

A study on the immunocytochemical localization of neurofascin in rat sciatic nerve

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We examined the localization of neurofascin (NF) in the sciatic nerve of rat. In the myelinated fibers, neurofascin localizes strongly in the nodal axolemma except the small central cleft and also expresses in the paranodes, and weakly in the Schmidt-Lanterman incisures. In the paranodes, NF localizes around the axolemma and it expresses in the apposing membrane of paranodal loops. Axoplasm, compact myelin and cytoplasm of Schwann cell do not express NF at all. In the Schmidt-Lanterman incisures, NF is expressed weakly along the Schwann cell membrane. We propose that neurofascin may be a plasmalemmal integral protein of Schwann cell in the paranode and plays some important roles for the maintenance of axo-glial junctions at the paranode. It may also have some roles for maintaining the structure of Schmidt-Lanterman incisure and have some relations with proteins localizing in the node.

Key words: neurofascin, axo-glial junction, Schmidt-Lanterman incisure, immunocytochemistry

Introduction

Neurofascin is an axon-associated member of the L1 subgroup of the immunoglobulin superfamily that is implicated in the processes during the development of the nervous system such as cell adhesion, cell migration, neurite outgrowth, and fasciculation [3, 10, 11, 14, 20]. Neurofascin is concentrated in developing fiber tracts at early stages of development [15, 17]. At later stages of development, neurofascin is more widely expressed in the nervous system [13]. Neurofascin is characterized by extracellular domains comprised of 6 immunoglobulin domains and 4 fibronectin type III domains and cytoplasmic domains containing an ankyrin-binding site localized to a highly

conserved stretch of amino acids. The intracellular segment of neurofascin as well as those of other members of the L1 subgroup interact with the cytoskeletal component ankyrin [4, 6, 7].

To elucidate the exact localization of neurofascin in the peripheral nerves is important to clarify the function of this molecule. In this report we have examined the ultrastructural localization of neurofascin in rat sciatic nerve, and we got some important findings of NF localization in the node, paranode, and Schmidt-Lanterman incisure. From our investigations we suppose there are some related functions with its specific localization in the peripheral nerves.

Materials and Methods

Animals

Twenty Sprague-Dawley rats (Daehan Lab. Animal Res. Center, Korea), 5 to 8 weeks old and weighing 150-200 gm, were provided with basal diet and tap water *ad libitum* during the experiment.

Immunofluorescence

Animals were anesthetised by inhalation of ether and perfused with 4% paraformaldehyde. Sciatic nerves were exposed in the upper thigh level and excised nerves were fixed with the same fixative for 2 hours at room temperature (RT). Nerves for longitudinal and cross sections were washed 3 × 15 min with PBS and treated with 5%, 10%, and 25% sucrose and embedded with OCT compound (Sakura Fine Tech., Japan). Ten µm sections were collected on TESPA (3-aminopropyltriethoxy-silane; Sigma-Aldrich, Korea) coated slides and allowed to dry for 2 hours at RT. Nerves for teased fiber were washed 4 × 15 min with PBS. Teased fibers were prepared by separating each sciatic nerve fiber with acupuncture needles. Teasing procedure was performed after soaking in the solution of 0.1% Triton X-100 for 3 hours after removing epineurium.

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Nerves were washed with PBS and blocked for 1 hour with 10% goat serum in 0.2% gelatin, 0.3% Triton X-100 in PBS (buffer A). Nerves were incubated overnight with 1 : 4000 goat anti-rabbit neurofascin (from Dr. Brophy, Univ. of Edinburgh) diluted in 4% goat serum, buffer A and washed 3×20 min with buffer A. Nerves were incubated in 1 : 200 goat anti-rabbit FITC (Vector, U.S.A) diluted in buffer A for 3 hours at RT and washed 4×5 min with PBS. After draining off most of PBS and coverslipped with Vectashield.

Immunoelectron microscopy

Animals were anesthetised by inhalation of ether and perfused with a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde. Each side of sciatic nerve was cut and fixed for 3 hours with the same fixatives. Fixed tissues were washed 3 times with 0.1 M phosphate buffer and dehydrated for 2 min with 30%, 50%, 70%, 90%, and 100% ethanol respectively. After infiltrating with a mixture of LR gold and ethanol, tissues were embedded with LR gold at -25°C under ultraviolet lamp. Thin sections were cut with ultramicrotome and collected on Formvar coated nickel grid and dried. Sections were blocked with PBS-Milk-Tween (0.1M PBS, 0.2% milk, 0.1% Tween 20) for 30 min and incubated with 1 : 200 goat anti-rabbit neurofascin for 12 hours at 4°C . After washing with PBS-BSA-Tween (0.1M PBS, 0.2% BSA, 0.1% Tween 20), sections were incubated with 1 : 50 15 nm gold particles conjugated with goat anti-rabbit IgG (British Biocell International, U.K.) for 2 hours at RT. Sections were washed with PB-Tween (0.1 M phosphate buffer, 0.1% Tween 20), and then fixed with 2.5% glutaraldehyde for 15 min, and stained with uranyl acetate-lead citrate and observed with JEOL 1200 EXII TEM under 60 Kv.

Results

Immunofluorescence

Strong expression of neurofascin was detected throughout the nerve fibers intermittently in both longitudinal sections and teased fibers of sciatic nerve (Fig 1A & 1B). This strong expression of NF was defined around the axonal circumference and their staining areas look like slender rectangular appearance with a small central cleft. With electron microscopical immunocytochemistry, the rectangular area was identified to be the node and paranode. Although nodes are stained strongly with NF, there is a small unstained central cleft in the node. Paranodes are stained strongly as well, but the strength of immunoreaction toward the internode was getting weaker. In addition to the strong immunoreactive regions of nodes and paranodes, very many weak expression sites of NF were also detected in the longitudinal sections and teased fibers. These

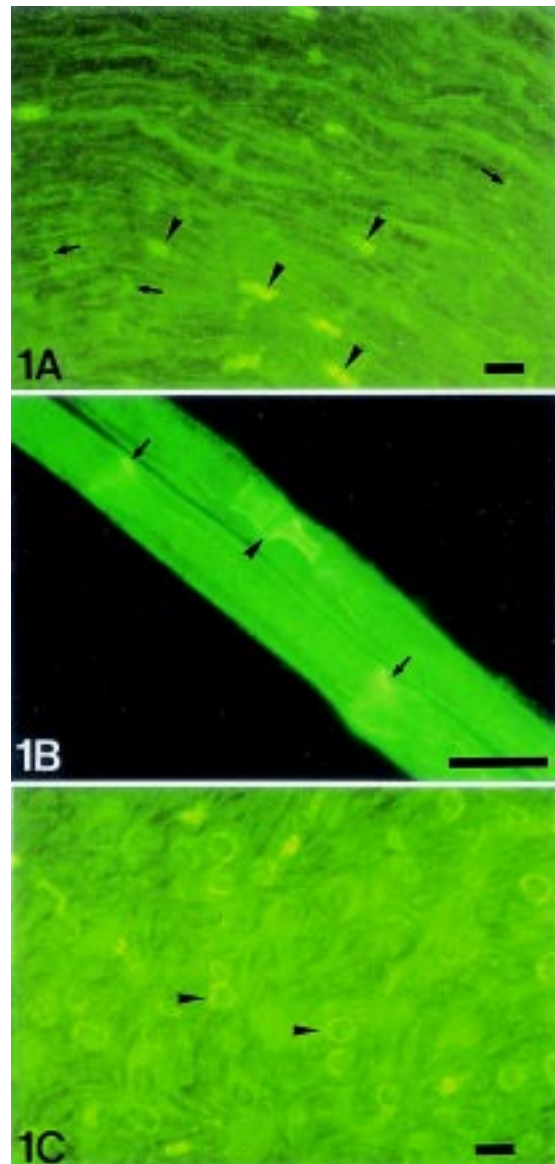


Fig 1. Immunofluorescence of NF in 8 weeks old rat sciatic nerve. (A) Longitudinal sectioned. NF is expressed in the nodes and paranodes (arrow heads) strongly and Schmidt-Lanterman incisures (arrows) weakly. (B) Teased fibers. A node of Ranvier and 2 Schmidt-Lanterman incisures are shown. The node and paranode (arrow head) express NF strongly except the small central cleft. Schmidt-Lanterman incisures (arrows) also express NF. (C) Cross sectioned. NF is expressed around the axonal circumference (arrow heads) strongly. Bar = $10\ \mu\text{m}$

narrow regions of NF expression were scattered throughout the internode and these structures were turned out to be the Schmidt-Lanterman incisures. In the cross sections, NF immunoreaction was clearly expressed around the axonal circumference and no immunoreaction were detected in axoplasm and compact myelin layers (Fig 1C).

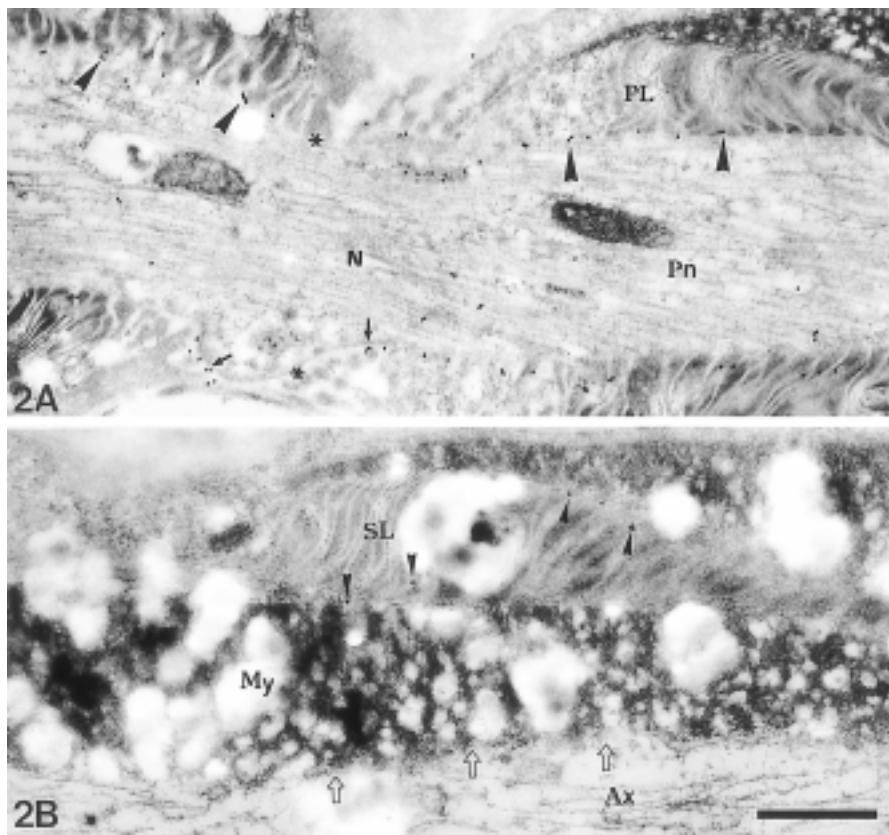


Fig 2. Post-embedding immunoelectron microscopy of NF expression in 8 weeks old rat sciatic nerve. (A) Node (N) and paranode (Pn). NF immunoreactive gold particles are localized in the nodal axolemma (arrows) except the small central cleft (asterisks). NF expression was revealed in the paranodal loops (PL; large arrow heads). (B) Internode. NF immunoreactive gold particles were localized only in the Schmidt-Lanterman incisures (SL; small arrow heads). There is no NF expression at the axolemma of internode (broken arrows). My; myelin sheath. Ax; axon. Bar = 500 nm

Immunoelectron microscopy

With electron microscopy, we identified NF immunoreactive gold particles were expressed in the nodal axolemma except a small central cleft of nodes (Fig 2A). NF immunoreactive gold particles were also detected in the membranes of paranodal loops as well. Besides the nodes and paranodes, NF immunoreactive gold particles were seen in the Schmidt-Lanterman incisures weakly (Fig 2B). There was no immunoreactive gold particles found in axoplasm, compact myelin, and internodal axolemma.

Discussion

Cell adhesion molecules (CAMs) play an important role in both the initiation and signaling of axon-glial contact. Among them, neurofascin is a chick neurite-associated surface glycoprotein implicated in axon extension [15] and this molecule is a powerful candidate for recognizing the axons that they ensheath during the development. In the CNS, neurofascin is strongly but transiently up-regulated in oligodendrocytes at the onset of myelinogenesis. After the initial surge of neurofascin expression in oligoden-

drocytes, there is a shift to a predominantly neuronal localization that persists into adulthood [1].

Neurofascin in adult rat brain includes polypeptides of 186 kD and 155 kD and a minor form of 140 kD confined to the cerebellum. Antibody that recognized 186, 155, and 140 kD neurofascin cross-react strongly with the node of Ranvier. Immunoblots of sciatic nerve revealed the 155 kD polypeptide as the major form of neurofascin, and thus a candidate for the isoform of neurofascin at the node of Ranvier [6].

In this report we describe the localization of neurofascin which recognizing 155 kD and 186 kD polypeptides in the sciatic nerve of rat. Davis et al. [5] reported the 186 kD neurofascin is the major form in adult brain and is present at specialized membrane domains including nodes of Ranvier and axonal initial segments, and an alternative form of 155 kD neurofascin localizes in paranodal region. In this study we identified the NF localizes strongly in the nodes and paranodal loops and it is in consistent with the result of Davis et al. [5]. We have also identified the NF was expressed in the nodal axolemma except the small central cleft. This data is not completely in agreement with

the result of Davis *et al.* [5]. They investigated the NF localization with immunofluorescence. In this report we also identified the small non-immunoreactive areas of NF in the central zone of the nodes. These two findings seem to be not very different in immunofluorescence, but the existence of NF at the large area of nodal axolemma in this study was obviously elucidated with post-embedding immunoelectron microscopy. From the findings of this study we suggest NF in the nodal axolemma may have some relations with other molecules localizing in the node, like ankyrin and voltage-dependent sodium channels.

NF immunoreaction in this study was strong in the paranodes and many immunoreactive gold particles localizes in the Schwann cell membrane of paranodal loops. This finding is in agreement with the study of Tait *et al.* [19] and it suggests NF may be a component of Schwann cell membrane protein and it is likely to interact with some axonal membrane proteins in the paranode.

We have firstly identified the NF was expressed in the Schmidt-Lanterman incisures, which are spirals of cytoplasm inserted between lamellae of the myelin sheath connecting the inner and outer layers of Schwann cell or oligodendroglial cytoplasm. Numerous investigations of normal and pathological peripheral nerve have focused on the Schmidt-Lanterman incisures [2, 8, 12, 16, 18], yet their precise role has not been determined. Ghabriel and Allt [8] suggested the possible roles of Schmidt-Lanterman incisure are metabolic maintenance of the myelin sheath, transport of metabolites through the sheath to the axon, a mechanism providing for longitudinal growth of myelin segments, and contribution to peristaltic movement of axoplasm. According to the suggestion of the metabolic functions of Schmidt-Lanterman incisure, NF may have some roles to maintain the myelin structures. We suggest the localization of NF in the Schmidt-Lanterman incisures may be important to stabilize the apposed Schwann cell membranes at the incisures and paranodes. And it may also be interesting to reveal the presence of incisures during the earliest stages of myelination and the initial expression of neurofascin in the incisure.

The cytoplasmic domains of neurofascin contain highly conserved region that associates with the membrane-skeletal protein ankyrin [4, 6, 7, 9]. In this study, we report the existence of neurofascin in the node, and then it may explain neurofascin has some relation with some other molecules including ankyrin, spectrin, voltage-dependent sodium channels, which are mainly localized in the node.

We examined there is no evidence of NF staining in the axoplasm, compact myelin, and Schwann cell cytoplasm, so it is obvious that neurofascin is not a constituent of myelin and has not any important roles in the axoplasm and cytoplasm of Schwann cell.

From these investigations of NF localization in rat sciatic nerve, we propose that neurofascin may be a

plasmalemmal integral protein of Schwann cell in the paranode and plays some important roles for the maintenance of axo-glial junctions at the paranode. It may also have some roles for maintaining the structure of Schmidt-Lanterman incisure and have some relations with proteins localizing in the node. Further study about the initial expression of neurofascin in peripheral nerves will be necessary to identify further the roles of this molecule in peripheral nerves.

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