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Regional differences in myelination of chick vestibulocochlear ganglion cells

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ABSTRACT

In vertebrates, vestibular and cochlear ganglion (VG and CG, respectively) cells are bipolar neurons with myelinated axons and perikarya. The time course of the myelination of the VG and CG cells during development of chick embryos was investigated. Chick VG and CG from embryonic day at 7–20 (E7–20) were prepared for a transmission electron microscopy, myelin basic protein immunohistochemistry, and real-time quantitative RT-PCR. In the VG cells, myelination was first observed on the peripheral axons of the ampullar nerves at E10, on the utricular and saccular nerves at E12, and on the lagenar and neglecta nerves at E13. In the VG central axons, myelination was first seen on the ampullar nerves at E11, on the utricular and saccular nerves at E13, and on the lagenar nerves at E13. In the CG cells, the myelination was first observed on the peripheral and central axons at E14. In both VG and CG, myelination was observed on the perikarya at E17. These results suggest that the onset of the axonal myelination on the VG cells occurred earlier than that on the CG cells, whereas the perikaryal myelination occurred at about the same time on the both types of ganglion cells. Moreover, the myelination on the ampullar nerves occurred earlier than that on the utricular and saccular nerves. The myelination on the peripheral axons occurred earlier than that on the central axons of the VG cells, whereas that on the central and peripheral axons of the CG cells occurred at about the same time. The regional differences in myelination in relation to the onset of functional activities in the VG and CG cells are discussed.

1. Introduction

The vestibulocochlear nerve contains mostly afferent fibers connecting the inner ear hair cells to the vestibular and cochlear nuclei in the brainstem. The somata (perikarya) of the fibers are located in vestibular and cochlear ganglion (VG and CG). These ganglion cells are bipolar sensory neurons with two processes (axons) that are not readily distinguishable with exception to their location (Fig. 1). The perikarya of the VG and CG cells are usually surrounded by more than two satellite cells, similar to other types of sensory and autonomic ganglia. Electron microscopic examinations have revealed that the satellite cells wrap the perikarya of the VG and CG cells with multiple layers of myelin (perikaryal myelin sheath) (Endo et al., 1993; Fermin and Cohen, 1984b; Rosenbluth, 1962; Sun et al., 1996). The bipolar perikarya are completely included within one internodal segment of the nerve fiber. The number of myelin lamellae covering the perikaryal internode segment is generally smaller than that covering the remaining internodes along the axonal fibers (axonal myelin sheath). Unlike the axonal myelin sheath, the perikaryal myelin sheath is produced by satellite cells and is composed of the following two types of myelin lamellae: a compact type consisting of lamellae with major dense lines; and a loose type consisting of multiple thin cytoplasmic sheets of satellite cells. It is generally believed that each internode of an axon in the peripheral nervous system is composed of a single Schwann cell, whereas the perikaryal internode may be myelinated by more than one satellite cell (Rosenbluth, 1962).

The formation of the myelin sheathes is an important event in the developmental process, because it is coupled with the onset of the rapid and synchronized axonal conduction. The axonal maturation and myelination influence conduction velocity, such that larger and/or thicker myelinated axons conduct faster impulses (Ritchie, 1982). The inner ear of the chick has one auditory sensory organ known as the basilar papilla and seven vestibular organs, i.e. three crista ampullae, two maculae, one lagena, and one macula neglecta. The vestibulocochlear nerve consists of vestibular and cochlear parts. The vestibular part is further divided into the superior, posterior, and lateral ampullar nerves and the utricular, saccular, and macula neglecta nerves, respectively. The cochlear

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part consists of the cochlear and lagenar nerves. Several studies have reported that axonal myelin first appears in the chick vestibulocochlear nerve at embryonic day 13 (E13; stage 39) and the perikaryal myelin in CG and VG at E17 (stage 43) and E20 (stage 46), respectively (Endo et al., 1993; Fermin and Cohen, 1984b; Kurosawa, 1967). However, Kurosawa (1967) demonstrated that the myelination occurred at E13 only in some nerve fascicles of the vestibulocochlear nerve, while other fascicles remained unmyelinated. These observations indicate that the time course of the myelination in the chick vestibulocochlear nerves has not yet been resolved. Furthermore, because these studies were performed on isolated nerve tissues, it was impossible to distinguish differences between the vestibular and cochlear nerves or between branches of the vestibular nerve.

Tract-tracing studies performed in multiple species have shown that the ganglion cells that innervate individual sensory organs are located in specific regions in the ganglion, and that their central axons enter the brainstem by separate and distinct pathways (Barmack, 2003; Cox and Peusner, 1990; Dickman and Fang, 1996; Highstein and Holstein, 2006). Accordingly, the branches of the vestibulocochlear nerve and their neurons are distinguishable in serial sections of the inner ear.

During the axonal myelination, myelinating cells coordinately regulate the expression of a unique set of genes that are required for synthesis of the myelin sheath (Garbay et al., 2000). One such genes, encoding myelin basic protein (MBP), is thought to play an important role in compaction of the myelin sheath. Since MBP is located on the cytoplasmic surface of the membrane, it plays a role in the formation of the major dense line. MBP has been widely used as a marker for the myelination (Abrahám et al., 2010; Scherer and Arroyo, 2002; Vincze et al., 2008). Thus, MBP immunohistochemistry of serial sections from the chick inner ear can be used to evaluate the myelination of the individual vestibular and cochlear nerves. Another gene, peripheral myelin protein 2 (P2) belonging to a family of fatty acid binding proteins may serve important functions in the generation and maintenance of the unique lipid composition of the myelin membrane (Garbay et al., 2000).

In the present study a transmission electron microscopy, MBP immunohistochemistry, and a real-time quantitative RT-PCR are used to determine the time course and regional differences of the myelination in the VG and CG cells during the development of chick embryos. The relationship between the timing of the myelination and function of VG and CG is discussed.

2. Materials and methods

2.1. Animals

Fertilized White Leghorn chicken eggs (Gallus domesticus) were obtained from a local supplier (Ghen Corporation, Gihu, Japan). Eggs were incubated at 37.5 °C for designated periods of time, and embryos were staged according to Hamburger and Hamilton (1951). A total of 190 embryos ranging from E7 (stage 30) to E20 (stage 46) were analyzed.

2.2. Electron microscopy

VGs and CGs with surrounding tissues were dissected from E8–E20 embryos (stage 34–46; $n = 5$ each). The specimens were immediately fixed in 2.5% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide at 4 °C for 1 h. The specimens were then dehydrated in a graded series of alcohol and embedded in Epoxy resin. For a light microscopy, 1-μm-thick sections were cut and stained with methylene blue. For transmission electron microscopy (Hitachi 7100; Hitachi, Tokyo, Japan), 70- to 80-nm-thick sections were cut and stained with uranyl acetate and lead citrate.

2.3. Immunohistochemistry

E7–E20 embryos (stage 31–46; $n = 5$ each) were removed from eggs and quickly decapitated. The isolated heads (E7–E14) and VGs and CGs (E15–E20) were fixed overnight at 4 °C in Bouin's solution without acetic acid. Fixed tissues were processed and embedded in paraffin according to standard procedures (Shirasawa et al., 2006). Serial coronal sections (7-μm thick) were prepared and mounted onto glass slides. The sections were immunohistochemically stained using avidin–biotin–peroxidase and double-labeling methods. Briefly, after deparaffinization in xylene and rehydration in a graded series of ethanol to water, the sections were treated with 0.3% H_2O_2 in methanol for 15 min to quench endogenous peroxidase activity. The sections were washed three times for 5 min each in phosphate-buffered saline (PBS), and then nonspecific binding sites were blocked by incubating for 1 h at room temperature in 10% normal serum from the species in which the secondary antibody was generated. After a brief rinse with PBS, sections were incubated overnight at 4 °C with the primary antibody. The primary and secondary antibodies were diluted in DAKO Antibody Diluent (Dako, Carpinteria, CA, USA). The following primary antibodies were used: rat monoclonal anti-MBP (1:1000; Chemicon Millipore, Billerica, MA, USA), and mouse monoclonal anti-neurofilament (NF, 1:2000; Chemicon Millipore). After extensive washing in PBS, the sections were incubated in a biotinylated anti-rat or anti-mouse secondary antibody (1:500; Dako) for 1 h at room temperature, followed by incubation in a streptavidin/peroxidase complex solution (Dako) for 30 min at room temperature. The sections were washed three times for 5 min in PBS and transferred to a Tris–HCl buffer (pH 7.4). Peroxidase activity was visualized using 0.002% 3,3′-diaminobenzidine tetrahydrochloride, 1% H_2O_2, and 1 mM ammonium nickel sulfate hexahydrate in a Tris–HCl buffer (pH 7.4). In order to ensure the consistency of staining-efficiency, the immunohistochemistry for all the stages was performed together and stopped at the same time. Sections were dehydrated through a graded series of ethanol, cleared in xylene, and cover-slipped. For double-labeling experiments, rat anti-MBP and mouse anti-NF were used as described above for the primary antibodies, and Alexa Fluor 488 goat anti-rat IgG and Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, Eugene, OR, USA) were used as the secondary antibodies. Sections were viewed on a Leica microscope (Leica, Tokyo, Japan) and photographed using a digital camera (Olympus DP70, Olympus Opt. Co., Tokyo, Japan). As a negative control, nearly adjacent sections were incubated without the primary antibody. No labeling was confirmed in these controls.

2.4. Estimation of mRNA levels by SYBR Green-based real-time quantitative RT-PCR

Total RNAs were prepared from the VG and CG of chick embryo (E10–E20, $n = 10$) by an RNA Isolation Kit (RNAspin mini, GE Healthcare UK Ltd., Buckinghamshire, UK) which included a genomic DNA digestive step by DNase I treatment. Expressions of MBP and P2 mRNAs during embryo development were determined by real-time RT-PCR. Total RNA (0.5 μg) was converted into cDNA by reverse transcription using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, San Mateo, CA, USA). Primer sequences used were as follows: MBP (GenBank accession no. NM_205280) forward: 5′-AGGACTGTCCCTCACCAGATTTAG-3′; reverse: 5′-CTTGTGAGCAGATTTGTGCTCATA-3′, P2 (GenBank accession no. XM_418309) forward: 5′-ATGTGTAACCGATTTGTGGGAACC-3′; reverse: 5′-TTTCCTGGTCGCTAAGCCCACACC-3′, and beta-actin (GenBank accession no. NM_205518) forward: 5′-ATGATGATATTGCTGCGCTCG-3′; reverse: 5′-ACCAACCATCACACCCTGATG-3′. Real-time PCR was performed in the Applied Biosystems 7500 Fast Real-Time PCR System with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. PCR conditions were 10 min at 95 °C, with 40 cycles of denaturation for 15 s at 95 °C, and an annealing-extension for 60 s at 60 °C. The relative mRNA expression of MBP and P2 at each stage was determined using the comparative threshold cycle method and normalizing the levels to those of action mRNA (Livak and Schmittgen, 2001). Experiments were performed in triplicate for each set of genes. The data were statistically analyzed by one-way ANOVA followed by the Bonferroni/Dunn test using StatView software (Hulinks, Inc., Tokyo, Japan). The p value was set at either <0.05 or <0.01.

3. Results

3.1. Overview of the VG and CG cells

For orientation purposes, the NF marker was used to establish a profile of the VG and CG development in the chick's inner ear at E12 (stage 38, Fig. 1). The chick's inner ear contains a single auditory sensory organ known as the basilar papilla and seven vestibular organs including three crista ampullae, two maculae, one lagene, and one macula neglecta. The lagena is located on the apical end of the cochlear duct. The lagenar ganglion and CG develop from a singular initial ganglion that divides at this stage. In NF-labeled coronal sections of the inner ear, the major features of VG and CG are apparent at five anteroposterior levels (Fig. 1A–E).

At level 1, the section was taken through the cochlear duct, and CG and lagenar ganglion were observed (Fig. 1A). The cochlear duct contained two distinct sensory end organs, the basilar papilla and lagena. Basilar papilla occupied most of the length of the cochlear