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Electron Tomographic Analysis of Cytoskeletal Cross-Bridges in the Paranodal Region of the Node of Ranvier in Peripheral Nerves

Guy A. Perkins¹, Gina E. Sosinsky^{1,2}, Sassan Ghassemzadeh¹, Alex Perez¹, Ying Jones¹, and Mark H. Ellisman^{1,2}

¹ National Center for Microscopy and Imaging Research, Center for Research in Biological Systems, University of California, San Diego 92093-0608

² Dept. of Neurosciences, University of California, San Diego 92093-0608

Abstract

The node of Ranvier is a site for ionic conductances along myelinated nerves and governs the saltatory transmission of action potentials. Defects in the cross-bridging and spacing of the cytoskeleton is a prominent pathologic feature in diseases of the peripheral nerve. Electron tomography was used to examine cytoskeletal-cytoskeletal, membrane-cytoskeletal, and heterologous cell connections in the paranodal region of the node of Ranvier in peripheral nerves. Focal attachment of cytoskeletal filaments to each other and to the axolemma and paranodal membranes of the Schwann cell via narrow cross-bridges was visualized in both neuronal and glial cytoplasm. A subset of intermediate filaments associates with the cytoplasmic surfaces of supramolecular complexes of transmembrane structures that are presumed to include known and unknown junctional proteins. Mitochondria were linked to both microtubules and neurofilaments in the axoplasm and to neighboring smooth endoplasmic reticulum by narrow cross-bridges. Tubular cisternae in the glial cytoplasm were also linked to the paranodal glial cytoplasmic loop juxtanodal membrane by short cross-bridges. In the extracellular matrix between axon and Schwann cell, junctional bridges formed long cylinders inking the two membranes. Interactions between cytoskeleton, membranes, and extracellular matrix associations in the paranodal region is likely critical not only for scaffolding, but also for intracellular and extracellular communication.

Keywords

cross-bridge; cytoskeleton; electron tomography; freeze substitution; high-pressure freezing; microfilaments; microtubules; neurofilaments; node of Ranvier; paranodal junctional bridge; paranode

1. Introduction

Node of Ranvier complexes are heterologous cellular specializations containing components from both neuron and glial cells. These nodes are found in both the central nervous (CNS) and peripheral nervous systems (PNS). The node of Ranvier aids the amplification and regeneration

Contact Person: Guy Perkins, National Center for Microscopy and Imaging Research, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0608, 858-534-7968 voice, 858-534-7497 fax.

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of action potentials in axons by facilitating segregation of ion channels into discrete domains and enabling the saltatory propagation of large ionic currents (Ellisman et al., 1982). Saltatory conduction permits a rapid propagation of action potentials without an increase in axonal bore width. The overall structure of the node of Ranvier appears to be highly developed in part to contain large ionic fluxes to compartmentalized domains. The formation of the node of Ranvier requires specific contacts between axons and the surrounding glial cells, generating specialized membrane sub-domains with distinct compositions of ion channels (Bonnon et al., 2007; Sosinsky et al., 2005). Much of the node's complexity stems from it being a unique heterologous system, requiring close interaction between two or more distinct cell types.

The term “node of Ranvier complex” often refers not only to the nodal gap that contains no myelin, but also to the paranodal regions with its uncompacted cytoplasm-containing terminal myelin loops. The nodal gap is bracketed by the paranodes and defined as the region in which the cytoplasm-encompassing margins of the uncompacted myelin lamellae abut the axon. The glial margin wraps around the paranodal axolemma in a helical fashion. Both intracellular and intercellular bridging or junctional molecules are abundant in the paranodal and juxtaparanodal regions (Ichimura and Ellisman, 1991). Macromolecular junctional complexes, form between the axolemma to the spiral paranodal loops. Another role of these junctions is to act as barriers to separate the sodium and potassium channels into the nodal, paranodal, and juxtaparanodal membranes where they are needed (Ellisman and Levinson, 1982; Mi et al., 1995; Mi et al., 1996; Mi et al., 1999; Rasband, 2004; Trimmer and Rhodes, 2004).

The three types of cytoskeletal filaments prevalent in the PNS are intermediate filaments (both glial filaments and neurofilaments), microtubules, and microfilaments. Neurofilaments are type IV intermediate filaments and are numerous in the axons of vertebrate neurons (Karp, 2002). They measure between 9-11 nm in diameter when assembled. These filaments are often arranged as loosely packed bundles oriented parallel to the axon length and can be extensively cross-linked (Nakagawa et al., 1995). The extent of neurofilament gene expression is correlated to axonal bore diameter, which controls the longitudinal resistance of the fiber, influencing the spacing between nodes of Ranvier and thereby how fast electrical signals traverse the axon (Lee and Cleveland, 1996; Marszalek et al., 1996). Microtubules are the “highways” of transport for materials moving rapidly within the axons of neurons (Li et al., 2004) and have a diameter of about 24 nm; they vary in length from several hundred nanometers to hundreds of micrometers in axons. Microfilaments, composed of F-actin, and found in the cytoplasm of the paranodal loops (Ichimura and Ellisman, 1991; Scherer, 1996; Trapp et al., 1989) measure about 7 nm in diameter and are likely also involved with transport. In addition, all three filament types seem to function as mechanical stabilizers of the cytoplasm to resist mechanical stress (Fuchs and Cleveland, 1998; Garcia and Cleveland, 2001).

Associated with the axon of the PNS, the glial Schwann cell provides myelin at the internode, lateral loops at the paranode, and lamellipodia (also called microvilli) that abut the axon at the node (Ellisman and Porter, 1980; Ellisman et al., 1987). Between the loops of the paranode and the axolemma in the extracellular matrix are linear arrays of heterogeneously sized particles of 8-16 nm in diameter that bridge this space (Ellisman, 1979; Wiley and Ellisman, 1980; Wiley-Livingston and Ellisman, 1981); we name these paranodal junctional bridges (**PJB**). They have also been called “septate junctions” or “septate-like junctions” (Ichimura and Ellisman, 1991; Sosinsky et al., 2005; Spiegel and Peles, 2002). These structures, which appear continuous, are comprised of heterologous macromolecules and appear to insert into both the glial and axonal plasma membranes.

The proteins in the PJB that link the loop membranes with the axolemma form a complex of heterodimers consisting of neurofascin 155 and caspr/paranodin (Boyle et al., 2001; Poliak et al., 2001; Scherer and Arroyo, 2002; Tait et al., 2000). In a unique heterologous handshake,

the glial cell contributes neurofascin 155 and the neuronal cell provides caspr. Contactin, residing in the axonal membrane, brings the other proteins to the handshake. Deficiency in either caspr, contactin, or neurofascin 155 produces a disorganization of the paranodal loops and a reduction in axon conduction velocity (Bhat et al., 2001; Bonnon et al., 2007; Boyle et al., 2001; Sherman et al., 2005). At the nodal/paranodal region in the myelinated axons of sciatic nerves of mice deficient in caspr, PJBs were missing and abnormal, swollen mitochondria were observed (Einheber et al., 2006). In contrast, mitochondria located in the axoplasm outside this region appeared normal, suggesting that the PJB influences mitochondrial function and perhaps transport in the axoplasm of the nodal/paranodal region of myelinated axons of peripheral nerves. The distribution of neurofascin 155 in the glial membrane is altered in one form of multiple sclerosis (MS), a demyelinating disease (Maier et al., 2007). This group discovered that efficient association of neurofascin 155 with lipid raft domains is required for the maintenance of the paranodal axo-glial junction and without this junction, demyelination occurs in MS. The PJB complex was seen previously in images of deep-etched rotary-shadowed preparations and described as 16-nm “pegs” that line up in ordered arrays (Ellisman, 1979; Ichimura and Ellisman, 1991; Wiley and Ellisman, 1980; Wiley-Livingston and Ellisman, 1981). In our reconstructions, the PJBs were only occasionally observed as discrete pegs, but more often formed a continuous rod of density. This appearance conforms to the role described for PJBs as septate-like junctions that serve in a paracellular capacity to block the diffusion of ions and channels between the paranodal and juxtaparanodal areas (Rios et al., 2003; Salzer, 2003).

This study extends the earlier work of Sosinsky and co-workers (Sosinsky et al., 2005), who provided a broad structural and modeling framework of the node from electron tomography structure determination. Here, we examine in greater detail the cross-bridges connecting cytoskeleton, membranes, and adjacent neuronal and glial cells in the paranodal region of the PNS. This is the first report of electron tomography being used to characterize these cross-bridges. The filamentous or spanning nature of the bridges, their focal attachment, and their association with membrane particles were visualized in three dimensions in tomographic reconstructions. We found that at the paranode, filaments extend from the linear array of PJBs into both loops and axoplasm thus forming an apparent filamentous link of the cytoskeleton in the paranodal loop with that in the axon. Membrane-cytoskeleton cross-bridges, sometimes connecting to the PJBs, were also found. The cross-bridges we have measured have thicknesses in the intermediate filament and microfilament range and also function to tether cytoplasmic organelles such as mitochondria and tubular cisternae (TC) and other membranous organelles such as autophagosomes and lysosomes to cytoskeleton or membrane. Combination of the 3D information contained in tomographic volumes support the construction of a model that include filaments with specialized orientations and attachments to axonal and glial components via short cross-bridges.

2. Materials and Methods

Conventional preservation with aldehyde-fixation of PNS samples from rat was accomplished as described previously (Perkins et al., 2001). Conventional electron microscopy of thin sections (~80 nm thick) was performed with a JEOL 1200 EX microscope operated at 80 kV.

2.1 High Pressure Freezing

The procedures for high pressure freezing of chemically fixed tissue followed by freeze substitution follow that described in detail in Sosinsky et al., (2008). Rats were first anaesthetized by intraperitoneal injection of Nembutal (35 mg per kg body weight), and whole blood was flushed out with oxygenated rat Ringers solution at 37° C. They were then fixed by intracardial perfusion with an aldehyde mixture composed of 2% paraformaldehyde and 2.5%

glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) at 37° C. The spinal root was dissected out, immersed in the same fixative and transferred to the high-pressure freezing (HPF) unit. These samples were trimmed and blotted (to remove excess water) before placing in the carrier. Samples were loaded into the carriers of the Bal-Tec HPM010 HPF unit (Bal-Tec, Liechtenstein) and operated according to manufacturers instructions. Carriers with a depth of 0.2 mm were used with thin bundles of spinal root to provide uniform ultrastructural preservation. The inert liquid, hexadecene, was added to the carrier to fill in the well, excluding air from surrounding the sample.

2.2 Freeze Substitution

After the samples were high-pressure frozen, they were freeze-substituted in dry acetone containing 0.1% tannic acid for 24 hrs at -90° C in a Leica EMASFS freeze substitution unit (FSU, Leica Microsystems, Bannockburn, IL), then in acetone containing 2% OsO₄ and 0.1% of uranyl acetate for the remaining time of the procedure. Both tannic acid and uranyl acetate were added not only to help stain the material but also to aid in membrane preservation. During this procedure, the water in the sample is completely extracted and replaced by the fixative, i.e., freeze-substituted. The temperature for substitution was maintained at -90° C for 3 days to ensure that the biological structures were cryo-immobilized during this substitution. The temperature was raised gradually at a rate of 2° C per hour to -60° C and held there for 8 hours because OsO₄ does not start to cross-link until around -70° C. The samples were then warmed to -20° C at a rate of 5° C per hour and held at this temperature for 12 hours. The carriers were then taken from the FSU, the tissue removed from the carriers and quickly washed several times in dry acetone at room temperature (RT) to remove the OsO₄ and uranyl acetate. Acetone above 70% can extract lipids at RT. Hence, the exposure time to it was minimized. The tissues were then incubated in a 50:50 mixture of acetone:Durcupan for 4 hour at RT followed by 100% Durcupan resin of 24 hrs infiltration with 3 changes. Resin polymerization was accomplished in a vacuum oven at 65-70 °C for 48 hours. The resin-embedded samples were removed from the oven and sections were cut using a Leica ultramicrotome. Sections were then stained 30 min in 2% aqueous uranyl acetate, followed by 15 min in lead salts. Fiducial cues consisting of 15 and 20 nm colloidal gold particles were deposited on opposite sides of the section.

2.3 Electron Tomography

Two tomograms from HPF/FS samples (both single-tilt) and two tomograms from conventionally prepared samples (1 single-tilt, 1 double-tilt) were analyzed. For each reconstruction, a series of images at regular tilt increments was collected with a JEOL 4000EX intermediate-voltage electron microscope (JEOL USA, Peabody, MA) operated at 400 kV. The specimens were irradiated before initiating a tilt series in order to limit anisotropic specimen thinning during image collection. Tilt series were recorded on film at 20,000 times magnification with an angular increment of 2° from -60° to +60° about an axis perpendicular to the optical axis of the microscope using a computer-controlled goniometer to increment accurately the angular steps. For the double-tilt series, the grid was subsequently rotated 90° and a second tilt series was collected with the same angular increment and range. The illumination was held to near parallel beam conditions and optical density maintained constant by varying the exposure time. The negatives were digitized with a Nikon CoolScan at 1800 dpi producing images of size 4033 × 6010 pixels. The final pixel size in our reconstructions was 1.41 nm. The IMOD package (Kremer et al., 1996) was used for rough alignment with the fine alignment and reconstruction performed using the TxBR package (Lawrence et al., 2006). One of the HPF/FS single-tilt series reconstructions (310 nm thickness) had a (global) resolution estimated by using the even/odd Fourier shell correlation (Cardone et al., 2005) and software provided by Alasdair Steven (NIH) to be 8.4 nm at a threshold of 0.3; the other two single-tilt reconstructions analyzed likely had similar resolution estimates because they had

similar thicknesses and angular increment and range. The double-tilt series reconstruction (215 nm thickness) had a resolution estimated to be 4.6 nm by the same criterion. Volume segmentation was performed by manual tracing in the planes of highest resolution with the program Xvoxtrace (Perkins et al., 1997b). The reconstructions were visualized using Analyze (Mayo Foundation, Rochester, MN) or the surface-rendering graphics of Synu (Hessler et al., 1996). These programs allow one to step through slices of the reconstruction in any orientation and to track or model features of interest in three dimensions. Measurements of structural features were made using NIH ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2004.) Movies of surface-rendered volumes and slices through the reconstructions were made using Amira (Mercury/TGS, San Diego, CA). For the averages shown in Fig. 3, we had to first select for cross-bridges of each type that appeared straight, not kinked along their lengths. Subvolumes of similar dimension were cut out and aligned together in 3D using Analyze. After alignment, multiple subvolumes were averaged using EMAN (Schmid and Booth, 2008; Tang et al., 2007) to increase the signal-to-noise ratio, enabling visualization of greater structural details.

3. Results

Electron tomography coupled with preservation by high-pressure freezing and freeze-substitution has enabled additional resolution of structural details in the paranodal region. This preparation has an advantage in preserving the fine structure of fluid space occupied by an aqueous environment, since the water molecules are replaced by substitution fluid while they are in the frozen state. Shrinkage and warping of membranes and cytoskeleton during the dehydration process are minimized.

3.1 Overall organization of the paranode

The paranodal region is rich in juxtapositions of membrane-bounded structures of the Schwann cell cytoplasm and neuronal axoplasm and includes linkages of cytoskeletons, organelles, with numerous examples of cross-bridges between them (Fig. 1). In the Schwann cell cytoplasm and the axoplasm there are extensive cytoskeletal networks and numerous organelles such as mitochondria, endoplasmic reticulum, autophagosomes, lysosomes, and tubular and cisternae that are linked to the cytoskeleton, sometimes forming anatomosing networks. The mitochondria in the axoplasm appear different from those normally seen in tissues because they are in the condensed state (Hackenbrock, 1966), a structural configuration that is now starting to become more recognized in the axoplasm far from somas and terminals (Perkins et al., unpublished results). Condensed mitochondria have a dark matrix that appears like a long twisted sausage in 3D and are thought to be indicative of greater ATP production. The crista junction (Frey and Mannella, 2000; Mannella, 2006; Perkins et al., 1997a) may be either a narrow tube or an elongated opening (Renken et al., 2002).

The use of electron tomography revealed a variety of cross-bridges (Fig. 2), including the presence of arrays of transcellular junctions, PJBs, traversing the paranodal glial-axonal juxtaposition (Fig. 2a, a'). This juxtaposition is constituted by the terminal cytoplasmic loops of the myelin forming Schwann cell, the extracellular matrix studded with septate junction-like structures that span the space between the glial and axonal membranes, and the axolemma. The PJBs not only span the cleft between cells, but also appear to insert into both glial and axonal membranes (Fig. 2a, h, a', h'). Tubular cisternae (TC) consist of tubules of smooth endoplasmic reticulum of various diameters and are closely associated with juxtapositional membranes in the glial loops. Filamentous cross-bridges of diameter similar to F-actin linking the TC to the loop membrane were revealed in tomographic volumes (Fig. 2b, c, b', c'). Focal attachments of cytoskeletal filaments to the paranodal membranes were visualized in both

cytoplasm of the glial loops and axon (Fig. 2d, d'). A subset of these filaments attaches to the transmembrane PJB (Fig. 2a').

Organelles are also connected to the cytoskeleton and occasionally to each other in the axoplasm. Mitochondria were seen connected by short tethers to neurofilaments (NF) (Fig. 2e, e') or microtubules (MT) (Fig. 2f, f'). These tethers had a diameter similar to that of neurofilaments. These filaments may be comprised of desmin or keratin (Toivola et al., 2005), which attach to mitochondria. Mitochondrial-ER cross-bridges were also observed (Fig. 2g, g'). Multiple cross-bridges of the same diameter and similar lengths were found connecting the two organelles. Microfilaments in the glial cytoplasm running more-or-less perpendicular to the filaments and microtubules in the axoplasm were found in the sub-volume closest to the juxtapositional loop membrane (Fig. 2h, h'). These actin filaments did not appear bundled, nor did they appear to have cross-bridges with each other, other cytoskeleton, or membranes. Cross-bridges are also common between neurofilaments (Fig. 2i, i') and between neurofilaments and microtubules (Fig. 2j, k, j', k'). These cross-bridges have a diameter about the same as neurofilaments. We noticed that a small subset of neurofilaments appear to terminate directly at the axolemma, including at the PJBs (data not shown).

Averages of tomographic subvolumes of cross-bridges enhanced the contrast of the sometimes faint individual examples of such bridges (Fig. 3). These averages have an increased signal-to-noise ratio, enabling visualization of greater structural details. A general difference in cross-bridge diameter between the NF-NF, NF-MT, and mito-MT averages on one hand and the NF-Axl and TC-PNL averages on the other hand is noticeable. In addition, a variation in diameter along the length of each bridge is evident, which might reflect variability in stain deposition.

3.1 Analysis of cytoskeletal elements in the paranodal region

Figure 4 is a 3D model that incorporates the structural information obtained in the present study. The associations of the major cytoskeletal components are shown: neurofilaments, glial filaments, microtubules, microfilaments, and cross-bridges of various sizes (Fig. 4a). The cross-bridges that connect neurofilaments to each other or to microtubules are few and far between. We found only one or two such bridges per connecting cytoskeletal components (Fig. 4b). This limited connectivity was also seen with the cross-bridging of neurofilaments to the axolemma. The PJBs are seen to be rod-like structures (Fig. 4c) of uneven lengths (mean $\pm$ std dev=45 $\pm$ 34 nm; range:16-128 nm) and arranged in nearly parallel arrays separated by remarkably uniform spacing (HPF/FS samples: mean $\pm$ std=19 $\pm$ 4 nm; conventional samples: mean $\pm$ std=21 $\pm$ 3 nm). The focal attachment of axoplasmic neurofilaments to the PJBs appears to be complemented by a similar attachment of glial cytoskeletal filaments on the glial side. Occasionally, instead of being connected to the axolemma via cross-bridges, neurofilaments would connect by terminating at this membrane, often at the site of a PJB (Fig. 4d). This site-specific link is suggestive of intercellular association between axon and glia. Tubular cisternae, possibly involved in regulating intracellular calcium levels, are linked to the loop membrane also by only a small number of cross-bridges (Fig. 4d). Mitochondria and ER were found positioned within a dense "jungle" of cytoskeleton (Fig. 4a), yet the connection of these organelles to the cytoskeleton was limited to either a single neurofilament or microtubule (Fig. 4e) and moreover, a single cross-bridge. Intercellular cytoskeletal connections, not always apparent in thin-section micrographs or in slices through the tomographic reconstruction, were revealed after volume segmentation. An example is an axoplasmic cross-bridge connected to a glial cross-bridge through more than 40 nm of intervening PJB rod (Fig. 4f). Whereas neurofilaments and microtubules predominate the axoplasm, microfilaments are more pronounced in the loop cytoplasm (Fig. 4g, h). What role the microfilaments play is not implied from ultrastructure revealed here as we observed no cross-bridges to each other, to other

cytoskeletal elements or any attachments to organelles or loop membranes (see supplemental movie).

Measurements of diameters uncovered two distinct classes of cross-bridges (Fig. 5). The range and number of measurements are found in Table 1. Those bridges connecting neurofilaments, microtubules, and organelles have different cross-bridging diameters to the second class—those connecting membranes: either filaments to the axolemma or between the TC and paranodal loop membrane. The two different classes are reflected in the probability of sampling from the same population values, p , derived from the Student's t -test. For example, $p=0.0014$, 0.000038 , 0.00020 , 1.2×10^{-10} , and 3.1×10^{-6} for NF-NF vs. TC-PNL, NF-MT vs. TC-PNL, NF-NF vs. NF-Axl, NF-MT vs. NF-Axl, and mito-MT vs. NF-Axl, respectively. No other t -test between cross-bridge types had $p<0.01$, which we used as the cutoff for statistical significance, including comparisons between the measured diameters from HPF/FS and conventional data. The lengths show similarity between NF-NF, NF-MT, and mito-MT cross-bridges (Fig. 5b). A notable length dissimilarity exists between the TC-PNL cross-bridge and all the NF cross-bridges, i.e., NF-NF, NF-MT, and NF-Axl cross-bridges ($p<0.01$ for all 3 comparisons) and is remarkable because the NF-Axl and TC-PNL mean diameters are similar. It may be that the links position the TC close to the PNL for the purpose of limited-diffusion calcium release or sequestration. The PJB diameter of 15 ± 2 nm (mean $\pm$ std dev; range 12–19 nm) for HPF/FS samples or 16 ± 4 nm (range 10–22 nm) for conventional samples is much greater than the fibrous cross-bridges (Fig. 5a) and emphasizes that PJBs are rods with an axis running lengthwise along the juxtapositional membranes, not between the membranes. By plotting cross-bridge diameter vs. length, we found some indication that there exists a linear correlation between four of them: NF-NF, NF-MT, NF-Axl, and TC-PNL (Fig. 5c, 5d) and more noticeable with the conventionally prepared samples. In other words, as the diameter of the cross-bridge increased, its length also tended to increase linearly. The one exception was the mito-MT cross-bridge, consistent with our observations that these are generally short-linkers. Because of the size of mitochondria, a thicker cross-bridge might be required.

4. Discussion

Highly organized cytoskeletal networks appear to be one way of imposing rigidity and compartmentalization, or alternatively, flexible spacing to an area of cellular specialization such as is found in the node of Ranvier. The filamentous cross-bridges, some thinner than 7 nm, might serve as spacing struts that are easily pushed apart by organelles translocating through the axoplasm or Schwann cell cytoplasm. We characterized the junctional bridge and cross-bridges that exist in the paranodal region, where they can be found, which components are cross-linked, the extent of cross-linking, and the structural features of these bridges. Analyzing tomographic volumes aided the understanding of the 3D relationship between cross-bridges and the components they link. We uncovered two classes of cross-bridges in the paranodal region. The cross-bridges of the first class have similar diameters to that of neurofilaments—9–11 nm (Karp, 2002). Intermediate filaments are constructed from a family of proteins, related by sequence, that number about 70 to date (Karp, 2002). Neurofilaments are built from 3 proteins in the type IV family of such filaments. There are six family types to draw on for cross-bridges that would have the same range of diameter and yet the cross-bridge constituents may not be from these families; it simply is not known. Likewise, even though the second cross-bridge class has filament diameter similar to that of microfilaments, it is not known if the constituent is actin. The PJB is not a fibrous connection, appearing like a thick rod, and may function more as a tight junction.

Cytoskeleton-membrane-transcellular interactions at the paranode were described previously (Ichimura and Ellisman, 1991). Support for the integrated operation of the juxtaposed neuronal and glial cells during the propagation of nerve impulses abounds (Bonnon et al., 2007; Carley

and Raymond, 1987; Endres et al., 1986; Halter and Clark, 1993; Lev-Ram and Grinvald, 1986; Lev-Ram and Grinvald, 1987; Lev-Ram and Ellisman, 1995; Murray and Steck, 1984; Rasband, 2006; Sosinsky et al., 2005; Wurtz and Ellisman, 1986). Equally compelling support for an axo-glial integration of function is provided by the disruption of the myelin structure or maintenance of the glial cell in Charcot-Marie-Tooth disease, a set of human hereditary neuropathies (Bergoffen et al., 1993; Martini, 2001), causing changes in axonal morphology and nerve impulse conduction. Pathological conditions in peripheral neuropathies are also found when constituent proteins of the node of Ranvier malfunction (Bergoffen et al., 1993; Griffin et al., 1996; Rasband et al., 2003; Sima, 1993), are deficiently expressed (Bhat et al., 2001; Boyle et al., 2001; Sherman et al., 2005; Weber et al., 1999) or after axonal ischemic injury (Waxman et al., 1992) and trauma (Maxwell et al., 1991).

4.1 Protein composition of the PJB

An additional observation is that cross-bridges to cytoskeleton in the cytoplasm and occasional neurofilaments terminations were found connected to PJBs. These cytoskeletal components might also serve to separate this septate-like junction with a nearly uniform spacing we report here. We found that similar filamentous cross-bridges exist in the glial cytoplasm and the axoplasm originating from the PJB that might be part of an intercellular link of glial and axonal cytoskeletons. We propose that the elegant architecture of the transcellular cytoskeletal network provides a mechanical link that stabilizes the two-cell spacing particularly for efficient action potential conduction.

4.2 Cytoskeletal cross-bridges serve to maintain the structural integrity of the paranode

We found three types of neurofilament cross-bridging. One type involved connecting to the axolemma via thin fibrous extensions, usually no longer than 100 nm. A second type of association was direct attachment of one end of the neurofilament to the axolemma. The third type of bridge was a cross-linking filament, about the same diameter as neurofilaments themselves, between neurofilaments or neurofilaments and microtubules (Fig. 5). The cross-bridges connecting to the axolemma had a diameter consistent with actin. Actin filaments are outnumbered by neurofilaments by about an order of magnitude in our tomographic volumes, consistent with the report that intermediate filaments can account for up to 85% of the total protein complement in fully differentiated cells (Fuchs and Weber, 1994). The cross-bridging of neurofilaments provides flexible resilience to the axoplasm in response to external stresses by reversibly cross-linking these filaments to the other cytoskeletal filaments and attachment sites at the axolemma (Fuchs and Cleveland, 1998). Part of the cross-bridging of neurofilaments to other neurofilaments and microtubules is likely accomplished by the long carboxyl tails of the neurofilament proteins NF-M and NF-H that protrude up to 30 nm from the filament backbone. Cross-bridging might also be through plectin, BPAG1n, and related proteins (Fuchs and Cleveland, 1998).

The cross-bridges between membranes and cytoskeleton reported here have both similar and dissimilar linkers to those reported previously by Ichimura and Ellisman (1991) who used three different methods for tissue preparation and three different electron microscopic techniques. Two cross-bridging types connecting neurofilaments with the glial-axonal junction were reported in the previous work. One is via 5-8 nm filaments equivalent to those we found that have a mean diameter of about 7 nm and a range of 6-9 nm (Table I). The slight difference in range of diameter could be due to the different contrasting mechanism—negative stain in the work of Ichimura and Ellisman and positive stain in our work. The other neurofilament cross-bridges previously reported were 3-5 nm short linkers. However, we did not observe these linkers and it may be that these fine filaments are just below the resolution or detection limit in our tomographic volumes. Another cross-bridge reported in the two studies was found connecting the tubular cisternae with the paranodal loop membrane (TC-PNL). However, in

the previous work, 3-5 nm fine linking filaments were observed whereas we found that the measured diameter was ~5-9 nm. Could it be that the positive stain is adding thickness to these filaments not present in the earlier work because of the negative contrast? Support for this interpretation is seen in fig. 3 where the stain accumulation is not uniform along the fiber lengths, giving the appearance of thicker and thinner regions. Further, the grains of uranyl acetate are about 1.5 nm across and so ~3 nm to the thickness of our positively stained cross-bridges may be attributable to stain deposition.

Mutations that weaken or disrupt the cytoskeletal network are implicated in a number of human disorders. For example, missense and splice-site mutations of tau, a protein that stabilizes microtubules, cause aberrant filamentous assembly that is involved with a variety of human cognitive diseases, including Alzheimer's disease, inherited frontotemporal dementia, and Parkinsonism (Garcia and Cleveland, 2001). Disruption of cross-bridging and abnormal accumulation of neurofilaments are pathologic for a series of motor neuron diseases, among which are infantile spinal muscular atrophy, familial and sporadic amyotrophic lateral sclerosis (ALS), and hereditary sensory motor neuropathy (Lee and Cleveland, 1996; Xu et al., 1993). The mechanism of ALS disease is thought to stem from disorganization of neurofilament connectivity that would disrupt the transportation of cargo along the axon (Collard et al., 1995; Fuchs and Cleveland, 1998).

The cross-bridges that we have analyzed are central to the cyto-architecture and structural integrity of the paranode. The continued work to identify the proteins that constitute these bridges, how they link with each other to form filaments or rods, and how they attach to membranes and cytoskeleton is anticipated to enhance discovery of functional interactions. The combination of high-resolution 3D structures of the paranodal region with the localization of specific macromolecules that populate this region is essential for subsequent modeling studies (Sosinsky et al., 2005) and for a better understanding of the structural and molecular bases for diseases affecting the node and paranode (Bergoffen et al., 1993; Bhat et al., 2001; Boyle et al., 2001; Griffin et al., 1996; Martini, 2001; Rasband et al., 2003; Sherman et al., 2005; Sima, 1993; Weber et al., 1999). The cytoskeletal filaments with their various cross-bridges, including those that are transcellular, are assumed to play an important role in maintaining the electrophysiological properties required for functioning of the node of Ranvier by establishing a mechanical link between the glial and neuronal cytoskeletons, thereby stabilizing the relationship between these cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HPF/FS	high-pressure freezing, freeze-substitution
PNS	peripheral nervous system
CNS	central nervous system
PJB	paranodal junctional bridges
TC	tubular cisternae

Figure 1.

Detailed three-dimensional information about the components of the paranode was obtained from HPF/FS samples. **(a)** Using conventional electron microscopy of rat spinal root, cross-bridges between cytoskeletal components, cytoskeleton and axolemma, and cytoskeleton and organelles were observed in thin sections of the node of Ranvier. **(b)** Annotation with black “pencil” of 46 such cross-bridges found in the image of (a). **(c)** Slice through a tomographic volume of spinal root axon and surrounding paranodal loops (PNL) of a Schwann cell. Example features are labeled that are shown in greater detail in subsequent figures. An array of paranodal junctional bridges (PJB) is indicated by 4 arrowheads. Examples of a tubular cisterna (TC), microtubule (MT), a loose bundle of neurofilaments (NF), endoplasmic reticulum (ER), a lysosome (L) and a mitochondrion (M) are labeled. The mitochondrion is in the condensed state with a crista junction observed on the left (arrow). Scale bar = 200 nm.

Figure 2.

Magnified views of sub-regions of slices through the four tomographic reconstructions analyzed provide detail of cross-bridges. Annotation with color of highlighted features are provided in the “primed” panels, e.g., a’. (a) PJBs span the region between the paranodal loop (PNL) membrane and the axolemma (arrowheads) and often appear as somewhat ordered two-dimensional arrays. (a’) cyan: PNL membrane, yellow: PJB, blue: axolemma. (b) A fibrous connection between a TC and the PNL (arrowhead) from a conventionally prepared specimen. (b’) red: axolemma, cyan: PNL membrane, blue: TC, yellow: TC-PNL cross-bridge. (c) Two closely positioned fibrous connections from a HPF/FS specimen extend from a TC to the membrane of the PNL (arrowheads). The location of the TC near the sites of rapid and frequent ionic movement suggests their involvement in sequestration and release of ions requisite to nodal functioning. (c’) blue: axolemma, cyan: PNL membrane, red: TC, yellow: TC-PNL cross-bridge. (d) A cross-bridge (small arrowhead) between two parallel neurofilaments (arrows) in the axoplasm and the axolemma (pair of large arrowheads). It is suggested that this element of spacing contributes to cell integrity by cross-bridging within the cytoplasm and to various membranes. (d’) cyan: axolemma, yellow: NF-Axl cross-bridge, blue: NF, red: NF-NF cross-bridge. (e) A mitochondrion (M) is seen connected to a neurofilament (arrowhead) by a narrow tether (arrow). (e’) cyan: mito outline, yellow: mito-NF cross-bridge, blue: NF. (f) Several short-linking bridges (arrow) connect a mitochondrion (M) to a microtubule (arrowhead). (f’) cyan: mito outline, yellow: mito-NF cross-bridge, red: MT. (g) A mitochondrion (M) and nearby smooth endoplasmic reticulum (ER) are connected by multiple cross-bridges (arrowheads); two are shown in this tomographic slice. (g’) cyan: mito outline, red: ER outline, yellow: mito-ER cross-bridge. (h) In the sub-volume of the PNL closest to the axolemma, F-actin strands (microfilaments) are observed (arrows point to cross-sections) running perpendicular to the neurofilaments and microtubules in the axoplasm. A PJB is also nearby. (h’) cyan: PNL membrane, yellow: PJB, red: microfilament cross-section, blue: axolemma. (i) Two cross-bridges between neurofilaments in the axoplasm (arrowheads). (i’) blue: NF, yellow: NF-NF cross-bridge. (j) A cross-bridge (arrowhead) between a neurofilament (NF; black arrow) and a microtubule (MT; white arrow) is also observed in the axoplasm. (j’) blue: NF, red: MT, yellow: NF-MT cross-bridge, green: PNL membrane, cyan: axolemma. (k) More

pronounced examples of NF-MT cross-bridges (arrowheads) from a tomographic slice of a conventionally prepared sample. (**k'**) blue: NF, red: MT, yellow: NF-MT cross-bridge. Scale bar = 100 nm for all panels.

Figure 3.

Comparison of cross-bridge structures by type after alignment and averaging of tomographic volumes. Images from a central slice through each averaged subvolume of **(a)** NF-NF, **(b)** NF-MT, **(c)** mito-MT, **(d)** NF-Ax1, and **(e)** TC-PNL types are shown. The variation in diameter along the length of each bridge is evident as well as the general difference in cross-bridge diameter between the first three and last two types of bridge. The numbers of subvolumes averaged together per cross-bridge type are 15, 17, 8, 11, and 15 for NF-NF, NF-MT, mito-MT, NF-Ax1, and TC-PNL, respectively. Scale bar = 10 nm.

Figure 4.

Segmentation of the volumes shown in Figs. 1-3 provides surface-rendered volumes used for visualization and dissection of connections and cross-bridges. Cytoskeletal networks provide a flexible scaffolding to impart structure to the cytoplasm and to resist stresses applied externally to the cell. **(a)** All segmented components are displayed. In the axoplasm, there were 32 MTs (thicker orange threads), 158 NFs (thinner green threads), 8 mitochondria and other membranous organelles (magenta) (outer membrane only shown), 5 fibrous connections between PJBs and NFs (magenta), and 5 fibrous extensions from PJBs into the axoplasm (red). In the paranodal loop region, there were 32 PNLs (alternating blue and cyan), 23 PJBs (yellow), 9 fibrous extensions from PJBs into the PNL (red), 7 TCs (dark green), and 24 F-actin fibers (microfilaments) (cyan). Scale bar = 200 nm. **(b)** Cross-bridges connect neurofilaments (NF) to each other and to microtubules (MT) in the axoplasm although none were observed between MTs. Three examples of NF-NF and two examples of NF-MT cross-bridges are shown. Only one or two cross-bridges between individual cytoskeletal threads were found. Cross-bridges were also found between NFs and the axolemma (arrowhead). **(c)** PJBs form nearly equidistant rows that connect the PNL membrane (blue) with the axolemma (not shown). Six PJBs (yellow) are shown of various lengths (arrowhead points to an example). Cross-bridges between PJBs and NFs were found (magenta filaments), but not between PJBs and MTs. Filamentous extensions originating from PJBs were observed (red filaments) that had no obvious connection

to a cytoskeletal element. **(d)** Cross-bridges (orange) connecting TCs in the PNL lumen to the PNL membrane (blue) were common. Extensions (cyan) from the PNL membrane adjacent to the axolemma into the PNL lumen were also observed. Occasionally, a NF would terminate at a PJB (arrowhead). **(e)** Small mitochondria (M) in the axoplasm connect to a single NF (green) by a narrow tether (blue). Nearby is a cross-bridge connecting an NF with an MT. **(f)** Connections across cells were found. A cross-bridge (cyan) connects a NF in the axon with a PJB (yellow) in the extracellular matrix. Farther down the same PJB, a filamentous extension (orange) into the paranodal loop of the Schwann cell is seen. **(g)** Actin microfilaments (cyan) in the PNL lumen run more or less perpendicular to the neurofilaments (green) and microtubules (not shown) in the axoplasm and also perpendicular to the cross-bridges (yellow). Scale bar = 100 nm. **(h)** View perpendicular to that of panel f showing the limited extent of microfilament lengths. Twelve microfilaments are shown in 4 PNLs.

Figure 5.

Cross-bridge diameters and lengths comparison between HPF/FS and conventionally prepared samples. **(a)** Measurements of diameters reveal two different classes of cross-bridges. A histogram of mean cross-bridge and fiber diameters for five morphologically distinct connections and actin (microfilament) is shown. The error bars represent the standard deviations of the measurements. The cross-bridges between neurofilaments, microtubules, and mitochondria are similar in diameter and consistent in width to intermediate filaments. In contrast, the cross-bridges between neurofilaments (NF) and the axolemma (Axl) and between the TC membrane and the PNL membrane are significantly narrower and similar in width to microfilaments **(b)** The cross-bridge lengths show greater variation than the diameters. The means (shown) ranged from 21 to 35 nm. The error bars represent the standard deviations of the measurements. The lengths are similar between NF-NF cross-bridges and NF-MT cross-bridges. The lengths are also similar between mito-MT cross-bridges and TC-PNL cross-bridges. Plot to correlate diameters and lengths of **(c)** HPF/FS samples and **(d)** conventional samples. There is some indication that there exists a linear correlation between NF-NF, NF-MT, NF-Axl, and TC-PNL diameters and lengths, more noticeable with the conventional samples. No linear relationship between Mito-MT cross-bridge diameters and lengths is present.

Table 1

Range of Cross-Bridge Diameters and Lengths

Cross Bridge Type	HPF Diameters (nm)	Number of Measurements	CAF Diameters (nm)	Number of Measurements	HPF Lengths (nm)	CAF Lengths (nm)
NF-NF	7.4-12.2 [§]	40	7.6-10.9 [§]	34	21.8-74.1 [§]	18.8-62.5 [§]
NF-MT	8.1-12.4	26	7.7-11.5	23	23.9-65.8	19.8-93.8
mito-MT	8.1-11.4	18	9.9-10.9	3	13.8-51.1	17.6-35.8
TC-PNL	5-8.7	18	5.4-9.2	21	9.6-45.7	8.5-84
NF-Axl	5.7-9.4	36	5.4-9.1	12	21.6-66.5	16.3-60.4
Actin	5.5-8.4	47	5.6-8.2	17		

[§] Minimum-maximum measured values

HPF = high pressure freezing with aldehyde fixation

CAF = conventional aldehyde fixation