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メタデータ	言語: English 出版者: Elsevier 公開日: 2020-07-31 キーワード (Ja): キーワード (En): cervical cord, GABAergic synapse, glutamic acid decarboxylase (GAD), potassium chloride co-transporter 2 (KCC2), vesicular GABA transporter (VGAT) 作成者: Kosaka, Yoshinori, Kin, Hidemichi, Tatetsu, Masaharu, Uema, Itsuki, Takayama, Chitoshi メールアドレス: 所属:
URL	http://hdl.handle.net/20.500.12000/46545

Distinct development of GABA system in the ventral and dorsal horns in the embryonic mouse spinal cord

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Sections: Development, Degeneration and Regeneration, and Aging

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ABSTRACT

In the adult brain, γ-amino butyric acid (GABA) is an inhibitory neurotransmitter, whereas it acts as an excitatory transmitter in the immature brain, and may be involved in morphogenesis. In the present study, we immunohistochemically examined the developmental changes in GABA signaling in the embryonic mouse cervical spinal cord. Glutamic acid decarboxylase and GABA were markers for GABA neurons. Vesicular GABA transporter was a marker for GABAergic and glycinergic terminals. Potassium chloride cotransporter 2 was a marker for GABAergic inhibition. We found five points: (1) In the ventral part, GABA neurons were divided into three groups. The first differentiated group sent commissural axons after embryonic day 11 (E11), but disappeared or changed their transmitter by E15. The second and third differentiated groups were localized in the ventral horn after E12, and sent axons to the ipsilateral marginal zone. There was a distal-to-proximal gradient in varicosity formation in GABAergic axons and a superficial-to-deep gradient in GABAergic synapse formation in the ventral horn; (2) In the dorsal horn, GABA neurons were localized after E13, and synapses were diffusely formed after E15; (3) GABA may be excitatory for several days before synapses formation; (4) There was a ventral-to-dorsal gradient in the development of GABA signaling. The GABAergic inhibitory network may develop in the ventral horn between E15 and E17, and GABA may transiently play crucial roles in inhibitory regulation

of the motor system in the mouse fetus; (5) GABA signaling continued to develop after birth, and GABAergic system diminished in the ventral horn.

Key words: cervical cord, GABAergic synapse, glutamic acid decarboxylase (GAD), potassium chloride co-transporter 2 (KCC2), vesicular GABA transporter (VGAT)

Abbreviations

ABC: avidin-biotin-peroxidase-complex
AF: anterior funiculus
CC: central canal
CNS: central nervous system
de: dendrite
DH: dorsal horn
E: embryonic day
G1-G4: GABAergic neuronal group 1-4
GABA: γ-amino butyric acid
GAD: glutamic acid decarboxylase
Gly: glycine
KCC2: potassium chloride co-transporter 2
LF: lateral funiculus
ML: mantle layer
MZ: marginal zone
PB: phosphate buffer
PF: posterior funiculus
VGAT: vesicular GABA transporter
VH: ventral horn
VZ: ventricular zone
WM: white matter

1. Introduction

In the adult central nervous system (CNS), γ-amino butyric acid (GABA) is a predominant neurotransmitter that mediates the hyperpolarization of membrane potential, negatively regulating the excitatory activity of neurons (Macdonald and Olsen, 1994; Olsen and Tobin, 1990). During development, on the other hand, GABA is an excitatory transmitter, serves as a trophic factor, and may play an important role in controlling morphogenesis (Ben-Ari, 2002; Ben-Ari et al., 2007; McCarthy et al., 2002; Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005). Furthermore, GABA may be also involved in neural regeneration after axon injury (Nabekura et al., 2002; Tatetsu et al., 2012).

In previous studies, we examined the developmental changes in GABA signaling within the cerebral and cerebellar cortexes (Takayama and Inoue, 2004a; Takayama, 2005; Takayama and Inoue, 2006; Takayama and Inoue, 2007; Takayama and Inoue, 2010). We found that (1) GABA may act as an excitatory transmitter for several days before the formation of GABAergic synapses, whereas it may act as an inhibitory transmitter after synapse formation, and (2) these developmental changes occurred in chronological order of laminar structure formation.

Next, we focused on the spinal cord, which has benefits for the investigation of developmental changes in GABA signaling. First, the spinal cord consists of well-organized

cytoarchitectural and functional groups (Grant and Koerber, 2004; Ribeiro-Da-Silva, 2004; Tracey, 2004). The location of neurons within the spinal cord contributes to the formation of appropriate connections between neurons, and these connections are important for mediating motor and sensory behaviors. (Alvarez et al., 2005; Kiehn, 2006; Sibilla and Ballerini, 2009; Vinay and Jean-Xavier, 2008; Zagoraiou et al., 2009). Since one spinal cord segment contains several different functional areas, including the ventral motor and dorsal sensory neuron groups, it is easy to examine the chronological and sequential order of the changes in GABA signaling among them. Second, the formation of cyto- and synaptic- architecture in the spinal cord proceeds during embryonic development prior to other brain regions (Gao and Ziskind-Conhaim, 1995; Sibilla and Ballerini, 2009; Wu et al., 1992). Therefore, the time course of the normal development of GABA signaling in the embryonic spinal cord would be helpful for the analysis of abnormalities in embryonic lethal gene-targeted mice, including the mice mentioned below. Third, it was demonstrated that formation of the GABAergic network during embryonic development is crucial for the expression of various spinal cord functions by the analysis of knockout mice, lacking key molecules involved in GABAergic transmission, such as glutamic acid decarboxylase (GAD) 67 (Condie et al., 1997; Ding et al., 2004; Ji et al., 1999), which is one of the synthetic enzymes of GABA (Barker et al., 1998; Martin and Rimvall, 1993; Varju et al., 2001), vesicular GABA transporter (VGAT) (Fujii et al., 2007;

Saito et al., 2010; Wojcik et al., 2006; Yamada et al., 2012), which transports both GABA and glycine (Gly) into synaptic vesicles (Fon and Edwards, 2001; McIntire et al., 1997; Reimer et al., 1998), $GABA_A$ receptor channel β3 subunit (Culiat et al., 1995), which is one of the major subunits in the embryonic spinal cord (Laurie et al., 1992), and potassium sodium chloride co-transporter 2 (KCC2) (Hubner et al., 2001), which reduces intracellular chloride ion concentration and shifts GABA action from excitation to inhibition (Ben-Ari, 2002; Owens and Kriegstein, 2002; Payne et al., 2003).

Previous studies reported the development of GABA signaling in the spinal cord, such as the distribution of GABA neurons and synapses (Phelps et al., 1999; Schaffner et al., 1993; Sibilla and Ballerini, 2009; Tran et al., 2003; Tran et al., 2004; Wu et al., 1992), $GABA_A$ receptor subunits (Laurie et al., 1992), and KCC2 (Delpy et al., 2008; Stein et al., 2004; Stil et al., 2009). However, little is known about their spatial and temporal relationship with the ontogeny of the spinal cord and mechanism underlining the abnormalities in knockout mice. To address these points, we examined the time course of the expression and localization of various molecules involved in GABAergic transmission. GABA and GAD were markers for GABA neurons, including cell bodies, dendrites, axons, and terminals. Immunohistochemistry for VGAT was performed to detect the terminals of both GABAergic and glycinergic synapses. KCC2 was a marker of GABAergic inhibition.

2. Results

2.1. Immunohistochemical localization of GAD, VGAT, and KCC2 in the adult cervical spinal cord

First, to examine GABA signaling in the mature mouse spinal cord, we performed immunohistochemistry for GAD, VGAT, and KCC2 in two-month-old mice. Immunolabeling of three molecules was observed within the gray matter, but axon bundles in the anterior, lateral, and posterior funiculi were negative (Fig. 1A-F). GAD immunohistochemistry demonstrated that GABAergic terminals, detected as GAD-positive dots, were few in the ventral horn, but numerous in the dorsal horn, and ascending, and descending axons did not contain GABAergic fibers in the white matter (Fig. 1A, B). VGAT immunohistochemistry demonstrated that the density of VGAT-positive dots in the ventral horn (Fig. 1C, D) was much higher than that of GAD-positive dots (Fig. 1A, B), suggesting that the glycinergic inputs may be dominant in the ventral horn. KCC2 was abundantly detected throughout the gray matter, suggesting that both GABA and glycine (Gly) may act as inhibitory transmitters for all neurons.

2.2. Developmental changes in GAD and GABA localization in the embryonic spinal cord

Second, to examine developmental changes in the distribution and shape of GABA neurons, embryonic mouse spinal cord was immunohistochemically stained with GAD and

GABA antibodies.

2.2.1 Ontogeny of GABA neurons in the embryonic mouse spinal cord

On embryonic day 10 (E10), no GAD immunolabeling was detected within the spinal cord (Fig. 2A). After E11, we observed four GABA neuron groups (Figs. 2B-F, 3A-D). On E13, GABA neurons were almost absent on the surface of the ventricular zone (Fig. 3B). These results indicated that GABA neurons were born between E10 and E13 in the spinal cord.

On E11, there were three GABA neuron groups; dorsal group, group 1 (G1, oval in Fig. 2B), middle group, group 2 (G2, upper square in Fig. 2B, 2C, 2D), and ventral group, group 3 (G3, lower square in Fig. 2B, 2C, 2D). G1 was the smallest of the three groups (oval in Fig. 2B), suggesting that G1 neurons were developed after G2 and G3 neurons. G1 neurons were localized at the dorsal end of the mantle layer and on the surface of the ventricular zone on E12 (square in Fig. 3A), scattered within the ventral part of the dorsal horn on E13 (square in Fig. 3B), and distributed throughout the dorsal horn on E15 (square in Fig. 3C). On E17, GAD immunolabeling markedly increased in intensity and density in the dorsal horn (Fig. 3D). G2 was the largest of the three groups (upper square in Fig. 2B), and many neurons were localized in the mantle layer (Fig. 2C, D), suggesting that G2 were developed prior to G1 and G3. The axons of G2 neurons (large arrows) ran between the motor neuron pool and

ventricular zone, crossed in front of the floor plate (asterisk), and entered the contralateral marginal zone (Fig. 2B, 2F). G2 neurons did not form cell masses after E12 (Fig. 3A), but they may have been localized within the ventral part. Their GABAergic commissural axons gradually decreased in number on E13 (Fig. 3B) and did not detected on E15 (Fig. 3C), suggesting that G2 neurons disappeared from the spinal cord or changed their transmitter. G3 (lower square in Fig. 2B) consisted of many neurons on the surface of the ventricular zone and a few neurons (arrowheads) in the mantle layer on E11 (Fig. 2E, 2F), suggesting that G3 neurons developed between G2 and G1 neurons. Their axons directly entered the ipsilateral marginal zone (small arrows in Fig. 2E, 2F). G3 neurons formed a neuronal mass in the ventral horn on E12 (oval in Fig. 3A) and E13 (oval in Fig. 3B). The fourth group (G4) of GABA neurons was first detected in the middle of the spinal cord on E12 (circle in Fig. 3A). G4 neurons formed a neuronal mass at the dorsal position of the G3 in the ventral horn on E13 (circle in Fig. B). G3 and G4 neurons intermingled in the ventral horn after E15 (Fig. 3C, 3D), and their axons exhibited a mesh-like structure on E17 (Fig. 3D).

2.2.2 GABA neurons in the ventral horn from E12 to E17

In the ventral horn, GABA neurons were first detected on E12 by GABA (Fig. 4A) and GAD immunohistochemistry (Fig. 4B). GABA and GAD filled up the neurons, including cell bodies, dendrites, and axons (arrowheads in Fig. 4A, 4B). GABAergic axons were

smooth, did not have varicosities, and often entered the ipsilateral marginal zone (arrows in Fig. 4A, B). On E13, GABA neurons (arrowheads) and axons (arrows) increased in number (Fig. 4C, D). The majority of axons were smooth, but some varicosities (ovals) were detected on the axons near the white matter (Fig. 4C, D). On E15, GABA- and GAD-positive axons also often entered the ipsilateral white matter (ovals) and exhibited a sparse plexus-like structure within the gray matter (Fig. 4E, F). Varicosities were clearly and diffusely discernible on GAD and GABA positive axons within the ventral horn, and immunolabeling between varicosities was low in intensity (Fig. 4E, F). On E17, the varicosities markedly increased in number and density, and often surrounded the cell bodies of large motor neurons (asterisks in Fig. 4G, H). Furthermore, GABA and GAD immunolabeling was confined to the varicosities, and the immunolabeling had almost disappeared from axons by E17 (Fig. 4G, 4H).

2.2.3 GABA neurons in the dorsal horn from E12 to E17

In the dorsal horn, we first observed GABA neurons (arrowheads) on E13. They were filled up by GABA (Fig. 5A) and GAD (Fig. 5B), and their axons were smooth. Although many GABAergic axons entered the white matter in the ventral part (Fig. 4A-D), only a few axons entered the posterior funiculus (arrows in Fig. 5A, B). On E15, GABA- and GAD-positive neurons (arrowheads) and their axons increased in number, but the majority of

axons were still smooth (Fig. 5C, D). On E17, GABAergic axons markedly increased in density, and varicosities were clearly discernible on the axons (Fig. 5E, F). After E15, GABA and GAD were gradually confined to the varicosities as detected in the ventral horn, but immunolabeling was still detected between varicosities on E17.

2.2.4. Developmental change in the marginal zone

On E11 and E12, moderate GAD immunolabeling was detected in the anterior part of the marginal zone (Fig. 2B, 3A). On E13, white matter, including anterior, lateral and posterior funiculi, was more densely stained (Fig. 3B). On E15 and E17, the immunolabeling intensity in anterior and lateral funiculi gradually decreased, whereas the posterior funiculus was still heavily labeled (Fig. 3C, 3D).

2.2.5. Electron microscopic localization of GAD in the marginal zone or white matter

Furthermore, to examine the characterization of GAD-positive profiles in the marginal zone or white matter, we performed electron microscopic analysis. GAD-immunolabeling was detected within some axons in the marginal zone (asterisks in Fig. 6A-D). Several GAD-positive axons contained flat vesicles and formed symmetrical synapses with dendrites (arrowheads in Fig. 6C-6E), indicating that GABAergic synapses were formed within the marginal zone or white matter on at least E13.

2.3. Developmental localization of VGAT

Third, to examine the localization of GABAergic and glycinergic terminals or varicosities of GABAergic and glycinergic axons, we performed immunohistochemistry for VGAT. A lower magnification view demonstrated that VGAT immunolabeling was first detected within the marginal zone on E11 (Fig. 7A) and continued to be localized within the white matter (Fig. 7B-E). In the ventral horn, obvious immunolabeling was observed after E15 (Fig. 7D) and markedly increased on E17 (Fig. 7E). The dorsal horn was weakly labeled on E17 (Fig. 7E). A higher magnification view demonstrated that VGAT immunolabeling exhibited fine dots during embryonic development (Fig. 7F-M). In the ventral part, VGAT-positive dots (arrows) were detected within the marginal zone on E11 (Fig. 7F) and E12 (Fig. 7G). On E13, few dots were detected on the axons within the ventral horn near the white matter (ovals in Fig. 7H). On E15, many VGAT-positive dots were diffusely detected on the axons (arrowheads) within the gray matter (Fig. 7I). On E17, the ventral horn was occupied by numerous dots, and the dots often surrounded the large cell bodies of motor neurons (asterisks in Fig. 7J).

In the dorsal part, numerous dots were observed within the developing posterior funiculus on E13 (Fig. 7K). In the dorsal horn, a few VGAT-positive dots were first detected on E15 (Fig. 6L), and the dots diffusely increased in density on E17 (Fig. 7M), but their number was still lower than that in the ventral horn on E17 (Fig. 7J, 7M).

2.4. Developmental localization of KCC2

Lastly, to examine the time course of the shift in GABA action from excitation to inhibition, we performed immunohistochemistry for KCC2. A lower magnification view demonstrated that weak KCC2 immunolabeling was first detected on the surface of the ventral spinal cord on E10 (Fig. 8A). On E11, KCC2 was slightly or moderately localized within the ventral horn (Fig. 8B). During development, KCC2 immunolabeling gradually increased in intensity, the stained area gradually spread to the dorsal part (Fig. 8C-F), and the gray matter was homogeneously labeled on E17 (Fig. 8F). However, white matter and ventricular zone were negative (Fig. 8A-F). A higher magnification view in the ventral part demonstrated that weak KCC2 immunolabeling was localized within perikarya of monolayer neurons (arrow) on the surface of the spinal cord on E10 (Fig. 8G). On E11, moderate or dense immunolabeling was detected in a few neurons beneath the marginal zone (arrows), but the immunolabeling in the neuropil of the ventral horn was weak (Fig 8H). On E12, moderate KCC2 immunolabeling was diffusely detected in the neuropil of the ventral horn, including the dendrites extending into the marginal zone (arrowheads in Fig. 8I). On E13 and E15, immunolabeling increased in intensity, and dendrites and cell bodies were clearly labeled (Fig. 8J, K). On E17, dense immunolabeling was localized at the cell membrane of somata and dendrites (Fig. 8L).

In the dorsal part, few neurons were labeled on the surface of the dorsal horn on E13 (arrows in Fig. 8M). On E15, KCC2-positive neurons covered the surface of the dorsal horn, whereas the dorsal horn was negative (Fig. 8N). On E17, sensory neurons were moderately labeled (Fig. 8O).

3. Discussion

In the present study, we morphologically investigated the development of GABA signaling in the embryonic mouse cervical spinal cord. The results are summarized in Figure 9 and Table 1.

3.1. Differentiation of GABA neurons in the embryonic spinal cord

3.1.1. Ontogeny of GABA neurons

The present results by GAD and GABA immunohistochemistry suggested the following chronological and sequential ontogeny of GABA neurons in the ventral and dorsal parts (Fig. 9). In the ventral half, G2 neurons, which were commissural neurons, were born between E10 and E11, were localized in the middle of the spinal cord, and disappeared or changed their transmitter by E15 (Figs. 2, 3). The G3 neurons were born around E11 on the ventral side of G2 (Fig. 2B), and was distributed in the ventral horn after E12 (Fig. 3A). The G4 neurons were born around E12 (Fig. 3A) and entered the ventral horn after E13 (Fig. 3B).

Both G3 and G4 neurons were intermingled in the ventral horn and sent axons into the ipsilateral marginal zone (Figs. 2F, 4A-H). Recent studies demonstrated that ventral spinal cord neurons are classified into V neuron groups by the expression of transcriptional factors (Briscoe and Ericson, 2001; Helms and Johnson, 2003; Jessell, 2000; Lee and Jessell, 1999; Lee and Pfaff, 2001; Poh et al., 2002). The G2 may be equivalent to V0 neurons, since V0 neurons are born near the *sulcus limitans*, send commissural axons into the contralateral marginal zone, and transiently release GABA (Moran-Rivard et al., 2001). G3 and G4 neurons may be equivalent to V1 (Alvarez et al., 2005; Siembab et al., 2010) and V2b neurons (Joshi et al., 2009; Lundfald et al., 2007), since they were born in a more ventral part than V0 neurons, send axons into the ipsilateral marginal zone, and released GABA. In the dorsal half, G1 neurons were born between E11 and E13 at the dorsal end of the ventricular zone (Fig. 9B) and localized in the dorsal horn after E13 (Fig. 3B).

3.1.2. Formation of GABAergic synapses

The staining patterns of VGAT immunohistochemistry were quite similar to those by GAD- and GABA-positive dots on the same embryonic days, indicating that not all but many VGAT-positive dots represented axon varicosities or presynaptic terminals on GABAergic axons (Figs. 4, 5, 7) in the embryonic spinal cord.

By the immunohistochemistry for GAD, GABA, and VGAT, we found the difference

in the formation of GABAergic synapses between ventral and dorsal parts. In the ventral part, G2 neurons disappeared by E15, and GABAergic inhibitory inputs may come from G3 and G4 neurons (Fig. 9). Many axons of G3 and G4 neurons entered the ipsilateral marginal zone or white matter, and GABAergic synapses were first formed at the axon varicosities, labeled by GAD (Fig. 6C-E) and VGAT (Fig. 7F, 7G) antibodies, within the marginal zone. The localization of axon varicosities gradually spread to the proximal site (Figs. 4C-H, 7H-J), suggesting that there were distal-to-proximal gradient in formation of varicosities, which were presynaptic terminals, in the axons of GABA neurons and superficial-to-deep direction in formation of GABAergic synapses in the ventral horn. In the dorsal part, in contrast, the majority of axons from GABA neurons were confined to the dorsal horn. GABAergic synapses gradually and diffusely increased after E17, and we could not find any direction in synapse formation similar to that in the ventral part.

3.2. Developmental shift in GABA actions

In the ventral part, many GABAergic fibers from G2 neurons were localized on E11 (Fig. 2B), and G3 were distributed on E12 (Fig. 3A), but abundant KCC2 expression was detected after E13 (Fig. 8, Table 1). In the dorsal horn, GABAergic fibers were detected after E13, but abundant KCC2 expression was detected after E17. These results indicated that GABA bound to the receptors on neurons that did not express enough KCC2, suggesting that

GABA may induce the depolarization of membrane potential for several days (Ben-Ari, 2002; Owens and Kriegstein, 2002; Payne et al., 2003). Furthermore, the abundant expression of KCC2 was almost concomitant with the onset of GABAergic synapse formation (Table 1). Taken together with these results, GABA may act as an excitatory transmitter for several days before GABAergic synapse were formed in the embryonic spinal cord, as detected in cerebral and cerebellar cortexes (Takayama and Inoue, 2004b; Takayama and Inoue, 2006; Takayama and Inoue, 2010), suggesting that these phenomena in the changes in GABA action may be common in the CNS.

3.3 Developmental changes in GABA signaling

Previously, we found a deep-to-superficial gradient in the maturation of GABA signaling in the cerebral cortex with special reference to the chronological and sequential order of laminar-structure formation (Takayama and Inoue, 2010). In the present study, we also found a ventral-to-dorsal gradient in the spinal cord, such as (1) localization of GABA neurons and GABAergic fibers as mentioned in *3.1.1.*, (2) formation of GABAergic synapses as mentioned in *3.1.2.*, and (3) KCC2-expression as mentioned in *3.2.* This gradient is considered to depend on the direction of neuronal maturation by a sonic hedgehog signal (Briscoe and Ericson, 2001; Jessell, 2000; Lee and Jessell, 1999), and maturation of GABA signaling proceeded in the ventral motor area prior to the dorsal sensory area.

Two types of knockout mice, lacking GAD67 (Condie et al., 1997; Ding et al., 2004; Ji et al., 1999) and VGAT (Fujii et al., 2007; Saito et al., 2010; Wojcik et al., 2006; Yamada et al., 2012), have common severe phenotypes, such as omophalocele, cleft palate, hunched posture, loss of movement, and respiratory failure, and cannot survive after birth. Furthermore, KCC2-knockout mice have similar phenotypes (Hubner et al., 2001). The present results demonstrated that presynaptic terminals, GAD- and GABA-positive varicosities, were distributed throughout the ventral horn, and motor neurons expressed abundant KCC2 on E15 and E17 (Table 1). Previous study revealed that $GABA_A$ receptor subunits were abundantly expressed in the motor neurons during embryonic development (Laurie et al., 1992), suggesting that the GABAergic inhibitory network were developed in the ventral motor area between E15 and E17. Taken together with these results, transient inhibitory action by GABA in the ventral horn may be crucial for the survival of newborn mice. Furthermore, motor dysfunction as a consequence of hyper-excitation by loss of GABAergic inhibition may underlie the many phenotypes in the above three knockout mice.

3.4. Development of GABAergic system after birth

The present study demonstrated that the ontogeny of GABA signaling proceeded markedly during embryogenesis. However, GABA signaling was still immature and continued to develop after birth as follows: (1) GABA synapses would be formed mainly in the dorsal

horn; (2) GABAergic synapses would massively reduce in number or shift to glycinergic synapses after birth (Nabekura et al., 2004); (3) GABAergic projection axons would retract or disappear from the white matter during postnatal development.

4. Materials and Methods

4.1. Animals

Two-month-old mice and pregnant mice (C57BL/6J) were used in the present study. Adult and pregnant mice were deeply anesthetized by intraperitoneal injection of chloral hydrate (3.5mg/10g body weight). Fetuses on embryonic day 10 (E10; E0 = mating day), E11, E12, E13, E15 and E17 were removed from the uterus of pregnant mice. At least five mice and fetuses were investigated for immunostaining at each stage.

These experiments were approved by the Animal Care and Use Committees of the University of the Ryukyus (No. 4683, No.5205) 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 and their suffering.

4.2 Antibody characterization

Table 2 lists all antibodies used in the present study. The specificities of antibodies were checked in previous studies (Takayama and Inoue, 2004a; Takayama and Inoue, 2006).

4.3 Tissue preparation

Fetuses on E10, E11 and E12 were immediately immersed in fixatives containing 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) or mixed solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in PB. Fetuses on E13, E15, E17 and adult mice were fixed by transcardial perfusion with the above two types of fixatives. After immersion in the same fixatives overnight, the fetus and spinal cord of adult mice were cryoprotected with 30% sucrose in PB. The cervical part of the fetus and cervical spinal cord of adult mice were cut into transverse sections at a thickness of 20μm by a cryostat and the sections were mounted on glass slides coated with gelatin.

4.4. Immunohistochemistry for KCC2, VGAT, and GABA

Sections of fetus fixed with 4% paraformaldehyde in PB were treated as follows: with methanol containing 0.3% H_2O_2 for 30 minutes, PB for 10 minutes, 3% normal goat serum in PB for one hour, and GAD67/65 antibody, KCC2 antibody (Takayama and Inoue 2006) or VGAT antibody (Takayama and Inoue 2004) overnight at room temperature. After rinsing three times with PB for 15 minutes, sections were visualized using the avidin-biotin-peroxidase complex (ABC) method (Histofine kit; Nichirei, Tokyo, Japan) (Hsu et al., 1981).

Sections of fetus fixed with mixed solution containing 4% paraformaldehyde and

0.5% glutaraldehyde in PB were treated as follows: with methanol containing 0.3% H_2O_2 for 30 minutes, PB for 10 minutes, 0.2% glycine in PB for 30 minutes, 1% sodium borohydrate in PB for 30 minutes (Kosaka et al., 1986), PB for 15 minutes, 3% normal goat serum in PB for one hour, and GABA antibody overnight at room temperature. After rinsing three times with PB for 15 minutes, sections were visualized using the ABC method (Hsu et al., 1981) as described in a previous paper (Takayama and Inoue, 2004a).

4.5. Electronmicroscopic analysis

For electron microscopic analysis, the cervical spinal cord on E13 was cut into transverse sections at a thickness of 300μm using a microslicer (Dosaka, Kyoto, Japan). The sections were treated with 3% normal goat serum for 1 hour, followed by the primary antibody against GAD overnight at room temperature. After visualization by the ABC method, sections were post-fixed by 1% glutaraldehyde in PB for 20 minutes and 1% OsO_4 in PB for 2 hours at 4°C, stained with 1% uranyl acetate aqueous solution overnight, and embedded in epoxy resin in the usual manner (Takayama and Inoue, 2004a). Ultra-thin sections were observed under an electron microscope (H-9500, Hitachi, Japan).

Acknowledgements

We are grateful to Makiko Moriyasu-Kuroki and Yukiji Yabiku in the Department of

Molecular Anatomy for their assistance with the preparation of this manuscript. We would also like to thank Jeongtae Kim and Masanobu Sunagawa in the Department of Molecular Anatomy for valuable discussion. This work was supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (Kiban C No. 20500310, No. 23500413), Uruma Science Foundation, and Takeda Science Foundation.

Contributions

The majority of the experiment was performed by Yoshinori Kosaka (YK). Preparation of materials and control studies were partly performed by Hidemichi Kin (HK), Masaharu Tatetsu (MT), Itsuki Uema (IU), and Chitoshi Takayama (CT). Figure plates and Tables were organized by YK and CT. The manuscript was written by CT and checked by all authors.

Figure legends

Figure 1 Immunohistochemical localization of GAD (A, B), VGAT (C, D), and KCC2 (E, F) in the two-month-old mouse spinal cord at lower (A, C, E) and higher (B, D, F) magnifications

Asterisk: cell body of large motor neuron, arrow: dendrites extending into white matter

AF: anterior funiculus, cc: central canal, DH: dorsal horn, LF: lateral funiculus, PF: posterior funiculus, VH: ventral horn

Figure 2 Immunohistochemical localization of GAD (A-C, E, F) and GABA (D) in the mouse cervical spinal cord on E10 (A) and E11 (B-F)

A: Immunohistochemistry for GAD on E10. No immunolabeling was detected in the spinal cord including the monolayer of neurons (small arrowheads) on the surface of the spinal cord.

B: Immunohistochemistry for GAD on E11 at lower magnification.

Three GAD-positive neuron groups, dorsal (G1, oval), middle (G2, upper square), and ventral (G3, lower square) groups, were detected.

C, D: Immunohistochemistry for GAD (C) and GABA (D) in the middle group (G2 in B) at higher magnification. GAD neurons sent commissural axons (large arrows in B, F), which crossed in front of the floor plate (asterisks in B), to the contralateral marginal zone (MZ).

E, F: Immunohistochemistry for GAD of the ventral group (G3 in B) at higher magnification. Neurons (large arrowheads) in the mantle layer (ML) sent axons (small arrows) into the ipsilateral marginal zone (MZ).

CC: central canal, DH: dorsal horn, LF: lateral funiculus, ML: mantle layer, MZ: marginal zone, PF: posterior funiculus, VH: ventral horn, VZ: ventricular zone

Figure 3 Developmental localization of GAD in the spinal cord on E12 (A), E13 (B), E15 (C), and E17 (D).

Arrow: GAD-positive commissural axons which crossed in front of the floor plate (asterisks), circle (G4): middle GAD-positive neuron group, which was newly formed on E12, oval (G3): ventral group equivalent to G3 in Fig. 2B, square (G1): dorsal group equivalent to G1 in Fig. 2B.

AF: anterior funiculus, CC: central canal, DH: dorsal horn, LF: lateral funiculus, ML: mantle layer, MZ: marginal zone, PF: posterior funiculus, VH: ventral horn, VZ: ventricular zone

Figure 4 Immunohistochemical localization of GABA (A, C, E, G) and GAD (B, D, F, H) in the developing ventral horn on E12 (A, B), E13 (C, D), E15 (E, F) and E17 (G, H) at higher magnification.

Asterisk: cell body of large motor neuron, arrow: GABA- and GAD-positive axon, arrowhead: GABA and GAD-positive cell body, oval: varicosities on GABA- and GAD-positive axons which entered the marginal zone (MZ) or white matter (WM)

Figure 5 Immunohistochemical localization of GABA (A, C, E) and GAD (B, D, F) in the developing dorsal horn on E13 (A, B), E15 (C, D) and E17 (E, F) at a higher magnification. arrow: GABA and GAD-positive axons which entered the posterior funiculus, arrowhead: cell body of GABA- and GAD-positive neurons.

MZ: marginal zone, PF: posterior funiculus

Figure 6 Electron micrographs of GAD-immunohistochemistry in the anterior funiculus on E13

Asterisk: GAD-positive axon, arrowhead: symmetric synapse between GAD-positive terminal and dendrite (de)

Figure 7 Immunohistochemical localization of VGAT in the developing spinal cord on E11 (A, F), E12 (B, G), E13 (C, H, K), E15 (D, I, L) and E17 (E, J, M)

A-E) Lower magnification photographs

F-J) Higher magnification photographs of the ventral part. Each location is shown in A-E.

K-M) Higher magnification photographs of the dorsal part. Each location is shown in C-E.

Arrow: VGAD-positive dot in the anterior marginal zone (F, G) and dorsal horn (L), asterisk: cell body of large neuron surrounded by VGAT-positive dots, oval (H) and arrowhead (I): VGAT-positive dots on axons entering the marginal zone (MZ) or white matter (WM).

AF: anterior funiculus, CC: central canal, DH: dorsal horn, LF: lateral funiculus, ML: mantle layer, MZ: marginal zone, PF: posterior funiculus, VZ: ventricular zone, VH: ventral horn, WM: white matter

Figure 8 Developmental localization of KCC2 in the embryonic spinal cord on E10 (A, G), E11 (B, H), E12 (C, I), E13 (D, J, M), E15 (E, K, N) and E17 (F, L, O)

A-F) Lower magnification photographs

G-L) Higher magnification photographs of the ventral part. Each location is shown in A-F.

M-O) Higher magnification photographs of the dorsal part. Each location is shown in D-F.

Arrow: KCC2-positive neurons on the surface of gray matter (G, H, M, N), arrowhead: KCC2-positive dendrites extending into the marginal zone, asterisk: large neuron expressing KCC2.

AF: anterior funiculus, CC: central canal, DH: dorsal horn, ML: mantle layer, MZ: marginal

zone, PF: posterior funiculus, VH: ventral horn, VZ: ventricular zone, WM: white matter

Figure 9 Schematic illustrations of developing GABA neurons in the spinal cord on E10 (A), E11 (B), E12 (C), E13 (D), E15 (E), and E17 (F)

G1-G4: GABAergic neuron groups

AF: anterior funiculus, CC: central cannal, DH: dorsal horn, LF: lateral funiculus, ML: mantle layer, MZ: marginal zone, PF: posterior funiculus, VH: ventral horn, VZ: ventricular zone

References

Alvarez, F.J., Jonas, P.C., Sapir, T., Hartley, R., Berrocal, M.C., Geiman, E.J., Todd, A.J., Goulding, M., 2005. Postnatal phenotype and localization of spinal cord V1 derived interneurons. J Comp Neurol. 493, 177-92.

Barker, J.L., Behar, T., Li, Y.X., Liu, Q.Y., Ma, W., Maric, D., Maric, I., Schaffner, A.E., Serafini, R., Smith, S.V., Somogyi, R., Vautrin, J.Y., Wen, X.L., Xian, H., 1998. GABAergic cells and signals in CNS development. Perspect Dev Neurobiol. 5, 305-22.

Ben-Ari, Y., 2002. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci. 3, 728-39.

Ben-Ari, Y., Gaiarsa, J.L., Tyzio, R., Khazipov, R., 2007. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev. 87, 1215-84.

Briscoe, J., Ericson, J., 2001. Specification of neuronal fates in the ventral neural tube. Curr Opin Neurobiol. 11, 43-9.

Condie, B.G., Bain, G., Gottlieb, D.I., Capecchi, M.R., 1997. Cleft palate in mice with a targeted mutation in the gamma-aminobutyric acid-producing enzyme glutamic acid decarboxylase 67. Proc Natl Acad Sci U S A. 94, 11451-5.

Culiat, C.T., Stubbs, L.J., Woychik, R.P., Russell, L.B., Johnson, D.K., Rinchik, E.M., 1995. Deficiency of the beta 3 subunit of the type A gamma-aminobutyric acid receptor causes cleft palate in mice. Nat Genet. 11, 344-6.

Delpy, A., Allain, A.E., Meyrand, P., Branchereau, P., 2008. NKCC1 cotransporter inactivation underlies embryonic development of chloride-mediated inhibition in mouse spinal motoneuron. J Physiol. 586, 1059-75.

Ding, R., Tsunekawa, N., Obata, K., 2004. Cleft palate by picrotoxin or 3-MP and palatal shelf elevation in GABA-deficient mice. Neurotoxicol Teratol. 26, 587-92.

Fon, E.A., Edwards, R.H., 2001. Molecular mechanisms of neurotransmitter release. Muscle Nerve. 24, 581-601.

Fujii, M., Arata, A., Kanbara-Kume, N., Saito, K., Yanagawa, Y., Obata, K., 2007. Respiratory activity in brainstem of fetal mice lacking glutamate decarboxylase 65/67 and vesicular GABA transporter. Neuroscience. 146, 1044-52.

Gao, B.X., Ziskind-Conhaim, L., 1995. Development of glycine- and GABA-gated currents in rat spinal motoneurons. J Neurophysiol. 74, 113-21.

Grant, G., Koerber, H.R., 2004. Spinal cord cytoarchitecture. In The rat nervous system. Vol., P. George, ed.^eds. elsevier, San Diego.

Helms, A.W., Johnson, J.E., 2003. Specification of dorsal spinal cord interneurons. Curr Opin Neurobiol. 13, 42-9.

Hsu, S.M., Raine, L., Fanger, H., 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem. 29, 577-80.

Hubner, C.A., Stein, V., Hermans-Borgmeyer, I., Meyer, T., Ballanyi, K., Jentsch, T.J., 2001. Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. Neuron. 30, 515-24.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet. 1, 20-9.

Ji, F., Kanbara, N., Obata, K., 1999. GABA and histogenesis in fetal and neonatal mouse brain lacking both the isoforms of glutamic acid decarboxylase. Neurosci Res. 33, 187-94.

Joshi, K., Lee, S., Lee, B., Lee, J.W., Lee, S.K., 2009. LMO4 controls the balance between excitatory and inhibitory spinal V2 interneurons. Neuron. 61, 839-51.

Kiehn, O., 2006. Locomotor circuits in the mammalian spinal cord. Annu Rev Neurosci. 29, 279-306.

Kosaka, T., Nagatsu, I., Wu, J.Y., Hama, K., 1986. Use of high concentrations of glutaraldehyde for immunocytochemistry of transmitter-synthesizing enzymes in the central nervous system. Neuroscience. 18, 975-90.

Laurie, D.J., Wisden, W., Seeburg, P.H., 1992. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci. 12, 4151-72.

Lee, K.J., Jessell, T.M., 1999. The specification of dorsal cell fates in the vertebrate central nervous system. Annu Rev Neurosci. 22, 261-94.

Lee, S.K., Pfaff, S.L., 2001. Transcriptional networks regulating neuronal identity in the developing spinal cord. Nat Neurosci. 4 Suppl, 1183-91.

Lundfald, L., Restrepo, C.E., Butt, S.J., Peng, C.Y., Droho, S., Endo, T., Zeilhofer, H.U., Sharma, K., Kiehn, O., 2007. Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord. Eur J Neurosci. 26, 2989-3002.

Macdonald, R.L., Olsen, R.W., 1994. GABAA receptor channels. Annu Rev Neurosci. 17, 569-602.

Martin, D.L., Rimvall, K., 1993. Regulation of gamma-aminobutyric acid

synthesis in the brain. J Neurochem. 60, 395-407.

McCarthy, M.M., Auger, A.P., Perrot-Sinal, T.S., 2002. Getting excited about GABA and sex differences in the brain. Trends Neurosci. 25, 307-12.

McIntire, S.L., Reimer, R.J., Schuske, K., Edwards, R.H., Jorgensen, E.M., 1997. Identification and characterization of the vesicular GABA transporter. Nature. 389, 870-6.

Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M.K., Burrill, J., Goulding, M., 2001. Evx1 is a postmitotic determinant of v0 interneuron identity in the spinal cord. Neuron. 29, 385-99.

Nabekura, J., Ueno, T., Okabe, A., Furuta, A., Iwaki, T., Shimizu-Okabe, C., Fukuda, A., Akaike, N., 2002. Reduction of KCC2 expression and GABAA receptor-mediated excitation after in vivo axonal injury. J Neurosci. 22, 4412-7.

Nabekura, J., Katsurabayashi, S., Kakazu, Y., Shibata, S., Matsubara, A., Jinno, S., Mizoguchi, Y., Sasaki, A., Ishibashi, H., 2004. Developmental switch from GABA to glycine release in single central synaptic terminals. Nat Neurosci. 7, 17-23.

Olsen, R.W., Tobin, A.J., 1990. Molecular biology of GABAA receptors. Faseb J. 4, 1469-80.

Owens, D.F., Kriegstein, A.R., 2002. Is there more to GABA than synaptic inhibition? Nat Rev Neurosci. 3, 715-27.

Payne, J.A., Rivera, C., Voipio, J., Kaila, K., 2003. Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci. 26, 199-206.

Phelps, P.E., Alijani, A., Tran, T.S., 1999. Ventrally located commissural neurons express the GABAergic phenotype in developing rat spinal cord. J Comp Neurol. 409, 285-98.

Poh, A., Karunaratne, A., Kolle, G., Huang, N., Smith, E., Starkey, J., Wen, D., Wilson, I., Yamada, T., Hargrave, M., 2002. Patterning of the vertebrate ventral spinal cord. Int J Dev Biol. 46, 597-608.

Reimer, R.J., Fon, E.A., Edwards, R.H., 1998. Vesicular neurotransmitter transport and the presynaptic regulation of quantal size. Curr Opin Neurobiol. 8, 405-12.

Represa, A., Ben-Ari, Y., 2005. Trophic actions of GABA on neuronal development. Trends Neurosci. 28, 278-83.

Ribeiro-Da-Silva, A., 2004. Substantia gelatinosa of the spinal cord. In The rat nervous system. Vol., G. Paxinos, ed.^eds. elsevier, San Diego.

Saito, K., Kakizaki, T., Hayashi, R., Nishimaru, H., Furukawa, T., Nakazato, Y., Takamori, S., Ebihara, S., Uematsu, M., Mishina, M., Miyazaki, J., Yokoyama, M., Konishi, S., Inoue, K., Fukuda, A., Fukumoto, M., Nakamura, K., Obata, K., Yanagawa, Y., 2010. The physiological roles of vesicular GABA transporter during embryonic development: a study using knockout mice. Mol Brain. 3, 40.

Schaffner, A.E., Behar, T., Nadi, S., Smallwood, V., Barker, J.L., 1993. Quantitative analysis of transient GABA expression in embryonic and early postnatal rat spinal cord neurons. Brain Res Dev Brain Res. 72, 265-76.

Sibilla, S., Ballerini, L., 2009. GABAergic and glycinergic interneuron expression during spinal cord development: dynamic interplay between inhibition and excitation in the control of ventral network outputs. Prog Neurobiol. 89, 46-60.

Siembab, V.C., Smith, C.A., Zagoraiou, L., Berrocal, M.C., Mentis, G.Z., Alvarez, F.J., 2010. Target selection of proprioceptive and motor axon synapses on neonatal V1-derived Ia inhibitory interneurons and Renshaw cells. J Comp Neurol. 518, 4675-701.

Stein, V., Hermans-Borgmeyer, I., Jentsch, T.J., Hubner, C.A., 2004. Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride. J Comp Neurol. 468, 57-64.

Stil, A., Liabeuf, S., Jean-Xavier, C., Brocard, C., Viemari, J.C., Vinay, L., 2009. Developmental up-regulation of the potassium-chloride cotransporter type 2 in the rat lumbar spinal cord. Neuroscience. 164, 809-21.

Takayama, C., Inoue, Y., 2004a. Extrasynaptic localization of GABA in the developing mouse cerebellum. Neurosci Res. 50, 447-58.

Takayama, C., Inoue, Y., 2004b. GABAergic signaling in the developing cerebellum. Anat Sci Int. 79, 124-136.

Takayama, C., 2005. GABAergic signaling in the developing cerebellum. Int Rev Neurobiol. 71, 63-94.

Takayama, C., Inoue, Y., 2006. Developmental localization of potassium chloride co-transporter 2 in granule cells of the early postnatal mouse cerebellum with special reference to the synapse formation. Neuroscience. 143, 757-67.

Takayama, C., Inoue, Y., 2007. Developmental localization of potassium chloride co-transporter 2 (KCC2) in the Purkinje cells of embryonic mouse cerebellum. Neurosci Res. 57, 322-5.

Takayama, C., Inoue, Y., 2010. Developmental localization of potassium chloride co-transporter 2 (KCC2), GABA and vesicular GABA transporter (VGAT) in

the postnatal mouse somatosensory cortex. Neurosci Res. 67, 137-48.

Tatetsu, M., Kim, J., Kina, S., Sunakawa, H., Takayama, C., 2012. GABA/glycine signaling during degeneration and regeneration of mouse hypoglossal nerves. Brain Res. 1446, 22-33.

Tracey, D., 2004. Acending and descending pathways in the spinal cord. In The rat nervous system. Vol., G. Paxinos, ed.^eds. elsevier, San Diego.

Tran, T.S., Alijani, A., Phelps, P.E., 2003. Unique developmental patterns of GABAergic neurons in rat spinal cord. J Comp Neurol. 456, 112-26.

Tran, T.S., Cohen-Cory, S., Phelps, P.E., 2004. Embryonic GABAergic spinal commissural neurons project rostrally to mesencephalic targets. J Comp Neurol. 475, 327-39.

Varju, P., Katarova, Z., Madarasz, E., Szabo, G., 2001. GABA signalling during development: new data and old questions. Cell Tissue Res. 305, 239-46.

Vinay, L., Jean-Xavier, C., 2008. Plasticity of spinal cord locomotor networks and contribution of cation-chloride cotransporters. Brain Res Rev. 57, 103-10.

Wojcik, S.M., Katsurabayashi, S., Guillemin, I., Friauf, E., Rosenmund, C., Brose, N., Rhee, J.S., 2006. A shared vesicular carrier allows synaptic corelease of GABA and glycine. Neuron. 50, 575-87.

Wu, W.L., Ziskind-Conhaim, L., Sweet, M.A., 1992. Early development of glycine- and GABA-mediated synapses in rat spinal cord. J Neurosci. 12, 3935-45.

Yamada, M.H., Nishikawa, K., Kubo, K., Yanagawa, Y., Saito, S., 2012. Impaired glycinergic synaptic transmission and enhanced inflammatory pain in mice with reduced expression of vesicular GABA transporter (VGAT). Mol Pharmacol. 81, 610-9.

Zagoraiou, L., Akay, T., Martin, J.F., Brownstone, R.M., Jessell, T.M., Miles, G.B., 2009. A cluster of cholinergic premotor interneurons modulates mouse locomotor activity. Neuron. 64, 645-62.

Table 1 Ontogeny of GABA neurons, formation of GABAergic synapses, and localization of KCC2 in the mouse spinal cord

Embryonic days		E10	E11	E12	E13	E15	E17
GABA neuron	mantle layer	-	+	++			
	ventral horn		-	+	++	++	++
	dorsal horn				+	++	++
GABAergic synapse	anterior funiculus		+	++	++	++	+
	posterior funiculus			+	++	++	++
	ventral horn		-	-	+/-	+	++
	dorsal horn				-	+/-	+
KCC2	ventral horn		-/+	+	++	++	++
	dorsal horn				-	-	+

-: negative
+/-: few or weak
+: moderate
++: many or dense

Table 2 Antibody characterization

Antigen	Immunogen	Manufacturer Species, Antibody type Reference	Dilution used
GABA	GABA conjugated with bovine serum albumin	Rabbit, polyclonal (Takayama and Inoue, 2004a)	Final concentration 0.3μg/ml
Glutamic acid decarboxylase (GAD)	Synthetic peptide aa 1-14 from C-terminals of rat [C]DFLIEEIERLGQDL	AB1511, Millipore Rabbit polyclonal	Final concentration 1:2000
Potassium chloride Co-transporter 2 (KCC2)	Synthetic peptide, aa 44-64 from N-terminals of mouse	Rabbit, polyclonal (Takayama and Inoue, 2006)	Final concentration 1μg/ml
Vesicular GABA Transporter (VGAT)	Synthetic peptide, aa 1022-1042 from N-terminals of mouse	rabbit, polyclonal (Takayama and Inoue, 2004a)	Final concentration 1μg/ml