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# Embryonic development of GABAergic signaling in the mouse spinal trigeminal nucleus interpolaris

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## HIGHLIGHTS

- Developmental changes in GABAergic transmission were investigated in the mouse SpVi.
- The SpVi may first receive GABAergic projection fibers from extra-nuclear area.
- GABAergic neurons were localized after E15 and may form synapses after E17.
- GABA action may shift from excitatory to inhibitory between E13 and E17.

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## ABSTRACT

In the mature central nervous system, γ-amino butyric acid (GABA) is an inhibitory neurotransmitter, whereas during development, GABA induces depolarization. To examine the embryonic development of GABAergic transmission in the mouse spinal trigeminal nucleus interpolaris (SpVi), which receives sensory input from the face and is important in survival of rodents, we performed immunohistochemistry for three related molecules: glutamic acid decarboxylase (GAD), a marker of GABAergic neurons; vesicular GABA transporter (VGAT), a marker of GABAergic and glycinergic vesicles; and potassium chloride co-transporter 2 (KCC2), which shifts GABA action from excitatory to inhibitory. GAD-positive longitudinal projection fibers, where VGAT-positive dots were localized, were clearly discernible until embryonic day (E)17, and were markedly decreased in number on postnatal day 0. GAD-positive neurons were detected after E15, and GAD- and VGAT-positive axon varicosities were observed after E17. KCC2 immunolabeling was first localized in the dendrites and cell bodies of several neurons in the lateral part of the SpVi on E13 and throughout the nucleus on E17. These results suggest that the SpVi may first receive GABAergic projection fibers from extra-nuclear area before birth, and GABAergic interneurons may form synapses within the SpVi after E17. In addition, GABA action may gradually shift from excitatory to inhibitory between E13 and E17.



## 1. Introduction

Gamma-amino butyric acid (GABA) is a predominant neurotransmitter in the mature central nervous system (CNS), and negatively regulates the excitatory activity of neurons [14,16]. In contrast, GABA induces depolarization of membrane potential during development and after axonal injury, and may be involved in controlling morphogenesis and regeneration [3,15,17,26].

Previously, we found common developmental changes in the expression pattern of molecules related to GABAergic transmission in various regions, such as spinal cord [9], cerebellar cortex [23,25], and cerebral cortex [22]. In our study, GABA and its synthetic enzyme, glutamic acid decarboxylase (GAD) [2], were distributed throughout axons before GABAergic synapses were formed. However, the vesicular GABA transporter (VGAT), which is a marker of GABAergic and glycinergic synaptic vesicles [5], was not detected in the axons, and potassium chloride co-transporter 2 (KCC2), which reduces intracellular chloride ion ($Cl^-$) concentration [18], was not localized in the dendrites and cell bodies. These results suggest that

*Abbreviations:* ABC, avidin–biotin–peroxidase complex; $Cl^-$, chloride ion; CNS, central nervous system; E, embryonic day; GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; KCC2, potassium chloride co-transporter 2; NKCC1, sodium potassium chloride co-transporter 1; P, postnatal day; PB, phosphate buffer; SpVi, spinal trigeminal nucleus interpolaris; VGAT, vesicular GABA transporter.

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before formation of GABAergic synapses, GABA may be extrasynaptically released and mediate depolarization of membrane potential. After synapses were formed, GABA and VGAT were confined to the presynaptic terminals, and KCC2 was abundantly expressed in postsynaptic neurons, suggesting that GABA may be synaptically released, and act as an inhibitory transmitter. Furthermore, we also found that exclusively in the spinal cord, GABAergic projection fibers in the white matter transiently innervated neurons in the gray matter during the embryonic period, but the fibers disappeared during postnatal development [9].

Next, we focused on the trigeminal nucleus, in particular the spinal trigeminal nucleus interpolaris (SpVi), which is the largest nucleus in the trigeminal nucleus complex. The trigeminal nucleus receives somatic sensory information from the face, such as pain, touch, and temperature, and sends axons to the contra-lateral thalamus in the mature rodents [28]. Furthermore, in newborn mice, the SpVi is the center of the suckling reflex, which is critical for milk intake [29]. Abnormal glutamatergic transmission in the trigeminal nucleus affects the suckling reflex and milk intake, and pups die within a day [11,13]. Since the trigeminal nervous system uses GABA as a neurotransmitter in the processing of sensory information, the maturation of the GABAergic network during embryonic development might also be critical for the survival of newborn mice. However, little is known about the development of the GABAergic signaling in the trigeminal nucleus.

In the present study, immunohistochemistry for three related molecules, GAD, VGAT, and KCC2 were performed to examine the time course of embryonic development of GABAergic transmission in the SpVi. We qualitatively investigated the developmental changes in localization of GABAergic neurons, formation of GABAergic synapses, and shift in GABA action as previously performed in the spinal cord [9], cerebellar cortex [23,25], and cerebral cortex [22]. GABAergic neurons and axons were detected as GAD-positive cells and fibers, respectively. GABAergic synapses, in particular presynaptic terminals, were detected as GAD- and VGAT-positive dots, because the staining patterns of VGAT immunohistochemistry were quite similar to the GAD- and GABA-positive dots observed during embryonic development in other regions, and electron microscopic analysis demonstrated that the GAD-positive structure formed synapses [9,22,24]. Changes in GABA action from excitatory to inhibitory were examined using KCC2 immunohistochemistry.

## 2. Materials and methods

### 2.1. Animals

We used mice (C57BL/6J) on embryonic day (E)13 (E0 = mating day), E15, E17, and postnatal day (P)0. At least three mice on P0 and three fetuses on each embryonic day were examined. The experiments were approved by the Animal Care and Use Committees of the University of the Ryukyus (No. 5539) and were performed in compliance with the Guide for the Care and Use of Laboratory Animals of the University of the Ryukyus. Every effort was made to minimize the number of animals used and their suffering.

### 2.2. Tissue preparation

Fetuses were removed from the uterus of pregnant mice, which were deeply anesthetized by an intraperitoneal injection of a mixed solution (10 μl/g body weight), containing 8% Nembutal and 20% ethanol in saline. Mice on P0 were anesthetized on ice. Mice and fetuses were fixed by transcardial perfusion with 4% paraformaldehyde in phosphate buffer (PB: 0.1 M, pH 7.4), and immersed in the same fixative overnight. Brains were removed from skulls and cryoprotected with 30% sucrose in PB for 2 days. The brainstems were cut into 20 μm-thick coronal sections using a cryostat. The sections were mounted on gelatin-coated glass slides.

### 2.3. Immunohistochemistry

Sections were incubated in methanol containing 0.3% $H_2O_2$ for 30 min, PB for 10 min, 3% normal goat serum in PB for 1 h, and GAD (1:2000, Millipore, Billerica, MA, USA), VGAT (1 μg/ml) [24], or KCC2 (1 μg/ml) [23] overnight at room temperature. After rinsing three times with PB for 15 min, sections were visualized using the avidin–biotin–peroxidase complex (ABC) method (Histofine kit; Nichirei, Tokyo, Japan) [6].

## 3. Results

### 3.1. Developmental localization of GAD, VGAT, and KCC2 at low magnification

We observed developmental changes in localization of three molecules, GAD, VGAT, and KCC2, in the SpVi using serial coronal sections containing the middle part of the hypoglossal nucleus and inferior olive complex in accordance with the atlases of developing mouse brain [8,19].

GAD immunohistochemistry demonstrated that on E13 (Fig. 1A) and E15 (Fig. 1B), the positive signals were observed at the longitudinal projection fibers in the transverse sections, namely axon bundles, which consisted of many GAD-positive axons running through the SpVi from the extra-nuclear area in the higher magnification photographs (Fig. 2A and B). On E17 (Fig. 1C), GAD-positive axon bundles were sparse in the lateral part, whereas GAD immunolabeling around the GAD-positive axon bundles gradually increased in intensity. On P0 (Fig. 1D), the intensity of GAD immunolabeling markedly increased in the neuropil, whereas GAD-positive axon bundles were not clearly discernible.

VGAT immunolabeling was also detected within the SpVi on E13 (Fig. 1E) and E15 (Fig. 1F). Because the VGAT-positive structure on E13 (Fig. 1E) was quite similar to GAD-positive axon bundles on the same embryonic day (Fig. 1A), VGAT immunolabeling might be co-localized with GAD-positive axon bundles. The immunolabeling at the axon bundles gradually decreased in intensity on E17 (Fig. 1G), and was not discernible on P0 (Fig. 1H). On P0 (Fig. 1H), VGAT immunolabeling was diffusely localized in the SpVi, but its intensity was lower compared with the adjacent medial area.

Moderate KCC2 immunolabeling was detected in the lateral part of the SpVi on E13 (Fig. 1I). On E15 (Fig. 1J), the majority of the SpVi was stained, but immunolabeling was weak in the dorsal and medial parts. On E17 (Fig. 1K) and P0 (Fig. 1L), the SpVi was diffusely labeled, and the intensity was similar to that of other parts of the medulla oblongata.

### 3.2. Developmental localization of GAD, VGAT, and KCC2 at high magnification

Intense GAD immunolabeling was localized in the axon bundles (thick arrows) on E13, but was not detected around the axon bundles (Fig. 2A). On E15 (Fig. 2B) and E17 (Fig. 2C), perikarya of neurons (arrowheads) were labeled, indicating that GABAergic interneurons were localized within SpVi after E15. On E15 (Fig. 2B), their axons (thin arrows) were smooth and diffusely stained. On E17 (Fig. 2C), GAD-positive axons of the interneurons increased in density, and were often divided into several branches (thin arrows). Furthermore, axon varicosities, which were identified as GAD-positive dots on the GAD-positive axons, were clearly discernible (thin arrows in Fig. 2C). On P0 (Fig. 2D), GAD-positive axons of the interneurons