Neuroscience 343 (2017) 459–471

DISTINCT DEVELOPMENT OF THE GLYCINERGIC TERMINALS IN THE VENTRAL AND DORSAL HORNS OF THE MOUSE CERVICAL SPINAL CORD

MASANOBU SUNAGAWA,[a] CHIGUSA SHIMIZU-OKABE,[a] JEONGTAE KIM,[a] SHIORI KOBAYASHI,[a] YOSHINORI KOSAKA,[a] YUCHIO YANAGAWA,[b] MASAYUKI MATSUSHITA,[c] AKIHITO OKABE[a] AND CHITOSHI TAKAYAMA[a]*

[a] *Department of Molecular Anatomy, School of Medicine, University of the Ryukyus, 207 Uehara 207, Nishihara, Okinawa 9030215, Japan*

[b] *Division of Neuroscience, Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine, Maebashi, Gunma 3718511, Japan*

[c] *Department of Molecular and Cellular Physiology, School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 9030215, Japan*

Abstract—In the spinal cord, glycine and γ-amino butyric acid (GABA) are inhibitory neurotransmitters. However, the ontogeny of the glycinergic network remains unclear. To address this point, we examined the developmental formation of glycinergic terminals by immunohistochemistry for glycine transporter 2 (GlyT2), a marker of glycinergic terminals, in developing mouse cervical spinal cord. Furthermore, the developmental localization of GlyT2 was compared with that of glutamic acid decarboxylase (GAD), a marker of GABAergic terminals, and vesicular GABA transporter (VGAT), a marker of inhibitory terminals, by single and double immunolabeling. GlyT2-positive dots (glycinergic terminals) were first detected in the marginal zone on embryonic day 14 (E14). In the ventral horn, they were detected at E16 and increased in observed density during postnatal development. Until postnatal day 7 (P7), GAD-positive dots (GABAergic terminals) were dominant and GlyT2 immunolabeling was localized at GAD-positive dots. During the second postnatal week, GABAergic terminals markedly decreased and glycinergic terminals became dominant. In the dorsal horn, glycinergic terminals were detected at P0 in lamina IV and P7 in lamina III and developmentally increased. GlyT2 was also localized at GAD-positive dots, and colocalizing dots were dominant at P21. VGAT-positive dots (inhibitory terminals) continued to increase until P21. These results suggest that GABAergic terminals first appear during embryonic development and may often change to colocalizing terminals throughout the gray matter during development. The colocalizing terminals may remain in the dorsal horn, whereas in the ventral horn, colocalizing terminals may give rise to glycinergic terminals.

Key words: γ-amino butyric acid, glutamic acid decarboxylase, glycine, glycine transporter 2, inhibitory synapse, vesicular GABA transporter.

INTRODUCTION

Glycine and γ-amino butyric acid (GABA) are the main inhibitory neurotransmitters in the mammalian central nervous system (Aprison, 1990; Olsen and Tobin, 1990; Macdonald and Olsen, 1994; Zafra et al., 1997; Legendre, 2001; Lopez-Corcuera et al., 2001). In the adult spinal cord, glycine is the predominant inhibitory neurotransmitter and regulates the neuronal activity.

We previously reported the developmental formation of the GABAergic network in the mouse spinal cord by immunohistochemistry for GABA, glutamic acid decarboxylase (GAD) (a synthetic enzyme of GABA), and GABA transporters (GAT-1 and GAT-3), which remove GABA from the synaptic cleft and transport GABA into presynaptic terminals and astrocytic processes (Kosaka et al., 2012; Kim et al., 2014). GABAergic terminals, detected as GAD-positive dots, were distributed throughout the gray matter during perinatal development, whereas they were sparse in the adult ventral horn. In contrast, inhibitory terminals, identified by immunohistochemistry for vesicular GABA transporter (VGAT), which is also known as vesicular inhibitory amino acid transporter (VIAAT) (Sagne et al., 1997; Bedet et al., 2000) and transports both GABA and glycine into synaptic vesicles, were densely and homogeneously distributed throughout the gray matter after birth until maturation. Thus, our previous results suggest that many GABAergic terminals switch to glycinergic terminals during postnatal development in the ventral horn (Kosaka et al., 2012). Previous immunohistochemical analyses have demonstrated that in the spinal cord and brain stem, glycinergic

*Corresponding author. Fax: +81-98-895-1401.
E-mail address: takachan@med.u-ryukyu.ac.jp (C. Takayama).

Abbreviations: ABC, avidin–biotin-peroxidase-complex; Ax, axon; CNBr, cyanogen bromide; CNS, central nervous system; den, dendrite; DH, dorsal horn; E, embryonic day; Ex, excitatory terminal; GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; GAT, GABA transporter; Gly, glycinergic presynapse; GlyT2, glycine transporter 2; GST, glutathione-S-transferase; IgG, immunoglobulin G; I–IX, laminae of spinal cord; kDa, kiloDalton; LSO, lateral superior olivary nucleus; P, postnatal day; PB, phosphate buffer; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sp, spine (of the dendrite); V, vacuole; VGAT, vesicular GABA transporter; VH, ventral horn; VIAAT, vesicular inhibitory amino acid transporter; WM, white matter.

http://dx.doi.org/10.1016/j.neuroscience.2016.12.032
0306-4522/

synapses appear before birth, increase in number during mouse postnatal development (Jursky and Nelson, 1996), and are abundantly distributed after maturation in mice and rats (van den Pol and Gorcs, 1988; Jursky and Nelson, 1995; Luque et al., 1995; Zafra et al., 1995; Zeilhofer et al., 2005). Electrophysiological analysis has demonstrated the postnatal transition from GABAergic to glycinergic transmission in the spinal cord (Gao et al., 2001) and lateral superior olivary nucleus (LSO) (Kotak et al., 1998) and the shift of neurotransmitter from GABA to glycine in a single terminal within the LSO (Nabekura et al., 2004). In addition, double immunolabeling analyses have demonstrated that glycine or glycine transporter 2 (GlyT2), which is localized at glycinergic terminals, and GABA or GAD are often colocalized in the same terminals and neurons (Todd et al., 1996; Tanaka and Ezure, 2004; Allain et al., 2006; Crook et al., 2006; Dufour et al., 2010a, 2010b; Rahman et al., 2015). Electrophysiological analyses have demonstrated that GABA and glycine are often coreleased (Jonas et al., 1998; Dumoulin et al., 2001; Nabekura et al., 2004; Dufour et al., 2010a,b; Ishibashi et al., 2013) during development and after maturation. Furthermore, GAD67-knockout mice have severe phenotypes such as loss of movement, omphalocele, cleft palate, and respiratory failure and cannot survive after birth (Condie et al., 1997; Ji et al., 1999; Ding et al., 2004). In contrast, mice lacking GlyT2, which reuptakes glycine from the synaptic cleft into the presynapse and is essential for glycinergic inhibitory transmission (Eulenburg et al., 2005; Betz et al., 2006), can survive after birth but suffer from neuromotor disorders such as impairment of motor coordination, strong tremor, and rigid muscular tonus, followed by death during the second postnatal week (Gomeza et al., 2003; Eulenburg et al., 2005; Latal et al., 2010). Taken together, GABAergic terminals are dominant during embryonic development, but glycinergic terminals increase during postnatal development and become dominant in the adult spinal cord. Nevertheless, the developmental formation of glycinergic synapses, particularly its spatial and temporal relationship with that of GABAergic synapses, have still not been clearly demonstrated.

To address the above point, we performed immunohistochemistry for GlyT2. GlyT2 is one of the most important molecules for glycinergic synaptic transmission and assumed to be a marker for glycinergic axon terminals (Poyatos et al., 1997; Zeilhofer et al., 2005) in the developing spinal cord, particularly the gray matter and ventral and dorsal horns where inhibitory local circuit terminals remain after maturation. Furthermore, we immunohistochemically stained the adjacent sections with antibodies against GAD and VGAT/VIAAT and also performed double staining for GlyT2 and GAD.

EXPERIMENTAL PROCEDURES

Animals

Male mice at postnatal day 0 (P0), P7, P14, and P21 and pregnant mice (C57Bl/6J, Japan SLC, Shizuoka, Japan) were used in this study. Newborn mice were anesthetized by hypothermia on ice. Other mice were deeply anesthetized by intraperitoneal injection of a mixed solution (10 μL/g body weight) containing 8% (v/v) pentobarbital sodium and 20% (v/v) ethanol in saline. Fetuses at E12 (E0 = mating day), E14, E16, and E18 were removed from the uteri of pregnant mice. At least five mice or fetuses obtained from at least three different mother mice were investigated for immunostaining at each stage.

These experiments were approved by the Animal Care and Use Committee 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 and their suffering.

Establishment of guinea-pig antibodies against GAD and VGAT

cDNA corresponding to amino acid residues 1–111 of mouse VGAT (AB080232.1, GI: 26665358) (McIntire et al., 1997; Sagne et al., 1997) was prepared by polymerase chain reaction using the pair of primers 5′-GGATCCATGGCCACCCTGCTCCGCAGCAAGCT and 5′-GAATTCGTGACC CCCGAACTCCCCACCAGCT. The cDNA was ligated to pGEX-6P-1 (GE Healthcare Bio-Sciences, USA). Glutathione-S-transferase (GST) fusion protein was expressed in *Escherichia coli* BL21 (Takara, Japan), extracted, and purified with glutathione-Sepharose (GE Healthcare Bio-Sciences). The fusion protein was cleaved from GST with PreScission protease, and GST was absorbed by glutathione-Sepharose.

For double staining with a rabbit anti-GlyT2 antibody, we raised antiserum in guinea pigs. Guinea pigs were immunized at 2-week intervals by subcutaneous injection of synthetic peptide ([C]DFLIEEIERLGQDL, 14 aa of the C-terminal) for GAD and recombinant protein (1022–1042 of the mouse VGAT) as above (200 μg/rabbit) for VGAT. The synthetic peptides were emulsified in an equal amount of complete Freund's adjuvant (DIFCO Laboratories, USA). Two weeks after the last injection, immunoglobulin G (IgG) fractions were purified from the antiserum using a Protein G Sepharose column (GE Healthcare Bio-Science). The specific IgG fraction binding to the synthetic peptide or recombinant protein used for the immunization was affinity-purified with CNBr-activated Sepharose 4B (GE Healthcare Bio-Science).

Antibody characterization

Characterizations of all antibodies used in the present study are listed in Table 1. The specificities of the new GAD and VGAT antibodies were checked by both western blotting and immunohistochemistry. For the western blotting against GAD, the lysed P2 fraction prepared from adult mouse spinal cord was extracted with buffer A (20 mM HEPES–NaOH, 150 mM NaCl, pH 7.4) containing 2% (v/v) Triton X-100. For the detection of VGAT, the lysed P2 fraction prepared from