area of fraction A expands gradually from medial to lateral as the embryonic stage proceeds from seven to nine days; (iii) fraction B is found for the first time in the eight-day-old preparation and its area expands with development of the embryo from eight- to 10-day-old stages; (iv) fraction C was not found in any of the preparations...
through the seven- to 10-day-old stages; (v) the area of fraction D expands as the developmental age proceeds from seven- to 10-day-old embryonic stage, in parallel with the expansion of the total GABA response area; and (vi) there are differences in the spatial distribution between fractions A, B and D. The area of fraction D is concentrated in the dorsal region.

To emphasize the early developmental history of the spatial distribution of fractions A, B and D, the locations of their maximum amplitudes were compared (Fig. 16). The data were obtained from four to seven different preparations for each of the indicated embryonic stages. In Fig. 16, an additional interesting feature of the locations of the maximum amplitudes of the fractions A, B and D is exhibited. In the seven- and eight-day-old preparations, the locations of the maximum amplitudes of fractions A and D were separated; fraction A was located on the medial region, and fraction D on the lateral side. In the eight-day-old preparations, the locations of the maximum amplitudes of fractions B and D were superimposed. However, surprisingly, in the nine- and 10-day-old preparations, the locations of the maximum amplitudes of fractions A and D exchanged places with one another, and the maximum amplitude positions of fractions A and B were nearly superimposed. These results indicate that, in the NTS, the dynamics of the development of the spatial distribution pattern of fractions A, B and D are not simple.

**DISCUSSION**

The experiments reported in this article show developmental profiles of GABA responses identified in
Embryonic GABA responses in brainstem

The NTS of the seven- to 10-day-old embryonic chick brainstem preparations. These experiments used a multiple-site optical recording technique employing a fast voltage-sensitive dye: the optical measurements allowed us to monitor the voltage responses of early embryonic stage neurons.\(^{19}\)

From experiments using various GABA receptor antagonists, three specific components were isolated in the GABA responses, which were assessed by means of the inhibitory effects of GABA on the EPSP-related slow optical signal, in seven- to 10-day-old embryonic brainstem preparations. The identified components were: component 1, resembling the GABA\(_A\) receptor function; component 2, resembling the GABA\(_B\) receptor function; and component 3, the pharmacology of which did not resemble that of any of the GABA\(_A\), GABA\(_B\), or GABA\(_C\) receptors. Hence, this component is presumed to be mediated by a novel type of GABA receptor, which we call the GABA\(_D\) receptor.

In experiments with a whole-cell recording technique on cultured postnatal rat hippocampal neurons, Yoon and Rothman\(^{17}\) have reported the effect of GABA on both presynaptic and postsynaptic sites. This issue suggests a possibility that the reduction of the slow optical signal could contain both the presynaptic and postsynaptic GABA responses. Unfortunately, at present at least, it is a formidable task to separate experimentally the presynaptic and postsynaptic GABA responses in younger embryonic neural tissues. In addition, it is difficult to apply directly the idea obtained in the cultured postnatal rat hippocampal neuron to the younger embryonic chick brainstem. In the experiment by Yoon and Rothman\(^{17}\) at a low concentration (1 \(\mu M\)), GABA near maximally stimulated transmitter release while in the present experiment, the effect of 1 \(\mu M\) GABA on the slow optical signal was not significant. Furthermore, even at a high concentration of GABA (300 \(\mu M\)), the presynaptic action potential signal was
Fig. 11. Optical signals detected from a seven-day-old embryonic preparation (positions I-9 and I-5), in normal Ringer’s solution, and in a Ringer’s solution containing GABA (50 µM), GABA (50 µM) and bicuculline (200 µM), GABA (50 µM) and picrotoxin (100 µM), or GABA (50 µM), picrotoxin (100 µM) and 2-hydroxyketocon (200 µM).

Fig. 12. Illustrations of the identification of fractions A (yellow), B (green), and D (red), which are pharmacologically separable. The illustrations were traced from the original recordings obtained from the positions of nine- and seven-day-old embryonic preparations. The experimental conditions are the same as those in Figs. 10 and 11.
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Table 1. Effects of antagonists of GABA<sub>A</sub> and GABA<sub>B</sub> receptors on GABA response.
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The concentrations of GABA and GABA antagonist used were 50 μM (GABA), 100 μM (picrotoxin), 10 μM (dihydroxyacetoxy), 100 μM (phentolamine), 100 μM (CCP2534), 200 μM (2-hydroxyacetoxy). The values are expressed as the fold-change (mean ± SEM). *p < 0.05.

The differences were estimated for each preparation, and the maximum values of the differences are given in the table.
not affected (Fig. 6). Accordingly, it is likely that, even though there is a small fraction of the presynaptic effect, the effect of GABA on the slow optical signal is mainly postsynaptic in origin.

The linear relationship between the voltage-sensitive dye optical signal and changes in extracellularly recorded membrane potential has been long established. It is further assumed that, in our multiplexed optical recording using a multi-element photodiode array, one photodiode sees the activities from many neurons and processes. The signals are therefore likely to represent the responses from many simultaneously active neurons and, hence, the amplitude of the slow optical signal is likely to be a function of the potential changes exhibited by each postsynaptic neuron times the number of postsynaptic neurons within the area imaged on to one detector.

However, considering that the slow signal is very slow (about 1 s duration) compared with intracellular EPSP electrode recordings by others in adult, juvenile, or cultured neurons and cultured neuron preparations, there may be some concern that the slow signal contains additional components, such as polysynaptic EPSPs. In a few experiments, we increased the microscope magnifications, and we could not discern significant differences in the duration of the slow signals. Therefore, in the range of the microscope magnifications used in the present experiment, the possibility of detecting delays in the signals among the multiple neurons within the receptive field by one detector was not considered. Thus, we interpret the long duration of the slow signal as due to slow synaptic transmission in the embryonic preparation. The slow optical signal appeared to be configurationally similar to the glial signal that was recorded from skate ocellar slices. Nonetheless, the slow signal was detected from the preparation stained with an extrinsic dye (RH-482) which is relatively insensitive to the glial cell membrane potential changes. Therefore, the possibility was ruled out that the slow signal was glial in origin.

This interpretation recalls some earlier results. We have demonstrated that the slow signal evoked by vagal stimulation in the embryonic brainstem is (i) decreased by continuous stimulation (synaptic fatigue), (ii) inhibited in Ca²⁺-free or Mg²⁺- or Cd²⁺-containing bathing solution, and (iii) eliminated in the presence of kynurenic acid. From these facts, we have concluded that the slow optical signal reflects a glutamate-mediated EPSP. Furthermore, the initial phase is reduced by CNQX and the later phase is reduced by APV, indicating non-NMDA- and NMDA-dependent components.

Three components

The three components identified in the present experiment, components 1, 2, and 3, correspond to fractions A, B, and D illustrated in Fig. 12, respectively, and the components are expressed in terms of fractional amplitudes. Therefore, in Fig. 15, the maps reflect the regional distribution patterns of the averaged amplitude of EPSPs modified by GABA_1A receptors, GABA_2A receptors, and/or the numbers of the GABA_1A, GABA_2A, and GABA_2B receptors. The data for the total GABA response are also presented in Fig. 15.

Accordingly, from the maps in Fig. 15, the following profiles arise: (1) Functionally, GABA_1A and GABA_2B receptors have already been generated in the seven-day-old embryonic stage in which the EPSP is first detected and, subsequently, GABA_1A receptors emerge in the eight-day-old embryonic stage. (2) During embryonic developmental stages from seven
to 10 days old, the GABA receptors appear to distribute over the entire region of the NTS. (3) The fraction related to the GABA$_D$ receptor accounts for the great majority of the total GABA receptors, and the fractions of GABA$_A$ and GABA$_B$ receptors are relatively small. (4) The developmental expansion of the GABA$_D$ fraction is parallel with that of the total GABA fraction. (5) GABA$_A$ receptors appear to be expressed initially in the medial region of the NTS. (6) Typical GABA$_B$ receptors, which have bicuculline-insensitive and picROTOX-sensitive characteristics, described in frog and rat retinal horizontal and bipolar cells, are not detectable in the seven- to 10-day-old embryonic NTS.

Furthermore, the maps suggest that the GABA$_A$ receptor function, which is coupled to chloride ion-selective channels, is differentiated prior to the GABA$_B$ receptor function, which is coupled to calcium or potassium ion channels via GTP binding proteins, during early development of the brainstem. Xia and Haddad examined differences in GABA$_A$ receptor density and distribution during postnatal development of the rat brainstem using quantitative receptor autoradiography, and found that GABA$_A$ receptors appeared by postnatal day 1 in almost all of the brainstem. However, considering our findings, we suppose that, in the rat brainstem, the function of the GABA receptors is also generated much earlier than the stage suggested by Xia and Haddad. They also concluded that brainstem functions rely more on GABA$_A$ receptors in early postnatal life than at a more mature stage. This idea seems to be of interest. Nonetheless, the present experiments, the embryological significance of the early appearance of GABA$_A$ and GABA$_B$ receptors is as yet unclear.

A possible profile of GABA$_D$ receptor

The most salient finding described in this article is the identification of component 3 (corresponding to fraction D). For this component, an additional profile is given in the experiments using GABA$_A$ and GABA$_B$ receptor agonists (Fig. 14). The results suggest that the hypothetical GABA$_D$ receptor is sensitive to both GABA$_A$ and GABA$_B$ receptor agonists.

Although at present it is very difficult to arrive at a complete profile of the GABA$_D$ receptors, the following three possibilities have been considered:

(1) The GABA$_D$ receptor is an uniquely new GABA receptor class which is distinct from any of the known GABA$_A$, GABA$_B$, and GABA$_C$ receptors.

(2) The GABA$_D$ receptor is an undifferentiated prototype of GABA$_A$ and GABA$_B$ receptors. Therefore, although the GABA$_D$ receptor

![Fig. 15. Regional distribution maps of GABA responses.](image-url)
Fig. 16. Comparison of the locations of the maximum amplitude of fractions A (square), B (triangle), and D (circle) observed in seven- to 10-day-old embryonic preparations. Each symbol indicates the relative position of the maximum amplitude area in each preparation. The symbols correspond to one preparation, but data from some preparations overlapped. The y-axes correspond to the midline of the slice preparations, and the dashed lines indicate the direction of changes in the relative location of the maximum amplitude area. The outlines of the preparations were traced with the averaged lines. The scales on the abscissa (x-axes) indicate the distance (in μm) from the midline (y-axes), and those on ordinate (y-axes) indicate the distance (in μm) from the x-axes to the dorsal direction so that the locations are represented on the orthogonal coordinates.

2. The third possibility derives from the concept of diversity of the GABA receptor subtype, and the following two cases are considered:
(a) The GABA<sub>B</sub> is an isomer of both GABA<sub>A</sub> and GABA<sub>B</sub> receptors. This isomer binds to agonists of both GABA<sub>A</sub> and GABA<sub>B</sub> receptors, but lacks a binding site for antagonists of GABA<sub>A</sub> and GABA<sub>B</sub> receptors.
(b) The GABA<sub>A</sub> receptor contains two distinct receptors. One is the isomer of GABA<sub>A</sub> receptor (D<sub>A</sub>) and the other is the isomer of GABA<sub>B</sub> receptor (D<sub>B</sub>). The former binds to agonists of GABA<sub>A</sub> receptor, but lacks a binding site for antagonists of GABA<sub>A</sub> receptors. The latter binds to agonists of GABA<sub>B</sub> receptors, but lacks a binding site for antagonists of GABA<sub>B</sub> receptors. These concepts, referred to as assumptions (a) and (b), are illustrated schematically in Fig. 17.

Based on sequence similarity, it is known that five different GABA<sub>A</sub> receptor subunit families have been identified, and that most of the subunit families have multiple subtypes (for reviews, see Refs. 46, 48, and 50). GABA<sub>A</sub> receptors probably belong to the family of G-protein-coupled receptors which are characterized by seven transmembrane spanning domains. However, GABA<sub>B</sub> receptors have not been cloned as yet, and hence their heterogeneity can only be inferred indirectly on the basis of different potencies of agonists and antagonists, as well as different transduction pathways. Therefore, in the comparison of molecular structures of GABA<sub>A</sub> and GABA<sub>B</sub> receptors, it is impossible to draw a molecular structural profile of the suggested GABA<sub>A</sub> receptor.

Several investigations have been devoted to the postnatal developmental approach to GABA receptors. However, little or no attention has been paid to GABA receptors in the developing early embryonic CNS.

Although the molecular profile and the physiological/embryological meanings of the GABA<sub>A</sub> receptor are not clear, we believe that this first report of a new type of GABA receptor in the early embryonic CNS marks a new direction in the investigation of GABA physiology and pharmacology. Molecular approaches are required to support the present results and conclusions. In this context, the present experimental results may provide new insights into GABA receptor studies.

CONCLUSIONS

The simplest interpretation of the present results is that, in the inhibitory effects of GABA on the vagal motoneural FSPs in the seven- to 10-day-old embryonic chick brainstems, three different components are contained. Component 1 is related to GABA<sub>A</sub> receptors and component 2 is related to GABA<sub>B</sub> receptors. Furthermore, component 3 is mediated by a possibly suggested GABA<sub>B</sub> receptor. Components 1 and 3 emerge in the seven-day-old embryo and components 2 and 3 are generated functionally at the eight-day-old embryonic stage. The GABA<sub>B</sub> receptor-related component has not been observed. There are differences in the spatial distribution among components 1, 2, and 3.
Fig. 17. Schematic of a profile of the asialomous GABA<sub>A</sub> receptors. See Discussion for details.

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REFERENCES

Embryonic GABA responses in brainstem


(Accepted 23 January 1987)
Optical characterization of a novel GABA response in early embryonic chick brainstem.

Momose Sato Y, Sato K, Hirot A, Sakai T, Yang XS, Kamino K.

Department of Physiology, Tokyo Medical and Dental University School of Medicine, Bunkyo-ku, Japan.

To examine the functional expression of embryonic GABA receptors, the inhibitory effects were studied of GABA (GABA responses) on the excitatory postsynaptic potentials evoked by vagal stimulus in seven- to 10-day-old embryonic chick brainstem slice preparations. A multiple-site optical recording technique was used, with a multiple element photodiode array system and a fast voltage-sensitive merocyanine-rhodamine dye (NK2761). First, in the GABA response, three components were pharmacologically identified: component 1, related to GABA(A) receptors; component 2, related to GABA(B) receptors; and component 3 which is insensitive to GABA(A) and GABA(B) antagonists, but is stimulated by both GABA(A) and GABA(B) agonists. Subsequently, the embryogenesis and early development of the three components were investigated, and early developmental maps of regional distribution patterns of the three components were constructed. Components 1 and 3 have already emerged in the seven-day-old embryonic brainstem preparation; component 2 appeared in the eight-day-old preparations. No component related to GABA(C) receptors was observed in the seven- to 10-day-old embryonic stages. From the pharmacological properties of component 3, we suggest that it is related to a new subtype, the GABA(D) receptor.

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