Axon initial segment cytoskeleton comprises a multiprotein submembranous coat containing sparse actin filaments

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The axon initial segment (AIS) of differentiated neurons regulates action potential initiation and axon–dendritic polarity. The latter function depends on actin dynamics, but actin structure and functions at the AIS remain unclear. Using platinum replica electron microscopy (PREM), we have characterized the architecture of the AIS cytoskeleton in mature and developing hippocampal neurons. The AIS cytoskeleton assembly begins with bundling of microtubules and culminates in formation of a dense, fibrillar–globular coat over microtubule bundles. Immunogold PREM revealed that the coat contains a network of known AIS proteins, including ankyrin G, spectrin βIV, neurofascin, neuronal cell adhesion molecule, voltage-gated sodium channels, and actin filaments. Contrary to existing models, we find neither polarized actin arrays, nor dense actin meshworks in the AIS. Instead, the AIS contains two populations of sparse actin filaments: short, stable filaments and slightly longer dynamic filaments. We propose that stable actin filaments play a structural role for formation of the AIS diffusion barrier, whereas dynamic actin may promote AIS coat remodeling.

Introduction

The axon initial segment (AIS) is a specialized compartment of polarized neurons that separates the somatodendritic domain from the axonal domain. The AIS functions as the site of synaptic input integration and action potential initiation because of high density clustering of voltage-gated ion channels (Kole et al., 2008). The AIS is also a major contributor to the maintenance of axon–dendrite polarity that is critical for establishment of the entire neuronal circuitry (Hedstrom et al., 2008; Sobotzik et al., 2009; Song et al., 2009). Neuronal injury can alter the properties of the AIS, whereas mutations in AIS proteins may contribute to pathogenesis of neurological disorders, such as epilepsy and schizophrenia (Buffington and Rasband, 2011; Kole and Stuart, 2012). Although much is known about its role in action potential initiation, it remains less clear how the AIS maintains neuron polarity, partly because of poor understanding of its cytoskeletal architecture.

Early electron microscopy studies using a thin-section technique revealed microtubule (MT) fascicles and a dense granular undercoating at the plasma membrane as two characteristic features of the AIS (Palay et al., 1968). Later studies showed clustering of various membrane and submembranous proteins, such as ankyrin G (AnkG; Kordeli et al., 1995), spectrin βIV (Berghs et al., 2000), voltage-gated ion channels (Devaux et al., 2004; Kole et al., 2008), and cell adhesion molecules (Davis et al., 1996), within the AIS that could correspond to the membrane undercoating. The adaptor protein AnkG is one of the earliest components localized to the AIS and is responsible for the recruitment of most other AIS-enriched proteins (Zhou et al., 1998; Jenkins and Bennett, 2001; Hedstrom et al., 2007) and for maintaining AIS structure and role in neuron polarity (Zhou et al., 1998; Hedstrom et al., 2008; Sobotzik et al., 2009).

AIS contribution to neuron polarity depends on formation of a barrier that prevents diffusional mixing of plasma membrane components between axons and dendrites (Kobayashi et al., 1992; Nakada et al., 2003; Song et al., 2009). This AIS function depends on actin cytoskeleton dynamics (Winckler et al., 1999; Nakada et al., 2003; Song et al., 2009).
Here we used platinum replica electron microscopy (PREM) to determine the molecular architecture of the AIS, with a special focus on the actin cytoskeleton, in dissociated hippocampal cultures at different stages of neuronal development. Our data reveal novel features of AIS architecture essential for understanding its assembly and functions.

Results

The AIS cytoskeleton comprises a dense submembranous coat

To determine the structure of the AIS cytoskeleton, we used PREM of detergent-extracted hippocampal neurons cultured for 7 d in vitro (DIV7; Fig. 1), when the AIS is considered to become fully assembled (Grubb and Burrono, 2010). Axons in these fully polarized neurons were distinguished from dendrites by their extended length. The cytoskeleton of both axons and dendrites largely consisted of MTs associated with few thin (3–6-nm) fibers that often appeared to cross-link the MTs and also included few neurofilaments (Fig. 1, C and E). The proximal axon of most neurons, however, had an unusual cytoskeletal structure characterized...
Assembly of the AIS coat during neuronal development

To understand how the AIS cytoskeleton assembles during neuronal maturation at a structural level, we examined the proximal axon by PREM from DIV3 to DIV21 (Fig. 3). In early DIV3 or DIV4 cultures (Fig. 3, A and B), few axons (longest neurites) contained a fibrillar coat in the proximal region (within 100 µm from the hillock; Fig. 3 G). Instead, most early axons (64%) displayed tight MT bundles, either continuous or intermittent (Fig. 3 B and Fig. S1), which were not observed in distal axon regions. Actin filaments were sparse in axons along most of their length, but abundant in the distal axon, growth cones, and occasional lateral protrusions, such as filopodia and patches (Gallo, 2013; Fig. S2).

At DIV7, ~80% of neurons displayed in their proximal axons a fibrillar coat mixed with occasional globules of various shapes and sizes (Fig. 3, C and G). The density of the coat varied broadly, from sparse fibrils with variable thicknesses (3–11 nm) and orientations (Fig. 3 C) to dense networks (Figs. 1 and 2). By DIV10 and DIV14, the density of the coat progressively increased and coat substructure gradually shifted from fibrillar to globular (Fig. 3, D, E, and G). In proximal axons at DIV21, the coat was dominated by tightly packed globules that completely covered the MTs (Fig. 3, F and G). However, fibrils were also occasionally observed in small spaces between globules (Fig. 3 F).
We next used PREM to define actin organization in the AIS of DIV10 neurons, when the coat is sufficiently dense, but still mostly fibrillar. Although actin filaments can be identified in PREM samples based on their thickness or using myosin subfragment 1 (S1) decoration, very short filaments may escape detection by either approach. Therefore, we used immunogold PREM to detect phalloidin-stained actin filaments with an antibody to the phalloidin-bound fluorophore. This labeling approach was both specific and complete, resulting in virtually full coverage of detectable actin filaments with gold particles in actin-rich protrusions, whereas MTs were unlabeled (Fig. S3).

In contrast, in the AIS of DIV10 neurons, the level of immunogold labeling was low, consistent with fluorescence microscopy data. The immunogold patterns could be divided into two categories. Some gold particles were linearly arranged along actin filaments 330 ± 242 nm (mean ± SD) in length and others were distributed as single particles or in small clusters (Fig. 5A), indicative of very short phalloidin-labeled actin filaments that could be analogous to short filaments in RBCs.

To obtain a clearer view of the AIS cytoskeleton, we examined axonal branch points. Neurons are known to form branches from their main axon shaft (Gallo, 2011). In our cultures, branches...
The AIS coat is a self-maintained scaffold

Major components of the AIS are resistant to detergent extraction and remain associated with the cytoskeleton (Garrido et al., 2003; Sánchez-Ponce et al., 2012), suggesting that they form a highly interlinked polymeric scaffold. However, dynamic actin filaments and/or MTs can play a role in maintaining coat integrity. Therefore, we examined effects of actin filament and/or MT depletion from DIV10 detergent-extracted neurons on AIS coat structure. Fluorescence microscopy showed a significant reduction of actin filaments after treatment of detergent-extracted cells with actin-severing protein gelsolin and of MTs after incubation of extracted cells in a cold calcium-containing buffer without altering AnkG staining (Fig. 6, A and B).

Phalloidin immunogold staining revealed the complete removal of long actin filaments after gelsolin treatment. However, many single or small clusters of gold particles were still present in the AIS coat, suggesting that short actin filaments are resistant to severing by gelsolin (Fig. 6 C, c1 and c2), possibly because of their interaction with stabilizing proteins in analogy to short actin filaments in RBCs. The AIS coat remained intact after gelsolin treatment in both main axonal shafts (Fig. 6 C, c1) and stretched regions (Fig. 6 C, c2). The coat structure also seemed well preserved after removal of MTs (Fig. 6 D) or after removal of both actin filaments and MTs (Fig. 6 E). These data indicate that the AIS coat maintains its structure independent of and actin-rich protrusions occasionally formed in the proximal axon (Fig. 5 B). Remarkably, the AIS coat covered not only the main axon, but also the beginning of the branch, and appeared to undergo “stretching” at branch junctions, suggesting that the coat has elastic properties. These stretched regions of the coat displayed fewer globules and more thin fibrils, than in the main axon (Fig. 5 B, b1). The fibrils were free of gold labeling and often oriented roughly in the direction of apparent stretch (Fig. 5 B, b2). These observations suggest that globules and fibrils in the AIS coat may partly represent the same molecules undergoing conformational change. An expanded coat at branch junctions could help to reveal more actin filaments in the AIS if their paucity was caused by poor penetration of labeling reagents. However, we observed approximately the same level and pattern of phalloidin gold staining as in shafts of main axons (Fig. 5, A and B).

To test an idea that actin filaments in the AIS can form directional tracks, we examined the orientation of actin filaments that had an appreciable length in DIV10 AISs using S1 decoration (Fig. 5 C). We found that filaments displayed no preferential orientation in the AIS if their paucity was caused by poor penetration of labeling reagents. However, we observed approximately the same level and pattern of phalloidin gold staining as in shafts of main axons (Fig. 5, A and B).
at axonal branch junctions, the coat typically was not spread as observed under control conditions (Fig. 5 B) and appeared sparse with many large gaps (Fig. 7 E). In other cases, the AIS coat appeared to peel off the MT bundles as a cohesive sheet (Fig. S4). These data suggest that dynamic actin filaments contribute to AIS coat elasticity and potentially to its interaction with MT bundles.

Molecular architecture of the AIS coat

To further understand the molecular organization of the AIS coat, we wanted to identify major AIS components by immunogold PREM. As a first step toward this goal, we confirmed by immunofluorescence microscopy that spectrin βIV, the cell adhesion molecules neurofascin and neuronal cell adhesion molecule (NrCAM), and voltage-gated sodium channels (Nav) all remained enriched in the AnkG-positive AIS of DIV14 neurons after detergent extraction (Fig. 8, A–D), a procedure used to prepare samples for PREM.

Because of molecular and structural similarities between the AIS coat and RBC membrane skeleton, we tested whether the AIS coat contains adducin, a specific component of RBC cytoskeleton, which caps barbed ends of short actin filaments in the RBC junctional complexes and stabilizes actin–spectrin interaction (Kuhlman et al., 1996). Consistent with previous studies (Kaiser et al., 1989; Xu et al., 2013), we found that adducin
immunofluorescence staining was present along dendrites and axons of DIV14 neurons. Surprisingly, however, adducin staining within the AIS was greatly diminished relative to other axonal regions (Fig. 8 E), suggesting that a mechanism of actin filament stabilization in the AIS is distinct from that in RBC junctional complexes despite structural similarities between AIS and RBC cytoskeletons. We next tested whether structural changes accompanying AIS maturation (Fig. 3) reflect different timing of arrival of various AIS components during AIS development. Immunofluorescence intensities of several AIS proteins (AnkG, spectrin βIV, neurofascin, NrCAM, and Nav) normalized to their respective maximal values over a period from DIV4 to DIV21 displayed a general upward trend (Fig. 8 F). However, neurofascin and especially NrCAM reached plateau relatively early, at DIV14 and DIV10, respectively, whereas spectrin βIV and Nav were recruited to the AIS with gelsolin (C), cold calcium (D), or both (E) and stained with phalloidin immunogold (C and D). The AIS coat maintains integrity in all cases. (C) Low-magnification image of the proximal axon. White outline shows axon boundary. On the bottom right side, the coat spreads away from the axon shaft, probably being stretched by an actin-based protrusion removed by gelsolin treatment. Boxes c1 and c2 are enlarged in corresponding panels. (C) Dense AIS coat along main axon and beginning of stretched region, where transversely oriented thin fibrils (blue) can be seen. (C) Expanded coat at the putative branch junction contains numerous fibrils. Immunogold phalloidin staining in both c1 and c2 reveals only short filaments (i.e., individual particles and small gold clusters). (D) Dense coat in the axon shaft after MT depletion. Long, thick filaments are neurofilaments. Actin filament staining is comparable to that in untreated neurons. Bars: (A and B) 10 µm; (C) 1 µm; (c1 and c2) 100 nm; (D and E) 200 nm.

Figure 6. Structure of AIS coat after actin filament and MT extraction. (A and B) Fluorescence microscopy of detergent-extracted DIV10 neurons stained with Alexa Fluor 647–phalloidin (F-actin) and antibodies to AnkG and β3 tubulin (MTs). (A) Control neuron. (B) Neuron treated first with cold calcium-containing buffer to depolymerize MTs and then with gelsolin to sever actin filaments. (C–E) PREM of DIV10 neurons treated with gelsolin (C), cold calcium (D), or both (E) and stained with phalloidin immunogold (C and D). The AIS coat maintains integrity in all cases. (C) Low-magnification image of the proximal axon. White outline shows axon boundary. On the bottom right side, the coat spreads away from the axon shaft, probably being stretched by an actin-based protrusion removed by gelsolin treatment. Boxes c1 and c2 are enlarged in corresponding panels. (C) Dense AIS coat along main axon and beginning of stretched region, where transversely oriented thin fibrils (blue) can be seen. (C) Expanded coat at the putative branch junction contains numerous fibrils. Immunogold phalloidin staining in both c1 and c2 reveals only short filaments (i.e., individual particles and small gold clusters). (D) Dense coat in the axon shaft after MT depletion. Long, thick filaments are neurofilaments. Actin filament staining is comparable to that in untreated neurons. Bars: (A and B) 10 µm; (C) 1 µm; (c1 and c2) 100 nm; (D and E) 200 nm.

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They occasionally appeared to extend from elongated globular clusters (Fig. 10, bottom) analogous to those containing AnkG (Fig. 9). Individual neurofascin- and NrCAM-positive structures appeared as filaments that also sometimes bent (Fig. 10, high mag). Neurofascin filaments were 58 ± 24 nm in length (mean ± SD) and 6.0–6.5 nm in width, similar to reported dimensions of purified neurofascin molecules (Davis et al., 1993), whereas NrCAM filaments were longer (145 ± 23 nm) and slightly thicker (7.5–9.5 nm), which was unexpected because both neurofascin and NrCAM have similar molecular masses (186 and 145 kD, respectively).

Individual structures labeled by a pan-Nav antibody were globular or donut shaped with a 12.5 ± 2.3-nm (mean ± SD) diameter (Fig. 10, high mag), similar to that of purified protein (Sato et al., 2001). These Nav channels often clustered with other donut shapes of similar or larger (17.7 ± 1.5 nm, mean ± SD) diameter (Fig. 10, yellow arrows), possibly representing Nav proteins that were not immunolabeled or other channels known to localize to the AIS (e.g., potassium or calcium channels). Remarkably, similar donut shapes were sometimes apparent as components of AnkG-positive elongated globular clusters (Figs. 9 and 10) or in close proximity to NrCAM filaments (Fig. 10), consistent with known ability of Nav channels to interact with these proteins (Zhou et al., 1998; Lemaillet et al., 2003; McEwen and Isom, 2004; McEwen et al., 2004). Consistent

To directly identify the AIS coat substructures, we used immunogold PREM with antibodies for several AIS proteins in DIV14 neurons, when both fibrils and globules are common. For all proteins examined, the identity of immunogold-associated structures in dense AIS regions was often ambiguous, requiring examination of regions where the coat was sparse or stretched. Accordingly, only a small fraction (~30%) of gold particles could be unambiguously interpreted.

Immunogold labeling of AnkG using an antibody against a C-terminally located epitope (Fig. 9) was positive in both fibril- and globule-rich regions of the coat (Fig. 9, A–D). Individual gold particles frequently labeled a tip of thin, often curved, filaments 139 ± 16 nm (mean ± SD) in length and 4.0–7.5 nm in width (Fig. 9 E). Other gold particles were associated with one end of elongated globular clusters of approximately the same length, but varying in shape and thickness throughout their lengths. These AnkG-based globular clusters could represent a thin flexible filament, like in the first category, associated with AnkG interaction partners.

Immunogold labeling of spectrin βIV, neurofascin, NrCAM, and Nav confirmed the presence of these proteins within the coat (Fig. 10, top). Individual spectrin βIV–positive structures comprised filaments 58 ± 20 nm in length (mean ± SD) and 4.5–6.5 nm in width that were either curved or fairly straight (Fig. 10, High Mag). They occasionally appeared to extend from elongated globular clusters (Fig. 10, bottom) analogous to those containing AnkG (Fig. 9). Individual neurofascin- and NrCAM-positive structures appeared as filaments that also sometimes bent (Fig. 10, high mag). Neurofascin filaments were 58 ± 24 nm in length (mean ± SD) and 6.0–6.5 nm in width, similar to reported dimensions of purified neurofascin molecules (Davis et al., 1993), whereas NrCAM filaments were longer (145 ± 23 nm) and slightly thicker (7.5–9.5 nm), which was unexpected because both neurofascin and NrCAM have similar molecular masses (186 and 145 kD, respectively).

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spectrin-rich structure is the membrane skeleton in RBCs, which represents an elastic meshwork of α/β spectrin heterotetramers connected at their ends by short (~35-nm) actin filaments at so-called junctional complexes (Byers and Branton, 1985). Actin filaments in junctional complexes are capped at both ends and stabilized along the length by tropomyosin (Fowler, 1996; An et al., 2007). The actin–spectrin meshwork in RBCs is attached to the plasma membrane primarily by ankyrin R, which interacts with both spectrin and transmembrane proteins, but components of junctional complexes and spectrin itself also participate in membrane binding (Mohandas and Gallagher, 2008). In artificially

with immunofluorescence data, adducin was not found in the AIS coat by immunoPREM (unpublished data). These observations demonstrate that the AIS coat comprises many proteins that are known to localize to the AIS, and that these proteins, in part, account for the globules and fibrils observed in the coat.

### Discussion

#### AIS coat substructure

The AIS is highly enriched with AnkG and spectrin βIV (Grubb and Burrone, 2010). The best characterized ankyrin- and spectrin-rich structure is the membrane skeleton in RBCs, which represents an elastic meshwork of α/β spectrin heterotetramers connected at their ends by short (~35-nm) actin filaments at so-called junctional complexes (Byers and Branton, 1985). Actin filaments in junctional complexes are capped at both ends and stabilized along the length by tropomyosin (Fowler, 1996; An et al., 2007). The actin–spectrin meshwork in RBCs is attached to the plasma membrane primarily by ankyrin R, which interacts with both spectrin and transmembrane proteins, but components of junctional complexes and spectrin itself also participate in membrane binding (Mohandas and Gallagher, 2008). In artificially

Figure 8. **Time course of accumulation of AIS proteins during neuron development.** (A–D) Major AIS proteins remain associated with the cytoskeleton after detergent extraction of DIV14 neurons and colocalize with AnkG. (E) Adducin is largely absent from the AnkG-containing AIS (arrowhead), but is abundant in dendrites and in the axon outside of the AIS (arrow). (F) Time course of protein accumulation in the AIS during neuronal development. Fluorescence intensities of individual immunostained proteins are expressed as a percentage of the maximal value over a period from DIV4 to DIV21 (DIV7 for adducin and DIV21 for other proteins) and plotted against the DIV. n values for DIV4, DIV6/7, DIV10, DIV14, and DIV21 are, respectively: 21, 29, 36, and 51 for spectrin βIV; 9, 18, 28, 6, and 34 for NrCAM; 22, 37, 24, 46, and 32 for neurofascin; 36, 29, 27, 28, and 64 for Nav; and 98, 123, 127, 146, and 190 for AnkG. For adducin, n = 10 for DIV4–14 and 9 for DIV21. Error bars represent standard deviations. Bars: (A–D) 20 µm; (E) 10 µm.

This figure shows the time course of accumulation of AIS proteins during neuron development. It includes images and a graph illustrating the expression of various proteins in the AIS during neuronal development. The graph displays the fluorescence intensities of individual immunostained proteins as a percentage of the maximal value over a period from DIV4 to DIV21. The proteins include spectrin βIV, NrCAM, neurofascin, Nav, and AnkG. Error bars represent standard deviations.
and differences between the AIS coat and the RBC membrane skeleton.

The AIS coat appears structurally similar to native membrane skeletons in RBCs and platelets (Hartwig and DeSisto, 1991; Moyer et al., 2010; Nans et al., 2011), but displays more abundant globules, especially in the mature state. Our immunopREM data suggest that the majority of these globules can correspond to voltage-gated ion channels. Although we tested here only Nav channels, other channels that are abundant in the AIS likely contribute to the globular appearance of the AIS coat. Ion channels can also be responsible for the globular appearance of AnkG-positive elongated clusters, as most ion channels are recruited to the AIS through direct interaction with AnkG. A subset of globular structures may correspond to recoiled elongated molecules.

expanded RBC cytoskeletons, spectrin molecules are typically seen at their maximal length of ~200 nm, whereas ankyrin R appears as globules along spectrin fibers (Byers and Branton, 1985). However, in native RBCs (Moyer et al., 2010; Nans et al., 2011) or platelets (Hartwig and DeSisto, 1991), the actin–spectrin meshwork appears more contracted, suggesting stress-dependent remodeling. Because spectrins and ankyrins are expressed ubiquitously, it is believed that similar actin–spectrin membrane skeletons provide structural support to the plasma membrane in other cell types. However, the structure of the membrane skeleton remained poorly characterized except in blood cells. In this study, we have extensively characterized the spatial and molecular architecture of the spectrin–ankyrin–rich submembranous cytoskeleton in the AIS of cultured hippocampal neurons and found both similarities and differences between the AIS coat and the RBC membrane skeleton.

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Figure 9.  AnkG organization in the AIS coat. Immunogold PREM of AnkG in DIV14 neurons. (A–D) Regions of the AIS coat along the main axon. Gold particles (12 nm; yellow) are present in small clusters or individually in dense regions (A). In stretched or sparse regions of the coat, AnkG immunogold associates with fibrillar networks (B) or globular clusters (C and D). (E) A gallery of individual AnkG-positive structures in the sparse or stretched AIS coat. Duplicate images are pseudocolored to show gold particles (yellow) associated with putative AnkG structures (red), such as long, thin, wavy filaments (e.g., top row) or elongated globular clusters of variable thickness and morphology. A donut-shaped putative ion channel (see Fig. 10) within an AnkG globular cluster is pseudocolored in blue. Bars: (A–D) 100 nm; (E) 20 nm.
Thin fibrils found in the AIS coat represent a mixed population formed by neurofascin, NrCAM, and extended spectrin and AnkG molecules. Fibrillar appearance of neurofascin and NrCAM is consistent with the presence of six immunoglobulin repeats and three to five fibronectin repeats in their extracellular domains (Davis et al., 1993; Bennett and Baines, 2001). Although neurofascin and NrCAM, as well as ion channels, appear to be intermingled with cytoplasmic proteins, this impression is likely caused by the absence of the plasma membrane in PREM samples, rather than reflecting native intracellular localization of these transmembrane proteins. The filamentous appearance of AnkG is conceivable because the 480-kD AnkG isoform is predicted to have a flexible tail that may extend up to 0.5 µm from the globular membrane-binding head (Bennett and Healy, 2009). Fibrillar components of the AIS mostly run along the axon in mature AISs, but at axonal branch junctions, they acquire less uniform orientation, suggesting a forced reorientation in response to tension exerted by the axonal branch. Although it remains unclear what force causes longitudinal alignment of fibers in the axon shaft and their reorientation at branch junctions, the fact that rearrangements are possible underscores the elastic nature of the AIS cytoskeleton and its ability to undergo

![Image of molecular composition of the AIS coat](image-url)
remodeling in a tension-dependent manner, similar to the membrane skeleton in RBCs (Nans et al., 2011).

Although we have identified several major components of the AIS coat, there are additional proteins that can contribute to the dense mass of fibrils and globules within the coat. They may represent several known AIS proteins (e.g., voltage-gated potassium or calcium channels or PSD93), as well as proteins that exist in the AIS but remain unknown. We also cannot exclude the possibility that the coat is partly impenetrable to gold particles, thereby underestimating the actual density of each protein.

**Development of the AIS cytoskeleton**

The specialized AIS skeleton is gradually assembled during neuron maturation with AnkG serving as a master organizer of the AIS (Bennett and Baines, 2001). Thus, it is expected that appearance of AnkG-based structures in the proximal axon should be a first sign of AIS assembly. Instead, we found that the first distinct feature of the proximal axon is MT bundling. In mature neurons, MT bundles become poorly visible by PREM as a result of high coat density, but they still span the AIS. MT bundles (fascicles) are a key signature of AISs in neurons (Palay et al., 1968). They may function as a filter for cytoplasmic proteins in mature AISs (Li et al., 2011) and to capture AIS structural components during AIS assembly.

The formation of MT bundles before arrival of AIS coat components does not contradict the idea of AnkG as a master organizer of the AIS. Indeed, AnkG itself can serve as a MT bundler during early stages of AIS development, because it can bind MTs and oligomerize (Bennett and Baines, 2001) and because AnkG knockout mice lack MT bundles (Sobotzik et al., 2009). Additional candidates to mediate MT bundling in the AIS are plus end–tracking proteins EB1 and EB3 because they (especially EB3) are enriched in the AIS, interact with AnkG, and induce MT bundles in a manner that is independent of plus end tracking (Leterrier et al., 2011).

A second feature of AIS assembly is accumulation of the fibrillar–globular meshwork that gradually coats MT bundles. The initial predominance of fibrils in the coat likely reflects accumulation of filamentous proteins that arrive early during AIS assembly, such as neurofascin, NrCAM, and, to a lesser extent, spectrin βIV. The subsequent accumulation of globules correlates with the late arrival of Nav channels and their donut-shaped morphology. Early arrival of neurofascin and NrCAM to the AIS is unexpected because neither of them is required for AIS assembly (Hedstrom et al., 2007), although neurofascin is needed for long term stabilization of the AIS and NrCAM clustering in mouse Purkinje cells (Zonta et al., 2011). Possibly, some early functions of these proteins in AIS assembly remain to be revealed.

**Actin cytoskeleton organization in the AIS**

We found two actin filament populations in the AIS: short stable filaments resistant to gelsolin or LatB treatment and longer dynamic filaments that are sensitive to these treatments. In RBCs, short actin filaments are connected by spectrin molecules within a regular triangular network (Byers and Branton, 1985). Subdiffraction fluorescence microscopy data suggested a similar actin–spectrin arrangement in axons of cultured neurons by showing that short adducin-positive filamentous forms periodic rings along the axon, including the AIS, and are connected by stretched and longitudinally aligned spectrin molecules (Xu et al., 2013). Although we indeed observed longitudinal fibrils in mature AISs, actin periodicity was not readily apparent in our phalloidin immunogold–stained samples. We doubt that actin rings were destroyed in our samples by detergent extraction because short actin filaments that supposedly form rings were sufficiently stable to survive not only extraction but also gelsolin or LatB treatment. Possibly, greater signal averaging combined with larger fields of view makes light microscopy better suited for revealing global features of actin organization, whereas higher resolution PREM can reveal small details.

We were also unable to document the presence of adducin in the AIS by light or electron microscopy, although it was readily detectable outside of the AIS. A reason for this discrepancy is currently unknown, especially because we used the same antibody and similar staining conditions. Regardless of these inconsistencies, because the periodic actin–spectrin arrangement detected by subdiffraction microscopy was observed throughout the axonal shaft and was not specific for the AIS, this structure unlikely plays a mechanistic role in the AIS polarity function. However, short actin filaments in the AIS can function similarly to junctional complexes in RBCs to maintain structural integrity of the coat.

In addition to short stable actin filaments, we also detected a small population of relatively long and dynamic actin filaments that were enriched by 30–50% in the AIS relative to adjacent axonal regions. In RBCs, short actin filaments in junctional complexes can be transiently uncapped and serve as seeds for polymerization of longer dynamic filaments (Byers and Branton, 1985), probably because adducin has relatively low affinity for barbed ends (Kuhlman et al., 1996). In the AIS coat, barbed ends of stable actin filaments should be capped by another barbed end capping protein, because adducin is not present there. However, these filaments can still be transiently uncapped in a regulated manner to serve as sites of actin polymerization to produce longer dynamic filaments.

**Actin functions in the AIS**

In cultured neurons, dynamic actin filamentous networks are required for maintaining the AIS diffusion barrier (Winckler et al., 1999; Nakada et al., 2003; Song et al., 2009; Rashband, 2010). Because short actin filaments in the AIS are not dramatically affected by LatB treatment, we speculate that the dynamic actin filaments with submicrometer lengths are more likely to be responsible for LatB-dependent barrier defects. The spatial organization of the dynamic actin population in the AIS is essential to models of AIS polarity function. The polarized trafficking model (Al-Bassam et al., 2012; Watanabe et al., 2012) proposes that the AIS forms a selective filter that relies on oriented actin tracks present within 2–3-µm actin patches, which, however, were not correlated with AIS markers (Watanabe et al., 2012). We also observed 1–2-µm actin patches in axons, but they corresponded to actin-rich axonal protrusions rather than to AIS-specific structures. In contrast, the submicrometer-sized actin
puncta were constantly detected by fluorescence microscopy in the AIS. They likely corresponded to dynamic actin filaments in PREM images. These actin filaments, however, do not display preferential orientation and thus cannot serve as directional tracks in the AIS. These results, together with overall sparse distribution of actin filaments in the AIS, do not support a selective filter model that depends on polarized orientation of filaments.

The picket fence model proposes that a dense actin meshwork in the AIS prevents mixing of lipids and plasma membrane proteins between the axon and dendrites (Nakada et al., 2003; Kusumi et al., 2012), but supporting structural data are available only from epithelial cells (Morone et al., 2006). Our data reveal an actin network of insufficient density to hinder diffusion of membrane proteins in the AIS. Instead, the submembranous AIS coat could serve in this capacity, as it is very dense, even though not particularly rich in actin. Short stable actin filaments can contribute to the formation of the AIS diffusion barrier by maintaining the structural integrity of the coat.

The specific role of dynamic actin filaments in the maintenance of AIS-dependent neuron polarity is not entirely clear. A clue was provided by our observation that the most obvious structural aberrations in the AIS coat after LatB treatment occurred at axonal branch junctions. Because the AIS coat typically appears more stretched at branch junctions, we infer that the AIS coat may experience additional mechanical challenge at these sites, which may be tolerated in the presence of actin dynamics, but not in its absence. Thus, we propose that mechanical stress (or a chemical signal) induces coat remodeling that depends on dynamic actin filaments. For example, during remodeling events, spectrin and/or other AIS components may detach from actin filaments and then recombine into a new configuration. Long dynamic filaments can facilitate reassembly of the meshwork, for example, by capturing unattached spectrin molecules, whereas in the absence resealing of the coat can fail and cause meshwork rupture. In the absence of mechanical or chemical signals, however, dynamic actin filaments may be dispensable whereas short stable filaments serve exclusively to maintain coat integrity.

We assume that the remodeling capability of the AIS coat may be important for covering a branch junction, but it is likely even more essential for other events in the AIS, such as passage of vesicles through the crowded AIS. Thus, cases where the AIS coat peels off the MT bundles after LatB treatment may reflect the inability of the coat to reseal after vesicle passage. A need for remodeling may explain the selectivity of cargo transport, if we assume that an acceptable cargo carries a recognition signal, which initiates a remodeling process that transiently opens a passage for the cargo. It would be interesting to test this idea in the future, although it is not the only scenario explaining LatB effects. Thus, dynamic actin filaments may participate in attaching the AIS coat to the plasma membrane and/or to MTs. It is also possible that global actin depolymerization triggers a mechanism for “opening” of the barrier to enhance supply of actin for other cellular activities, such as growth cone dynamics.

Conclusions

We used PREM to show that the AIS of mature neurons comprises a dense submembranous coat that is not present elsewhere in the neuron. We found neither a dense actin meshwork nor oriented tracks within the AIS, but revealed two subpopulations of actin: short stable and longer dynamic filaments. Although our data are in general consistent with the view that the AIS coat is analogous to that of the membrane skeleton of RBCs, we additionally revealed that dynamic actin filaments are important for providing remodeling capability to the AIS coat in stressed conditions. Based on this data, we propose that the dense fibrillar–globular coat functions as a diffusion barrier at the AIS to prevent mixing of axonal and dendritic proteins, whereas signal-triggered coat remodeling is dependent on dynamic actin filaments and allows for selective passage of vesicles during establishment of neuron polarity.

Materials and methods

Cell culture

Rat hippocampal cells (provided by M.A. Dichter, University of Pennsylvania, Philadelphia, PA) were isolated and dissected as described previously (Wilcox et al., 1994). In brief, hippocampi were dissected from brains of Sprague-Dawley rat embryos at embryonic day 18–20 and dissociated into individual cells by incubating in a trypsin-containing solution. The cells were then washed and plated at low density (60,000 cells/ml) on poly-l-lysine-coated (1 mg/ml) glass coverslips subsequently used to find cells imaged in PREM images. These actin filaments, however, do not disappear; critical point dried; coated with platinum and carbon; and transferred onto electron microscopic grids for observation. Detergent extraction to expose cytoskeletons was done as described for fluorescence microscopy. For correlative PREM, cells were cultured on glass-bottomed dishes containing coverslips that were coated with carbon through a finder grid to introduce fiducial marks, which were subsequently used to find cells imaged by fluorescence microscopy for PREM analysis. For myosin S1 decoration, the cell-moving coverslips were coated with carbon through a finder grid to introduce fiducial marks, which were subsequently used to find cells imaged by fluorescence microscopy for PREM analysis. For myosin S1 decoration,
Fig. S1 shows MT bundling in the proximal axon of DIV3 neurons. Fig. S2 based protrusions, such as filopodia. Fig. S3 shows PREM of immunogold-labeled actin structures in neurons and non-neuronal cells. Fig. S4 shows how the AIS coat breaks and detaches from MTs after LatB treatment. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201401045/DC1.

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Statistics and data analysis

Measurements were done using MetaMorph or Photoshop software packages and repeated for at least two independent experiments. Statistic analysis was performed using Excel (Microsoft) or Instat (GraphPad Software) software. Fluorescence intensities for phalloidin, adducin, and AIS proteins were determined after background subtraction. For phalloidin staining, cells were considered neutrally oriented, that is, orthogonal to the axon axis. Proximal orientation, respectively. Filaments that were not within this range were considered proximally, that is, orthogonal to the axon axis. Analysis of S1-decorated filaments was performed as described previously (Shutova et al., 2012).

In brief, detergent-extracted unfixed cells were incubated with 0.4 µg/ml S1 antibody (gift of Y. Goldman, University of Pennsylvania, Philadelphia, PA) in buffer containing 50 mM NaCl, 0.1 mM CaCl2, and 2 µM taxtacetax for 10 min at room temperature and fixed with 2% glutaraldehyde. For depletion of actin filaments and MTs, MT-depleted preparations were subsequently treated with gelson. For immunogold staining of AnkG, spectrin βIV, neurofascin, and NCAM, neurons were detergent extracted, fixed with 0.2% glutaraldehyde, quenched with NaNb4 for 10 min, incubated first with primary antibody (1:10 or 1:5) for 45 min, and then with 12 nm of colloidal gold–conjugated anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) after which cells were again fixed with 2% glutaraldehyde for 20 min. Immunogold staining of Nav was done similar to other AIS proteins except that the primary antibody was added after detergent extraction and before fixation; then cells were fixed, quenched, and incubated with secondary antibody. For phalloidin immunogold staining, cells were exposed for regular PREM except that phalloidin was omitted from the extraction buffer. Then cells were fixed with 0.2% glutaraldehyde, quenched with NaNb4 for 10 min, blocked with 10% donkey serum, and sequentially incubated with Alexa Fluor 488–phallolin (1:50) for 45 min, a rabbit anti–Alexa Fluor 488 primary antibody (Molecular Probes) for 45 min, and 18 nm of colloidal gold–conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) after which cells were again fixed with 2% glutaraldehyde in 0.2 M sodium cacodylate for 20 min.

Samples were examined using a transmission electron microscopy (JEM 1011; JEOL USA) operated at 100 kV. Images were acquired by a charge-coupled device camera (ORIUS 832.10W; Gatan) and presented in inverted contrast. Gold particles in replica electron microscopy images were identified at high magnification after contrast enhancement to distinguish them from other bright objects in the samples. Color labeling and image overlays were performed using Adobe Photoshop (Adobe Systems), as described previously (Shutova et al., 2012).

Online supplemental material

Fig. S1 shows MT bundling in the proximal axon of DIV3 neurons. Fig. S2 shows actin structures in axons of DIV3 neurons decorated with S1. Fig. S3
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