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Developmental localization of calcitonin gene-related peptide in dorsal sensory axons and ventral motor neurons of mouse cervical spinal cord

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ABSTRACT

Calcitonin gene-related peptide (CGRP) is a 37-amino-acid neuropeptide, synthesized by alternative splicing of calcitonin gene mRNA. CGRP is characteristically distributed in the nervous system, and its function varies depending on where it is expressed. To reveal developmental formation of the CGRP network and its function in neuronal maturation, we examined the immunohistochemical localization of CGRP in the developing mouse cervical spinal cord and dorsal root ganglion. CGRP immunolabeling (IL) was first detected in motor neurons on E13, and in ascending axons of the posterior funiculus and DRG neurons on E14. CGRP-positive sensory axon fibers entered Laminae I and II on E16, and Laminae I through IV on E18. The intensity of the CGRP-IL gradually increased in both ventral and dorsal horns during embryonic development, but markedly decreased in the ventral horn after birth. These results suggest that CGRP is expressed several days after neuronal settling and entry of sensory fibers, and that the CGRP network is formed in chronological and sequential order. Furthermore, because CGRP is markedly expressed in motor neurons when axons are vastly extending and innervating targets, CGRP may also be involved in axonal elongation and synapse formation during normal development.

1. Introduction

Calcitonin gene-related peptide (CGRP) is a neuropeptide, consisting of 37 amino acids, and synthesized by alternative splicing of calcitonin gene mRNA (Amara et al., 1982; Rosenfeld et al., 1983). It is characteristically localized in the central and peripheral nervous systems, such as primary sensory fibers, autonomic nerves, motor neurons, and dorsal root ganglion (DRG) neurons (Ishida-Yamamoto and Tohyama, 1989). CGRP function varies depending on regions where it is expressed. CGRP in autonomic nerves innervating cardiovascular organs acts as a powerful vasodilator (Brain et al., 1985; Marshall et al., 1986; Preibisz, 1993). In Aδ and C sensory fibers, CGRP may be involved in the conduction of noxious stimulation, temperature sensation, and pain (Bennett et al., 2000; Satoh et al., 1992; Yu et al., 2009). CGRP may also be involved in the re-extension of axons and play an important role in repair mechanism after nerve injury (Chen et al., 2010; Zheng et al., 2008). Zheng et al. reported that the intensity of CGRP immunolabeling (IL) transiently increased in Laminae I–IV of the dorsal horn (DH) and motor neurons of the ventral horn (VH) in the lumbar spinal cord after sciatic nerve crush injury and sciatic nerve transection injury (Zheng et al., 2008). CGRP expression also increased in cranial motor nerves, such as oculomotor, trochlear, abducens, trigeminal, facial, and hypoglossal nerves after axonal injury, and then gradually decreased to the control level by day 56 after the surgical procedure (Fukuoka et al., 1999; Makwana et al., 2010). We also detected the same phenomenon in the spinal cord and brain stem (Kim et al., unpublished data). In particular, after facial nerve injury, CGRP-IL was observed in the roots and the internal genu of facial nerves, which were CGRP-negative in normal mice, indicating that CGRP expression markedly increased in facial motor neurons during facial nerve regeneration.

Abbreviations: AF, anterior funiculus; CGRP, calcitonin gene-related peptide; cc, central canal; cu, cuneate fasciculus; DH, dorsal horn; DRG, dorsal root ganglion; E, embryonic day; gr, gracile fasciculus; IL, immunolabeling; LF, lateral funiculus; P, postnatal day; PB, phosphate buffer; PF, posterior funiculus; VH, ventral horn.

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We subsequently focused on the developmental localization of CGRP in the cervical spinal cord, and hypothesized that an increase of CGRP-expression might lead to a massive extension of axons during not only axonal regeneration but also embryonic development as speculated in rats by Matteoli et al. (1990). There have been numerous studies concerning the localization of CGRP since Amara's report (Amara et al., 1982; Cortes et al., 1990; Gibson et al., 1988; Kawai et al., 1985; Kresse et al., 1995; Kruger et al., 1988; Skofitsch and Jacobowitz, 1985), and its ontogenetic changes in the spinal cord have been demonstrated in the rat, mouse, human, and sheep (Funakoshi et al., 2003; Marti et al., 1987; Matteoli et al., 1990; Nitsos et al., 1994). Funakoshi et al. reported developmental expression and localization of CGRP in lumbar and sacral spinal cord during the prenatal period, but they focused only on autonomic networks, and did not mention the CGRP in the sensory and somatic motor system (Funakoshi et al., 2003). Matteoli et al. demonstrated the ontogenetic changes in the rat motor neurons, but they only focused on rat motor neurons (Matteoli et al., 1990). Other studies mentioned CGRP as one of the many neuropeptides and did not precisely report the developmental localization of CGRP (Marti et al., 1987; Nitsos et al., 1994). In the present study, to reveal the formation of the CGRP network in the mouse spinal cord and its function in neuronal maturation, we examined the developmental localization of CGRP in both sensory and motor systems by immunohistochemistry, and focused on two points, (i) the chronological order of the ontogeny of CGRP-positive neurons and fibers including DRG neurons, dorsal sensory fibers, and motor neurons, and (ii) the spatial and temporal relationship between changes in expression level and embryonic and postnatal maturation of mouse spinal cord.

2. Materials and methods

2.1. Animals

C57BL/6J mice on embryonic day 12 (E12, E0 = mating day), E13, E14, E16, E18, postnatal day 0 (P0), P7, P21, and P60 (as adult) were used in this study. We examined at least three mice on each embryonic and postnatal day. The experiments were approved by the Animal Care and Use Committees of the University of the Ryukyus (No. 5762) and were performed in compliance with the Guide for the Care and Use of Laboratory Animals of the University of the Ryukyus. Procedures for the care and handling of animals conformed to current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996). 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. P0 mice were anesthetized on ice. Postnatal mice on P7 to P60 were anesthetized by the injection of the same mixed solution as pregnant mice. Fetuses on E12 were immediately immersed in fixatives containing 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). Fetuses older than E13 and postnatal mice were fixed by transcardial perfusion with the same fixative. The spinal cord, spinal roots, and DRG were removed from postnatal mice on P7 to P60. After immersion in the same fixative overnight at 4 °C, the fetuses, new born mice, and spinal cord with DRG were cryoprotected with 30% sucrose in PB for more than 2 days, and the cervical part was cut into 20 μm-thick transverse sections with 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 rabbit anti-CGRP (1:40,000, C8198, Sigma–Aldrich, St. Louis, MO, USA) (McCarthy et al., 2015) overnight at room temperature. After rinsing three times with PB for 15 min, sections were visualized using the avidin-biotin-peroxidase complex method (Histofine kit; Nichirei, Tokyo, Japan) (Hsu et al., 1981).

Density of the CGRP-positive fibers in the dorsal region, and intensity of CGRP-IL in the motor neurons and DRG neurons were determined as follows. According to the micro-photographs of CGRP immunohistochemistry in the spinal cord of three mice (objective lens, 10×), intensity and density were scored by three of the authors, **JK**, **SK**, and **CT**. Maximum level in the spinal cord during development was determined as "strong positive (+++)", and no-staining was determined as "negative (−)". The staining level between strong positive and negative was divided into two levels; weak positive (+) and moderate positive (++). We calculated the average of three authors' scores.

3. Results

3.1. Developmental localization of CGRP at lower magnification

There was no CGRP-IL in the VH, DH, and DRG on E12 (Fig. 1A). Weak IL was first observed in the motor neurons of the VH on E13 (dashed circle in Fig. 1B), but was negative in the DH and DRG. On E14 (Fig. 1C), weak CGRP-IL was detected in the most dorsal part of the posterior funiculus (PF) and the dorsolateral region of DH. Some large motor neurons were clearly stained in the lateral part of the VH, and some DRG neurons were shown to be CGRP-positive. On E16, dorsal roots, intermediate zone, and PF were moderately stained (Fig. 1D). In the DH, CGRP-positive axon fibers were distributed in the Laminae I and II. In the VH, CGRP-positive motor neurons increased in number and were more densely stained compared with those on E14. In the DRG, many neurons were heavily labeled, and CGRP-positive axon fibers were markedly increased in number. On E18, CGRP-positive sensory axons were more densely stained, and distributed in Lamina I through V in the DH (Fig. 1E). In the VH, CGRP-positive motor neurons were localized at the ventro-medial, ventro-lateral, and dorso-lateral region of the VH. In the DRG, not all but many neurons and their central and peripheral axons were heavily labeled with a CGRP antibody.

On P0, CGRP-IL was also localized in the dorsal roots, dorsal sensory fibers in the laminae I–V of the DH, motor neurons in the VH, and sensory neurons in the DRG (Fig. 2A). The structures stained by CGRP immunohistochemistry were the same as that on E18, and the intensity of CGRP-IL was not obviously decreased (Fig. 1E). On P7, the immunohistochemistry in the DH and PF was not changed, but the CGRP-IL in the motor neurons markedly decreased (Fig. 2B). After P7, immunohistochemical staining did not obviously change (Fig. 2C and D). Many CGRP-positive sensory fibers were detected in dorsal roots, PF, and lamina I through V of the DH, and weak IL was detected in the motor neurons.

3.2. Developmental localization of CGRP in the dorsal part and DRG at higher magnification

3.2.1. Posterior funiculus

On E14, a few CGRP-positive axon bundles were detected between the gracile fasciculus and cuneate fasciculus, which

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